Chemical Systems Biology Reveals Mechanisms of Glucocorticoid Receptor Signaling

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Abstract

Glucocorticoids display remarkable anti-inflammatory activity, but their use is limited by on-target adverse effects including insulin resistance and skeletal muscle atrophy. We used a chemical systems biology approach, Ligand Class Analysis (LCA), to examine ligands designed to modulate glucocorticoid receptor activity through distinct structural mechanisms. These ligands displayed diverse activity profiles, providing the variance required to identify target genes and coregulator interactions that were highly predictive of their effects on myocyte glucose disposal and protein balance. Their anti-inflammatory effects were linked to glucose disposal but not muscle atrophy. This approach also predicted selective modulation *in vivo*, identifying compounds that were muscle sparing or anabolic for protein balance and mitochondrial potential. LCA defined the mechanistic links between the ligand-receptor interface and ligand-driven physiological outcomes, a general approach that can be applied to any ligand-regulated allosteric signaling system.

Introduction

Glucocorticoids (GCs) are among the most prescribed medicines due to their remarkable anti-inflammatory effects. Efforts to develop improved GCs have been hampered by poor understanding of the structural and molecular mechanisms through which glucocorticoid receptor (GR) ligands drive diverse phenotypic outcomes such as effects on inflammation, glucose disposal, or skeletomuscular atrophy[1,2]. This phenomenon is called selective modulation, and is a common feature of allosteric signaling through GR and other nuclear receptor superfamily transcription factors[3], G protein-coupled receptors, and other small molecule drug targets.

The adrenal gland secretes GCs in a circadian fashion and in an acute response to nutrient deprivation or other stressors. In skeletal muscle, GCs stimulate catabolic signaling that opposes the insulin/PI3K/AKT signaling pathway, inhibits glucose uptake, and releases amino acids by inhibiting protein synthesis and stimulating protein degradation[2]. Upon GC binding, GR dissociates from inhibitory heat shock protein complexes, translocates to the nucleus, oligomerizes upon binding to DNA, and recruits an ensemble of coregulators, including chromatin-remodeling and histone-modifying enzymes that regulate gene expression. Several GR coregulators and target genes have been identified in skeletal muscle[4-7], but factors that drive selective modulation are unclear.

We developed a chemical systems biology approach called Ligand Class Analysis (LCA) to identify molecular mechanisms that drive ligand-selective signaling[8-10]. With LCA, we characterize a series of related compounds that display a full range of variance in phenotypes such as insulin-mediated glucose uptake or protein synthesis and use that variance to define molecular predictors of the phenotype. This enables us to identify ligand-dependent gene expression and protein-interaction profiles that predict the molecular phenotypes of ligand

classes that perturb receptor structure differently[8-10]. We then overexpress or knockdown predictive genes or interacting proteins to identify factors required for ligand-dependent phenotypes.

In this work, we evaluated the physiological effects of GCs during overnight nutrient deprivation when they coordinate acute metabolic adaptations, including glucose disposal and protein balance. We tested a GC series with different modifications on the steroidal scaffold[11], providing distinct strategies to perturb GR structure and dynamics. Using a machine learning approach, we found that GCs selectively modulate protein balance in skeletal muscle versus glucose disposal and the inflammatory response. This identified coregulators and target genes that drive these activities, and enabled discovery of orally available GCs with muscle-sparing activity profiles in vivo.

Results

A GC-profiling platform for effects on skeletal muscle

We designed a series of GCs to test three different structural effects of PF-0251802 (PF802) (**1**), a selective modulator, the pro-drug of which reached clinical trials (**Extended Data Fig. 1a**)[12,13]. PF802 (**1**) drives antagonism by disrupting the dexamethasone (Dex) (**2**)-bound GR conformation (**Extended Data Fig. 1b**), using its benzyl group which is similar to the carbon-11 (C11) substitution in the antagonist, RU-486 (**3**) (**Extended Data Fig. 1c**). To increase affinity, PF802 (**1**) has a trifluoromethyl group attached at the steroid C17-equivalent position, while RU-486 (**3**) and momethasone furoate (**4**) have other substitutions at this site (**Extended Data Fig. 1c-d**). PF802 (**1**) also contains a methylpyridinyl acetamide group attached at the steroid C3-equivalent position. This group presumably enters the solvent channel underneath the coregulator

87 binding site, as first seen with deacylcortivazol (**5**) (**Extended Data Fig. 1e**), to alter the shape of
88 AF2, and the ensemble of coregulators that bind this surface, thus driving selective modulation
89 as we previously showed for the estrogen receptor[9,14].

90 To understand how these different substitutions control GR activity, we generated 22
91 related compounds based on a steroidal core, which enabled us to direct substitutions within the
92 ligand-binding pocket (**Extended Data Fig. 1f**). These included substitutions of the
93 methylpyridinyl acetamide at C3 to perturb the AF2 surface (**6-12**)(**Fig. 1a**); substitutions at C11
94 to drive agonism/antagonism (**13-20**) (**Fig. 1b**); substitutions at C17 to optimize affinity (**21-24**)
95 (**Fig. 1c**); and other modifications (**Fig. 1d**). We have published the synthesis and preliminary
96 structure-activity relationships for these compounds[11].

97 We developed a compound profiling platform to examine how ligand-regulated GR
98 nuclear localization, structure, and target gene expression in skeletal muscle control glucose
99 disposal and protein balance (**Fig. 1e**), and whether these effects could be dissociated from anti-
100 inflammatory effects such as the suppression of IL-6 secretion. We assayed ligand-induced
101 nuclear translocation of a GFP-GR fusion protein, and probed for ligand-selective changes in
102 surface structure by characterizing the GR-interaction profiles of 154 peptides derived from
103 nuclear receptor coregulators and other interacting proteins using the MARCoNI FRET assay[15].
104 We also completed nascent RNA expression profiling of C2C12 myotubes, one hour after
105 vehicle or Dex (**2**) treatment and selected 28 differentially expressed genes as potential
106 regulators of glucose disposal and protein balance (see **Supplementary Information,**
107 **Supplementary Table 1** for selection rationale). We compared ligand-dependent regulation of
108 these genes in C2C12 myotubes using the nanoString nCounter for direct multiplexed transcript
109 counting without PCR amplification, enabling highly quantitative and reproducible assays[16].

110 These datasets comprise the independent variables used in a machine learning approach to
111 identify ligand-selective regulators of glucose disposal and protein balance in skeletal muscle,
112 the dependent variables.

113 To assay physiologically relevant effects of GCs on skeletal muscle, myotubes were
114 treated in the context of nutrient deprivation and a brief insulin challenge. One of the barriers to
115 understanding GC action in skeletal muscle is that effects on protein balance required 50–100
116 μM Dex (**2**) *in vitro* [17], despite a ~5 nM Kd for GR. Effects on protein balance and Glut4
117 translocation also vary *in vivo*, depending upon the stressor. For example, GCs induce skeletal
118 muscle atrophy and insulin resistance during fasting, while exercise has opposite effects[18,19]. We
119 found that overnight serum-deprivation enabled robust GC-induced signal to noise after a brief
120 insulin challenge with maximal effects of Dex (**2**) in the 1-10 nM range (**Methods, Extended**
121 **Data Fig. 2a–g**). This suggests that GR acts by inhibiting the insulin/PI3K/AKT signaling
122 pathway[20].

123 We developed a compound profiling assay for phosphorylation of AKT at Thr308
124 (pAKT), due to its joint role in coordinating protein synthesis and glucose disposal (**Extended**
125 **Data Fig. 2a–b**). For effects of compounds on glucose disposal, we measured the rate limiting
126 step—insulin-mediated translocation of the glucose transporter, Glut4, to the cell surface.
127 (**Extended Data Fig. 2c**). For protein balance, we developed two assays: 1) Degradation: GC-
128 induced protein degradation assayed by the release of tritiated phenylalanine (**Extended Data**
129 **Fig. 2d**); and 2) Synthesis: effects of the ligands on insulin-stimulated protein synthesis assayed
130 using the SUnSET assay, which measures the incorporation of puromycin into nascent
131 polypeptide chains (**Extended Data Fig. 2e**). We assessed the relationship between protein
132 balance and mitochondrial potential (Ψm) measured by high content imaging after staining with

133 a MitoTracker dye, where accumulation of the dye is dependent on membrane potential, and
134 modulated by the GR ligands. (**Extended Data Fig. 2f–h**). We also compared ligand-dependent
135 effects on IL-1β-induced secretion of IL-6 by A549 lung cells, a standard assay for the
136 inflammatory response. Ligand activity in these assays were used as dependent variables to
137 identify predictive coregulator interactions and target genes, and selective modulators.

138 We identified two clusters of dependent variables, connected by the effects of the ligands
139 on pAKT. In the first cluster, effects of the ligands were significantly intercorrelated between
140 effects on Synthesis, Degradation, pAKT, and ψm, suggesting transcriptional control via a
141 common set of coregulators and target genes (**Fig. 1f**, **Extended Data Fig. 2i**, **Supplementary**
142 **Dataset** shows the Pearson correlation matrix and P values). In the second cluster, GC effects on
143 IL-6 and Glut4 were correlated to pAKT and GR nuclear translocation (**Fig. 1f**). However, GC
144 effects on pAKT and GR nuclear translocation were not correlated (**Extended Data Fig. 2j**),
145 indicating that some ligand-dependent outcomes of GR signaling (e.g. Glut4, IL-6) involve a
146 graded response to the amount of nuclear GR, while others require a threshold amount of nuclear
147 GR. These results suggest that GCs modulate different transcriptional signaling pathways—
148 coregulators and target genes—that coordinate some processes but allow others to be dissociated.
149

150 **Machine learning defines top predictors of GC action**

151 We performed predictive modeling of each GR-mediated phenotype (dependent variable) using
152 *Random Forest*, a classification and regression tree algorithm[21] (**Fig. 1e**). To identify a
153 statistically significant set of gene expression and peptide interaction predictors for each
154 phenotype, we used *Boruta*, a Random Forest-based wrapper algorithm[22]. We iteratively
155 compared the importance of each feature (gene or peptide) in the dataset to a "shadow" dataset

156 obtained by shuffling the dependent and independent variables. In each of 500 iterative decision
157 rounds, a feature was selected if it was more significant than all the shadow features. We then
158 identified a minimal set of predictors by applying a forward selection strategy. The set of
159 significant features were tested one-by-one, starting with the most significant, for their ability to
160 improve the model. Features that improved the model were retained, while those that worsened
161 the model were removed. With this approach, we were able to predict between 47%
162 (Degradation) and 84% (Glut4) of the variance in the dependent variables (**Fig. 2a**,
163 **Supplementary Table 2**, **Supplementary Dataset**).

164 This analysis revealed the degree to which target genes and interacting peptides
165 functioned as selective predictors, or coordinated effects of GCs across the different biological
166 processes (**Fig. 2a–b, Supplementary Table 2**, **Extended Data Fig. 3a–b**). We used linear
167 regression to define the statistical power of individual predictors, where the r^2 statistic
168 (mathematically equivalent to the square of the Pearson correlation) defines the percent of
169 variance in the dependent variable that is predicted by the independent variable. Ligand
170 interaction patterns with GR and NCOR1 or NCOR2 were selective for predicting Synthesis,
171 while NCOA1 and NCOA2 interaction profiles and expression of the *Bcl2l2* selectively
172 predicted Glut4. In contrast, expression of *Sgk1* and *Socs2* were common regulators of both
173 processes (**Fig. 2b**, **Extended Data Fig. 3b**). *Bcl2l1* encodes Bcl-xL, a mitochondrial, anti-
174 apoptotic BCL-2 family protein that inhibits glucose metabolism and mitochondrial-mediated
175 secretion of insulin by pancreatic β-cells [23], suggesting that it also contributes to glucose disposal
176 in skeletal muscle.

177 Ligand-mediated NCOR2 peptide interaction with GR significantly predicted Synthesis,
178 but not Glut4 or the top Glut4-predictive target genes (**Fig. 2c**, **Supplementary Table 3**). There

179 was a robust connection between the coregulator peptides that predicted Glut4, showing very low
180 r^2 with the protein balance assays, but very high predictive power for the specific genes that best
181 predicted Glut4 activity (**Fig. 2c**). While the MARCoNI assay is best viewed as a probe for
182 ligand-regulated effects on surface structure, all of the peptides in **Figure 2c** represent bona fide
183 nuclear receptor interaction motifs. Here we exploited the variance in ligand activity to identify
184 common and selective transcriptional networks underlying GR-mediated phenotypes in skeletal
185 muscle.

186 The variance in ligand activity profiles also allowed LCA to identify signal in what
187 would typically be considered noise, such as the *Bcl2l1* and *Socs2* genes where most of the
188 compounds had very little activity individually, but collectively proved highly predictive (**Fig.
189 2b, Extended Data Fig. 3ab**). Much of the gene expression data displayed an inflection point
190 (**Extended Data Fig. 3c**). When we removed the data below the inflection point, *Fkbp5, Blc2l1*,
191 *Tsc22d3* still significantly predicted Glut4 translocation (**Extended Data Fig. 3d**), suggesting
192 that insulin-mediated glucose disposal can be fine-tuned by very small changes in target gene
193 expression. We call these super-resolution analyses, similar to what we did with super-resolution
194 X-ray crystallography, where comparing many structures bound to a series of compounds
195 identified, within the noise of an individual crystal structure, ligand-induced perturbations that
196 drive biological outcomes[8]. Thus, subtle changes in gene expression are sufficient to identify
197 transcriptional networks underlying GR-mediated phenotypes using this approach.

198 We also found that substitutions on the steroidal scaffold that were designed to
199 differentially perturb GR structure modulated distinct transcriptional networks. A comparison of
200 ligand-dependent effects on Glut4 revealed a dramatic increase in predictive power by the C11-
201 substituted compounds compared to the whole compound set, while the C3-substituted

202 compounds exhibited a widespread reduction in r^2 (**Fig. 2d–e**, **Extended Data Fig. 3e**, compare
203 to **Fig. 2b**). This was evident despite their similar variances in the skeletal muscle profiling
204 assays (**Extended Data Fig. 3f**), supporting the idea that C3- and C11-substituted compounds
205 modulate GR-mediated phenotypes via different transcriptional networks and structural
206 mechanisms, which we explore below with molecular dynamics simulations. This approach,
207 LCA, demonstrates that looking at different classes of compounds, here comparing C3- and C11-
208 substituted GCs, reveals different structural and signaling mechanisms to achieve their biological
209 effects.
210

211 **Validation of individual predictors with gene perturbation studies**

212 FKPB5 is a good candidate to coordinate metabolic effects of GCs at an organismal level, as it
213 facilitates pAKT dephosphorylation by the phosphatase, PHLPP1[24]. In addition, *Fkbp5*-knockout
214 mice show greater skeletal muscle insulin sensitivity and reduced adiposity on a high fat diet[25].
215 We electroporated *GFP* and *Fkbp5* or an atrophy-inducing control (*Foxo1*) expression plasmid
216 into contralateral tibialis anterior (TA) muscles. The *Fkbp5*- or *Foxo1*-transduced muscles were
217 approximately 10% smaller than control (**Fig. 3a**). Electroporation with *Foxo1* or *Fkbp5*
218 inhibited insulin-induced *de novo* protein synthesis and pAKT *in vivo* (**Fig. 3b-c**,
219 **Supplementary Fig. 1a**), demonstrating that *Fkbp5* regulates protein balance in skeletal muscle.
220 Knockdown of *Ncor1* or *Ncor2* enhanced Dex-dependent inhibition of Synthesis (**Fig.**
221 **3d**, **Supplementary Fig. 1b**), consistent with the peptide binding data showing that GR
222 interaction with NCOR1 and NCOR2 peptides were stimulated by anabolic compounds (**Fig.**
223 **2b**). In contrast, knockdown of *Ncoa6* and *Pelp1* fully reversed the inhibitory effect of Dex (**2**),
224 while knockdown of *Ncoa22*, but not *Ncoa1*, partially reversed this effect (**Fig. 3d**,

225 **Supplementary Fig. 1b-c**). In contrast, pAKT was regulated by *Ncoa1*, as well as *Pelp1* (**Fig.**
226 **3e**). These data support a model where GR recruits specific coregulators to distinct target gene
227 sets that can have overlapping or different effects on skeletal muscle metabolism and protein
228 balance.
229

230 **Selective GR modulators with improved activity profiles**

231 We identified two promising GCs that selectively modulate inflammation and protein balance
232 (**Fig. 4a–d**). Compounds **13-15** in the C11 series inhibited IL-6 with efficacies comparable to
233 Dex (**2**) (**Extended Data Fig. 4a**). Among these, SR11466 (**15**) is a partial GR agonist (EC50 =
234 0.1 nM) that showed no effects on protein balance, and a slight improvement (i.e. less inhibition)
235 compared to Dex (**2**) in the Glut4 assay (**Fig. 4a–c**, **Extended Data Fig. 4b-c**). We also
236 identified SR16024 (**18**) as a full GR antagonist (IC50 = 1.4 nM) that stimulated Synthesis but
237 did not suppress Degradation or increase Glut4 translocation (**Fig. 4b-c**, **Extended Data Fig. 4b-**
238 **c**). **15** and **18** increased ψm, with the latter doubling ψm (**Fig. 4d, Extended Data Fig. 4d**).

239 As a further test for selective modulation, the compounds were profiled in primary human
240 osteoblasts for effects on mineralization during 4 weeks of differentiation from mesenchymal
241 stem cells. **15** showed a slight improvement in the inhibition of mineralization compared to Dex
242 (**2**) and PF802 (**1**), while **18** showed a better profile (**Fig. 4e**). We also noticed SR15421 (**9**), a
243 lower affinity compound with EC50 of 240 nM (**Extended Data Fig. 4c**), that was anti-
244 inflammatory with less impact on mineralization (**Fig. 4e, Supplementary Fig. 2**). **9** is part of
245 the C3-substituted series, where modifying the position of the methyl and the nitrogen on the
246 methylpyridine had profound effects on inhibition of IL-6 secretion (**Fig. 4e**, **Supplementary**
247 **Fig. 2**). Dose response curves reveal a moderately, but significantly improved effect of **15** on

248 mineralization compared to Dex (**2**) (2-way ANOVA, drug $p = 0.0018$, drug x time $p = 1.4 \times 10^{-4}$), while **9** and **18** inhibited mineralization only at the 10 μM dose (**Fig. 4f**). The mineralization data showed very low correlations with the other assays, except for Synthesis (Pearson $r = 0.54$, $p = 4 \times 10^{-3}$), supporting the joint regulation of bone and muscle mass by GCs. These data demonstrate that it is possible to find GCs with improved bone- and muscle-sparing properties compared to Dex (**2**) using our in vitro profiling platform.

254 We opted to test the two high affinity ligands further in animal studies based on their favorable pharmacokinetics and on-target mechanism of action (**Fig. 5a–b**, **Extended Data Fig. 5a–f**, **Extended Data Fig. 6a, Supplementary Fig. 3**). In vivo, **15** strongly suppressed LPS-induced TNFα levels in the blood, while **18** was not inhibitory (**Fig. 5c**). We also assessed loss of lean mass following a larger dose of LPS, which was significantly worsened by Dex (**2**). Both **15** and **18** were significantly better than Dex (**2**), while **18** was protective against loss of lean mass compared to vehicle (**Fig. 5d**). The differential in vivo effects of **15** on inflammation and proteostasis demonstrate that it is a bona fide selective modulator, while **18** displayed stronger muscle sparing activities.

263 A lactate tolerance test was then administered to probe for differences in liver gluconeogenesis following an overnight fast. Here again, Dex (**2**) caused significantly greater loss of lean mass after the fast; both **15** and **18** were significantly better than Dex (**2**) (**Extended Data Fig. 6b**); and **18** attenuated the loss of overall body weight from the fast (**Extended Data Fig. 6c**). The lactate tolerance test showed that Dex (**2**) and **15** increased the glucose production rate, while **18** did not (**Fig. 5e**), suggesting that selective GC effects on glucose uptake in skeletal muscle and gluconeogenesis in the liver may utilize common signaling mechanisms, such as ligand-selective coregulator recruitment, to integrate organismal stress responses.

271

272 **Structural features of GR linked to suppression of IL-6**

273 C3 isomers with differences in the location of the methyl and nitrogen in the pyridine ring
274 showed large differences in anti-inflammatory effects, demonstrating that the methylpyridinyl
275 acetate binding under the AF-2 surface is an important allosteric regulator of GR. To understand
276 the mechanism, we performed all atom molecular dynamics (MD) simulations comparingthe
277 isomers **7** and **9**, which differ only in the placement of the methyl on the pyridine (**Fig. 1a**) but
278 showed very different effects on suppression of IL-6 (**Supplementary Fig. 2**), where **9**
279 suppresses inflammation but **7** does not. Molecular docking demonstrated that the compounds
280 bound similarly to Dex (**2**), with the pyridine extending off the A-ring into a solvent channel, in
281 between helix 3 (h3) and h5 (**Methods, Extended Data Fig. 7a**). In three independent 1 μs
282 simulations of ligand-bound and ligand-free (apo) GR LBD, we observed no significant
283 structural changes in the conformation of helix 12 (h12). However, ligand anti-inflammatory
284 activity (Dex (**2**) > **9** >**7** > apo) was associated with a multi-Å decrease in the distance between
285 the C-terminus of h11 and the N-terminus of h3, and a decrease in the heterogeneity of this
286 h3/h11 interface (**Fig. 6a–c**). We also assessed patterns of correlated motion and found that the **7**
287 showed less correlation between residues h3 and h11/h12 residues compared to Dex (**2**), with **9**
288 again showing intermediate effects (**Extended Data Fig. 7b**).

289 We used dynamical network analysis[26] to further probe for effects of the C3 substitutions
290 on correlated motion between h12 and the solvent channel, by connecting pairs of nodes with
291 their edges if they have satisfied a distance requirement (<4.5 Å) for ≥75% of the simulation
292 time. Edge distance is inversely proportional to the pairwise correlations between two nodes;
293 thus, a short path length indicates a strong correlation/communication. A pair of distal nodes are

294 connected by the optimal (shortest) path and the suboptimal (longer) paths with the length
295 defined as the sum of edge distances along the path. We analyzed the top 1,000 suboptimal paths
296 between residue Glu755 and Arg614 as two nodes to study the allosteric communication
297 between h12 and the C-terminus of h5, where the modifications on the steroidal A ring extend to
298 the solvent channel. With apo GR, this communication was through h11 and along h5, while
299 with Dex (**2**) it wrapped around the ligand-binding pocket from h12 to h3 and then through the
300 β-sheet (**Fig. 6d**), enabled by the Dex-induced closing of the distance between h3 and h11 (**Fig.**
301 **6b**). With Dex (**2**), this was associated with much longer path lengths (**Fig. 6e**), representing
302 overall weaker allosteric communication. With **9**, there were less of these extended path lengths,
303 while **7** more closely resembled the apo GR (**Fig. 6d-e**). An analysis of residues contacting the
304 C3-substituted pyridine showed that differential positioning of the Arg611 side chain on h5,
305 which can form h-bonds with the pyridine or A-ring ketone of Dex (**2**), can drive the orientation
306 of the steroid core to control the dynamics and surface structure and associated receptor activity
307 (**Fig. 6f, Extended Data Fig. 7c–e**).
308

309 **Discussion**

310 We used structure-based design and LCA to reveal how GCs modulate different transcriptional
311 networks (coregulators and target genes) to physiologically control glucose disposal and protein
312 balance during acute nutrient deprivation, and how different classes of these ligands with
313 substitutions at C3 or C11 utilize these signaling pathways. By examining closely related
314 compounds, we determined that protein balance and Ψm were intercorrelated but not correlated
315 with effects on Glut4 translocation and suppression of IL-6. Using a physiologically relevant
316 skeletal muscle profiling platform optimized to study insulin signaling during nutrient

317 deprivation, we identified SR11466 (**15**) as a partial agonist that was not catabolic,
318 demonstrating in vivo selective modulation of anti-inflammatory activity. **15** was slightly
319 anabolic for protein balance, while maintaining strong anti-inflammatory activity in vivo. Such
320 compounds may be useful treatments for cachexia, muscular dystrophies, back pain or
321 osteoarthritis. We also identified SR16024 (**18**) as a highly anabolic GC that inhibits fasting- and
322 LPS-induced weight loss, while stimulating Ψm and protein synthesis in response to insulin.
323 Further studies are needed to evaluate the long-term effects of **18**, but GR antagonists have been
324 tested clinically for depression[27], and may have efficacy in muscular dystrophies and
325 enzalutamide-resistant prostate cancers that have switched from androgen to glucocorticoid
326 dependency[28]. While preliminary, the lack of SR15421 (**9**) effects on mineralization suggests that
327 it may also be possible reduce the osteoporotic effects of agonist GCs using physiologically
328 relevant profiling assays.

329 The unbiased nature of LCA enabled the identification of a number of unexpected
330 signaling and biophysical properties of GR. Insulin receptor signaling pathways control anabolic
331 effects and glucose disposal, which can be disconnected by transcriptional control of pathway-
332 selective regulators, often with subtle changes in gene expression. Covariance among assays
333 reflects common underlying signaling mechanisms driven by common receptor conformations,
334 coregulators, and target genes. The correlation between effects on bone and skeletal muscle was
335 not surprising, but the connection between anti-inflammatory and metabolic effects of GCs in
336 different cell types was unexpected and may point to an organismal level of evolutionary
337 connection between underlying signaling pathways. GC coordination of the inflammatory
338 response often coincides with immune differentiation[29,30], which requires metabolic adaptations
339 [31,32]. This suggests that GCs may use a common set of coregulators to coordinate anti-

340 inflammatory effects with modulation of metabolism. LCA provides a mechanism to identify
341 common signaling mechanisms among different cell types and processes and how they are
342 utilized by structurally distinct classes of ligands.

343 GC-regulated transcription is controlled via underlying biophysical properties of GR,
344 such as oligomerization, nuclear translocation, and on/off rates of interactions with an ensemble
345 of response elements, coregulators, and collaborating transcription factors. We have shown here
346 that LCA predicts that ligand-selective surface conformers—identified from a structural peptide
347 interaction assay—can predict differential effects of the ligands, which we validated with
348 knockdown of selected coregulators. We and others have observed a similar dichotomy where
349 NCOA2 is required for NF-κB-induced cytokine production, but then switches to a repressor
350 upon recruitment of GR to the promoter[33,34]. This is representative of a more general phenomena
351 of receptors using many coregulators to regulate a single gene, but also some shared and some
352 differential coregulator usage across genes[14,35,36]. The selective association of GR nuclear
353 translocation with glucose disposal and not protein balance was also surprising. This suggests
354 that GCs modulate insulin dependent Glut4 translocation via a graded transcriptional program
355 that is highly sensitive to the amount of nuclear GR, and control protein balance through
356 transcriptional responses that require a threshold amount of nuclear GR. It is remarkable that
357 changing the position of a single methyl in the solvent channel drove such divergent effects of
358 the C3 compounds on inflammation, while the molecular dynamics simulation revealed this
359 region to be an important allosteric regulator of GR structure and function. Collectively our
360 finding show that complex biology can be simulated by a set of simple biophysical models[9,37]
361 and their importance identified with LCA.
362

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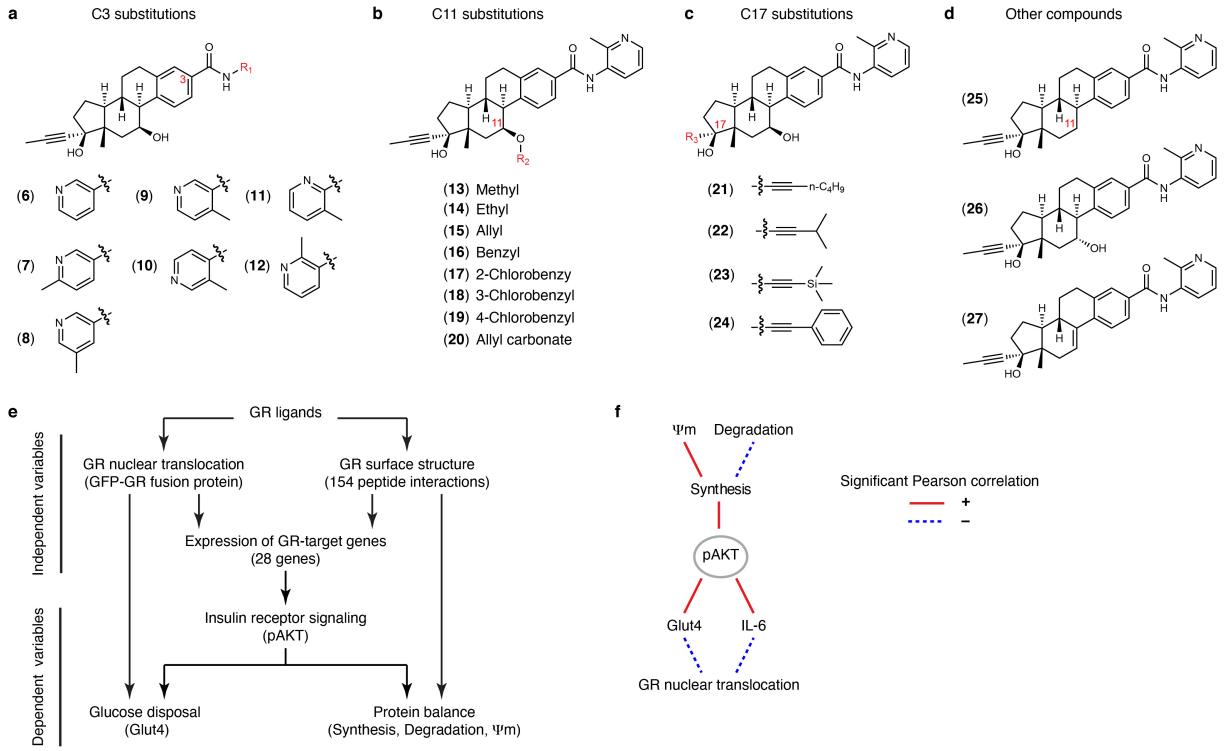
373 **Figures**



374

375 **Figure 1. Structure-based design approach and glucocorticoid profiling platform.**

376 **a-d**) Glucocorticoids used in this study.

377 **e**) Compound profiling and computational strategy. Effects on the independent variables were

378 used in a machine learning approach, Random Forest, to identify predictors of skeletal muscle

379 phenotypes, the dependent variables, described in **Figure 2**, **Supplementary Table 2,** and

380 below.

381 **f**) Relationships among GR-mediated phenotypes. Lines indicate significant Pearson correlation

382 between variables using Bonferroni $p_{Adj} < 0.0071$.

383

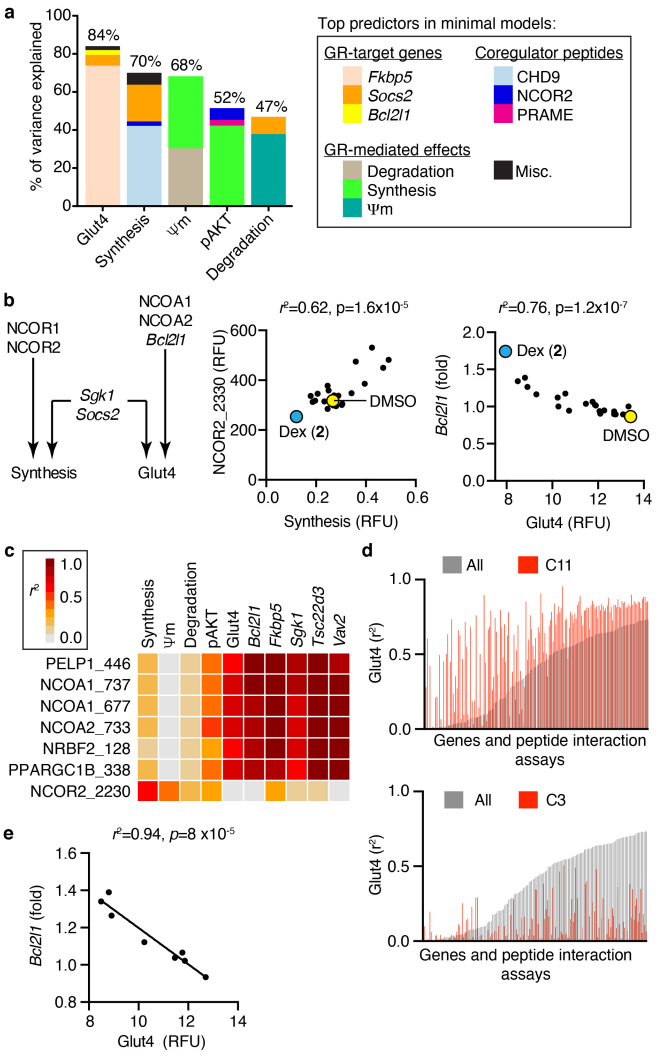


Figure 2. Machine learning reveals top predictors of selective modulation and common signaling

a) Composition of minimal predictive models defined by machine learning. The predictive capacity of the model (y-axis) for a GR-mediated phenotype i.e. dependent variable (x-axis), is also indicated. See **Supplementary Table 2** for the full list of predictors.

b) Linear regression (scatter plots) demonstrates their predictive power (r^2), and its associated p-value. Each point represents the effects of a distinct ligand.

c) Linear regression comparing the predictive power of the indicated peptide interactions for the indicated phenotypes and target genes. See **Supplementary Table 3** for r^2 values.

394 **d**) The Glut4-predictive power, r^2, of target genes and peptide interactions observed with all
395 compounds (**1-3** and **6-27**) were rank ordered, and then compared to the r^2 observed within the
396 C11-substituted (**13–20**) or C3-substituted (**6-12**) compound series.
397 **e**) Linear regression demonstrating the Glut4-predictive power of *Bcl2l1* within the C11-
398 substituted compounds series (**13–20**). Each datapoint represents the effects of a distinct C11-
399 substituted compound on Glut4 translocation and *Bcl2l1* expression.
400 See also **Methods**, **Supplementary Dataset**, and **Extended Data Fig. 3.**
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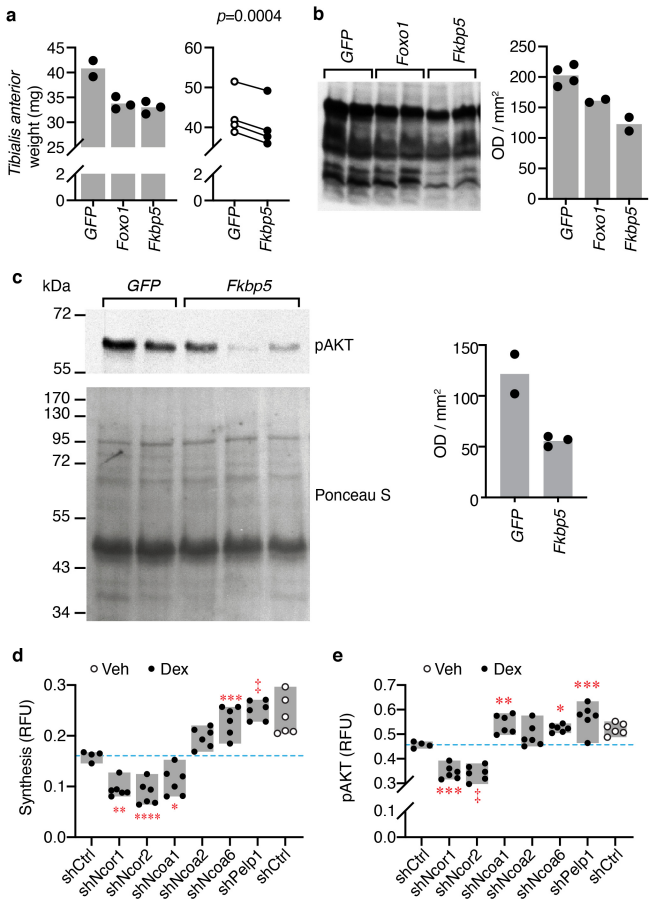

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406 **Figure 3. Functional validation of the predictive target gene, *Fkbp5*, and GR coregulators**

407 **a**) Weight of Tibias Anterior (TA) muscles transduced with *GFP* (control), *Fkbp5,* or *Foxo1*

408 genes in 2 independent experiments. Left panel, bars represent the mean, and n = 3 except for

409 *GFP* where n = 2 TA muscles per condition. Right panel, each pair of datapoints represent TA

410 muscles from the same mouse, n = 4 mice. Also see **Methods.**

411 **b**–**c**) Whole lysates of transduced TA muscles were analyzed by Western blot and subsequent

412 quantitation. **b**) In vivo SUnSET assay for Synthesis. Bars represent the mean; n = 2, except for

413 *GFP* where n = 4 biologically independent samples. **c**) Insulin-induced pAKT levels. Bars

414 represent the mean; for *GFP*, n = 2 and for *Fkbp5*, n = 3 biologically independent samples. Also

415 see **Supplementary Fig. 1a**.

416 **d**) SUnSET assay or **e)** in-cell Western for pAKT in C2C12 myotubes expressing the indicated
417 shRNAs. Boxes represent the range; n = 6, except for Dex/shCtrl where n = 4 biologically
418 independent samples. 1-way ANOVA, Sidak's multiple comparisons test, adjusted p-values,
419 *p_{adj} = 0.0235, **p_{adj} = 0.0018, ***p_{adj} = 0.0007, ****p_{adj} = 0.0004, ‡p_{adj} < 0.0001. **e**) Insulin-
420 induced pAKT levels in C2C12 myotubes expressing the indicated shRNAs. Bars represent the
421 range; n = 6, except for Dex/shCtrl where n = 4 biologically independent samples. 1-way
422 ANOVA, Sidak's multiple comparisons test, adjusted p-values, *p_{adj} = 0.0385, **p_{adj} = 0.0045,
423 ***p_{adj} = 0.0001, ‡p_{adj} < 0.0001. Also see **Supplementary Fig. 1b**.
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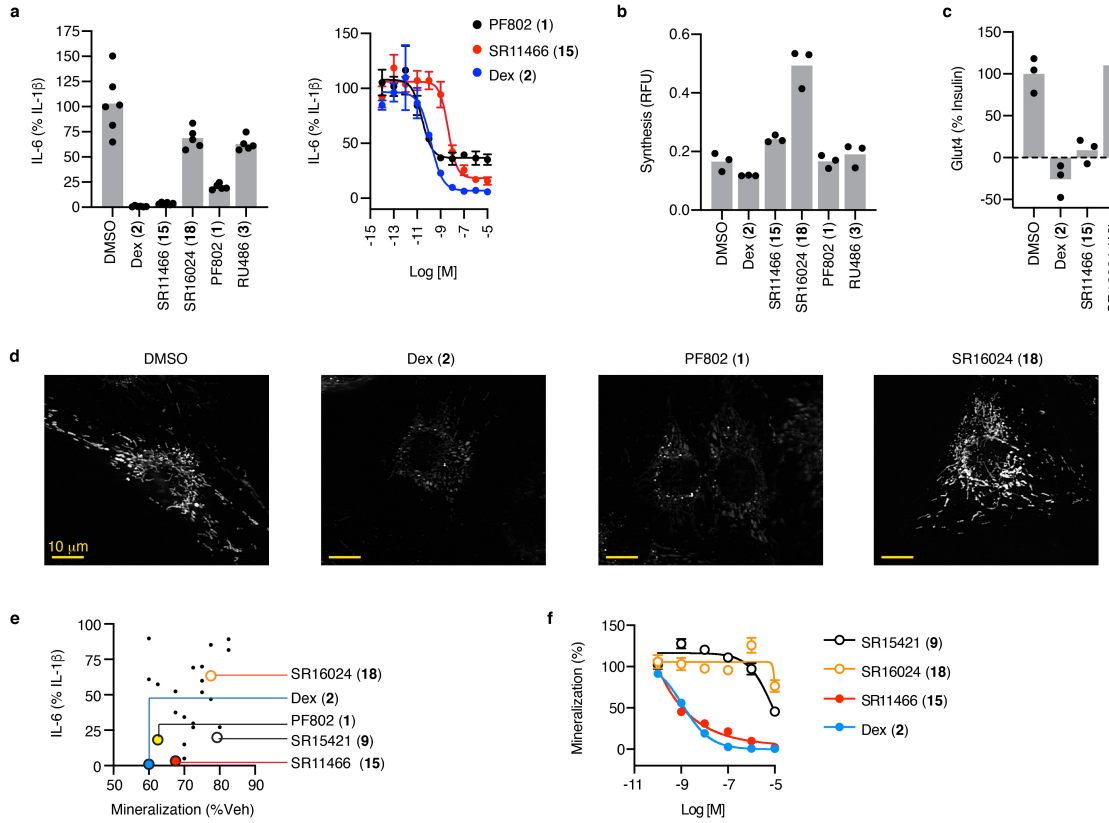

Figure 4. In vitro characterization of selective GR modulators with muscle-sparing activities.

a) IL-1β-induced secretion of IL-6 by A549 cells treated with the indicated compounds were compared by AlphaLISA. Left panel bars represent the mean; right panel datapoints represent mean ± SEM; n = 3, except for the vehicle (DMSO) where n = 6 biologically independent samples.

b) Effects of the indicated compounds on Synthesis in C2C12 myotubes were compared by SUnSET assay. Bars represent the mean; n = 3 biologically independent samples.

c) SR16024 (**18**) does not inhibit myotube surface expression of Glut4. Effects of the indicated compounds on Glut4 in L6 myotubes. Bars represent the mean; n = 3 biologically independent samples.

440 **d**) C2C12 myoblasts treated with the indicated compounds were stained with MitoTracker dye.
441 Images are representative of 18 images per condition i.e. 2 independent experiments with similar
442 results x 3 fields per well x 3 biologically independent wells per condition.
443 **e**) The effects of all tested compounds on IL-1β-induced secretion of IL-6 by A549 cells (y-axis)
444 and primary human osteoblast mineralization (x-axis). Datapoints represent the mean effects of a
445 distinct compound, For IL-6, n = 3 and for mineralization, n = 4 biologically independent
446 samples.
447 **f**) Dose curves of indicated compounds in the mineralization assay. Data are mean ± SEM, n = 4
448 biologically independent samples. Also see **Extended Data Fig. 2**, **Extended Data Fig. 4,** and
449 **Methods**.
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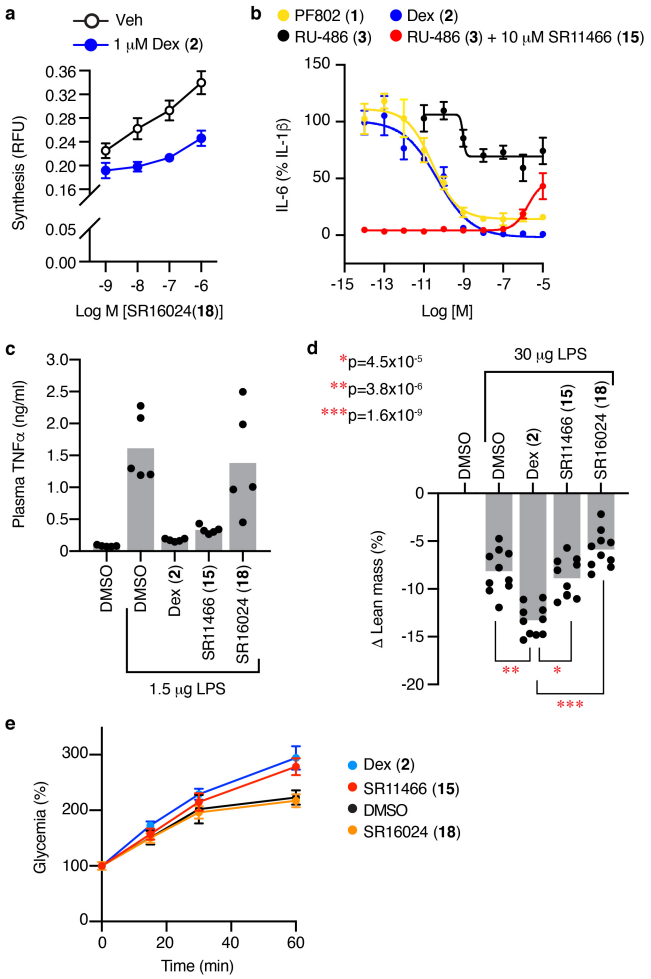

Figure 5. In vivo compound profiling and validation of on-target mechanism of action

a) Effect of the indicated compounds on Synthesis were compared by SUnSET assay in C2C12 myotubes. Datapoints are mean ± SEM, n = 4 biologically independent samples.

b) IL-1β-induced IL-6 production by A549 cells treated with the indicated compounds was compared by AlphaLISA. Datapoints are mean ± SEM, n = 3 biologically independent samples.

c) SR11466 (**15**) blocks the LPS-induced inflammatory response in mice. Plasma TNFα levels of mice treated with the indicated GR ligands (10 mg/kg Dex or SR16024, or 50 mg/kg SR1166) overnight before a 1-hr LPS challenge of 1.5 mg per mouse. Bars represent the mean, n = 5 mice per group.

d) Changes in lean mass of mice treated as described in panel **c** were determined by whole-body

462 NMR after an additional 18-hr LPS treatment of 30 mg per mouse. Bars represent the mean, n =
463 10 mice per group (5 x 2 experiments). 1-way ANOVA, Sidak's multiple comparisons test,
464 adjusted p-values, *p_{adj} = 4.5 x10^{-5}, **p_{adj} = 3.8 x10^{-6}, ***p_{adj} = 1.6 x10^{-9}.
465 **e**) Glucose production was compared by lactate-tolerance test (LTT) after overnight fast. Data
466 are mean ± SEM, n = 5 mice per group. See also **Extended Data Fig. 5 and Methods.**
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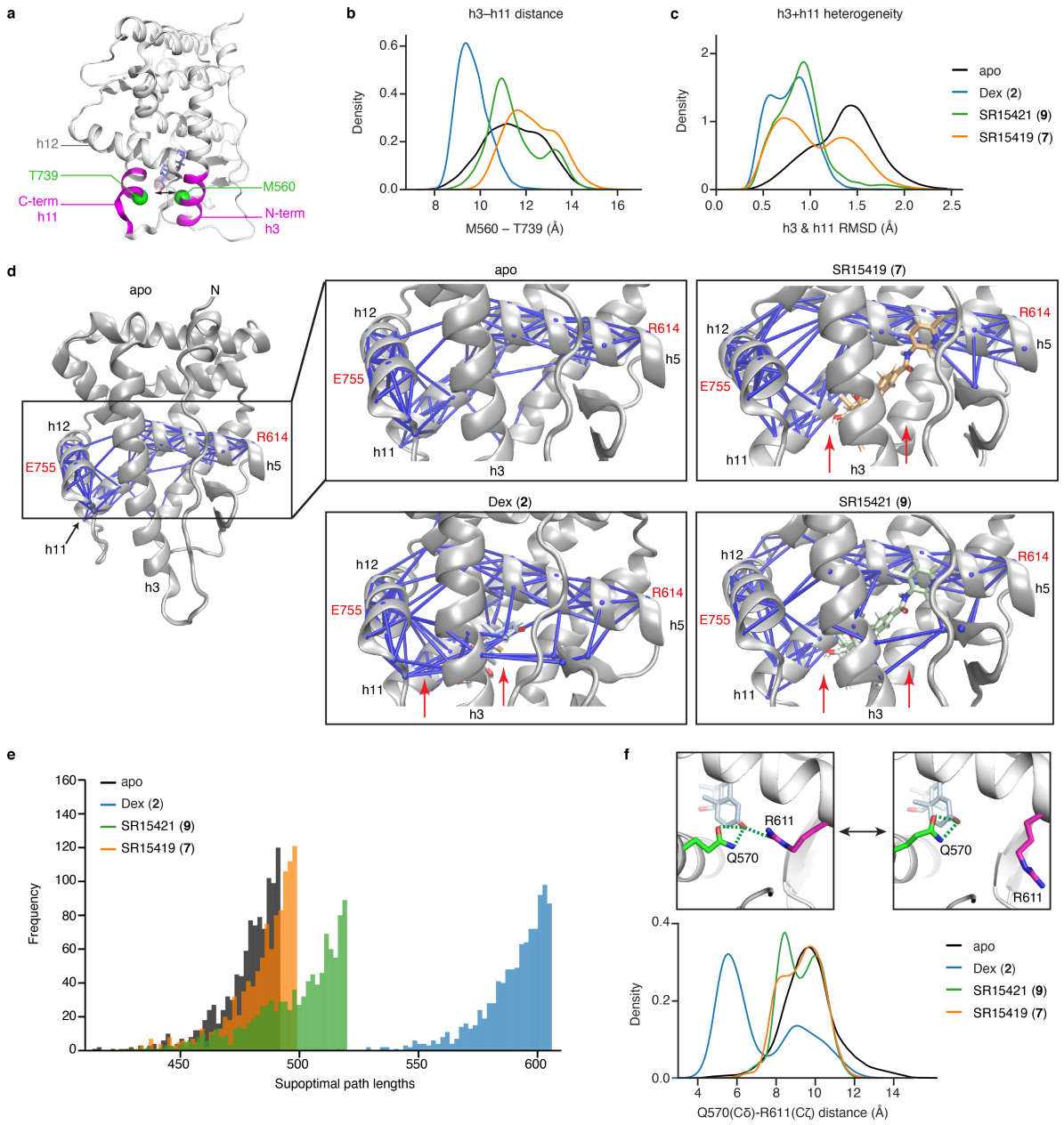

Figure 6. C3 substitutions in the steroid scaffold alter allosteric communication between ligand and activity.

a) Ribbon diagram of the GR-LBD illustrates the C-terminus of helix 11 (h11) and N-terminus of h3 where we observed ligand-induced conformational effects in three 1 μs molecular dynamics simulations.

b) Distance distribution plots of the Cα distance between Met560 (h3) and Thr739 (h11).

476 **c**) Backbone Cα, C', N, and O RMSD distribution plots of the h3 and h11 regions colored
477 magenta in (**a**).
478 **d**) Dynamical network analysis of suboptimal pathways for correlated motion between E755 and
479 R614. Red arrows indicate pathways found with Dex-bound GR.
480 **e**) Histogram showing the suboptimal pathlengths with the indicated ligands.
481 **f**) Distance distribution plots of Q570 (Cδ) and R611 (Cζ) side chain atom distances as a proxy
482 to determine the relative populations inward R611 conformations that can interact with ligand.
483 Inserts show R611 inward (left) and outward (right) side chain conformations extracted from the
484 Dex-bound simulations.
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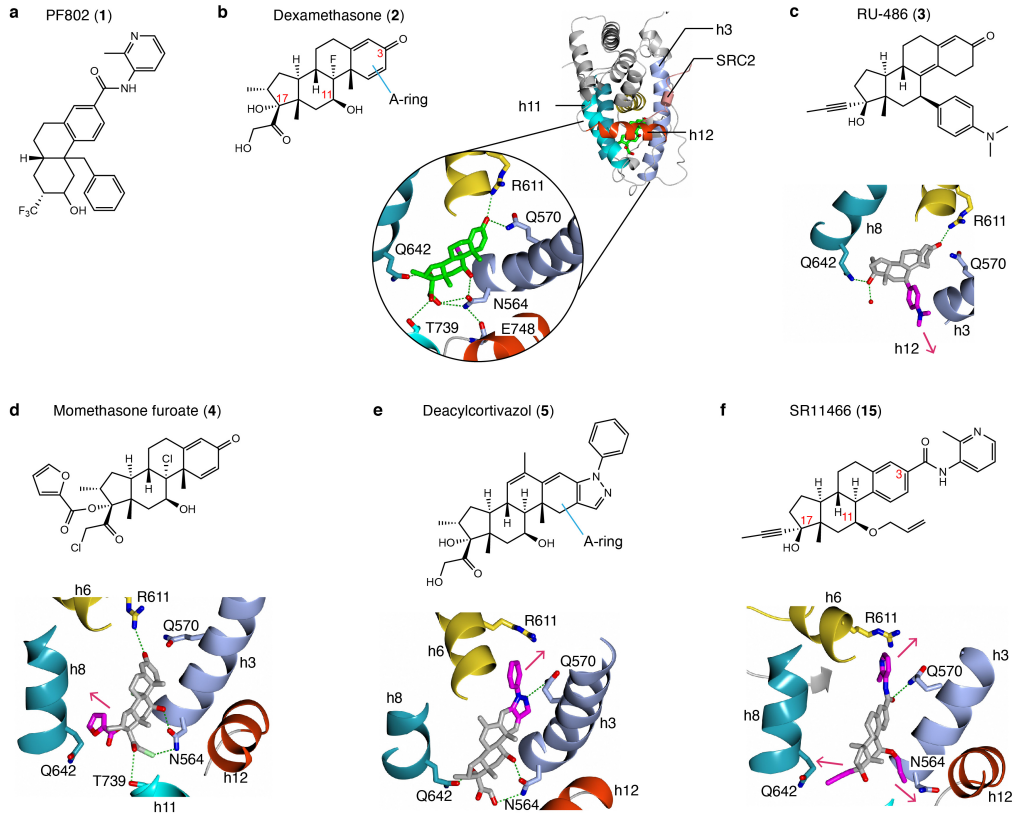

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489 **Extended Data Fig. 1. Structure-based design of GR ligands**

490 **a**) Chemical structure of the selective GR modulator, PF802.

491 **b**) Crystal structure of the dexamethasone (Dex) bound GR ligand-binding domain (LBD). Helix

492 12 is colored red and the NCOA2 coregulator peptide binding in the AF2 binding surface is

493 colored coral. Carbons-3, -11, and -17 are indicated in the chemical structure (1M2Z.pdb).

494 **c**) The bulky dimethylanaline group attached at C11 in RU-486 displaces h12 from the agonist

495 position to disrupt the AF2 surface and generate antagonism (1NHZ.pdb).

496 **d**) Substitutions at C3, as seen with the furoate group in momethasone furoate or the propyne in

497 RU-486 target a small internal pocket to increase affinity (4P6W.pdb).

498 **e**) Substitutions at C3 of the steroidal A-ring enter the solvent channel underneath the AF2

499 surface, potentially changing the shape of the surface and the ensemble of interacting

500 coregulators (3BQD.pdb).

501 **f**) Model of SR11466 (**15**) bound to the GR LBD

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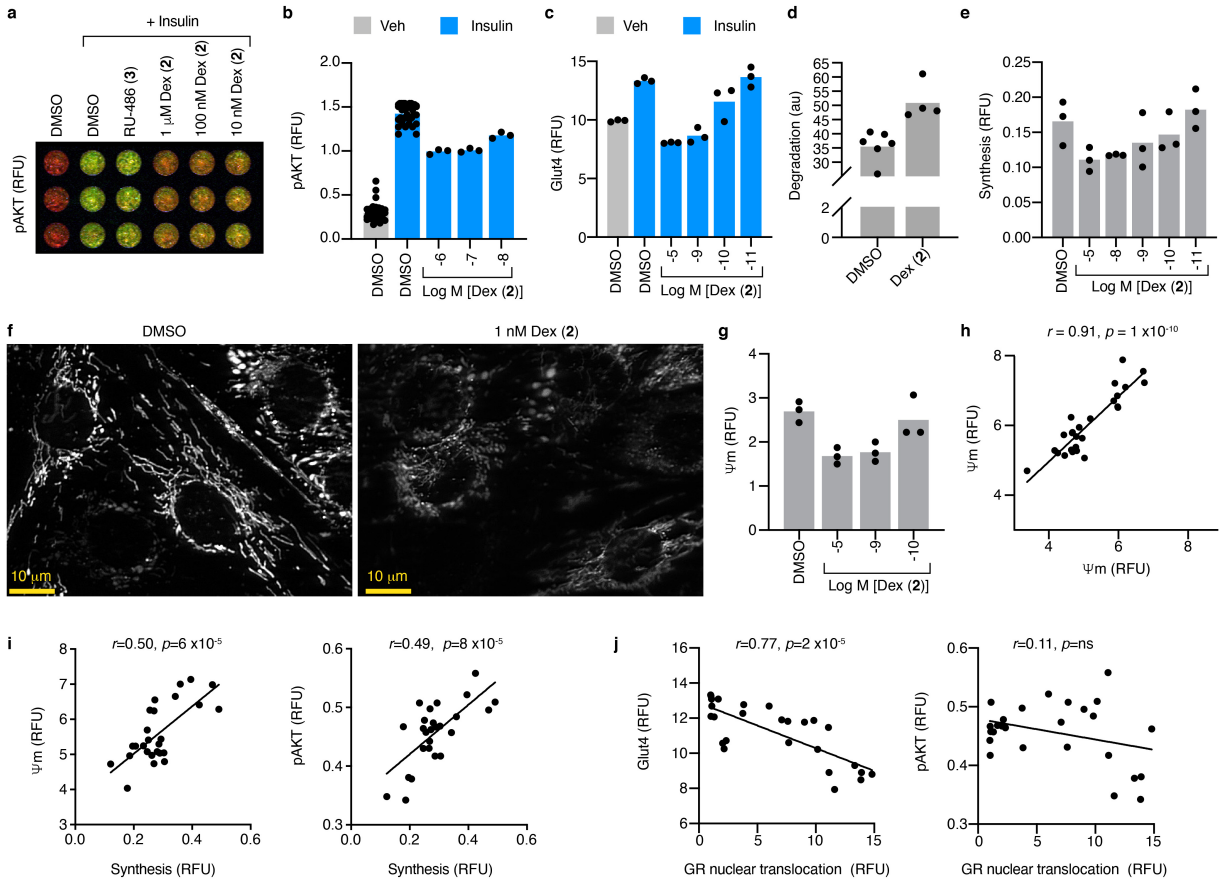


Extended Data Fig. 2. Quantitative phenotyping assays for GC action in skeletal muscle

a-e) Myotubes were nutrient-deprived, pre-treated with DMSO, RU-486, or Dex, and treated with insulin as outlined in **Methods**.

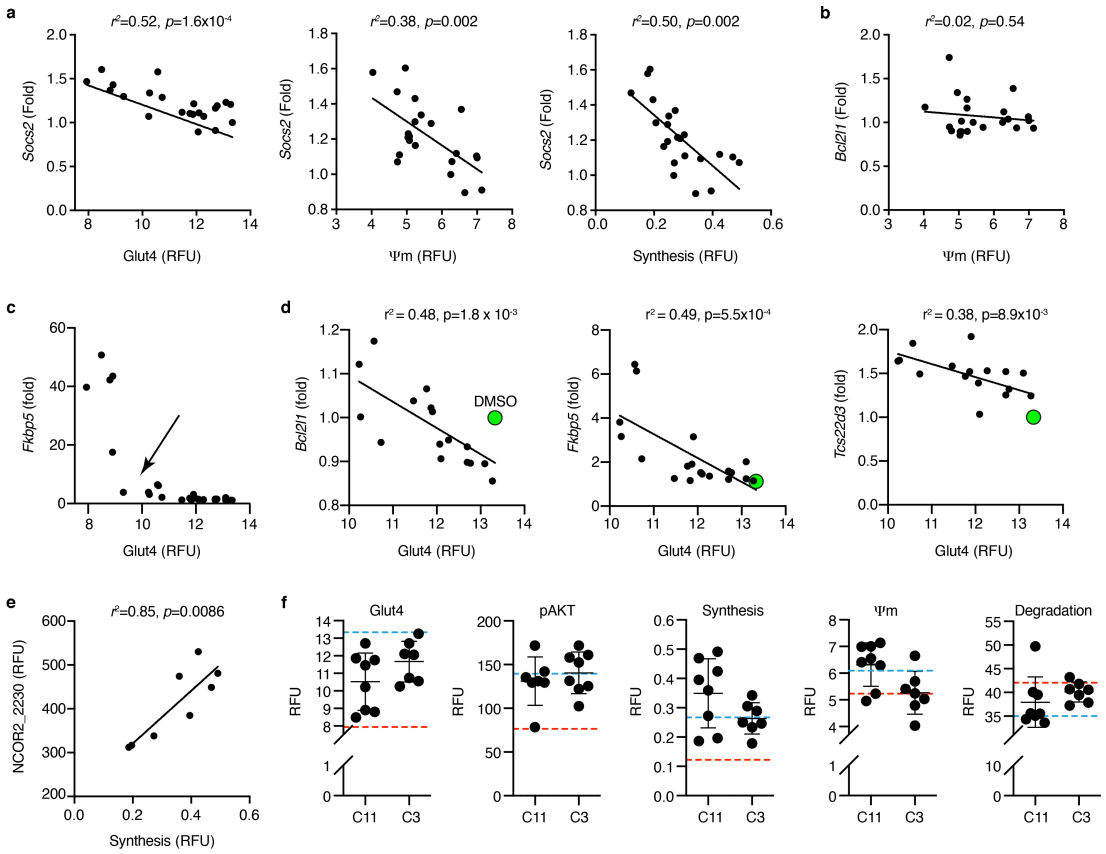
a) Effect of insulin on pAKT levels in C2C12 myotubes were compared by In-Cell Western assay (ICW) 48 h after treatment with RU-486 or Dex.

b) Quantitation of pAKT in C2C12 myotubes compared by ICW. Bars represent the mean; n = 3, except for DMSO where n = 36 biologically independent samples.

c) ICW for surface expression of Glut4 on L6 myotubes. Bars represent the mean; n = 3 biologically independent samples.

d) C2C12 myotubes were assayed for protein degradation by release of tritiated phenylalanine. Bars represent the mean; for DMSO, n = 6, and for Dex, n = 4 biologically independent samples.

524 **e**) ICW for protein synthesis by insulin-induced incorporation of puromycin into C2C12
525 myotube surface proteins. Bars represent the mean; n = 3 biologically independent samples.
526 **f-g**) High-content imaging and analysis of C2C12 myoblasts stained with MitoTracker™ dye.
527 Images are representative of 18 images per condition; i.e. 3 fields x 3 biologically independent
528 samples per condition in each of 2 independent experiments.
529 **h**) Assay reproducibility from screening 22 compounds on two separate occasions. The Pearson
530 correlation coefficient, r, and its associated p-value is indicated. Each datapoint represents the
531 mean effect of a distinct compound. Also see **Methods**.
532 **i–j**) Linear regression demonstrates the predictive power (r^2), and associated p-value for the
533 indicated variables. **i**) Ψm and pAKT predict Synthesis ($p = 6 \times 10^{-5}$ and 8×10^{-5}, respectively). **j**)
534 GR nuclear translocation selectively predicts Glut4 ($p = 2 \times 10^{-5}$) but not pAKT. Each datapoint
535 represents the effects of a distinct ligand.



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537 **Extended Data Fig. 3. Relationships among specific genes, peptide interaction assays, and**

538 **GR-mediated phenotypes**

539 **a-e**) Linear regression was performed for the indicated assay pairs, where each point represents a

540 different compound.

541 **a**) Effects of the ligands on *Socs2* expression predicts Glut4 translocation, ψm, and insulin-

542 stimulated protein synthesis.

543 **b**) Ligand-dependent expression of *Bcl2l1*, which encodes the mitochondrial anti-apoptotic

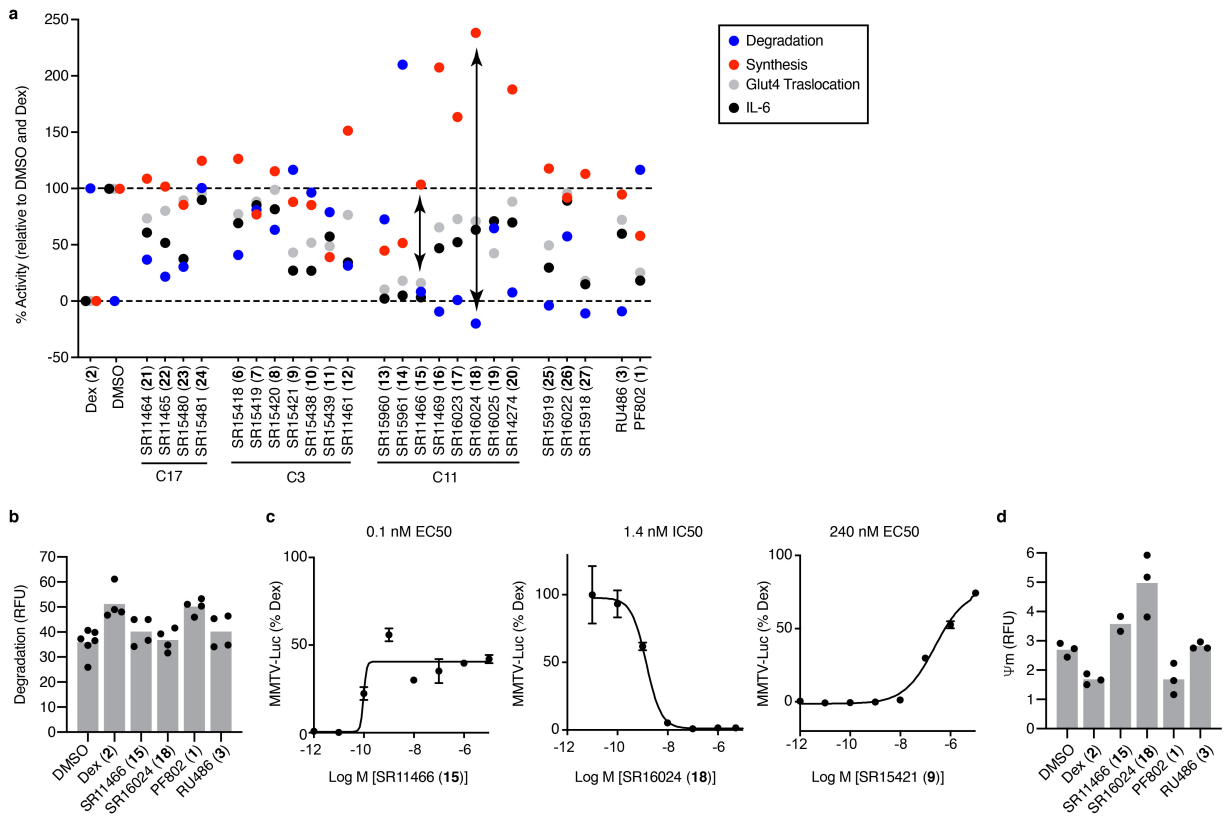
544 protein, Bcl-xL does not predict effects of on ψm.

545 **c**) *Fkbp5* expression as a predictor of Glut4 translocation shows an inflection point (arrow).

546 **d**) The Glut4 data was truncated below the inflection point shown in **c**).

547 **e**) GR interaction with an NCOR2 peptide predicts protein synthesis in the C11 subset of ligands.

f) The C11- and C3-substituted compounds showed similar variance in the skeletal muscle profiling assays. Blue dashed line, vehicle; red dashed line, Dex. Each datapoint represents the mean effect of a distinct compound, n=3 biologically independent samples. The error bars represent the mean ± SD of each compound series. For C11, n=8 distinct compounds; for C3, n=7 distinct compounds. See also **Fig. 2a-e**



Extended Data Fig. 4. Compound structure-activity relationships.

a) Individual compound data for protein degradation, insulin-stimulated protein synthesis, and Glut4 translocation in myotubes, as well as effects on IL-1β-stimulated secretion of IL-6 by A549 cells. Lead compounds are indicated with arrows. Among the 3 compounds with full suppression of IL-6 (**13,14,15**), only **15** did not inhibit protein synthesis or stimulate protein degradation. **18** showed the greatest anabolic effects, with stimulation of protein synthesis and inhibition of protein degradation.

b) Protein degradation in myotubes assayed as described in **Extended Data Fig. 2** and **Methods**. Bars represent the mean, n = 4, except for DMSO where n = 6 biologically independent samples.

c) 293T cells were co-transfected with a GR expression plasmid and MMTV-luciferase reporter. The next day cells were treated with the indicated compounds for 24 h and probed for luciferase

572 activity. For SR16024, was the cells were cotreated with 1 nM Dex. Data are mean ± SEM, n = 3
573 biologically independent samples.
574 **d**) Mitochondrial potential of myotubes assayed as described in **Extended Data Fig. 2** and
575 **Methods**. Bars represent the mean; n = 3, except for **15** where n = 2 biologically independent
576 samples.
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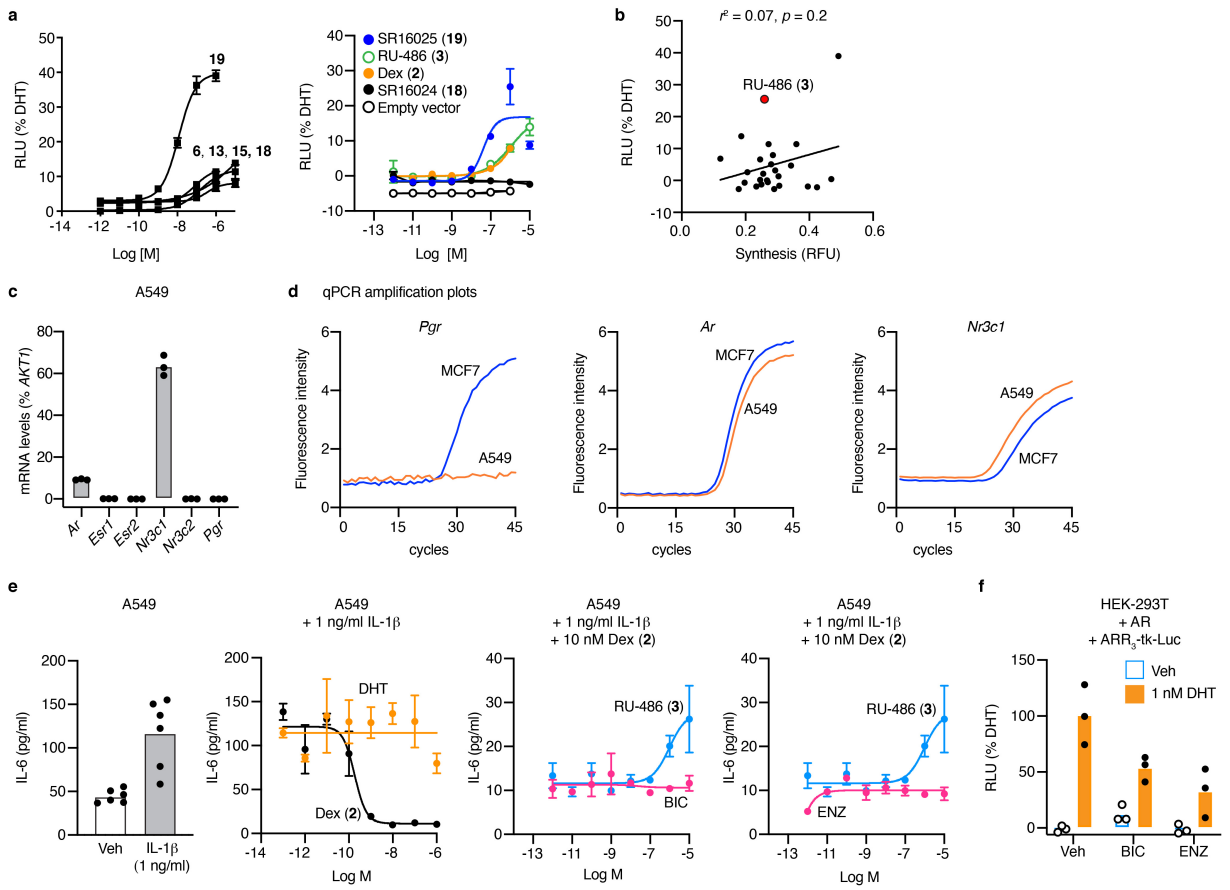

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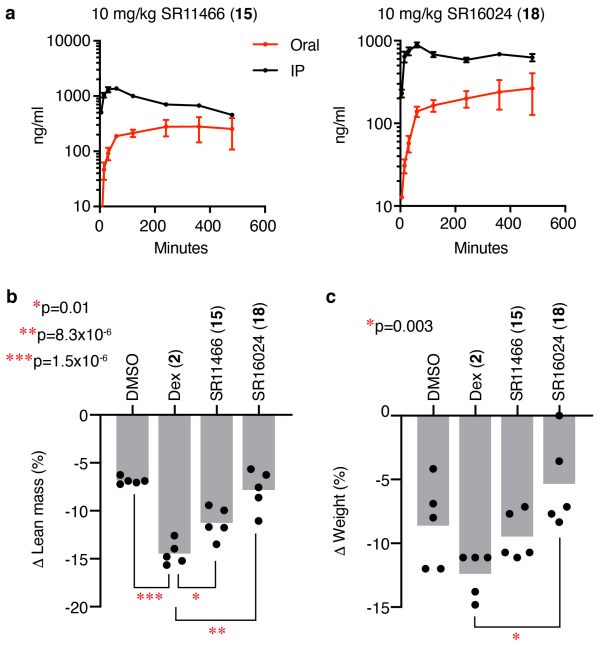
585 **Extended Data Fig. 5. On-target mechanism of action studies**

586 **a**) Reporter activity in steroid-deprived 293T cells co-transfected with an androgen-responsive
587 ARR_3-tk-luc reporter and an androgen receptor (AR) expression plasmid or empty vector
588 control, and then treated with the indicated compounds for 24 h. Dose curves for compounds that
589 stimulated AR activity (left) and the indicated compounds (right) are shown. None of the
590 compounds showed activity with the empty vector control. **18** and **19** are isomers differing only
591 in the position of the chlorine on the benzyl substitution. Datapoints are mean ± SEM; n = 3
592 biologically independent samples.

593 **b**) Linear regression demonstrating that ligand-specific AR activity profiles do not correlate with
594 protein synthesis.

595 **c**) Expression of steroid receptor mRNAs in A549 cells. Only *Ar* which encodes AR, and *Nr3c1*

596 which encodes GR were detected by qPCR. Bars represent the mean; n = 3 independent samples.
597 Also see **Supplementary Fig. 3**.
598 **d**) Representative qPCR amplification plots for *Pgr*, *Ar*, and *Nr3c1* in A549 versus MCF7 cells.
599 *Pgr*, which encodes the progesterone receptor, is not expressed in A549 cells.
600 **e**) AR antagonists do not reverse the effects of Dex on IL-6 secretion. IL-6 levels in A549 cell
601 media were measured by AlphaLISA after overnight exposure to the indicated conditions. DHT,
602 5α-dihydrotestosterone; BIC, bicalutamide; ENZ, enzalutamide. For the controls (left), bars
603 represent the mean; n = 6 biologically independent samples. For dose curves, datapoints are
604 mean ± SEM; n = 3 biologically independent samples.
605 **f**) Luciferase assay showing the effects of 1 nM DHT, 1 μM BIC and 1 μM ENZ on AR activity,
606 demonstrating that the antagonists have cellular activity. Bars represent the mean; n = 3
607 biologically independent samples.



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609 **Extended Data Fig. 6. In vivo compound profiling**

610 **a**) Mouse pharmacokinetics studies of the indicated compounds. Data are mean ± SEM; n = 3

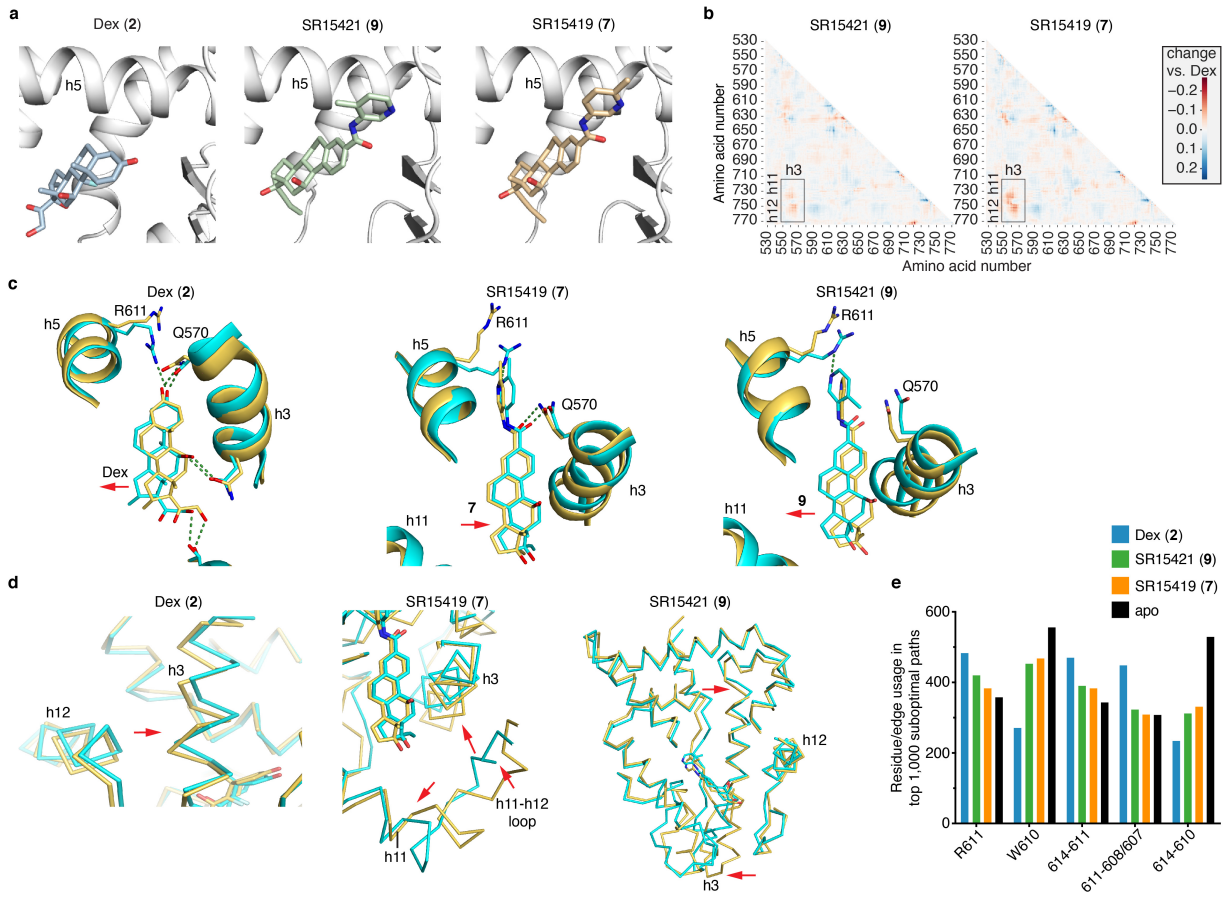
611 biologically independent samples.

612 **b-c**) Changes in the lean mass and body weights of male C57BL/6 mice treated with (10 mg/kg

613 Dex or SR16024, or 50 mg/kg SR11466) and fasted overnight. Bars represent the mean; n = 5

614 mice per group (in each of 2 independent experiments). 1-way ANOVA, Sidak's multiple

615 comparisons test, adjusted p-values are indicated.



Extended Data Fig. 7. Docking and molecular dynamics simulations

a) Ribbon diagram of GR LBD bound to the indicated ligands. **7** and **9** were docked with Autodock Vina.

b) Differential analysis of correlated motion between Cα atoms from the simulations with the indicated ligands subtracted from Dex.

c) Formation of a hydrogen bond with R611 differentially shifts the position of the ligands.

d) Formation of the hydrogen bond R611-induced changes in surface structure (red arrows). With Dex, there was a shift in h12 and the C-terminus of h3. With **7**, the C-terminus of h11 and N-terminus of h3 were shifted further apart, and away from h12. This destabilization of the h12 interface with h3 and h11 explains why this compound is an antagonist, a mechanism we have called "indirect antagonism." With **9**, there was a rotation of both ends of h3.

628 **e**) Usage of amino acid residue and edge in the suboptimal pathways between h12 E755 and h5
629 R614, demonstrating that Dex preferentially utilized R611 instead of W610 as a pathway for
630 correlated motion. Also see **Methods.**
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