Supplementary materials for J.T. Bridgham, E.A. Ortlund, J.W. Thornton, "An epistatic ratchet constrains the direction of glucocorticoid receptor evolution."

CONTENTS

Table S1. Data collection and refinement statistics for the AncGR2-dexamethasone crystal structure.

	AncGR2 – DEX
Resolution (highest shell)	2.5 Å (2.50– 2.59 Å)
Space Group	$P6_1$
Unit Cell Dimensions	a=104.2, b=104.2, c=144.2 α=β= 90.0, γ= 120.0
No. of Reflections	30813
R^a_{sym} (highest shell)	11.5% (44.2%)
Completeness (highest shell)	100.0% (100.0%)
Ave. Redundancy (highest shell)	7.3 (7.3)
I/σ	23.4 (5.2)
Monomers per asymmetric unit (AU)	2
No. of protein atoms/AU	4427
No. of ligand atoms/AU	56
No. of waters/AU	157
$R^b_{working}$ (R^c_{free})	19.9% (25.8%)
Ave. B-factors, Å^2	
Protein	39.5
Ligand	23.9
Water	41.7
r.m.s. deviations	
Bond lengths, Å	0.012
Bond angles, °	1.385

[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.

Fig. S1. Amino acid changes that occurred in the interval between AncGR1 and AncGR2. A) The aligned maximum likelihood sequences of AncGR1 and AncGR2 ligand-binding domains are shown, with selected extant human corticosteroid receptors (GR, glucocorticoid receptor; MR, mineralocorticoid receptor). Replacements in group W are in cyan, groups X, Y, and Z are in green. Other sites differing between AncGR1 and AncGR2 are in purple, and unchanged sites are in black. Secondary structural elements are labeled: H, helix; β, β-strand, AF-H, activation function helix. B) Sites that changed between AncGR1 and AncGR2 are shown, with additional sequences to show patterns of conservation. Dots signify states identical to first sequence in each of the two sections of the alignment. Upper section shows AncGR2 and extant sequences that descend from it (teleost and tetrapod GRs). Bottom alignment shows sequences that do not descend from AncGR2 and lack GR's cortisol-specificity (AncGR1, MRs, agnathan CRs, and elasmobranch GR). Green, sites in groups X, Y, and Z. Blue, sites in group W. Of sites not in groups X, Y, and Z, only the group W sites are conserved in the AncGR1-like state in all or all but one of the AncGR1-like receptors. Sites are numbered by amino acid position in AncGR1-LBD and separated into groups of ten for clarity.

A)

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                                          Z   Y
AncGR1      SLISILEVIEPEVLYAGYDSSLPDTTNRLLSSLNRLGGRQMVSVVKWAKALPGFRNLHLD
AncGR2      TLISLLEVIEPEVLYSGYDSTLPDTSTRLMSTLNRLGGRQVVSAVKWAKALPGFRNLHLD
HumanGR     TLVSLLEVIEPEVLYAGYDSSVPDSTWRIMTTLNMLGGRQVIAAVKWAKAIPGFRNLHLD
HumanMR     SPVMVLENIEPEIVYAGYDSSKPDTAENLLSTLNRLAGKQMIQVVKWAKVLPGFKNLPLE
              H1                                H3

                                      W       W       Y       ZXW    X   W
AncGR1      DQMTLLQYSWMSLMAFSLGWRSYKHSNGNMLYFAPDLIFNEERMQQSAMYDLCQGMRKIS
AncGR2      DQMTLLQYSWMFLMAFSLGWRSYKQSNGNMLCFAPDLVINEERMQLPYMYDQCQQMLKIS
HumanGR     DQMTLLQYSWMFLMAFALGWRSYRQSSANLLCFAPDLIINEQRMTLPCMYDQCKHMLYVS
HumanMR     DQITLIQYSWMCLSSFALSWRSYKHTNSQFLYFAPDLVFNEEKMHQSAMYELCQGMHQIS
              H4          H5                  β3      β4   H6         H7

AncGR1      VEFVRLQVTYEEYLCMKVLLLLSTVPKDGLKSQATFDEIRMSYIKELGKAIVKKEGNSSQ
AncGR2      SEFVRLQVSYDEYLCMKVLLLLSTVPKDGLKSQAVFDEIRMTYIKELGKAIVKREGNSSQ
HumanGR     SELHRLQVSYEEYLCMKTLLLLSSVPKDGLKSQELFDEIRMTYIKELGKAIVKREGNSSQ
HumanMR     LQFVRLQLTFEEYTIMKVLLLLSTIPKDGLKSQAAFEEMRTNYIKELRKMVTKCPNNSGQ
              H7          H8                              H9

                                W                   Y
AncGR1      NWQRFYQLTKLLDSMHDLVGGLLQFCFYTFVQSKTLSVEFPEMLVEIISNQLPKVMAGMA
AncGR2      NWQRFYQLTKLLDSMHEMVGGLLQFCFYTFVN-KSLSVEFPEMLAEIISNQLPKFKAGSV
HumanGR     NWQRFYQLTKLLDSMHEVVENLLNYCFQTFLD-KTMSIEFPEMLAEIITNQIPKYSNGNI
HumanMR     SWQRFYQLTKLLDSMHDLVSDLLEFCFYTFRESHALKVEFPAMLVEIISDQLPKVESGNA
                          H10                                AF-H

AncGR1      KPLLFHQK
AncGR2      KPLLFHQK
HumanGR     KKLLFHQK
HumanMR     KPLYFHRK
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B)

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                                     11111  1111111112  2222222
                       12222344 7899900011  1223567991  1123333
                     -1450569104 1417856714  6080413671  2444589
AncGR2                TLSTSTMTVA FQCVILPYQQ  LSSDVTREMN  -SAFKSV
HumanGR               .VASTW.... ...I...C.H  ...EL...VD  -T.YSNI
RatGR                 .VASAW.... ...I...C.H  ...EL...VD  -T.YSNI
AstatotilapiaGR1      ..A.F..... ..........  .....S...N  -......
TroutGR2              .......... ..........  .....S...N  -......
ParalichthysGR        .......... .......F..  .N.......N  -T.....
DicentrachusGR        .....S.... .......F..  .N.......N  -T.....
AstatotilapiaGR2      .....S.... .......F..  .N.E.....N  -T...N.
TroutGR               .......... ..........  .T.......N  -......

AncGR1                SIASTNLSMV SHYIFQSALG  RVTETSKDLQ  STVVMMA
HagfishSR1            PV...T..LL GN........  .ESDSTQN..  .LS.LH.
LampreySR1            T.ST.A..LI G..V.....E  .E..C.T.I.  .VAHHE.
HumanMR               .V..AE.T.. C..V......  HL..ANC..E  .A..EN.
ChickenMR             .V..AE.T.. C.........  HLS.ANC..E  .A..EN.
RatMR                 .A..AE.T.. C..V......  .L..ANC..E  .A..EN.
XenopusMR             .A.TAE.... C.........  QL..ANS..E  .A..EI.
TroutMRa              .C.T.D.... C..V......  .Q.QANA..E  .A..ENT
TroutMRb              .C.T.D.... C..V......  .Q.QANA..E  .A..ENT
AstatotilapiaMR       .CS..D...M C.........  .Q.DANA..E  .A..ELT
SkateMR               .........I C..V.R....  QI..SNNE.E  .A..T.T
SkateGR               ....P..G.. ..F....T..  G.A.......  .......
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Fig. S2. Crystal structure of AncGR2-dexamethasone complex. Helices (numbered) are shown in bronze; AF-H, activation function helix. Dexamethasone is shown in purple, with coactivator peptide hTIF2 in yellow.

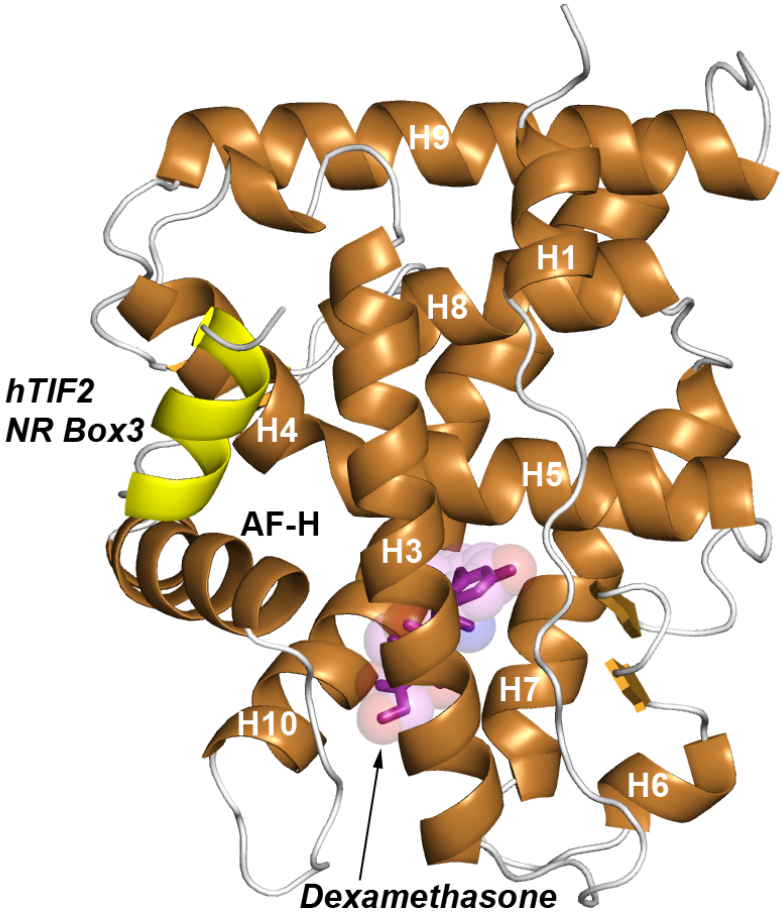

Fig. S3. 2Fo-Fc omit electron density for dexamethasone in the AncGR2 ligand binding pocket

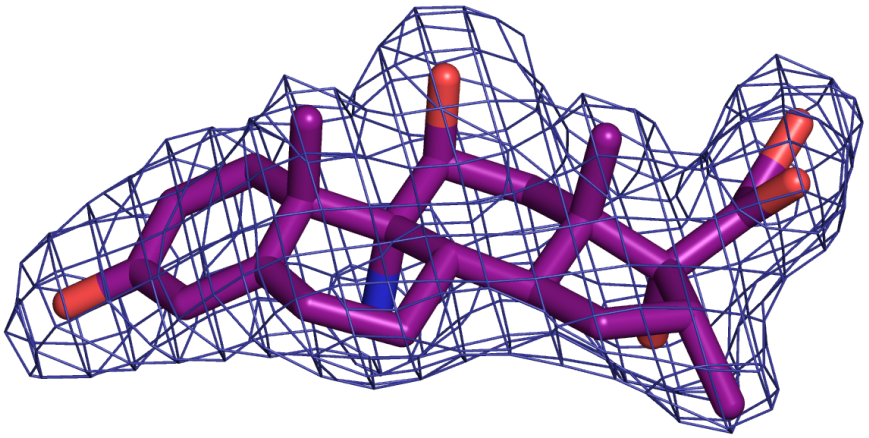

Fig. S4. Effect of reversing single and combined group w mutations on AncGR2 reversibility. Fold-activation of a luciferase reporter by variants of the AncGR2 ligand binding domain relative to vehicle-only control are shown with increasing concentrations of aldosterone (solid blue), DOC (dotted blue), and cortisol (purple).

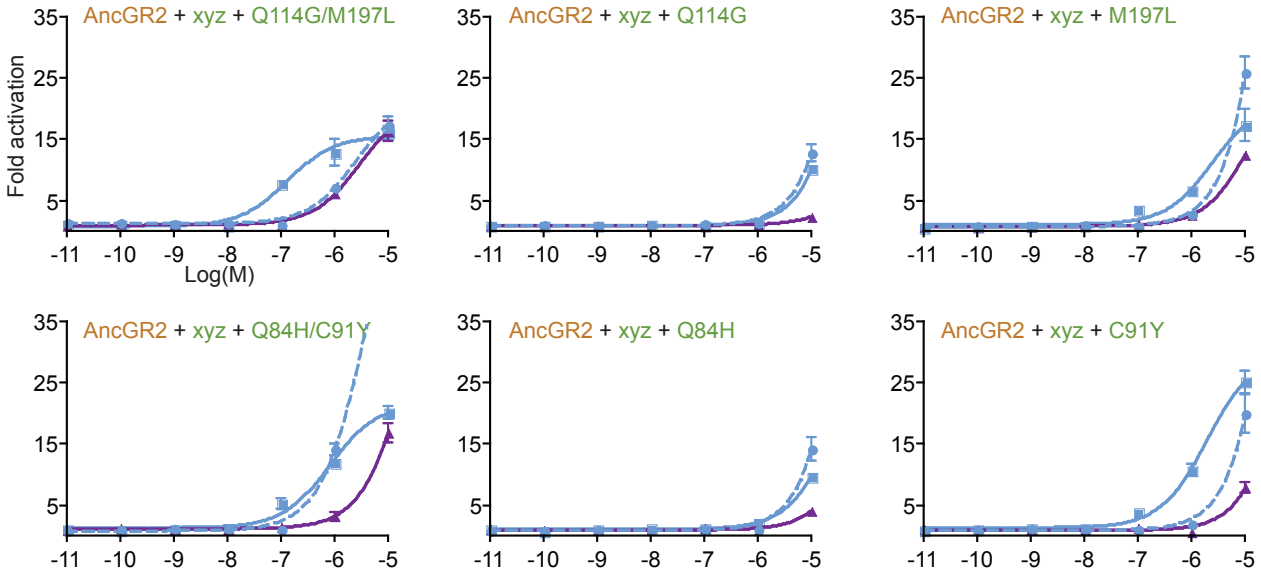

Fig. S5. Dose response curves for all combinations of substitution sets X, Y, Z, and W in AncGR2 background (left) and AncGR1 (right). Derived states at X,Y,Z and W positions are upper-case, and ancestral states are lower case. Solid blue, aldosterone; dashed blue, DOC: purple, cortisol.