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1 Aldosterone and Dexamethasone Activate African Lungfish Mineralocorticoid Receptor:
2 Increased Activation After Removal of the Amino-Terminal Domain

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31 **ABSTRACT.** Aldosterone, the main physiological mineralocorticoid in humans and other
32 terrestrial vertebrates, first appears in lungfish, which are lobe-finned fish that are forerunners of
33 terrestrial vertebrates. Aldosterone activation of the MR regulates internal homeostasis of water,
34 sodium and potassium, which was critical in the conquest of land by vertebrates. We studied
35 transcriptional activation of the slender African lungfish MR by aldosterone, other
36 corticosteroids and progesterone and find that aldosterone, 11-deoxycorticosterone, 11-
37 deoxycortisol and progesterone have half-maximal responses (EC50s) below 1 nM and are
38 potential physiological mineralocorticoids. In contrast, EC50s for corticosterone and cortisol
39 were 23 nM and 66 nM, respectively. Unexpectedly, truncated lungfish MR, consisting of the
40 DNA-binding, hinge and steroid-binding domains, had a stronger response to corticosteroids and
41 progesterone than full-length lungfish MR, indicating that the N-terminal domain represses
42 steroid activation of lungfish MR, unlike human MR in which the N-terminal domain contains an
43 activation function. BLAST searches of GenBank did not retrieve a GR ortholog, leading us to
44 test dexamethasone and triamcinolone for activation of lungfish MR. At 10 nM, both synthetic
45 glucocorticoids are about 4-fold stronger than 10 nM aldosterone in activating full-length
46 lungfish MR, leading us to propose that lungfish MR also functions as a GR.

47

48 **Keywords:** Lungfish, Lobe-finned Fish, Terrestrial Vertebrates, Aldosterone evolution;
49 mineralocorticoid receptor evolution; evolution

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52 **INTRODUCTION**

53 The mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) belong to the
54 nuclear receptor family, a diverse group of transcription factors that arose in multicellular
55 animals [1–3]. The MR and GR have key roles in the physiology of humans and other terrestrial
56 vertebrates and fish [4–11]. The MR and GR evolved from an ancestral corticoid receptor (CR)
57 in a jawless fish (cyclostome), which has descendants in modern lampreys and hagfish [12–14].
58 A distinct MR and GR first appear in cartilaginous fishes (Chondrichthyes) [1,13,15–17], which
59 diverged from bony vertebrates about 450 million years ago [18,19].

60 Aldosterone is the main physiological mineralocorticoid in humans and other terrestrial
61 vertebrates [5,6,9,20–23]. Aldosterone activation of the MR in the kidney regulates salt and

62 water homeostasis by promoting sodium and water reabsorption and potassium secretion, a
 63 mechanism that conserves salt and water. Thus, it is puzzling that aldosterone is a potent
 64 transcriptional activator of lamprey and hagfish CRs [15], skate MR [16] and elephant shark MR
 65 [13,24] because aldosterone is not synthesized by lampreys [15], cartilaginous fishes or ray
 66 finned fishes [25]. Aldosterone first appears in lungfish [26–28], which are lobe-finned fish that
 67 are forerunners of terrestrial vertebrates [29–31]. The key phylogenetic position of lungfish in
 68 the transition of vertebrates from water to land [27,29,30,32] and the role of the MR in
 69 maintaining internal electrolyte homeostasis [5,8,33,34] motivated us to investigate the response
 70 of the slender African lungfish MR to aldosterone, cortisol and other corticosteroids (Figure 1),
 71 and to hypothesize that this MR would be activated by progestins, which also activate elephant
 72 shark MR [24], ray-finned fish MR [35–39] and chicken MR [24,40].

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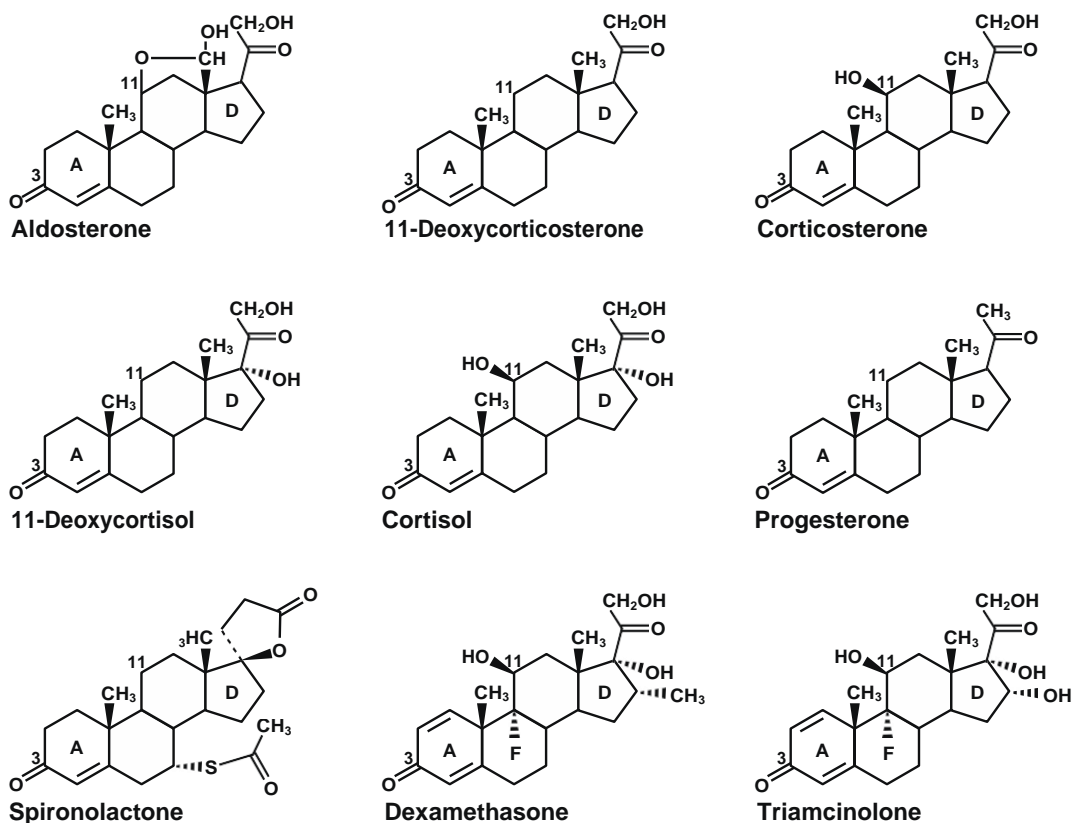


Figure 1. Structures of Corticosteroids, Dexamethasone, Triamcinolone, Progesterone and Spironolactone. Aldosterone and 11-deoxycorticosterone are mineralocorticoids [4]. 11-deoxycortisol is a mineralocorticoid in lamprey [41,42]. Cortisol and corticosterone are glucocorticoids in terrestrial vertebrates and ray-finned fish [43,44]. Dexamethasone and triamcinolone are synthetic glucocorticoids. Progesterone is female reproductive steroid that

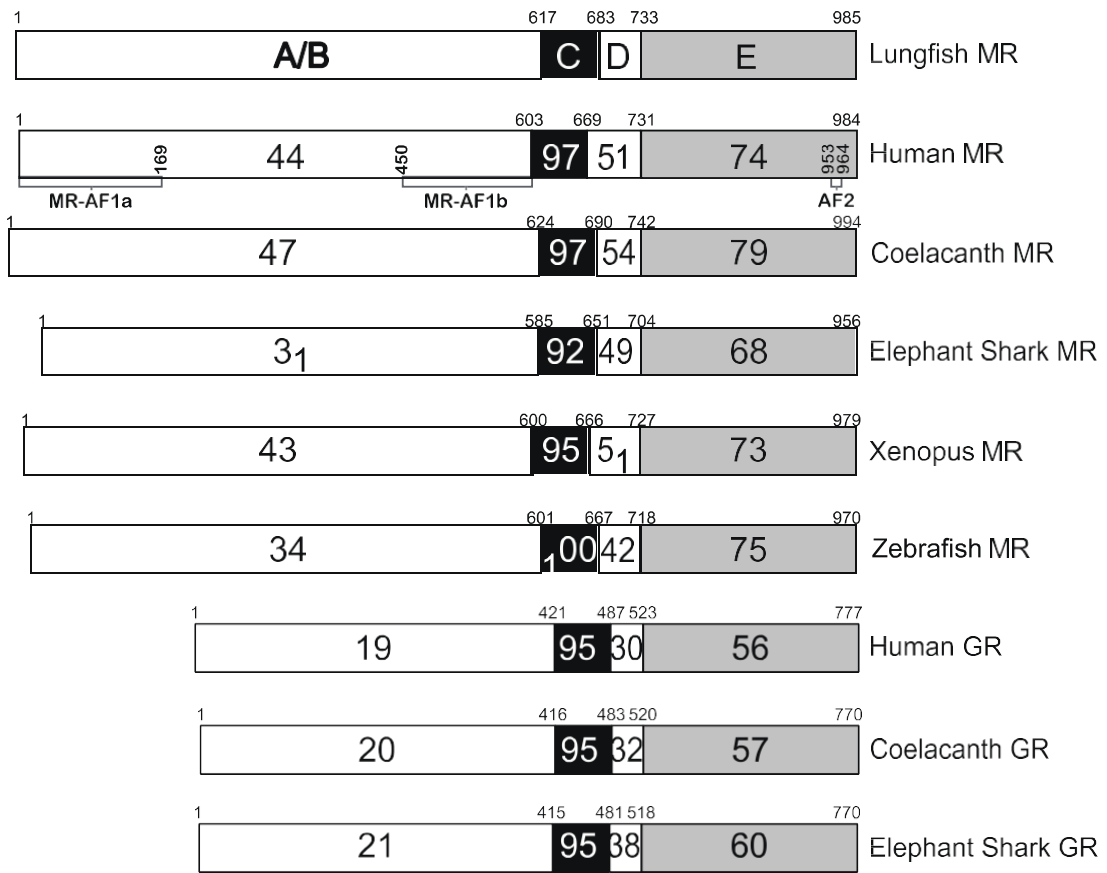
80 also is important in male physiology [45,46]. Spironolactone is a mineralocorticoid antagonist in
81 humans [47,48].

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83 Our investigation also uncovered an unexpected role of the N-terminal domain (NTD) of
84 lungfish MR in inhibiting transcriptional activation by steroids. Like other steroid receptors,
85 lungfish MR is a multi-domain protein, consisting of an NTD (domains A and B), a central
86 DNA-binding domain (DBD) (domain C), a hinge domain (D) and a C-terminal ligand-binding
87 domain (LBD) (domain E) [49–51] (Figure 2). The NTD in the human MR contains an
88 activation function domain (AF1), which is split into two segments [49–51]. (Figure 2). As
89 described below, we find that in contrast to human MR [24,49,51,52], the NTD in full-length
90 lungfish MR reduces steroid-mediated activation of lungfish MR, compared to truncated lungfish
91 MR-CDE in cells transfected with a 3X-Tyrosine Amino Transferase (TAT3) promoter [53].

92 We also find that lungfish MR is activated by dexamethasone. At 10 nM, dexamethasone
93 activates full-length lungfish MR and truncated lungfish MR with a signal that is 4-fold and 6-
94 fold stronger, respectively, than that of 10 nM aldosterone. This strong response to
95 dexamethasone and the absence of a lungfish GR sequence after a BLAST search of GenBank
96 leads us to propose that lungfish MR also functions as a GR.

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98

99 **Figure 2. Comparison of the functional domains of lungfish MR to corresponding domains**
100 **in selected vertebrate MRs (human, coelacanth, elephant shark, *Xenopus*, zebrafish) and**
101 **GRs (human, coelacanth, elephant shark). Lungfish MR and human MR have 97% and 74%**
102 **identity in DBD and LBD, respectively. Lungfish MR and elephant shark MR have 92% and**
103 **68% identity in DBD and LBD, respectively. This strong conservation of the DBD and LBD**
104 **contrasts with the low sequence identity of 44% and 47% between their NTDs. There are**
105 **similar % identities between corresponding domains in lungfish MR and other MRs.**

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107 **RESULTS AND DISCUSSION**

108 **Transcriptional activation of full-length and truncated lungfish MR by corticosteroids,** 109 **progestins and dexamethasone.**

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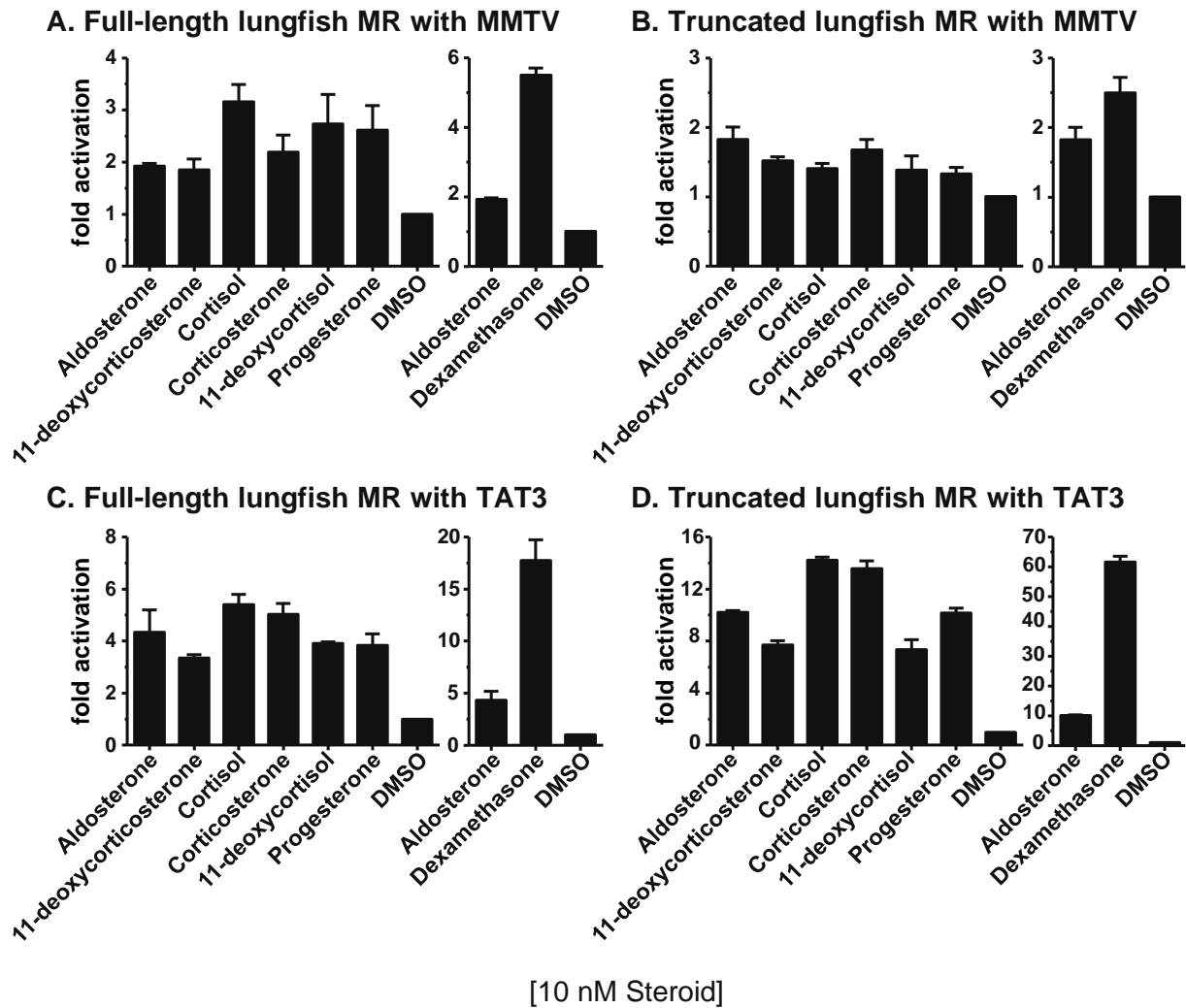
We screened a panel of steroids (Figure 1) at 10 nM for transcriptional activation of full-length and truncated lungfish MR containing the CDE domains (MR-CDE) using two promoters: 2X-Mouse Mammary Tumor Virus (MMTV) [54,55] and TAT3 [53], which along with plasmids for both lungfish MRs were transfected into HEK293 cells.

114 As shown in Figure 3A, there was about 2 to 3-fold activation by 10 nM aldosterone,
115 other corticosteroids or progesterone of full-length lungfish MR using the MMTV-luc reporter
116 and less steroid activation of lungfish MR-CDE (Figure 3B, suppl. Figure 1B).

117 Interestingly, compared to activation of full-length lungfish MR with the MMTV
118 promoter (Figure 3A, suppl. Figure 1A), transcriptional activation of full-length lungfish MR
119 with a TAT3 promoter and 10 nM aldosterone, other physiological corticosteroids or
120 dexamethasone increased by about 1.5 to 2-fold (Figure 3C, suppl. Figure 1C). Unexpectedly,
121 lungfish MR-CDE with the TAT3 promoter had an additional 2-fold increase in activation by all
122 corticosteroids (Figure 3D, suppl. Figure 1D). Progesterone activated lungfish MR in accord
123 with the prediction of Fuller et al. [37,39,56]. Together, these experiments show that removal of
124 the NTD increases corticosteroid and progesterone activation of lungfish MR in the presence of
125 the TAT3 promoter, which is stronger than the MMTV promoter.

126 Our results with dexamethasone, which activates human MR in vitro [52,57–59], were
127 unexpected. To our surprise, compared to aldosterone, dexamethasone was about 3-fold and 6-
128 fold more active, respectively, in activating full-length lungfish MR (Figure 3C, suppl. Figure
129 1C) and truncated lungfish MR (Figure 3D, suppl. Figure 1D) with the TAT3 promoter.
130 Moreover, both cortisol and corticosterone have stronger fold-activation than does aldosterone of
131 lungfish MR using the TAT3 promoter at equimolar concentrations. Under these conditions,
132 lungfish MR appears to have a GR-like response to steroids.

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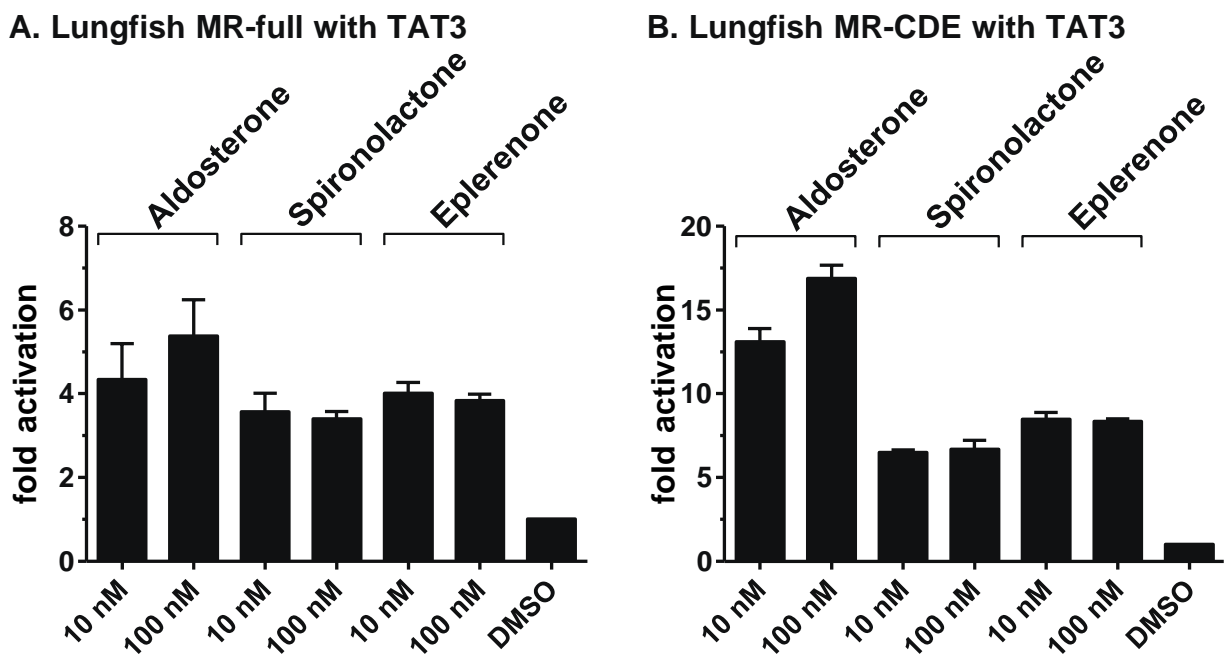
Figure 3. Ligand specificity of full-length and truncated lungfish MR.

Plasmids for full-length lungfish MR or truncated lungfish MR (MR-CDE) were expressed in HEK293 cells with an MMTV-luciferase reporter or a TAT3-luciferase reporter. Transfected cells were treated with either 10 nM aldosterone, cortisol, 11-deoxycortisol, corticosterone, 11-deoxycorticosterone, progesterone, dexamethasone or vehicle alone (DMSO). Results are expressed as means \pm SEM, n=3. Y-axis indicates fold-activation compared to the activity of control vector with vehicle alone as 1. A. Full-length lungfish MR with MMTV-luciferase. B. Truncated lungfish MR (MR-CDE) with MMTV-luciferase. C. Full-length lungfish MR with TAT3-luciferase. D. Truncated lungfish MR (MR-CDE) with TAT3-luciferase.

150 **Spironolactone and eplerenone are transcriptional activators of lungfish MR.**

151 Because spironolactone, an antagonist of human MR, activates elephant shark MR [24],
152 zebrafish MR [37,40,60] and trout MR [38], we investigated spironolactone for activation of full-
153 length lungfish MR and truncated lungfish MR-CDE. We also studied activation by eplerenone,
154 another antagonist of human MR [61]. As shown in Figure 4, both spironolactone and
155 eplerenone activated lungfish MR with a TAT3 promoter, and there was a further increase in
156 fold-activation by both steroids of lungfish MR-CDE.

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159 **Figure 4. Spironolactone and eplerenone activation of full-length and truncated lungfish**
160 **MR.** Plasmids for full-length lungfish MR or truncated lungfish MR (MR-CDE) were expressed
161 in HEK293 cells with a TAT3-luciferase reporter. Transfected cells were treated with either 10
162 nM or 100 nM aldosterone, spironolactone or eplerenone or vehicle alone (DMSO). Results are
163 expressed as means \pm SEM, n=3. Y-axis indicates fold-activation compared to the activity of
164 control vector with vehicle alone as 1. A. Full-length lungfish MR with TAT3. B. Truncated
165 lungfish MR (MR-CDE) with TAT3.

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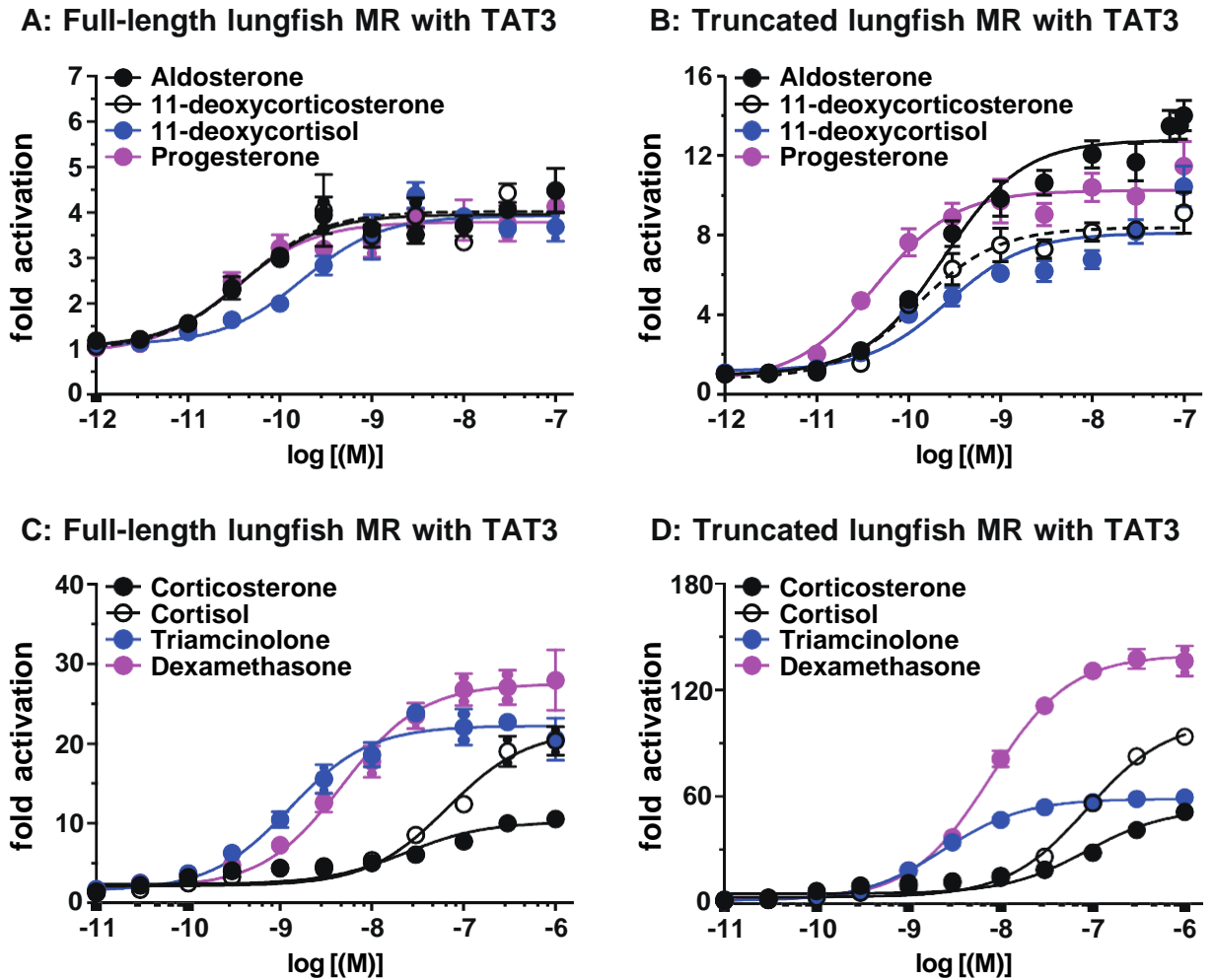
167 Concentration-dependent activation by corticosteroids and progestins of full-length and
168 truncated lungfish MR.

169 To gain a quantitative measure of corticosteroid and progestin activation of full-length
170 and truncated lungfish MR, we determined the concentration dependence of transcriptional
171 activation by corticosteroids and progestins of full-length lungfish MR and lungfish MR-CDE
172 using TAT3 (Figure 5). This data was used to calculate a half maximal response (EC50) for
173 steroid activation of lungfish MR with a TAT3 promoter (Table 1). For full-length lungfish MR,
174 the four lowest EC50s were for aldosterone (0.04nM), 11-deoxycorticosterone (0.04 nM), 11-
175 deoxycortisol (0.17nM) and progesterone (0.03nM). These low EC50s are consistent with a
176 physiological role for one or more of these steroids as ligand for lungfish MR. In contrast,
177 corticosterone and cortisol, two physiological corticosteroids in terrestrial vertebrates, had EC50s
178 of 23.1nM and 66.1nM, respectively. Two synthetic glucocorticoids, dexamethasone and
179 triamcinolone, had EC50s of 4.7nM and 1.3nM, respectively.

180 For truncated lungfish MR, there were similar low EC50s for aldosterone (0.24nM), 11-
181 deoxycorticosterone (0.013nM), 11-deoxycortisol (0.27nM) and progesterone (0.04nM). EC50s
182 for corticosterone and cortisol were 85.5nM and 86.7nM, respectively. EC50s for
183 dexamethasone and triamcinolone were 7.7nM and 2.4nM, respectively.

184 Overall, these results reveal that the EC50s of aldosterone, 11-deoxycorticosterone, 11-
185 deoxycortisol and progesterone for full-length lungfish MR and lungfish MR-CDE are similar
186 and that one or more of these steroids could be a physiological mineralocorticoid in lungfish.
187 Although EC50s for full-length lungfish MR of triamcinolone and dexamethasone were at least
188 10-fold higher than that of aldosterone, deoxycorticosterone, 11-deoxycortisol and progesterone,
189 compared to these steroids, dexamethasone and triamcinolone have a several fold higher
190 activation of full-length and truncated lungfish MR (Figure 5). Consistent with data in Figure 3,
191 deletion of the NTD to form truncated lungfish MR-CDE increased fold-activation by
192 aldosterone, the other corticosteroids, progesterone, dexamethasone and triamcinolone.
193 However, deletion of the NTD did not have a large effect on their EC50s.

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197 **Fig. 5. Concentration-dependent transcriptional activation by corticosteroids, progesterone,**
 198 **dexamethasone and triamcinolone of full length and truncated lungfish MR.** Plasmids for
 199 full-length lungfish MR or truncated lungfish MR, were expressed in HEK293 cells with a
 200 TAT3-luciferase promoter. Cells were treated with increasing concentrations of either
 201 aldosterone, cortisol, corticosterone, 11-deoxycorticosterone, 11-deoxycortisol, progesterone,
 202 dexamethasone and triamcinolone or vehicle alone (DMSO). Results are expressed as means \pm
 203 SEM, n=3. Y-axis indicates fold-activation compared to the activity of control vector with
 204 vehicle (DMSO) alone as 1. A. Aldosterone, 11-deoxycorticosterone, 11-deoxycortisol and
 205 progesterone with full-length lungfish MR with TAT3-luc. B. Aldosterone, 11-
 206 deoxycorticosterone, 11-deoxycortisol and progesterone with truncated lungfish MR (Domains
 207 CDE) with TAT3-luc. C. Cortisol, corticosterone, dexamethasone and triamcinolone with full-
 208 length lungfish MR with TAT3-luc. D. Cortisol, corticosterone, dexamethasone and
 209 triamcinolone with truncated lungfish MR (Domains CDE) with TAT3-luc.

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214 **Table 1. EC50 values for steroid activation of full-length and truncated lungfish MR with the TAT3**
 215 **promoter.**

	Aldosterone	11-deoxycorticosterone	11-deoxycortisol	Progesterone
	EC50	EC50	EC50	EC50
MR-full length	0.04 nM	0.04 nM	0.17 nM	0.03 nM
95% confidence interval	0.02-0.07 nM	0.02-0.09 nM	0.1-0.3 nM	0.02-0.06 nM
MR-CDE	0.24 nM	0.13 nM	0.27 nM	0.044 nM
95% confidence interval	0.17-0.35 nM	0.08-0.2 nM	0.14-0.53 nM	0.026-0.076 nM

216

	Corticosterone	Cortisol	Triamcinolone	Dexamethasone
	EC50	EC50	EC50	EC50
MR-full length	23.1 nM	66.1 nM	1.3 nM	4.7 nM
95% confidence interval	11.3-47.1 nM	44.5-98.4 nM	0.9-1.9 nM	3.3-6.9 nM
MR-CDE	85.5 nM	86.7 nM	2.4 nM	7.7 nM
95% confidence interval	60.0-121.8 nM	74.3-101.2 nM	2.1-2.8 nM	6.6-9.1 nM

217
 218
 219 **Transcriptional activation of full-length and truncated human MR and full-length and**
 220 **truncated elephant shark MR by corticosteroids and progestins.**

221 To gain an evolutionary perspective on activation of lungfish MR by steroids, we
 222 screened a panel of steroids, at 10 nM, for transcriptional activation of full-length human and
 223 elephant shark MRs and truncated human and elephant shark MR-CDEs using two reporters:
 224 MMTV-luc and TAT3-luc.

225 **Comparison of human MR and lungfish MR.**

226 Overall, compared to lungfish MR, fold activation of human MR was significantly higher
 227 for aldosterone and other corticosteroids. For example, compared to 2-fold activation by
 228 aldosterone of full-length lungfish MR with the MMTV promoter (Figure 3A), activation of full-
 229 length human MR by aldosterone was about 70-fold with the MMTV promoter (Figure 6A).
 230 Although fold-activation by steroids for truncated human MR (Figure 6B) decreased compared
 231 to full-length human MR (Figure 6A), activation by aldosterone and other corticosteroids of
 232 truncated human MR with the MMTV promoter (Figure 6B) was about 7-fold higher than for
 233 truncated lungfish MR (Figure 3B).

234 Unlike for lungfish MR, deletion of the NTD in human MR resulted in a loss of
235 activation by aldosterone and other corticosteroids for human MR-CDE with both promoters
236 (Figure 6A-D), consistent with the presence of two activation function domains in the NTD
237 (Figure 2) [49–52]. The relative loss of activation of human MR was greater with the MMTV
238 promoter than with the TAT3 promoter. For example, at 10 nM aldosterone, activation of full-
239 length human MR with the MMTV reporter was 70-fold (Figure 6A), which decreased to 14-fold
240 for human MR-CDE (Figure 6B). In contrast, at 10 nM aldosterone, fold-activation of human
241 MR-CDE with the TAT3 promoter was about 75% of activity for full-length human MR (Figure
242 6C, D). However, 11-deoxycorticosterone and 11-deoxycortisol lost substantial activity for
243 human MR-CDE with the MMTV and TAT3 promoters (Figure 6D).

244 There also was higher fold-activation by aldosterone of full-length and truncated human
245 MR with the TAT3 promoter (Figure 6C, D) compared to full-length and truncated lungfish MR
246 (Figure 3C, D). Aldosterone activation of full-length human MR with the TAT3 promoter
247 (Figure 6C) was about 45-fold higher than that for full-length lungfish MR with the TAT3
248 promoter (Figure 3C). Aldosterone activation of human MR-CDE with the TAT3 promoter
249 (Figure 6D) was about 15-fold higher than that for lungfish MR-CDE (Figure 3D).

250 The relative activation by aldosterone and dexamethasone of human MR and lungfish
251 MR was reversed. Aldosterone was more active than dexamethasone in stimulating transcription
252 by full-length human MR and human MR-CDE with the TAT3 promoter (Figure 6C, D). In
253 contrast, for lungfish MR dexamethasone was more active than aldosterone for full-length
254 lungfish MR and lungfish MR-CDE with the TAT3 promoter (Figure 3C, D).

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257 Comparison of elephant shark MR and lungfish MR.

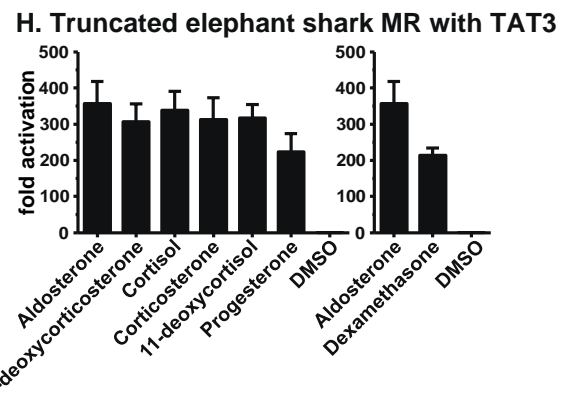
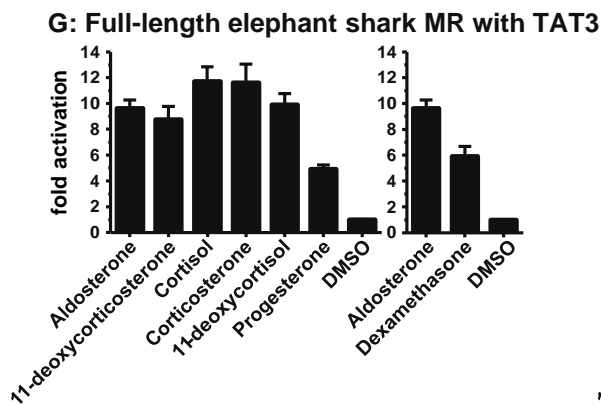
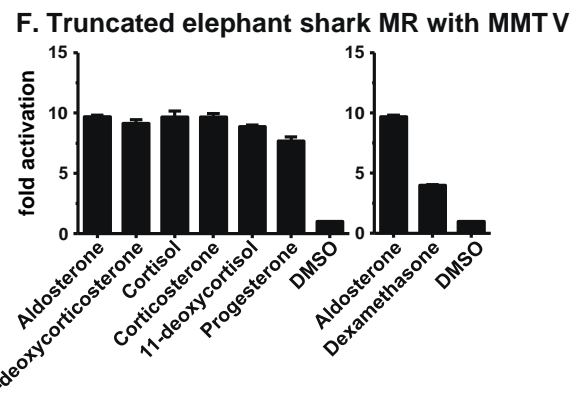
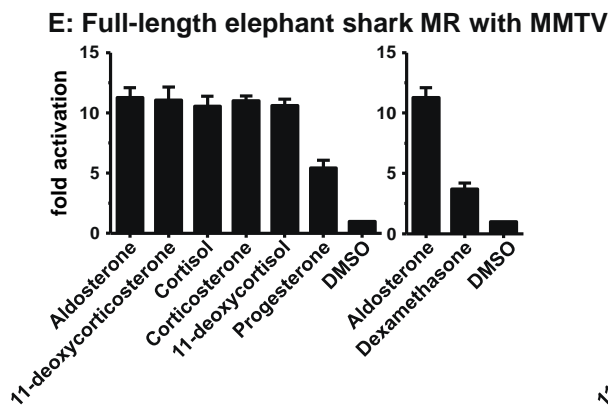
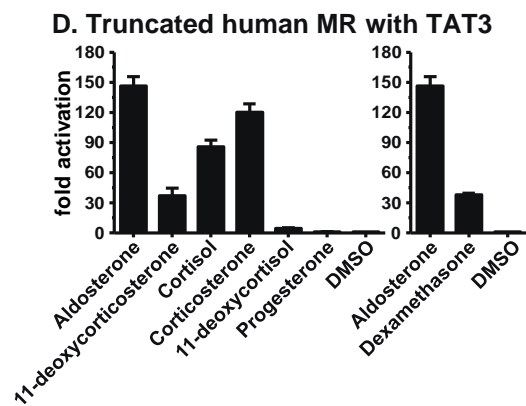
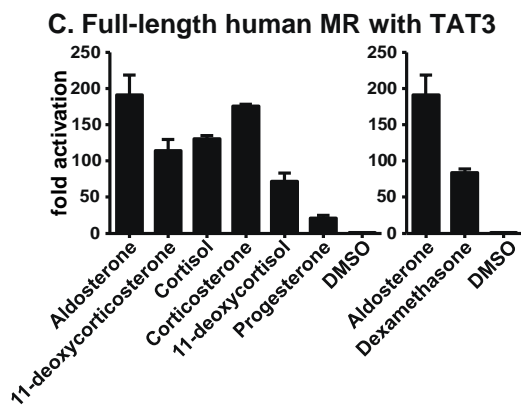
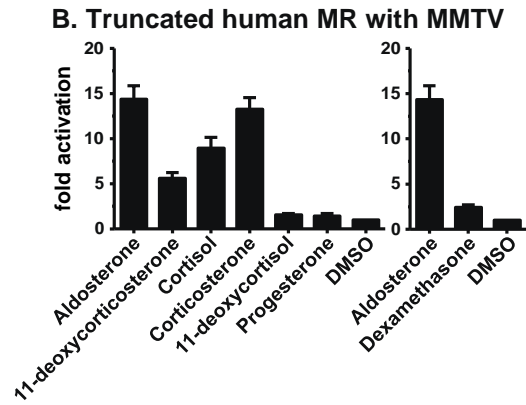
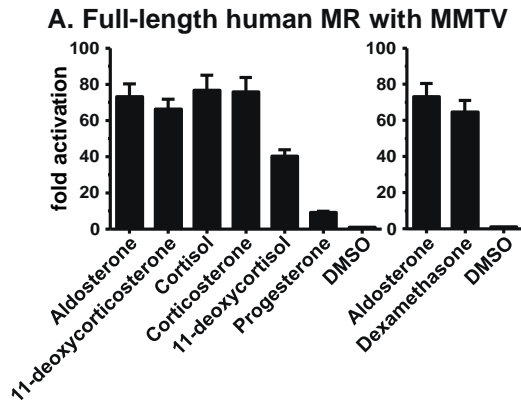
258 Activation by corticosteroids and progesterone of elephant shark MR with the MMTV
259 promoter has some similarities with their activation of lungfish MR. Like lungfish MR,
260 corticosteroids have a similar activation of about 10-fold for full-length and truncated elephant
261 shark MR, with little difference in potency among the corticosteroids. However, unlike lungfish
262 MR, aldosterone is stronger than dexamethasone in activating full-length and truncated elephant
263 shark MR with the MMTV promoter (Figure 6E, F).

264 At a 10 nM steroid concentration, aldosterone and other corticosteroids activated full-
265 length elephant shark MR with the TAT3 promoter by 9 to 12-fold (Figure 6G), which was
266 similar to activation with the MMTV promoter (Figure 6E). Activation of full-length elephant
267 shark MR by progesterone was about 5-fold with the TAT3 and MMTV promoters (Figure 6E,
268 G). Aldosterone was about 2-fold more active than dexamethasone.

269 However, deletion of the NTD from elephant shark MR resulted in a significant increase
270 in activation by steroids in the presence of the TAT3 promoter (Figure 6H). Thus, truncated
271 elephant shark MR with the TAT3 promoter was activated from 300 to 350-fold by aldosterone
272 and other corticosteroids and about 200-fold by progesterone and dexamethasone (Figure 6H),
273 indicating that like lungfish MR, the NTD in elephant shark inhibits activation by corticosteroids.
274 However, unlike lungfish MR, compared to aldosterone, dexamethasone was less active for full-
275 length and truncated elephant shark MR with the MMTV promoter and truncated elephant shark
276 MR with the TAT3 promoter.

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280 **Figure 6. Ligand specificity of full-length and truncated human MR and elephant shark**
281 **MR.** Plasmids for full-length human and elephant shark MR or truncated human and elephant
282 shark MR (MR-CDE) were expressed in HEK293 cells with an MMTV-luciferase reporter or a
283 TAT3-luciferase reporter. Transfected cells were treated with either 10 nM aldosterone, cortisol,
284 11-deoxycortisol, corticosterone, 11-deoxycorticosterone, progesterone, dexamethasone or
285 vehicle alone (DMSO). Results are expressed as means \pm SEM, n=3. Y-axis indicates fold-
286 activation compared to the activity of control vector with vehicle alone as 1. A. Full-length
287 human MR with MMTV-luciferase. B. Truncated human MR (MR-CDE) with MMTV-
288 luciferase. C. Full-length elephant shark MR with TAT3-luciferase. D. Truncated elephant
289 shark MR (MR-CDE) with TAT3-luciferase.

290

291 **Does lungfish contain a separate GR gene?**

292 We used sequences of human GR, coelacanth GR and elephant shark GR as probes in a
293 BLAST search of GenBank and did not retrieve a lungfish GR sequence. The absence of a
294 lungfish GR ortholog coupled with the strong response of lungfish MR to dexamethasone and
295 triamcinolone (Figure 5) leads us to propose that lungfish MR also functions as a GR.

296 Dobzhansky's aphorism "Nothing in Biology Makes Sense Except in the Light of
297 Evolution" [62] explains the importance of the evolution of aldosterone in lungfish because
298 aldosterone activation of the kidney MR in terrestrial vertebrates regulates sodium, potassium
299 and water transport, which is critical in maintaining internal electrolyte homeostasis in terrestrial
300 vertebrates [30,34,63–65] an activity that was important in the transition from water to land.
301 Here we report that aldosterone, 11-deoxycorticosterone, and progesterone have EC50s below 1
302 nM for lungfish MR (Table 1), which makes these steroids potential physiological ligands for
303 lungfish MR. Another potential physiological steroid is 11-deoxycortisol, which is a steroid for
304 the CR in Atlantic sea lamprey [41,42]. 11-deoxycortisol has EC50 of 0.17 nM for full-length
305 lungfish MR (Table 1).

306 A functional advantage of 11-deoxycorticosterone, 11-deoxycortisol and progesterone as
307 ligands for the MR is that they lack an 11 β -hydroxyl group, and thus, like aldosterone, they are
308 inert to 11 β -hydroxysteroid dehydrogenase-type 2, unlike cortisol and corticosterone [66–69].
309 Indeed, this inertness to 11 β -hydroxysteroid dehydrogenase-type 2 and the low EC50s of these
310 steroids for lungfish MR suggests that more than one corticosteroid and progesterone [39] may
311 be physiological mineralocorticoids in the presence of 11 β -hydroxysteroid dehydrogenase-type 2.
312 In tissues that lack 11 β -hydroxysteroid dehydrogenase-type 2, cortisol or corticosterone may be
313 the physiological “glucocorticoid” ligands for lungfish MR.

314 Like ray-finned fish MRs [24,37,38,60] and elephant shark MR [24,39], lungfish MR is
315 activated by spironolactone (Figure 4), and, as reported here, by eplerenone [47,48,61].

316 We also find important differences between the response of lungfish MR and human MR
317 to aldosterone, 11-deoxycorticosterone, 11-deoxycortisol and progesterone, indicating that
318 further selectivity for aldosterone in human MR occurred during the evolution of terrestrial
319 vertebrates [13,15,37,40,52,69,70].

320 An unexpected difference between lungfish MR and human MR is the substantial
321 increase in fold-activation by steroids of lungfish MR after deletion of the NTD, in contrast to
322 human MR in which the NTD contains an activation function domain (Figure 2) [49–52]. The
323 NTDs on human MR and lungfish MR have 44% sequence identity (Figure 2). This sequence
324 difference may account for the different effect of the NTDs on corticosteroid activation of human
325 MR and lungfish MR. Deletion of the NTD in elephant shark MR also resulted in a substantial
326 increase in fold-activation by corticosteroids and progesterone using the TAT3 promoter (Figure
327 6), but not for the MMTV promoter [17]. These data with lungfish MR and elephant shark MR
328 suggest that early in the evolution of the MR there was an allosteric interaction between the LBD

329 and NTD [71,72] that repressed steroid activation of the MR, and that the activation function in
330 the NTD as found in human MR [49–52] evolved later in terrestrial vertebrates, along with
331 changes in steroid specificity, such loss of MR activation by progesterone [37,39,70]. The
332 different responses of full-length and truncated lungfish MR, human MR and elephant shark MR
333 with the MMTV and TAT3 promoters indicate that the NTD and the promoter are important
334 regulators of steroid activation of these MRs. Corticosteroid activation of these MRs in the
335 presence of other promoters merits investigation.

336 The stronger response of lungfish MR to dexamethasone compared to aldosterone and the
337 absence a lungfish GR ortholog sequence are puzzling. At a 10 nM concentration, fold-
338 activation by dexamethasone and triamcinolone is substantially higher than that of cortisol,
339 corticosterone, as well as aldosterone, 11-deoxycorticosterone, 11-deoxycortisol and
340 progesterone for lungfish MR (Figure 5). One explanation is that lungfish MR also has a GR
341 function.

342

343 **MATERIALS AND METHODS**

344 **Chemical reagents**

345 Aldosterone, cortisol, corticosterone, 11-deoxycorticosterone, 11-deoxycortisol and
346 progesterone, spironolactone and eplerenone were purchased from Sigma-Aldrich. For reporter
347 gene assays, all hormones were dissolved in dimethyl-sulfoxide (DMSO); the final DMSO
348 concentration in the culture medium did not exceed 0.1%.

349

350 **Animal**

351 A slender spotted African lungfish, *Protopterus dolloi*, was purchased from a local
352 commercial supplier. Lungfish were anesthetized in freshwater containing 0.02% ethyl 3-
353 aminobenzoate methane-sulfonate from Sigma-Aldrich, and tissue samples were quickly
354 dissected and frozen in liquid nitrogen. Animal handling procedures conformed to the guidelines
355 set forth by the Institutional Animal Care and Use Committee at the University of Tokyo.

356

357 Molecular cloning of lungfish mineralocorticoid receptor

358 Two conserved amino acid regions, GCHYGV and LYFAPD of vertebrate MRs were
359 selected and degenerate oligonucleotides were used as primers for PCR. First-strand cDNA was
360 synthesized from 2 µg of total RNA isolated from the liver after amplification, and an additional
361 primer set (CKVFFK and LYFAPD) was used for the second PCR. The amplified DNA
362 fragments were subcloned with TA-cloning plasmid pGEM-T Easy vector, sequenced using a
363 BigDye terminator Cycle Sequencing-kit with T7 and SP6 primers, and analyzed on the 3130
364 Genetic Analyzer (Applied Biosystems). The 5'- and 3'-ends of the mineralocorticoid receptor
365 cDNAs were amplified by rapid amplification of the cDNA end (RACE) using a SMART RACE
366 cDNA Amplification kit. Genbank accessions for this lungfish MR are: Nucleotide ID:
367 LC630795 and Protein ID: BCV19931.

368 Construction of plasmid vectors

369 The full-length and truncated MRs were amplified by PCR with KOD DNA polymerase. The
370 PCR products were gel-purified and ligated into pcDNA3.1 vector (Invitrogen). The truncated
371 MR proteins were designed to possess methionine and valine residues at the N-terminus and
372 contain a DNA-binding domain, a hinge-region, and a ligand-binding domain. The truncated
373 MRs were amplified by PCR with KOD DNA polymerase by using the following primers:

374 lungfish MR forward primer (5'-
375 CAAGCTTACCATGGTGTGTCTGGTGTGTGGTGACGAAG-3' containing *HindIII* site) and
376 lungfish MR reverse primer (5'-CCTACTTCCTGTGAAAGTACAATGAC -3' containing stop
377 codon), human MR forward primer (5'-
378 CGGATCCACCATGGTGTGTTTGGTGTGTGGGGATGAG-3' containing *BamHI* site) and
379 human MR reverse primer (5'-CTCACTTCCGGTGGAAGTAGAGCGGC -3' containing stop
380 codon). The amplified DNA fragments were subcloned with TA-cloning plasmid pGEM-T Easy
381 vector and sequenced, and then subcloned into pcDNA 3.1 vector by using *HindIII-NotI* sites for
382 lungfish MR truncated form or *BamHI-NotI* sites for human MR truncated form. Mouse
383 mammary tumor virus-long terminal repeat (MMTV-LTR) was amplified from pMSG vector by
384 PCR, and inserted into pGL3-basic vector containing the *Photinus pyralis* luciferase gene. 3X-
385 Tyrosine Amino Transferase (TAT3) promoter containing reporter vector named pGL4.23-
386 TAT3-Luc was constructed as described previously [53]. All cloned DNA sequences were
387 verified by sequencing.

388

389 **Transactivation assay and statistical methods**

390 Transfection and reporter assays were carried out in HEK293 cells, as described previously
391 [40,73]. All experiments were performed in triplicate. The values shown are mean \pm SEM from
392 three separate experiments, and dose-response data, which were used to calculate the half
393 maximal response (EC50) for each steroid, were analyzed using GraphPad Prism. Supplement
394 Figure 1 shows an ANOVA analysis for Figure 3. Fold activation levels were analyzed using
395 one-way ANOVA for each treatment with Tukey's multiple comparisons test.

396

397 DECLARATION OF INTEREST

398 We have no conflict of interest.

399

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403

404 AUTHOR CONTRIBUTIONS

405 Y.K., S.O., and M.E.B. carried out the research and analyzed data. S.H. aided in the collection
406 of animals. X.L. constructed plasmid DNAs used in this study. Y.K. and M.E.B. conceived and
407 designed the experiments. Y.K. and M.E.B. wrote the paper. All authors gave final approval for
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409

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652 Supplemental Figure 1: Ligand specificity of full-length and truncated lungfish MR.

653 Plasmids for full-length lungfish MR or truncated lungfish MR (MR-CDE) were expressed in
654 HEK293 cells with an MMTV-luciferase reporter or a TAT3-luciferase reporter. Transfected
655 cells were treated with either 10 nM aldosterone, cortisol, 11-deoxycortisol, corticosterone, 11-
656 deoxycorticosterone, progesterone, dexamethasone or vehicle alone (DMSO). Results are
657 expressed as means \pm SEM, n=3. Y-axis indicates fold-activation compared to the activity of
658 control vector with vehicle alone as 1. A. Full-length lungfish MR with MMTV-luciferase. B.
659 Truncated lungfish MR (MR-CDE) with MMTV-luciferase. C. Full-length lungfish MR with
660 TAT3-luciferase. D. Truncated lungfish MR (MR-CDE) with TAT3-luciferase. *P<0.05,
661 **P<0.01, ***P<0.001 compared with each steroid treatment.