

1 **Structural mechanism of calcium-mediated hormone recognition and G β**
2 **interaction by the human melanocortin-1 receptor**

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4 Shanshan Ma^{1,2}, Yan Chen^{1,3}, Antao Dai⁴, Wanchao Yin¹, Jia Guo^{1,2}, Dehua Yang^{1,2,4}, Fulai
5 Zhou¹, Yi Jiang^{1,2}, Ming-Wei Wang^{1,2,3,4,5,6} and H. Eric Xu^{1,2,5}

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7 ¹CAS Key Laboratory of Receptor Research, Shanghai Institute of Materia Medica, Chinese
8 Academy of Sciences, Shanghai 201203, China;

9 ²University of Chinese Academy of Sciences, Beijing 100049, China;

10 ³School of Pharmacy, Fudan University, Shanghai 201203, China;

11 ⁴The National Center for Drug Screening, Shanghai Institute of Materia Medica, Chinese
12 Academy of Sciences, Shanghai 201203, China;

13 ⁵School of Life Science and Technology, ShanghaiTech University, Shanghai 201210, China.

14 ⁶School of Basic Medical Sciences, Fudan University, Shanghai 200032, China.

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16 Correspondence: Ming-Wei Wang (mwwang@simm.ac.cn) or H. Eric Xu

17 (eric.xu@simm.ac.cn)

18 These authors contributed equally: Shanshan Ma, Yan Chen.

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21 **Melanocortins are peptide hormones critical for stress response, energy homeostasis,**
22 **inflammation, and skin pigmentation. Their functions are mediated by five G protein-**
23 **coupled receptors (MC1R to MC5R), predominately through the stimulatory G protein**
24 **(Gs). MC1R, the founding member of melanocortin receptors, is mainly expressed in**
25 **melanocytes and is involved in melanogenesis. Dysfunction of MC1R is associated with**
26 **the development of melanoma and skin cancer. Here we present three cryo-electron**
27 **microscopy structures of the MC1R-Gs complexes bound to endogenous hormone α -**
28 **MSH, a marketed drug afamelanotide, and a synthetic agonist SHU9119. These**
29 **structures reveal the orthosteric binding pocket for the conserved HFRW motif among**
30 **melanocortins and the crucial role of calcium ion in ligand binding. They also**
31 **demonstrate the basis of differential activities among different ligands. In addition,**
32 **unexpected interactions between MC1R and the G β subunit were discovered from these**
33 **structures. Together, our results provide a conserved mechanism of calcium-mediated**
34 **ligand recognition, specific mode of G protein coupling, and a universal activation**
35 **pathway of melanocortin receptors.**

36

37 The melanocortin system is composed of five melanocortin receptors (MC1R to MC5R), four
38 melanocortin-related peptide hormones, and two endogenous antagonists agouti and agouti-
39 related peptide (AgRP)¹. Melanocortins, with a highly conserved His-Phe-Arg-Trp (HFRW)
40 sequence motif and consisting of adrenocorticotrophic hormone (ACTH) and three
41 melanocyte-stimulating hormones (α -MSH, β -MSH, and γ -MSH) (**Fig. 1a**), are derived from
42 tissue-specific posttranslational processing of pro-opiomelanocortin (POMC)^{2,3}. POMC is a
43 precursor of polypeptide hormones, mainly secreted by the anterior pituitary, hypothalamus
44 and brainstem⁴. The activity of POMC neurons is up-regulated by leptin and down-regulated
45 by ghrelin, respectively⁵. Leptin, a satiety hormone, inhibits AgRP neurons and depolarizes
46 POMC neurons to increase the expression of POMC and α -MSH. α -MSH, a 13-residue
47 peptide hormone, was first identified in 1957 and is best known for maintaining energy
48 homeostasis and protecting skin from ultraviolet radiation via augment of skin pigmentation⁶⁻
49 ⁸. Consequently, leptin decreases food intake and body weight by activating downstream
50 signaling of the melanocortin system, while ghrelin, the hunger hormone, which is opposite to

51 leptin, increases food intake and body weight by inhibiting melanocortin system signaling^{9,10}.
52 Dysregulation of melanocortins, leptin and ghrelin is associated with high risks of anorexia,
53 cachexia and obesity¹¹⁻¹³.
54
55 Activation of melanocortin receptors by cognate ligands induces a cascade of signal
56 transduction through coupling to the stimulatory G protein (Gs) and arrestin¹⁴. MC1R to
57 MC5R are among the shortest receptors in class A G protein-coupled receptors (GPCRs) that
58 show distinct tissue-specific expression and physiological function. MC1R is mainly
59 expressed in melanocytes and melanoma cells, and plays crucial roles in regulation of
60 melanogenesis, skin pigmentation, and inflammation^{15,16}. Abnormal functions of MC1R are
61 linked to the development of melanoma and non-melanoma skin cancer¹⁷⁻²⁰. MC2R is mostly
62 located in the adrenal cortex and crucial for the hypothalamus-pituitary-adrenal (HPA) axis.
63 Defective MC2R signaling causes a lethal disease called familial glucocorticoid deficiency
64 (FGD)²¹. MC3R and MC4R, widely expressed in both the central nervous system and
65 peripheral tissues, participate in the leptin-melanocortin signaling axis and are responsible for
66 energy homeostasis, blood pressure, and inflammation. Selective ligands targeting MC3R and
67 MC4R are promising drug candidates for obesity or anorexia²²⁻²⁴. MC5R is commonly seen in
68 peripheral tissues and regulates exocrine gland secretion such as lacrimal, preputial and
69 harderian glands²⁵. However, structural basis for the complex interplay between
70 melanocortins and MC1R-MC5R is largely unknown, except for the recent studies on
71 MC4R²⁶⁻²⁸.
72
73 Given the important physiological functions of the melanocortin system, diverse synthetic
74 ligands have been developed for therapeutic applications (**Fig. 1a**). Afamelanotide is the first
75 synthetic α -MSH analog that has high affinity for MC1R²⁹, and it has been approved as
76 ScenesseTM by European Medicines Agency (EMA) for the prevention of phototoxicity in
77 patients with erythropoietic protoporphyria³⁰. SHU9119, a cyclic α -MSH analog, is a partial
78 agonist for MC1R and MC5R but acts as an antagonist for MC3R and MC4R^{31,32}. Currently,
79 only the inactive crystal structure of MC4R bound to SHU9119 and the active cryo-electron
80 microscopy (cryo-EM) structures of the MC4R-Gs complexes are available²⁶⁻²⁸. The limited

81 structural information of the melanocortin system has hindered our understanding of the
82 detailed mechanism by which various endogenous and synthetic peptides exert their
83 differentiated actions. Here we present three cryo-EM structures of the MC1R-Gs complexes
84 bound to α -MSH, afamelanotide and SHU9119 with a global resolution of 3.0 Å, 2.7 Å and
85 3.1 Å, respectively. The structures provide a paradigm for studying signal transduction of the
86 melanocortin system and multiple structural templates for rational design of novel therapeutic
87 agents targeting melanocortin receptors.

88

89 **RESULTS**

90 **Cryo-EM structures of MC1R-Gs complexes**

91 For cryo-EM studies, we co-expressed the full-length human MC1R, human dominant
92 negative G α s, human G β and human G γ in High Five insect cells (**Supplementary**
93 **information, Fig. S1a-b**). The structures of α -MSH, afamelanotide and SHU9119 bound
94 MC1R-Gs complexes were determined at a resolution of 3.0 Å, 2.7 Å and 3.1 Å, respectively
95 (**Supplementary information, Fig. S1c-e, S2 and Table S1**). In addition, a subset of
96 afamelanotide bound MC1R-Gs complex without Nb35 were extracted and the structure was
97 determined at a resolution of 2.9 Å (**Supplementary information, Fig. S1e and Table S1**).
98 The high-quality EM maps allowed unambiguous model refinement of MC1R, the Gs
99 heterotrimer and three bound peptide ligands α -MSH afamelanotide and SHU9119. Besides, a
100 calcium ion was well defined in the EM maps (**Supplementary information, Fig. S3 and**
101 **Table S1**).

102

103 Similarly, an annular detergent micelle surrounding the transmembrane domain (TMD) of
104 MC1R was observed in all three structures mimicking the phospholipid bilayer. The receptors
105 exhibit a nearly identical conformation with a large opening in the extracellular side of TMD
106 (**Fig. 1b-e**). Different from other class A GPCRs, the extracellular loop 2 (ECL2) of MC1R is
107 extremely short and its ECL3 forms an ordered helix (**Fig. 2**). Three peptides adopt a U shape
108 conformation in the extracellular end of the TMD with a similar orientation. In addition, the
109 well-defined calcium ion near TM3 is positioned to stabilize MC1R ligand binding.

110

111 **Orthosteric peptide binding pocket**

112 The overall structures of the three MC1R-Gs complexes are highly similar with root mean
113 square deviation (RMSD) values of 0.70 Å for the C α atoms between α -MSH and
114 afamelanotide bound MC1R, and 0.87 Å for the C α atoms between α -MSH and SHU9119
115 bound MC1R (**Fig. 2a**). All three peptides adopt a U shape conformation in the extracellular
116 end of TMD, with the benzene ring of Phe^{M7/F7} and the naphthalene ring of D-Nal^{U7}
117 penetrating deeply into the TMD core (superscript M refers to α -MSH, F to afamelanotide and
118 U to SHU9119, residue numbers are based on α -MSH) (**Fig. 2b-d**). The interactions of α -
119 MSH, afamelanotide and SHU9119 with MC1R bury a total interface area of 2085 Å², 1986
120 Å² and 1790 Å², respectively (**Fig. 2e-g**). The smaller interface area between SHU9119 and
121 MC1R might explain why SHU919 is a weaker agonist than α -MSH and afamelanotide for
122 MC1R (**Supplementary information, Fig. S4a and Table S5**). The highly conserved HFRW
123 motif of melanocortins is at the center of the U shape pocket and provides the major contacts
124 for binding to MC1R (**Fig. 3**).

125

126 The orthosteric peptide-binding pocket can be divided into three parts based on the
127 conformation of α -MSH (**Fig. 2**). The first part is formed by the N-terminal residues 1-5,
128 which is parallel to the plane between TM2 and TM3. The second part is the critical HFRW
129 motif (residues 6-9), which is inserted deeply into the TMD core and interacts with TM1 to
130 TM7. The third part is formed by the C-terminal residues 10-13, which is in proximity to
131 TM6, TM7, and ECL3 (**Fig. 2**). Extensive hydrophobic and polar interactions are observed
132 between MC1R and three peptides (**Fig. 3 and supplementary information, Table S2-S4**)
133 and the majority of residues involved in peptide binding are conserved in melanocortin
134 receptors. For example, His⁶ of peptides packs against F45^{1.39} (superscripts denote the
135 Ballesteros-Weinstein numbers³³) forming a conserved π - π interaction (**Fig. 3a-c**). The
136 positively charged side chain of Arg⁸ of peptides forms hydrogen bonds with the negatively
137 charged side chains of D117^{3.25} or D121^{3.29} (**Fig. 3d-f**). In order to correlate these structural
138 observations with signaling profiles, various mutants were constructed to detect cAMP
139 responses of MC1R (**Fig. 3j and Supplementary information, Fig. S4b-d, Table S5-S6**).

140 The majority of alanine mutations in the orthosteric peptide-binding pocket reduced both

141 potency and efficacy of ligand-stimulated cAMP accumulation mediated by MC1R. Notably,
142 there is a considerable divergence in the basal activities of different MC1R constructs,
143 consistent with the constitutive activity of MC1R reported previously³⁴⁻³⁶. Therefore, the
144 decline in pEC₅₀ values and cAMP responses of MC1R mutants elicited by three peptides
145 reveal an important role of these residues in ligand binding and receptor activation (**Fig. 3j**
146 **and Supplementary information, Fig. S7**).

147

148 Of note is the observation of an extremely short ECL2 in the three MC1R-Gs complexes,
149 different from a longer ECL2 in the β_2 AR-Gs complex, where it forms a lid covering the
150 extracellular top of TMD (**Supplementary information, Fig. S4e**)³⁷. In the case of GPR52,
151 ECL2 can fold into the transmembrane bundle and function as a built-in ‘agonist’ to activate
152 the receptor³⁸. In addition, TM2 and ECL1 of MC1R move outwards compared to β_2 AR. In
153 the MC1R-Gs complex structures, ECL3 forms an ordered helix, in which two cysteines,
154 C267^{ECL3} and C273^{ECL3}, make a disulfide bond instead of the canonical disulfide bond
155 between TM3 and ECL2 seen in other class A GPCRs (**Fig. 3d-f**). Such a unique feature of
156 the MC1R structure allows a broader opening in the extracellular side of TMD to
157 accommodate larger peptide ligands and a calcium ion. Extending ECL2 by a nine-residue
158 insertion (3×GSA) between D184^{ECL2} and H185^{ECL2} or disruption of the ECL3 disulfide bond
159 through mutations of C267^{ECL3}A and C273^{ECL3}A decreased both pEC₅₀ values and potencies
160 of the three peptides (**Supplementary information, Fig. S4f-g and Table S5-S6**).

161

162 **Differential activities of peptide ligands**

163 The three peptide ligands (α -MSH, afamelanotide and SHU9119) used in this study display
164 differential activities toward different melanocortin receptors, which can be readily explained
165 by our structural observations. Specifically, afamelanotide, which has D-Phe^{F7} instead of
166 Phe^{M7} in α -MSH, has a higher affinity for melanocortin receptors. SHU9119 with D-Nal^{U7}
167 displays a partial agonism for MC1R and MC5R but acts as an antagonist for MC3R and
168 MC4R. Structural analysis of the ligand-binding pocket of MC1R reveals that the change of
169 Phe^{M7} causes slightly different orientations of nearby residues, resulting in different
170 interactions between MC1R and the peptides (**Fig. 3 and Fig. 4a-b**). For example, the

171 hydroxyl group on the carboxyl group of Trp^{M9} forms a hydrogen bond with the nitrogen on
172 the imidazole ring of H260^{6,54}, which is absent in the afamelanotide bound MC1R-Gs
173 complex (**Fig. 3d-e**). H260^{6,54}A mutation decreases the affinity of α -MSH for MC1R, without
174 affecting that of afamelanotide (**Supplementary information, Fig. S4d, g and Table S5-S6**).
175 Besides, in comparison with the inactive SHU9119-MC4R complex, the benzene ring of
176 Phe^{M7} in the active α -MSH-MC1R-Gs complex inserts into the TMD and induces a downward
177 shift of F257^{6,51} and F280^{7,35} of MC1R, which makes steric clash with the toggled switch
178 residue W254^{6,48} and pushes W254^{6,48} into the active position (**Fig. 4a**). The rearrangement of
179 the toggled switch residue W254^{6,48} is a molecular hallmark to start a cascade of
180 conformational changes during receptor activation. However, the cyclic structure of
181 SHU9119, which is different from non-cyclic peptides of α -MSH and afamelanotide, makes
182 more compact interactions with MC1R and a smaller shift of F257^{6,51} and F280^{7,35} (**Fig. 4b**),
183 providing a basis for the partial agonism of SHU9119 toward MC1R, in which α -MSH and
184 afamelanotide are full agonists.

185

186 In addition, when comparing the structures of SHU9119 bound MC1R with SHU9119 bound
187 MC4R, several differences are observed in their ligand-binding pockets despite that most
188 pocket residues are conserved. Notably, D-Nal^{U7} of SHU9119 was constrained in the TMD
189 core by L133^{3,36} and F261^{6,51} of MC4R in the inactive SHU9119-MC4R complex structure²⁶.
190 However, L133^{3,36} of MC4R corresponds to M128^{3,36} in MC1R. The side chain of M128^{3,36}
191 moves upward to interact with D-Nal^{U7}, causing a severe steric clash with the toggled switch
192 residue W254^{6,48} and a subsequently downward movement of W254^{6,48} (**Fig. 4c**). M128^{3,36}L
193 mutation of compromised SHU9119-stimulated cAMP response of MC1R (**Supplementary**
194 **information, Fig. S5a and Table S5**), consistent with that L133^{3,36}M mutation of MC4R
195 converted SHU9119 from an antagonist to a partial agonist^{27,39}. Together, these results provide
196 a basis of SHU9119 as an agonist for MC1R and as an antagonist for MC4R.

197

198 **Role of calcium ion**

199 Extensive evidence reveal that the divalent ion is of crucial importance for melanocortin
200 signaling. Calcium ion assists melanocortins in binding to their cognate receptors with a

201 better effect than magnesium ion⁴⁰⁻⁴². Zinc ion activates MC1R and MC4R by acting as an
202 agonist or allosteric modulator^{43,44}. A well-resolved electron density of calcium ion was
203 observed in the MC1R-Gs complexes at the same position as that of the SHU9119 bound
204 MC4R structure (**Fig. 5a-c**). The Ca²⁺-binding pocket is conserved within the orthosteric
205 peptide binding pocket, consisting of E^{2.60}, D^{3.25}, and D^{3.29} from melanocortin receptors (**Fig.**
206 **5d**) as well as Glu/Asp⁵, Phe⁷ and Arg⁸ from melanocortins. Declined cAMP responses and
207 peptide affinities for MC1R with mutations of E94^{2.60}A, D117^{3.25}A and D121^{3.29}A are likely
208 the consequence of destroying both peptide and calcium ion binding pockets (**Supplementary**
209 **information Fig. S5c-d and Table S5-S6**). The affinity of α -MSH for MC1R increases when
210 Ca²⁺ concentrations are elevated (**Supplementary information Fig. S5b**). Specifically,
211 addition of 0.5 mM Ca²⁺ shifted cAMP response curve to the left upon stimulation with α -
212 MSH and SHU9119 (500-fold) or afamelanotide (10-fold) (**Supplementary information Fig.**
213 **S5e**), pointing to an allosteric modulation role of Ca²⁺.

214

215 It is noteworthy that D^{3.25} of melanocortin receptors corresponds to highly conserved C^{3.25}
216 which forms a canonical disulfide bond with cysteine of ECL2 in other class A GPCRs (**Fig.**
217 **5d**). However, the extremely short ECL2 and the calcium-binding pocket of MC1R preclude
218 the possibility of a disulfide bond between ECL2 and TM3. Instead, two cysteines of ECL3
219 form a conserved disulfide bond in melanocortin receptors, which was absent in other class A
220 GPCRs (**Supplementary information, Fig.S5f**). These distinct features demonstrate that the
221 calcium-binding pocket is both conserved and unique in all five melanocortin receptors.

222

223 **Activation of MC1R**

224 The active SHU9119-MC1R-Gs complex reported here together with that of previous inactive
225 structure of SHU9119-MC4R complex reveal large conformational changes upon receptor
226 activation (**Fig. 6a**). At the extracellular side, ligand binding induced an inward movement of
227 TM1 by 1.7 Å at F45^{1.39} and an outward movement of TM2 by 2.2 Å at L101^{2.67} (**Fig. 6b**). At
228 the cytoplasmic side, TM3, TM4 and TM7 moved inwards slightly, TM5 extended by four
229 helices and moved inwards to interact with Gs, while TM6 moved outwards by 13.4 Å at
230 L237^{6.31} (**Fig. 6c**). The pronounced outward movement of TM6 in MC1R is consistent with

231 that seen among activated Gs coupled receptors.

232

233 As mentioned above, the Phe⁷ of melanocortin peptide ligands interacts with M128^{3.36},
234 inducing a downward movement of the toggled switch residue W254^{6.48} and a subsequently
235 downward movement of F250^{6.44} (**Fig. 6d-e**). In contrast to the conserved P^{5.50}I^{3.40}F^{6.44} motif,
236 M199^{5.50} in melanocortin receptors fits better in α -helical conformation than P^{5.50}, generating a
237 straight helix without the bulge as observed in the β_2 AR-Gs complex (**Fig. 6e and**
238 **supplementary information, Fig. S6a**). Structural superimposition with the inactive
239 structure of SHU9119-MC4R complex reveals that M199^{5.50} in the active MC1R-Gs complex
240 changes its orientation to induce an inward movement of TM5.

241

242 In addition, the highly conserved D^{3.49}R^{3.50}Y^{3.51} motif in class A GPCRs is shown to be critical
243 for receptor activation (**Fig. 6f**). Upon activation, Y143^{3.51} moves inwards to form
244 hydrophobic interactions with TM5 and a hydrogen bond with H209^{5.60}. The side chain of
245 R142^{3.50} stretches out straightly, breaking the salt bridge with D141^{3.49} and pushing TM6 away
246 from the TMD core. Meanwhile, R142^{3.50} packs against Tyr391 of Gas (**Fig. 7a**) and
247 contributes to stable interactions with Y207^{5.58} and Y298^{7.53}. The DRY motif links the
248 cytoplasmic ends of TM3, TM5, TM7 and G α_s , playing a direct role in stabilizing the active
249 state of MC1R.

250

251 Melanocortin receptors contain an aspartate (D^{7.49}) instead of an asparagine (N^{7.49}) at the
252 conserved N^{7.49}P^{7.50}xxY^{7.53} motif as seen in most class A GPCRs (**Fig. 6g**). D294^{7.49}N
253 mutation exhibited a nearly equivalent cAMP response of MC1R stimulated by α -MSH, while
254 D294^{7.49}A mutation remarkably impaired the peptide's ability to activate MC1R, suggesting
255 that both DPxxY and NPxxY motifs could effectively govern the transition of GPCRs from
256 inactive to active states (**Supplementary information, Fig. S6e and Table S5**). Taken
257 together, MC1R activation involves a cascade of conformational changes through
258 rearrangement of the toggle switch W^{6.48}, P(M)I(L)F, DRY and N(D)PxxY motifs present in
259 most class A GPCRs.

260

261 **Unique features of G protein coupling by MC1R**

262 The massive conformational changes in the cytoplasmic side of TMD is supposed to
263 accommodate $\alpha 5$ helix of Gas, which is the primary structural element interacting with
264 MC1R. There is negligible difference in Gs coupling among α -MSH, afamelanotide and
265 SHU9119 bound MC1R, and the interactions of Gs with MC1R bury a total surface area of
266 3252 \AA^2 , 3166 \AA^2 and 3044 \AA^2 , respectively. $\alpha 5$ helix of Gas inserts into the cytosolic core
267 surrounded by TM3, TM5, TM6, TM7 and ICL2 (**Fig. 7a**). R142^{3.50} packs against Tyr391 of
268 Gas, stabilized by van der Waals interaction. H301^{7.56} forms a salt bridge with Glu392 of Gas
269 and mutating H301^{7.56} to alanine impairs the basal cAMP activity of MC1R (**Supplementary**
270 **information, Fig. S6f and Table S5**). The extension of TM5 allows further interactions with
271 Gas (**Fig. 7a-c**). M210^{5.61}, R213^{5.64}, H217^{5.68} and I221^{5.72} of TM5 make substantial polar and
272 hydrophobic interactions with $\alpha 5$ helix of Gas. The C terminus of TM5 directly contacts $\alpha 4$
273 helix, $\alpha 4$ - $\beta 6$ loop and $\beta 6$ sheet of Gas (**Fig. 7c**). Alanine mutations of the C-terminal residues
274 of TM5 result in a significant reduction of cAMP responses (**Supplementary information,**
275 **Fig. S6f and Table S5**).

276

277 Furthermore, the intracellular loops facilitate additional interactions with Gs to stabilize the
278 complex. Typically, ICL2 adopts a 3^{10} -helix conformation and inserts into the groove formed
279 by αN - $\beta 1$ hinge, $\beta 2$ - $\beta 3$ loop and $\alpha 5$ helix of Gas (**Fig. 7b**). It was reported that the binding of
280 ICL2 to Gas induces sequential activation of Gs to release GDP⁴⁵. The side chain of L150^{ICL2}
281 is enclosed by the hydrophobic interactions with His41, Val217, Phe376 and Ile383 of Gas.
282 L150^{ICL2}A mutant dramatically suppressed the ability of MC1R to couple Gs to elicit cAMP
283 response (**Supplementary information, Fig. S6f and Table S5**). Different from class B
284 GPCRs, ICL1, rather than helix 8, forms van der Waals interactions with Arg52 and Phe335
285 of G β (**Fig. 7d**)⁴⁶. Mutating residues of ICL1 to alanine destabilizes the complex and impairs
286 the cAMP response of MC1R (**Supplementary information, Fig. S6g and Table S5**).

287

288 Interestingly, compared to the afamelanotide bound MC1R-Gs-Nb35-scFv16 complex (the
289 Nb35^{plus} complex), Gs in the absence of Nb35 (the Nb35^{minus} complex) adopts a relatively
290 loose conformation as Nb35 interacts with both Gas and G β (**Fig. 7e**). The αN helix of Gas

291 moved upwards by 5.7 Å despite the nearly overlapping $\alpha 5$ helix. The most surprising
292 observation is that G β from the Nb35^{minus} complex is in proximity to MC1R to make direct
293 contacts with E304^{8.49} and R307^{8.52} of helix 8 (**Fig. 7e and supplementary information, Fig.**
294 **S6b-c**). Deletion of helix 8 deprived the ability of the three peptides to activate MC1R and
295 dual mutations of E304^{8.49}A and R307^{8.52}A considerably weakened the Gs coupling, which
296 was consistent with that the mutations in the corresponding residues of G β decreased the
297 efficacy of G protein activation induced by three peptides α -MSH, afamelanotide and
298 SHU9119 (**Supplementary information, Fig. S6h-i and Table S5**). Such a rearrangement
299 results in a higher interface area of 3764 Å² between MC1R and Gs in the Nb35^{minus} complex.
300 No obvious shift was observed on peptide binding and receptor activation between the
301 Nb35^{plus} and the Nb35^{minus} complexes.

302

303 **DISCUSSION**

304 In this paper, we present three active cryo-EM structures of the MC1R-Gs complexes bound
305 to α -MSH, afamelanotide or SHU9119. These structures reveal a unique orthosteric peptide-
306 binding pocket in the extracellular side of MC1R, where a wide opening is observed and
307 allowed by the extremely short ECL2 and the ordered ECL3 to hold relatively large peptide-
308 hormones. In this pocket, three peptides adopt a similar U-shape conformation, with the
309 highly conserved HFRW motif among melanocortins lying at the bottom. It is noteworthy that
310 this motif makes major contacts with MC1R and provides indispensable energy to stabilize
311 the binding with MC1R. The binding mode between three peptides and MC1R is similar to
312 that of recently reported setmelanotide-MC4R-Gs complex (**Supplementary information,**
313 **Fig. S6j**), indicating a conserved mechanism of ligand recognition by different family
314 members of melanocortin receptors²⁷.

315

316 Structural superimposition of the three MC1R-Gs complexes shows that the critical residue
317 Phe in the HFRW motif contributes to the differential activities among three melanocortin
318 peptides. Substitution of Phe^{M7} with D-Phe^{F7} or D-Nal^{U7} affects the orientation of nearby
319 residues and the extent of peptide insertion into the binding pocket, thereby distinguishing the
320 detailed interactions between different peptides and MC1R. Accordingly, mutations in this

321 binding pocket led to different effects of the peptides used with afamelanotide being the
322 strongest agonist as it was least affected. Particularly, we demonstrate that M128^{3,36} of MC1R,
323 instead of L133^{3,36} of MC4R, is a key residue that converts SHU9119 from an antagonist of
324 MC4R to a partial agonist of MC1R.

325

326 Notably, Ca²⁺ was observed in all three MC1R-Gs complexes. Sequence alignment and
327 structural comparison among melanocortin receptors and with other class A GPCRs highlight
328 a unique and conserved calcium-binding pocket consisting of E^{2,64}, D^{3,25} and D^{3,29} of
329 melanocortin receptors and the backbone of melanocortin peptides. Specifically, the existence
330 of Ca²⁺ excludes the canonical disulfide bond between TM3 and ECL2 seen in other class A
331 GPCRs. Depletion of Ca²⁺ or disruption of the calcium-binding pocket reduced the potencies
332 and efficacies of cAMP responses elicited by three peptides and mediated by MC1R,
333 indicating a crucial role of the calcium ion in ligand recognition and MC1R activation.

334

335 It is known that the residues involved in the receptor activation and Gs coupling are
336 conserved, delineating a universal mechanism among class A GPCRs. However, except for
337 the interactions between ICL1 and G β as seen in other GPCR-Gs complexes^{38,47,48},
338 unexpected interactions between helix 8 of MC1R and G β were found in the Nb35^{minus}
339 complex. Structural superimposition of the Nb35^{minus} complex with the Nb35^{plus} complex
340 reveals that the Gs heterotrimer protein adopts a relatively loose conformation and G β is
341 closer to the receptor to make direct interactions with helix 8 of MC1R in the absence of
342 Nb35. Mutations in helix 8 and G β both markedly reduced the potency of Gs coupling by
343 MC1R, suggesting that the interaction between helix 8 and G β is important for G protein
344 coupling. To date, such interactions have only been observed in D1R and some class B
345 GPCRs⁴⁶⁻⁵⁷. The residues from helix 8 of MC1R that form interactions with the G β subunit
346 are conserved in D1R, implying that G β interaction may be a shared feature of G protein
347 recruitment by certain GPCRs (**Supplementary information, Fig. S6b-d**). Since most class
348 A GPCR-Gs complex structures were solved with Nb35 and the interaction between the G β
349 subunit and helix 8 in MC1R was only observed in the Nb35^{minus} complex, it is suggested that
350 other class A GPCRs could also interact with the G β subunit through their helix 8.

351

352 **MATERIALS AND METHODS**

353 **Constructs of MC1R and Gs**

354 The full-length human MC1R was cloned into pFastBac vector (Invitrogen) with its native
355 signal peptide replaced by the haemagglutinin (HA) signal peptide followed by a 10 × His tag
356 and cytochrome b562RIL (BRIL) as a fusion partner. To facilitate expression and purification,
357 the C terminus of MC1R was fused with a 15-amino-acid polypeptide linker
358 (GSSGGGGSGGGGSSG) and a LgBiT (Promega).

359

360 Human G α s was constructed based on miniGs (PDB: 5G53) deleting switch III and including
361 eight mutations (G49D, E50N, L63Y, A249D, S252D, L272D, I372A and V375I)⁵⁸. Two
362 additional dominant-negative mutations (G226A and A366S) were introduced to G α s to
363 decrease the affinity of nucleotide binding and increase the stability of the heterotrimeric G
364 protein⁵⁹. The N terminus (M1-K25) and α -helical domain (AHD, G67-L203) of G α s were
365 replaced by the N terminus (M1-M18) and AHD (G60-K180) of human G α i, which was
366 initially designed to bind scFv16 and Fab_G50^{60,61}.

367 Human G β with a C-terminal 15-amino-acid polypeptide linker followed by a HiBiT (peptide
368 86, Promega) and human G γ were cloned into pFastBac vector, respectively⁶². scFv16 was
369 constructed into the same vector with an N-terminal GP67 signaling peptide.

370

371 **Preparation of Nb35**

372 Nb35 was expressed and purified according to previously described methods⁶³. The purified
373 Nb35 was concentrated and stored in -80°C.

374

375 **Expression and purification of the MC1R-Gs complex**

376 Recombinant viruses of MC1R, G α s, G β , G γ and scFv16 were generated using Bac-to-Bac
377 baculovirus expression system (Invitrogen) in *sf9* insect cells (Expression Systems). High
378 FiveTM cells (ThermoFisher) at a density of 2×10^6 cells/mL were transfected with above five
379 baculoviruses at a ratio of 1:1:1:1:1. The cells were cultured for 48 h at 27°C after infection
380 and collected by centrifugation at 2×1000 rpm for 20 min. Notably, the α -MSH-MC1R-Gs

381 and SHU9119-MC1R-Gs complexes were expressed without scFv16.

382

383 The cell pellets were suspended in 20 mM HEPES, 100 mM NaCl, 100 μ M TCEP, pH 7.4,

384 supplemented with protease inhibitor cocktail (EDTA-Free) (Bimake) and centrifuged at

385 30,000 \times g for 30 min. The pellets were lysed in the same buffer supplemented with 40 mM

386 imidazole, 10 mM MgCl₂ and 5 mM CaCl₂, and the complex formation was initiated by

387 addition of 25 mU/mL Apyrase (Sigma), 20 mg/mL Nb35 and 10 μ M peptide (GenScript).

388 The lysate was incubated for 1.5 h at room temperature (RT) followed by addition of 0.5%

389 (w/v) lauryl maltose neopentylglycol (LMNG, Anatrace) and 0.1% (w/v) cholesterol

390 hemisuccinate (CHS, Anatrace) for 3 h at 4°C to solubilize the membrane. The supernatant was

391 isolated by centrifugation at 65,000 \times g for 30 min and incubated with Ni-NTA beads (Smart

392 Life Science) for 2 h at 4°C. The resin was collected by centrifugation at 500 \times g for 10 min

393 and loaded onto a gravity flow column. The resin was then washed with 30 column volumes

394 of 20 mM HEPES, 100 mM NaCl, 40 mM imidazole, 100 μ M TCEP, 4 μ M peptide, 2 mM

395 CaCl₂, pH 7.4, 0.01% (w/v) LMNG, 0.01% (w/v) GDN and 0.004% (w/v) CHS before bound

396 material was eluted with the same buffer containing 250 mM imidazole. The complexes were

397 concentrated using a 100-kD Amicon Ultra centrifugal filter (Millipore) and loaded onto

398 Superdex 200 10/300 GL column (GE Healthcare) with running buffer containing 20 mM

399 HEPES, 100 mM NaCl, 100 μ M TCEP, 4 μ M peptide, 2 mM CaCl₂, pH 7.4, 0.00075% (w/v)

400 LMNG, 0.00025% (w/v) GDN and 0.0002% (w/v) CHS. The monomeric peak fractions were

401 collected and concentrated to 4-6 mg/mL for electron microscopy experiments. Protein

402 concentration was determined by absorbance at 280 nm using a Nanodrop 2000

403 Spectrophotometer (ThermoFisher).

404

405 **Cryo-EM data acquisition**

406 For preparation of cryo-EM grids, 3 μ L of the purified MC1R-Gs complex was applied to a

407 glow-discharged holey carbon EM grid (Quantifoil, Au 300 R1.2/1.3) in a Vitrobot chamber

408 (FEI Vitrobot Mark IV). The Vitrobot chamber was set to 100% humidity at 4°C. The sample-

409 coated grids were blotted before plunge-freezing into liquid ethane and stored in liquid

410 nitrogen for data collection. Cryo-EM imaging was performed on a Titan Krios equipped with

411 a Gatan K3 Summit direct electron detector in the Center of Cryo-Electron Microscopy
412 Research Center, Shanghai Institute of Materia Medica, Chinese Academy of Sciences
413 (Shanghai, China). The microscope was operated at 300 kV accelerating voltage, at a nominal
414 magnification of 81,000 \times , corresponding to a pixel size of 1.045 Å. In total, 4,600 movies of
415 α -MSH-MC1R-Gs and 4,600 movies of afamelanotide-MC1R-Gs complexes were obtained at
416 a dose rate of about 22.3 electrons per Å² per second with a defocus range from -0.5 to -3.0
417 μ m. The total exposure time was 3.6 s and the intermediate frames were recorded in 0.1
418 intervals, resulting in an accumulated dose of 80 electrons per Å² and a total of 36 frames per
419 micrograph. For the SHU9119-MC1R-Gs complex, a total of 6,024 movies were collected
420 with a modified pixel of 1.071 Å. The images were obtained at a dose rate of about 22.3
421 electrons per Å² per second with a defocus range from -0.5 to -3.0 μ m. The total exposure
422 time was 3.2 s and the intermediate frames were recorded in 0.089 intervals, resulting in an
423 accumulated dose of 70 electrons per Å² and a total of 36 frames per micrograph.

424

425 **Cryo-EM data processing**

426 Dose-fractionated image stacks were subjected to beam-induced motion correction and dose-
427 weighting using MotionCor2.1⁶⁴. Contrast transfer function parameters for each micrograph
428 were determined by Gctf v1.18⁶⁵. Further data processing was performed with RELION-3.1-
429 beta2⁶⁶.

430

431 For the datasets of α -MSH-MC1R-Gs and afamelanotide-MC1R-Gs complexes, particle
432 selection, two-dimensional (2D) classification and three-dimensional (3D) classification were
433 performed on a binned dataset with a pixel size of 2.09 Å. For α -MSH-MC1R-Gs complex,
434 semi-automated selection yielded 4,151,805 particle projections that were subjected to three
435 rounds of reference-free 2D classification to discard false positive particles or particles
436 categorized in poorly defined classes, producing 2,281,404 particle projections for further
437 processing. A well-defined subset of 454,593 particle projections was selected after four
438 rounds of 3D classification and subsequently subjected to 3D refinement, CTF refinement,
439 and Bayesian polishing. The final map has an indicated global resolution of 3.0 Å for α -MSH
440 -MC1R-Gs complex at a Fourier shell correlation of 0.143. For afamelanotide-MC1R-Gs

441 complex, semi-automated selection yielded 3,968,825 particle projections that were subjected
442 to three rounds of reference-free 2D classification to discard false positive particles or
443 particles categorized in poorly defined classes, producing 2,068,327 particle projections for
444 further processing. Two subsets of 814,298 particle projections and 469,220 particle
445 projections were selected after two rounds of 3D classification. Further 3D classifications,
446 focusing on the alignment on the receptor, produced two good subsets of 460,989 particles
447 and 312,962 particles, respectively, which were subsequently subjected to 3D refinement,
448 CTF refinement, and Bayesian polishing. The final maps have an indicated global resolution
449 of 2.7 Å for afamelaonotide-MC1R-Gs-Nb35-scFv16 complex and 2.9 Å for afamelaonotide-
450 MC1R-Gs-scFv16 complex at a Fourier shell correlation of 0.143.

451

452 For the datasets of SHU9119-MC1R-Gs complex, particle selection, 2D classification and 3D
453 classification were performed on a binned dataset with a pixel size of 2.142 Å. Semi-
454 automated selection yielded 4,337,394 particle projections that were subjected to three rounds
455 of reference-free 2D classification to discard false positive particles or particles categorized in
456 poorly defined classes, producing 2,162,470 particle projections for further processing. A
457 well-defined subset of 502,722 particle projections was selected after four rounds of 3D
458 classification and subsequently subjected to 3D refinement, CTF refinement, and Bayesian
459 polishing. The final map has an indicated global resolution of 3.1 Å for SHU9119-MC1R-Gs
460 complex at a Fourier shell correlation of 0.143.

461

462 The maps were subsequently post-processed in DeepEMhancer⁶⁷. Local resolution was
463 determined using the ResMap with half maps as input maps and surface coloring of the
464 density map was performed using UCSF Chimera^{68,69}.

465

466 **Model building and refinement**

467 The crystal structure of SHU9119-MC4R complex (PDB: 6W25) was used as the initial
468 model of MC1R for model rebuilding and refinement against the electron microscopy map²⁶.

469 The cryo-EM structure of V2R-Gs complex (PDB: 7DW9) was used to generate the initial
470 model of Gs, Nb35 and scFv16⁷⁰. For the structure of afamelaonotide-MC1R-Gs and

471 SHU9119-MC1R-Gs complexes, the coordinates of α -MSH-MC1R-Gs complex were used as
472 an initial template. The models were docked into the electron microscopy density maps using
473 UCSF Chimera followed by iterative manual adjustment and rebuilding in Coot^{69,71}. Real
474 space refinement and rosetta refinement were performed using ISOLDE and Phenix software
475 package^{72,73}. All residues were checked for fitting in electron density, Ramachandran and
476 rotamer restraints. The model statistics was validated using the module ‘comprehensive
477 validation (cryo-EM)’ in Phenix. Finally, MC1R from L36^{ECD} to T308^{8.53}, α -MSH (residues
478 Y2-V11), afamelanotide (residues Y2-V11), the full-length SHU9119, and the calcium ion
479 were well defined in the EM maps. However, the fusion partner BRIL, LgBiT, ICL3 of MC1R
480 and AHD of G α s showed very poor density in the EM maps and were omitted from the final
481 models. Structural figures were prepared in UCSF Chimera, UCSF ChimeraX and PyMOL
482 (<https://pymol.org/2/>)⁷⁴. The final refinement statistics are provided in **Table S1**.

483

484 **Cell culture and transfection**

485 Chinese hamster ovary (CHO-K1) cells were cultured in F12 (Gibco) containing 10% (v/v)
486 fetal bovine serum (FBS, Gibco) at 37°C in 5% CO₂. Human embryonic kidney 293 cells
487 (HEK293) were maintained in DMEM (Gibco) supplemented with 10% (v/v) FBS, 1 mM
488 sodium pyruvate (Gibco), 100 units/mL penicillin and 100 μ g/mL streptomycin at 37°C in 5%
489 CO₂. For cAMP and MC1R expression level assays, CHO-K1 cells were seeded into 6-well
490 cell culture plates at a density of 5×10^5 cells per well. For whole cell binding assay, HEK293
491 cells were seeded into 96-well poly-D-lysine-treated cell culture plates at a density of 3×10^4
492 cells per well. After overnight incubation, cells were transfected with different MC1R
493 constructs using FuGENE[®] HD transfection reagent (Promega) for cAMP accumulation assay,
494 or Lipofectamine 2000 transfection reagent (Invitrogen) for binding and flow cytometry
495 assays, respectively. Following 24 h culturing, the transfected cells were ready for detection.

496

497 **cAMP accumulation assay**

498 α -MSH, afamelanotide and SHU9119 stimulated cAMP accumulation was measured by
499 LANCE Ultra cAMP kit (PerkinElmer). Twenty-four hours post-transfection, CHO-K1 cells
500 were washed and seeded into 384-well microtiter plates at a density of 3,000 cells per well.

501 Then they were incubated with different concentrations of ligands in stimulation buffer
502 (calcium and magnesium free HBSS buffer (Gibco), 5 mM HEPES (Gibco), 0.1% BSA
503 (Abcone) and 0.5 mM IBMX (Abcone)) for 40 min at RT. Eu-cAMP tracer and ULight-anti-
504 cAMP were diluted by cAMP detection buffer and added to the plates separately to terminate
505 the reaction. Plates were incubated at RT for 40 min and read according to the protocol using
506 an EnVision multilabel reader (PerkinElmer) with the emission window ratio of 665 nm over
507 620 nm. Data were normalized to the wild-type (WT) receptor.

508

509 For assessing the effect of calcium ion on cAMP signaling, CHO-K1 cells were dissociated by
510 0.02% (w/v) EDTA and washed three times with calcium and magnesium free HBSS buffer.
511 Then the cells were resuspended and stimulated with different concentrations of ligands in
512 Ca^{2+} free stimulation buffer consisting of aforementioned stimulation buffer supplemented
513 with 1 mM EGTA, or with additional 1.5 mM CaCl_2 in Ca^{2+} free stimulation buffer ($[\text{Ca}^{2+}] \sim$
514 0.5 mM)²⁶. The rest steps were essentially the same as described above.

515

516 **Whole cell binding assay**

517 Radiolabeled ligand binding assays were performed using the whole cell method. In brief,
518 HEK293 cells were harvested 24 h after transfection, washed twice and incubated with
519 blocking buffer (F12 supplemented with 25 mM HEPES and 0.1% BSA, pH 7.4) for 2 h at
520 37°C. The homogeneous competition binding experiments were conducted by incubating
521 constant concentration of [¹²⁵I]-[Nle⁴, D-Phe⁷]- α -MSH (30 pM, PerkinElmer) with serial
522 dilution of unlabeled ligands [α -MSH (2.38 pM to 5 μM); afamelanotide (2.38 pM to 5 μM);
523 and SHU9119 (2.38 pM to 5 μM)] in binding buffer (DMEM supplemented with 25mM
524 HEPES and 0.1% BSA). For the effect of divalent cations on ligand binding, the cells were
525 incubated with 1 mM EGTA (PBS supplemented with 1% BSA) for 2 h to neutralize divalent
526 cations in medium before addition of 30 pM [¹²⁵I]-[Nle⁴, D-Phe⁷]- α -MSH and varying
527 concentrations of CaCl_2 and MgCl_2 . The reactions were carried out for 3 h at 37°C and
528 terminated by washing three times with ice-cold PBS. The bound radioactivity was measured
529 with a MicroBeta2 plate counter (PerkinElmer) using a scintillation cocktail (OptiPhase
530 SuperMix, PerkinElmer).

531

532 **NanoBiT assay**

533 HEK293A cells (G protein knockout) were seeded into 10-cm plates at a density of 3×10^6
534 cells per plate and transfected with the plasmid mixture containing 2 μ g WT MC1R, 1 μ g
535 Gas-LgBiT, 5 μ g WT G β or G β with mutations F292A and D312A, and 5 μ g SmBiT-G γ using
536 Lipofectamine 3000 transfection reagent (Invitrogen). After 24 h, the cells were transferred to
537 poly-D-lysine coated 96-well plates at a density of 50,000 cells/well and grown overnight
538 before incubation in NanoBiT buffer (calcium and magnesium free HBSS buffer,
539 supplemented with 10 mM HEPES and 0.1% BSA, pH 7.4) in 37°C for 30 min. Then 10 μ L
540 coelentrastazine-h (Yeasen Biotech) was added to each well at a working concentration of 5 μ M
541 followed by incubation for 2h at room temperature. The luminescence signal was measured
542 using an EnVision plate reader (PerkinElmer) at 30 s interval for 4 min as baseline, and then
543 read for 10 min after addition of ligand. Data were corrected to baseline measurements and
544 then the vehicle control to determine ligand-induced changes in response. Dose-response
545 values were obtained from the area-under-the-curve of elicited responses by each ligand.

546

547 **Receptor expression**

548 Membrane expression of MC1R was determined by flow cytometry to detect the N-terminal
549 Flag tag on the WT and mutated receptor constructs transiently expressed in CHO-K1 cells.
550 Briefly, approximately 2×10^5 cells were blocked with PBS containing 5% BSA (w/v) at RT
551 for 15 min, and then incubated with 1:300 anti-Flag primary antibody (diluted with PBS
552 containing 5% BSA, Sigma) at RT for 1 h. The cells were then washed three times with PBS
553 containing 1% BSA (w/v) followed by 1 h incubation with 1:1000 anti-mouse Alexa Fluor
554 488 conjugated secondary antibody (diluted with PBS containing 5% BSA, Invitrogen) at RT
555 in the dark. After washing three times, cells were resuspended in 200 μ L PBS containing 1%
556 BSA for detection by NovoCyte (Agilent) utilizing laser excitation and emission wavelengths
557 of 488 nm and 530 nm, respectively. For each sample, 20,000 cellular events were collected,
558 and the total fluorescence intensity of positive expression cell population was calculated. Data
559 were normalized to WT receptor and parental CHO-K1 cells.

560

561 **Data analysis**

562 Dose-response data were analyzed using Prism 8 (GraphPad). Non-linear curve fit was
563 performed using a three-parameter logistic equation [log (agonist vs. response)]. All data are
564 presented as means \pm S.E.M. of at least three independent experiments. Statistical
565 significance was determined by Dunnett's test.

566

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580

581 **AUTHOR CONTRIBUTIONS**

582 S.M. designed the expression constructs, optimized and purified the MC1R-Gs protein
583 complexes, prepared the cryo-EM grids, collected the cryo-EM images, performed the
584 structure determination and model building, participated in the preparation of the constructs
585 for functional assays, analyzed the structures, prepared the figures and wrote the manuscript;
586 Y.C. prepared the constructs for functional assays, performed the cAMP accumulation and
587 MC1R surface expression assays, participated in the figure preparation; A.D. performed the
588 whole cell binding assay; W.Y. designed the Gas construct and participated in the cryo-EM
589 grids preparation. J.G. and F.Z. participated the data analysis and manuscript editing; D.Y.
590 performed the data analysis and participated in the manuscript editing; Y.J. participated in

591 project supervision and manuscript editing; M.-W.W. oversaw the work of C.Y., A.D. and
592 D.Y. and participated in the manuscript writing; H.E.X. conceived and supervised the project,
593 analyzed the structures, and wrote the manuscript with input from all co-authors.

594

595 **CONFLICT OF INTERESTS**

596 The authors declare no conflict of interests.

597

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- 793

794 **FIGURE LEGENDS**

795

796 **Fig. 1 Cryo-EM structures of the MC1R-Gs complexes.** **a** The left panel shows differential
797 activities of α -MSH, afamelanotide and SHU9119 on MC1R and MC4R. The thickness of
798 lines indicates the strength of affinity. The right panel is the sequence alignment of
799 melanocortins with two synthetic peptides afamelanotide and SHU9119. The C-terminal
800 residues of ACTH were omitted for clarify and the highly conserved HFRW motif is marked
801 by purple stars. **b-e** Cryo-EM density maps (left panel) and cartoon representation (right
802 panel) of the α -MSH-MC1R-Gs-Nb35 complex (**b**), SHU9119-MC1R-Gs-Nb35 complex (**c**),
803 afamelanotide-MC1R-Gs-Nb35-scFv16 complex (**d**), and afamelanotide-MC1R-Gs-scFv16
804 complex (**e**). α -MSH is shown in olive, SHU9119 in dark orange, afamelanotide in magenta
805 and rosy brown in both complexes. The corresponding MC1R is shown in medium purple, hot
806 pink, orange red and salmon, respectively. Calcium ion is shown in lime, G α s in gold, G β in
807 cornflower blue, G γ in light sea green, Nb35 in dark gray, and scFv16 in purple.

808

809 **Fig. 2 The orthosteric peptide binding pocket of MC1R.** **a** Structural comparison of α -
810 MSH bound MC1R, afamelanotide bound MC1R and SHU9119 bound MC1R. ECD and
811 ECL3 of MC1R were omitted for clarify and the alignment was based on the receptor. **b-g**
812 Side views (**b-d**) and top views (**e-g**) of the orthosteric binding pocket in α -MSH bound
813 MC1R complex (**b, e**), afamelanotide bound MC1R complex (**c, f**) and SHU9119 bound
814 MC1R complex (**d, g**). Calcium ion is displayed in sphere and colored in lime. The EM
815 density maps of α -MSH, afamelanotide, SHU9119 and calcium ion are shown at 0.08
816 threshold.

817

818 **Fig. 3 Molecular recognition of α -MSH, afamelanotide and SHU9119 by MC1R.** **a-f** Two
819 different views of the detailed interactions between α -MSH and MC1R (**a, d**), afamelanotide
820 and MC1R (**b, e**), SHU9119 and MC1R (**c, f**). **a-c** show interactions of His⁶ and Phe^{M7}/D-
821 Phe^{F7}/D-Nal^{U7} in peptide ligands with MC1R. **d-f** depict interactions of Arg⁸ and Trp⁹ in
822 peptide ligands with MC1R. C267^{ECL3} of MC1R forms a conserved disulfide bond with
823 C273^{ECL3}. Hydrogen bonds are shown as red dash lines. **g-i** Schematic diagrams of the

824 interactions between α -MSH and MC1R (**g**), afamelanotide and MC1R (**h**), SHU9119 and
825 MC1R (**i**). The highly conserved residues in peptides are surrounded by black circles. **j**
826 Effects of mutations in the orthosteric binding pocket of MC1R on α -MSH, afamelanotide
827 and SHU9119 induced cAMP accumulation. Values are presented as means \pm S.E.M. from at
828 least three independent experiments performed in quadruplicate. All data were analyzed by
829 one-way ANOVA and Dunnett's test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$.
830 NS, not significant (comparison between the wild-type (WT) MC1R and its mutants).

831

832 **Fig. 4 Structural basis of differential activities among melanocortin peptide ligands. a-b**

833 Structural comparison of α -MSH bound MC1R complex (**a-b**), afamelanotide bound MC1R
834 complex (**a**), SHU9119 bound MC1R complex (**b**) and SHU9119 bound MC4R complex
835 (PDB: 6W25, **a-b**). The conformational changes of W254^{6,48}, F257^{6,51} and F280^{7,35} upon
836 MC1R activation are emphasized. The alignment was based on the receptors and MC1R
837 residue numbers are colored in medium purple. **c** Comparison of SHU9119-binding pocket in
838 MC1R and MC4R. M128^{3,36} of MC1R forms steric clash with D-Nal^{U7} and W254^{6,48}, making
839 SHU9119 a partial agonist for MC1R. Hydrogen bonds are shown as red dash lines.

840

841 **Fig. 5 The calcium binding pocket of melanocortin receptors. a-c** Expanded views of the

842 calcium ion binding pocket in α -MSH bound MC1R complex (**a**), afamelanotide bound
843 MC1R complex (**b**), SHU9119 bound MC1R and MC4R complexes (**c**). The alignment was
844 based on the structures of MC1R and MC4R. **d** Sequence alignment of melanocortin
845 receptors with other class A GPCRs from different branches of the rhodopsin family. The
846 residues involved in the calcium binding pocket are highlighted in green and the cysteines
847 forming conserved disulfide bonds are highlighted in yellow.

848

849 **Fig. 6 Activation of MC1R by peptide ligands. a-c** Structural alignment of the SHU9119

850 bound MC1R and MC4R complexes (PDB: 6W25). The alignment was based on the
851 structures of MC1R and MC4R, which are colored hot pink and dark gray, respectively. **a** side
852 view; **b** extracellular view; **c** intracellular view. **d-g** Conformational changes of the conserved
853 "micro-switches" upon receptor activation. **d** Toggle switch; **e** MLF motif; **f** DRY motif; **g**

854 DPxxY motif. The conformational changes of residue side chains are shown as arrows.

855 Hydrogen bonds are shown as red dash lines.

856

857 **Fig. 7 G protein coupling of MC1R.** **a** The interactions between MC1R (medium purple)
858 and $\alpha 5$ helix of *G α s* (gold) in the cavity at the cytoplasmic region of MC1R. **b** The
859 interactions between ICL2 of MC1R and *G α s*. ICL2 inserts into the groove formed by α N- β 1
860 hinge, β 2- β 3 loop and $\alpha 5$ helix of *G α s*. **c** The interactions between the C terminus of TM5 and
861 $\alpha 4$ helix, $\alpha 4$ - $\beta 6$ loop and $\beta 6$ sheet of *G α s*. **d** The interactions between ICL1 of MC1R and *G β* .
862 **e** Comparison of *Gs* protein coupling between afamelanotide-MC1R-*Gs*-Nb35-scFv16
863 complex and afamelanotide-MC1R-*Gs*-scFv16 complex. The alignment was based on the
864 receptor. Differences are in the α N helix of *G α s* and the interactions between helix 8 of
865 MC1R and *G β* .

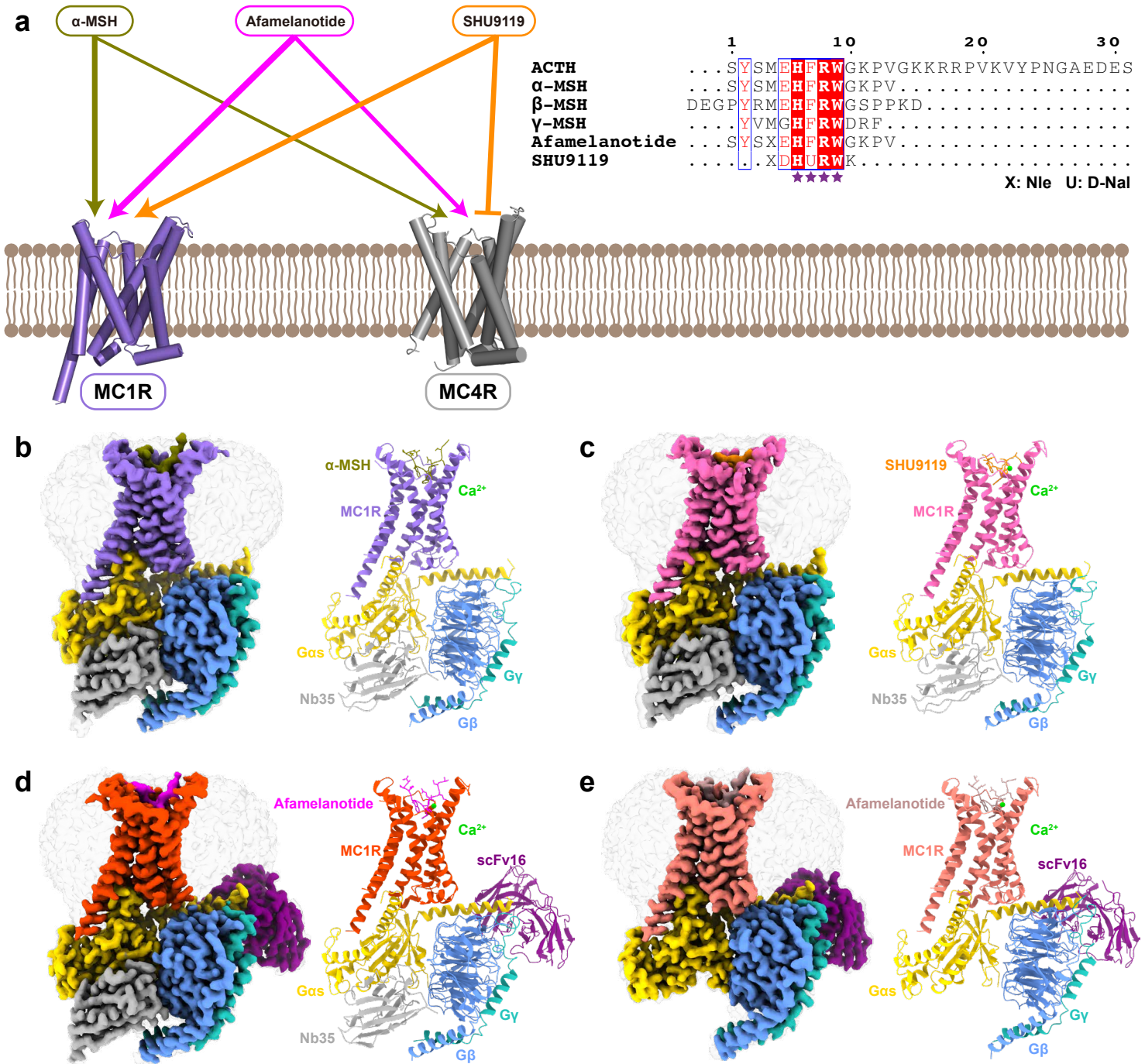


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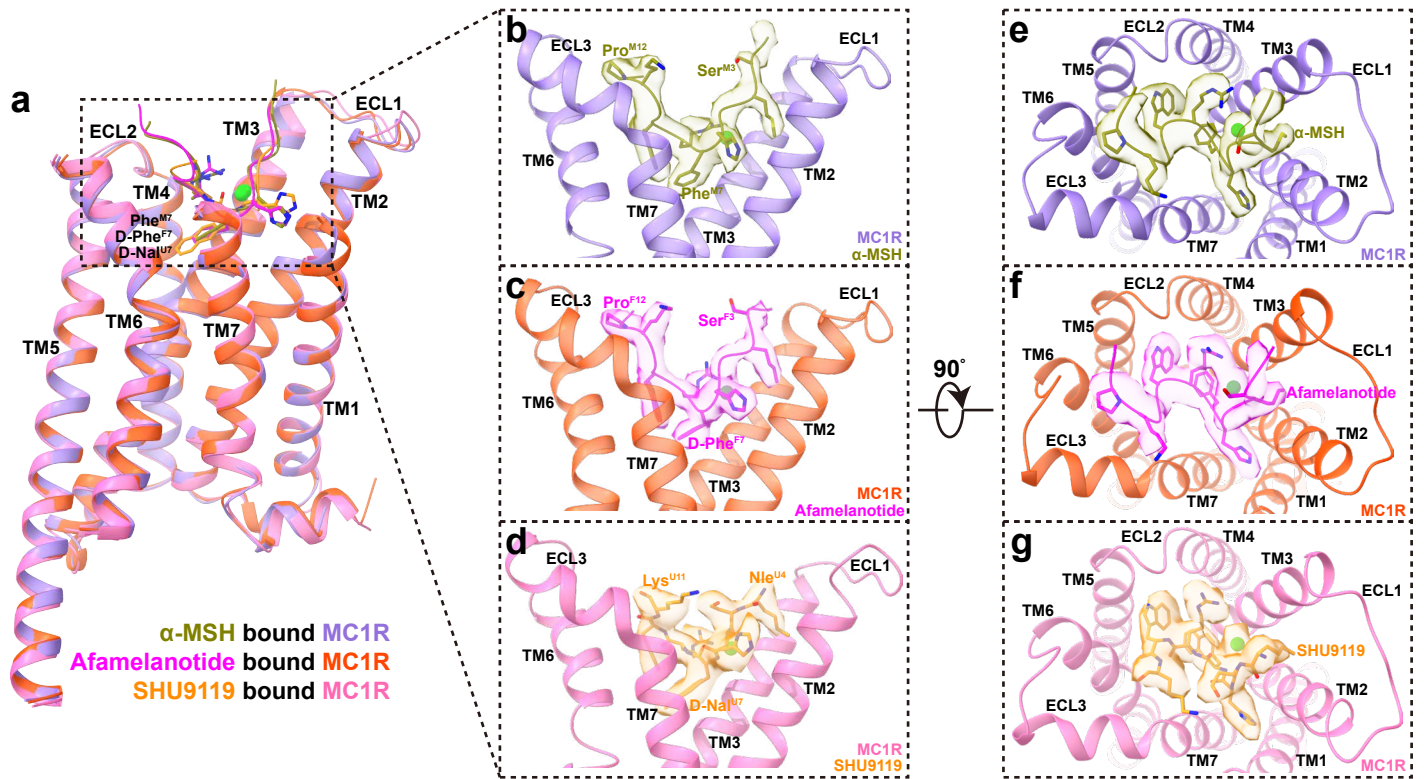


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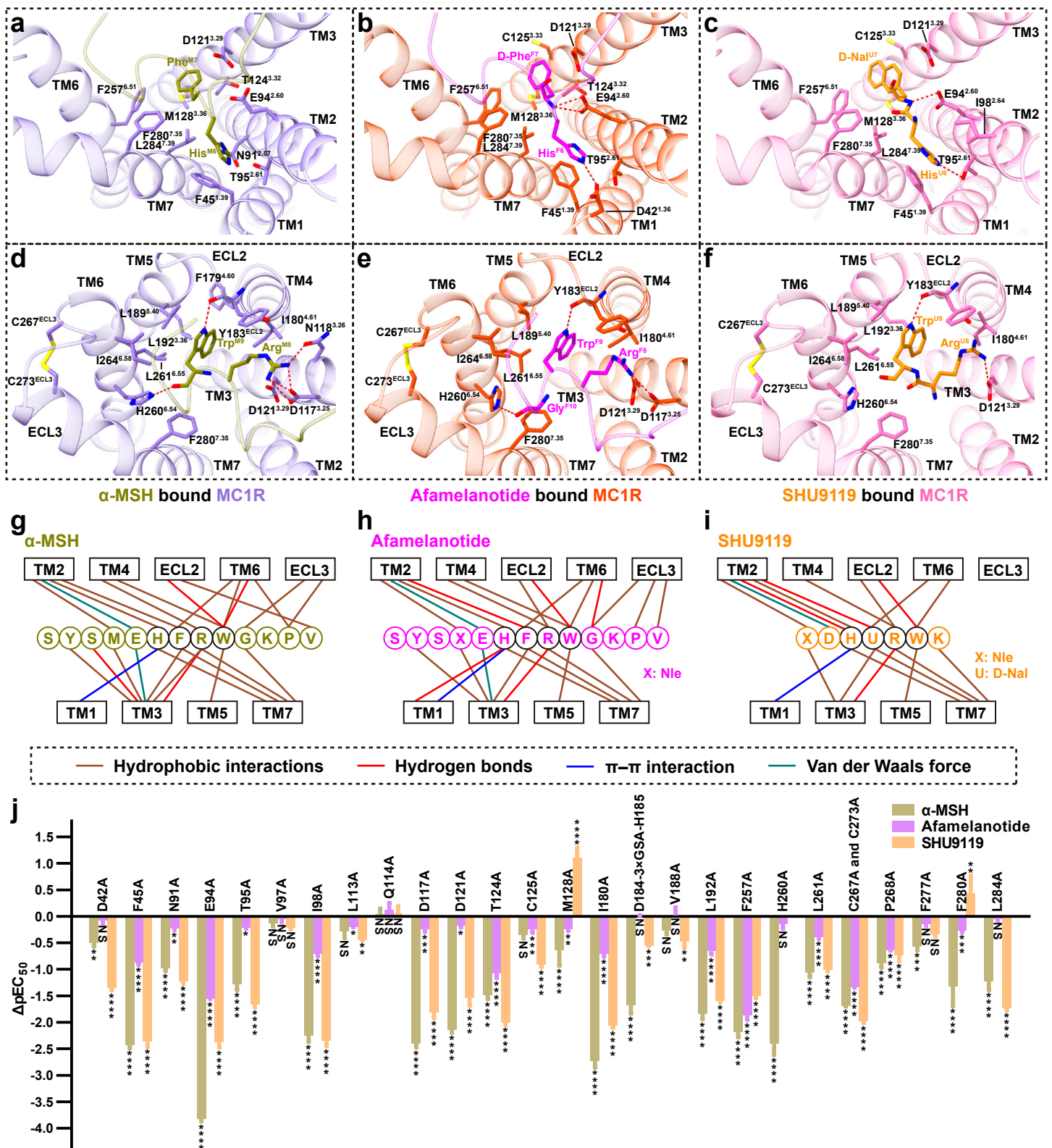


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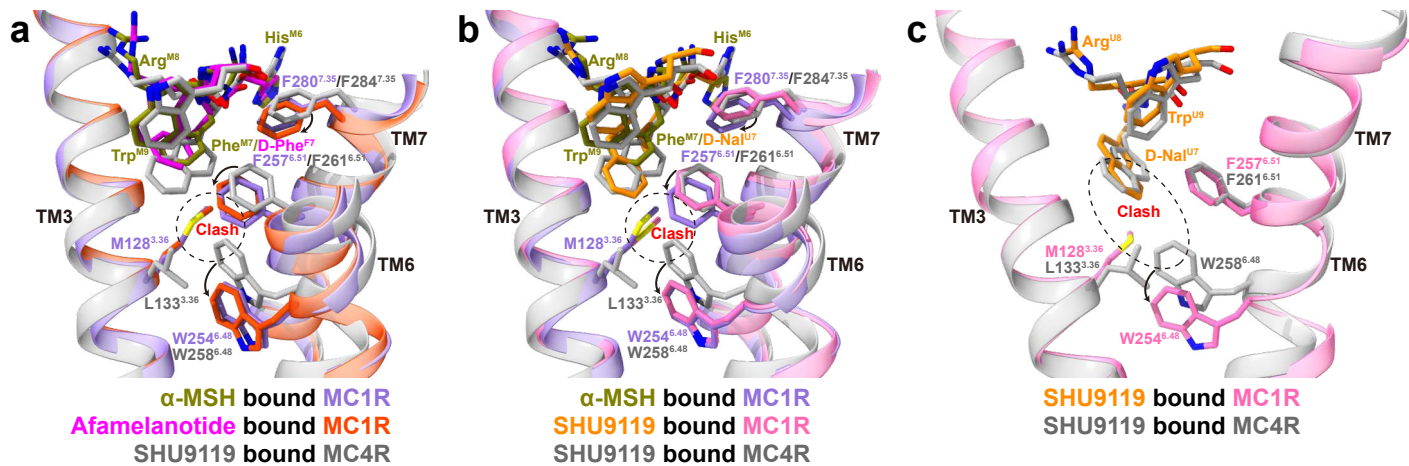


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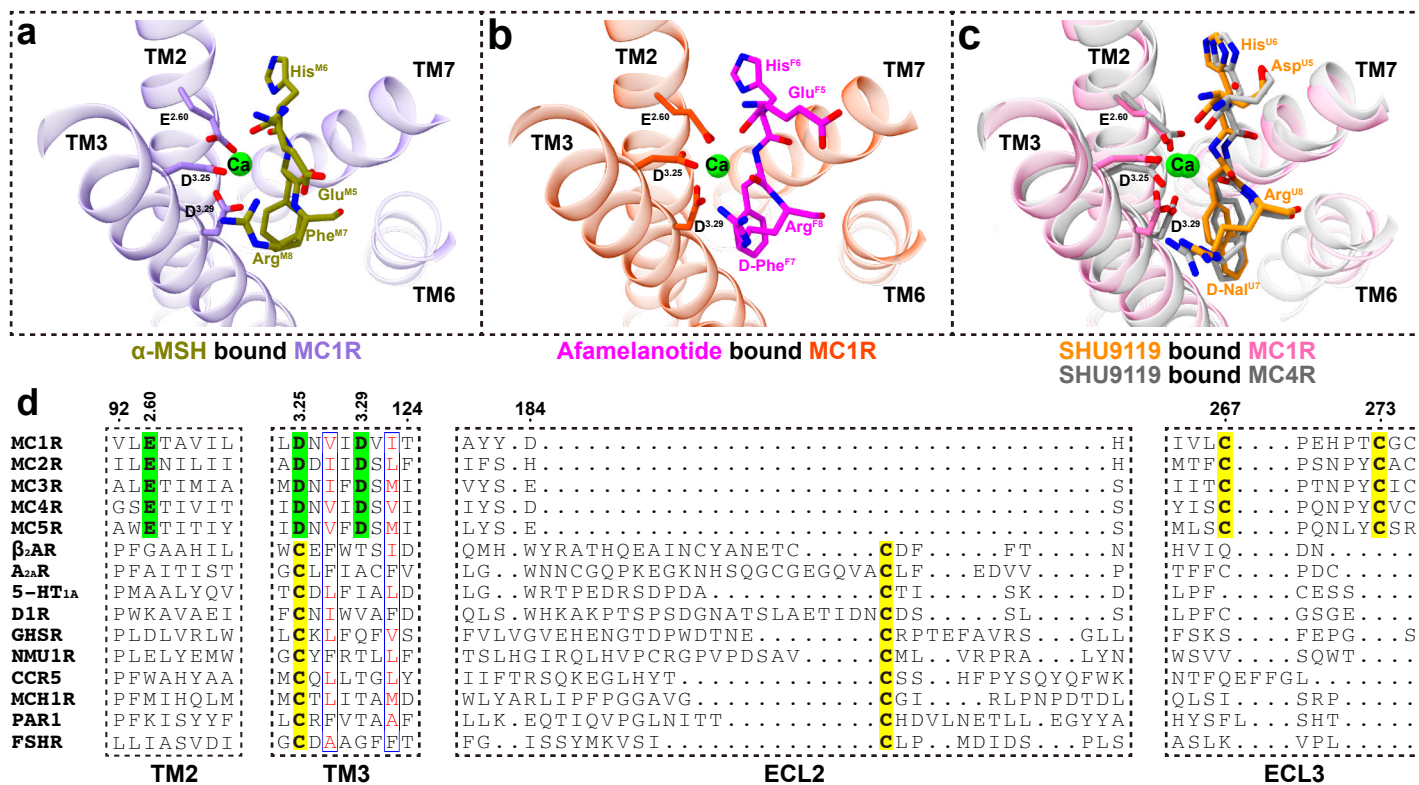


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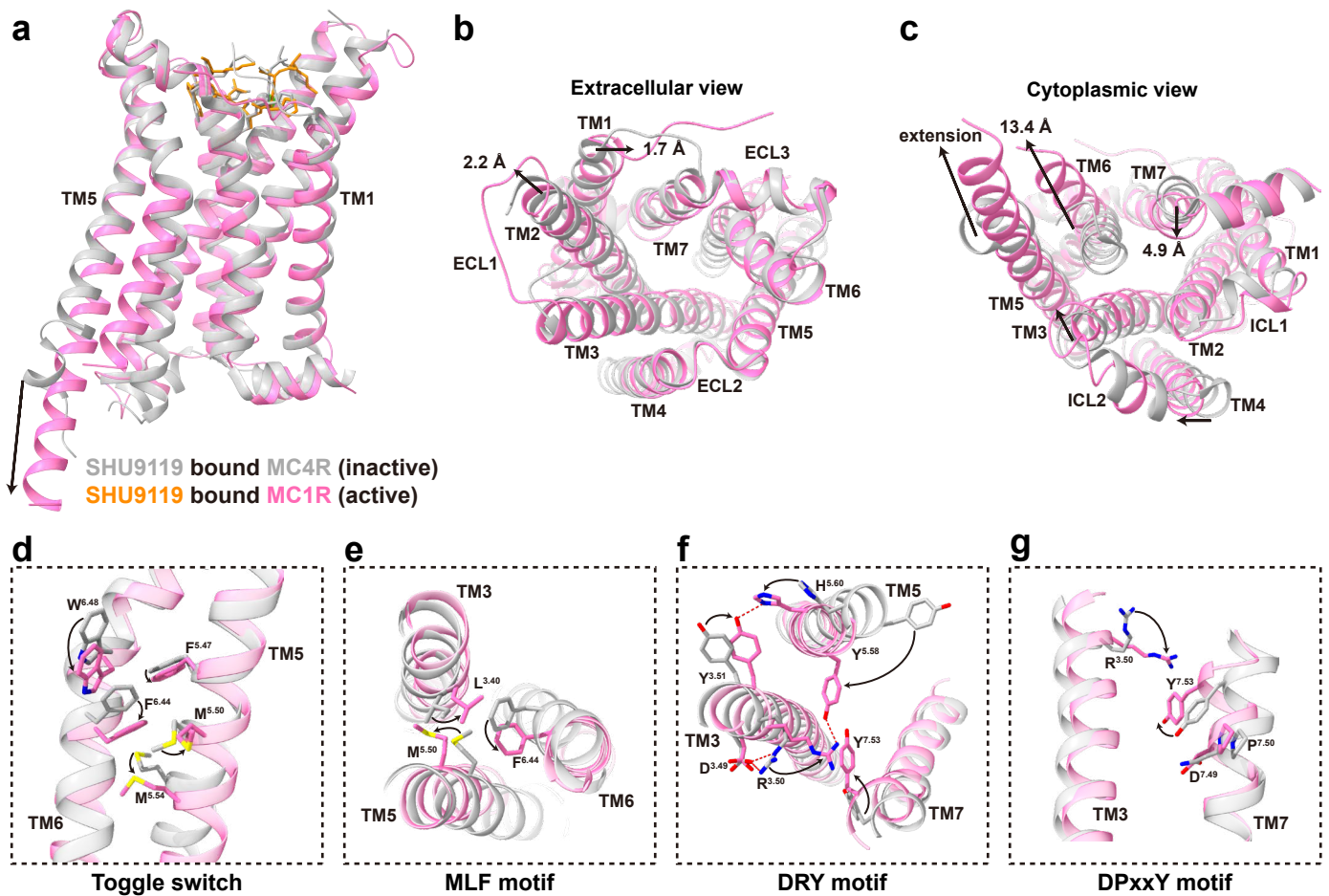


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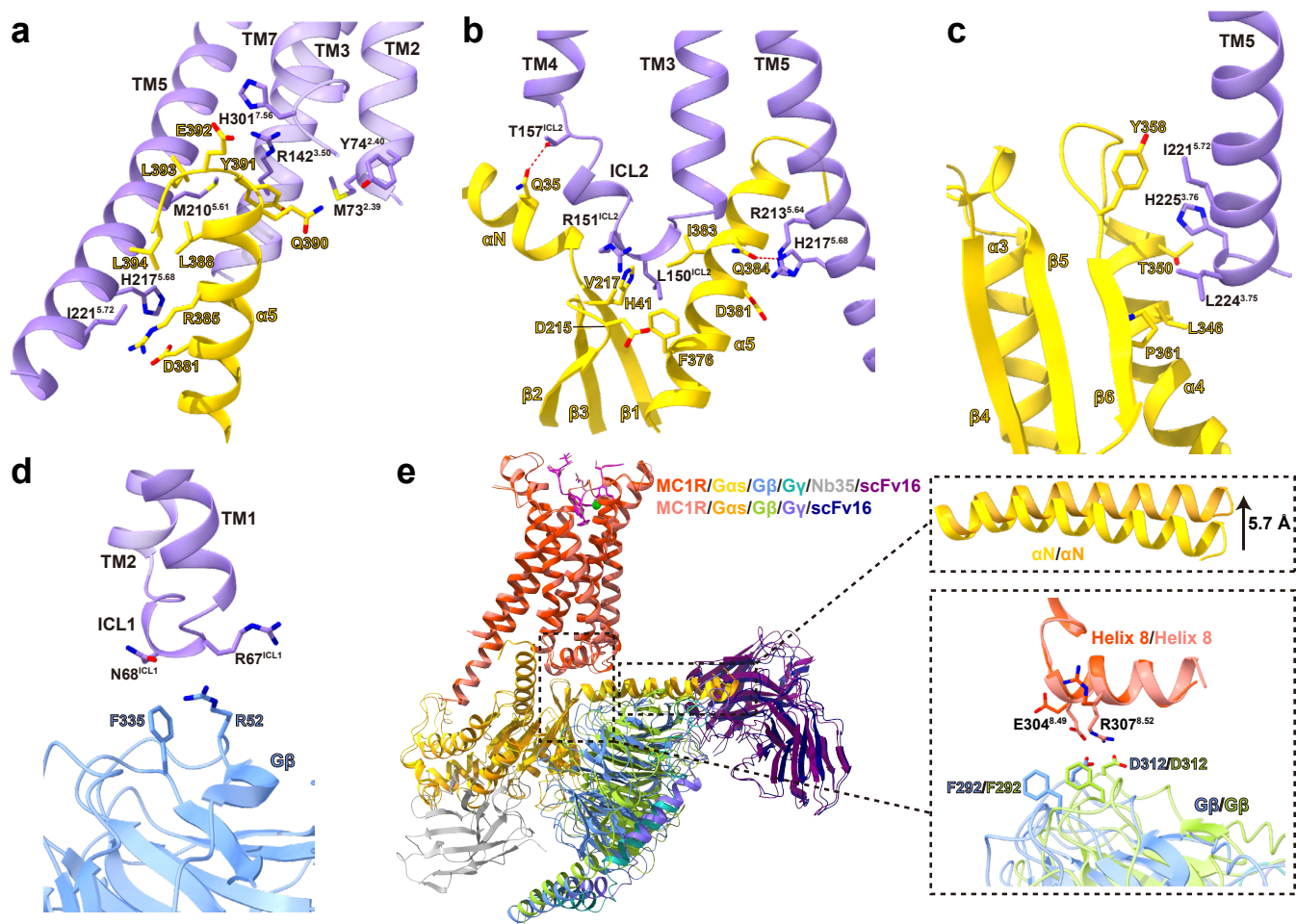


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