SUPPLEMENTARY MATERIAL
FOR ORTLUND, BRIDGHAM, REDINBO AND THORNTON

CONTENTS

MATERIALS AND METHODS

Ancestral sequence inference and synthesis. Ancestral protein sequences of AncCR, AncGR1, and AncGR2 were inferred by maximum likelihood (*1*) using PAML 3.15 software on the maximum likelihood phylogeny of 60 amino acid sequences of extant steroid and related receptors (see ref. (*2*) for details and support measures). For ancestral reconstruction, the JTT+G model (supported with 100% posterior probability in the Bayesian analysis) was assumed. Ancestral sequences of the ligand-binding domain (LBD) and carboxy-terminal extension (CTE) were back-translated assuming human codon bias, synthesized (GenScript, Piscataway, NJ), and verified by sequencing. Plausible alternate states were defined as those with posterior probability greater than 0.20 (see Supplemental data and discussion). Mutagenesis to explore alternate sequence reconstructions and to recapitulate evolutionary substitutions was performed using the QuikChange kit (Stratagene, La Jolla, CA) and verified by sequencing.

Functional assays. Reconstructed ancestral receptor LBDs (including the carboxy-terminal extension, CTE) were fused to human GR hinge (amino acids 460-503) and subcloned in GAL4-pSG5-DBD (gift of D. Furlow). For extant receptors, hinge+LBD+CTE was amplified by polymerase chain reaction using high-fidelity Phusion enzyme (NEB, Beverly, MA), verified by sequencing, and subcloned into GAL4-pSG5-DBD. Templates for tetrapod GR and MR were from *Homo sapiens* and teleost GR was from *Astatotilapia burtoni*, provided by B. Darimont, R. Evans and R. Fernald, respectively. Elasmobranch GR was isolated from *Leucoraja erinacea* as described (*2*).

Chinese hamster ovary (CHO-K1) cells were transfected using 1 ng of receptor plasmid, 100 ng of a UAS-driven firefly luciferase reporter (pFR-luc), and 0.1 ng of the constitutive phRLtk Renilla luciferase reporter in 96-well plates using Lipfectamine Plus in Optimem (Invitrogen, Carlsbad, CA). After four hours incubation, transfection medium was replaced with phenol red-free αMEM supplemented with 10% dextran-charcoal-stripped fetal bovine serum (Hyclone, Logan, UT). After overnight recovery, cells were incubated in triplicate with hormone for 24 hours, then assayed by luminometry using the Dual-Glo luciferase system (Promega, Madison, WI). Firefly luciferase activity was normalized by Renilla luciferase. Dose-response relationships were estimated using non-linear regression, with sensitivity reported as the EC50 (Prism Software, Graphpad Software Inc., San Diego, CA). Receptor-ligand combinations are reported as "No activation" if the calculated EC50 is >5000 nM or maximal activation was <2-fold over vehicle alone.

Protein Expression, Crystallization, and Structural Analysis. AncCR-LBD cDNA (residues 1-247), was cloned into pMALCH10T (a gift of J. Tesmer). Mutation C71S was introduced to enhance protein expression and solubility. AncCR C71S was expressed as a maltose-binding protein/TEVfusion protein in BL21(DE3) pLys cells in the presence of 50 μM ligand using standard methods, and purified using affinity chromatography (HIS-Select, Sigma, St. Louis, MO). Following TEV cleavage, the tagged fusion protein was removed by an additional nickel affinity column and AncCR was polished via gel filtration. Pure AncCR LBD was dialyzed against 200 mM sodium chloride, 50 μM HEPES (pH 7.8) and 50 μM CHAPS, and concentrated to 3-7 mg/mL. Mutants were

introduced using QuikChange (Stratagene, LaJolla, CA) methods confirmed by sequencing.

Crystals of AncCR-LBD with ligand were grown by hanging drop vapor diffusion at 22 °C from solutions containing 0.75 μL of protein at 3-7 mg/mL protein and 0.75 μL of the following crystallant: 9.5%-15% PEG 3350, 5% glycerol, and 50 mM Bis-Tris, pH 6.4. The addition of a 19 amino acid peptide derived from NR box 1 of human SHP ($^{+}H_3N$-QGAASRPAILYALLSSSLK-CO_2^{-}, synthesized by SynPep, Dublin, CA) was required for crystal formation. Crystals were cryoprotected in crystallant containing 20% glycerol and were flash-cooled in liquid N_2. Data to 1.9, 1.95 and 2.4 Å resolution were collected at 100 K for the AncCR – aldosterone, AncCR – deoxycorticosterone and AncCR – cortisol complexes, respectively. All data were collected either at Stanford Synchrotron Radiation Laboratory beamline 11-1 or the South East Regional Collaborative Access Team at the Advanced Photon Source, and were processed and scaled with HKL2000 (Table S1) (*3*). Initial phases for the AncCR-cortisol complex were determined using the structure of the mineralocorticoid (MR) LBD (2AA2) as a molecular replacement search model. Subsequent structures were solved using the best available AncCR structure for initial phases. All structures were refined using COOT v.0.9 (*4*) and CNS v.1.1 (*5*). The AncCR structures with aldosterone, DOC, and cortisol have PDB accessions 2Q1H, 2Q3Y and 2Q1V. AncGR1 and AncGR2 were expressed and purified using similar methods; despite repeated efforts under a variety of conditions, neither crystallized in a morphology suitable for X-ray analysis.

The homology model for AncGR1 was initially generated using the functionally similar AncCR-cortisol crystal structure as a guide. Residue replacements were

performed in the program COOT using rotamers that approximated the side chain positions in either AncCR or in human GR (1M2Z). The initial AncGR2 homology model was generated using the Swiss-Model homology modeling server (*6*), using the functionally similar human GR (1M2Z) in complex with dexamethasone as a template. Rotamers were again corrected by hand using COOT. These models were then subject to 200 rounds of energy minimization in the program to correct geometry. Robustness of structural inferences to uncertainty in the ancestral reconstructions was examined by modeling plausible alternative reconstructions (PP > 0.20) into the AncCR crystal structure or the AncGR1 and AncGR2 homology models, using rotamers that approximate the side chain positions in the ML AncCR reconstruction or in the human MR and GR. Structures were rendered for display using Pymol software (DeLano Scientific, Palo Alto, CA, USA).

Fig. S1. Crystal structures of the AncCR LBD with cortisol and DOC. A) Overall structure of the AncCR LBD in complex with cortisol (purple, with red oxygen atoms) and **B)** DOC (orange, with red oxygen atoms) and a peptide derived from the NR Box1 of human SHP (red). Helices are labeled, including the activation function helix, AF-H.

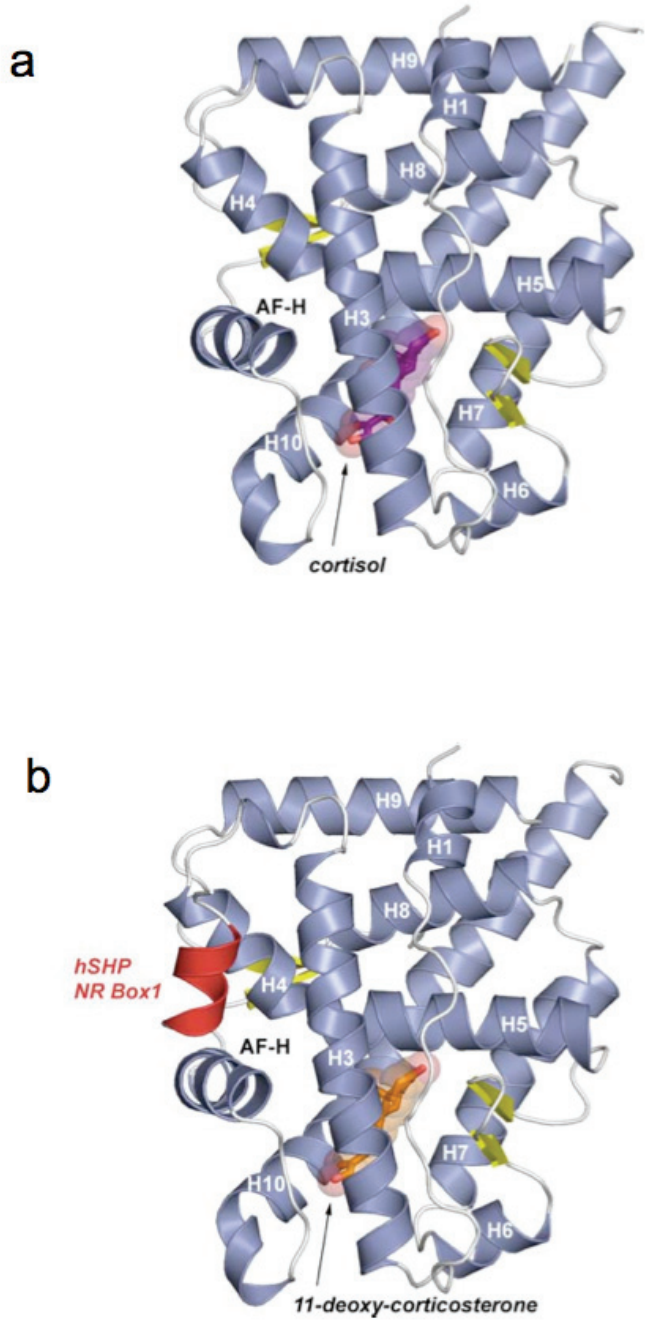


Fig. S2. Electron density for hormones in the AncCR ligand pocket. F_o-F_c simulated annealing omit density contoured at 2σ for bound aldosterone, cortisol and DOC at 1.9, 1.95 and 2.4 Å resolution, respectively.

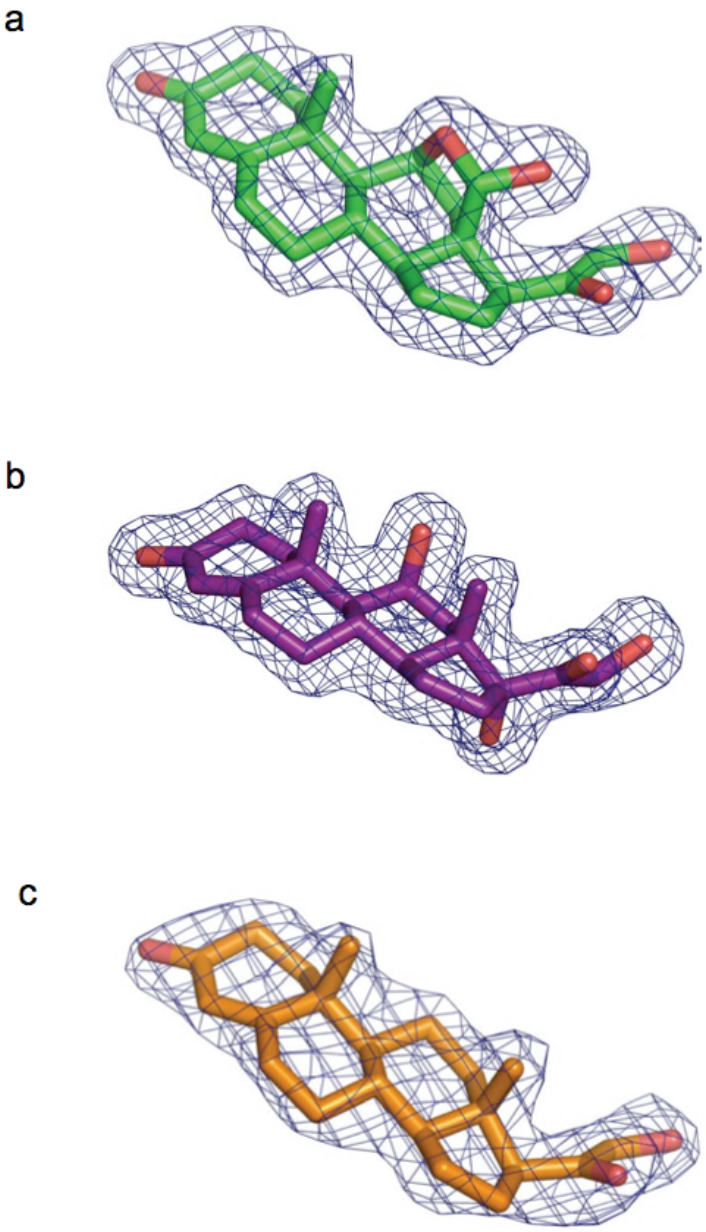


Fig S3. Conserved H-bond activation network in AncCR. The network of conserved hydrogen bonds between the ligand, helix 3, helix 10, and the activation helix is shown for AncCR (blue, panel a) and the human MR (purple, panel b) in complex with cortisol (green). Key side chains making stabilizing contacts are shown, with hydrogen-bonds as dotted red lines. Oxygen and nitrogen atoms are red and blue, respectively.

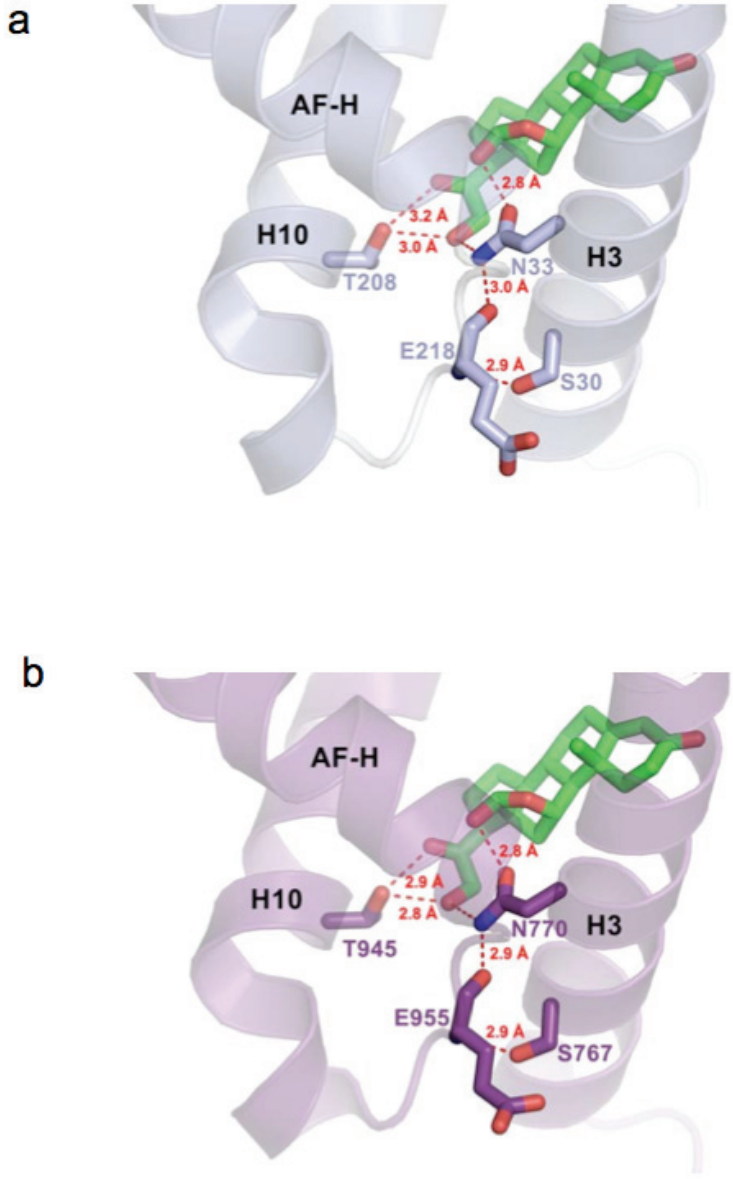
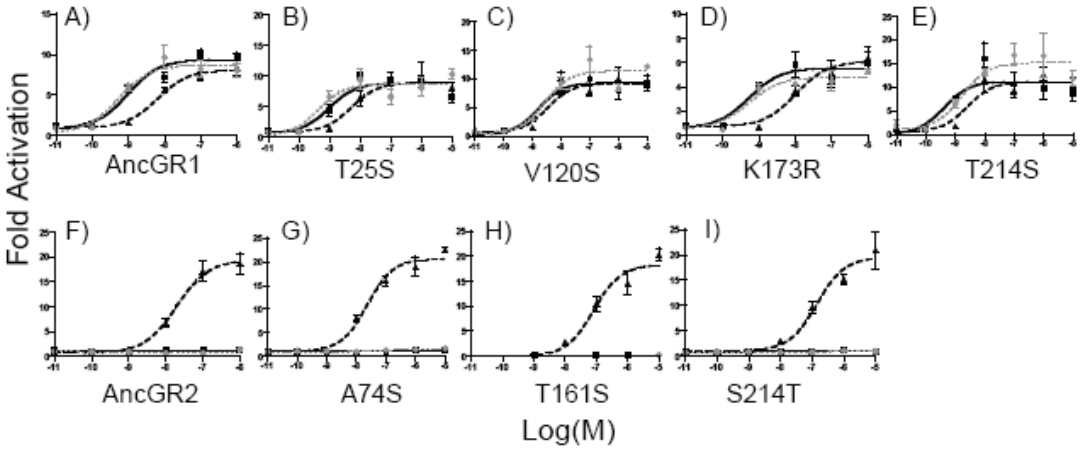


Fig. S4. Timing of the GR's functional shift is robust to alternative reconstructions. Sites having alternative ancestral states with posterior probability > 0.20 were defined as ambiguously reconstructed. A) AncGR1 maximum likelihood reconstruction activates transcription in response to aldosterone (squares, black line), DOC (circles, gray line), and cortisol (triangles, dotted line) in a luciferase reporter assay. B-E) Alternate AncGR1 reconstructions do not change function. Of AncGR1's alternate reconstructed states, all but four are found in extant receptors that are sensitive to aldosterone and DOC, indicating that these residues are not sufficient to abolish response to these ligands. These four states (T25S, V120S, K173R, T214S) were introduced into AncGR1 by directed mutagenesis and assayed for transcriptional response. F) AncGR2 maximum likelihood reconstruction is sensitive only to cortisol. G-I) Alternate AncGR2 states do not change function. Of AncGR2's alternate reconstructed states, all but two are found in extant receptors that are sensitive only to cortisol, indicating that these amino acids are not sufficient to confer aldosterone/DOC response. We introduced the others (Y161S and S214T) into AncGR2, but neither changed the response to ligand. One additional alternate reconstruction was in a position that contacts ligand (A74S); introduction of this residue also did not change AncGR2 function. EC50 values are listed in the table. NA, no activation, defined as <2-fold maximal activation and/or EC50>1000 nM.



		Aldosterone	Cortisol	DOC
A	AncGR1	0.83nM	6.1nM	0.57nM
B	AncGR1 T25S	0.96nM	5.2nM	0.42nM
C	AncGR1 V120S	1.6nM	3.3nM	2.7nM
D	AncGR1 K173R	0.53nM	10.0nM	0.53nM
E	AncGR1 T214S	0.4nM	2.6nM	1.7nM
F	AncGR2	NA	29nM	NA
G	AncGR2 A74S	NA	23nM	NA
H	AncGR2 T161S	NA	82nM	NA
I	AncGR2 S214T	NA	140nM	NA

Fig. S5. Amino acid sequence alignment of ancestral receptor LBDs. The sequences of extant and reconstructed ancestral corticosteroid receptors are shown in aligned format. Secondary structural elements are underlined and annotated below the sequences. Blue, substitutions between AncCR and AncGR1; orange, changes between AncGR1 and AncGR2; green, sites that changed between AncCR and AncGR1 and again between AncGR1 and AncGR2. Stars indicate ligand contacting residues. Helices are labeled as in ref. (*7*) .

Fig. S6. Structural map of sequence changes in the AncGR1-AncGR2 interval. Sites that changed from one state in AncGR1 to a different state in AncGR2 are shown mapped on the backbone of the AncGR1 model. Function-changing substitutions in the X, Y, and Z groups are shown with blue spheres for the α-carbon (light, medium, and dark blue, respectively); red spheres show other substitutions.

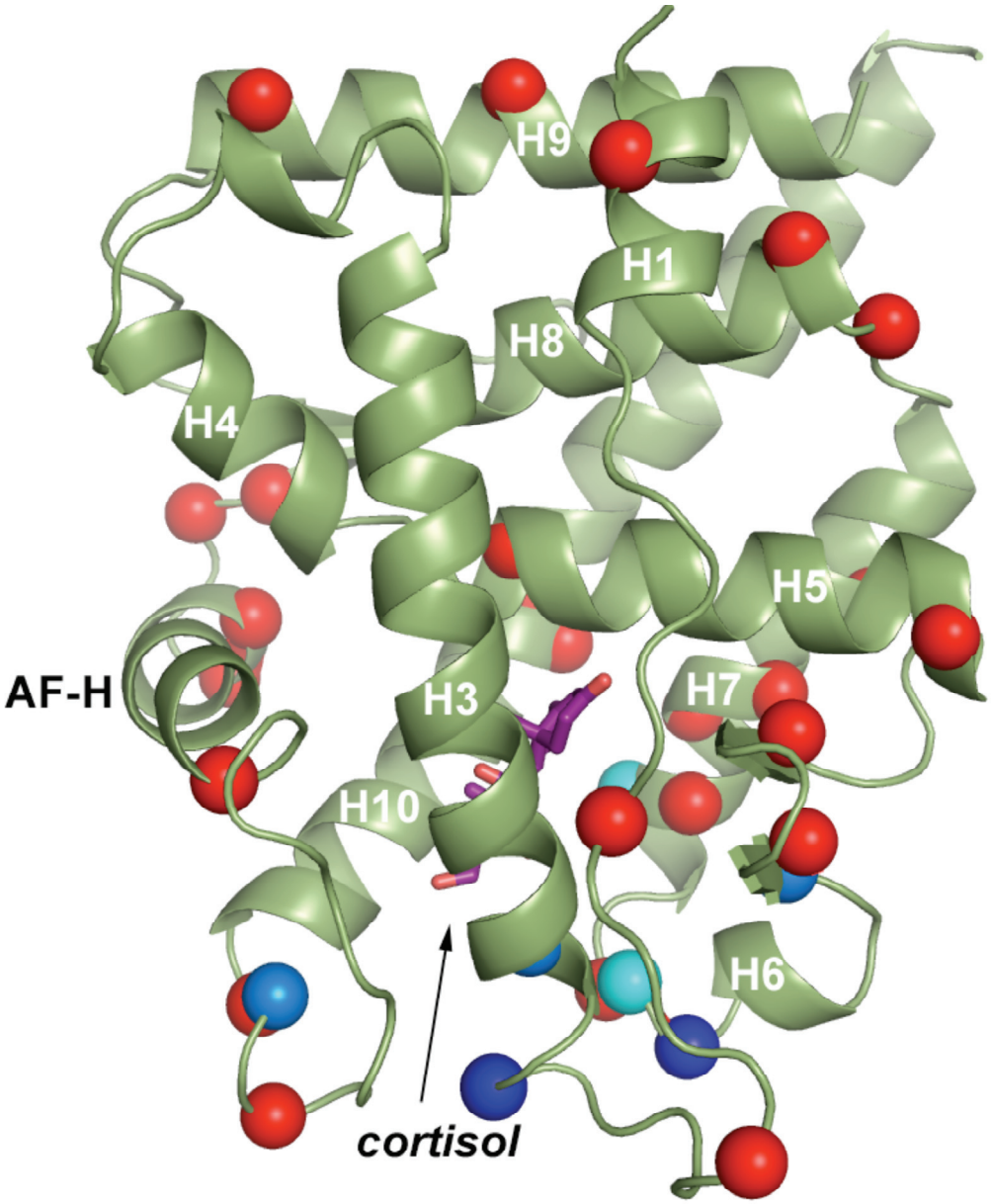


Fig. S7. Effect of historical mutations on extant receptors. **A)** Two historical substitutions (S106P and L111Q) that switch ligand preference from aldosterone to cortisol in AncGR1 were introduced into the human MR (numbers correspond to the homologous position in the human sequence). Activation of a luciferase reporter gene by the wild-type and mutant receptor ligand-binding domains is shown in the presence of aldosterone (green), cortisol (purple), and DOC (orange). **B)** The reverse historical substitutions were introduced into the human GR. Bars show SEM for three replicates.

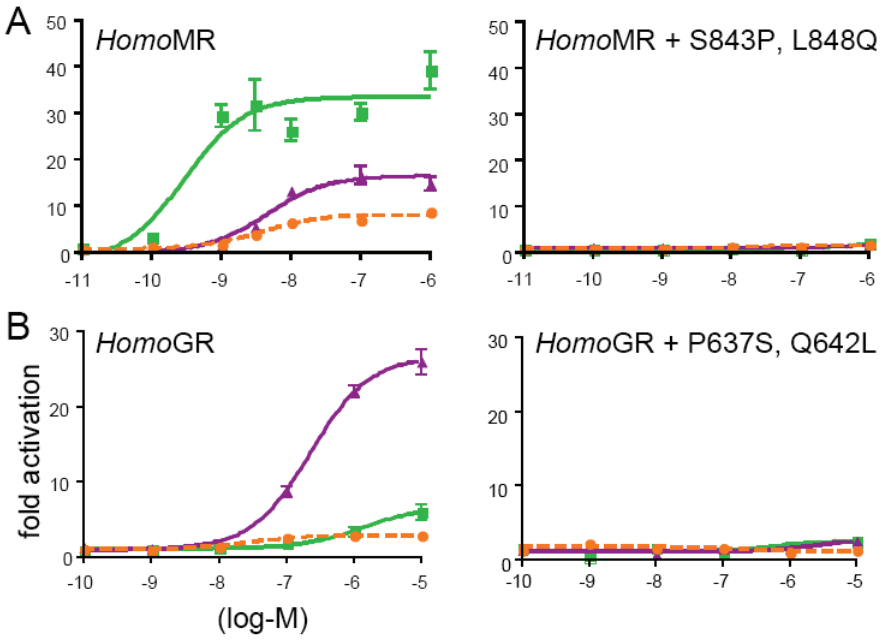


Fig. S8. Superposition of AncCR, AncGR1, and AncGR2 structures. The backbones of the AnCR-LBD crystal structure (blue) and the AncGR1 (green) and AncGR2 (yellow) models, all with cortisol, are shown superimposed upon each other. Helices are labeled, including AF-H, the activation-function helix.

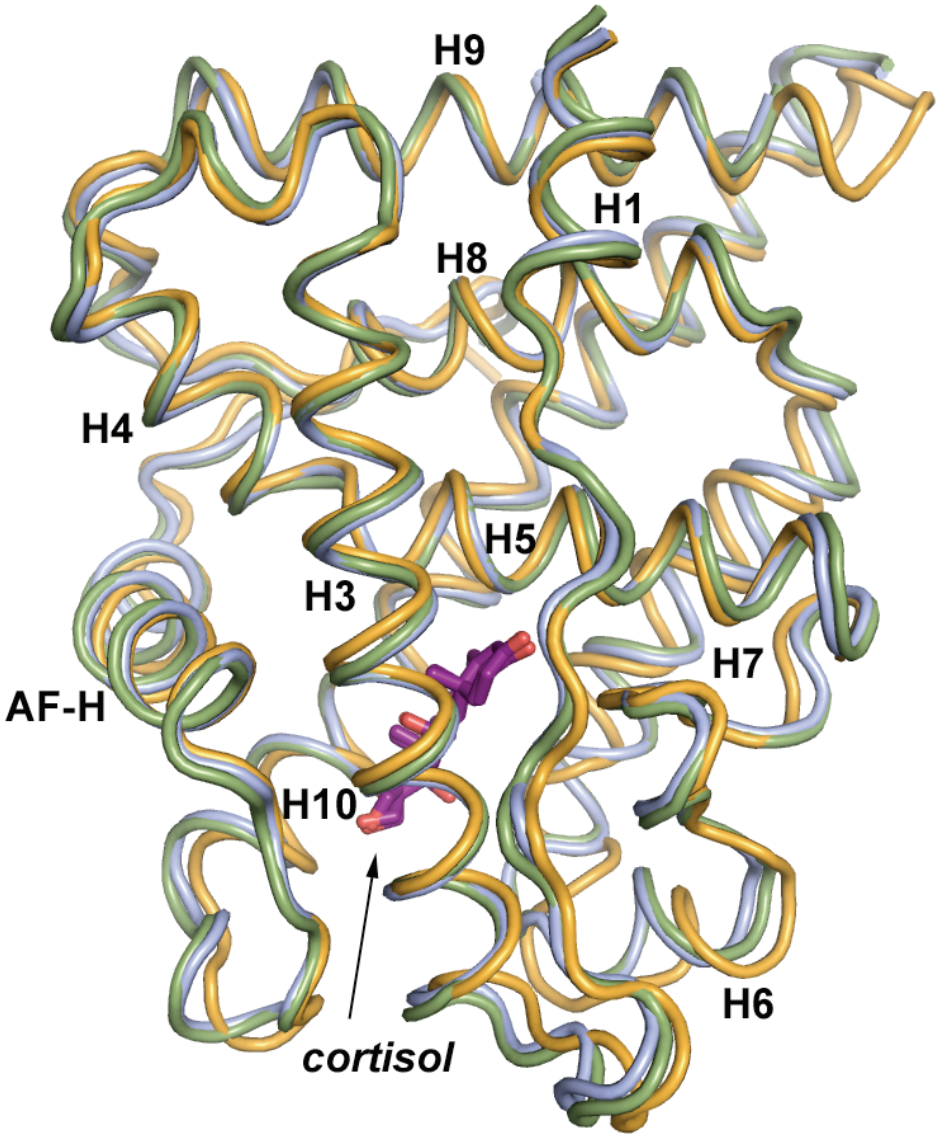


Fig. S9. Reversing the permissive substitution Y27R does not change the function of AncGR1. Left panel, transcriptional activation by AncGR1 (which contains an arginine at position 27) in the presence of corticosteroids. Right, activation by a mutant AncGR1 with tyrosine at position 27. Receptor sensitivity to aldosterone (green), cortisol (purple), and DOC (orange) is not changed substantially by the mutation. Fold-increase in activation of luciferase reporter gene is shown in response to increasing hormone concentrations. Error bars show s.e.m. for three replicates.

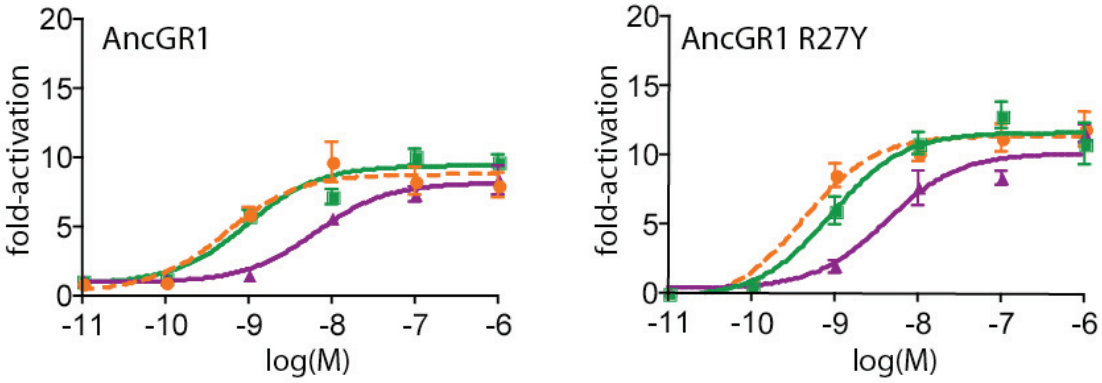


Fig. S10. Phylogeny of the corticosteroid receptors. Complete BMCMC phylogeny of the corticoid receptors and other steroid receptors. Branch labels indicate posterior probabilities. The tree is rooted to minimize gene duplications; other rootings require additional duplications and losses. Corticoid receptors in bold. Circles; blue, AncCR, green, AncGR1 and yellow, AncGR2. For analysis parameters, see Methods.

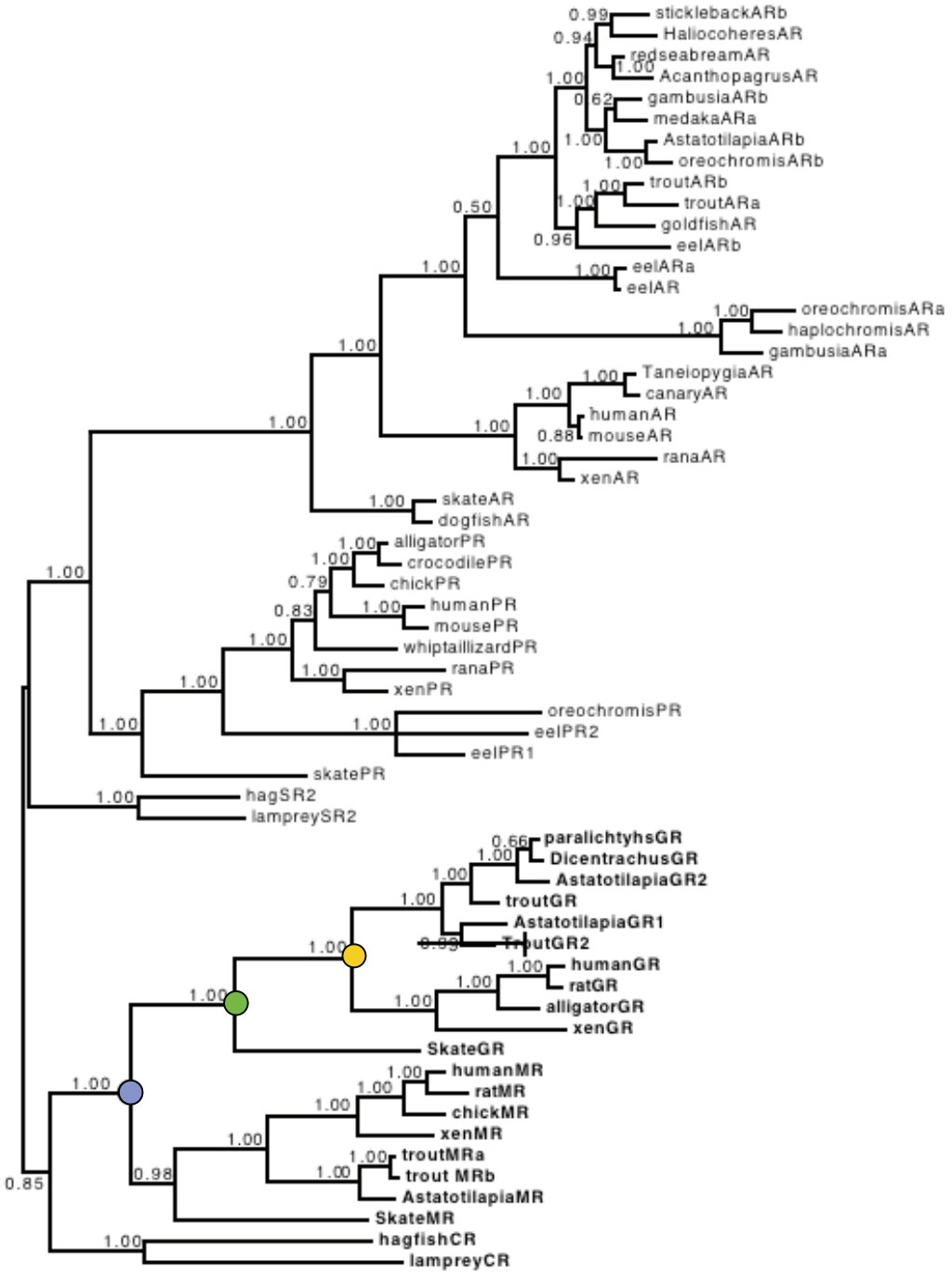


Table S1. Data collection and refinement statistics for AncCR crystal structures.

	AncCR – Aldo	AncCR – DOC	AncCR – Cort
Resolution (highest shell)	1.90 Å (1.90 – 1.97 Å)	2.4 Å (2.49 – 2.4 Å)	1.95Å (2.02 – 1.95 Å)
Space Group	$P4_32_12$	$P4_32_12$	$P4_32_12$
Unit Cell Dimensions	a=79.7, b=79.7, c=113.9 $\alpha=\beta=\gamma=90.0$	a=78.9, b=78.9, c=112.5 $\alpha=\beta=\gamma=90.0$	a=79.9, b=79.9, c=114.6 $\alpha=\beta=\gamma=90.0$
No. of Reflections	29610	14500	27603
R^a_{sym} (highest shell)	3.7% (37.0%)	8.9% (30.1%)	8.0% (36.1%)
Completeness (highest shell)	99.9% (100.0%)	99.7% (98.7%)	99.9% (99.6%)
Ave. Redundancy (highest shell)	10.1 (10.2)	9.3 (9.2)	7.6 (6.5)
I/σ	63.8 (7.5)	23.6 (10.1)	17.5 (5.2)
Monomers per asymmetric unit (AU)	1	1	1
No. of protein atoms/AU	1987	2065	1987
No. of ligand atoms/AU	26	24	26
No. of waters/AU	161	101	181
$R^b_{working}$ (R^c_{free})	20.7% (22.0%)	19.6% (24.0%)	20.7% (23.3%)
Ave. B-factors, Å^2			
Protein	31.3	38.9	29.5
Ligand	19.5	25.7	17.4
Water	41.1	42.6	38.8
r.m.s. deviations			
Bond lengths, Å	0.005	0.006	0.005
Bond angles, °	1.005	1.100	1.100

[a] $R_{sym} = \Sigma|I - \langle I\rangle|/ \Sigma|I|$, where I is the observed intensity and <I> is the average intensity of several symmetry-related observations.

[b] $R_{working} = \Sigma||Fo|-|Fc||/ \Sigma|Fo$, where Fo and Fc are the observed and calculated structure factors, respectively.

[c] $R_{free} = \Sigma||Fo|-|Fc||/ \Sigma|Fo$ for 7% of the data not used at any stage of the structural refinement.

Table S2. Maximum likelihood reconstruction and posterior probabilities of AncGR1. Columns show residue position (numbered as in AncCR), maximum likelihood state and posterior probability (PP). LBP: * residues in the ligand binding pocket that contact hormone. Alternate state lists plausible amino acids other than the ML reconstruction, if any, with posterior probability > 0.2. Final column shows extant aldosterone-activated receptors, if any, that contain the alternate states. AncGR1 has GenBank accession EF631975.

Site#	ML state	PP	LBP	Alternate state (if PP>0.2)	PP of alt state	Aldo-activated receptors with alternate state
-2	P	1.000				
-1	S	0.814				
1	L	0.744				
2	I	0.624		V	0.28	human MR + 4others
3	S	0.997				
4	I	0.869				
5	L	1.000				
6	E	0.995				
7	V	0.368				
8	I	1.000				
9	E	1.000				
10	P	1.000				
11	E	0.888				
12	V	0.904				
13	L	0.654		V	0.22	human MR + 9 others
14	Y	1.000				
15	A	0.964				
16	G	1.000				
17	Y	1.000				
18	D	1.000				
19	S	0.876				
20	S	0.754		T	0.23	trout MR and xenopus MR
21	L	0.969				
22	P	1.000				
23	D	0.999				
24	T	1.000				
25	T	0.538		S	0.32	None
26	N	0.930				
27	R	0.878				
28	L	1.000				
29	L	0.969	*			
30	S	0.983				
31	S	0.849				
32	L	1.000	*			
33	N	1.000	*			
34	R	0.908				

Site#	ML state	PP	LBP	Alternate state (if PP>0.2)	PP of alt state	Aldo-activated receptors with alternate state
35	L	1.000	*			
36	G	0.980	*			
37	G	0.994				
38	R	0.716				
39	Q	1.000	*			
40	M	0.990				
41	V	0.823				
42	S	0.899				
43	V	0.976				
44	V	1.000				
45	K	1.000				
46	W	1.000				
47	A	1.000				
48	K	1.000				
49	A	0.839				
50	L	0.997				
51	P	1.000				
52	G	1.000				
53	F	1.000				
54	R	1.000				
55	N	0.956				
56	L	1.000				
57	H	1.000				
58	L	0.999				
59	D	1.000				
60	D	1.000				
61	Q	1.000				
62	M	1.000				
63	T	0.987				
64	L	1.000				
65	L	0.981				
66	Q	1.000				
67	Y	0.999				
68	S	1.000				
69	W	1.000	*			
70	M	1.000	*			
71	S	0.455		C	0.38	human MR + 9 others
72	L	1.000				
73	M	1.000	*			
74	A	0.723	*			
75	F	1.000				
76	S	0.813				
77	L	0.998	*			
78	G	0.864				
79	W	1.000				
80	R	1.000	*			

Site#	ML state	PP	LBP	Alternate state (if PP>0.2)	PP of alt state	Aldo-activated receptors with alternate state
81	S	1.000				
82	Y	1.000				
83	K	0.980				
84	H	0.980				
85	S	0.660		T	0.32	human MR + 8others
86	N	1.000				
87	G	0.971				
88	N	0.503		S	0.28	skate GR
89	M	1.000				
90	L	1.000				
91	Y	0.794				
92	F	1.000	*			
93	A	1.000				
94	P	1.000				
95	D	1.000				
96	L	1.000				
97	I	0.889				
98	F	0.997				
99	N	1.000				
100	E	1.000				
101	E	0.885				
102	R	1.000				
103	M	1.000				
104	Q	0.968				
105	Q	0.974				
106	S	0.992				
107	A	0.484		T	0.36	skate GR
108	M	1.000	*			
109	Y	0.998				
110	D	0.881				
111	L	0.992	*			
112	C	1.000				
113	Q	0.578		K	0.33	skate GR
114	G	0.968				
115	M	1.000	*			
116	R	0.406		Q	0.27	xenopus MR and skate MR
117	K	0.486		Q	0.32	human MR + 7others
118	I	0.994				
119	S	0.963				
120	V	0.278		S	0.21	None
121	E	0.954				
122	F	1.000				
123	V	0.741				
124	R	0.924				
125	L	1.000				
126	Q	0.993				

Site#	ML state	PP	LBP	Alternate state (if PP>0.2)	PP of alt state	Aldo-activated receptors with alternate state
127	V	0.988				
128	T	0.470		S	0.31	hagfish CR
129	Y	0.946				
130	E	0.922				
131	E	1.000				
132	Y	0.976				
133	L	1.000				
134	C	1.000				
135	M	1.000				
136	K	1.000				
137	V	0.741		A	0.26	skate GR
138	L	1.000				
139	L	1.000				
140	L	1.000				
141	L	1.000				
142	S	0.983				
143	T	1.000				
144	V	0.755		I	0.25	tree shrew MR, sheep MR and skate GR
145	P	1.000				
146	K	0.986				
147	D	0.938				
148	G	1.000				
149	L	1.000				
150	K	1.000				
151	S	0.999				
152	Q	1.000				
153	A	0.986				
154	T	0.370		A	0.23	human MR +7tohers
155	F	1.000				
156	D	0.995				
157	E	1.000				
158	I	0.962				
159	R	1.000				
160	M	0.975				
161	S	0.506		N	0.31	human MR + 9others
162	Y	1.000				
163	I	1.000				
164	K	0.997				
165	E	1.000				
166	L	1.000				
167	G	0.967				
168	K	0.946				
169	A	0.972				
170	I	0.993				
171	V	0.869				
172	K	0.961				

Site#	ML state	PP	LBP	Alternate state (if PP>0.2)	PP of alt state	Aldo-activated receptors with alternate state
173	K	0.566		R	0.41	None
174	E	0.986				
175	G	0.742				
176	N	1.000				
177	S	0.981				
178	S	0.903				
179	Q	1.000				
180	N	0.959				
181	W	1.000				
182	Q	0.979				
183	R	1.000				
184	F	1.000				
185	Y	1.000				
186	Q	1.000				
187	L	1.000				
188	T	1.000				
189	K	1.000				
190	L	1.000				
191	L	1.000				
192	D	1.000				
193	S	1.000				
194	M	1.000				
195	H	1.000				
196	D	0.838				
197	L	0.944				
198	V	1.000				
199	G	0.692		E	0.28	trout MR and skate GR
200	G	0.972				
201	L	1.000	*			
202	L	1.000				
203	Q	0.964				
204	F	1.000	*			
205	C	1.000	*			
206	F	1.000				
207	Y	0.995				
208	T	1.000	*			
209	F	1.000				
210	V	0.795				
211	Q	0.819				
212	S	0.995				
213	K	0.948				
214	T	0.419		A / S	0.36 / 0.22	A in human MR + 9other, S not present in aldo+
215	L	0.997				
216	S	0.993				
217	V	0.997	*			
218	E	0.999				

Site#	ML state	PP	LBP	Alternate state (if PP>0.2)	PP of alt state	Aldo-activated receptors with alternate state
219	F	1.000	*			
220	P	1.000				
221	E	0.999				
222	M	1.000				
223	L	0.999	*			
224	V	0.678		A	0.31	human MR + 9others
225	E	1.000				
226	I	1.000				
227	I	1.000				
228	S	0.998				
229	N	0.962				
230	Q	1.000				
231	L	0.999				
232	P	1.000				
233	K	0.999				
234	V	0.903				
235	M	0.469		T	0.21	skate MR
236	A	0.905				
237	G	1.000				
238	M	0.897				
239	A	0.900				
240	K	1.000				
241	P	0.965				
242	L	1.000				
243	L	0.980				
244	F	1.000				
245	H	1.000				
246	Q	0.618				
247	K	0.999				
	mean	0.9303				

Table S3. Maximum likelihood reconstruction and posterior probabilities of AncGR2. Columns show residue position (numbered as in AncCR), maximum likelihood state and posterior probability (PP). LBP: * residues in the ligand binding pocket that contact hormone. Alternate state lists plausible amino acids other than the ML reconstruction, if any, with posterior probability > 0.2. Seventh column shows extant aldosterone-insensitive receptors, if any that contain the alternate state. Penultimate column shows extant aldosterone-activated receptors, if any, that contain the alternate states. Final column shows whether the ML state is present in any aldosterone-activated receptors. AncGR2 has genbank accession EF631976.

Site#	ML state	PP	LBP	Alternate state (PP> 0.2)	PP of alt state	Aldo-insensitive receptors with alternate state	Aldo-activated receptors with alternate state	ML state in aldo-activated receptors?
-2	P	1.000						
-1	T	0.968						
1	L	0.692		M	0.28	trout GR, haplochromis GRalph, and halibut GR	0	yes
2	I	0.457		V	0.36	human GR +7 others	Human MR, tree shrew MR	yes
3	S	1.000						
4	L	0.977						
5	L	1.000						
6	E	0.968						
7	V	0.535		A	0.41	trout GR, haplochromis GRalph, and halibut GR	0	no
8	I	1.000						
9	E	1.000						
10	P	1.000						
11	E	0.931						
12	V	0.891						
13	L	0.659		I	0.28	trout GR, haplochromis GRalph, and halibut GR	0	yes
14	Y	1.000						
15	S	0.973						
16	G	1.000						
17	Y	1.000						
18	D	1.000						
19	S	0.993						
20	T	0.518		S	0.47	sq monkey GR, trout GR, haplochromis GRalph, and halibut GR	lampreyCR troutMR and xenopus MR	yes
21	L	0.992						
22	P	1.000						

Site#	ML state	PP	LBP	Alter-nate state (PP> 0.2)	PP of alt state	Aldo-insensitive receptors with alternate state	Aldo-activated receptors with alternate state	ML state in aldo-activated receptors?
23	D	1.000						
24	T	0.999						
25	S	0.613		T	0.36	human GR +5others	5 MRs	no
26	T	0.500						
27	R	0.995						
28	L	0.999						
29	M	0.994	*					
30	S	0.853						
31	T	0.872						
32	L	1.000	*					
33	N	1.000	*					
34	R	0.934						
35	L	1.000	*					
36	G	1.000	*					
37	G	1.000						
38	R	0.985						
39	Q	1.000	*					
40	V	0.993						
41	V	0.902						
42	S	0.948						
43	A	0.990						
44	V	1.000						
45	K	1.000						
46	W	1.000						
47	A	1.000						
48	K	1.000						
49	A	0.975						
50	L	0.956						
51	P	1.000						
52	G	1.000						
53	F	1.000						
54	R	1.000						
55	N	0.999						
56	L	1.000						
57	H	1.000						
58	L	1.000						
59	D	1.000						
60	D	1.000						
61	Q	1.000						

Site#	ML state	PP	LBP	Alternate state (PP> 0.2)	PP of alt state	Aldo-insensitive receptors with alternate state	Aldo-activated receptors with alternate state	ML state in aldo-activated receptors?
62	M	1.000						
63	T	1.000						
64	L	1.000						
65	L	1.000						
66	Q	1.000						
67	Y	0.979						
68	S	1.000						
69	W	1.000	*					
70	M	0.996	*					
71	F	0.995						
72	L	1.000						
73	M	1.000	*					
74	A	0.711	*	S	0.23	trout GR, haplochromis GRalph, and halibut GR	human MR + 9others	yes
75	F	1.000						
76	S	0.731		A	0.23	human GR +8others	human MR + 6others	yes
77	L	1.000	*					
78	G	0.999						
79	W	1.000						
80	R	1.000	*					
81	S	1.000						
82	Y	1.000						
83	K	0.765						
84	Q	0.987						
85	S	0.692		T	0.24	xenopus GR	human MR + 8others, node 69 is S	yes
86	N	0.999						
87	G	0.999						
88	N	0.928						
89	M	0.991						
90	L	1.000						
91	C	0.833						
92	F	1.000	*					
93	A	1.000						
94	P	1.000						
95	D	1.000						

Site#	ML state	PP	LBP	Alter-nate state (PP> 0.2)	PP of alt state	Aldo-insensitive receptors with alternate state	Aldo-activated receptors with alternate state	ML state in aldo-activated receptors?
96	L	1.000						
97	V	0.603		I	0.37	human GR +5others	haplochromis MR and xenopus MR	yes
98	I	0.998						
99	N	1.000						
100	E	0.999						
101	E	0.909						
102	R	1.000						
103	M	1.000						
104	Q	0.498		K	0.33	trout GR, haplochromis GRalph, and halibut GR	0	yes
105	L	0.989						
106	P	0.998						
107	Y	0.659						
108	M	1.000	*					
109	Y	0.938						
110	D	0.965						
111	Q	0.994	*					
112	C	1.000						
113	Q	0.496		K	0.29	human GR +6others	0	yes
114	Q	0.766						
115	M	1.000	*					
116	L	0.988						
117	K	0.927						
118	I	0.988						
119	S	0.986						
120	S	0.926						
121	E	0.998						
122	F	0.990						
123	V	0.782						
124	R	0.990						
125	L	1.000						
126	Q	1.000						
127	V	0.990						
128	S	0.979						
129	Y	0.970						
130	D	0.656		E	0.34	human GR +8others	human MR + 6others	yes

Site#	ML state	PP	LBP	Alter-nate state (PP> 0.2)	PP of alt state	Aldo-insensitive receptors with alternate state	Aldo-activated receptors with alternate state	ML state in aldo-activated receptors?
131	E	1.000						
132	Y	1.000						
133	L	1.000						
134	C	1.000						
135	M	1.000						
136	K	1.000						
137	V	0.971						
138	L	1.000						
139	L	1.000						
140	L	1.000						
141	L	0.999						
142	S	0.999						
143	T	1.000						
144	V	0.910						
145	P	1.000						
146	K	1.000						
147	D	0.922						
148	G	1.000						
149	L	1.000						
150	K	1.000						
151	S	1.000						
152	Q	1.000						
153	A	0.993						
154	V	0.611		L	0.20	human GR + 8others	0	yes
155	F	1.000						
156	D	0.997						
157	E	1.000						
158	I	1.000						
159	R	1.000						
160	M	0.999						
161	T	0.733		S	0.25	0	skate GR and lamprey CR	hagfish CR
162	Y	1.000						
163	I	1.000						
164	K	1.000						
165	E	1.000						
166	L	1.000						
167	G	1.000						

Site#	ML state	PP	LBP	Alter-nate state (PP> 0.2)	PP of alt state	Aldo-insensitive receptors with alternate state	Aldo-activated receptors with alternate state	ML state in aldo-activated receptors?
168	K	0.999						
169	A	0.999						
170	I	0.999						
171	V	0.986						
172	K	0.996						
173	R	0.964						
174	E	0.996						
175	G	0.758						
176	N	1.000						
177	S	0.998						
178	S	0.992						
179	Q	1.000						
180	N	0.998						
181	W	1.000						
182	Q	1.000						
183	R	1.000						
184	F	1.000						
185	Y	1.000						
186	Q	1.000						
187	L	1.000						
188	T	1.000						
189	K	1.000						
190	L	1.000						
191	L	1.000						
192	D	1.000						
193	S	1.000						
194	M	1.000						
195	H	1.000						
196	E	0.956						
197	M	0.577		V	0.20	human GR + 8others	0	no
198	V	1.000						
199	G	0.686		E	0.29	human GR + 10others	0	yes
200	G	0.947						
201	L	1.000	*					
202	L	1.000						
203	Q	0.909						
204	F	0.997	*					
205	C	1.000	*					
206	F	1.000						

Site#	ML state	PP	LBP	Alter-nate state (PP> 0.2)	PP of alt state	Aldo-insensitive receptors with alternate state	Aldo-activated receptors with alternate state	ML state in aldo-activated receptors?
207	Y	0.983						
208	T	1.000	*					
209	F	1.000						
210	V	0.828						
211	N	0.541		D	0.41	human GR + 8others	0	no
212	deletion							
213	K	0.999						
214	S	0.747		T	0.22	human GR + 8others	node 69, skateGR	no
215	L	0.951						
216	S	0.999						
217	V	0.967	*					
218	E	1.000						
219	F	1.000	*					
220	P	1.000						
221	E	0.998						
222	M	1.000						
223	L	1.000	*					
224	A	0.977						
225	E	1.000						
226	I	1.000						
227	I	1.000						
228	S	0.995						
229	N	0.999						
230	Q	1.000						
231	L	0.973						
232	P	1.000						
233	K	1.000						
234	F	0.797						
235	K	0.357		S	0.29	human GR + 7others	0	no
236	A	0.852						
237	G	1.000						
238	S	0.519		N	0.36	human GR + 8others	0	no
239	V	0.870						
240	K	1.000						
241	P	0.968						
242	L	1.000						
243	L	0.999						
244	F	1.000						

Site#	ML state	PP	LBP	Alter-nate state (PP> 0.2)	PP of alt state	Aldo-insensitive receptors with alternate state	Aldo-activated receptors with alternate state	ML state in aldo-activated receptors?
245	H	1.000						
246	Q	0.988						
247	K	0.999						
	mean	0.947						

Table S4. Effect of permissive substitutions on AncGR1 ligand sensitivity. Group Z consists of two substitutions (Q105L and N26T). These were introduced individually and in combination into the AncGR1 XY background, which includes group X substitutions (S106P, L111Q) and group Y (L29M, F981, S212Δ). Mutant receptors were then assessed for hormone responsiveness in a luciferase reporter transcription assay. Table shows the EC50 for each ligand – the concentration required for half-maximal activation. NA, no activation, defined as <2-fold maximal activation at up to 1000 nM hormone.

Receptor	aldosterone	cortisol	DOC
AncGR1 XY + Q105L + N26T	NA	308 nM	6400 nM
AncGR1 XY + Q105L	NA	1190 μM	NA
AncGR1 XY + N26T	NA	2200 μM	NA
AncGR1 XY	NA	6250 μM	NA

Table S5. List of sequences used for phylogenetic analysis and reconstruction.

Sequence name (on phylogeny)	Genbank ID	Species
AcanthopagrusAR	28974723	Acanthopagrus schlegelii
AlligatorGR	22135433	Alligator mississippiensis
AlligatorPR	41386653	Alligator mississippiensis
AstatilapiaARb	19879685	Astatotilapia burtoni
AstatotilapiaGR1	20563131	Astatotilapia burtoni
AstatotilapiaGR2	20563133	Astatotilapia burtoni
AstatotilapiaMR	20563137	Astatotilapia burtoni
CanaryAR	414734	Serinus canaria
ChickMR	30315964	Gallus gallus
ChickPR	130893	Gallus gallus
CrocodilePR	2564207	Crocodylus siamensis
DicentrachusGR	44889863	Dicentrarchus labrax
DogfishAR	37909992	Squalus acanthias
EelARa	7446192	Anguilla japonica
EelARb	5821400	Anguilla japonica
EelPR1	6716126	Anguilla japonica
EelPR2	19386568	Anguilla japonica
GambusiaARa	56783596	Gambusia affinis
GambusiaARb	56783598	Gambusia affinis
GoldfishAR	20135660	Carassius auratus
HagfishCR	DQ382336	Myxine glutinosa
HagfishSR2	DQ382337	Myxine glutinosa
HalicoheresAR	12061008	Halichoeres trimaculatus
HaplochromisAR	4583413	Astatotilapia burtoni
HumanAR	4557331	Homo sapiens
HumanGR	121069	Homo sapiens
HumanMR	126885	Homo sapiens
HumanPR	4505767	Homo sapiens
LampreyCR	13919634	Petromyzon marinus
LampreySR2	13919636	Petromyzon marinus
MedakaARa	37359712	Oryzias latipes
MouseAR	113831	Mus musculus
MousePR	130895	Mus musculus
OreochromisARa	12248398	Oreochromis niloticus
OreochromisARb	12248400	Oreochromis niloticus
OreochromisPR	31339298	Oreochromis niloticus
ParalichthysGR	6016116	Paralichthys olivaceus
RanaAR	46576435	Rana catesbeiana
RanaPR	46577033	Rana dybowskii
RatGR	19343485	Rattus norvegicus
RatMR	126886	Rattus norvegicus
RedseabreamAR	3668185	Pagrus major
SkateAR	DQ382340	Raja erinacea
SkateGR	DQ382338	Raja erinacea
SkateMR	DQ382339	Raja erinacea
SkatePR	DQ382341	Raja erinacea
SticklebackARb	29568404	Gasterosteus aculeatus
TaeniopygiaAR	22417219	Taeniopygia guttata
TroutARa	3551156	Oncorhynchus mykiss
TroutARb	3551158	Oncorhynchus mykiss
TroutGR1	1730254	Oncorhynchus mykiss

TroutGR2	40548814	Oncorhynchus mykiss
TroutMRa	53766435	Oncorhynchus mykiss
TroutMRb	53766437	Oncorhynchus mykiss
WhiptaillizardPR	1195594	Cnemidophorus uniparens
XenopusAR	4038480	Xenopus laevis
XenopusGR	1730255	Xenopus laevis
XenopusMR	2500914	Xenopus laevis
XenopusPR	11991857	Xenopus laevis

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