

1 Functional characterization of melanocortin 2 receptor (Mc2r) from a lobe-finned
2 fish (*Protopterus annectens*) and insights into the molecular evolution of
3 melanocortin receptors

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24 Lungfish Mc2r
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ABSTRACT

Recent studies from our group on melanocortin 2 receptors (Mc2r) from basal families of actinopterygians have served to resolve that Mrap1 dependence and ACTH selectivity are features of even the most basal ray-finned fishes. However, there have been no studies on Mc2r function of the basal sarcopterygians, the lobe-finned fishes, represented by the extant members coelacanths and lungfishes. Here, we offer the first molecular and functional characterization of an Mc2r from a lobe-finned fish, the West African lungfish (*Protopterus annectens*). Plasmids containing cDNA constructs of lungfish (lf) Mc2r and Mrap1 were expressed in mammalian and zebrafish cell lines. Cells were then stimulated by human ACTH(1-24) and melanocyte stimulating hormone (α -MSH), as well as alanine-substituted analogs of hACTH(1-24) targeting residues within the H⁶F⁷R⁸W⁹ and K¹⁵K¹⁶R¹⁷R¹⁸P¹⁹ motifs. Activation of lfMc2r was assessed using a cAMP-responsive luciferase reporter gene assay. In these assays, lfMc2r required co-expression with lfMrap1, was selective for ACTH over α -MSH at physiological concentrations of the ligands and was completely inhibited by multiple-alanine substitutions of the HFRW (A⁶⁻⁹) and KKRRP (A¹⁵⁻¹⁹) residue. Single- and partial-alanine substitutions of the HFRW and KKRRP motifs varied in their impacts on receptor-ligand affinity from having no effect to completely inhibiting lfMc2r activation. This characterization of the Mc2r of a lobe-finned fish fulfills the last major extant vertebrate group for which Mc2r function had yet to be characterized. In doing so, we resolve that all basal bony vertebrate groups exhibit Mc2r function that substantially differs from that of the cartilaginous fishes, indicating that rapid and dramatic shift in Mc2r function occurred between the radiation of cartilaginous fishes and the emergence of bony fishes. We support this interpretation with a molecular clock analysis of the melanocortin receptors, which demonstrates the uniquely high rate of sequence divergence in Mc2r. Much remains to be understood regarding the molecular evolution of Mc2r during the early radiation of vertebrates that resulted in the derived functional characteristics of Mrap1 dependence and exclusive selectivity for ACTH.

KEYWORDS

molecular evolution; ACTH; Mc2r; Mrap; lungfish

1. Introduction

Generally, all vertebrates share a similarly arranged hypothalamus-pituitary-adrenal/interrenal (HPA/I) axis to stimulate corticosteroid production, wherein the adrenal gland of tetrapods is homologous to the interrenal tissue associated with the kidneys of amphibians and fishes (Bouyoucos et al., 2021). Stimulation of the HPA/I axis in gnathostomes, such as during stress, includes the melanocortin 2 receptor (Mc2r) of the adrenal/interrenal receiving a pituitary-derived signal, adrenocorticotrophic hormone (ACTH) (Dores and Chapa, 2021). Once stimulated by ACTH, Mc2r activation initiates corticosteroid biosynthesis in the adrenal/interrenal cells.

Mc2r is one member of a larger family of five melanocortin receptors (Mc[1–5]r) in gnathostomes (Cone, 2006). In mammals, all melanocortin receptors can be activated by ACTH, and Mc1r, Mc3r, Mc4r, and Mc5r can also be activated by other melanocortin ligands, such as melanocyte stimulating hormone alpha (α -MSH) (Cone, 2006). However, mammalian Mc2r is unique from other Mcr counterparts in that Mc2r does not bind MSH-sized ligands and is exclusively selective for ACTH (Mountjoy et al., 1992). Additionally, whereas Mc1r, Mc3r, Mc4r, and Mc5r in mammals can traffic to the plasma membrane and bind melanocortin ligands on their own, the Mc2r requires the co-expression of an accessory protein, the melanocortin 2 receptor accessory protein (Mrap1) for trafficking to the plasma membrane (Metherell et al., 2005; Ramachandrappa et al., 2013). Without chaperoning by Mrap1, the mammalian Mc2r can neither traffic to the plasma membrane nor be activated by its ACTH ligand (Hinkle and Sebag, 2009; Webb and Clark, 2010).

These unique functional properties of the Mc2r (Mrap1 dependence and ACTH selectivity) have been observed in the Mc2rs of all bony vertebrates (superclass Osteichthyes) studied to date, including ray-finned fishes and tetrapods (Dores and Chapa, 2021). However, these functional traits appear to have been derived throughout vertebrate evolution, as the Mc2rs of non-Osteichthyans function differently. Studies on the melanocortin receptors from sea lamprey (*Petromyzon marinus*), a member of the most basal group of vertebrates, the jawless vertebrates (class Agnatha), have shown the lamprey Mcrs do not require Mrap for trafficking or activation and are promiscuously activated by all types melanocortin ligands (Haitina et al., 2007; Zhu et al., 2019). Additionally, the Mc2rs of cartilaginous fishes, the basal group of jawed vertebrates including elasmobranchs and holocephalans, exhibit a range of dependence on Mrap1

87 chaperoning, and can be activated by either ACTH or α MSH with varying degrees of efficacy
88 (Dores and Chapa, 2021).

89 Seeking to better understand the evolution of Mc2r function between the cartilaginous
90 fishes and the more derived bony vertebrates, our group has made an effort to characterize the
91 function of Mc2r in the most basal bony vertebrates. The rationale for this endeavor has been to
92 examine whether Mc2rs in the most basal bony vertebrates exhibit some intermediate function
93 between the Mrap1-independent and promiscuous Mc2rs of cartilaginous fishes and the Mrap1-
94 dependent and ACTH-specific Mc2rs of modern bony vertebrates. To this end, in recent studies,
95 we have characterized the function of Mc2rs from gar (Wong and Dores, 2022), bowfin (Dores
96 et al., 2022), paddlefish (Dores et al., 2022), sturgeon (Shaughnessy et al., 2023), and bichir
97 (Shaughnessy et al., 2022). The Mc2rs of all of these basal actinopterygian taxa exhibit strict
98 Mrap1 dependence for trafficking and activation and exclusive ACTH selectivity. Thus, the
99 Mc2rs of basal actinopterygians do not appear to demonstrate any intermediate function. Missing
100 from our recent studies has been the examination of an Mc2r from a lobe-finned fish, a basal
101 representative of the sarcopterygian side of the bony vertebrate superclade.

102 Here, we present the first molecular and functional characterization of an Mc2r from a
103 lobe-finned fish, the West African lungfish (*Protopterus annectens*; hereafter simply referred to
104 as “lungfish”). Our hypothesis for these studies, based on the results of our recent works
105 investigating the function of other basal osteichthyan Mc2rs, was that the lungfish (lf) Mc2r
106 would exhibit derived functional properties such as Mrap1 dependence and ACTH selectivity. In
107 our molecular characterization, we analyzed the primary sequence structure of lfMc2r and
108 lfMrap1 in comparison to orthologues from other vertebrate models. In our functional assays, we
109 expressed Mc2r and Mrap1 from lungfish and other vertebrate models in mammalian cells and
110 assessed receptor activation by melanocortin ligands using a cAMP-responsive luciferase
111 reporter gene assay. With this characterization of the Mc2r of a lobe-finned fish fulfilling the last
112 major extant vertebrate group for which Mc2r function had yet to be characterized, we
113 synthesize our results in the context of other recent investigations from our group. Further, we
114 present additional molecular analyses regarding the sequence divergence of vertebrate
115 melanocortin receptors to support a broader discussion on the evolution of Mc2r function in
116 vertebrates.

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2. Materials and Methods

2.1 Sequences and in silico analyses

Nucleotide and translated protein sequences of the lungfish *mc2r* (Accession No. XP_043923917) and *mrp1* (Accession No. XP_043927725) were obtained from the National Center for Biotechnology Information (NCBI) GenBank (Assembly No. GCA_019279795). Additionally, a selection of other gnathostome Mc2rs were obtained via NCBI (**Supplementary File 1**).

For our comparative structural analysis, we aligned lfMc2r with Mc2rs from basal actinopterygian species. As no Mrap1 sequences from these species were available on NCBI, we aligned lfMrap1 with other sarcopterygian species. The alignments of the primary (amino acid) sequences for lungfish (lf), polypterus (*Polypterus senegalus*; ps), and sturgeon (*Acipenser ruthenus*; ar) Mc2rs and lungfish, chicken (*Gallus gallus*; c), and human (*Homo sapiens*; h) Mrap1s were produced using a common motif-based approach as previously described (Dores et al., 1996). Transmembrane topology of lfMc2r was predicted using the TMHMM tool (Version 1.0.24) from the DTU Bioinformatics Server (<https://dtu.biolib.com/DeepTMHMM>). The larger multiple sequence analyses that were used to build the Mc2r phylogeny and perform molecular clock analyses were carried out on primary sequences using Clustal Omega (Sievers et al., 2011).

Molecular clock analyses were carried out on primary sequences for all 5 melanocortin receptor subtypes (Mc1r, Mc2r, Mc3r, Mc4r, Mc5r) from balanced selection of species across the gnathostome phylogeny (**Supplementary File 2**). Phylogenetic construction and calculation of maximum likelihood distances were performed on MEGA11 software (Kumar et al., 2008) using the maximum likelihood (ML) method and a Jones-Taylor-Thornton matrix-based model (JTT+G), where +G indicates that heterogenous substitution rates across sites was assumed according to a gamma distribution ($G = 1.0250$) (Jones et al., 1992; Yang, 1993). A JTT+G substitution model was selected among 55 substitution models analyzed as it was considered to best describe the substitution pattern within our sequence set (*i.e.*, JTT+G had the lowest Bayesian information criterion score in our analysis). Taxa divergence times were calculated based on the species divergences times presented in TimeTree of Life resource (<https://www.timetree.org>) (Kumar et al., 2022) and presented as relative to human. Pairwise ML distances were plotted against taxa divergence times for each receptor subtype. The rate of sequence divergence was determined by the slope using a linear regression analysis.

2.2 Cells

Chinese hamster ovary (CHO) cells (ATCC, Manassas, VA) were cultured in Kaighn's modification of Ham's F12 media (ATCC) made complete by supplementing with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 100 µg/mL normacin, and maintained in a humidified incubator with 95 % air and 5 % CO₂ at 37 °C. CHO cells were selected for this project because this cell line does not express endogenous *mcr* (Noon et al., 2002; Sebag and Hinkle, 2007) or *mrp* genes (Reinick et al., 2012a).

Zebrafish embryonic fibroblast (ZF4) cells (ATCC, Manassas, VA) were cultured in L-15 media (ATCC) made complete by supplementing with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 100 µg/mL normacin, and maintained in a humidified incubator with ambient CO₂ at 28 °C.

2.3 Peptides

Human adrenocorticotrophic hormone (hACTH[1-24]) and α-MSH (N-acetyl-ACTH[1-13]-NH₂) were purchased from Sigma-Aldrich Inc. (St. Louis, MO). These peptides have a high degree of primary sequence identity to the corresponding melanocortin peptides from the lungfish genome. The α-MSH sequences for human and lungfish are 100 % identical, and only one neutral amino acid substitution differentiates human and lungfish ACTH(1-24)—at residue position 20, lungfish ACTH contains an Ile where human ACTH contains a Val. As Ile and Val are functionally neutral substitutions and residue positions 20-24 in hACTH(1-24) do not affect interaction with Mc2r (Kovalitskaya et al., 2007), the commercially available human analogues of α-MSH and ACTH were considered suitable proxies for lungfish peptides in our functional assays characterizing lfMc2r. For the cAMP reporter gene assay, hACTH(1-24) and α-MSH were used to stimulate transfected cells at concentrations from 10⁻¹² M to 10⁻⁶ M. Alanine-substituted analogs of hACTH(1-24) in the H⁶F⁷R⁸W⁹ and K¹⁵K¹⁶R¹⁷R¹⁸P¹⁹ motifs (**Table I**) were synthesized by New England Peptide, (Gardiner, MA) and were also used at concentrations from 10⁻¹² M to 10⁻⁶ M.

2.4 Reporter gene assay

The coding sequences for *mc2r* and *mrp1* were individually inserted into a pcDNA3(+) expression vectors (GenScript; Piscataway, NJ). The cAMP reporter gene construct, comprised of a *luciferase* gene promoted by a cAMP-responsive element (*cre-luciferase*) (Chepurny and Holz, 2007) was provided by Dr. Patricia Hinkle (University of Rochester, Rochester, NY).

The reporter gene assay involves transfecting cDNA constructs of receptor and/or accessory protein along with the cDNA construct of the luciferase reporter into CHO cells, then stimulating with ligand to measure receptor activation. Transfections were performed using the Amaxa Cell Line Nucleofector II System (Lonza, Switzerland) with the Solution T transfection kit (Lonza). Approximately 2 µg of cDNA per 1×10^5 cells was used for transfection, with a molar ratio of lfMc2r to lfMrp1 of 1:3. The transfected cells were plated in an opaque 96-well plate (Costar 3917, Corning Inc., Kennebunk, ME) at a final density of 1×10^5 cells per well..

After 48 hours of post-transfection incubation, the transfected CHO cells were stimulated with a range of concentrations of ligand (either hACTH(1-24), α -MSH, or alanine-substituted hACTH(1-24) analogs (**Table I**), as well as ligand-free control) in serum-free media, which was identical to the CHO cell culture media described above only without supplementation with fetal bovine serum. Each concentration of ligand (or the ligand-free control) was replicated in triplicate.

Following a 4 h incubation with the ligand-containing stimulating media, the stimulating media was removed, and the luciferase substrate reagent (BrightGLO, Promega, Madison, WI) was added to each well following the manufacturer protocol for a 5 min incubation. After incubation with the luciferase substrate, luminescence was immediately measured using a BioTek Synergy HT plate reader (Winooski, VT). To account for any background levels of cAMP production, the average luminescence reading for the ligand-free control wells for each assay was subtracted from the luminescence readings for the ligand-containing wells. The final, background-corrected luminescence values were fitted to the Michaelis-Menton equation to obtain EC_{50} values for each dose-response curve. To better visually compare EC_{50} values, data were normalized to set the calculated V_{max} for each assay to a value of 100.

The reporter gene assay run with ZF4 cells was performed identically to the assay performed in CHO cells with only slight modifications (doubling of incubation times) to account for the lower incubation temperature. Transfected ZF4 cells were stimulated 96 h post-

transfection and stimulated ZF4 cells were assessed for luciferase luminescence after 8 h in stimulating media.

2.5 Calculations and statistics

All assays were performed in triplicate, and all data is presented as mean $\pm$ standard error. Statistical differences ($\alpha = 0.05$) between the EC₅₀ values were determined using the extra sum-of-squares *F* test. Statistics were conducted and figures were prepared using GraphPad Prism software (GraphPad Inc, La Jolla, CA, USA).

3. Results

3.1 Structural analysis of *lfMc2r* and *lfMrp1*

The primary sequence of *lfMc2r* aligned closely with that of both *psMc2r* and *arMc2r* (representing basal orders from the actinopterygian side of the osteichthyan clade). These basal osteichthyan *Mc2r* orthologs were aligned with relatively few gap insertions (mostly single-residue gaps), and the overall primary sequence similarity of the three orthologs was 58% (**Fig. 1A**). The three orthologs have the highest primary sequence divergence in the N-terminal domain (24%), EC2 domain (0%), and the C-terminal domain (35%), and the highest similarity in the IC1 and IC2 domains (100% and 83%, respectively).

In its functional domains, the *Mrp1* ortholog for lungfish closely resembled the two other sarcopterygian *Mrp1* orthologs, from chicken and human, including the alignment of a putative N-linked glycosylation site, the Y¹⁴E¹⁵Y¹⁶Y¹⁷ and the δ^{18} D¹⁹Y²⁰ δ^{21} motifs (residue numbers corresponding to hMRAP1) located in the N-terminal domain (Dores and Chapa, 2021), and the transmembrane domain (Sebag and Hinkle, 2007) (**Fig. 1B**). The only gap insertion was in N-terminal domains of hMRAP1 and cMRAP1, to facilitate alignment with the longer N-terminal domain of *lfMc2r*. The overall sequence similarities between the three *Mrp1* orthologs was only ~25 %, but the sequence similarities were moderate within the N-terminal and transmembrane domains (47% and 48%, respectively). Low (6%) sequence similarity was observed in the C-terminal domains , which is characteristic of osteichthyan *Mrp1* orthologs as the C-terminal domain is not known to be functionally important (Liang et al., 2011; Sebag and Hinkle, 2009, 2007).

3.2 Pharmacological Analysis of lfMc2r

Given the common origin of Sarcopterygii and Actinopterygii (Brazeau and Friedman, 2015), and the observation that psMc2r (a basal actinopterygian Mc2r) requires co-expression with an osteichthyan Mrap1 for functional activation (Shaughnessy et al., 2022), we predicted that lfMc2r would have a similar reliance on co-expression with Mrap1. To test this, lfMc2r was expressed either alone or with lfMrap1 and stimulated with hACTH(1-24). Expression of lfMc2r alone did not result in activation at any of the concentrations of hACTH(1-24) (**Fig. 2A**). However, a robust response to stimulation by hACTH(1-24) in the nanomolar range was observed when the lfMc2r was co-expressed with lfMrap1 (**Fig. 2A**). To determine whether lfMc2r could be activated by α -MSH, lfMc2r was co-expressed with lfMrap1 and stimulated with either hACTH(1-24) (serving as a positive control) or α -MSH. Here, lfMc2r showed only minor stimulation to α -MSH compared to hACTH(1-24) and only at the highest concentration of 10^{-6} M (**Fig. 2B**).

Since previous studies had evaluated the role of residues in the “message” motif ($H^6F^7R^8W^9$) and the “address” motif ($K^{15}K^{16}R^{17}R^{18}P^{19}$) of various osteichthyan Mc2r orthologs (Barlock et al., 2014; Davis et al., 2013; Liang et al., 2013; Schwyzer, 1977), the activation of lfMc2r (co-expressed with lfMrap1) was evaluated using the set of alanine-substituted analogs of hACTH(1-24) (**Table I**). Activation of lfMc2r was completely inhibited by a complete alanine substitution of the $H^6F^7R^8W^9$ motif ($A^6A^7A^8A^9$), whereas single-alanine substitutions of the $H^6F^7R^8W^9$ motif resulted in a range of inhibition of lfMc2r activation (**Fig. 2C**). The $A^6F^7R^8W^9$ analog had no effect on lfMc2r activation compared to the $H^6F^7R^8W^9$ control (comparison of EC_{50} values: $P = 0.102$), but the $H^6A^7R^8W^9$ analog resulted in a 10-fold reduction in affinity ($P < 0.001$; **Table I**). The $H^6F^7A^8W^9$ and $H^6F^7R^8A^9$ analogs resulted in 100- and 1,000-fold reductions in affinities, respectively (for both, $P < 0.001$).

Similar to the alanine substitution studies on the $H^6F^7R^8W^9$ motif, complete alanine substitution of the $K^{15}K^{16}R^{17}R^{18}P^{19}$ motif nearly completely inhibited activation of lfMc2r (co-expressed with lfMrap1), and partial alanine substitution resulted in partially inhibited lfMc2r activation (**Fig. 2D**). The two partial alanine-substituted analogs tested ($A^{15}A^{16}R^{17}R^{18}P^{19}$ and $K^{15}K^{16}A^{17}A^{18}A^{19}$) both resulted in a 10-fold reduction in affinity (for both, $P < 0.001$).

Co-transfection of lfMc2r and lfMrap1 into ZF4 cells and stimulation by hACTH(1-24) resulted in similar characteristics of activation as was observed in CHO cells. In ZF4 cells,

activation of lfMc2r was only possible when the receptor was co-expressed with lfMrap1, and the Log(EC₅₀) value of lfMc2r for hACTH(1-24) (Log(EC₅₀) = -9.0; 95% CI = -10.0 to -8.4) was similar to that observed CHO cells (**Fig. 3**).

3.3 Analysis of molecular evolution of Mc2r

In our phylogenetic analysis of the melanocortin receptor family, each receptor subtype was grouped in a distinct clade (**Fig. 4**). Within the Mc2r clade, the Mc2rs from the chondrichthyans were grouped in a basal sub-clade to the osteichthyan Mc2rs, which distinctly grouped into the Actinopterygii and Sarcopterygii sub-clades. Within the sarcopterygian Mc2r sub-clade, the lungfish Mc2r occupied the basal position (**Fig. 4** and **Fig. 6**).

Generally, the Mc1r and Mc2r subtypes had higher sequence divergence compared to Mc3r, Mc4r, and Mc5r (**Fig. 5A**). For each subtype, the sequence divergence was generally higher at longer taxa divergence times (*i.e.*, there was a positive correlation between sequence divergence and taxa divergence times) (**Fig. 5A**). The rate of sequence divergence was similar between Mc1r, Mc3r, Mc4r, and Mc5r at ~0.0004 substitutions site⁻¹ My⁻¹, but was 3-fold higher for Mc2r at ~0.0012 substitutions site⁻¹ My⁻¹ (**Fig. 5B**). Within Mc2r, the rate of divergence varied across sites. Lower than average divergence rates were found in the TM1, IC1, TM2, TM3, IC2, and TM7 regions, and higher divergence rates were found in the EC1, EC2, TM5, and IC3 regions (**Fig. 5C-D**).

4. Discussion

In the present study, we described the structural and functional characteristics of the ACTH receptor, Mc2r, from the last remaining major extant vertebrate taxa for which Mc2r function had not yet been investigated, the ancient sarcopterygians, the lobe-finned fishes, as represented here by the lungfish. This work fulfills a decade-long investigation by our group into characterizing the function and pharmacology of vertebrate Mc2rs and testing hypotheses regarding the progression of melanocortin ligand-receptor relationships throughout vertebrate evolution (**Fig. 6**). In doing so, we have shown the acquisition of ACTH selectivity and Mrap1-dependence to be distinctly derived features of Mc2r function.

Among the cartilaginous fishes (class Chondrichthyes), studies by our lab have described Mc2r orthologs with varying degrees of ligand selectivity and interaction with Mrap1. For

example, the Mc2r from the holocephalan, the elephant shark (*Callorhinchus milii*), does not require Mrap1 for activation or membrane-trafficking and can be activated by either ACTH or α -MSH (Barney et al., 2019; Reinick et al., 2012b). These characteristics of the elephant shark Mc2r are similar to the apparent functional characteristics of the agnathan melanocortin receptors (Mca and Mcb) (Haitina et al., 2007; Zhu et al., 2019). Unlike the elephant shark Mc2r, orthologs of Mc2rs from elasmobranchs, including stingray (*Dasyatis akajei*) (Dores et al., 2018; Takahashi et al., 2016), whale shark (*Rhincodon typus*) (Hoglin et al., 2022), and dogfish (*Squalus suckleyi*) (Boyucos et al., manuscript under revision), do appear to require co-interaction with an Mrap to traffic to the plasma membrane, although they do not require Mrap1 for activation. Like the elephant shark Mc2r, elasmobranch Mc2rs can be activated by either ACTH or α -MSH at physiological concentrations of ligand, although they do tend to have a greater affinity for ACTH compared to α -MSH.

Among Actinopterygii, studies on the Mc2rs from taxa within the neopterygian lineage, such as the zebrafish (*Danio rerio*) (Agulleiro et al., 2010; Dores et al., 2016b), sea bass (*Dicentrarchus labrax*) (Agulleiro et al., 2013), rainbow trout (*Oncorhynchus mykiss*) (Dores et al., 2016b; Liang et al., 2015, 2011), bowfin (*Amia calva*) (Hoglin et al., 2023), and gar (*Lepisosteus oculatus*) (Wong and Dores, 2022), have all described Mc2r orthologs which are exclusively selective for ACTH and dependent on interaction with Mrap1 for both trafficking and activation. Recently, we have shown that these derived functional characteristics of the Mc2rs of neopterygian bony fishes are also present in the more ancient actinopterygian lineages, including Chondrostei, sturgeons (*Acipenser oxyrinchus* and *Acipenser ruthenus*) (Shaughnessy et al., 2023) and paddlefish (*Polyodon spathula*) (Dores et al., 2022), and the most basal extant actinopterygian lineage, Cladistia, represented by the bichir (*Polypterus senegalus*) (Shaughnessy et al., 2022). Together, these studies illustrating that the Mc2rs from taxa across the Actinopterygii phylogeny are exclusively selective for ACTH and dependent on Mrap1 for trafficking and activation indicate that a common ancestor of the Actinopterygii also possessed a highly selective Mc2r that requires Mrap1.

Similar to the Mc2r of actinopterygians, the sarcopterygian Mc2rs also demonstrate exclusivity for ACTH ligands and requisite co-expression with Mrap1 for membrane-trafficking and activation. These functional characteristics of Mc2r were first shown in mammalian models (Hinkle and Sebag, 2009; Metherell et al., 2005; Roy et al., 2007; Sebag and Hinkle, 2009,

2007), but have also been shown in other major extant tetrapod groups, including birds (*Gallus gallus*) (Barlock et al., 2014), reptiles (*Anolis carolinensis*) (Davis et al., 2013), and amphibians (*Xenopus tropicalis*) (Davis et al., 2013). Prior to the present study on lungfish, no pharmacological analyses had been conducted on the Mc2r orthologs of the non-tetrapod sarcopterygians, the lobe-finned fishes, such as the lungfishes (class *Dipnoi*) or the coelacanth (*Latimeria* spp.) which are the two most basal extant lineages of Sarcopterygii.

Structurally, the lungfish Mc2r and Mrap1 share many important features with actinopterygians and more derived sarcopterygians that explain the similarities in function. For example, there is a high degree of primary sequence conservation in portions of TM2-EC1-TM3 region and TM6, which have been implicated as the common H⁶F⁷R⁸W⁹ binding site for all melanocortin ligands (Chen et al., 2007; Pogozeva et al., 2005). Additionally, there is conservation of the phenylalanine in TM5 (Phe186 on lfMc2r; **Fig. 1A**), which serves as the contact site with the transmembrane domain of Mrap1 to facilitate trafficking (Davis et al., 2022). The lungfish Mrap1 exhibits the Y²³E²⁴Y²⁵Y²⁶ motif that is common to all Mraps (Dores and Chapa, 2021), and is followed by an apparent δ -D-Y- δ activation motif (D²⁷D²⁸Y²⁹I³⁰ on lfMrap1). Although this D²⁷D²⁸Y²⁹I³⁰ motif of lfMrap1 does not have an aromatic (δ) residue at position 27, it does have a Tyr residue at position 29, which has been shown to be critical for the activation of several osteichthyan Mc2r orthologs (Dores et al., 2022, 2016b; Sebag and Hinkle, 2009).

Consistent with the functional characteristics of Mc2rs of all other osteichthyans studied to date, the Mc2r of the lungfish also appears to be highly selective for an ACTH ligand and dependent on a cooperative interaction with Mrap1. By reproducing these results in a zebrafish cell line (in addition to our mammalian cell line), we confirmed that there were no potential confounding impacts of incubation temperature and cellular background on our functional studies of non-mammalian Mc2rs and Mrap1s. The alanine substitution experiments manipulating the “message” (H⁶F⁷R⁸W⁹) and “address” (K¹⁵K¹⁶R¹⁷R¹⁸P¹⁹) motifs on ACTH demonstrated that the lungfish Mc2r exhibits similar ligand interactions to other osteichthyans. For example, it has been shown in bowfin (*Amia calva*) and whale shark (*Rhincodon typus*) that the alanine substitution of all four H⁶F⁷R⁸W⁹ residues completely inhibits activation of Mc2r, with W⁹ appearing to be the single residue with the greatest effect on activation (Hoglin et al., 2023). Likewise, the requirement of an intact K¹⁵K¹⁶R¹⁷R¹⁸P¹⁹ “address” motif supports the

hypothesis that osteichthyan Mc2r orthologs are activated by a two-step process of involving the K¹⁵K¹⁶R¹⁷R¹⁸P¹⁹ “address” motif interacting with Mc2r first to open up a binding site for the H⁶F⁷R⁸W⁹ “message” motif to activate Mc2r (Dores and Chapa, 2021).

This current work on the lobe-finned fish Mc2r, together with our recent work on the bichir Mc2r (Shaughnessy et al., 2022), establishes that an ancestral osteichthyan likely exhibited a highly selective Mc2r that requires Mrap1, indicating that the functional evolution of Mc2r occurred relatively quickly between the chondrichthyan and osteichthyan lineages.

The present study and previous works from our laboratory have mapped how major shifts in Mc2r function occurred throughout the radiation of vertebrates (**Fig. 6**), while the function of the other melanocortin receptors (Mc1r, Mc3r, Mc4r, and Mc5r) remained conserved (Dores et al., 2016a, 2014). The unique functional evolution of Mc2r among the family of melanocortin receptors is likely a result of the much higher rate of spontaneous substitutions within the Mc2r gene (Schiöth et al., 2005) compared to Mc1r, Mc3r, Mc4r, and Mc5r. By our analysis, that difference in rate of sequence divergences has been approximately 3-fold (**Fig. 5B**). That Mc2r sequence divergence is so strongly positively correlated with taxa divergence time (**Fig. 5A**), implies that it is still evolving at a high rate. This could explain not only why major functional differences exist between the Mc2rs of chondrichthyans and osteichthyans, but also why functional differences in Mc2r exist within the chondrichthyans as well—Mc2r is a rapidly evolving gene. Importantly, the rate of sequence divergence was concentrated to specific regions of the Mc2r protein, wherein particularly high rates of divergence were found at EC1, EC2-TM5, IC3, TM6-EC3 (**Fig. 5C-D**). Many residues within these domains have already been described as impacting ligand recognition, ligand binding, and/or interaction with Mrap1 and have been implicated as causes for familial glucocorticoid deficiency, a disorder caused by dysfunction of Mc2r (Fridmanis et al., 2017).

Important questions remain regarding the functional evolution of Mc2r that cannot be answered solely by examining the Mc2rs of extant vertebrate taxa: When did exclusive selectivity for ACTH and dependence on Mrap1 emerge as a functional characteristic of Mc2r? Does the lack of ACTH selectivity and Mrap1 dependence exhibited by the Mc2rs of extant chondrichthyans represent the functional qualities of a hypothetical ancestral gnathostome or are these functional characteristics unique to the Mc2rs of cartilaginous fishes? Similar questions could be asked regarding the presence of an activation motif in Mrap1: Did the osteichthyans

gain an activation motif in Mrap1, or did the chondrichthyans lose it? To test such questions relating molecular structure and function, a 'Functional Synthesis' of evolutionary biology has emerged (Dean and Thornton, 2007; Storz et al., 2015). Experimental approaches within the framework of the Functional Synthesis powerfully combine the predictive power of molecular phylogenetic modelling with the explanatory power of functional molecular analyses. Using the computational method of ancestral gene reconstruction, the coding sequences of various ancestral states of Mc2r and Mrap1 can be deduced and evaluated for their functional characteristics. Manipulation of primary structure in key functional motifs of extant and ancestral Mc2rs or Mrap1s can be performed to determine the precise genetic changes that caused the evolutionary shifts in the complex functional relationship between Mc2r, its accessory protein Mrap1, and its ACTH and α -MSH ligands.

In conclusion, we have characterized the structure and function of Mc2r in the last remaining major vertebrate group occupying a key phylogenetic position regarding the evolution of Mc2r, with that group being the basal sarcopterygians, the lobe-finned fishes. The present work and previous works from our laboratory have mapped the shifts in Mc2r function through the radiation of vertebrates, and charted a path forward for resolving the precise mechanisms for how sequence divergence in key domains and at key residue positions resulted in the molecular evolution of Mc2r. Future studies within the Functional Synthesis are poised to answer questions such as: How did complexity arise in the functional interactions of Mc2r and Mrap1? Did the functional evolution of Mc2r and Mrap1 proceed by many small-in-effect genetic mutations or by a few large-in-effect mutations? Could there have been multiple genetic evolutionary paths to the same derived functional characteristics of Mc2r and Mrap1? Future research on the evolution of melanocortin signaling in the HPI axis should seek to employ principles and the experimental framework of the Functional Synthesis to ultimately resolve the functional co-evolution of Mc2r and Mrap1.

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428

429 **Declaration of Competing Interest**

430 The authors declare that they have no known competing financial interests or personal
431 relationships that could have appeared to influence the work reported in this paper.

432

433 **Data availability**

434 All data is contained within the manuscript.

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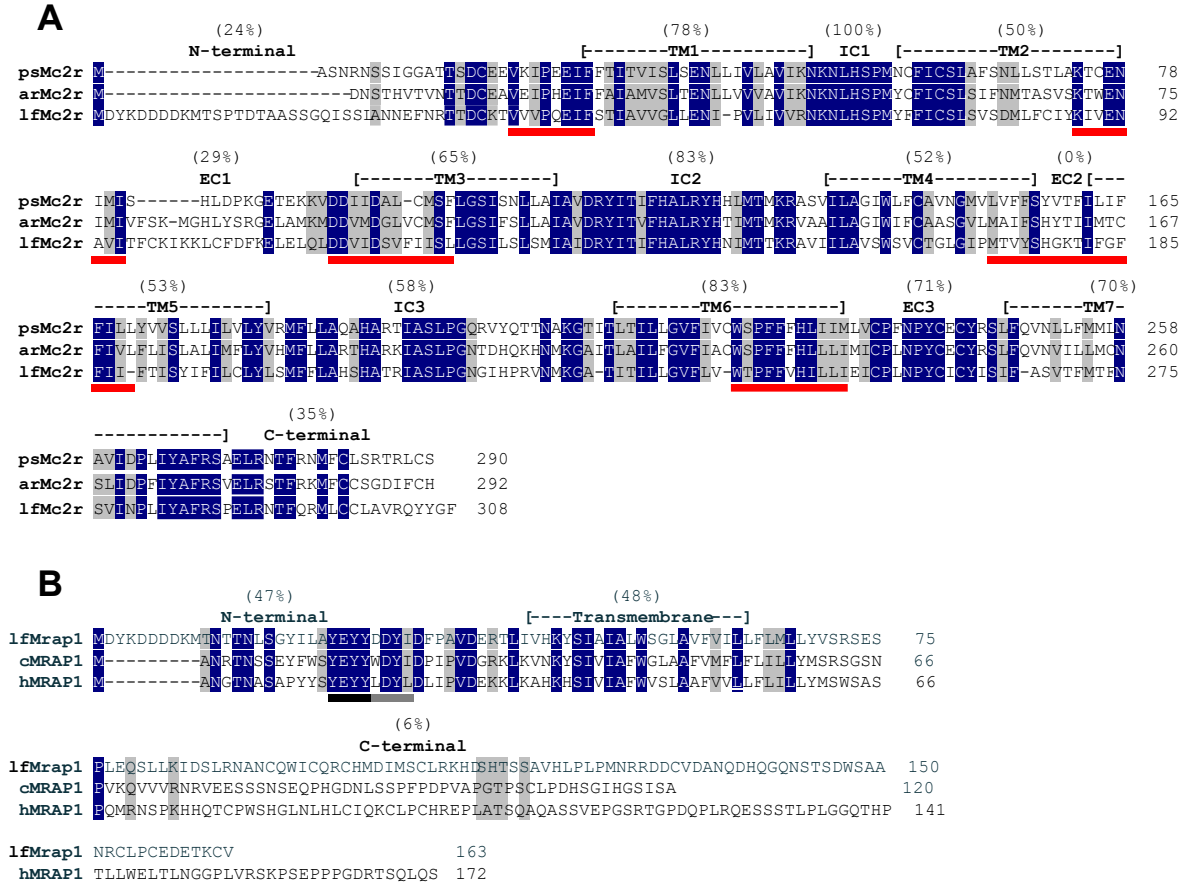
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613 Figure 1



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616 **Figure 1: Alignment lungfish (*Protopterus annectens*; lf) Mc2r (A) and Mrap1 (B) with**

617 **selected osteichthyan Mc2r and Mrap1 sequences. Colors indicate sequence similarity (gray)**

618 **and identity (blue). Structural domains (bolded) and their sequence similarities (percentages**

619 **within parentheses) are indicated above alignments. In A: Red bars indicate regions containing**

620 **residues involved in the H⁶F⁷R⁸W⁹ forming the ligand binding pocket and determining ligand**

621 **recognition (Fridmanis et al., 2017). In B: the black bar indicates the Y¹⁴E¹⁵Y¹⁶Y¹⁷ motif and the**

622 **following gray bar indicates the δ^{18} D¹⁹Y²⁰ δ^{21} activation motif. Sequence name abbreviations: ps,**

623 ***Polypterus senegalus* (bichir); ar, *Acipenser ruthenus* (sterlet sturgeon); c, chicken (*Gallus***

624 ***gallus*); h, human (*Homo sapiens*). Domain abbreviations: TM, transmembrane domain; IC,**

625 **intracellular loop; EC, extracellular loop.**

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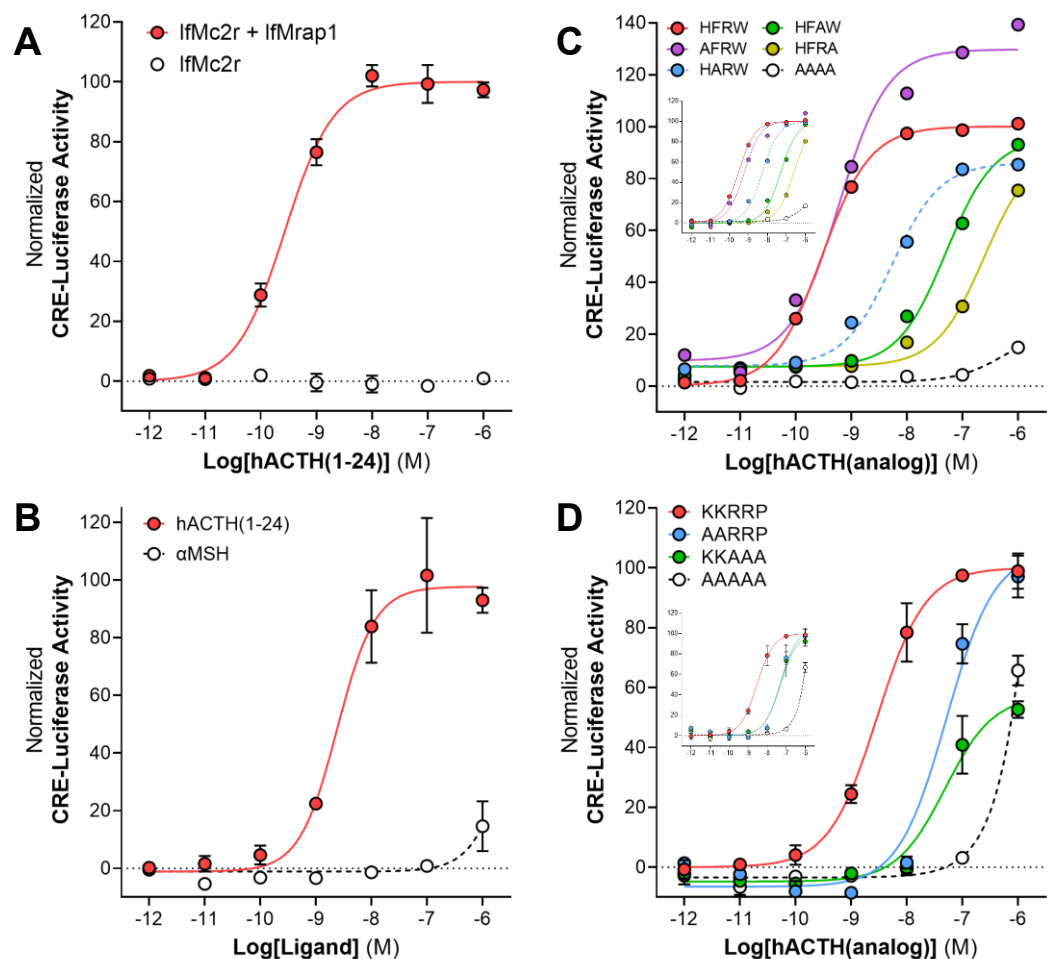
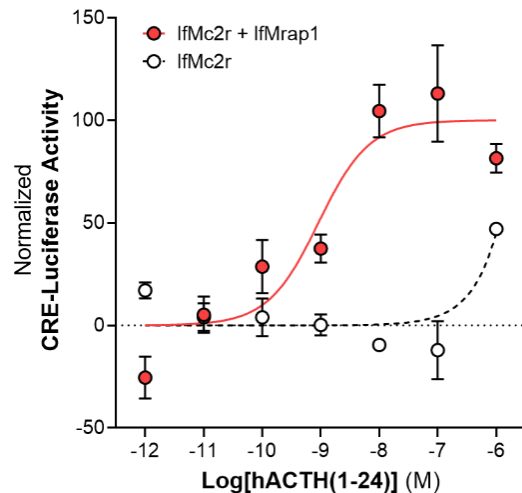
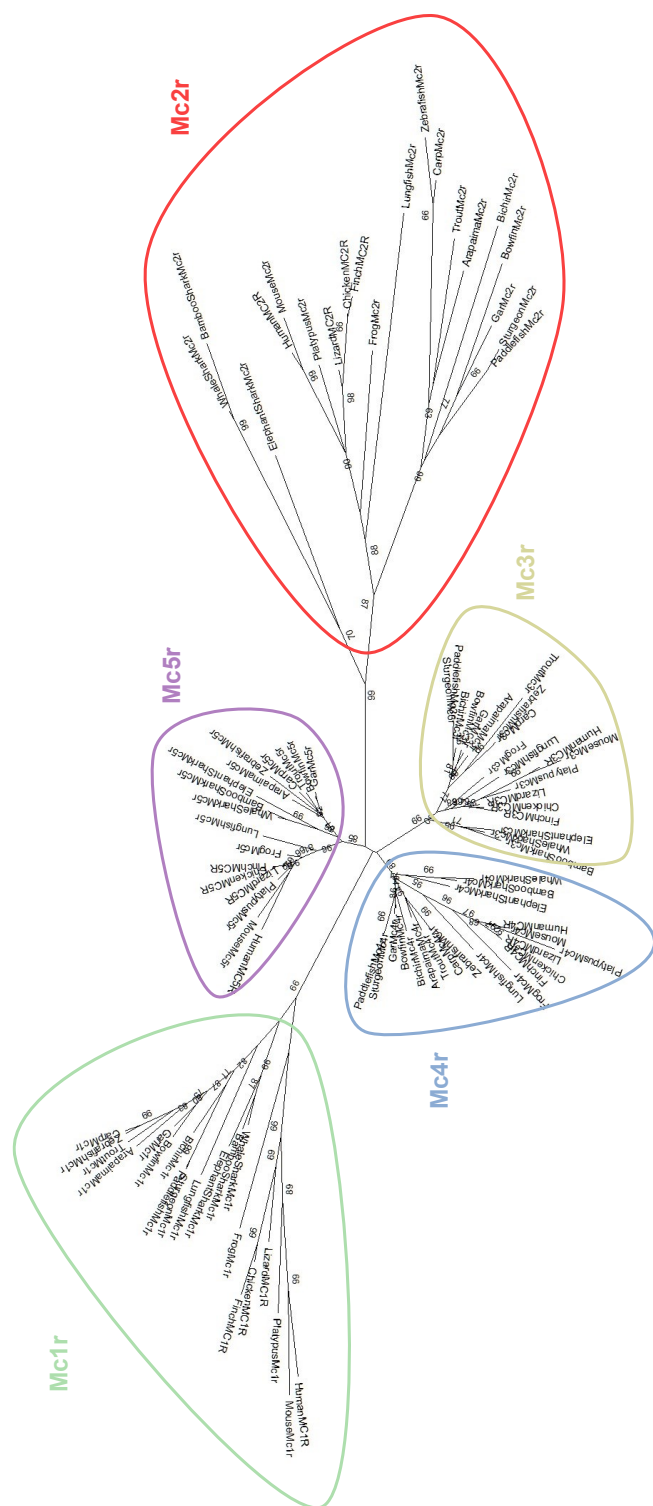


Figure 2: Pharmacology of lungfish (*Protopterus annectens*; lf) Mc2r expressed in Chinese hamster ovary (CHO) cells. (A) Dose-response stimulation by human (h) ACTH(1-24) of lfMc2r co-expressed with or without lfMrap1. (B) Dose-response stimulation by either hACTH(1-24) or α-MSH of lfMc2r co-expressed with lfMrap1. (C) Dose-response stimulation by alanine substitutions in the H⁶F⁷R⁸W⁹ motif in hACTH(1-24). (D) Dose-response stimulation by alanine substitutions in the K¹⁵K¹⁶R¹⁷R¹⁸P¹⁹ motif in hACTH(1-24). Stimulation is presented as normalized (to the V_{max} of the unaltered hACTH(1-24) positive control) cAMP-responsive luciferase activity, with lines representing the fitted dose-response curve (three-parameter polynomial). For clearer comparison of EC₅₀ values, insets depict curves wherein the V_{max} of each curve is set to 100. Data are presented as mean ± standard error (n = 3). See text for additional methodological details.

642 Figure 3



643
644 **Figure 3: Pharmacology of lungfish (*Protopterus annectens*; lf) Mc2r expressed in zebrafish**
645 **embryonic fibroblast (ZF4) cells.** Dose-response stimulation by human (h) ACTH(1-24) of
646 lfMc2r co-expressed with or without lfMrp1. See text for additional methodological details.
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Figure 4: Molecular phylogeny of Mc1r, Mc2r, Mc3r, Mc4r, and Mc5r protein sequences by maximum likelihood method and a Jones-Taylor-Thornton matrix-based model. See text for additional methodological details.

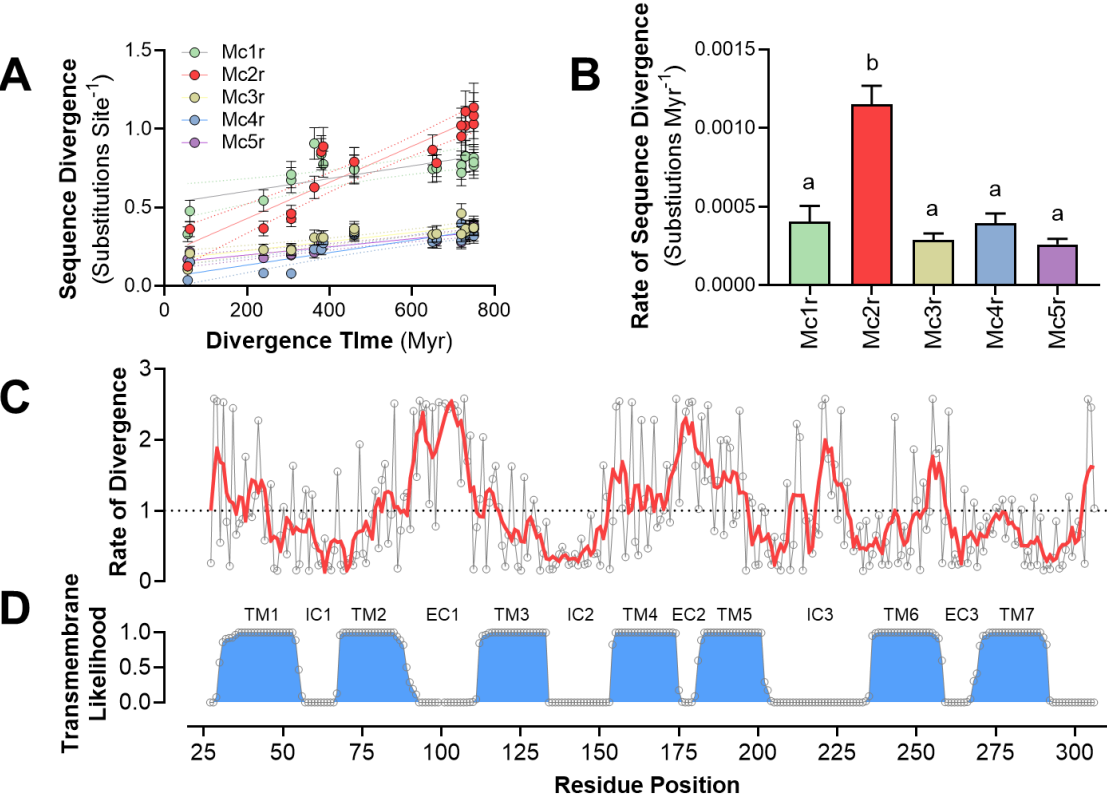


Figure 5: Molecular clock analyses of melanocortin receptors. (A) Molecular clock analysis of gnathostome melanocortin receptors comparing sequence divergence (pairwise distance) and geological divergence time from humans. (B) Rate of sequence divergence as described by the slope of the linear regression in A. (C) Position-specific rate of divergence in Mc2r. Gray circles and lines depicts rate at each position. Red line depicts a smoothed curve of the averages of four-position bins. (D) Transmembrane domain predictions at each residue of lungfish (*Protopterus annectens*) Mc2r, which, for clarity, has been positionally aligned with the positions-specific divergence rates of vertebrate Mc2r presented in C. See text for additional methodological details.

Figure 6

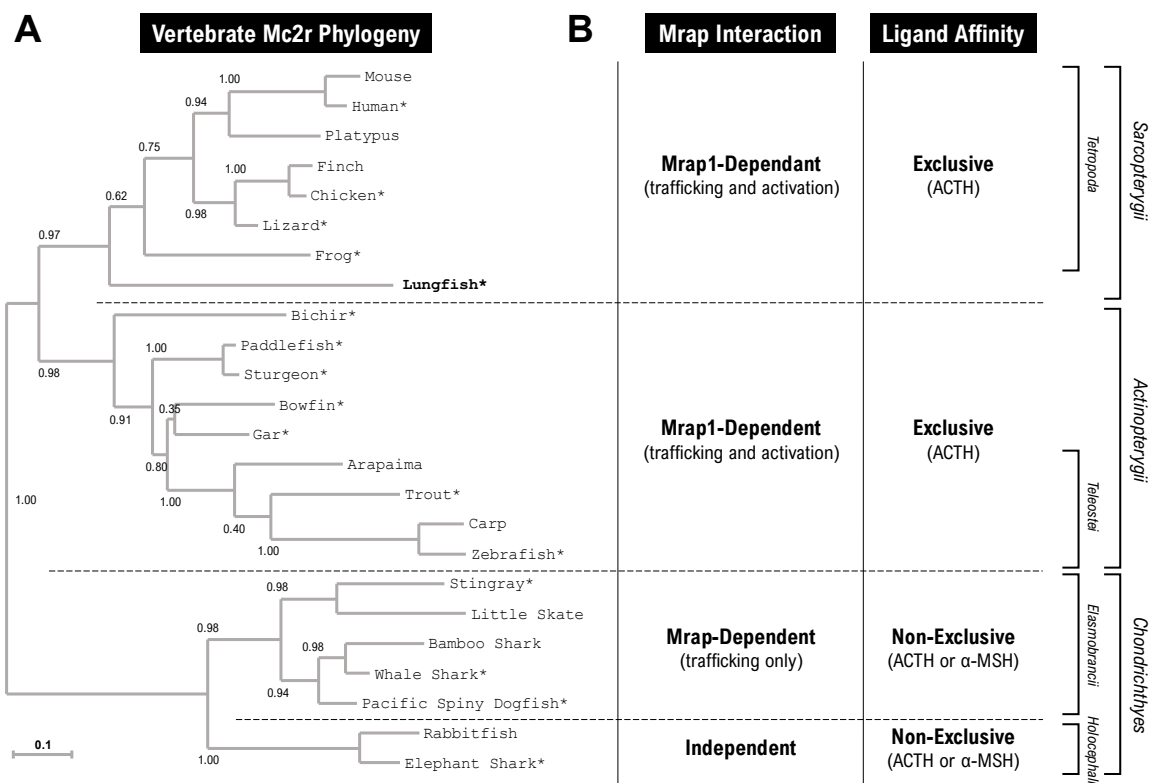


Figure 6: The functional evolution of Mc2r across vertebrates. (A) Molecular phylogeny of vertebrate Mc2r protein sequences by maximum likelihood (bootstrap values indicated at nodes). (B) Current understanding of the functional characteristics of Mc2r in various vertebrate taxonomic groups. Asterisks indicate taxa for which our laboratory has conducted pharmacological studies of Mc2r (see text for citations).

Table I: Sequences and affinities for alanine-substituted analogs of hACTH(1-24)

Analog	Peptide Sequence	Log(EC ₅₀) (M)	P
HFRW	SYSME HFRW GKPVGKKRRPVKVYP	-9.5 (-9.7, -9.3)	–
AFRW	SYSME AFRW GKPVGKKRRPVKVYP	-9.226 (-9.6, -8.9)	0.102
HARW	SYSME HARW GKPVGKKRRPVKVYP	-8.266 (-8.4, -8.1)	<0.001
HFAW	SYSME HFAW GKPVGKKRRPVKVYP	-7.295 (-7.5, -7.1)	<0.001
HFRA	SYSME HFRA GKPVGKKRRPVKVYP	-6.607 (-6.8, -6.3)	<0.001
AAAA	SYSME AAAA GKPVGKKRRPVKVYP	–	–
KKRRP	SYSMEHFRWGKPVG KKRRP VKVYP	-8.534 (-8.7, -8.3)	–
AARRP	SYSMEHFRWGKPVG AARRP VKVYP	-7.306 (-7.6, -7.0)	<0.001
KKAAA	SYSMEHFRWGKPVG KKAAA VKVYP	-7.287 (-7.5, -7.1)	<0.001
AAAAA	SYSMEHFRWGKPVG AAAAA VKVYP	–	–

In all experiments, IfMc2r was co-expressed with IfMap1. Analogs HFRW and KKRRP are the native peptides for hACTH(1-24) (positive controls). EC₅₀ values are derived from an activation curve containing 8 doses at 3 replicates per dose and presented as: mean (95 % confidence interval). *P* value reflects comparison of EC₅₀ value to positive control.