

1 **A basal actinopterygian melanocortin receptor: molecular and functional**
2 **characterization of an Mc2r ortholog from the Senegal bichir (*Polypterus***
3 ***senegalus*)**

4

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16 **RUNNING HEAD**

17

18 Bichir Mc2r

19 **ABSTRACT**

20

21 In bony vertebrates, melanocortin 2 receptor (Mc2r) specifically binds
22 adrenocorticotropic hormone (ACTH) and is responsible for mediating anterior pituitary
23 signaling that stimulates corticosteroid production in the adrenal gland/interrenal cells.
24 In bony fishes Mc2r requires the chaperoning of an accessory protein (Mrap1) to traffic
25 to the membrane surface and bind ACTH. Here, we evaluated the structure and
26 pharmacological properties of Mc2r from the Senegal bichir (*Polypterus senegalus*),
27 which represents the most basal bony fish from which an Mc2r has been
28 pharmacologically studied to date. In our experiments, cDNA constructs of the Mc2r
29 from the Senegal bichir (sbMc2r) and various vertebrate Mrap1s were heterologously
30 co-expressed in Chinese hamster ovary (CHO) cells, stimulated by ACTH or
31 melanocyte-stimulating hormone (α -MSH) ligands, and assessed using a luciferase
32 reporter gene assay. When expressed without an Mrap1, sbMc2r was not activated by
33 ACTH. When co-expressed with Mrap1 from either chicken (*Gallus gallus*) or bowfin
34 (*Amia calva*), sbMc2r could be activated in a dose-dependent manner by ACTH, but not
35 α -MSH. Co-expression of sbMrap2 with sbMc2r resulted in no detectable activation of
36 the receptor. Collectively, these results demonstrate that sbMc2r has pharmacological
37 properties similar to those of Mc2rs of later-evolved bony fishes, such as Mrap1
38 dependence and ACTH selectivity, indicating that these qualities of Mc2r function are
39 ancestral to all bony fish Mc2rs.

40

41 **OTHER KEY WORDS**

42

43 endocrinology, evolution, stress, Mc2r, Mrap1, chondrostean fish

44 **1. INTRODUCTION**

45

46 Melanocortin receptors are members of the large rhodopsin family of seven-pass
47 transmembrane G protein-coupled receptors (Cone, 2006; Ramachandrappa et al.,
48 2013). Among bony vertebrates, there are five known melanocortin receptors (Mc1r,
49 Mc2r, Mc3r, Mc4r, and Mc5r) which exhibit varying degrees of selectivity to the
50 proopiomelanocortin (POMC)-derived peptides adrenocorticotropin hormone (ACTH)
51 and melanocyte-stimulating hormone (α -MSH) (Nakanishi et al., 1979). Mammalian
52 Mc1r, Mc3r, Mc4r, and Mc5r can all be activated by both ACTH and α -MSH ligands
53 (Cone, 2006). However, Mc2r can only be activated by ACTH and is designated as the
54 "ACTH receptor" (Mountjoy et al., 1992).

55 As a receptor for ACTH, Mc2r is important in mediating hypothalamic-pituitary-
56 adrenal/interrenal (HPA/HPI) signaling, wherein the adrenal gland of later-evolved
57 tetrapods is homologous to the interrenal tissue associated with the kidney of
58 amphibians and fishes. In the HPI axis of fishes, corticotropin releasing factor (CRF) is
59 secreted from the hypothalamus to the pituitary causing the release of ACTH into the
60 vascular system (Wendelaar Bonga, 1997). At the interrenal cells, ACTH binds to Mc2r
61 to initiate steroid biosynthesis resulting in the production and release of corticosteroids
62 (Dores and Garcia, 2015). Comparative pharmacological investigations on Mc2r have
63 described many functional qualities that appear to have been derived throughout
64 vertebrate evolution (Dores and Chapa, 2021). Of the bony vertebrates (class
65 Osteichthyes) studied to date, Mc2r exclusively binds ACTH and requires cooperative
66 interaction with a melanocortin receptor accessory protein (Mrap1) for membrane
67 trafficking and activation by ACTH (Dores and Chapa, 2021). However, these qualities
68 of bony vertebrate Mc2r function appear to be derived (Dores and Chapa, 2021).

69 In the group of jawed cartilaginous fishes (class Chondrichthyes), comprised of
70 the elasmobranchs (i.e., sharks, skates, rays) and holocephalans (i.e., chimaeras)
71 (Nelson et al., 2016), the reliance of Mc2r on Mrap1 chaperoning and modulation is less
72 apparent or altogether absent. The Mc2r ortholog of the stingray (*Dasyatis akajei*)
73 (srMc2r) can be activated by both ACTH and α -MSH at non-physiological
74 concentrations. Although co-expression with Mrap1 is not required for srMc2r activation,

75 it does greatly increase the sensitivity of srMc2r to stimulation by ACTH (Dores et al.,
76 2018; Takahashi et al., 2016). Similar observations were made for the Mc2r ortholog of
77 the whale shark (*Rhincodon typus*) (wsMc2r), for which it was explained that Mrap1 had
78 no effect on wsMc2r activation, but enhanced trafficking of wsMc2r to the plasma
79 membrane (Hoglin et al., 2022). Together, these studies in elasmobranchs suggest that
80 the lack of selectivity for ACTH and the increased sensitivity of Mc2r for ACTH by Mrap1
81 chaperoning are shared features of Mc2rs in elasmobranchs. Similar studies have been
82 performed in the elephant shark (*Callorhincus milli*), a representative of the sister group
83 to the elasmobranchs, the holocephalans. Like in the elasmobranchs, the Mc2r ortholog
84 of the elephant shark (esMc2r) can be activated by both ACTH and α -MSH, but the
85 sensitivity of esMc2r to ACTH was not appreciably affected by co-expression with
86 Mrap1 (Barney et al., 2019).

87 Conspicuously absent from previous comparative studies on Mc2r orthologs
88 were representatives from the subclass Chondrostei (e.g. bichir, ropefish, paddlefish,
89 sturgeon) (Nelson et al., 2016). In the present study, we sought to evaluate the
90 pharmacological properties of the Mc2r from the Senegal bichir (*Polypterus senegalus*)
91 (sbMc2r). Bichirs are members of the family Polypteridae (order Polypteriformes), which
92 represent the most basal order of ray-finned fishes (class Actinopterygii) (Carroll, 1988).
93 As there have been no functional studies on Mc2r from any of the lobe-finned fishes
94 (the basal representatives of the sister class of bony vertebrates, Sarcopterygii), sbMc2r
95 represents an Mc2r that may be more closely related to the Mc2rs of cartilaginous
96 fishes than any other Mc2r studied to date (Betancur et al., 2017). Indeed, functional
97 studies of Mc2rs from the most basal bony vertebrates (including bichir and lobe-finned
98 fishes) may facilitate an enhanced understanding of how the functional qualities of the
99 bony vertebrate Mc2r arose. Our primary hypothesis was that sbMc2r would exhibit
100 functional qualities characteristic of the Mc2rs of more derived actinopterygians,
101 including ACTH selectivity and Mrap1 dependence. However, we have made the
102 observation that a sequence for an *mrap1* gene is apparently absent from the recently
103 published *P. senegalus* genome assembly (Bi et al., 2021). This observation has raised
104 the possibility that sbMc2r may have properties more similar to the cartilaginous fish
105 Mc2r, which lacks Mrap1-dependance and is capable of being activated by either ACTH

106 or α -MSH (Hoglin et al., 2022; Reinick et al., 2012a). Hence, this study tested an
107 alternative hypothesis that sbMc2r may be an Mrap1-independent Mc2r ortholog that is
108 capable of being activated by either ACTH or α -MSH.

109

110 2. MATERIALS AND METHODS

111

112 2.1 Sequence discovery and analyses

113 The genome assembly of *P. senegalus* is available from the National Center for
114 Biotechnology Information (NCBI) GenBank (assembly ASM1683550v1; Bi et al., 2021).
115 Using the BLAST (Basic Local Alignment Search Tool) from NCBI, we surveyed the *P.*
116 *senegalus* assembly by querying with nucleotide sequences of *mc2r*, *mrap1*, and *mrap2*
117 orthologues from several species of basal bony fishes for which sequences for these
118 genes are available, including spotted gar (*Lepisosteus oculatus*) and bowfin (*Amia*
119 *calva*). Amino acid sequences for sbMc2r and sbMrap2 were deduced using the
120 Translate tool from ExPASy (<https://www.expasy.org>). Hypothetical membrane
121 topology of sbMc2r was predicted using the TMHMM tool from the DTU Bioinformatics
122 Server (<https://www.bioinformatics.dtu.dk>). Multiple sequence alignment and
123 phylogenetic analyses were carried out using a selection of gnathostome Mc2r and
124 Mrap amino acid sequences available from NCBI GenBank. Sequence alignments were
125 performed using the Clustal Omega multiple sequence alignment tool available from the
126 European Bioinformatics Institute (<https://www.ebi.ac.uk/tools/msa/>), which was also
127 used to obtain percent sequence identities. Multiple sequence alignments were
128 arranged using BioEdit software (Hall, 1999), with modifications to ensure the alignment
129 of functional motifs following previously described methods (Dores et al., 1996).
130 Phylogenetic analyses using the maximum parsimony method (1,000 bootstrap
131 replicates) were implemented using MEGA10 software (Kumar et al., 2008).

132 Accession numbers for the amino acid sequences used in our analyses were:
133 elephant shark Mc2r (FAA00704), whale shark Mc2r (XP_020380838), stingray Mc2r
134 (BAU98231), gar Mc2r (XP_006636159), rainbow trout Mc2r (ABV23494), carp Mc2r
135 (CAE53845), zebrafish Mc2r (AAO24743), lungfish Mc2r (XP_043923917), frog Mc2r
136 (XP_002936118), chicken Mc2r (AGR42637), mouse Mc2r (NP_001288301), human

137 Mc2r (NP_001278840), whale shark Mrap1 (XP_020375601), whale shark Mrap2
138 (XP_020377388), chicken Mrap1 (XR_001470382), chicken Mrap2 (XP_046770071),
139 mouse Mrap1 (NP_084120), and mouse Mrap2 (NP_001346884). Sequences for
140 bowfin (bf) Mc2r, Mrap1, and Mrap2 were obtained as a gift from the authors of the
141 recently published bowfin genome (Thompson et al., 2021).

142

143 *2.2 Cell Culture, Transfection, and Reporter Gene Assay*

144 Using previously described methods (Hoglin et al., 2022; Liang et al., 2011;
145 Reinick et al., 2012b), sbMc2r receptor activation by melanocortin ligands was analyzed
146 by transfecting cDNA constructs of the bichir *mc2r* and various vertebrate *mrap1s* into
147 Chinese hamster ovary (CHO) cells, then using a luciferase reporter gene assay to
148 indirectly measure cAMP production resulting from receptor activation by melanocortin
149 ligands. CHO cells were selected for this project because this cell line does not
150 endogenously express Mc2r or Mrap1 proteins (Noon et al., 2002; Reinick et al., 2012b;
151 Sebag and Hinkle, 2007).

152 Transfection was performed using cDNA constructs that were commercially
153 obtained as inserts on a pcDNA3.1+ expression vector (GenScript; Piscataway, NJ).
154 Plasmid vectors (2 µg per 1×10^5 cells) were transiently transfected into CHO cells
155 (ATCC; Manassas, VA) using a Solution T kit for the Amaxa Nucleofector 2b system
156 (Lonza; Portsmouth, NH). This system is quoted by the manufacturer as delivering
157 efficiencies of ~80% (<http://bioscience.lonza.com>), and has been empirically shown to
158 produce such efficiencies (Hannes et al., 2010; Maasho et al., 2004). Our lab has used
159 this system to successfully transfet CHO cells for many years (Liang et al., 2011;
160 Reinick et al., 2012a, 2012b), and we confirm the success of our transfections using
161 positive controls for each functional assay we run. Although the particular arrangement
162 of *mc2r* and *mrap* constructs that were co-transfected varied by experiment, all
163 transfections included the co-transfection of a cAMP reporter construct (*luciferase* gene
164 promoted by a cAMP responsive element (CRE-Luciferase); transfected at 2.5 µg per
165 1×10^5 cells) (Chepurny and Holz, 2007), totaling a maximum of 3 simultaneous
166 transfections for any experiment. After transfection, CHO cells were seeded in triplicate
167 wells in opaque 96-well cell culture plates (Cat. No. 3912; Corning Life Sciences;

168 Manassas, VA) at a density of 3×10^5 cells cm^{-2} and incubated at 37 °C with 5% CO_2 for
169 2 d in a DMEM/F12 media (Cat. No. 11320-033; Gibco, UK) with 10% fetal calf serum
170 and 1% penicillin-streptomycin. After 48 h in incubation post-transfection, the culture
171 media was removed, and transfected cells were stimulated with either human ACTH(1–
172 24) (hereafter referred to as ACTH) or α -MSH (New England Peptide, Gardiner, MA)
173 diluted to concentrations ranging from 10^{-6} – 10^{-12} M in serum-free DMEM/F12 media,
174 then placed back into incubation for an additional 4 h. After stimulation, media was
175 removed and replaced with a luciferase substrate (BrightGLO; Promega; Madison, WI).
176 The luminescence generated after 5 min was measured spectrophotometrically by a
177 BioTek Synergy HT microplate reader using Gen5 software (Agilent Technologies;
178 Santa Clara, CA). Luminescence readings (in relative light units) were corrected by
179 subtracting the average luminescence values of an unstimulated (0 M ligand) control for
180 each unique transfection.

181

182 2.3 Calculations and statistics

183 The dose-response curve of receptor activation was analyzed using non-linear
184 regression (three-parameter polynomial; $\log([\text{ligand}])$ vs luminescence). From the fitted
185 curves, values for half-maximal effective concentration (EC_{50}) and maximal response
186 (V_{\max}) were obtained. Values for EC_{50} and V_{\max} were compared using the extra-sum-of-
187 squares F test ($\alpha = 0.05$). All statistics and figure preparation were performed using
188 Prism 9 software (GraphPad Inc., La Jolla, CA). All data are presented as mean \pm
189 standard error ($n = 3$).

190

191 3. RESULTS

192

193 3.1 Sequence analyses

194 In our survey of the *P. senegalus* genome, we were able to identify a sequence
195 for *mc2r* (XM_039738597). However, no *mrap1* ortholog was detected in the *P.*
196 *senegalus* genome, although we could identify an accessory protein sequence that
197 NCBI BLAST analysis identified as an *mrap2* ortholog (XM_039747869). The deduced
198 amino acid sequence of sbMc2r aligned with a selection of other vertebrate Mc2r

199 sequences, exhibiting high sequence similarity in the 7 transmembrane domains that
200 are characteristic of Mc2r proteins (Fig. 1). In our analysis of vertebrate Mc2r orthologs,
201 the highest sequence similarities were within the mammals (mouse and human; 89%
202 sequence similarity) and the cyprinids (carp and zebrafish; 83%), whereas the lowest
203 sequence similarities were between the stingray and the cyprinids (39–41%) (Fig. 2A).
204 As expected, the sequence similarity of sbMc2r to other vertebrate Mc2rs fell within
205 these two extremes. The sbMc2r ortholog had highest similarity to the Mc2r in gar
206 (65%), and sbMc2r had a 55% sequence similarity to human MC2R and a 46%
207 sequence similarity to srMc2r (Fig. 2A). In a molecular phylogenetic comparison, Mc2r
208 sequences of sarcopterygians, actinopterygians, and chondrichthyes all formed
209 respective monophyletic clades, with the sbMc2r occupying the basal position among
210 the Mc2rs of actinopterygians (Fig. 2B).

211 The amino acid sequence for sbMrap2 was aligned with a selection of
212 gnathostome Mrap1 and Mrap2 sequences (Fig. 3A). All gnathostome Mrap sequences
213 had high sequence similarity in the reverse topology motif in the N-terminal domain and
214 the trafficking motif in the transmembrane domain, but only the bony vertebrate Mrap1
215 sequences contained the putative activation motif consisting of a δ -D-Y- δ residue
216 sequence (Dores and Chapa, 2021) (Fig. 3A). The sbMrap2 sequence was missing this
217 putative activation motif (Fig. 3A). In a phylogenetic analysis, the Mrap1 and Mrap2
218 sequences formed distinct monophyletic clades, with the sbMrap2 sequence being
219 represented as most closely related to the bowfin Mrap2 (Fig. 3B).

220

221 *3.2 Receptor activation studies*

222 To test the hypothesis that sbMc2r was dependent on chaperoning by an Mrap,
223 we performed receptor activation studies on sbMc2r expressed either alone or with the
224 co-expression of various vertebrate Mrap1s or the sbMrap2. When expressed alone
225 (without co-expression of an Mrap), sbMc2r was not activated by any concentration of
226 ACTH ligand (Fig. 4A). The positive control for this experiment was bfMc2r co-
227 expressed with bfMrap1, which responded in a robust manner to stimulation by ACTH
228 (Fig. 4A).

Given these results, we tested the hypothesis that sbMc2r does indeed require co-expression with an Mrap1 ortholog for functional activation. This experiment was performed using various vertebrate Mrap1 orthologues, including bfMrap1 and chMrap1 orthologs, which contain the δ -D-Y- δ activation motif, and wsMrap1, which lacks the δ -D-Y- δ activation motif. Co-expression of sbMc2r with various vertebrate Mrap1s resulted in activation of sbMc2r by ACTH, but this effect was dependent on the particular Mrap1 being co-expressed (Fig. 4B). When sbMc2r was co-expressed with wsMrap1, no activation of sbMc2r by ACTH was observed. When sbMc2r was co-expressed with either bfMrap1 or chMrap1, a characteristic activation of sbMc2r by ACTH was observed that fit a classic dose-response curve (bfMrap1: $R^2 = 0.91$; chMrap1: $R^2 = 0.90$) (Fig. 4A). The EC₅₀ values of sbMc2r modulated by bfMrap1 and chMrap1 were as follows: bfMrap1: EC₅₀ = 9.0×10^{-10} M (95% CI: 4.3×10^{-10} to 1.8×10^{-9} M); chMrap1: EC₅₀ = 1.0×10^{-8} (95% CI: 3.9×10^{-9} to 2.3×10^{-8} M). These EC₅₀ values were determined to be significantly different from each other ($F_{1,38} = 13.7$; $P < 0.001$). Although the maximal luciferase activity (V_{max}) was consistently marginally higher when sbMc2r was co-expressed with bfMrap1 compared with chMrap1, the V_{max} for each was not significantly different ($F_{1,38} = 1.78$; $P = 0.190$).

The results of experiments presented in Fig. 4A-B demonstrated the requirement of Mrap co-expression for sbMc2r function. Due to our ability to detect an *mrap2* ortholog, but not an *mrap1* ortholog, in the *P. senegalus* genome, we evaluated whether sbMrap2 could facilitate activation of sbMc2r (Fig. 4C). In this experiment, although sbMc2r co-expressed with bfMrap1 (the positive control) was activated following stimulation with ACTH, sbMc2r co-expressed with sbMrap2 did not respond to stimulation with ACTH (Fig. 4C).

Finally, previous studies have shown that neopterygian and tetrapod Mc2r orthologs are exclusively selective for activation by ACTH, whereas cartilaginous fish Mc2r orthologs could be activated by either ACTH or α -MSH with varying degrees of efficacy (Dores and Chapa, 2021). To evaluate the ligand selectivity of sbMc2r, the receptor was co-expressed with bfMrap1 and stimulated with either ACTH or α -MSH ligands (Fig. 5). As in our previous experiments, sbMc2r co-expressed with bfMrap1 characteristically responded to stimulation by ACTH (the positive control) (Fig. 5).

260 However, no activation of sbMc2r co-expressed with bfMrap1 was observed at any
261 concentration of α -MSH (Fig. 5).

262

263 **4. DISCUSSION**

264

265 In the present study, we have provided a molecular and functional
266 characterization of the most basal Mc2r from a bony vertebrate studied to date, the
267 Mc2r from a polypterus species, the Senegal bichir. As will be discussed, we have
268 demonstrated that the sbMc2r was selectively activated by ACTH (compared to α -MSH)
269 and dependent on the cooperative interaction of Mrap1 for functional activation. These
270 results increase the resolution of our view of how derived Mc2r function arose
271 throughout the radiation of the vertebrates, indicating that a major shift in Mc2r function
272 occurred at the root of the radiation of class Osteichthyes.

273 Although many similarities exist across the five-member family of melanocortin
274 receptors (Ramachandrappa et al., 2013), Mc2r has many unique features due to the
275 considerably more rapid sequence divergence of this gene (Schiöth et al., 2005). All
276 melanocortin receptors are coupled to cAMP/PKA pathways and activated by POMC-
277 derived peptides, however only Mc2r has been shown to require chaperoning by Mrap1
278 and exclusive selectivity for ACTH (Ramachandrappa et al., 2013). Importantly, these
279 unique functional properties of Mc2r are not universal and seem to only be present in
280 the Mc2rs of the most derived chordates (Dores and Chapa, 2021).

281 Recent investigations in our lab and others have renewed an interest in better
282 understanding the evolutionary origins of a stress-responsive HPI axis responsible for
283 corticosteroid production by studying basal vertebrates (Bouyoucos et al., 2021).
284 Although it has been shown that jawless and cartilaginous fishes, as well as basal bony
285 fishes, produce corticosteroids in response to stress (Close et al., 2010; McCormick et
286 al., 2020; Rai et al., 2015; Ruiz-Jarabo et al., 2019; Shaughnessy et al., 2020;
287 Shaughnessy and McCormick, 2021), whether an ACTH- and Mc2r-mediated HPI axis
288 exists in these basal vertebrate lineages remains unknown (Bouyoucos et al., 2021;
289 Roberts et al., 2014). Whereas the Mc2rs of all bony vertebrates studied to date appear
290 to display Mrap1 dependence and ACTH selectivity, the Mc2r of cartilaginous fishes

291 have variable Mrap1 dependance and no ACTH selectivity (Barney et al., 2019; Dores
292 and Chapa, 2021; Hoglin et al., 2022). Thus, there appears to have been an important
293 shift in Mc2r function during the emergence of bony vertebrates. By studying the Mc2r
294 of *polypterus* in the present study, we aimed to determine whether sbMc2r, as the most
295 basal bony vertebrate Mc2r studied to date, exhibited any ancestral (i.e., cartilaginous
296 fish-like) features of Mc2r function, such as Mrap1 independence or lack of ACTH
297 selectivity. In doing so, we aimed to further resolve the timing of acquisition of the
298 derived features of Mc2r function in bony vertebrates.

299 Our analyses demonstrated that sbMc2r unambiguously exhibited only the
300 derived features of Mc2r function, including an obligatory dependance on Mrap1 for
301 activation and an exclusive selectivity for ACTH over α -MSH. These results indicate that
302 the acquisition of these derived features of Mc2r function occurred at the root of the
303 bony vertebrates, prior to the radiation of Polypteriformes. Indeed, the presence of
304 these features of Mc2r function in both the actinopterygian and sarcopterygian lineages
305 was already a good indication that these derived functional qualities of Mc2r would be
306 represented in the common ancestor to all bony vertebrates (Dores and Chapa, 2021).
307 However, functional analyses of the most basal members of these two primary bony
308 vertebrate lineages have been needed to add support for this hypothesis. The present
309 study provides such evidence from the actinopterygian lineage. It will be important to
310 also analyze the functional properties of the Mc2r from the most basal members of
311 Sarcopterygii, such as the lobe-finned fishes which include the lungfishes and the
312 coelacanth.

313 The functional evolution of vertebrate Mc2r is likely related to the functional
314 evolution of Mrap1. In the present study, it was not simply the case that sbMc2r could
315 be activated when co-expressed with any vertebrate Mrap1. Whereas sbMc2r could be
316 activated by ACTH when co-expressed with bowfin or chicken Mrap1, sbMc2r was
317 unable to be activated when co-expressed with the Mrap1 of whale shark. This is likely
318 explained by the putative activation motif $\delta^{18}D^{19}Y^{20}\delta^{21}$ (residue numbers indicated for
319 mouse Mrap1) (Chan et al., 2009; Malik et al., 2015; Sebag and Hinkle, 2009; Webb
320 and Clark, 2010). This activation motif is present in the bfMrap1 and chMrap1 but
321 absent in wsMrap1. The δ -D-Y- δ motif is specific to osteichthyan Mrap1s, and directly

322 follows the highly conserved Y-E-Y-Y motif contained in nearly all Mrap1 and Mrap2
323 sequence structures (Fig. 3A) (Dores and Chapa, 2021). In a previous study from our
324 lab, we demonstrated that wsMc2r can be activated without co-expression with an
325 Mrap, but only at supraphysiological concentrations of ACTH (Hoglin et al., 2022). This
326 contrasts with the functional qualities of sbMc2r demonstrated in the present study,
327 where sbMc2r could not be activated by any concentration of ACTH without Mrap1 co-
328 expression. Interestingly, sensitivity of wsMc2r to ACTH is substantially improved by co-
329 expression with either wsMrap1 or wsMrap2, which are both missing a δ -D-Y- δ
330 activation motif (Hoglin et al., 2022). This has been explained as wsMrap1 and
331 wsMrap2 helping to increase the trafficking of the wsMc2r to the cell surface (Hoglin et
332 al., 2022). It is due to the appearance in bony vertebrates of the requirement of Mrap1
333 co-expression for Mc2r activation by ACTH, coupled with the appearance of the δ -D-Y- δ
334 activation motif in Mrap1 of bony vertebrates, that it has been suggested that Mc2r and
335 Mrap1 have been co-evolving since the emergence of jawed vertebrates (Dores et al.,
336 2016, 2014; Dores and Chapa, 2021; Västermark and Schiöth, 2011).

337 In the present study, the dependence of sbMc2r on co-expression with a bony
338 vertebrate Mrap1 indicates that an *mrap1* gene containing a δ -D-Y- δ activation motif
339 likely exists in the genome of *P. senegalus*, despite our current inability to detect any
340 such *mrap1* in the published *P. senegalus* assembly. It's possible that the assembly of
341 the bichir genome is either incomplete (less than 100% coverage) or fragmented in such
342 a way that makes the *mrap1* sequence impossible or difficult to detect. A similar
343 phenomenon occurred with *Xenopus tropicalis*, for which an Mc2r was identified and
344 functionally characterized as being Mrap1-dependant (Liang et al., 2011) many years
345 before the genomic discovery of a *X. tropicalis* Mrap1 orthologue (Tai et al., 2022).

346 In summary, we have presented a molecular and functional characterization of
347 the Mc2r of the Senegal bichir, which represents an Mc2r from the most basal bony
348 vertebrate studied to date. The sbMc2r was obligatorily dependent on the cooperative
349 interaction with a bony vertebrate Mrap1, and sbMc2r had exclusive selectivity for
350 ACTH over α -MSH. These results indicate that an Mrap1 likely exists for *P. senegalus*,
351 despite its apparent absence from the available genome. Furthermore, our results add
352 needed support to the hypothesis that the acquisition of Mrap1 dependence and ACTH

353 selectivity with respect to Mc2r function occurred at the root of the radiation of the class
354 Osteichthyes, though this hypothesis of ancestral Mc2r function requires more empirical
355 testing from basal sarcopterygians.

356

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362

363 **DISCLOSURES**

364

365 The authors declare that they have no known competing financial or personal interests
366 regarding the studies presented in this manuscript.

367

368 **AUTHOR CONTRIBUTIONS**

369

370 **CAS:** conceptualization, methodology, investigation, formal analysis, data curation,
371 visualization, writing (original draft, editing, review of final draft), funding acquisition

372 **MFJ:** investigation, writing (review of final draft)

373 **RMD:** conceptualization, methodology, investigation, writing (editing, review of final
374 draft), supervision, project administration, funding acquisition

375

376 **FIGURE LEGENDS**

377

378 **Figure 1: Multiple alignment of Mc2r amino acids sequences from a selection of
379 jawed vertebrates, including bichir Mc2r.** Shading indicates identical (black) and
380 molecularly similar (grey) residues. Horizontal bars indicate the 7 transmembrane
381 regions; arrows indicate continuation of the indicated regions. See Materials and
382 Methods for details of analyses and sequence accession numbers.

383

384 **Figure 2: Sequence similarities and phylogenetic relationships of Mc2r amino**
385 **acid sequences from a selection of jawed vertebrates.** (A) Amino acid sequence
386 similarities of gnathostome Mc2rs are presented as percent identity (shading intensity
387 reflects sequence similarity). (B) Molecular phylogeny of gnathostome Mc2rs, where the
388 clade of Chondrichthyes was rooted as an outgroup. Bichir Mc2r is highlighted by black
389 dot. See Materials and Methods for details of analyses and sequence accession
390 numbers.

391

392 **Figure 3: Molecular characterization of Mrap amino acid sequences from a**
393 **selection of jawed vertebrates, including bichir Mrap2.** (A) Multiple sequence
394 alignment of gnathostome Mrap1s and Mrap2s. Shading indicates identical (black) and
395 molecularly similar (grey) residues. Horizontal bars indicate the activation motif (AM),
396 reverse topology motif (RTM), and the membrane trafficking motif (MTM); arrows
397 indicate continuation of the indicated regions. (B) Molecular phylogeny of gnathostome
398 Mrap1s and Mrap2s. See Materials and Methods for details of analyses and sequence
399 accession numbers.

400

401 **Figure 4: Mrap1-dependance of bichir Mc2r.** (A) Dose-response stimulation of bowfin
402 (bf) Mc2r (co-expressed with bfMrap1) and bichir (sb) Mc2r (expressed without an
403 Mrap) by human ACTH(1–24). (B) Dose-response stimulation by ACTH of sbMc2r
404 expressed with and without various vertebrate Mrap1s (bfMrap1; chicken, chMrap1;
405 whale shark, wsMrap1). (C) Dose-wise stimulation by ACTH of sbMc2r co-expressed
406 with either bfMrap1 or sbMrap2. In all panels, data are presented as mean \pm standard
407 error ($n = 3$) and lines represent fitted dose-response curve (three-parameter
408 polynomial).

409

410 **Figure 5: ACTH selectivity of bichir Mc2r.** Dose-wise stimulation by either human
411 ACTH(1–24) or α -MSH of bichir Mc2r co-expressed with bowfin Mrap1. Data are
412 presented as mean \pm standard error ($n = 3$) and line represents fitted dose-response
413 curve (three-parameter polynomial).

414

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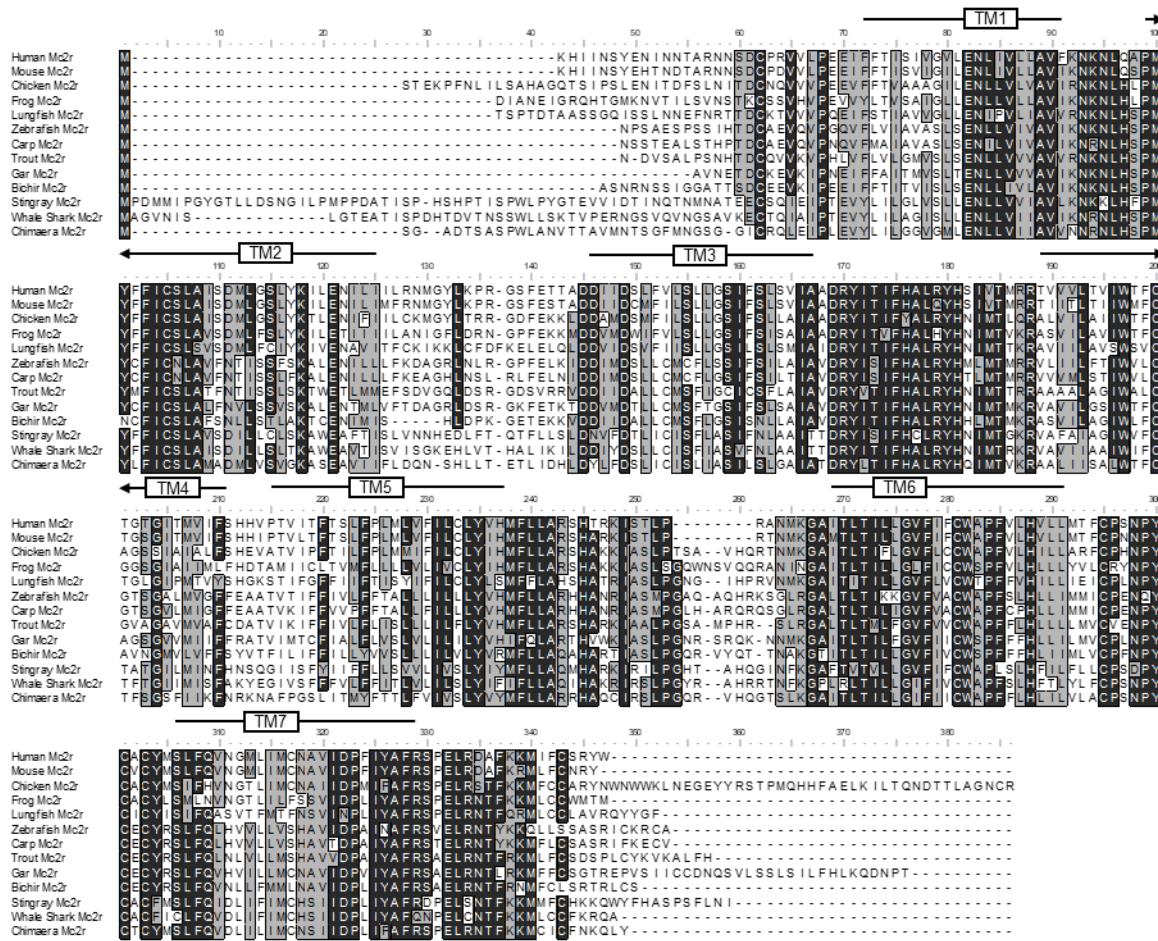
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577 **Figure 1**

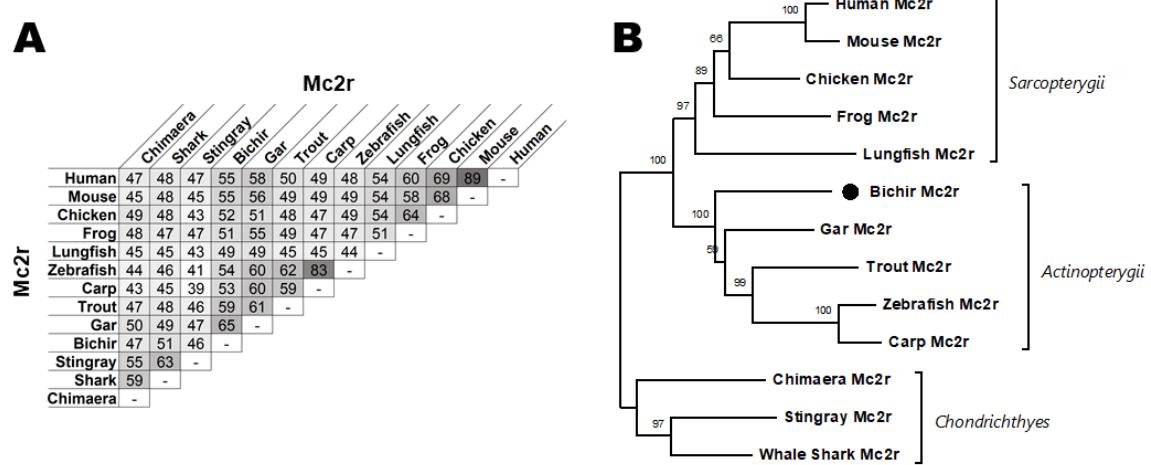
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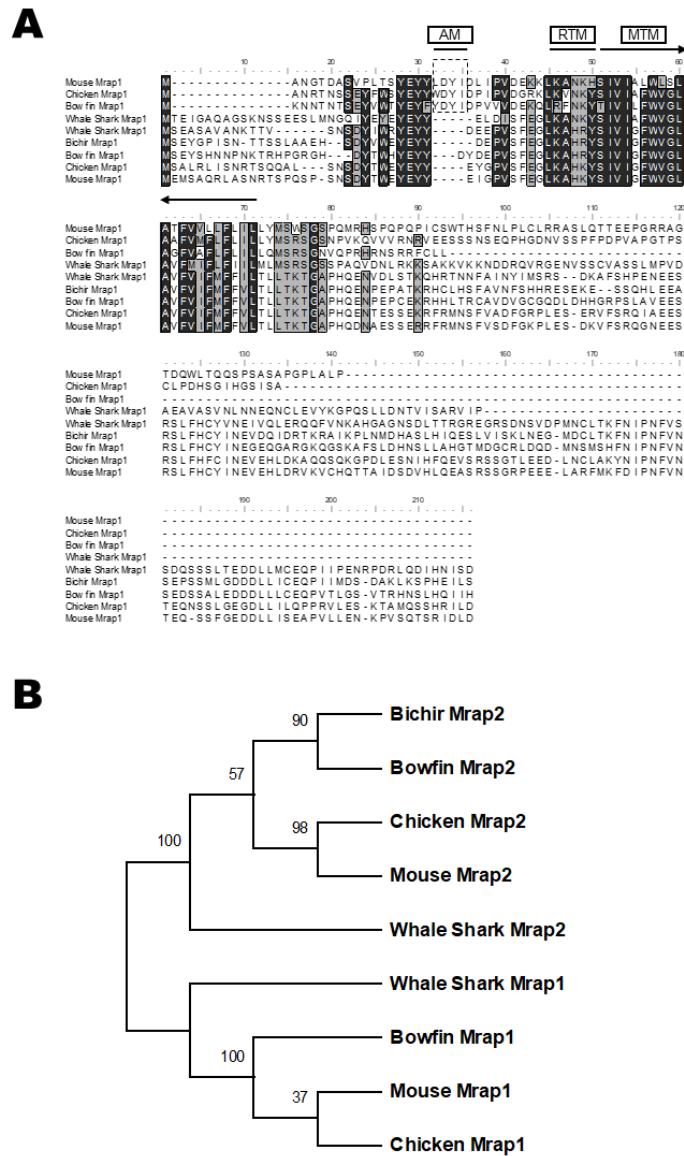
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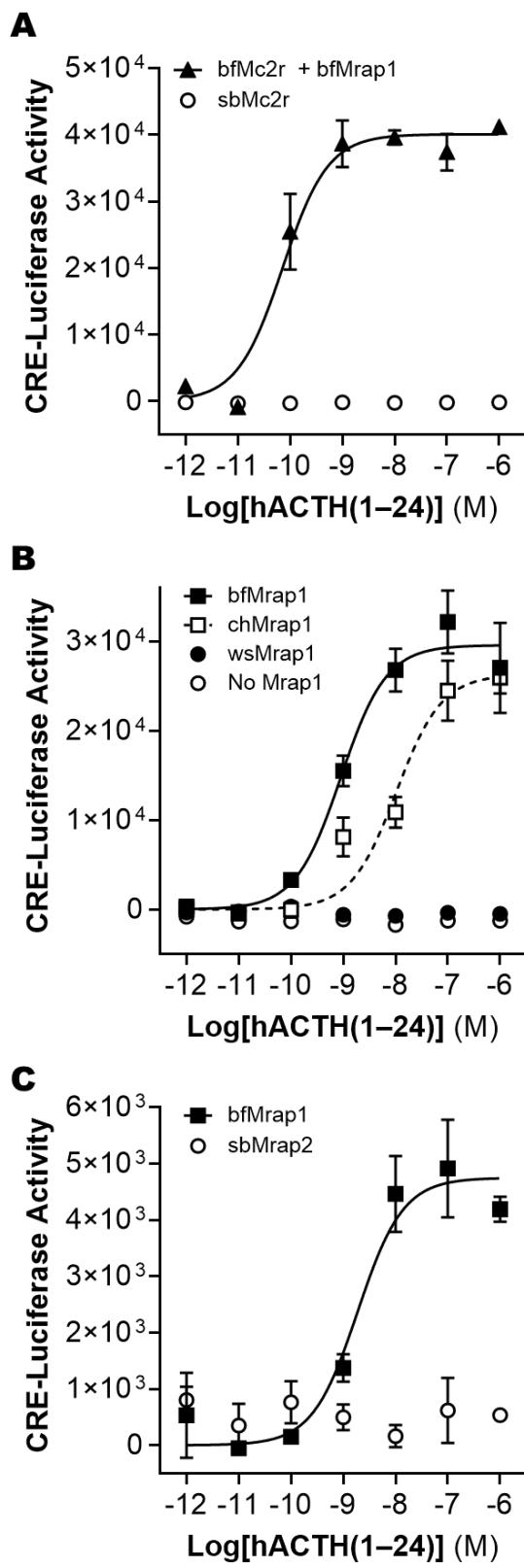
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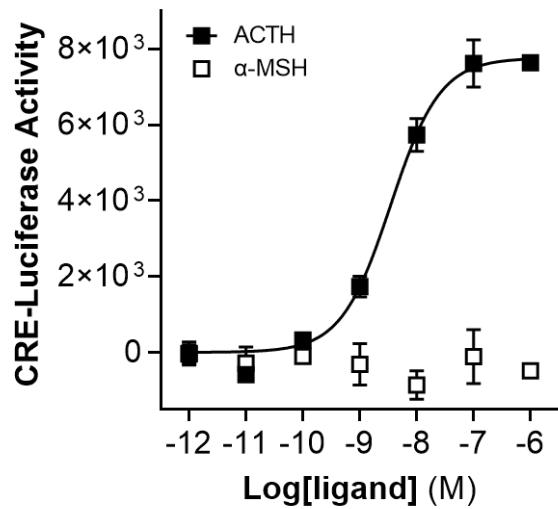
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586 **Figure 4**



588 **Figure 5**



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