

1 **Title: Identification of a Binding Site for Small Molecule Inhibitors Targeting Human**
2 **TRPM4**

3
4 **Authors**

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15 **Abstract:**

16 Transient receptor potential (TRP) melastatin 4 (TRPM4) protein is a calcium-activated
17 monovalent cation channel associated with various genetic and cardiovascular disorders.
18 The anthranilic acid derivative NBA is a potent and specific TRPM4 inhibitor, but its binding
19 site in TRPM4 has been unknown, although his information is crucial for drug development
20 targeting TRPM4. We determined the cryo-EM structures of full-length human TRPM4
21 embedded in native lipid nanodiscs in an unbound, a state bound to NBA, and a new
22 anthranilic acid derivative known as IBA-bound state. We found that the small molecules
23 NBA and IBA were bound in a pocket formed between the S3, S4, and TRP helices and the
24 S4-S5 linker of TRPM4. Our structural data and results from patch clamp experiments
25 enable validation of a binding site for small molecule inhibitors, paving the way for further
26 drug development targeting TRPM4.

27 **Main text**

28 **Introduction**

29 Transient receptor potential (TRP) ion channels are a superfamily of cation channels
30 involved in various physiological functions, including sensory perception, cellular
31 homeostasis, and ion transport ^{1,2}. Mutations in the genes encoding TRP ion channels that
32 lead to their dysfunction or dysregulation, have been implicated in numerous diseases and
33 genetic disorders. Therefore, TRP channels are attractive therapeutic targets for drug
34 development ^{3,4}.

35 The TRPM (melastatin-like transient receptor potential) sub-family member, TRPM4, is a
36 calcium-activated non-selective monovalent cation channel. The voltage-dependent
37 activation of TRPM4 by intracellular calcium drives a current due to monovalent cations flux
38 such as Na⁺ and K⁺ through the channel, leading to plasma membrane depolarization
39 facilitating, in many cell types, calcium uptake via other calcium-permeable channels ^{5,6}.
40 TRPM4 activity regulates physiological processes such as cardiac conduction, smooth
41 muscle contraction, insulin secretion, and immune responses ⁷⁻⁹. Genetic defects in *TRPM4*
42 have been found in patients with inherited cardiac conduction disorders. Furthermore,
43 alterations of TRPM4 function have been linked to diabetes, hypertension, and cancer ¹⁰⁻¹⁶.
44 Thus far, several small molecules identified and developed as potent and selective TRPM4
45 inhibitors are used as research tools to investigate the potential of TRPM4 as a therapeutic
46 target and are promising candidates for drug development ¹⁷. These molecules include 9-
47 phenanthrol, flufenamic acid and the anthranilic acid derivatives CBA (4-chloro-2-[2-(2-
48 chloro-phenoxy)-acetylamino]-benzoic acid) and NBA (4-chloro-2-(2-(naphthalene-1-yloxy)
49 acetamido) benzoic acid ¹⁸⁻²¹. NBA and CBA are thus far the most selective and potent
50 TRPM4 inhibitors reported; however, it remains unknown how these drugs bind and inhibit
51 TRPM4 activity ¹⁹. To facilitate drug development targeting TRPM4, detailed structural
52 information revealing the mode of binding these molecules to TRPM4 is essential.

53 Cryo-electron microscopy (cryo-EM) enabled the structure determination of several TRP
54 channels and revealed the druggable sites in these channels by structure determination in
55 the presence of antagonists and drug molecules ^{3,17}. These structures have revealed
56 different drug-binding pockets around the TRP channels' transmembrane domains (TMD).
57 For example, the synthetic molecule icilin binds into a hydrophobic pocket of the Voltage
58 Sensor Like Domain (VSLD) in TRPM8 and the binding of the small molecule inhibitor NDNA
59 (*N*-(3,4-dimethoxybenzylidene)-2-(naphthalen-1-yl)acetohydrazide) into the cavity between
60 the S1-S4 domain and pore domain of TRPM5 ^{22,23}. These two examples revealed different
61 drug-binding sites within the same subfamily of TRP channels. Interestingly, lipid molecules
62 can share binding sites with drug molecules and inhibit drug binding and activity, as
63 demonstrated in the binding of 2-ABP (2-aminoethyl diphenylborinate) into the VBP
64 (Vallanoid Binding Pocket) of TRPV2, which could be inhibited by binding an endogenous
65 cholesterol molecule ²⁴.

66 Structures of detergent-isolated TRPM4 studied by cryo-EM have revealed the binding sites
67 of the cholesterol homolog CHS (Cholesteryl Hemisuccinate) used to purify TRPM4,
68 suggesting potential endogenous cholesterol binding sites ²⁵⁻²⁸. However, to date there are
69 no reported structures of TRPM4 bound to specific inhibitors. It is also possible that the
70 addition of CHS required for stabilizing TRPM4 during detergent isolation could occlude
71 potential drug-binding sites. To address this problem, we have isolated TRPM4 in its native
72 lipid environment using SMA (Styrene Maleic Anhydride) nanodiscs and determined its high-
73 resolution structure in the presence and absence of the small molecules NBA and a new
74 derivative IBA (4-chloro-2-[2-(3-iodophenoxy)-acetylamino]-benzoic acid) which has a similar
75 potency to NBA (**Figure 1; Figure 2a-c and Supplementary Fig. 1- 8**) ²⁹. These structures
76 reveal the mode of binding and inhibition of two potent and specific TRPM4 inhibitors, paving
77 the way for future drug development and facilitating further investigations of TRPM4 as a
78 therapeutic target for treating related maladies.

79 **Results**

80 **Structures of HsTRPM4 in its native lipid environment**

81 We determined cryo-EM structures of full-length human TRPM4 in endogenous lipid
82 nanodiscs in apo (HsTRPM4_{apo}), IBA (HsTRPM4_{IBA}) and NBA (HsTRPM4_{NBA}) bound states
83 at overall resolutions of 3.7, 3.6 and 4.5 Å respectively (**Figure 1; Figure 2; Supplementary**
84 **Fig. 4- 8; Supplementary Table 1**). All structures obtained were identical to previously
85 determined cryo-EM structures of TRPM4 in detergent micelles and reconstituted nanodiscs
86 ^{25–28}. However, we found that in native nanodiscs, densities for the cytosolic regions of
87 TRPM4 were poorly resolved than previously determined structures resulting in lower-
88 resolution 3D reconstructions in these regions, which could be a result of TRPM4
89 solubilization using SMA. This effect was more pronounced in the cytosolic N-terminal TRPM
90 homology regions 1 and 2 (MHR1/ 2), with the density in these regions being much less
91 defined (**Supplementary Fig. 1c; Supplementary Fig. 2a and b**). Therefore, 3D
92 reconstructions could not be obtained for these regions (**Supplementary Fig. 2; 4- 6**).
93 These results suggest that SMA solubilization results in a less stable TRPM4 structure,
94 which could result from the differences in lipid composition or binding compared with
95 structures of TRPM4 in detergent micelles and reconstituted nanodiscs. Indeed, examination
96 of the TMD revealed that although the structural arrangement of the transmembrane helices
97 was identical, the lipid arrangement was strikingly different from previously determined
98 TRPM4 structures ^{25–28}. Notably, the published structure of HsTRPM4 in a reconstituted lipid
99 nanodisc as well as cryo-EM density maps of detergent-solubilized HsTRPM4 and
100 MmTrpm4 from this study, revealed ordered densities for annular phospholipids and three
101 CHS molecules, one of which (CHS-2) occludes the drug binding site (**Supplementary Fig.**
102 **2 and 3; Supplementray Table 2**) ²⁶.
103 On the other hand, in our structures, ordered densities for annular lipids are markedly
104 reduced, with primarily three densities for cholesterol molecules bound to TRPM4 observed
105 (**Figure 1a -d; Supplementary Fig. 2a and b**). Of the three, two cholesterol molecules

106 (CHR-1 and CHR-2) bind in similar positions to CHS molecules in a previous structure in a
107 reconstituted nanodisc (**Supplementary Fig. 2a and b**). One of these molecules (CHR-1)
108 interacts with the S6 helix close to the channel pore, and another (CHR-2) in a pocket
109 formed between S3 and S4 helices and the S4-S5 linker equivalent to the VBP of TRPV2;
110 however, the density for this cholesterol molecule is weak, suggesting low occupancy
111 (**Figure 1e -f**). The third cholesterol molecule (CHR-3) in our structures binds between the
112 S5 and S2 helix from an adjacent protomer differing from the previously reported position of
113 CHS at the pre-S1 elbow (**Supplementary Fig. 2a -c**). The presence of less density for
114 annular lipids in the native nanodiscs was an important factor that enables assignment of
115 density for the drugs in the HsTRPM4_{IBA} and HsTRPM4_{NBA} structures (**Supplementary Fig.**
116 **8**).

117 **The binding site of the small molecule inhibitors NBA and IBA in HsTRPM4**

118 Analysis of the HsTRPM4_{IBA} and HsTRPM4_{NBA} structures revealed discrete densities for the
119 IBA and NBA molecules located at the periphery of the inner membrane leaflet in the pocket
120 formed between S3 and S4 helices, S4-S5 linker and TRP helix (**Figure 2d- i**). The
121 presence of the ligands in this pocket suggests that these ligands can displace the
122 endogenous cholesterol molecule (CHR-3) present in the HsTRPM4_{apo} structure, which is
123 possibly not tightly bound as judged by the weaker density map for this molecule compared
124 to other bound cholesterol molecules (**Figure 1e and f**). Ligand binding induces only very
125 subtle conformational changes in the structure of HsTRPM4, with the overall structures
126 remaining broadly similar (**Figure 2j -l**). The HsTRPM4 ion conduction pore is also in the
127 closed state in all three structures. Although changes in the pore radius profile are observed
128 in the IBA-bound structure, these are due to side chain rotamer differences of a few residues
129 lining the pore. These changes are negligible as the pore remains in a closed state (**Figure**
130 **2l**). Interestingly, apparent density for Ca²⁺ could be observed in cryo-EM density maps of
131 our structures of TRPM4 in native nanodiscs, which suggests the possible binding of
132 endogenous Ca²⁺ ions however this did not result in an open state of the channel

133 (**Supplementary Fig. 9**). Both IBA and NBA share a common anthranilic acid moiety but
134 differ in that NBA possesses an additional ring of its naphthalene substituent, making it
135 bulkier and more hydrophobic than the smaller 3-iodophenyl ring of IBA (**Figure 2e and h,**
136 **Supplementary Fig. 8**). In both HsTRPM4_{IBA} and HsTRPM4_{NBA} structures, the hydrophilic
137 anthranilic acid moiety containing acidic, amide and chloride groups faces towards the
138 cytosol and interacts with charged amino acid side chains from TRPM4 (**Figure 3a and b;**
139 **Supplementary Fig. 7d and e**). Notably, the anthranilic acid moiety interacts with His908
140 from the S4 helix, Tyr1057, Gln1061, and Arg1064 from the TRP helix as well as with
141 Ser924 from the S4-S5 linker, and Ser863 from the S3 helix in both structures (**Figure 3a**
142 **and b**). These interactions between the anthranilic acid moiety and residues of TRPM4
143 reveal the chemical basis behind the specific binding of the anthranilic acid derivatives to
144 TRPM4.

145 NBA and IBA have a similar potency for TRPM4 inhibition, possibly by making additional
146 unknown interactions in the binding pocket^{19–20}. Our structures show that the naphthalene
147 substituent and the 3-iodophenyl ring of NBA and IBA bind into a hydrophobic pocket
148 between the S3 and S4 helices of TRPM4 (**Figure 4a and b; Supplementary Fig. 7d and**
149 **e**), making hydrophobic interactions with the residues that decorate this pocket, including
150 Val901, Val904 and Leu907 (**Figure 3b and c**). Therefore, these structures reveal a
151 rationale for the potency and specificity of NBA and IBA and pave the way for designing
152 chemical modifications of these molecules to enable increased targeting of the binding
153 pocket to develop more potent and specific inhibitors.

154 **Validation of the NBA and IBA binding site in HsTRPM4**

155 To disrupt the drug-protein interactions in the hydrophilic region of the drug binding site, we
156 introduced the HsTRPM4 mutations Ser863Ala, His908Ala, Ser924Ala, Lys925Ala,
157 Tyr1057Ala, Gln1061Ala, Arg1064Ala, Arg1064Gly, Arg1064Ser, and the double variant
158 Ser863Ala/ Ser924Ala. Additional Val901Trp and Val904Trp are space-filling mutations that

159 disrupt the hydrophobic pocket—introducing these mutations aimed to reduce the drug's
160 inhibitory effect compared to wildtype HsTRPM4. As proof of concept, the experiments were
161 done with NBA only since it shares a binding site similar to that of IBA. First, the expression
162 of the different variants was investigated using the western blot approach (**Supplementary**
163 **Fig. 10 a and b**). Only Ser863Ala and Gln1061Ala variants showed a significantly reduced
164 expression compared to the wildtype channel (**Supplementary Fig. 10 a and b**). In parallel,
165 functional experiments revealed that after activation of the HsTRPM4 function by adding 300
166 μM of free calcium, almost all variants decreased in function except Ser863Ala, Ser924Ala,
167 Arg1064Ala, and Arg1064Gly (**Supplementary Fig. 10c, 11 and 12**). Due to the reduction of
168 activation in these mutants, we wondered if this decrease in function observed may be due
169 to an alteration of the calcium sensitivity of those variants. The calcium sensitivity curves
170 performed on some loss-of-function HsTRPM4 variants (Val904Trp, Tyr1057Ala,
171 Gln1061Ala, Arg1064Ser, and the double variant Ser863Ala/ Ser924Ala) show that the
172 different EC_{50} s for calcium are not higher (less sensitivity to calcium-free) compared to
173 wildtype HsTRPM4 suggesting that this decrease of function may not entirely be to a
174 reduction of calcium sensitivity (**Supplementary Fig. 13a**). On the contrary, the different
175 EC_{50} s of the variants are smaller than the wildtype EC_{50} , suggesting an increase in this
176 calcium sensitivity (**Supplementary Fig. 13a**). Following those experiments and knowing
177 that the inhibitory effect mediated by NBA will be investigated on these variants in the
178 presence of calcium (to activate them), control experiments were performed using HsTRPM4
179 wildtype channels to explore the potential influence of NBA on calcium sensitivity. The
180 results showed no drastic difference in the EC_{50} s was observed, suggesting that NBA does
181 not alter the calcium sensitivity of the wildtype channels (**Supplementary Fig. 13b**). The
182 absence of the effect of NBA and amino acid mutations on calcium sensitivities leads us to
183 perform the final experiment investigating the consequences of modifying those amino acids
184 on the inhibitory effect mediated by NBA. As a proof of concept, the mutations Ser863Ala,
185 Val901Trp, His908Ala, Ser924Ala, Arg1064Ala, Arg1064Gly, and the double variant
186 Ser863Ala/ Ser924Ala were investigated. Compared to wildtype HsTRPM4, NBA dose-effect

187 curves on single variants of the HsTRPM4 channel show a drastic reduction (one log of
188 difference) of the IC_{50} s for the NBA (**Figure 3e**). Compared to the wildtype construct, a more
189 pronounced decrease in the efficiency of NBA (two logs of difference) is observed in the
190 presence of double mutation (**Fig. 3e; Figure 4; Supplementary Figure 14**). In addition, a
191 control experiment has been performed using a mutant of HsTRPM4 linked to cardiac
192 dysfunction and already reported in the literature; the mutant HsTRPM4 T677I³⁰. This
193 variant has been selected based on the presence outside the putative NBA binding of the
194 mutated amino acid and because this point mutation did not alter the biophysical properties
195 of the channel³⁰. This mutation did not alter the IC_{50} of the NBA, as observed with the NBA
196 binding pocket variants (**Figure 3f and g**). Overall, these data strongly support the notion
197 that those amino acids of HsTRPM4 are part of the binding pocket for NBA/ IBA determined
198 from our cryo-EM structures.

199 **Discussion**

200 We have utilized cryo-EM to determine the high-resolution structures of HsTRPM4 bound to
201 specific inhibitors. Importantly, we find that using SMA to isolate HsTRPM4 in its
202 endogenous lipid nanodisc was necessary to unequivocally assign densities for the inhibitor
203 molecules. This finding supports the relevance of previous attempts to isolate TRPM4 in its
204 native lipid environment but unfortunately yielded lower resolution structures³¹. High-
205 resolution structures of TRPM4 have previously been determined in detergent micelles and
206 in reconstituted lipid nanodiscs, both of which require detergent isolation of the protein from
207 the cell and, by so doing, introduce artefacts to the lipid environment of the protein. An
208 essential factor is that the added CHS, to maintain protein stability in detergent extraction,
209 binds in specific sites in the protein, which may or may not represent actual cholesterol
210 binding. We observed apparent differences in the binding arrangement of annular lipids to
211 TRPM4 from the structural data when isolated with SMA. Most striking is that a CHS
212 molecule completely occupies the drug binding site, suggesting that obtaining a structure or
213 performing biochemical experiments with detergent-CHS isolated protein and these

214 inhibitors does not represent the endogenous scenario. Indeed, in our previous attempts to
215 determine the structure of detergent-isolated TRPM4 in complex with NBA/ IBA, the
216 identification of the inhibitor binding site was ambiguous. This finding also shows the
217 importance of the composition and binding arrangement of the lipid annulus to the function
218 and inhibition of the target membrane protein. This study shows that using SMA would be
219 advantageous for the structure determination of drugs with membrane proteins, particularly
220 in cases where the drug binds in the TMD exposed to lipid molecules.

221 Although there are no structures of TRPM4 in its open state available, movement of the TRP
222 helix is required for the activation of the TRPM2 channel and the relative movement of the
223 TRP helix to S1-S4 helices is shown to be required for desensitization of the TRPM8
224 channel^{32,33}. These findings hint towards a role of TRP helix movement in regulating the
225 channel pore of the TRPM subfamily. The binding of NBA/ IBA to TRPM4 may restrict the
226 movement of the TRP helix, maintaining the TRPM4 channel pore in a closed state even in
227 the presence of activation stimuli, but structures of HsTRPM4 in the open state would be
228 necessary to determine whether this is the case or not. The role of the bound cholesterol
229 molecule in the same pocket remains unclear. This cholesterol could also regulate channel
230 function in response to changes in the membrane environment.

231 Our study has identified the binding pocket for inhibiting TRPM4 for the anthranilic acid
232 derivatives (**Figure 5**). These drugs bind in a pocket between S3 and S4 helices, S4-S5
233 linker and TRP helix. However, detailed insights into the mechanism of inhibition will require
234 structure determination of TRPM4 in its open state, which so far has been elusive. It is clear
235 that the addition of Ca²⁺ ions is not sufficient to obtain the open state with purified TRPM4 in
236 lipid nanodiscs, and the presence of a membrane potential may be necessary. Our future
237 studies will seek to address this. Nonetheless, the identification of a binding pocket for
238 TRPM4 inhibitors presents a significant milestone towards further development of new
239 potent and even more specific drugs targeting the TRPM4 ion channel for therapeutic
240 intervention.

241 **Acknowledgements**

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244 Nazarov, Bertrand Beckert and members of the DCI-Lausanne from EPFL, UNIL, and
245 UNIGE for their support. We thank Kelvin Lau, Florence Pojer, Laurence Durrer and Soraya
246 Quinch the PTPSP at EPFL for their support with protein expression.

247

248 **Author Contributions Statement**

249 HS and HA conceptualized the project. BE and DN performed cryoEM sample preparation
250 and structure determination with support from the DCI-Lausanne. PA, AH, SG, DR and MB
251 performed biochemistry and patch clamp experiments. CEG. and ML designed the target
252 molecule IBA and synthesis. CEG. performed the synthesis of NBA and IBA. HS and HA
253 acquired funding. Project administration and supervision was done by BE and JR. Writing
254 original draft was done by BE. Manuscript review & editing was done by DN, BE, JR, ML, HA
255 and HS. All authors read and approved the manuscript.

256

257 **Competing Interests Statement**

258 The authors declare no competing interests.

259 **Supplementary Table 1**

Data collection and processing	HsTRPM4apo (EMD-19057) (PDB 8RCR)	HsTRPM4IBA (EMD-19061) (8RCU)	HsTRPM4NBA (EMD-19069) (PDB 8RD9) (EMD-19060, local)
Nominal Magnification	165kx	165kx	165kx
Voltage (kV)	300	300	300
Recorded Micrographs	6 429	7 500	11 851
Electron exposure (e ⁻ /Å ²)	50	50	50
Defocus range (µm)	0.8-2.5	0.8-2.5	0.8-2.5
Pixel size (Å)	0.726	0.726	0.726
Symmetry imposed	C4	C1/C4	C4
Initial particle images (no.)	1 719 323	1 149 090	1 805 182
Final particle images (no.)	16 308	20 345	15 954
Map resolution (Å)	3.67	3.59	4.50
FSC threshold	0.143	0.143	0.143
Map resolution range (Å)	30-3.3	30-3.2	30-3.6
Map sharpening B factor (Å ²)	-69.7	-52.2	-108.1
Refinement			
Initial model used (PDB code)	6BQV	6BQV	6BQV
Model composition			
Non-hydrogen atoms	8	9	8
Protein residues	2420	2420	2422
Ligands	CHR: 12	IBA: 4 CHR: 8	NBA: 4 CHR: 8
B factors (Å ²)			
Protein	41.23/251.46/135.2	0.00/93.44/38.86	142.10/427.50/239.20
Ligand	1 63.19/103.12/79.00	15.44/136.15/47.75	20.00/33.04/22.07
R.m.s. deviations			
Bond lengths (Å)	0.012 (146)	0.003 (0)	0.004 (0)
Bond angles (°)	0.801 (7)	0.721 (0)	0.925 (10)
Validation			
MolProbity score	1.93	2.04	2.23
Clashscore	14.67	16.76	24.33
Poor rotamers (%)	0.00	0.19	0.05
Ramachandran plot			
Favored (%)	96.16	95.49	94.82
Allowed (%)	3.71	4.34	4.76
Disallowed (%)	0.13	0.17	0.42

260

261 **Supplementary Table 1 | Cryo-EM data collection, refinement and validation statistics**

262 **for SMA solubilized HsTRPM4 samples.**

263

264 **Supplementary Table 2**

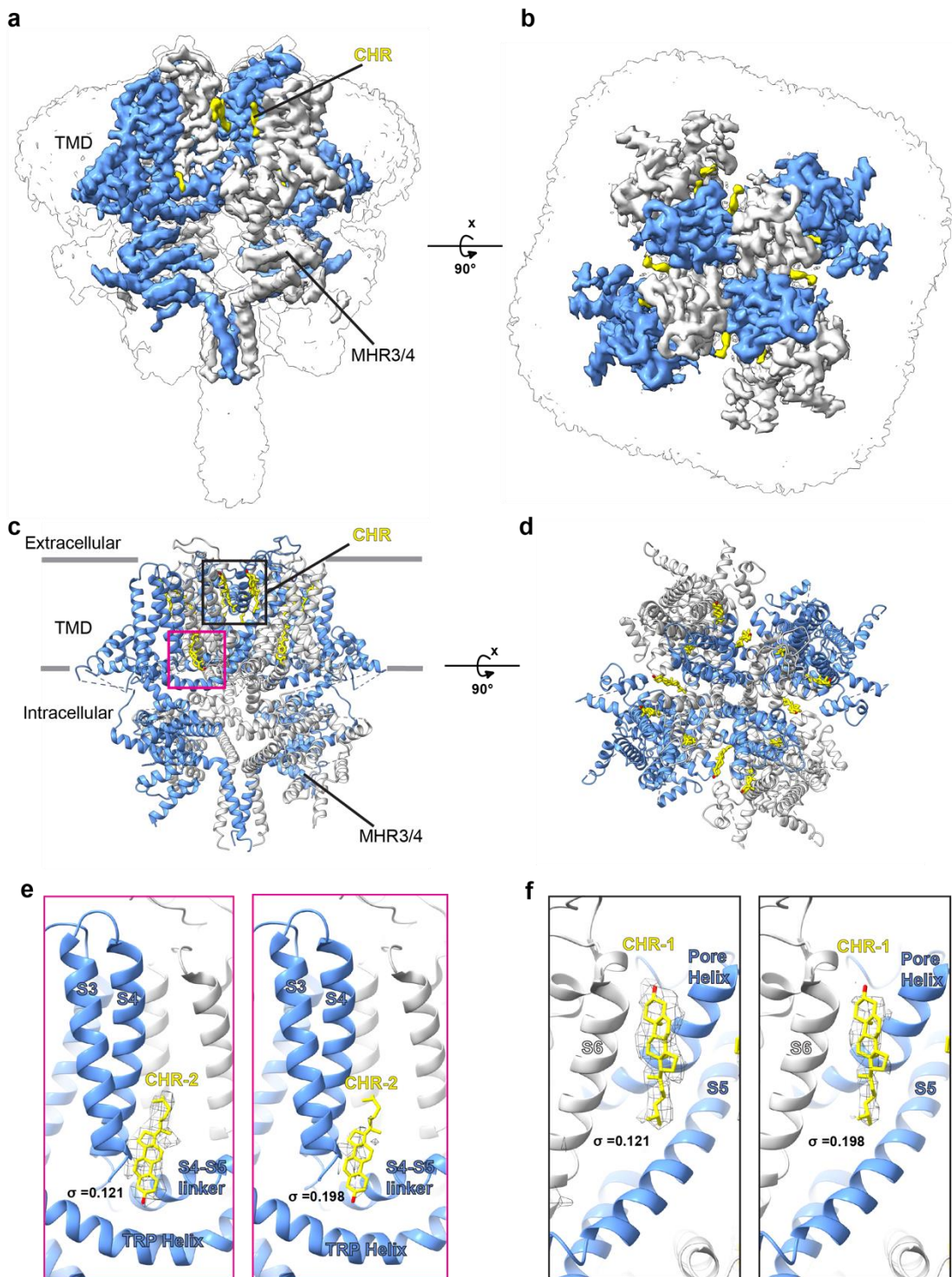
Data collection and processing	HsTRPM4+Ca ²⁺ EMD-19072	HsTRPM4+NBA+Ca ²⁺ EMD-19073	MmTRPM4+Ca ²⁺ EMD-19074
Nominal Magnification	96kx	96kx	165kx
Voltage (kV)	300	300	300
Recorded Micrographs	11 877	10 219	8 313
Electron exposure (e ⁻ /Å ²)	40	40	60
Defocus range (µm)	0.8-2.5	0.8-2.5	0.8-2.5
Pixel size (Å)	0.83	0.83	0.726
Symmetry imposed	C4	C4	C4
Initial particle images (no.)	4 430 540	2 774 028	4 340 237
Final particle images (no.)	128 316	40 061	100 227
Map resolution (Å)	3.04	2.98	2.87
FSC threshold	0.143	0.143	0.143
Map resolution range (Å)	30-2.6	30-2.5	30-2.5

265

266 **Supplementary Table 2 | Cryo-EM data collection for DDM/CHS solubilized HsTRPM4**
267 **and MmTRPM4 samples.**

268

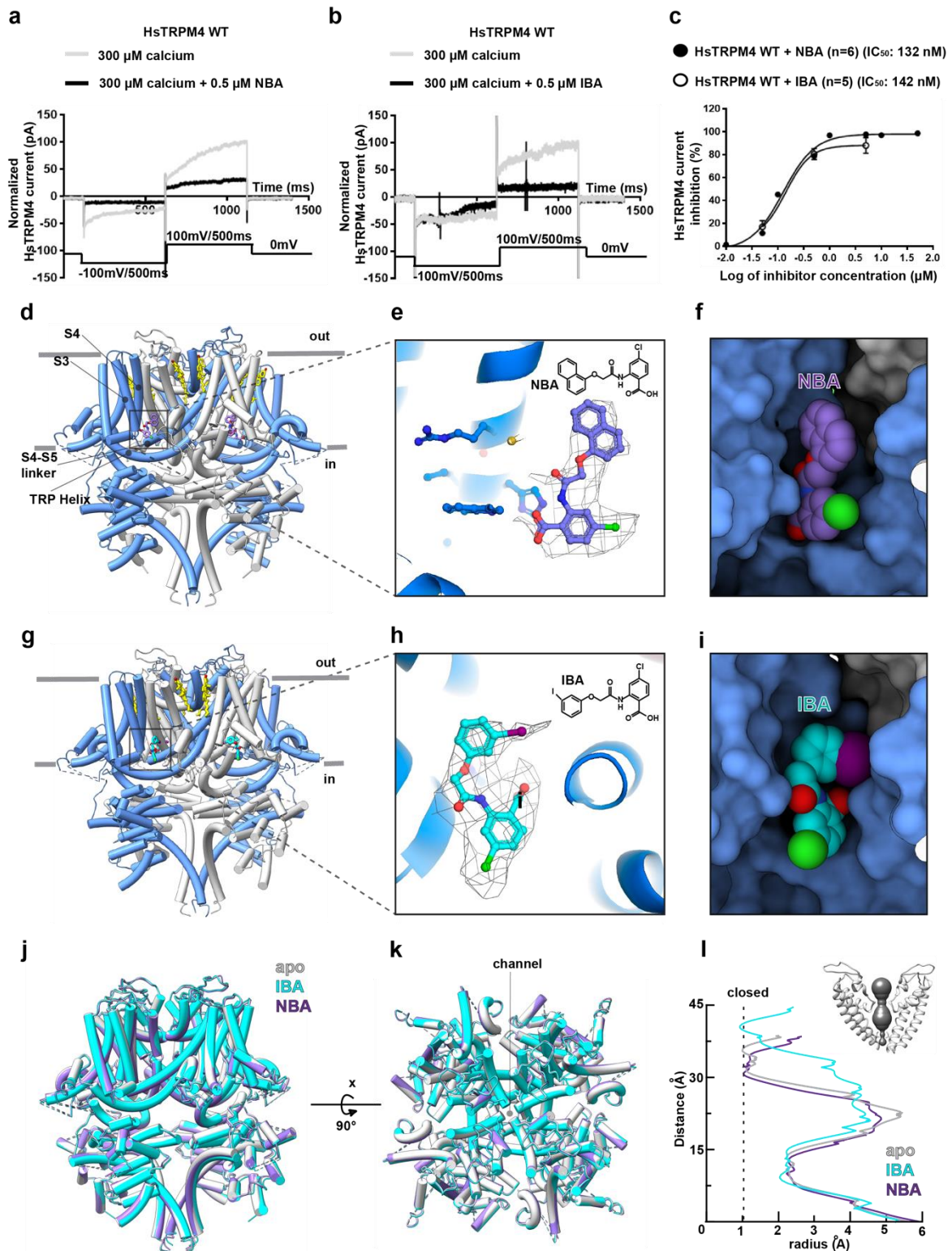
269



270

271 **Figure 1:** Overall structure of HsTRPM4_{apo} in native lipid nanodiscs. (a and b) Cryo-EM
 272 densities in surface representation of HsTRPM4_{apo} in two views. Alternating protomers
 273 of TRPM4 are shown in blue and white. (c and d) Cartoon representation of the
 274 HsTRPM4_{apo} corresponding to the cryo-EM densities in a and b. The positions of

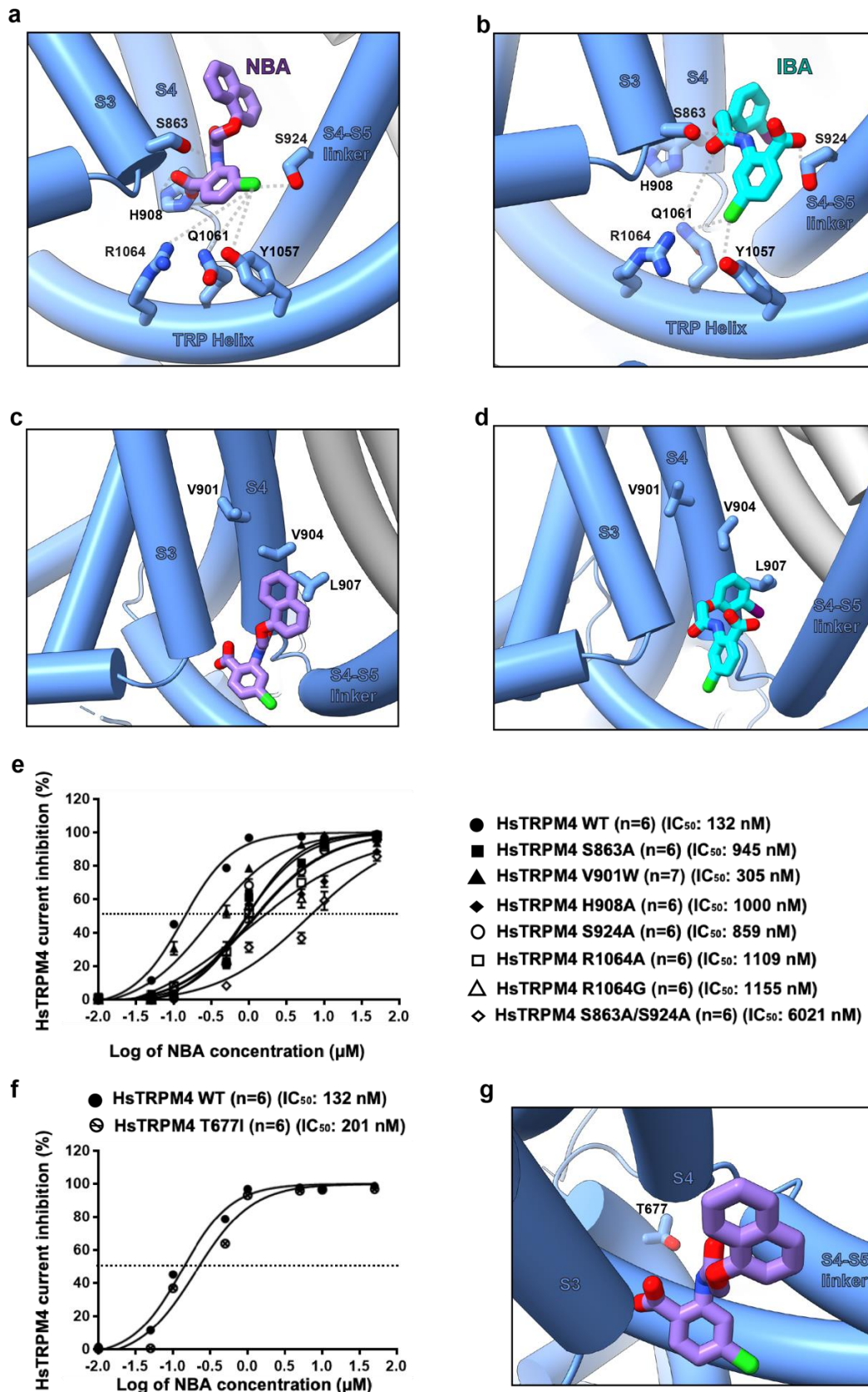
275 bound cholesterol molecules (CHR) are indicated. The black box indicates the
276 position of CHR-1 and CHR-3 shown in yellow. In contrast, the pink box indicates the
277 position of CHR-2. (e) Cryo-EM density in mesh representation for CHR-2 (f) Cryo-
278 EM density in mesh representation for CHR-1. In e and f, the density threshold level
279 is indicated by σ .
280



281

282 **Figure 2:** Structures of HsTRPM4_{NBA} and HsTRPM4_{IBA}. (a) Representative sodium
283 calcium-activated HsTRPM4 current traces of wildtype (WT) HsTRPM4 in the
284 presence of 300 μM of calcium (grey line) and in the presence of both calcium and

285 0.5 μ M of NBA (black line). (b) In the presence of 300 μ M of calcium (grey line) and
286 both calcium (grey line) and 0.5 μ M of IBA (black line). (c) NBA and IBA dose-
287 response curves of wildtype (WT). (n): number of cells. (d) The structure of
288 HsTRPM4_{NBA} with NBA shown in purple stick representation in the binding site. (e)
289 Zoom-in of NBA with the cryo-EM density in mesh representation. (f) NBA in sphere
290 representation is shown in the HsTRPM4 binding pocket shown in surface
291 representation. (g) The structure of HsTRPM4_{IBA} with IBA shown in cyan stick
292 representation in the binding site. (h) Zoom-in of NBA with the cryo-EM density in
293 mesh representation. (i) IBA in sphere representation is shown in the HsTRPM4
294 binding pocket in surface representation. (j) Superimposition of HsTRPM4_{apo} in light
295 gray, HsTRPM4_{NBA} in purple and HsTRPM4_{IBA} in cyan. Two views of the structures
296 are shown. (k) Pore radius for HsTRPM4_{apo}, HsTRPM4_{NBA} and HsTRPM4_{IBA}, all
297 calculated using MOLE. In the top right corner is the pore-forming helices in
298 HsTRPM4_{apo}. The pore profile is shown as a space-filling model (grey).
299
300

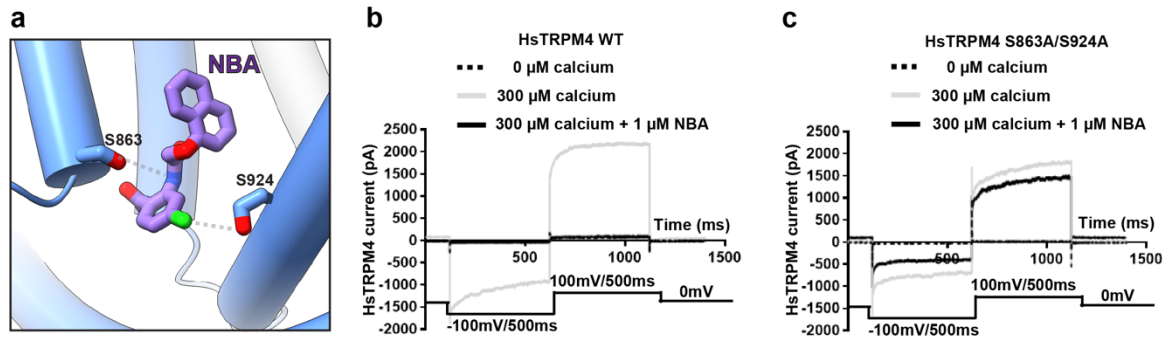


301

302 **Figure 3:** Amino acid interactions in the drug binding pocket. (a) Amino acid residues of

303 HsTRPM4 involved in interactions with the anthranilic acid moiety of NBA (in purple) and (b)

304 IBA (cyan) are shown. (c) Amino acid residues of HsTRPM4 involved in hydrophobic
305 interactions with NBA (in purple) and (d) IBA (in cyan) are shown. (e- h) Effect of HsTRPM4
306 variants on NBA inhibition. (e) NBA dose-response curves of wildtype (WT) and a few
307 variants of the predicted binding pocket of HsTRPM4 channel generated in this study. (n):
308 number of cells. (f and g) NBA dose-response curves of wildtype (WT) and a T677I variant
309 outside the drug binding pocket of the HsTRPM4 channel. (n): number of cells.
310
311



312

313 **Figure 4:** A double mutation in the drug-binding pocket alleviates inhibition. (a) Locations of

314 S863 and S924 in the drug binding pocket (b and c) Representative traces of wildtype (WT)

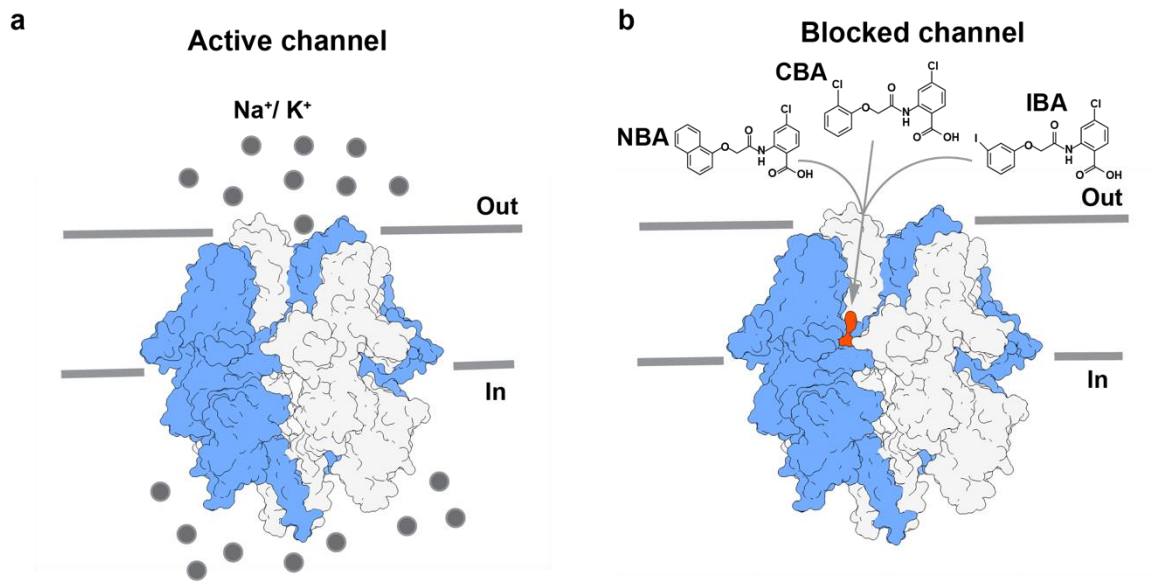
315 and variant HsTRPM4 currents: S863A/S924A in the absence of calcium (dotted black line),

316 in the presence of calcium (grey line) and in the presence of calcium and 1 μM of NBA (black

317 line).

318

319



320

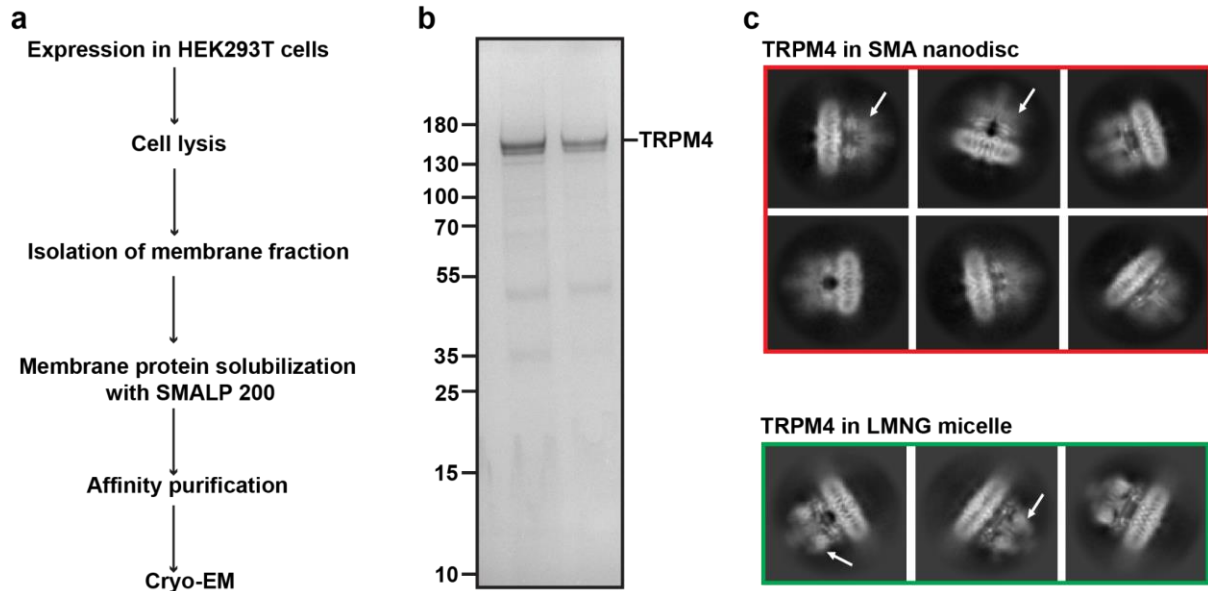
321 **Figure 5:** The binding of anthranilic acid drugs such as NBA, CBA and IBA in the TRPPM4

322 drug binding site leads to channel blocking, as shown in a and b.

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328 **Supplementary Fig. 1:** Purification of TRPM4 in SMA-extracted native lipid nanodiscs. (a)

329 Biochemical workflow for the isolation of and purification of HsTRPM4 in native lipid

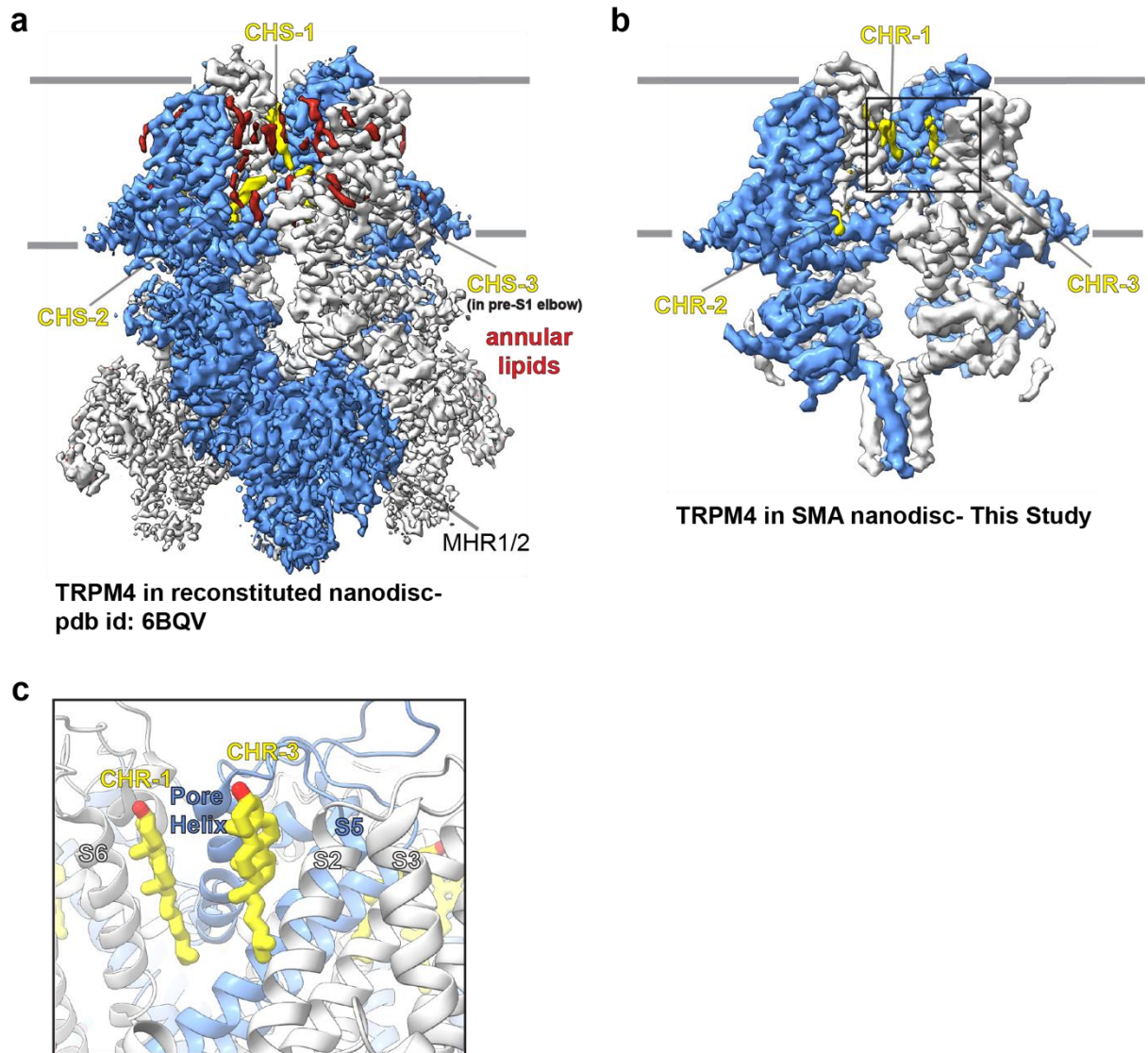
330 nanodiscs using SMA (SMALP-200). B) SDS-PAGE gel of purified HsTRPM4 following

331 affinity purification. (c) Cryo-EM 2D class averages of TRPM4 in SMA-extracted native lipid

332 nanodiscs and in LMNG detergent micelles. White arrows indicate the positions of MHR3/4

333 in the TRPM4 2D classes.

334



335

336 **Supplementary Fig. 2:** Comparison of the annular lipid environment between the cryo-EM

337 maps of HsTRPM4 in reconstituted lipid nanodiscs ²⁶ (a) and in SMA-extracted native lipid

338 nanodiscs (b) CHS represents cholesteryl hemisuccinate in (a) and CHR represents

339 cholesterol in (b). (c) Location of CHR-1 and CHR-3 is shown from the cryo-EM structure of

340 HsTRPM4 in native lipid nanodiscs.

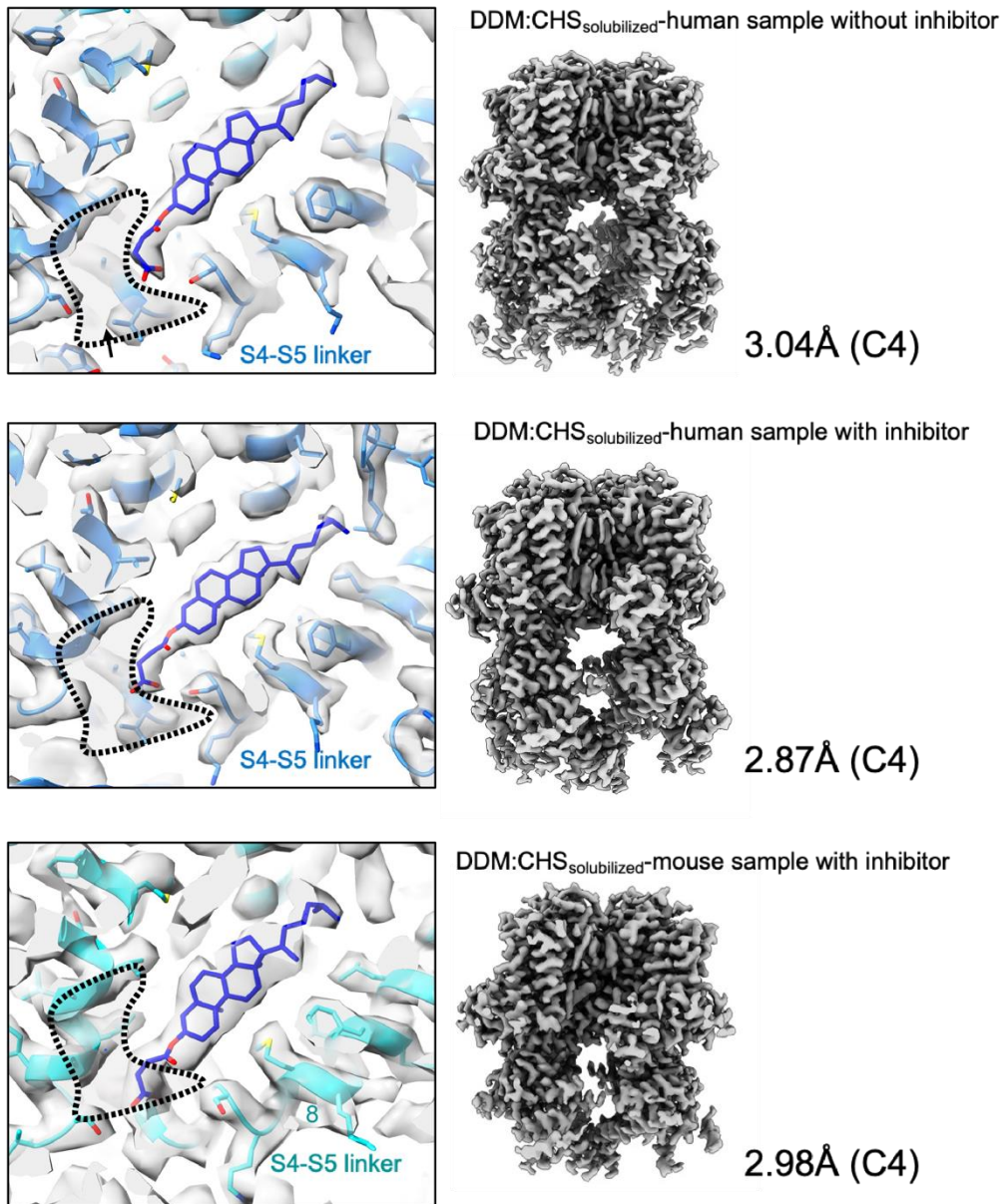
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CHR/IBA/NBA binding site (DDM:CHS_{solubilized} samples) vs pdb:6BQV

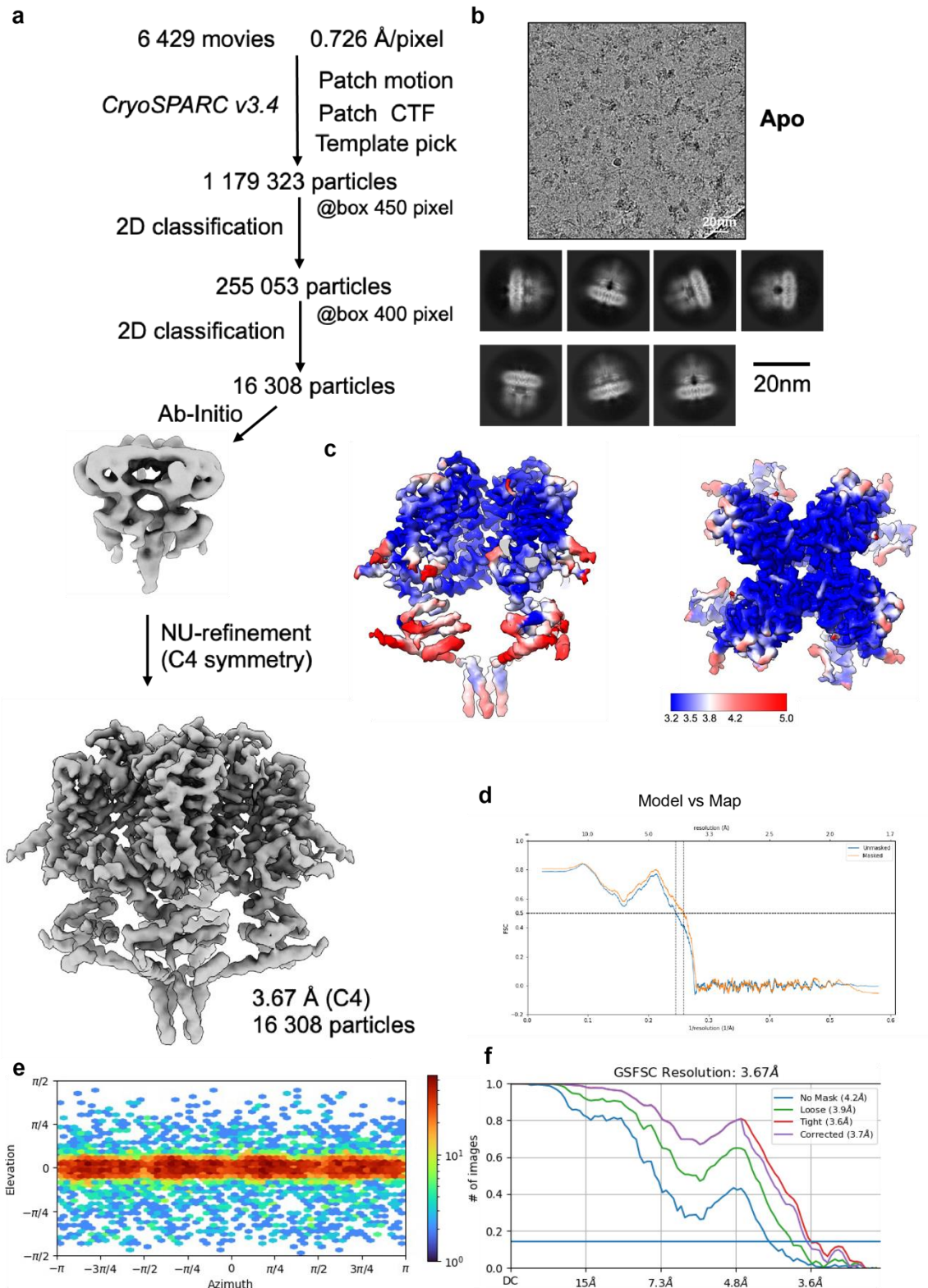


345

346 **Supplementary Fig. 3:** CryoEM maps of detergent-solubilized TRPM4 generated in this
347 study highlighting the presence of CHS in the drug binding site when fitted with the published
348 model (pdb-id; 6BQV)

349

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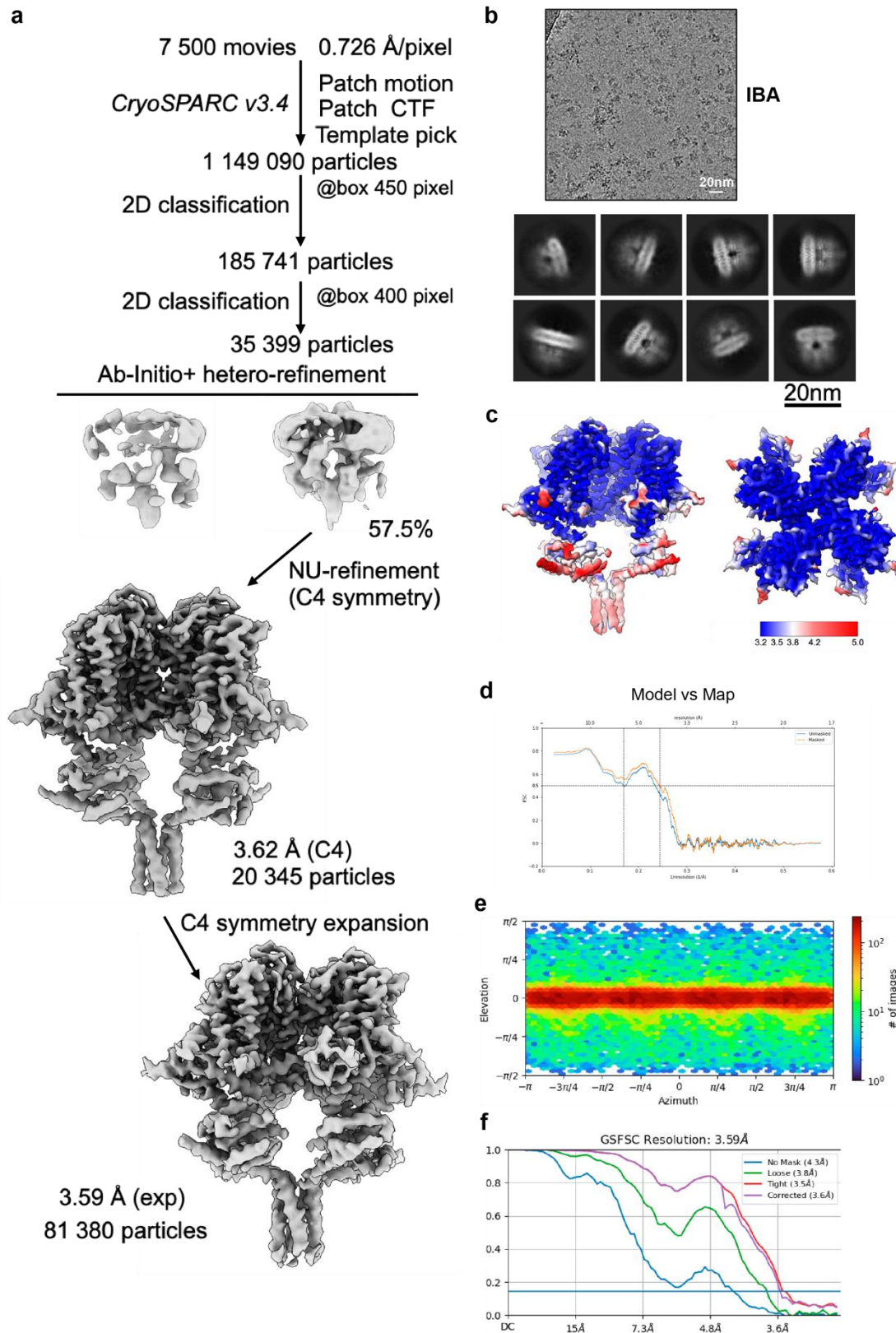


351

352 **Supplementary Fig. 4:** Cryo-EM data processing workflow and map resolution (a) The

353 image processing workflow of HsTRPM4_{apo}. (b) micrograph and 2D classes (c) Local

354 resolution (d) Model vs map FSC curves. (e) particle direction distribution. (f) FSC curves.

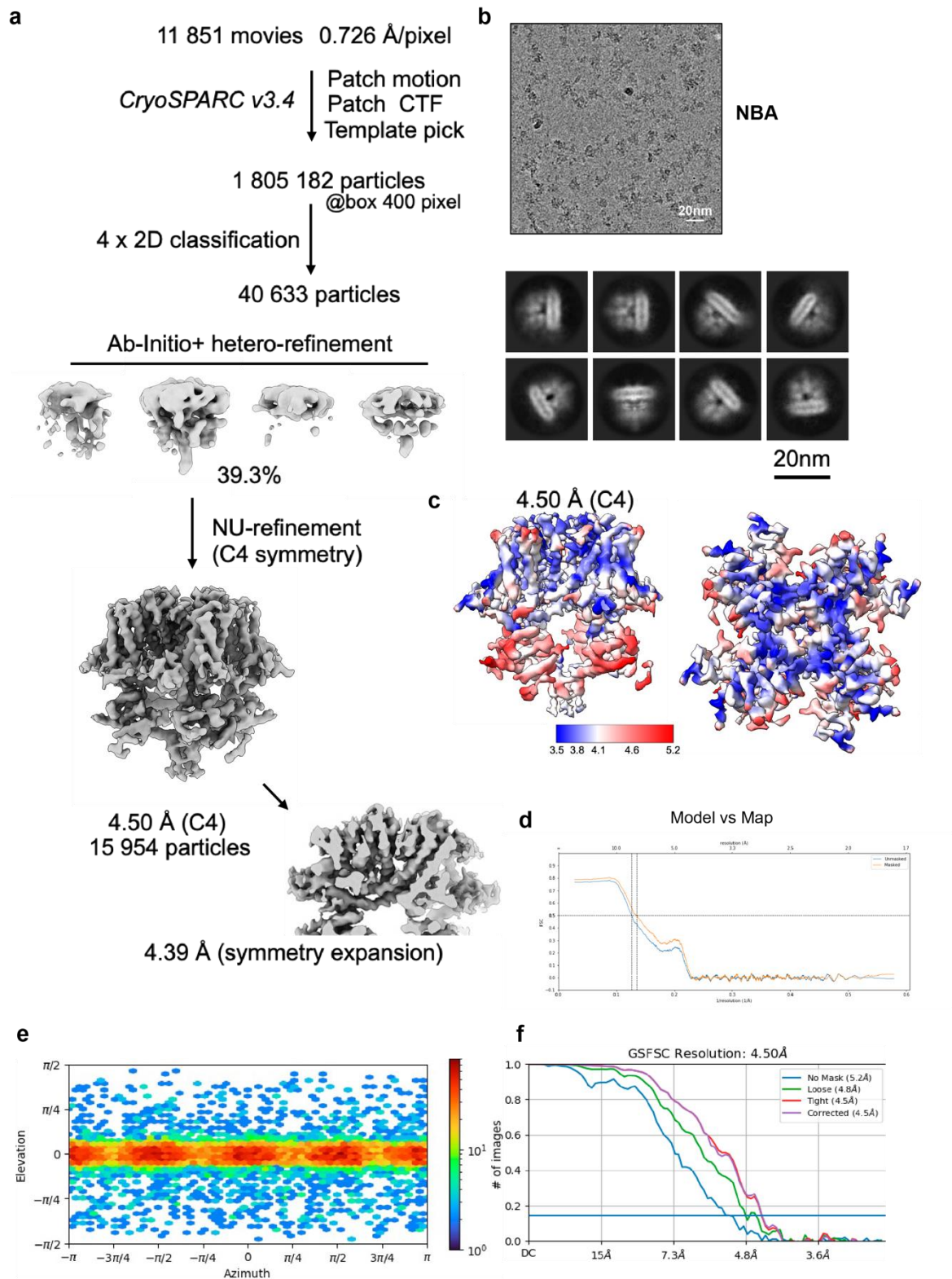


355

356 **Supplementary Fig. 5:** Cryo-EM data processing workflow and 3D reconstructions (a) The

357 image processing workflow of HsTRPM4_{IBA}. (b) Micrograph and 2D classes. (c) Local

358 resolution. (d) Model vs map FSC curves. (e) particle direction distribution. (f) FSC curves.

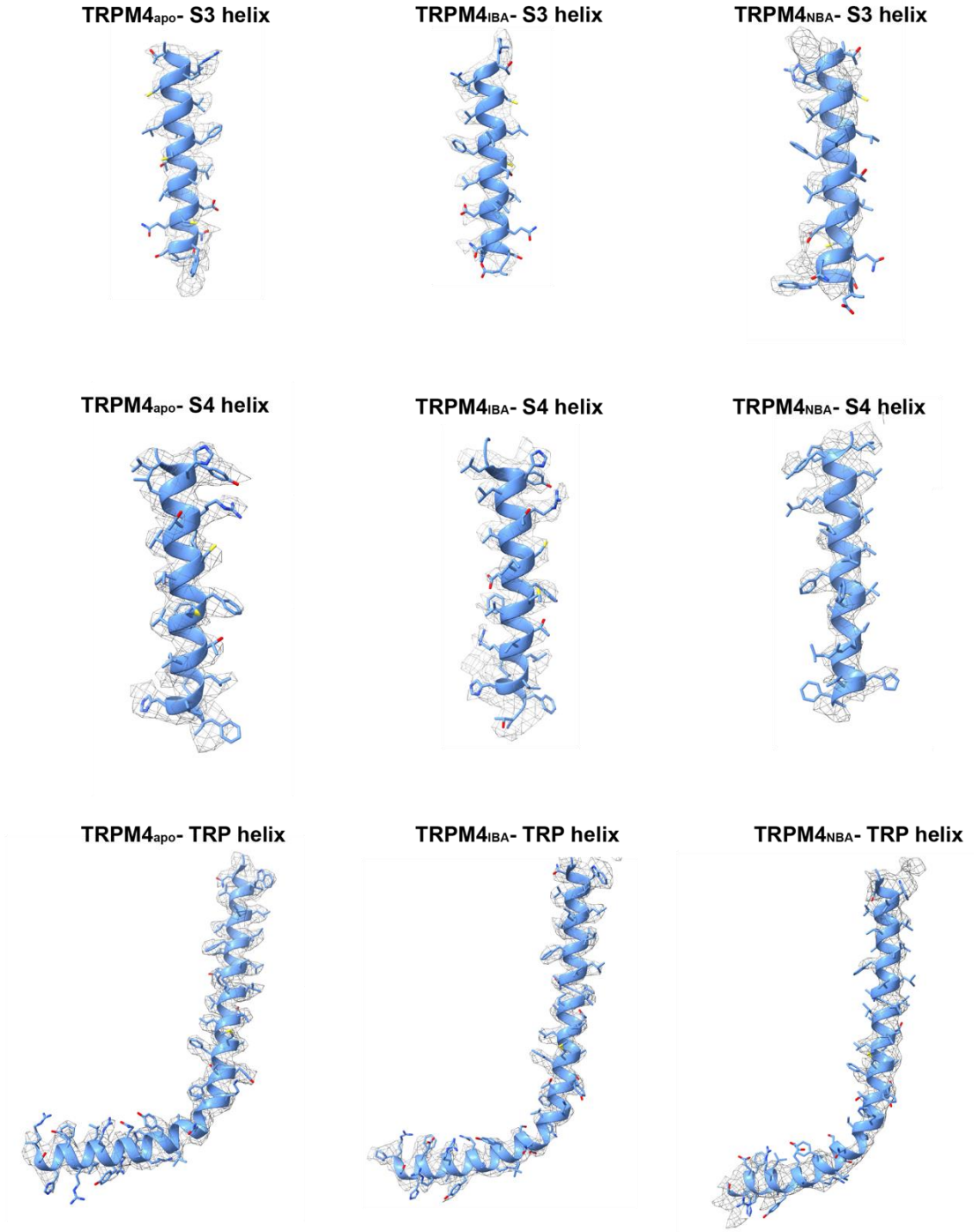


359

360 **Supplementary Fig. 6:** Cryo-EM data processing workflow and 3D reconstructions. (a) The

361 image processing workflow of HsTRPM4_{NBA}. (b) Micrograph and 2D classes. (c) Local

362 resolution. (d) Model vs map FSC curves. (e) particle direction distribution. (f) FSC curves.



363

364 **Supplementary Fig. 7:** Zoom-in view of the cryo-EM maps of structural elements

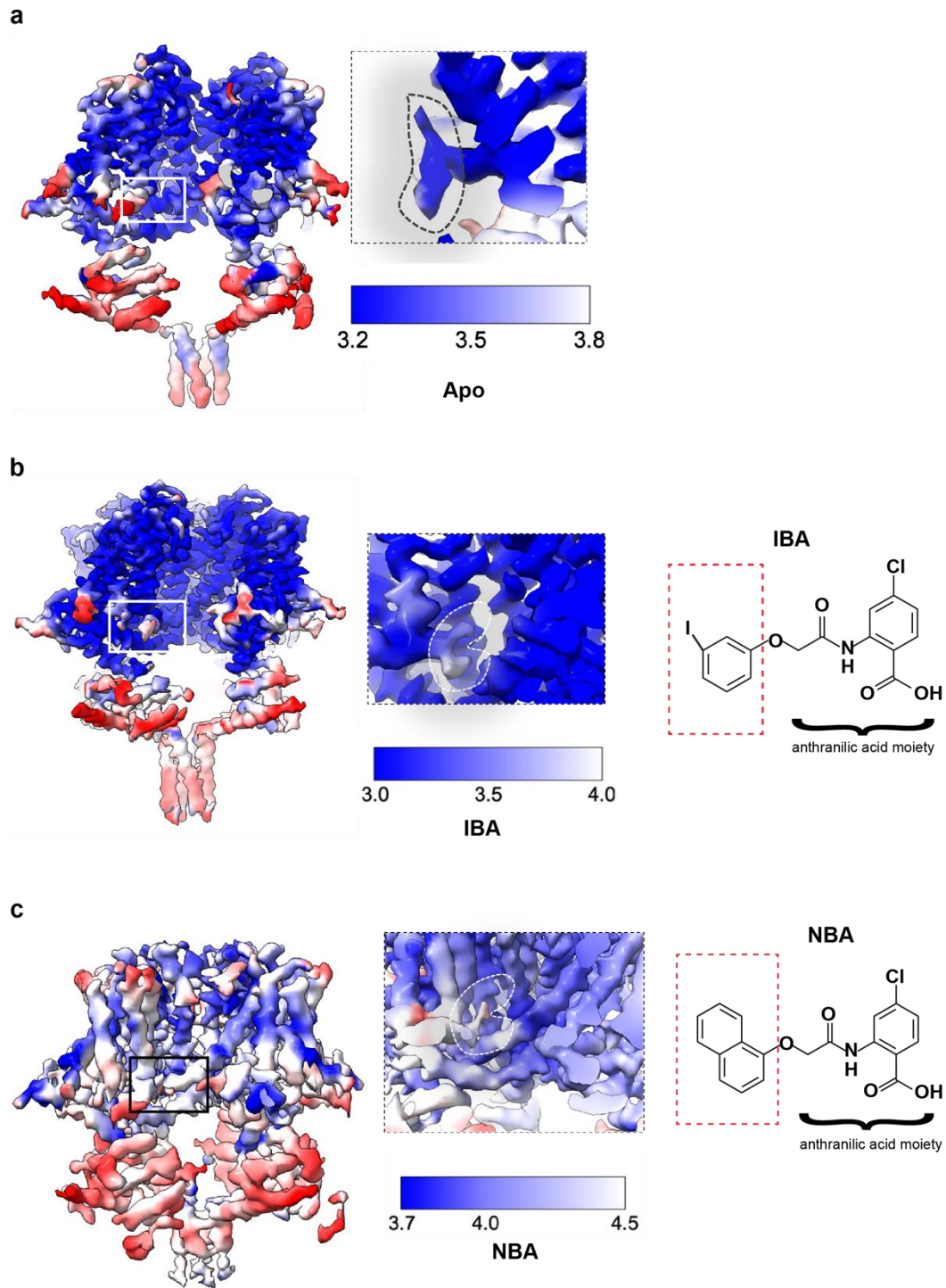
365 HsTRPM4_{apo}, HsTRPM4_{IBA} and HsTRPM4_{NBA}.

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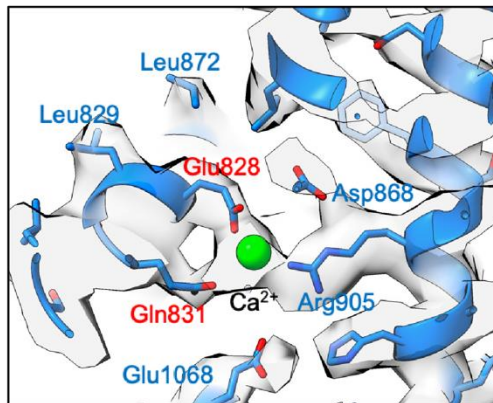


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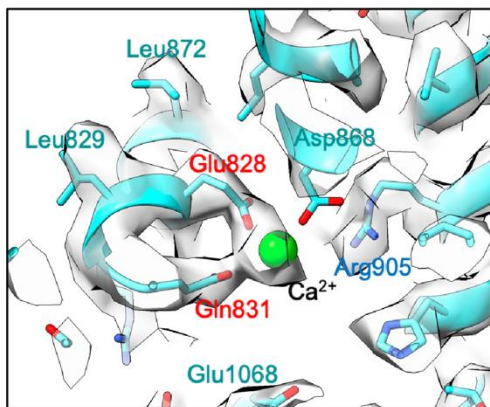
371 **Supplementary Fig. 8:** Local resolution maps of SMA solubilized HsTRPM4 generated in this
372 study. (a) Map for the HsTRPM4_{apo} with the density contained in the drug binding site
373 highlighted. (b) Map for HsTRPM4_{IBA} with the density contained in the drug binding site
374 highlighted. The chemical structure of IBA is shown on the right. (c) Map for HsTRPM4_{NBA} with

375 the density contained in the drug binding site highlighted. The chemical structure of NBA is
376 shown on the right.
377

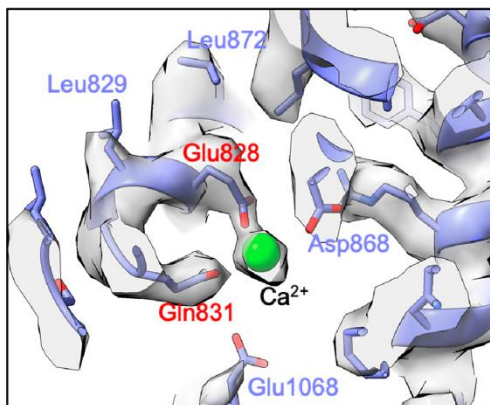
SMA_{solubilized}-human sample without extra Ca²⁺(CHS free)



DDM:CHS_{solubilized}-mouse sample + Ca²⁺

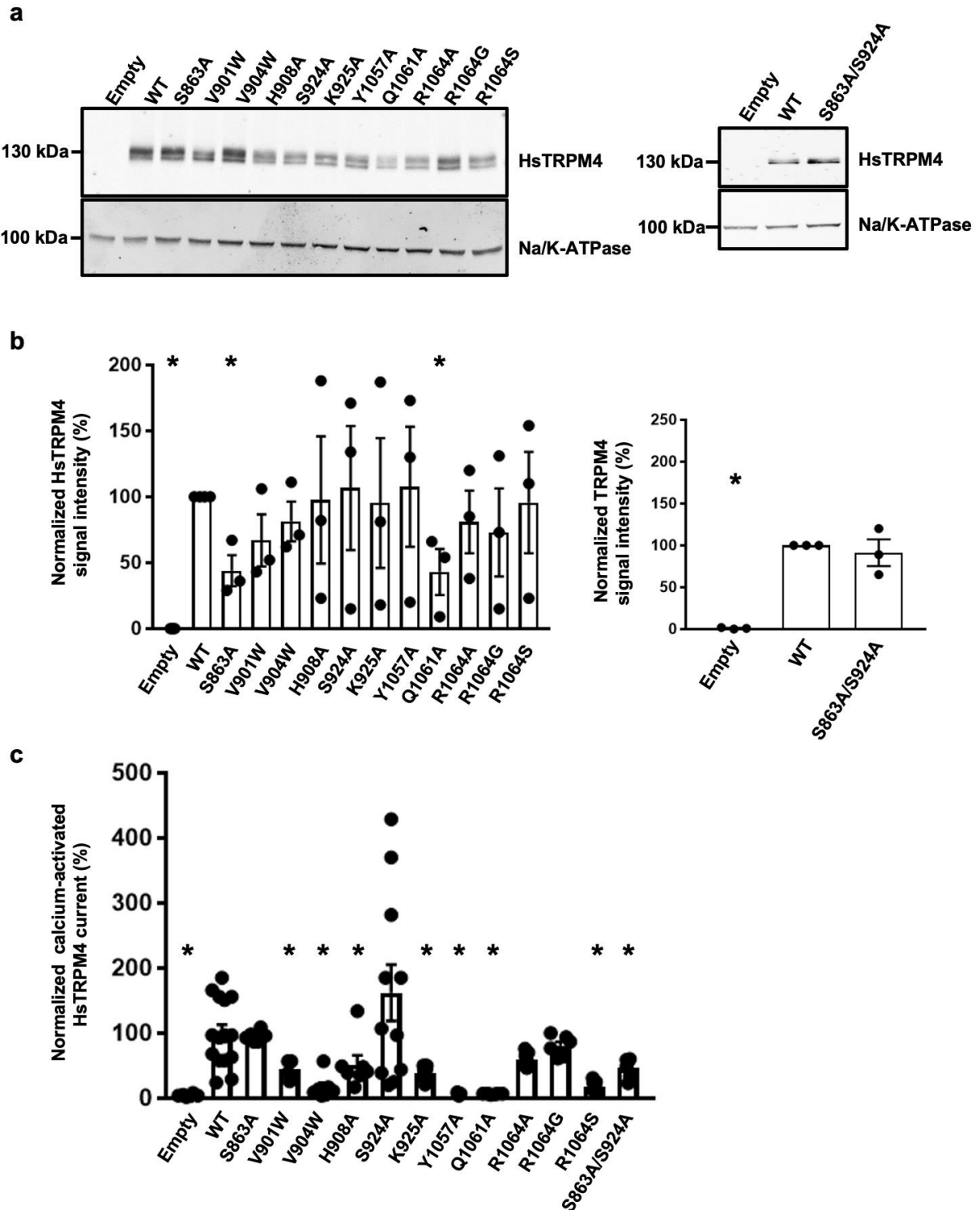


DDM:CHS_{solubilized}-human sample + Ca²⁺



378

379 **Supplementary Fig. 9:** Identification of cryo-EM densities for Ca²⁺ ions in the maps generated
380 in this study. The published HsTRPM4 model (pdb-id: 6BQV)²⁶ is fitted into each map. Clear
381 density is observed for Ca²⁺ in the map for SMA solubilized human TRPM4 sample without
382 addition of extra calcium which is comparable to the density shown for detergent solubilized
383 mouse and human TRPM4 with calcium added to the sample.



384

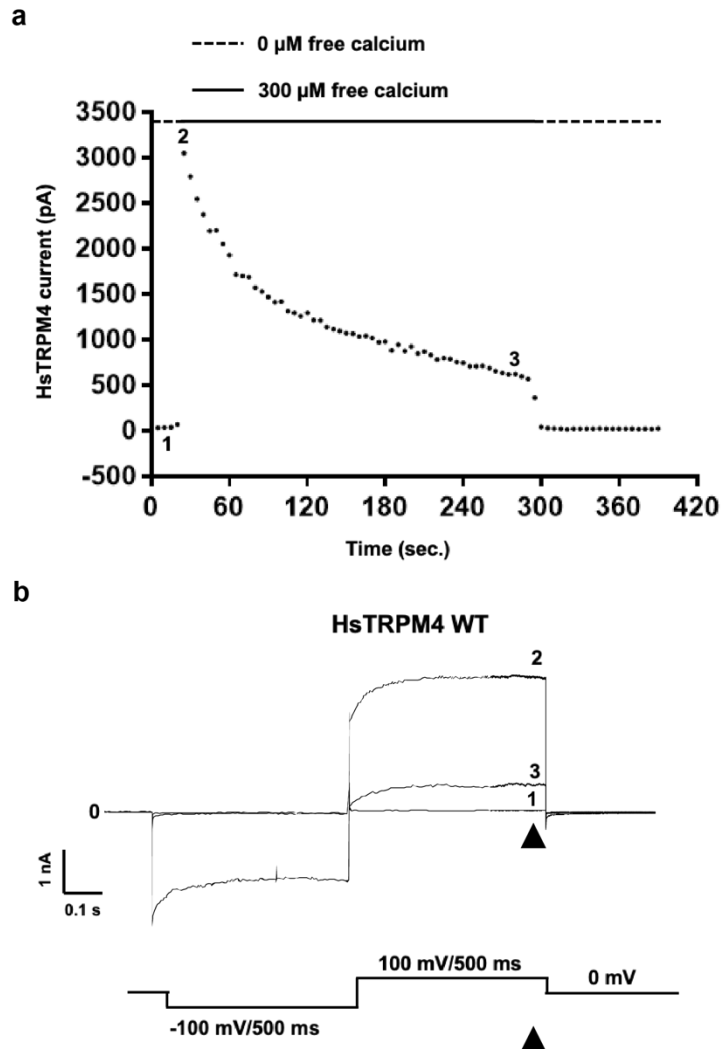
385 **Supplementary Fig. 10:** Expression of HsTRPM4 variants. (a) Western blot showing the

386 expression of wildtype (WT) and HsTRPM4 variants expressed in HEK293 cells. (b)

387 Graphical plot of relative western blot intensities between wildtype (WT) and HsTRPM4

388 variants. (*) represents the p-value < 0.05 compared to the wildtype condition. Experiments

389 have been done in triplicate. (c) Graphical plot of normalized recorded calcium-activated
390 TRPM4 currents for the wildtype (WT) and HsTRPM4 variants. (*) represents the p-value <
391 0.05 compared to the wildtype condition. At least 5 cells per condition have been recorded.
392
393



394

395 **Supplementary Fig. 11:** Time course of wildtype sodium calcium-activated HsTRPM4

396 current. (a) Example of a time course of sodium calcium-activated HsTRPM4 wildtype (WT)

397 current. Each point corresponds to the amplitude of the current measured at the end of the

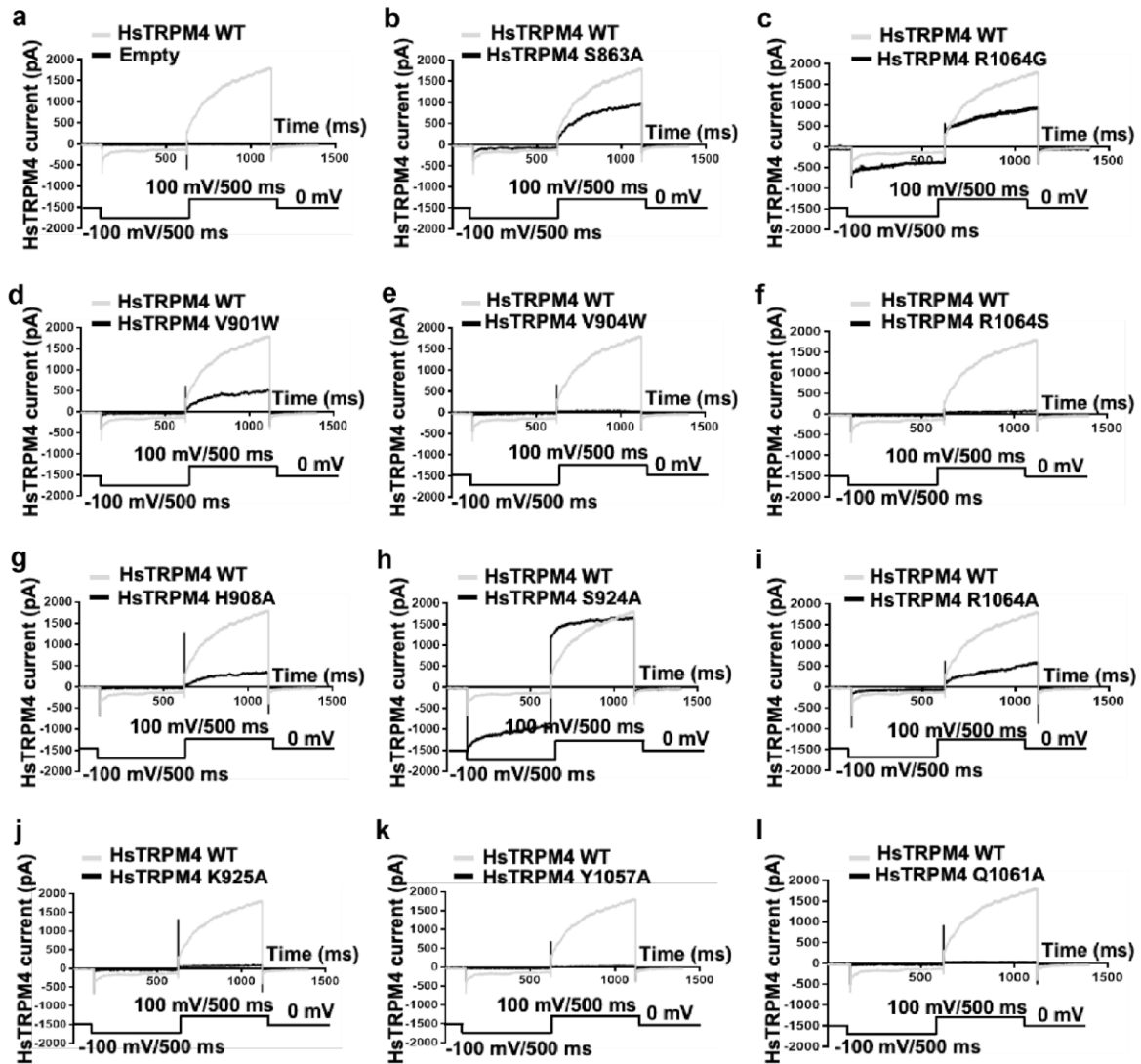
398 second pulse of the protocol shown in (b) (black triangles). (b) Currents from three different

399 time points 1, 2, and 3 from the time course shown in (a).

400

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404 **Supplementary Fig. 12:** Sodium calcium-activated HsTRPM4 currents. (a to f)

405 Representative traces of wildtype (WT) and variants HsTRPM4 currents: S863A, V901W,

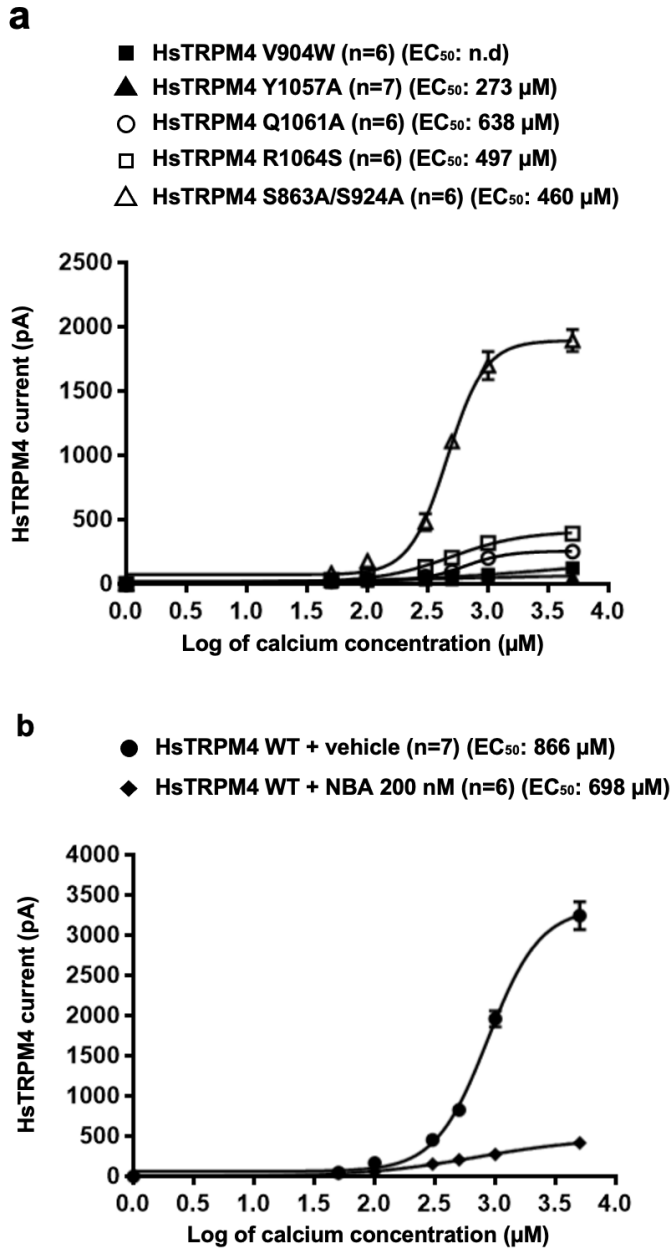
406 V904W, H908A, and S924A. (g to l) Representative traces of wildtype (WT) and variants

407 HsTRPM4 currents: K925A, Y1057A, Q1061A, R1064A, R1064G, and R1064S.

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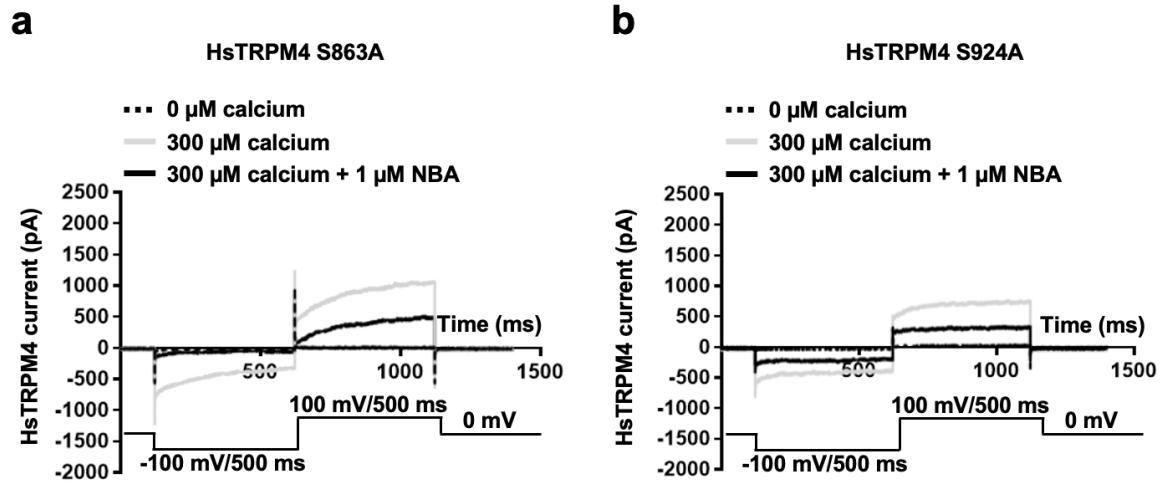
412 **Supplementary Fig. 13:** Calcium sensitivity curves of wildtype and variant HsTRPM4. (a)

413 Calcium sensitivity curves of a few loss-of-function HsTRPM4 variants. (n): number of cells.

414 n.d: not determinable. (b) Effect of NBA (200 nM) on the calcium sensitivity on wildtype (WT)

415 HsTRPM4 channels.

416



417

418 **Supplementary Fig. 14:** Sodium calcium-activated HsTRPM4 currents. (a and b)

419 Representative traces of variants HsTRPM4 current: S863A and S924A in the absence of
420 calcium (dotted black line), in the presence of calcium (grey line) and in the presence of
421 calcium and 1 μM of NBA (black line).

422

423

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491 TRPM8 channel. *Science* **363**, (2019).
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495 **Methods**

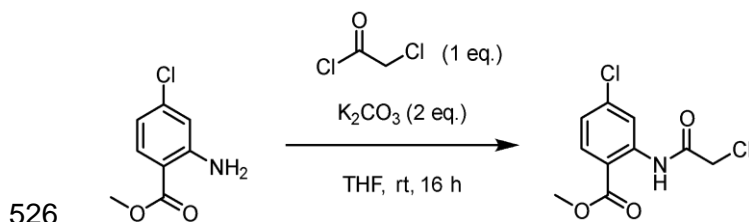
496 **Synthesis of 4-Chloro-2-(2-(3-iodophenoxy)acetamido)benzoic acid (IBA)**

497 *General Remarks*

498 Reagents and organic solvents were purchased from commercial suppliers and used without
499 further purification. Deionized water produced in house or commercially available Milli-Q®
500 water was used depending on the application. Aqueous solutions of sodium hydroxide,
501 hydrogen chloride, saturated ammonium chloride, saturated sodium chloride (brine) were
502 prepared with deionized water. Thin layer chromatography (TLC) was performed using
503 Macherey-Nagel ALUGRAM® Xtra SIL G/UV₂₅₄ plates coated with 0.20 mm silica gel 60
504 containing fluorescent indicator. High pressure liquid chromatography (HPLC) was
505 performed using a Thermo Fisher Scientific UltiMate 3000 RSLCnano System composed of
506 a DIONEX UltiMate 3000 Pump, a DIONEX UltiMate 3000 Sampler, a DIONEX UltiMate
507 3000 Column Compartment and a DIONEX UltiMate 3000 Diode Array Detector. HPLC
508 measurements were conducted using Milli-Q® water (+ 0.1 % TFA) and acetonitrile (+ 0.1 %
509 TFA) as eluents and an Acclaim™ 120 C18 column (Thermo Scientific™). Flash column
510 chromatography (LC) was performed using the Teledyne Isco CombiFlash®Rf+ system.
511 Teledyne Isco RediSep®Rf dry load cartridges were used for the preparation of dry loads. If
512 not stated otherwise, dry loads were prepared on silica gel. Teledyne Isco Silica RediSep®Rf
513 prepacked silica flash columns of two sizes (24g and 80g) were used. Nuclear magnetic
514 resonance spectroscopy (NMR) was performed at the Departement of Chemistry,
515 Biochemistry and Pharmaceutical Sciences, Universität Bern (Furrer Group) using a Bruker
516 AVANCE III HD 300 GA spectrometer with a magnetic field of 7.05 Tesla and operating
517 frequencies of 300.13 MHz for ¹H measurements and 75.48 MHz for ¹³C measurements.
518 High resolution mass spectrometry (HRMS) was performed by the mass spectrometry
519 service (Schürch group) at the Departement of Chemistry, Biochemistry and Pharmaceutical
520 Sciences, Universität Bern. The measurements were performed using electrospray
521 ionization (ESI) and a ThermoScientific LTQ Orbitrap XL mass spectrometer with high mass
522 resolution ($m/\Delta m > 100'000$) and accuracy ($\Delta m < 3\text{ppm}$). The previously published synthetic

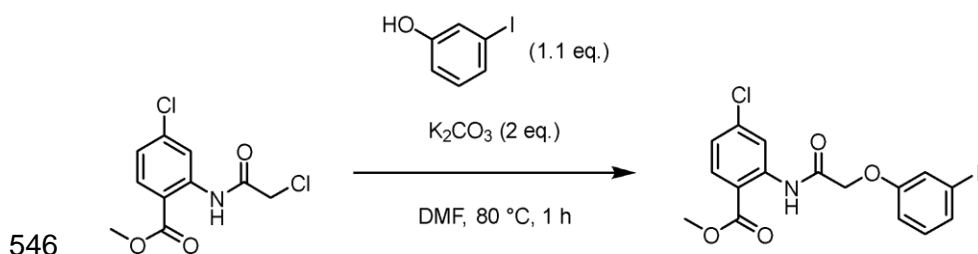
523 procedure (Ozhathil *et al.*, 2018) to generate similar anthranilic anilide compounds was
524 slightly adapted.

525 *Methyl 4-chloro-2-(2-chloroacetamido) benzoate*



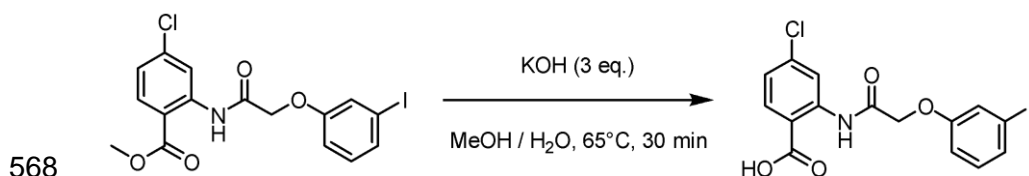
527 Methyl 2-amino-4-chlorobenzoate (1.7876 g, 9.6308 mmol) and potassium carbonate
528 (2.6620 g, 19.2616 mmol, 2 eq.) were dissolved in tetrahydrofuran (150 mL) and stirred for
529 10 minutes at room temperature. Not all potassium carbonate dissolved completely. The
530 mixture was then cooled in an ice bath and chloroacetyl chloride (1.0919 g, 9.6676 mmol,
531 0.77 mL, 1 eq.) was added dropwise via syringe. This mixture was stirred at 0 °C for 10
532 minutes and further stirred at room temperature for 16 hours. The reaction mixture showed a
533 pale pink color and was monitored by thin layer chromatography (eluent: cyclohexane / ethyl
534 acetate, 4:1). After full conversion of the starting material, water was added to the reaction
535 mixture and the product was extracted with ethyl acetate. The combined organic phases
536 were then washed with brine, dried over magnesium sulphate, filtered through celite and the
537 volatiles were evaporated under reduced pressure. The crude pale-yellow and solid product
538 was purified by flash column chromatography (eluent: cyclohexane / ethyl acetate, gradient
539 from 0 % to 20 % ethyl acetate). The product (white powder) was dried *in vacuo*. Yield
540 Methyl 4-chloro-2-(2-chloroacetamido) benzoate quant., 2.5 g, 9.5387 mmol. ¹H NMR (300
541 MHz, DMSO-*d*₆) δ 11.44 (s, 1H), 8.52 (d, *J* = 2.2 Hz, 1H), 8.00 (d, *J* = 8.6 Hz, 1H), 7.33 (dd,
542 *J* = 8.6, 2.2 Hz, 1H), 4.48 (s, 2H), 3.89 (s, 3H). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 166.75,
543 165.67, 140.29, 138.75, 132.51, 123.77, 119.84, 115.57, 52.82, 43.35. HRMS (ESI) *m/z*
544 [M+H]⁺ calculated for C₁₀H₉Cl₂NO₃ 262.0032, found 262.0037.

545 *Methyl 4-chloro-2-(2-(3-iodophenoxy)acetamido)benzoate*



Methyl 4-chloro-2-(2-chloroacetamido) benzoate (0.3270 g, 1.2475 mmol) was dissolved in dimethylformamide (6 mL) and potassium carbonate (0.3465 g, 2.5073 mmol, 2 eq.) was added. This mixture was stirred for 10 minutes at room temperature. Not all potassium carbonate dissolved completely. 3-Iodophenol (0.3019 g, 1.3723 mmol, 1.1 eq.) was then dissolved in dimethylformamide (6 mL) and added via syringe to the reaction mixture which was stirred at 80 °C. The color of the reaction mixture changed to deep brown. The reaction was monitored by thin layer chromatography (eluent: cyclohexane / ethyl acetate, 4:1). Upon full conversion of the starting material after 60 minutes, water was added to the mixture, resulting in a brown and cloudy suspension. The product was extracted with ethyl acetate and the combined organic phases were then washed with brine, dried over magnesium sulphate, filtered through celite and the volatiles were evaporated under reduced pressure. The crude product was purified by flash column chromatography (eluent: cyclohexane / ethyl acetate, gradient from 0 % to 20 % ethyl acetate) and the purified product (white powder) was dried *in vacuo*. Yield Methyl 4-chloro-2-(2-(3-iodophenoxy)acetamido)benzoate 84 %, 0.4653 g, 1.0442 mmol. ¹H NMR (300 MHz, DMSO-*d*₆) δ 11.76 (s, 1H), 8.70 (d, *J* = 2.1 Hz, 1H), 8.01 (d, *J* = 8.6 Hz, 1H), 7.48 (t, *J* = 1.9 Hz, 1H), 7.40 (ddd, *J* = 6.4, 2.5, 1.6 Hz, 1H), 7.30 (dd, *J* = 8.6, 2.2 Hz, 1H), 7.15 – 7.09 (m, 2H), 4.79 (s, 2H), 3.91 (s, 3H). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 167.24, 166.87, 157.61, 140.57, 138.94, 132.56, 131.50, 130.66, 123.67, 123.34, 119.33, 114.75, 114.64, 95.04, 67.35, 52.76. HRMS (ESI) *m/z* [M+H]⁺ calculated for C₁₆H₁₃ClINO₄ 445.9651, found 445.9647.

4-Chloro-2-(2-(3-iodophenoxy)acetamido)benzoic acid (IBA)



569 Methyl 4-chloro-2-(2-(3-iodophenoxy)acetamido)benzoate (0.4531 g, 1.0168 mmol) was
570 dissolved in methanol (100 mL) and potassium hydroxide (0.1744 g, 3.1075 mmol, 3 eq.)
571 was dissolved in Milli-Q® water (20 mL). The aqueous potassium hydroxide solution was
572 added to the reaction mixture which was stirred at 65° C and progress monitored by thin
573 layer chromatography (eluent: dichloromethane / methanol, 9:1). Upon full conversion of the
574 starting material after 30 minutes, the reaction mixture was cooled down to room temperate.
575 Aqueous hydrochloric acid solution (1 M, ca. 100 mL) was then added, resulting in an
576 immediate precipitation of the product, which was filtered off using a glass filter frit (Por. 4)
577 and the solid white product was dried *in vacuo*. Yield 4-Chloro-2-(2-(3-
578 iodophenoxy)acetamido)benzoic acid 79 %, 0.3466 g, 0.8030 mmol. Purity (HPLC): 98.52 %
579 (at 254 nm). ¹H NMR (300 MHz, DMSO-*d*₆) δ 14.08 (s, 1H), 12.25 (s, 1H), 8.77 (d, *J* = 2.1
580 Hz, 1H), 8.03 (d, *J* = 8.6 Hz, 1H), 7.46 (m, 1H), 7.38 (dt, *J* = 7.0, 1.7 Hz, 1H), 7.27 (dd, *J* =
581 8.6, 2.2 Hz, 1H), 7.19 – 7.08 (m, 2H), 4.77 (s, 2H). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 168.78,
582 167.24, 157.58, 141.12, 138.69, 133.05, 131.48, 130.55, 123.74, 123.06, 118.78, 114.94,
583 114.29, 95.06, 67.21. HRMS (ESI) *m/z* [M-H]⁻ calculated for C₁₅H₁₁ClINO₄ 429.9349, found
584 429.9348.

585
586 **Description of drugs**

587 Stock solutions of 4-chloro-2-(2-(naphthalene-1-yloxy)acetamido)benzoic acid (NBA) and the
588 iodo-modified congener; 4-chloro-2-(2-(3-iodo-phenoxy)acetamino)benzoic acid (IBA) were
589 dissolved in 100% DMSO to a final concentration of 10 mM. IBA was synthesised to
590 increase the electron densities when using the Cryo-EM approaches. Although, as proof of
591 concept, only NBA has been used in the functional experiments presented in this study, the
592 inhibitory efficacy of IBA has been evaluated and is the same as of NBA. NBA and IBA
593 solutions were freshly made before each experiment. The lipophilicity of these drugs (cLog

594 *P*) has been calculated using the algorithms on Swiss ADME from the Swiss Institute of
595 Bioinformatics (<http://www.swissadme.ch/>). The Log *P* corresponds to the ratio of the
596 concentration of the compound at equilibrium between organic (octanol) and aqueous
597 phases. A negative Log *P* means the compound is hydrophilic, and a positive value for Log
598 *P* denotes a more lipophilic compound. At pH = 7.4, the NBA and IBA compounds (both
599 protonated (carboxylic acid) and deprotonated (carboxylate) forms) tend to be lipophilic. NBA
600 is slightly more lipophilic than IBA (consensus cLog *P* protonated/deprotonated: NBA:
601 3.59/3.32; IBA: 3.25/3.05).

602
603

604 **Plasmid constructs design and cloning**

605

606 For this study, the wild-type codon-optimized *H. sapiens* and *M. musculus* TRPM4 genes
607 coding full-length TRPM4 were synthesized, fused to consecutive C-terminal HA
608 (hemagglutinin)- and FLAG-tags and cloned into pCDNA-3.1 plasmid for expression in
609 HEK293 cells by the CMV (cytomegalovirus) promoter (GenScript Biotech). Mutant variants
610 were generated by site directed mutagenesis (GenScript Biotech).

611
612

613 **Protein expression and purification**

614 The full-length *H. sapiens* TRPM4 and *M. musculus* TRPM4 were expressed and purified
615 from HEK293F cells grown in suspension. For expression, HEK293F cells were transfected
616 with 1 mg of plasmid containing the TRPM4 gene per liter of cells using PEI
617 (Polyethylenimine). The cultures were grown at 37°C and 5% CO₂ for 48 hours. The cultures
618 were harvested by centrifugation at 3,000 xg for 30 mins at 4°C washed in 1x PBS followed
619 by another round of centrifugation. The pellets were carefully resuspended in lysis buffer
620 containing HEPES-NaOH, pH 7.5, 200 mM NaCl and supplemented with cComplete™ EDTA-
621 free Protease Inhibitor Cocktail (Roche). 4 tablets of protease inhibitor cocktail were added
622 per 100ml of buffer. Following resuspension, the cells were lysed by sonication for a total of
623 1 min in 10 sec On and OFF cycles and an amplitude of 35%. After sonication the

624 membrane fraction was harvested by centrifugation using a Optima XPN-100 ultracentrifuge
625 (Beckman Coulter) with the Ti45 rotor and spun at $70,560 \times g$ for 30mins at 4°C . The
626 resulting pellets were stored at -80°C . For solubilization of the membrane fraction with
627 SMALP200, 12 g of pellet was resuspended in 30 ml of solubilization buffer containing
628 HEPES-NaOH, pH 7.5, 200 mM NaCl and 0.5% SMALP200 (CubeBiotech GmbH)
629 supplemented with 2 tablets of cOmplete™ EDTA-free Protease Inhibitor Cocktail (Roche).
630 The resuspended pellet was homogenized manually in a 40 ml Kimble glass homogenizer
631 (Sigma). The tube containing the mixture was then placed in a bottle with a stir bar and left
632 to incubate at 4°C for 1 hour 30 mins. The homogenate was clarified by centrifugation for 30
633 min at $70,560 \times g$ at 4°C in an Optima XPN Ultracentrifuge (Beckman Coulter) using a Ti-45
634 rotor. The supernatant, which contained soluble FLAG-tagged HsTRPM4 mixed with 1 ml of
635 Anti-FLAG® M2 affinity gel (Millipore, Billerica, MA) pre-equilibrated with wash buffer
636 containing 25 mM HEPES-NaOH, pH 7.5, 200 mM NaCl. The beads were washed with 100
637 mL of wash buffer containing 25 mM HEPES-NaOH, pH 7.5, 200 mM NaCl and eluted with 4
638 mL elution buffer containing 25 mM HEPES-NaOH, pH 7.5, 200 mM NaCl, 120 $\mu\text{g}/\text{ml}$ of
639 3xFLAG peptide, followed by concentration on a 100 K Amicon Ultra-15
640 concentrators (Millipore, Billerica, MA) to an absorbance at 280 nm of 1.0 to prepare
641 cryo-EM grids.

642 For detergent solubilization, 12 g of pellet was resuspended in 30 ml of solubilization
643 buffer containing HEPES-NaOH, pH 7.5, 200 mM NaCl, 1% n-Dodecyl-beta-
644 Maltoside (DDM) and 0.1% Cholesteryl Hemissuccinate (CHS)
645 (Avanti Pola lipids) supplemented with 2 tablets of cOmplete™ EDTA-free Protease
646 Inhibitor Cocktail (Roche). The resuspended pellet was homogenized manually in a
647 40 ml Kimble glass homogenizer (Sigma). The tube containing the mixture was then
648 placed in a bottle with a stir bar and left to incubate at 4°C for 2 hours. The
649 homogenate was clarified by centrifugation for 30 min at $70,560 \times g$ at 4°C in an Optima
650 XPN Ultracentrifuge (Beckman Coulter) using a Ti-45 rotor. The supernatant, which

651 containing soluble FLAG-tagged HsTRPM4 mixed with 1ml of Anti-FLAG® M2 affinity gel
652 (Millipore, Billerica, MA) pre-equilibrated with wash buffer containing 25 mM HEPES-NaOH,
653 pH 7.5, 200 mM NaCl and 1% DDM/ 0.1% CHS. The beads were washed with 100 mL of
654 wash buffer containing 25 mM HEPES-NaOH, pH 7.5, 200 mM NaCl and 1% DDM/ 0.1%
655 CHS and eluted with 4 mL elution buffer containing 25 mM HEPES-NaOH, pH 7.5, 200 mM
656 NaCl, 120 µg/ml of 3xFLAG peptide and 1% DDM/ 0.1% CHS. The purified protein was run
657 on a Superose 6 gel filtration column pre-equilibrated with 0.005% Lauryl Maltose Neopentyl
658 Glycol (LMNG)/ 0.0005% CHS and the peak fraction was concentrated on a 100 K Amicon
659 Ultra-15 concentrators (Millipore, Billerica, MA) to an absorbance at 280 nm of 1.0 to prepare
660 cryo-EM grids.

661

662 **SDS-PAGE Analysis**

663 An SDS-PAGE analysis was performed to assess the purity of purified proteins. 15 µL of
664 protein sample was supplemented with 5 µL of 4X NuPAGE LDS Sample Buffer (Thermo
665 Scientific). Samples were incubated at 95°C for 10 minutes before loading on a 4-12%
666 SurePAGE™ Bis-Tris precast gels (Witec AG). Spectra™ Prestained Protein Ladder
667 (Thermo Scientific) (10 to 180 kDa) was also loaded on the gel to run as a size marker. Gels
668 were run in 1X Tris-MOPS SDS running buffer (Witec AG) at 200 V for 30 minutes, washed
669 briefly in MilliQ water and stained for 2 hours with QuickBlue Protein Stain (LuBioScience
670 GmbH) with shaking. Gels were washed in MilliQ water before imaging on an iBright FL1500
671 Imaging System (Thermo Scientific).

672

673 **Cryo-EM sample preparation and data collection**

674 Purified TRPM4 was incubated with a final concentration of 0.2mM IBA for TRPM4_{IBA} and
675 NBA for TRPM4_{NBA} for 30mins at room temperature before freezing cryo-EM grids. Cryo-EM
676 grids were prepared by applying 3 µl of concentrated sample onto 400-mesh R1.2/1.3
677 UltraAuFoil grids (Quantifoil Micro Tools GmbH), which had rendered hydrophilic by glow

678 discharging at 15mA for 60seconds with a PELCO EasyGlow device (TED PELLA, INC).
679 The sample were immediately blotted and plunge frozen into liquid ethane using a Vitrobot
680 Mark IV plunge freezer (Thermo Fisher Scientific). Cryo-EM data were collected using the
681 automated data acquisition software EPU (Thermo Fisher Scientific) on a Titan Krios G4
682 transmission electron microscope (Thermo Fisher Scientific), operating at 300kV and
683 equipped with a cold-FEG electron source, a SelectrisX energy filter and a Falcon4 direct
684 detection camera. Images were recorded in counting mode at a nominal magnification of
685 165kx, corresponding to a physical pixel size of 0.726 Å at the sample level. Datasets were
686 collected at a defocus range of 0.8 to 2.5 µm with a total electron dose of 60 e⁻/Å². Image
687 data were saved as Electron Event Recordings (EER).

688

689 **Cryo-EM image processing, model building, and refinement**

690 The cryo-EM image processing was performed using cryoSPARC v3.4³⁴.
691 The patch-based motion correction (cryoSPARC implementation) was used for aligning the
692 EM movie stacks and applying dose-dependent resolution weighting to recorded movies.
693 CTF estimation was performed using the patch-based option as well. For the data of the
694 HsTRPM4_{apo}, a total of 6,429 movies at 0.726 Å per pixel were collected, and 1000 particles
695 were manually picked and used for one round of 2D classification for template creation.
696 Template-based automated particle picking was then used on the recorded image data,
697 which resulted in a set of 1'719'323 particles at a size of 450 pixels. Two rounds of 2D
698 classification were performed for the initial step of particle cleaning resulting in 255'053
699 particles in the first round and 16'308 in the second round. Ab-initio and non-uniform
700 refinement yielded one 3D reconstruction with a map at 3.67 Å overall resolution in C4
701 symmetry ([Supplementary Fig. 4](#)).

702

703 For the data of the HsTRPM4_{IBA}, a total of 7,500 movies at 0.726 Å per pixel were collected.
704 2D classes from the HsTRPM_{apo} dataset were used for template-based picking. Template-
705 based automated particle picking resulted in a set of 1'149'090 particles at a size of 450

706 pixels. Two rounds of 2D classification were performed for the initial step of particle cleaning
707 resulting in 185'741 particles at 400 pixels in the first round and 35'399 particles in the
708 second round. Ab-initio and hetero-refinement refinement yielded two 3D reconstructions.
709 One reconstruction representing 57.5% of particles was selected for further non-uniform
710 refinement resulting in a map at 3.62 Å overall resolution in C4 symmetry. Following
711 symmetry expansion, the overall resolution of the map could be improved to an overall
712 resolution of 3.62 Å ([Supplementary Fig. 5](#)).

713

714 For the data of the HsTRPM4_{NBA}, a total of 11,851 movies at 0.726 Å per pixel were
715 collected. 2D classes from the HsTRPM_{apo} dataset were used for template-based picking.
716 Template-based automated particle picking resulted in a set of 1'805'182 particles at 400
717 pixels. Four rounds of 2D classification were performed for the initial step of particle cleaning
718 resulting in 40'633 particles. Ab-initio and hetero-refinement refinement yielded four 3D
719 reconstructions. One reconstruction representing 15'954 particles was selected for further
720 non-uniform refinement resulting in a map at 4.50 Å overall resolution in C4 symmetry from
721 15,954 particles ([Supplementary Fig. 6](#)).

722

723 Cryo-EM data was also collected for DDM/CHS solubilized mouse and human TRPM4.
724 MmTRPM4 data was collected with 5mM Calcium chloride present in the sample. Two
725 datasets were collected for DDM/CHS solubilized HsTRPM4 with 5mM Calcium chloride
726 present in one sample and both 5mM Calcium chloride and 0.2mM NBA present in the other.
727 Details of the data collection are included ([Supplementary Table 2](#)).

728

729 Atomic models for HsTRPM_{apo}, HsTRPM4_{IBA} and HsTRPM4_{NBA} structures mainly were built
730 in Coot 0.9.4³⁵, using a model PDB id: 6BQV as an initial model. Real-space refinement for
731 all built models was performed using Phenix, version 1.19.2-4158 by applying a general
732 restraints setup³⁶.

733

734 **Cell Culture**

735 TsA-201 cells were cultured with Dulbecco's Modified Eagle's culture Medium DMEM
736 (Gibco, Basel, Switzerland) supplemented with 10% FBS, 0.5% penicillin, and streptomycin
737 (10,000 U/mL) at 37°C in a 5% CO₂ incubator.

738

739 **Transfections**

740 Sixty mm dishes (BD Falcon, Durham, North Carolina, USA) at 80% of confluence were
741 transiently transfected using Xtreme Gene 9™ transfection reagent (Sigma Aldrich Merck,
742 Switzerland) and following the instructions of the manufacturer. In brief, 1,000 ng of either
743 empty vector or HsTRPM4 WT or variants of HsTRPM4 (TRPM4 S863A, TRPM4 V901W,
744 TRPM4 V904W, TRPM4 H908A, TRPM4 S924A, TRPM4 K925A, TRPM4 Y1057A, TRPM4
745 Q1061A, TRPM4 R1064A, TRPM4 R1064G, TRPM4 R1064S or TRPM4 S863A/S924A)
746 was mixed with a solution containing 100 ng of a reporter gene coding for GFP and 1,000 ng
747 of empty vector. The expression of GFP was used to evaluate the transfection efficiency
748 procedure, and the empty vector was added to reach a certain amount of total cDNA to
749 ensure an efficient transfection. These final cDNA solutions (2,100 ng) were mixed with 210
750 µL of opti-MEM (Gibco, Basel, Switzerland) and 6.3 µL of Xtreme Gen9™ reagent (ration
751 1/3). After 30 minutes at room temperature, the cDNA solutions were applied to the cells.
752 Forty-eight hours post-transfection, the cells were harvested for the western blot
753 experiments.

754 **Western-blot**

755 The expression of the human TRPM4 channel was assessed in whole-cell lysates. First,
756 cells were washed with PBS 1X and then lysed for 1 hour at 4 °C in lysis buffer (50 mM
757 HEPES pH 7.4, 1.5 mM MgCl₂, 150 mM NaCl, 1 mM EGTA pH 8, 10% glycerol, 1% Triton X-
758 100, and Complete® protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany)).
759 The pellet was discarded after centrifugation at 4 °C, 16,000 g for 15 minutes. Protein
760 concentrations of each lysate sample were measured in triplicate by Bradford assay and

761 interpolated by a bovine serum albumin (BSA) standard curve. Samples were denatured at
762 30 °C for 37 minutes before loading on a gel. Twenty µg of protein for each sample was run
763 at 150 V for 1 hour on 9% polyacrylamide gels. The Turbo Blot dry blot system (Biorad,
764 Hercules, CA, USA) was used to transfer the samples to a nitrocellulose membrane. All
765 membranes were stained with Ponceau as a qualitative check for equivalent total protein
766 loading. Membranes were then rinsed twice with TBS 1X and blocked with 5% BSA in TBS
767 1X for 1 hour. After this blocking step, the membranes were incubated for 2 hours with rabbit
768 anti-human TRPM4 antibody (epitope: ¹¹³⁷CRDKRESDSERLKRTSQKV¹¹⁵⁵, Pineda, Berlin,
769 Germany) diluted 1:1,000 in TBS 1X + 0.1% tween and mouse anti-Na⁺/K⁺ ATPase antibody
770 (Abcam ab 7671) diluted 1:1,000 in TBS 1X + 0.1% tween. The membranes were washed 4
771 times in TBS 1X + 0.1% tween before incubating with fluorescent secondary antibodies.
772 Secondary antibodies IR Dye 800 CW, anti-rabbit diluted (1:20,000) in TBS 1X + 0.1%
773 tween and IR Dye 700 CW, anti-mouse diluted (1:20,000) in TBS 1X + 0.1% tween (LI-COR
774 Biosciences, Lincoln, NE, USA) were added for 1 hour. After 4 washes with TBS 1X + 0.1%
775 tween and 3 washes in TBS 1X, membranes were scanned with the FUSION FX Spectra®
776 Infrared Imaging System (VILBER smart imaging, Marne-la-Vallée, France) to detect
777 fluorescent protein. Subsequent quantitative analysis of protein content was achieved by
778 measuring and comparing band densities (equivalent to fluorescence intensities of the
779 bands) using the *Evolution-Capt software* (VILBER smart imaging, Marne-la-Vallée, France).
780 The background was first subtracted for each band (human TRPM4 and Na⁺/K⁺ ATPase),
781 then TRPM4 intensity was divided by the intensity of the Na⁺/K⁺ ATPase band (for a given
782 sample) and normalized for comparison.

783 **Electrophysiology**

784 **Transfections**

785 Thirty-five mm dishes (BD Falcon, Durham, North Carolina, USA) at 80% of confluence were
786 transiently transfected using JetPEI™ transfection reagent (Polyplus transfection, Illkirch,
787 France) and following the instructions of the manufacturer. In brief, 500 ng of either empty

788 vector or HsTRPM4 WT or variants of HsTRPM4 (TRPM4 T677I, TRPM4 S863A, TRPM4
789 V901W, TRPM4 V904W, TRPM4 H908A, TRPM4 S924A, TRPM4 K925A, TRPM4 Y1057A,
790 TRPM4 Q1061A, TRPM4 R1064A, TRPM4 R1064G, TRPM4 R1064S or TRPM4
791 S863A/S924A) was mixed with 200 ng of a reporter gene coding for GFP. Expression of
792 GFP was used to identify transfected cells during patch clamp experiments. Coding DNAs
793 (cDNAs from HsTRPM4 and GFP) were mixed with 4 μ l of JetPEI™ and 46 μ l of 150 mM
794 NaCl. After 15 minutes at room temperature, the cDNA solutions were applied to the cells.
795 Twenty-four hours post-transfection, the cells were plated at low density in a new 35 mm
796 dish coated with poly-l-lysine. The cells were patched 24 hours post-plating (48 hours post-
797 transfection).

798

799 **Inside-out patch clamp**

800 Electrophysiological recordings were performed in the inside-out patch-clamp configuration
801 with patch pipettes (1 and 2 μ m tip opening) pulled from 1.5 mm borosilicate glass capillaries
802 (Zeitz-Instruments GmbH, München, Germany) using micropipette puller P 97 (Sutter
803 Instruments, Novato, CA, United States). The tips were polished for 2–4 M Ω pipette
804 resistance in the bath solution. The pipette solution contained 150 mM NaCl, 10 mM
805 HEPES, and 2 mM CaCl₂ (pH 7.4 with NaOH). The initial bath solution with 0 calcium
806 contained 150 mM NaCl, 10 mM HEPES, and 2 mM HEDTA (pH 7.4 with NaOH). After
807 reaching the inside-out configuration, different solutions were perfused at the intracellular
808 side of the membrane patch using a modified rapid solution exchanger (Perfusion Fast-Step
809 SF-77B; Warner Instruments Corp. CT, United States).

810 For calcium sensitivity experiments. The first solution, applied for 3 to 5 sweeps to reach a
811 stable current, is the bath solution (150 mM NaCl, 10 mM HEPES, and 2 mM HEDTA; pH
812 7.4 with NaOH) with 0 free Ca²⁺. Then, to activate TRPM4 channels, a solution (150 mM
813 NaCl, 10 mM HEPES; pH 7.4 with NaOH) containing different amounts of free Ca²⁺ (50 μ M,
814 100 μ M, 300 μ M, 500 μ M, 1000 μ M, 5000 μ M) was applied. The effect of the calcium was
815 quantified when the calcium-activated current reached stability. Finally, the bath solution with

816 0 μM free Ca^{2+} was used to quantify the potential leak current that can occur during such
817 recordings.

818 For drug dose-response experiments. The first solution, applied for 3 to 5 sweeps to reach
819 the stable current, is the bath solution (150 mM NaCl, 10 mM HEPES, and 2 mM HEDTA;
820 pH 7.4 with NaOH) with 0 μM free Ca^{2+} . Then, after stabilization of the current, a solution
821 (150 mM NaCl, 10 mM HEPES; pH 7.4 with NaOH) containing 300 μM free Ca^{2+} without
822 compounds was applied to activated HsTRPM4 channels and recorded the calcium-
823 activated HsTRPM4 current until its stabilization. Then, a solution (150 mM NaCl, 10 mM
824 HEPES; pH 7.4 with NaOH) containing 300 μM free Ca^{2+} and the compound at different
825 concentrations (10 nM, 50 nM, 100 nM, 500 nM, 1000 nM, 5000 nM, 10'000 nM, 50'000 nM)
826 were applied. The effect of the drug was quantified when the calcium-activated current
827 reached stability. Finally, the bath solution with 0 μM free Ca^{2+} was used to quantify the
828 potential leak current that can occur during such recordings. No investigation and
829 comparison has been done concerning the kinetic of block and washout, and only "stable"
830 currents have been used to calculate the percentage of inhibition.

831 HsTRPM4 sodium currents calcium-activated were recorded with a Multiclamp 700B
832 amplifier (Molecular Devices, Sunnyvale CA, United States) controlled by Clampex 10 via
833 Digidata 1332A (Molecular Devices, Sunnyvale, CA, United States). Data were low-pass
834 filtered at 5 kHz and sampled at 10 kHz. Experiments were performed at room temperature
835 (20–25°C). The holding potential was 0 mV. The stimulation protocol consisted of two pulses
836 totaling 1,000 ms for measuring steady-state currents repeated at 0.2Hz (1 sweep every 5
837 sec.). The first pulse was at -100 mV for 500 ms, and the second was at $+100$ mV for
838 500 ms. For analysis, the effect of the compounds on sodium current calcium-activated has
839 been calculated by averaging the last 100 ms of the second sweep at $+100$ mV (from the
840 stable phase). Electrophysiology data were exported and analyzed using Prism7.05
841 GraphPad™ software (GraphPad by Dotmatics, San Diego, CA, USA). Concentration-
842 response curves were fitted using the log(inhibitor) vs. response - Variable slope (four

843 parameters) equation ($Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogIC50} - X) * \text{HillSlope}))}$) where X
844 corresponds to the log of concentration and Y current recorded in pA.

845 **Data analyses and statistics**

846 Data are represented as means \pm SEM. Statistical analyses were performed using
847 Prism7.05 GraphPad™ software (GraphPad by Dotmatics, San Diego, CA, USA). An
848 unpaired nonparametric t-test followed by a Mann-Whitney U post-test was used to compare
849 two unpaired groups. $p < 0.05$ was considered significant. No multi-group comparison has
850 been performed in this study. Western blots have been done in triplicate, and at least 6 cells
851 have been used for the different doses in electrophysiology experiments.

852

853 **Data visualization**

854 Gel images were processed and prepared on ImageJ (Version 1.53k). Figures were
855 rendered using PyMOL, UCSF Chimera, UCSF ChimeraX³⁷, and Adobe Illustrator
856 (<https://adobe.com/products/illustrator>).

857

858 **Data and Code Availability**

859 The reconstructed maps are available from the EMDB database under access codes
860 HsTRPM4apo (EMD-19057), HsTRPM4IBA (EMD-19061), HsTRPM4NBA (EMD-19069)
861 (EMD-19060, local). The atomic models are available in the PDB database, access codes
862 HsTRPM4apo (PDB 8RCR), HsTRPM4IBA (8RCU) and HsTRPM4NBA (PDB 8RD9). The
863 raw cryo-EM movies data of this work are available under access codes EMPIAR-xxxxx,
864 EMPIAR-xxxxx, and EMPIAR-xxxxx.

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