N-terminal Domain Regulates Steroid Activation of Elephant Shark Glucocorticoid and Mineralocorticoid Receptors

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Abstract. Orthologs of human glucocorticoid receptor (GR) and human mineralocorticoid receptor (MR) first appear in cartilaginous fishes. Subsequently, the MR and GR diverged to respond to different steroids: the MR to aldosterone and the GR to cortisol and corticosterone. We report that cortisol, corticosterone and aldosterone activate full-length elephant shark GR, and progesterone, which activates elephant shark MR, does not activate elephant shark GR. However, progesterone inhibits steroid binding to elephant shark GR, but not to human GR. Together, this indicates partial functional divergence of elephant shark GR from the MR. Deletion of the N-terminal domain (NTD) from elephant shark GR (truncated GR) reduced the response to corticosteroids, while truncated and full-length elephant shark MR had similar responses to corticosteroids. Swapping of NTDs of elephant shark GR and MR yielded an elephant shark MR chimera with full-length GR-like increased activation by corticosteroids and progesterone compared to full-length elephant shark MR. Elephant shark MR NTD fused to GR DBD+LBD had similar activation as full-length MR, indicating that the MR NTD lacked GR-like NTD activity. We propose that NTD activation of human GR evolved early in GR divergence from the MR.

34 **Running title:** AF1 in the NTD Regulates Shark GR Activation.

35

36 **Introduction**

37 Glucocorticoids, such as cortisol and corticosterone, have diverse physiological activities
38 in humans and other terrestrial vertebrates, including regulating glucose metabolism, cognition,
39 immunity and the response to stress [1–5]. The physiological actions of glucocorticoids are
40 mediated by the glucocorticoid receptor (GR), which belongs to the nuclear receptor family, a
41 diverse group of transcription factors that also contains receptors for mineralocorticoids (MR),
42 progestins (PR) androgens (AR) and estrogens (ER) [6–8].

43 The GR is closely related to the MR [9]; phylogenetic analysis indicates that the GR and
44 MR evolved from a corticoid receptor (CR) that evolved in a jawless vertebrate in an ancestor of
45 modern lamprey and hagfish [7,10–13]. Distinct orthologs of human GR and human MR first
46 appear in cartilaginous fishes, the oldest group of extant jawed vertebrates (gnathostomes) that
47 diverged from bony vertebrates about 450 million years ago [14]. Since the emergence of the
48 GR and MR in cartilaginous fishes, these steroid receptors have diverged to respond to different
49 corticosteroids. For example, human MR is activated by aldosterone, the physiological
50 mineralocorticoid in humans, at a concentration at least ten-fold lower than by cortisol [15–18].
51 However, aldosterone has little activity for human GR, which is activated by cortisol and
52 corticosterone (Figure 1) [6,12,16,19,20].

53 The multi-domain structure of the GR is important in regulating steroid activation of the
54 GR. Like other steroid receptors, the GR consists of an N-terminal domain (NTD) (domains A
55 and B), a central DNA-binding domain (DBD) (domain C), a hinge domain (D) and a C-terminal
56 ligand-binding domain (LBD) (domain E) [5,8,19,21,22] (Figure 2). The GR NTD is noteworthy
57 for containing an activation function domain (AF1) that interacts with the DBD to provide a
58 human GR NTD-DBD protein with constitutive activity [23–26]. AF1 also strongly stimulates
59 transcriptional activation by glucocorticoids of the GR in humans [19,24,25,27], other terrestrial
60 vertebrates [21,27–31] and ray-finned fish [21,32]. Interaction of the NTD with coactivators as
61 well as post-transcriptional modification of the NTD also regulates transcriptional activation of
62 the GR [5,22,30,33,34]. It is not known when strong activation by the NTD of vertebrate GRs
63 evolved.

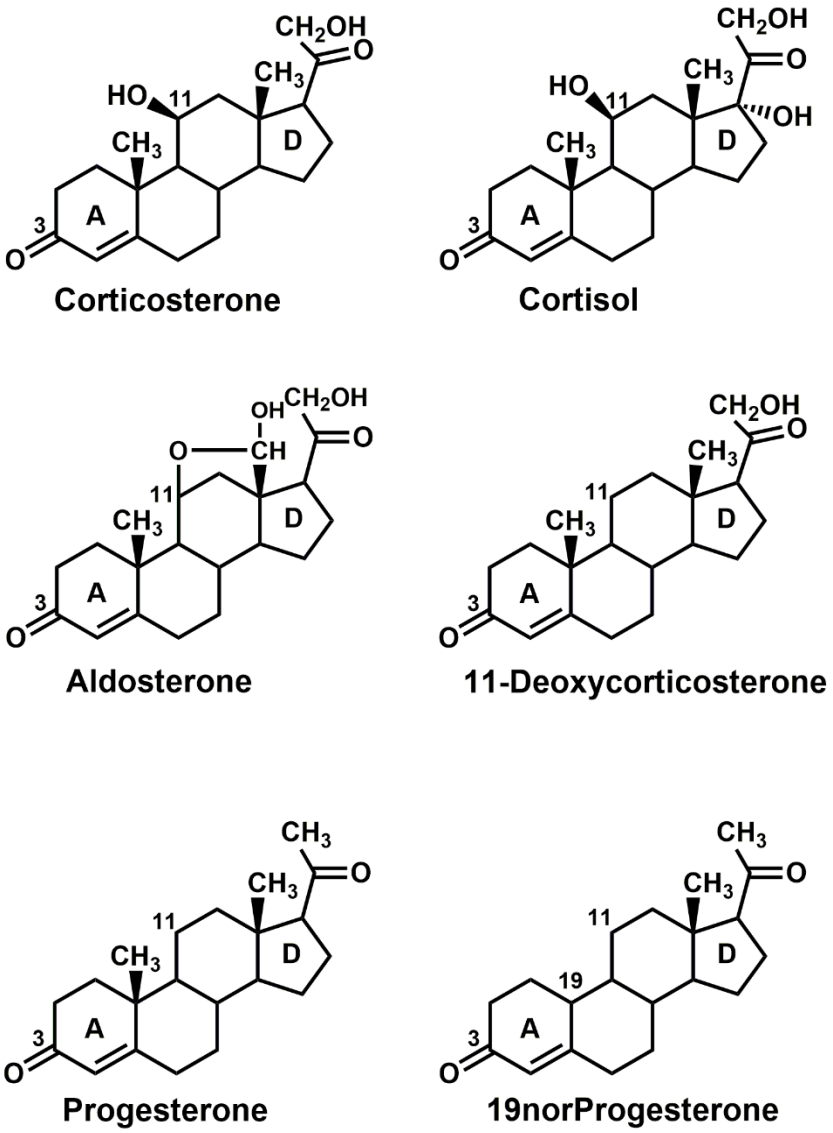

Fig. 1. Structures of Corticosteroids and Progesterone. Cortisol and corticosterone are physiological glucocorticoids in terrestrial vertebrates and ray-finned fish [7,10,35]. Aldosterone, 11-deoxycorticosterone are physiological mineralocorticoids [10,12,36,37]. Progesterone is female reproductive steroid that also is important in male physiology [38,39].

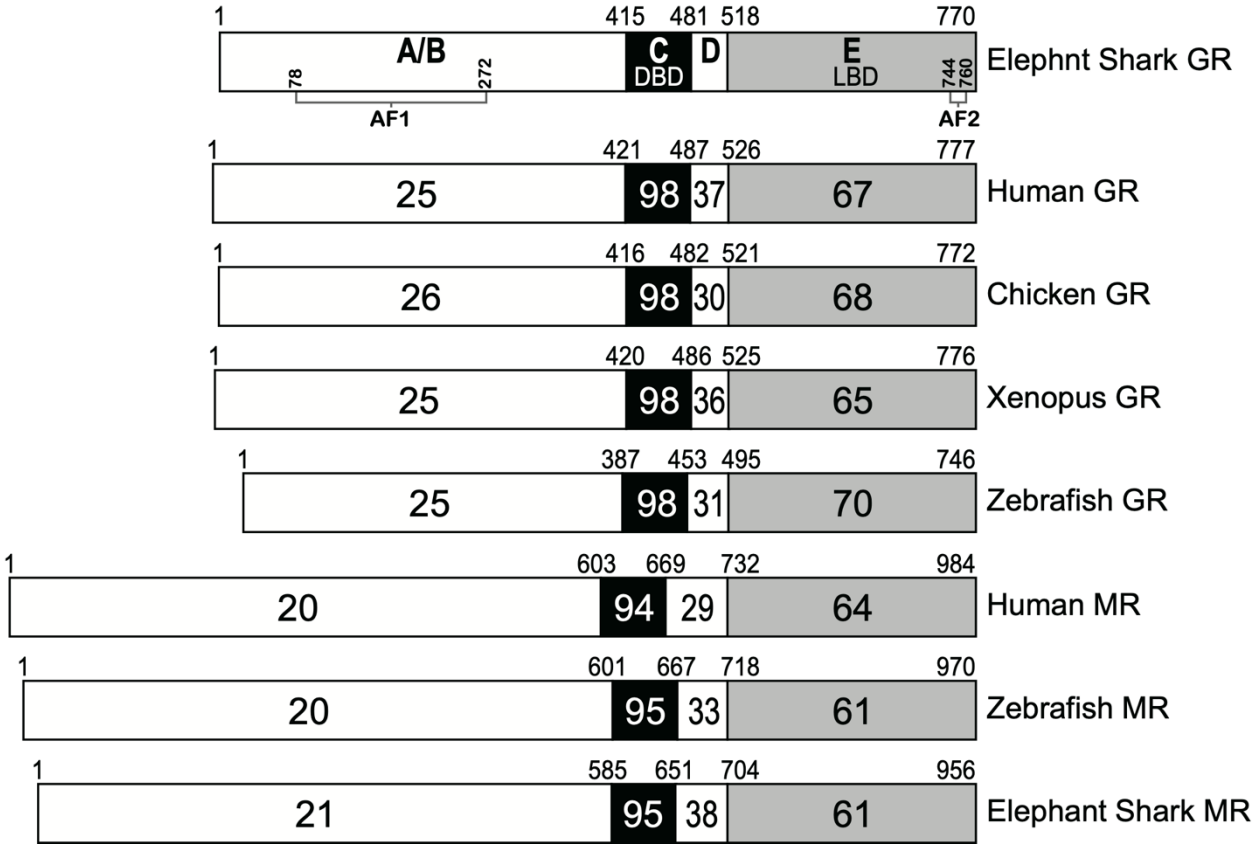

Fig.2. Comparison of domains in elephant shark GR with vertebrate GRs and MRs. GRs from elephant shark, zebrafish, *X. laevis*, chicken and humans and MRs from elephant shark, humans and zebrafish are compared. The functional NTD (A/B), DBD (C), hinge (D) and LBD (E) domains are schematically represented with the numbers of amino acid residues and the percentage of amino acid identity depicted. AF1 [5] and activation function 2 (AF2) [5] domains are shown in human GR.

To investigate corticosteroid signaling via the GR, in the light of the evolution [40], we decided to study activation of full-length and truncated elephant shark (*Callorhinchus milii*) GR by a panel of corticosteroids, which were used in a previous study of elephant shark MR [41].Although corticosteroid activation of another cartilaginous fish, skate GR, has been reported [42], this GR consisted of skate LBD fused to a GAL4-DBD. This GR lacked skate NTD and DBD, two domains that are important in AF1 activation of human GR [5,19,23–26]. The absence of data on steroid activation of a full-length cartilaginous fish GR prompted our investigation of the role of the NTD and DBD on transcriptional activation of a cartilaginous fish GR to determine if AF1 activity evolved in the NTD in a GR in a basal vertebrate. As the

90 sequences of the NTDs of elephant shark and human GRs are poorly conserved, with 25%
91 sequence identity (Figure 2), it was not evident that there would be strong AF1 activity in the
92 NTD in elephant shark GR. We also investigated the evolution of GR specificity for
93 glucocorticoids through a comparison of steroid activation of full-length elephant shark GR to
94 elephant shark MR and human GR [16,19–21,23,27].

95 Here we report that full-length elephant shark GR is activated by cortisol and
96 corticosterone, which activate human GR [16,19,20,27]. Unexpectedly, aldosterone and 11-
97 deoxycorticosterone, two mineralocorticoids, also activated elephant shark GR. These two
98 mineralocorticoids have little activity for human GR [15,16,27], which is selective for cortisol
99 and corticosterone [16,20,27]. Elephant shark GR is not activated by either progesterone or
100 19norprogesterone, in contrast to elephant shark MR, which also is activated by cortisol and
101 corticosterone [41]. However, we find that progesterone and 19norprogesterone inhibit
102 corticosterone activation of elephant GR, while neither progestin inhibits cortisol activation of
103 human GR.

104 We find that the NTD in elephant shark GR is a strong activator of steroid mediated
105 transcriptional activation. Corticosteroid activation of truncated elephant shark GR, in which the
106 NTD is deleted, was less than 10% of that of full-length GR, in contrast to truncated elephant
107 shark MR, in which deletion of the NTD did not have a major effect on corticosteroid activation
108 [41]. To investigate further the specificity of the each NTD for activation of the GR and MR, we
109 studied chimeras of elephant shark GR and MR in which the GR NTD was fused to elephant
110 shark MR DBD-LBD and the MR NTD was fused to GR DBD-LBD. In elephant shark GR
111 NTD fused to MR DBD-LBD activation by cortisol and corticosterone increased by over 10-
112 fold, compared to full length elephant shark MR indicating that this GR NTD contains AF1
113 activity. Interestingly, the chimera of GR NTD MR DBD-LBD had increased activation by
114 progesterone and 19norprogestrone. In contrast, in MR NTD fused to GR DBD-LBD
115 corticosteroid activation was reduced by over 90%. These data indicate allosteric regulation by
116 the NTD evolved in elephant shark GR soon after the divergence of the GR from its common
117 ancestor with the MR.

118

119

120 **Results**

121 **Functional domains on elephant shark GR and other vertebrate GRs.**

122 In Figure 2, we compare the functional domains of elephant shark GR to corresponding
123 domains in selected vertebrate GRs (human, chicken, Xenopus, zebrafish) and MRs (human,
124 zebrafish and elephant shark). Elephant shark GR and human GR have 98% and 67% identity in
125 DBD and LBD, respectively. Elephant shark GR and MR have 95% and 61% identity in DBD
126 and LBD, respectively. This strong conservation of the DBD and LBD contrasts with the low
127 sequence identity between the NTD in elephant shark GR and the NTD in other GRs and MRs.
128 The NTD of elephant shark GR has only 21% sequence identity with elephant shark MR.

129

130 **Effect of corticosteroids and progesterone on transcriptional activation of full-length**
131 **elephant shark GR and MR.**

132 We screened a panel of steroids (cortisol, corticosterone, 11-deoxycorticosterone,
133 aldosterone, progesterone, 19norProgesterone) at 10^{-8} M for activation of full-length elephant
134 shark GR (Figure 3A). For comparison, activation of full-length elephant shark MR by these
135 steroids is shown in Figure 3B. Cortisol, corticosterone, 11-deoxycorticosterone, and
136 aldosterone activated full-length elephant shark GR (Figure 3A) and MR (Figure 3B). However,
137 fold-activation of full-length elephant shark GR by corticosteroids at 10^{-8} M was over 10-fold
138 higher than for full-length elephant shark MR. For example, 10^{-8} M corticosterone activated full-
139 length GR by 225-fold and full-length MR by 8-fold. Interestingly, neither progesterone nor
140 19norProgesterone activated elephant shark GR, while these steroids activated elephant shark
141 MR, as previously reported [41].

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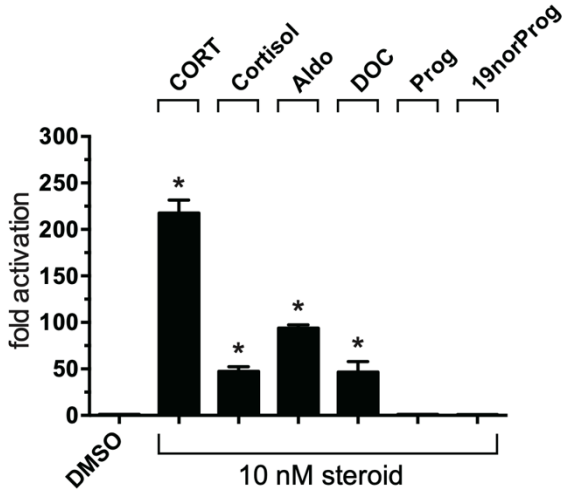
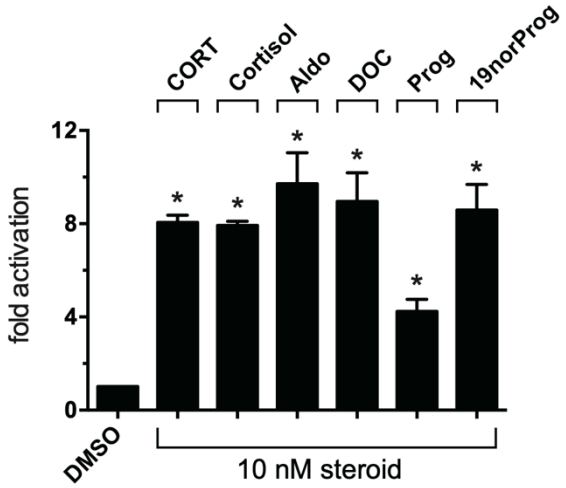
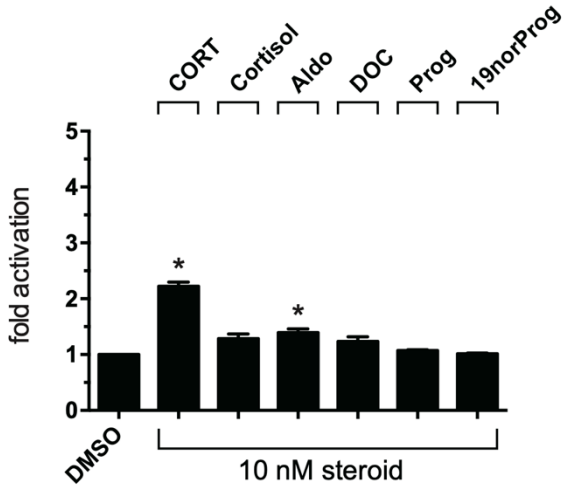
A: elephant shark GR-full



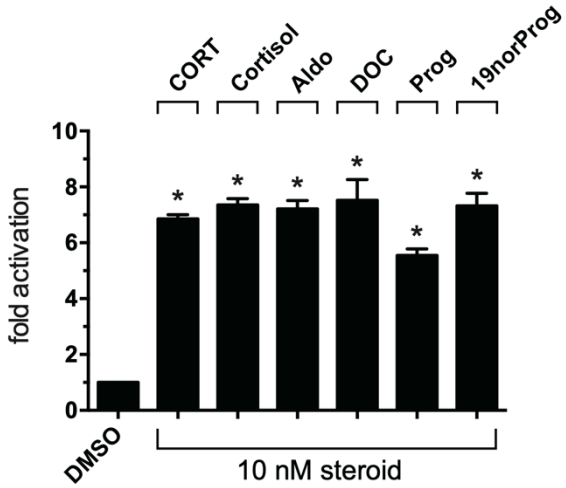
B: elephant shark MR-full



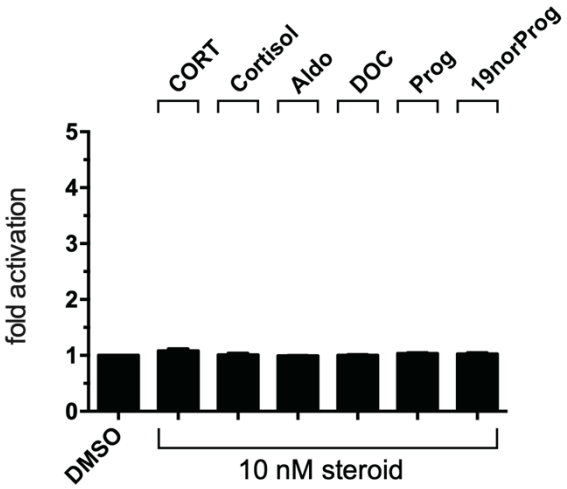
C: elephant shark GR-DBD-hinge-LBD



D: elephant shark MR-DBD-hinge-LBD



E: GAL4-DBD + elephant shark GR-hinge-LBD



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145 **Fig. 3. Ligand specificity of full-length and truncated elephant shark GR and MR.**
146 Plasmids for full-length elephant shark GR or MR or truncated elephant GR or MR, containing
147 the DBD (C domain), D domain and LBD (E domain), were expressed in HEK293 cells with an
148 MMTV-luciferase reporter. Transfected cells were treated with either 10 nM cortisol,
149 corticosterone (CORT), aldosterone (Aldo), 11-deoxycorticosterone (DOC), progesterone (Prog),
150 19norProgesterone (19norProg) or vehicle alone (DMSO). Results are expressed as means ±
151 SEM, n=3. Y-axis indicates fold-activation compared to the activity of control vector with
152 vehicle (DMSO) alone as 1. A. Full-length elephant shark GR. B. Full-length elephant shark
153 MR. C. Truncated elephant shark GR. D. Truncated elephant shark MR. E. GAL4-
154 DBD+elephant shark GR-hinge-LBD.

155

156 **Effect of corticosteroids and progesterone on transcriptional activation of truncated**
157 **elephant shark GR and MR.**

158 We investigated the role of the NTD in the response to corticosteroids and progestins at
159 10^{-8} M, of truncated elephant shark GR and MR, in which the NTD was deleted (Figure 3C, D).
160 Figure 3C shows that activation of truncated elephant shark GR by 10^{-8} M corticosterone
161 decreased by over 90%, compared to full-length elephant shark GR (Figure 3A), and there was
162 no significant activation of truncated GR by 10^{-8} M of either cortisol or 11-deoxycorticosterone,
163 both of which activated full-length GR. This indicates that NTD in elephant shark GR contains
164 an AF1 domain. Progesterone did not activate truncated elephant shark GR. In contrast, at 10^{-8}
165 M, corticosteroids and progesterone had similar levels of activation of truncated elephant shark
166 MR (Figure 3D) and full-length MR (Figure 3B), indicating that there is a major difference
167 between NTD activation of transcription of elephant shark GR and MR.

168 To investigate the effect of the elephant shark DBD on activation of elephant shark GR
169 and MR, we replaced the DBD with GAL4 DBD, which has been used for analysis of
170 transcription of the GR and MR [27,28,42,43]. As shown in Figure 3E, there was only a low
171 level of activation by 10 nM corticosterone and aldosterone of GAL4 DBD GR hinge-LBD, and
172 no activation by 11-deoxycorticosterone and cortisol. In contrast, GAL4 DBD MR LBD is
173 activated by aldosterone, 11-deoxycorticosterone and other corticosteroids [41].

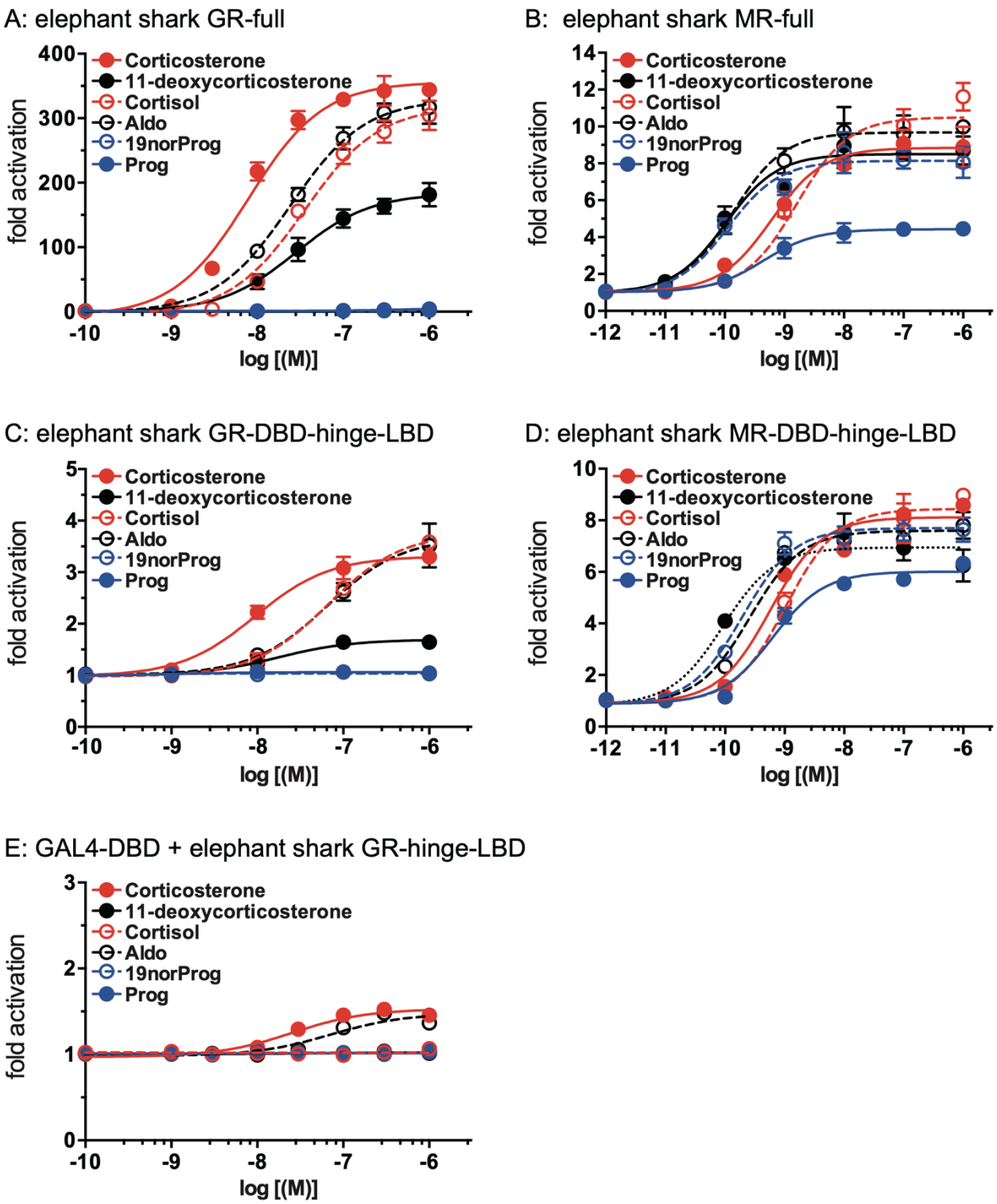
174

175 **Concentration-dependent activation by corticosteroids and progesterone of full-length and**
176 **truncated elephant shark GR and MR.**

177 To gain a quantitative measure of NTD activation of elephant shark GR, we determined
178 the concentration dependence of transcriptional activation by corticosteroids and progesterone of
179 full-length and truncated elephant shark GR (Figure 4A, B) and for comparison of elephant shark
180 MR (Figure 4C, D). This data was used to calculate a half maximal response (EC50) for steroid
181 activation of elephant shark GR and MR (Tables 1 and 2). The graphs for cortisol, aldosterone
182 and 11-deoxycorticosterone for elephant shark GR do not saturate. Thus, the EC50 values for
183 these steroids shown in Table 1 are approximate and underestimate the true EC50.
184 Corticosterone and cortisol, two physiological glucocorticoids in mammals, had EC50s of 7.9
185 nM and approximately 35 nM, respectively, for full-length elephant shark GR (Table 1). For
186 comparison, the EC50s of corticosterone and cortisol are 0.61 nM and 1.6 nM, respectively, for
187 full-length elephant shark MR (Table 2) [41]. However, aldosterone and 11-
188 deoxycorticosterone, two physiological mineralocorticoids in mammals, had EC50s of
189 approximately 24 nM and 28 nM, respectively, for elephant shark GR. Activation by these
190 steroids of elephant shark GR indicates that it retains some properties of its MR paralog. For
191 comparison, the EC50s of aldosterone and 11-deoxycorticosterone are 0.14 nM and 0.1 nM,
192 respectively, for full-length elephant shark MR (Table 2) [41].

193 The EC50 of corticosterone for truncated elephant shark GR was 9.4 nM. The EC50s of
194 cortisol and aldosterone for truncated elephant shark GR were approximately 66 nM and 65 nM
195 respectively. In contrast, the EC50s of corticosterone and aldosterone for GAL4-DBD+GR-
196 hinge-LBD were approximately 25 nM and 71 nM, respectively, and cortisol and 11-
197 deoxycorticosterone did not activate GAL4-DBD+GR-hinge-LBD. The higher EC50 for
198 corticosterone and the loss of activation by cortisol and 11-deoxycorticosterone, when elephant
199 shark DBD is replaced with GAL4-DBD indicates that the DBD is important in transcriptional
200 activation of elephant shark GR.

201 All four corticosteroids, progesterone and 19norProgesterone had EC50s for truncated MR
202 (DBD+LBD) that were either similar to or lower than that of full-length MR (Table 2). EC50s
203 for Gal4-DBD+MR-hinge-LBD were either similar to or lower than that of full-length MR.


A: elephant shark GR-full
B: elephant shark MR-full
C: elephant shark GR-DBD-hinge-LBD
D: elephant shark MR-DBD-hinge-LBD
E: GAL4-DBD + elephant shark GR-hinge-LBD
Corticosterone
11-deoxycorticosterone
Cortisol
Aldo
19norProg
Prog
fold activation
log [(M)]

204

205

206

207 **Fig. 4. Concentration-dependent transcriptional activation by corticosteroids and**
208 **progesterone of full length and truncated elephant shark GR and MR.** Plasmids for full-
209 length elephant shark GR or MR or truncated elephant GR or MR, were expressed in HEK293
210 cells with an MMTV-luciferase reporter. Cells were treated with increasing concentrations of
211 either cortisol, corticosterone, aldosterone, 11-deoxycorticosterone, progesterone,
212 19norProgesterone or vehicle alone (DMSO). Results are expressed as means ± SEM, n=3. Y-
213 axis indicates fold-activation compared to the activity of control vector with vehicle (DMSO)
214 alone as 1. A. Full-length elephant shark GR. B. Full-length elephant shark MR. C. Truncated
215 elephant shark GR (Domains CDE). D. Truncated elephant shark MR (Domains CDE). E.
216 GAL4-DBD+elephant shark GR-hinge-LBD.

217

218 The dependence of the level of cortisol and 11-deoxycorticosterone activation on the
219 NTD in elephant shark GR (Figures 4A, C) is similar to that of human GR, in which deletion of
220 the NTD reduces cortisol and 11-deoxycorticosterone fold-activation by over 90% [19,27]. The
221 diminished activation of GAL4-DBD-elephant shark GR-hinge-LBD (Figure 4E) indicates that
222 the DBD also is important in fold-activation of elephant shark GR. These results indicate that
223 allosteric signaling between the NTD and DBD-LBD in elephant shark GR is critical for its
224 response to corticosteroids. This contrasts to elephant shark MR, in which truncated MR and
225 full-length elephant shark MR have similar levels of activation by corticosteroids and
226 progesterone (Figures 4B, D). Together this indicates that an activation function in the GR NTD
227 and higher EC50s for some corticosteroids that also activated elephant shark MR evolved early
228 in the divergence of the GR from its MR kin.

229

230 Table 1. EC50 values for steroid activation of full-length and truncated elephant shark GR

	Cortisol	Corticosterone	Aldo	DOC	Prog	19norProg
	EC50 (M)	EC50 (M)	EC50 (M)	EC50 (M)	EC50 (M)	EC50 (M)
Elephant shark GR-full	35 nM	7.9 nM	24nM	28 nM	–	–
95% confidence intervals	27-46 nM	6.1-10.0 nM	19.0-31 nM	19-42 nM	–	–
% Relative Induction	88 %	100 %	92 %	53 %	–	–
Elephant shark GR-DBD-hinge-LBD	66 nM	9.4 nM	65 nM	17 nM	–	–
95% confidence intervals	55-80 nM	7.5-12 nM	44-95 nM	8.6-34 nM	–	–
% Relative Induction	112 %	100 %	109 %	30 %	–	–
Elephant shark Gal4-DBD-GR-hinge-LBD	–	25 nM	71 nM	–	–	–
95% confidence intervals	–	14-45 nM	31-165 nM	–	–	–
% Relative Induction	–	100 %	81 %	–	–	–
Skate GR [Gal4-DBD-GR-hinge-LBD]#	139 nM	58 nM	11 nM	306 nM	N.D.	N.D.
Elephant shark Chimera MR-NTD+GR-DBD-hinge-LBD	93 nM	13 nM	43 nM	60 nM	–	–
95% confidence intervals	58-150 nM	8.0-21 nM	28-67 nM	28-128 nM	–	–
% Relative Induction	108 %	100 %	88 %	20 %	–	–

231 # Values obtained from Mol Biol Evol. 12, 2643-2652 (2008)

232 Percent relative induction for GR is compared to maximal response to corticosterone.

233 – = no activation. N.D. = not determined

234 Aldo = aldosterone, DOC =11-deoxycorticosterone, Prog = progesterone, 19norProg = 19norProgesterone

235

236 Table 2. EC50 values for steroid activation of full-length and truncated elephant shark MR

	Cortisol	Corticosterone	Aldo	DOC	Prog	19norProg
	EC50 (M)	EC50 (M)	EC50 (M)	EC50 (M)	EC50 (M)	EC50 (M)
Elephant shark MR-full	1.6 nM	0.61 nM	0.14 nM	0.1 nM	0.45 nM	0.11 nM
95% confidence intervals	0.92-2.8 nM	0.34-1.1 nM	0.069-0.26 nM	0.54-0.2 nM	0.21-0.94 nM	0.06-0.22 nM
% Relative Induction	118 %	88 %	100 %	86 %	38 %	79 %
Elephant shark MR-DBD-hinge-LBD	1.1nM	0.58 nM	0.26 nM	0.1 nM	0.63 nM	0.2 nM
95% confidence intervals	0.67-1.7 nM	0.39-0.86 nM	0.15-0.45 nM	0.05-0.16 nM	0.44-0.9 nM	0.13-0.31 nM
% Relative Induction	117 %	111 %	100 %	77 %	78 %	98 %
Elephant shark Gal4-DBD-MR-hinge-LBD*	0.19 nM	0.1 nM	0.04 nM	0.02 nM	0.48 nM	0.02 nM
95% confidence intervals	0.08-0.2 nM	0.08-0.1 nM	0.02-0.05 nM	0.01-0.03 nM	0.39-0.57 nM	0.01-0.02 nM
% Relative Induction	79 %	90 %	100 %	81 %	40 %	98 %
Skate MR [Gal4-DBD-MR-hinge-LBD]#	1.04 nM	0.09 nM	0.07 nM	0.03 nM	N.D.	N.D.
Elephant shark Chimera GR-NTD+MR-DBD-hinge-LBD	0.3 nM	0.1 nM	0.05 nM	0.03 nM	0.2 nM	0.04 nM
95% confidence intervals	0.2-0.44 nM	0.09-0.16 nM	0.04-0.07 nM	0.02-0.04 nM	0.17-0.27 nM	0.03-0.06 nM
% Relative Induction	100 %	101 %	100 %	97 %	81 %	95 %

237 *Values obtained from Sci Signal. 12, eaar2668 (2019)

238 # Values obtained from Mol Biol Evol. 12, 2643-2652 (2008)

239 Percent relative induction for MR is compared to maximal response to aldosterone.

240 N.D. = not determined.

241 Aldo = aldosterone, DOC =11-deoxycorticosterone, Prog = progesterone, 19norProg = 19norProgesterone

242 **Progesterone binds to, but does not activate, full-length elephant shark GR.**

243 Neither progesterone nor 19norProgesterone activated transcription by elephant shark GR
244 (Figure 3A), although these steroids are transcriptional activators of elephant shark MR (Figure
245 3B) [41]. The lack of progesterone activation of elephant shark GR could be due to decreased
246 affinity of progesterone for elephant shark GR or to loss of activation of elephant shark GR after
247 binding progesterone. We find that progesterone and 19norProgesterone inhibit activation of
248 elephant shark GR by 10 nM corticosterone (Figure 5A) indicating that elephant shark GR
249 recognizes progesterone. A parallel study showed that neither progesterone nor
250 19norProgesterone inhibited activation of human GR by 10 nM cortisol (Figure 5B). This
251 indicates that the loss of activation by progesterone preceded the loss of progesterone binding to
252 the GR, which occurred later in vertebrate evolution.

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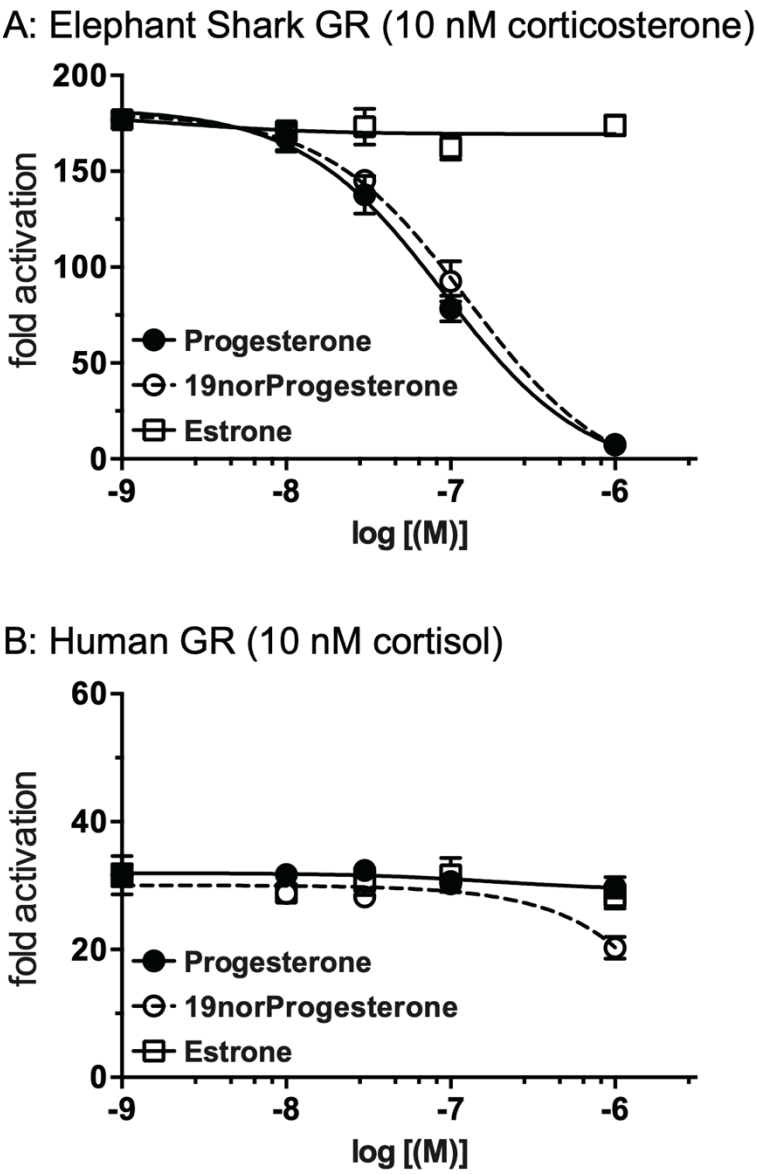

Fig. 5. Progesterone inhibits corticosterone activation of full-length elephant shark GR. Plasmids encoding full length elephant GR or human GR were expressed in HEK293 cells. Cells with elephant shark GR and human GR were treated with 10 nM corticosterone and cortisol respectively and increasing concentrations of either progesterone, 19norProgesterone or estrone. Y-axis indicates fold-activation compared to the activity of control vector with vehicle (DMSO) alone as 1.
A. Elephant shark GR. B. Human GR.

264 **Corticosteroid activation of chimeras in which the NTD is swapped between elephant shark**
265 **GR and MR.**

266 To investigate further the origins of NTD activation of transcription by the GR, we
267 studied corticosteroid activation of chimeras of elephant shark GR and MR, in which the GR
268 NTD was fused to MR DBD-hinge-LBD and the MR NTD was fused to GR DBD-hinge-LBD
269 (Figure 6). We find that despite low sequence similarity between the NTDs in elephant shark
270 GR and MR, their NTDs had dominant effects on transcription of the DBD-hinge-LBD in each
271 chimera (Figure 6, Tables 1 and 2). Thus, in the GR NTD-MR DBD-hinge-LBD chimera (GR
272 NTD fused to MR DBD-hinge-LBD), cortisol and corticosterone increased activation by over
273 30-fold, compared to full length elephant shark MR (Figures 3B, 4B and 6B). Moreover, the GR
274 NTD-MR DBD-hinge-LBD chimera also had a higher level of activation in the presence of
275 progesterone and 19norProgestrone. In addition, the EC50s for corticosteroids and progestins
276 were lower in the GR NTD-MR DBD-hinge-LBD chimera indicating that the NTD affects the
277 affinity of steroids for the chimera, as well as the level of transcriptional activation (Table 2).

278 In contrast, the MR NTD reduced activation by corticosterone and other corticosteroids
279 of the MR NTD-GR DBD-hinge-LBD chimera by over 90% compared to full-length GR
280 (Figures 3A, 4A and 6A, Table 1). These data indicate that increased activation by the NTD in
281 elephant shark GR evolved soon after it diverged from the MR.
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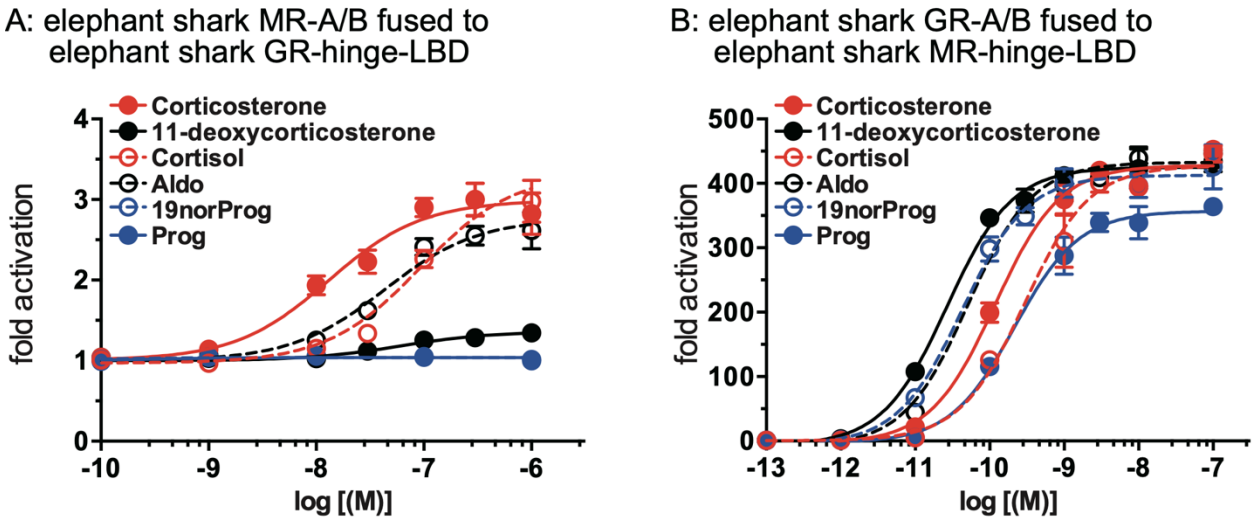

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285 **Fig. 6. Concentration-dependent transcriptional activation by corticosteroids of chimeras**
286 **of GR and MR.** Plasmids for chimeric GR or MR were expressed in HEK293 cells with an
287 MMTV-luciferase reporter. Cells were treated with increasing concentrations of either cortisol,
288 corticosterone, aldosterone, 11-deoxycorticosterone, progesterone, 19norProgesterone or vehicle
289 alone (DMSO). Results are expressed as means ± SEM, n=3. Y-axis indicates fold-activation
290 compared to the activity of control vector with vehicle (DMSO) alone as 1. A. Chimera of MR-
291 A/B fused to GR-DBD-hinge-LBD. B. Chimera of GR-A/B fused to MR-DBD-hinge-LBD.

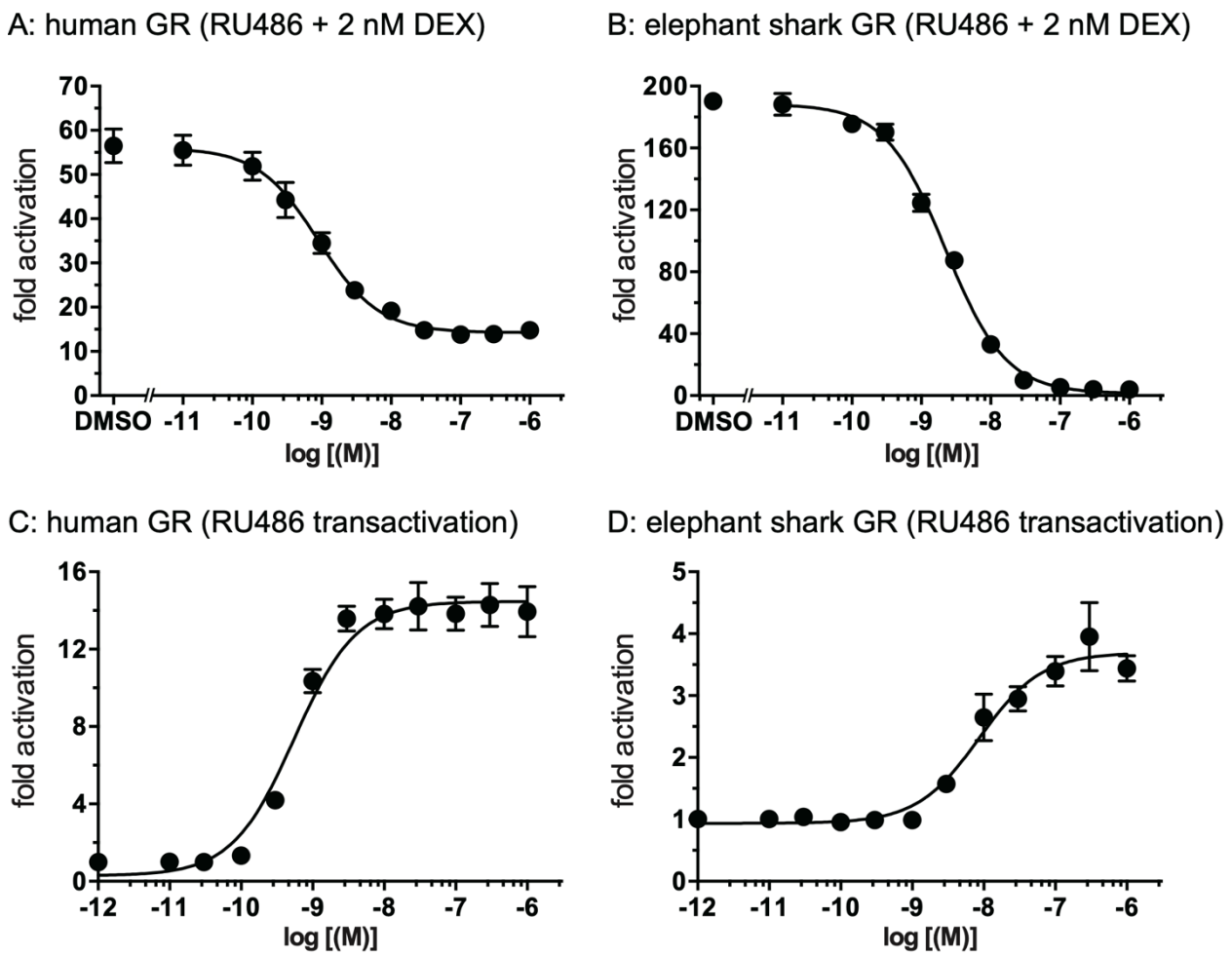
292

293 **RU486 is a glucocorticoid antagonist of full-length elephant shark GR.**

294 To gain another measure of the similarities and differences between elephant shark GR
295 and human GR, we investigated the effect of RU486 on transcriptional activation of elephant
296 shark GR. RU486, originally developed as a progesterone antagonist [44,45] also is used as an
297 antagonist for glucocorticoid activation of human GR [44], although at high concentrations
298 (>100nM), RU486 is a weak GR agonist. We found that RU486 inhibited activation by 2 nM
299 dexamethasone of elephant shark and human GR (Figure 7A, B). At 0.9 nM, RU486 inhibited
300 dexamethasone activation of human GR by 50% (Figure 7A). At 2.2 nM, RU486 inhibited
301 dexamethasone activation of elephant shark GR by 50% (Figure 7B).

302 Concentration dependent activation by RU486 of elephant shark GR and human GR,
303 shown in Figure 7C and D, was used to calculate EC50 values for RU486. The EC50 of RU486
304 for elephant shark GR is 8.7 nM and for human GR is 0.55 nM. Maximal transcriptional
305 activation by RU486 of elephant shark GR (Figure 7D) is considerably lower than for activation
306 of human GR (Figure 7C). Nevertheless, although RU486 is not as active towards elephant
307 shark GR as towards human GR, the responses of elephant shark GR to RU486 mimic those of
308 human GR.

309



310

311 **Fig. 7. RU486 inhibition of dexamethasone activation of elephant shark GR and human**
312 **GR.** Plasmids encoding full length elephant GR or human GR were expressed in HEK293 cells.
313 Cells with elephant shark GR or human GR were treated with 2 nM dexamethasone + increasing
314 concentrations of RU486 (panels A, B) or increasing concentrations of RU486 (panels C, D).
315 Results are expressed as means ± SEM, n=3. Y-axis indicates fold-activation compared to the
316 activity of control vector with vehicle (DMSO) alone as 1.

317

318 **Discussion**

319 Sequence analysis revealed that the MR and GR are kin [9,13]. A distinct GR and MR
320 first appear in cartilaginous fish, which occupy a key position at an ancestral node from which
321 ray-finned fish and terrestrial vertebrates diverged [14,46,47]. Genomic analyses reveal that
322 elephant shark genes are evolving slowly [14]. Thus, analysis of steroid activation of elephant
323 shark GR can provide insights into the evolution of steroid activation of the ancestral GR early in
324 its divergence from the MR. As a consequence, we studied activation of elephant shark GR by

325 physiological glucocorticoids (cortisol, corticosterone) and mineralocorticoids (aldosterone, 11-
326 deoxycorticosterone), as well as progesterone, which we compared to similar experiments with
327 elephant shark MR [41]. Because the NTD has a strong AF1 for human GR [19,23–26], unlike
328 human MR [19,41], we also studied elephant shark GR, in which the NTD was deleted. After
329 finding that corticosterone activation of truncated elephant shark GR declined by over 90% from
330 the activity of full-length elephant shark GR, we investigated the evolution of the NTD in
331 chimeras of elephant shark GR and MR, in which their NTDs were swapped. Despite low
332 sequence identity between the GR and MR NTDs, AF1 in elephant shark GR NTD could transfer
333 its activity to elephant shark MR, when it was substituted for the MR NTD. This indicates that a
334 strong AF1 appeared early in the evolution of the GR in a basal jawed vertebrate (Figure 6,
335 Tables 1 and 2).

336 Overall, we find that the response to corticosteroids and RU486 [44,48] by elephant shark
337 GR has some similarities and some differences to human GR [15,19,21,27], as well as to
338 elephant shark MR [41]. In comparison to corticosteroid activation of elephant shark MR,
339 corticosteroids have higher EC50s for elephant shark GR, indicating reduced sensitivity to
340 corticosteroids [Tables 1 and 2]. Interestingly, the level of transcriptional activation by
341 corticosteroids of elephant shark GR is over 10-fold higher than for elephant shark MR (Figure
342 3A, B). In this respect, elephant shark GR and MR are similar to human GR and human MR in
343 which cortisol has a higher EC50, as well as a higher level of transcriptional activation for
344 human GR, compared to human MR [15,16,19,20,49].

345 Activation of elephant shark GR by aldosterone is a notable difference with human GR.
346 Although 10nM aldosterone has little activity for human GR (Figure 3A) [15,27], aldosterone
347 and cortisol have a similar level of activation of elephant shark GR. Aldosterone, the
348 physiological mineralocorticoid in terrestrial vertebrates, also is a weaker activator of truncated
349 skate GR than of truncated skate MR [42] and of lamprey CR [7], which is ancestral to the GR
350 and MR [7,13]. Thus, elephant shark GR retains some responses, albeit diminished, of elephant
351 shark MR and of lamprey CR [7]. We conclude that elephant shark GR is transitional from
352 elephant shark MR towards human GR in specificity for glucocorticoids and mineralocorticoids.

353 A novel property of elephant shark MR is that it is activated by progesterone and
354 19norProgesterone [41,50]. Neither steroid activates elephant shark GR. Unexpectedly,

355 progesterone and 19norProgesterone inhibit activation of elephant shark GR by corticosterone
356 (Figure 5A), which suggests that progestins may influence activation of elephant shark GR.
357 Neither progesterone nor 19norProgesterone inhibit activation of human GR by cortisol (Figure
358 5B). The timing of the loss of recognition of progestins by the GR needs further study.

359 The evolution of a distinct GR and MR in cartilaginous fish has provided an opportunity
360 to investigate early events in the divergence of these two steroid receptors from their common
361 ancestor. Cartilaginous fish, including elephant shark, also contain a PR and AR, which
362 diverged from a common ancestor. The PR is a close relative of the MR. Comparison of steroid
363 activation of elephant shark PR and AR with each other and with the MR and GR could provide
364 valuable insights into their evolution as important transcription factors in vertebrates.

365 **Materials and Methods**

366 **Chemical reagents**

367 Aldosterone, cortisol, corticosterone, 11-deoxycorticosterone, progesterone and
368 19norProgesterone were purchased from Sigma-Aldrich. For the reporter gene assays, all
369 hormones were dissolved in dimethylsulfoxide (DMSO) and the final concentration of DMSO in
370 the culture medium did not exceed 0.1%.

371 **Construction of plasmid vectors**

372 The full-coding regions and DBD-LBD domains of the GR and MR from *C. milii* were
373 amplified by PCR with KOD DNA polymerase (TOYOBO Biochemicals, Osaka, Japan). The
374 PCR products were gel-purified and ligated into pcDNA3.1 vector (Invitrogen) [51].

375 **Transactivation Assay and Statistical Methods**

376 Hek293 were used in the reporter gene assay. Transfection and reporter assays were
377 carried out as described previously [51,52]. All transfections were performed at least three
378 times, employing triplicate sample points in each experiment. The values shown are mean ±
379 SEM from three separate experiments. Data from studies of the effect of different corticosteroid
380 concentrations on transcriptional activation of elephant shark GR and MR were used to calculate
381 EC50s for steroid activation of elephant shark GR and MR using GraphPad Prism. Comparisons
382 between two groups were performed using t-test, and all multi-group comparisons were

383 performed using one-way ANOVA followed by Bonferroni test. $P < 0.05$ was considered
384 statistically significant.

385 **Genbank Accessions for Domain Comparisons**

386 Elephant shark GR (XP_007899521), human GR (BAH02307), chicken GR
387 (ABB05045), X. laevis GR (NP_001081531), Zebrafish GR (NP_001018547), elephant shark
388 MR (XP_007902220), human MR (NP_000892), zebrafish MR (NP_001093873).

389

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396 and designed the experiments. M.E.B. wrote the paper. All authors gave final approval for
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