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Journal
Nature Reviews Molecular Cell Biology, 18(3)

ISSN
1471-0072

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Publication Date
2017-03-01

DOI
10.1038/nrm.2016.152

Peer reviewed

Published in final edited form as:

Nat Rev Mol Cell Biol. 2017 March ; 18(3): 159–174. doi:10.1038/nrm.2016.152.

Glucocorticoid receptor control of transcription: precision and plasticity via allostery

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Abstract

The glucocorticoid receptor (GR) is a constitutively expressed transcriptional regulatory factor (TRF) that controls many distinct gene networks, each uniquely determined by particular cellular and physiological contexts. The precision of GR-mediated responses seems to depend on combinatorial, context-specific assembly of GR-nucleated transcription regulatory complexes at genomic response elements. In turn, evidence suggests that context-driven plasticity is conferred by the integration of multiple signals, each serving as an allosteric effector of GR conformation, a key determinant of regulatory complex composition and activity. This structural and mechanistic perspective on GR regulatory specificity is likely to extend to other eukaryotic TRFs.

Control of gene transcription is critical for development, physiology and homeostasis. Thus, aberrant transcription regulation commonly drives disease processes. Transcriptional regulatory factors (TRFs) have a critical role in this process by recognizing specific DNA sequences to activate or repress the expression of specific genes. One of the best-characterized metazoan TRFs is glucocorticoid receptor (GR), the founding member of the nuclear receptor superfamily — proteins that evolved to bind specific small lipophilic signalling molecules[1].

Expressed in nearly all vertebrate cells, GR directly up- and downregulates thousands of genes distinct to the cell type, governing various aspects of development, metabolism, stress response, inflammation and other key tissue and organismal processes. GR is encoded by the nuclear receptor subfamily 3 group C member 1 (*NR3C1*) gene, which is located on chromosome 5 (5q31) and is closely related to its paralogues *NR3C2*, which encodes mineralocorticoid receptor (MR), *NR3C3*, which encodes progesterone receptor (PR), and *NR3C4*, which encodes androgen receptor (AR). These four nuclear receptors share a common domain structure consisting of an amino-terminal domain (NTD), a zinc-finger

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Competing interests statement

The authors declare no competing interests.

DNA-binding domain (DBD), a hinge region and a carboxy-terminal ligand-binding domain (LBD). Embedded within these domains are regions that confer regulatory activity; for GR, these are denoted as activation function domain 1 (AF1; also known as fra/isactivating domain 1, taul and τ1), tau2 (also known as *trans*activating domain 2 and τ2) and AF2 (FIG. 1a). While the DBD and LBD are highly conserved between GR, MR, PR and AR, the N-terminal AF1 domains and the surrounding NTD regions within these four proteins are more divergent in sequence and size. In addition, alternative splicing and translational start sites produce multiple isoforms of the NR3C family members. The predominant and best-studied isoform of GR, GRα, is a 777-amino-acid polypeptide in humans. Other less abundant and less well-characterized, but likely functional, isoforms of GR have been described and are reviewed in REFS 2.3. For example, a GRβ isoform carries LBD alterations that change its ligand binding properties and may compete with GRα in certain experimental settings[4].

GR activity is gated by the endogenous steroid hormone cortisol in humans, or by exogenous glucocorticoid drugs, such as dexamethasone. In the absence of ligand, apo-GR is monomeric in the cytoplasm, where it associates with molecular chaperone complexes containing heat shock protein 90 (HSP90) and HSP70 as well as other factors[5]; this interaction promotes high-affinity hormone binding while inactivating other receptor activities, such as nuclear localization and DNA binding. Glucocorticoid binding provokes conformational changes in GR that activate multiple functional domains, including nuclear-localization sequences within the hinge and LBD regions. After translocation into the nucleus, GR associates with specific genomic glucocorticoid response elements (GREs)[6] and nucleates the assembly of transcription regulatory complexes containing GR, other TRFs and co-regulatory factors, which together activate or repress the transcription of glucocorticoid-responsive genes[7,8] (FIG. 1b). Although GR forms stable complexes with DNA *in vitro*, it seems to exchange within seconds *in vivo*[9,10], implying that distinct GR molecules establish initial genomic contact and regulate transcription, and that GR is actively disassembled from chromatin *in vivo*[11,12]. The role of these striking *in vivo* dynamics in the mechanisms of regulation is not known.

GR operates in a context-specific manner — it regulates gene networks that are precisely determined in a given context, yet displays remarkable plasticity as a function of cell type and physiological state (recently reviewed in REF. 13), leading to diverse outcomes. For example, GR-mediated gene expression governs apoptosis in the context of haematopoietic T cells[14] but increases adipogenesis, lipolysis and differentiation in adipose cells[15]. How can both precision and plasticity of GR-regulated transcription be achieved? Addressing this apparent paradox of precision and plasticity is in fact the overarching challenge for all eukaryotic transcription regulation. GR provides a striking framework in which to address this challenge because it is expressed ubiquitously in vertebrate cells and the GR-regulated gene networks are strongly cell type-specific.

Here, we first discuss how GR interacts with the genome *in vivo* and *in vitro*, highlighting the context specificity of the *in vivo* interactions. Second, we document the importance of context in specifying GR activity and discuss how different ‘surfaces’ of this TRF, the accessibility of which is modulated by the context-specific cues, seem to define the ‘regulatory logic’ of GR function. Third, we consider how four classes of signals — DNA

binding, ligand binding, post-translational modifications (PTMs) and interaction with other, non-GR TRFs — are integrated to affect GR structure and function. Fourth, we examine multiple classes of co-regulatory factors that associate with GR interaction surfaces to assemble transcription regulatory complexes and impose enzymatic actions that modulate transcription. Finally, we present a model that accounts for the precision and plasticity of eukaryotic transcription regulation. In this model, TRFs act as scaffolds whose conformations are altered through allostery by signalling inputs, and co-regulators serve both as readers that associate with surfaces induced on TRF scaffolds by those allosteric signals and as enzymes that modulate target gene transcription.

GR-genome interactions

TRFs, including GR, obtain a portion of their regulatory specificity by interacting with specific genomic loci. These interactions can be direct GR-DNA contacts, or GR can associate with other transcription regulators that are separately bound to DNA. Specific genomic occupancy by a TRF is typically highly context specific, with patterns of binding differing substantially in distinct cellular and physiological settings. Importantly, locus-specific GR binding is not a sufficient determinant of regulatory activity. In this section, we focus on outlining the direct and indirect interactions that GR establishes with the genome (FIG. 2); see Supplementary information S1 (table) for a summary of experimental techniques referred to in this section.

Direct and indirect binding in vitro

DBDs of GR and other nuclear receptors contain two highly conserved subdomains, each with four Cys residues coordinating a single zinc ion, followed by an amphipathic helix and a peptide loop. The helix of the first subdomain contains the proximal box (P box), bearing three residues that make base-specific contacts in the major groove of the binding sequence. The second subdomain helix makes nonspecific contacts with the DNA helix backbone and minor groove, whereas the peptide loop provides distal box (D box) residues important for GR dimerization[16,17].

The DBD positions GR at specific genomic sites by at least three classes of GR-DNA interactions and at least one class of GR-protein interaction (FIG. 2A). The best-characterized GR-DNA interaction is through the canonical GR-binding sequence (GBS) (FIG. 2Aa,B,D,F), which is composed of two pseudo-palindromic hexameric AGAACA repeats, separated by a three-base- pair spacer[17]. The ‘DNA-reading helix’ utilizes the side chains of Arg447, Lys442 and Val443 to make three base-specific contacts within the major groove of each GBS half site (FIG. 2B.D). GR binding in this head-to-head fashion stabilizes interactions between two sister GR DBDs, which promotes GR-GR and GR-DNA interactions, creating positive cooperativity[18,19]. Five amino acids within the D box provide critical protein-protein contacts, stabilizing the GR DBD dimer on DNA. Mutational disruption of the dimerization loop (D-loop) conformation can affect GR’s regulatory activity[20]. For example, Ala458 makes a hydrogen bond with Ile483 on the dimer partner, and mutation of the Ala to Thr has been shown to alter GR activity in a gene-specific manner[20–22]. Although this mutation was initially thought to be devoid of dimerization

potential, it does still forms dimers on DNA but with diminished cooperativity[19]. In addition, a relatively weak LBD-LBD interaction (1.5 μM using GR LBD fragments in the presence of ligand and co-regulator peptide)[23] may contribute to GR dimerization, but the biological significance of this contact has not been established.

The second class of GR-DNA interaction is a recently discovered inverted-repeat GBS (IR-GBS) (FIG. 2Ab,C,E), which is characterized as an alternative GR-binding motif containing $CTCC(N)_{0–2}GGAGA$[24]. Structural studies of the GR DBD-IR-GBS revealed that GR molecules bind these non-identical sites on opposite sides of DNA, separated by a one-base-pair spacer in a head-to-tail fashion[25] (FIG. 2C). One monomer makes three contacts within a high-affinity binding site mediated by Lys442 and Val443, which are the same side chains that participate in recognizing the DNA in the GR DBD-GBS structure. Arg447 establishes hydrogen bonds with a guanine in canonical GBS structures, but in the IR-GBS structure Arg447 is prevented from making these base-specific interactions owing to steric clashes with a thymine[25]. The other monomer uses only Arg447 to make one base-specific contact to a guanine. As the two GR monomers bind to opposite sides of the DNA at IR-GBSs, they are not in direct contact through the DBD dimerization interface.

Biochemical analyses reveal that GR binds IR-GBS elements with negative cooperativity, whereby binding of one monomer dramatically reduces the propensity of a second monomer to bind. This contrasts strongly with the cooperative binding seen at canonical GBSs[25]. Furthermore, nuclear magnetic resonance (NMR) studies indicate that the D-loop residues display significant changes in chemical environment consistent with dimerization when bound to a canonical GBS, whereas they are unaffected on binding to an IR-GBS[26]. As with canonical GBSs, it has not been examined whether LBD-LBD interactions participate in GR binding to IR-GBSs. Taken together, we infer from these *in vitro* studies that monomeric GR most likely binds these elements *in vivo*. Other 3-keto steroid receptors, MR, PR and AR, can all bind to a canonical GBS, but only GR is able to bind an IR-GBS[26]. Even the MR DBD, which shares 90% sequence identity with the GR DBD, cannot bind or repress transcription from an IR-GBS, owing to epistatic mutations that occurred during the evolution of the steroid receptor family that limit this function in all steroid receptors except GR[26].

In a third class of GR-DNA interactions (FIG. 2Ac), not yet characterized structurally, selective binding of GR to canonical half site DNA sequences (consensus AGAACA) has been reported[21,27]. This binding may be facilitated by secondary interactions between GR and other non-GR TRFs bound to DNA proximal to the GBS, although there is no evidence for enrichment of particular TRF motifs contiguous with these half site GBSs and the understanding of which TRFs are involved in the regulation of gene expression at such composite GREs is still limited. Finally, GR can occupy specific genomic regions without directly binding DNA (FIG. 2Ad). In this mechanism, known as tethering, the DBD makes protein-protein contacts with other TRFs, such as activator protein 1 (AP-1) or nuclear factor-κB (NF-κB), specifically bound at their cognate sequence motifs[28–31] (FIG. 2Ad).

Contextual genomic occupancy in vivo

The four classes of sequence-specific GR-DNA interactions characterized *in vitro* (FIG. 2A) occur at high frequency in mammalian genomes. Chromatin immuno- precipitation followed by sequencing (ChIP-Seq)[32] is a robust, sensitive and fairly accurate (albeit not to single-nucleotide resolution) method to identify genomic segments occupied by GR *in vivo*, here termed GR-occupied regions (GORs) (for details of the technique see Supplementary information S1 (table)). It is clear from these approaches that GORs, although numerous, overlie only a small fraction of the potential sites predicted from *in vitro* studies, and that they are highly context specific. For example, only 0.5% of 11,666 GORs identified in mouse liver cells were found in common in four other mouse cell types examined[33.34], and 83% of them were unique to liver[35]. Although differences in methodological and statistical approaches may complicate meta-analyses, dramatically different GORs among different cell types have been commonly noted[13].

GORs typically reside proximal both to GR-regulated genes and to genes not regulated by GR[21,36], so mere proximity is uninformative either about regulatory function or about which genes are regulated by which functional GORs. What is clear is that GORs do not necessarily regulate the gene closest in linear proximity within the genome, and that non-GR-regulated genes commonly reside between a GOR and the nearest GR-regulated gene. Provisional evidence suggests that most GORs reside >10 kb from a glucocorticoid-responsive gene, and that many GORs are 10–100 kb or more upstream or downstream relative to the transcription start sites of genes they are thought to regulate (FIG. 3a), and can reside within introns[37]. Such action at a distance is well documented, heavily studied and essentially not understood for metazoan transcription regulators.

How particular GREs and promoters interact, and what drives the specificity of one GRE-promoter interaction over others, is a focus of current debate, but one or more of several proposed gene-looping mechanisms (reviewed in REF. 38) are likely involved.

The determinants of GOR context specificity are not fully understood, but some positive and negative correlates have been uncovered. For example, DNase I-hypersensitive sites (DHSs) (see Supplementary information S1 (table)), which are thought to identify ‘open chromatin’ regions depleted of nucleosomes but occupied by TRFs and other non-nucleosomal proteins, were found in whole-genome analyses in multiple cell types to pre-exist at up to 95% of GORs formed on hormone treatment[34]. DHSs vastly out-number GORs (DHSs comprise ~2.5% of the genome, whereas GORs comprise 0.02–0.05% of the genome in the cell types tested)[34], and it is interesting to speculate that DHSs at eventual GORs reflect non-GR TRFs pre-bound at eventual composite GREs. Similar results were obtained using formaldehyde-assisted isolation of regulatory elements and deep sequencing (FAIRE seq; see Supplementary information S1 (table)). In this case, pre-existing FAIRE seq signal indicative of open chromatin increased on glucocorticoid stimulation, suggesting that GR interacts with pre-existing open chromatin and then further alters the chromatin environment[39]. By contrast, negative regulatory DNA sequence (NRS) motifs were identified proximal to a subset of GBSs as anti-correlates to GR genomic occupancy. Here, GBSs that were not occupied by GR via ChIP-Seq experiments were found to have an over-representation of proximal NRS motifs[40]. Interestingly, NRSs did not affect DNase I

sensitivity. Rather, NRSs seemed to be enriched in para-speckle proteins, which were speculated to repress transcription by blocking GR from binding to its canonical site.

GRE context and regulatory logic

The context specificity of GORs demonstrates that DHSs, recognition sequences (GBSs) identified *in vitro* and non-GR TRFs with which GR interacts are insufficient to localize or predict the localization of GR *in vivo*. A dramatic example is the IR-GBS-containing GORs, which are widespread and highly occupied in mouse fibroblasts[24] but are detected only sparingly in certain other cell types[41–43]. The relationship of a GOR to a functional GRE is similarly complicated by context. Transient reporter assays, in which a plasmid bearing a genomic fragment underlying a GOR is inserted adjacent to a minimal promoter and reporter (typically luciferase) gene and is transfected into a GR-expressing cell line (see Supplementary information S1 (table)), have been widely used to assess GOR regulatory capacity, and therefore to infer GRE activity. However, the transient conditions for a newly introduced plasmid are surely different from the chromosomal and cellular settings of the endogenous GOR, and the strong context specificity of GR-mediated gene regulation raises concerns about the validity of GRE activity inferred using that approach.

Genome editing using zinc-finger nucleases or transcription activator-like effector nucleases (TALENs) provides, in principle, routes for assaying GRE activity *in situ*. Unfortunately, the technical complexity of these methods, together with incomplete appreciation of the overriding importance of context, has left these approaches largely unused. As a result, only one GRE has been validated at its endogenous locus *in vivo*. In that case, a short deletion introduced into the first intron of the mouse circadian clock gene period circadian homologue 2 (*Per2*) fortuitously covered a GOR ~25kb down-stream from the transcription start site and produced allele-specific loss of glucocorticoid-mediated induction of *Per2* expression in mesenchymal stem cells[44]. With the emergence of clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein (Cas) technologies[45], precise genome editing will rapidly become the ‘gold standard’ for validation of GRE activity in any chosen cell type or physiological context. Interestingly, underscoring the importance of context, the *Per2* GRE identified in mouse mesenchymal stem cells seems not to be functional in human lung carcinoma cells, as assessed by CRISPR-driven deletion (K. Ehmsen, personal communication).

As mentioned above, the genes and gene networks regulated by GR within different cell types are distinct. For example, FIG. 3b shows the vastly different sets of target genes regulated by the synthetic glucocorticoid dexamethasone in two different cancer cell lines. Most GR target genes are unique to one cell line, demonstrating striking cell-context specificity. Indeed, 62 genes regulated in both cells lines are differentially responsive (activated in one cell line, repressed in the other), showing that even genes regulated by GR in both cell lines can be controlled by distinct mechanisms.

Genetic strategies have also been used to identify GR surfaces that are engaged in the regulation of gene expression in different contexts. For instance, a triple point mutant, denoted 30iiB (in which Glul98 is changed to Lys, Phel99 to Leu and Trp213 to Arg),

abrogated activation by the AF1 domain, and single point mutants within the DBD dimerization domain (Ala458 to Thr) and AF2 (Glu755 to Arg) knocked out activation by those domains individually for genes tested in U2OS cells. These three surfaces, together with a naturally occurring splice variant, GRγ, which inserts a single amino acid (Arg452) into the lever arm region of the DBD, were found in U2OS cells to function in gene-specific patterns. For example, GR-mediated activation of one set of genes depended solely on AF2, whereas another set used a combination of AFl, DBD and AF2 (REFS 21,46,47). As each altered GR region contains protein interaction domains, it is reasonable to assume that the different patterns of domain use reflect assembly of distinct regulatory complexes at the GREs controlling the different sets of genes.

Beginning with context determinants defined in the genome and in GR, what can be inferred about the regulatory logic that confers such specificity yet permits facile plasticity when conditions are altered? By manipulating ligand dose, duration of treatment and other parameters that affect relative levels of GR activity, one study uncovered ‘toggle switch’-like behaviour in U2OS cell GREs in which a set of genes was activated at low levels of GR activity and underwent a dramatic shift to repression at a transition point as GR activity increased[48]. This stereotyped regulatory mode has been called incoherent type 1 feed-forward loop (I1-FFL) logic[49] and suggests that GREs that regulate these differentially responsive genes initiate two arms of a regulatory circuit, yielding net activation or repression as the level of GR activity is altered by various context determinants. Different FFL network motifs initiated by GR have been described in macrophages[50]. These findings imply that some, perhaps many, GREs may contain molecular switches that readily confer plasticity in a highly context-sensitive manner. Thus, the GRE seems to emerge as the regulatory logic module, driven by multiple signals and conferring specificity while enabling plasticity. Below, we suggest a conceptual and mechanistic basis for such a regulatory logic for GR, and likely for other eukaryotic TRFs.

Allosteric effectors of GR

If, as outlined above, regulatory specificity is determined by the assembly of distinct regulatory complexes, each attuned to a particular gene, cell and physiological context, what are the molecular determinants of context, and how are their effects communicated to GR? Extending a few examples into a general conclusion, current data suggest that four classes of cellular signals interact with GR, allosterically altering its conformation. The first two classes, the hormonal ligands and covalent PTMs, represent the endpoints of various signal transduction pathways that communicate physiological context to the receptor. A third signal class is the extensive array of DNA sequences bound specifically by GR; these differ at different GREs, and thus these signals impart gene-specific context to the receptor. Finally, the particular cell-specific repertoire of TRFs, including the variability in levels of their expression and activities, are reflections of cell type. Thus, the differences in the expression of TRFs that interact with GR convey cell-specific, allosteric signals. As described above, GR-TRF interactions occur at composite GREs and their special subset, the tethering GREs.

DNA-binding sequences

High-throughput studies have identified thousands of proteins that interact with DNA[51], and TRFs are the most common group with sequence-specific DNA-binding activity. Specific DNA sequences not only serve as platforms for binding but also seem to act as direct allosteric effectors of TRFs[18,19,52].

Although the oligomeric state of GR (monomeric, dimeric or higher order) at different points in signalling *in vivo* is a matter of current debate[42,43,53,54], it is established that full-length, ligand-bound GR is a monomer in solution *in vitro*, even at high concentrations[55.56]. At canonical GBSs, DNA binding increases the local concentration and favourable orientation of protein- protein interactions between two DBDs, resulting in productive dimerization[17]. Indeed, not only does GR make sequence-specific interactions with each hexameric half site of the DNA but also the three-base-pair spacer between the half sites, at least at certain GBSs, drives cooperative DNA binding and dimerization[18]. Moreover, allosteric changes provoked by one half site sequence can be transduced intermolecularly across the dimer interface, conformationally altering the dimer partner[19]. Spacer sequences can alter DNA shape, resulting in conformational changes that originate from the DNA-reading helix, allosterically propagate through the lever arm and alter the conformation of the receptor's D box, ultimately affecting GR transcriptional activity[19]. Such conformational distinctions can be seen in comparisons of different GR DBD-GBS structures (FIG. 2F). Finally, recent work has shown that sequences at the +8 and −8 positions flanking the GBS that alter DNA conformation also affect GR DBD structure, as assessed by NMR analysis *in vitro* and using a zinc-finger nuclease-generated, genomically integrated GBS-reporter system and endogenous GR *in vivo* [57]. Collectively, these studies suggest that DNA sequence-specific conformational states of GR result in the generation or stabilization of distinct patterns of GR surfaces, which serve as interaction platforms, driving alternative transcriptional outcomes.

Additionally, NMR analyses revealed allosteric communications between the dimerized GR monomers at canonical GBSs[19], and molecular dynamics simulations (for further details of the method, see Supplementary information S1 (table)) suggested the existence of allosteric regulation between monomers at IR-GBSs (although this might not be relevant *in vivo*, as IR-GBS elements may be occupied by only monomeric GR in the *in vivo* scenario[26]). In both cases, DNA acts as a ligand to impart allosteric changes that could affect affinity for co-regulators and ultimately regulatory out-comes, implying that the allosteric transitions extend into the NTD and LBD. So far, structural studies of GR in the context of allosteric modulation by DNA have been limited to isolated domains, but work with a full-length vitamin D receptor (VDR) and retinoid X receptor (RXR) heterodimer confirms allosteric communication throughout nuclear receptor complexes on DNA binding[58]. Indeed, hydrogen-deuterium exchange mass spectrometry (HDX-MS) on the full-length, ligand-bound VDR-RXR-DNA complex revealed conformation changes on binding of ligands, DNA and co-regulators. Changes within the VDR DNA-binding sequence (VBS) read through the VDR DBD had far-reaching intramolecular allosteric effects that altered the solvent accessibility of regions within the sister LBD of the complexed RXR molecule[58].

Future work with full- length GR will be essential to fully describe the allosteric consequences of differential GBS binding.

LBD-binding ligands

The NR3C family members, GR, MR, PR and AR, evolved from a common ancestor and share high sequence similarity within their LBDs; however, key architectural differences of each LBD produce strict ligand selectivity[59]. Differences in the aromatization of the A ring of the ligand (see FIG. 4a for ligand ring naming and carbon numbering), driven in part by the 3-oxo group interaction with polar residues within NR3 family LBDs distinguish endogenous NR3C family member 3-keto ligands from related NR3A family oestrogen receptor (ER) 3-hydroxy-specific ligand recognition[59,60] (FIG. 4a). The first crystal structure of the GR LBD complexed with a ligand (dexarnethasone) displayed an intricate network of polar and nonpolar interactions, which together define ligand selectivity — every polar atom within dexarnethasone directly interacts with the GR LBD[23].

Despite strong binding specificity, dexarnethasone occupies only ~65% of the GR ligand-binding pocket (leaving >200 $Å^3$ excess volume within the 590-$Å^3$ binding pocket[23]); similarly, the endogenous ligand cortisol binds specifically but fails to fill the binding pocket[61] (FIG. 4b). The additional volume within the binding pocket offers potential space for interaction with alternative modulatory ligands. Moreover, LBD structures bound to cortisol and an alternative GR ligand, RU-486, (FIG. 4c,d) reveal extensive structural malleability within the LBD, which seems to enable interaction with a wide range of potential ligands[62] that could confer different allosteric changes, resulting in ligand-specific alterations in regulatory outcomes. The capacities of functional GR ligands to fill only a portion of the binding pocket or to alter the shape of the pocket challenge and, at the same time, liberate the concept and practice of ligand design. Using the dexamethasone-occupied pocket as a guide, arylpyrazole compounds were developed as nonsteroidal GR ligands and shown indeed to differ from dexarnethasone in their phenotypic and molecular actions in several target cell types[63]. Notably, selective GR modulators (SGRMs) that preserve the anti-inflammatory and immunosuppressive actions of standard glucocorticoids but do not show the adverse effects that accompany chronic glucocorticoid therapeutic regimens have long been sought without success. Hence, discovery of molecules that achieve pre-selected, context-specific regulatory outcomes has proven difficult; we suggest in our concluding remarks a targeted approach.

In summary, GR ligands are critical physiological and pharmacological context inputs, conferring allosteric transitions[64] that affect GR-associated molecular chaperone affinities or functions and activate GR nuclear- localization signals and DNA-binding activity. Even subtle modifications of GR ligand chemistry or dose can affect gene-specific GOR formation[48.63], or regulatory complex composition and/or function (for example, altered histone acetyltransferase (HAT) activity) without affecting GOR formation itself[63].

PTMs

Covalent PTMs of TRFs are conferred as the endpoints of cell signalling pathways that provide physiological context information distinct from that provided by non-covalently

associated hormonal ligands. PTMs can confer allosteric transitions, create or inactivate protein interaction surfaces, and affect TRF protein localization, stability, DNA binding, ligand response and regulatory activity in a context-specific manner. GR can be modified by several PTMs at distinct sites[65–69] (FIG. 5). Here, we summarize some of these PTMs and their effects on GR action.

Phosphorylation.—Phosphorylation (generally on Ser, Thr or Tyr) of nuclear receptors is involved in ligand binding, nuclear localization, DNA binding, and modulating interactions with co-regulators[68,70]. GR maintains a basal level of phosphorylation, but additional sites are phosphorylated on ligand treatment[71–73]. To date, there are seven experimentally confirmed phosphorylation sites on GR clustered within the NTD: Ser113, Ser134, Serl41, Ser203,Ser211, Ser226 and Ser404 (REF. 65). These residues are conserved among humans, mice and rats[73]. Although the enzymes responsible *in vivo* are uncertain, these sites can be modified *in vitro* by cyclin-dependent kinases (CDKs), mitogen protein kinases (MAPKs), JUN N-terminal kinases (JNKs) and glycogen synthase kinase 3 (GSK-3)[74], implying that multiple signaling pathways communicate with GR. Mutational analysis of particular sites led to mixed reports of the effects of phosphorylation on the transcription regulatory activities of GR[75,76], consistent with strong gene-, cell- and physiology-specific context dependence, as expected.

In general, phosphorylation of GR increases the protein half-life, and mutation to Ala at several different phosphorylation sites, one site at a time, each resulted in rapid protein degradation[67,76]. Phosphorylation of sites Ser203, Ser211 and Ser226, which are located within AF1, are predicted to affect exposure of protein surfaces critical for cofactor interactions[77,78]. Phosphorylation of Ser211 results in increased recruitment of GR to GORs and subsequent regulation of GR target genes[79]. Mutation of Ser203 prevents phosphorylation at Ser226, suggesting interdependence of those modifications[72,80]. Upon ligand binding, Ser203 is phosphorylated and GR is selectively partitioned to the nucleus. Mutation of that site precludes nuclear accumulation and subsequent GR-dependent gene regulation[80], whereas Ser226 phosphorylation, presumably via JNK signalling pathways, results in increased nuclear export, thus decreasing gene regulation through GR[68]. Ser203 is phosphorylated *in vitro* by CDK and MAPK[74,81]. This apparent integration across different signalling pathways may contribute to the differences in transcriptional outcomes observed in different cellular and physiological contexts[70,80]. Another site, Ser404, phosphorylated *in vitro* by GSK-3, seems to be hypo-phosphorylated in nuclear fractions, but loss of Ser404 phosphorylation changes the conformation of GR, alters cofactor recruitment and transcriptional responses, and increases glucocorticoid-induced apoptosis[82,83].

Ubiquitylation.—The 8.5-kDa ubiquitin polypeptide is covalently attached to Lys residues of a target protein and promotes protein turnover by targeting the protein to the proteasome for degradation. Cells treated with pro- teasome inhibitors showed enhanced GR regulatory activity[67,84] and increased *in vivo* DNA occupancy time, indicating that ubiquitylation is involved in regulating GR stability[11,12]. Furthermore, ubiquitylation of GR at Lys419 (REF. 85) was shown to stimulate GR nuclear export and subsequent degradation[86].

Sumoylation.—The small ubiquitin-related modifier 1 (SUMO-1) polypeptide, when covalently linked to Lys residues of target proteins, can alter their stability, localization or transcription regulatory activity[67,87]. GR can be sumoylated at three sites: Lys277 and Lys293 within the NTD and Lys703 within the LBD; these modifications have been shown to have effects on GR activity that are highly context dependent[66,88]. Genome-wide profiling of GR sumoylation mutants revealed enhanced GR recruitment to DNA, specifically at genes involved in cell growth, proliferation and survival[89].

Although its functions are context dependent, sumoylation has most commonly been linked to transcription repression[90,91]. One recent example concerns the repression of inflammatory genes[92,93] bearing IR-GBS- containing GORs. Specifically, sumoylation at Lys293 within the NTD is required for IR-GBS-mediated repression, but mutations of the other SUMO sites have no effect. Sumoylation of Lys293 also promotes the recruitment of co-regulators silencing mediator of retinoic and thyroid receptors (SMRT) and nuclear receptor co-repressor (NCoR) but does not affect GR-mediated activation from certain genes linked to canonical GBS- containing GORs[92]. It seems that GR sumoylation not only facilitates the assembly of repressive regulatory complexes but also assists in the binding of GR to weaker associated sites, at least at the IR-GBS-containing GORs examined. Furthermore, loss of this sumoylation site results in diminished GR-mediated repression at certain AP-1 and NF-κB tethering sites[93], apparently owing to inhibition of regulatory complex assembly.

Acetylation.—GR is acetylated at Lys494 and Lys495 within the hinge region at a common acetylation motif, KXKK (where X is any amino acid). This motif is conserved among the 3-keto steroid receptors, suggesting that this acetylation may be important for certain general functions of these receptors. The HAT proteins circadian locomoter output cycles protein kaput (CLOCK) and BMAL1 (encoded by *ARNTL*) were inferred from cell-based assays to acetylate GR at these Lys residues[94]. KXKK acetylation reduced the affinity of GR binding to canonical GBSs *in vitro* and also reduced its ability to regulate transcription in transfection assays. The regulation of this modification by CLOCK is critical for circadian rhythm maintenance[95]. GR is deacetylated *in vitro* by histone deacetylase 2 (HDAC2), which seems to be important for repression of NF-κB-regulated genes[96].

Nitrosylation.—*S*-Nitrosylation involves the covalent attachment of a nitric oxide to a thiol group on a Cys residue. Nuclear receptors contain two zinc-finger domains, with four Cys residues each. Nitric oxide can target these residues and cause the release of bound Zn^{2+}. For GR, this modification has been shown to inhibit ligand binding[69]. Exposure of COS-7 cells to exogenous nitric oxide sources has been shown to inhibit DNA binding and dimerization of nuclear receptor complexes such as VDR-RXR[97], but to date this has not been studied for GR.

Composite GRE-bound non-GR TRFs

The fourth class of allosteric effectors that alter GR activity are non-GR TRFs bound at composite GREs.Correlative studies suggest that composite GREs are 0.5–2-kb genomic segments containing one or more of the four classes of GR-binding motifs described above, clustered with binding sequences for non-GR TRFs[36]. In the first-described composite GRE,

GR interacted both with GBS DNA and with an AP-1 factor bound contiguously, and either activated or repressed transcription, depending on the subunit composition of the AP-1 factor[28,98,99]. A simple interpretation is that particular combinations of non-GR TRFs bound at composite GREs could bias GR occupancy at those elements, and by directly interacting with GR confer allosteric effects, modulating GR activity. Occupancy of the non-GR TRF-binding sites at a given composite GRE will differ in different cell types, owing to cell context-specific differential absolute and relative expression and activities of the respective TRFs, thereby providing a context- dependent signal for transcription regulation mediated by GR.

Co-regulators as GR signalling readers

TRFs such as GR nucleate the assembly of large (~10^2 polypeptides and non-coding RNAs) transcription regulatory complexes, typically including other TRFs and distinct collections of co-regulatory factors, which confer structural or functional changes on the transcription machinery or chromatin, thereby positively or negatively modulating target gene mRNA production. Roughly 300 co-regulators have been identified, many of which are themselves multifactor complexes, and shown to exhibit a wide range of functions[100,101]. Which co-regulators interact with GR depends not only on co-regulator availability in a given cell type but also on the integrated effects of the four classes of signals that communicate context information to GR discussed above. Hence, co-regulators can be viewed as readers of GR-mediated signalling — it is the co-regulators that convert the integrated, signal-driven allosteric transitions at distinct receptor surfaces into context-specific transcription regulatory actions. Originally classified as co-activators and co-repressors, many co-regulators have been recognized to both activate and repress genes in a context-specific manner[102]. Here, we classify co-regulators based on their mechanisms of action rather than on the regulatory out-come in any particular context, and we discuss those classes shown to date to interact with GR (TABLE 1).

Basis of GR-co-regulator interactions

Structural analysis has provided deep insight into the interaction of co-regulators with regulatory domain AF2 near the GR C terminus, a highly conserved 12-α-helix and 4-β-sheet motif that folds into a 3-layer helical bundle[103]. AF2 of nuclear receptors is minimally comprised of helices 3, 4 and 12 (REFS [104],105), with which nuclear receptor co-regulators interact through highly conserved LXXLL motifs (so called nuclear receptor boxes)[106.107] or through (L/I)XX(I/V)I or LXXX(I/L) XXX(I/L) motifs (referred to as co-repressor nuclear receptor (CoRNR) boxes)[108] (FIG. 4c,d). By contrast, the entire NTD of GR, which includes the AF1 domain, is highly disordered. Interaction of steroid receptor co-regulator 2 (SRC-2; also known as NCoA-2, TIF2 and GRIP1) and TATA box-binding protein with AF1 stabilizes it and increases its α-helical content[109.110] (similar disordered regions are commonly directed to fold into well-ordered functional domains in other TRFs and other proteins[111]); little else is known of the structure and dynamics of the functional domain or of the AF1- co-regulator interfaces. Still less is known structurally about the tau2 domain within the GR hinge domain (FIG. 1 a), which interacts with co-regulator HIC-5 (also known as TGFB1I1)[112,113] (TABLE 1). Importantly, because systematic mutagenesis and

mapping in multiple contexts has not been carried out, we are far from understanding even the number of functional surfaces that potentially might form within any given GR domain.

Functional classes of GR co-regulators

TABLE 1 displays five functional classes of co-regulators reported to interact with GR, together with specific examples from each class (for a more complete description of co-regulators, see REFS 102,114,115). As this is an active field of research, additional GR-associating co-regulator classes are probably yet to be discovered. These co-regulators typically interact with GR bound by one of the standard, cortisol-like glucocorticoid ligands, such as cortisol itself or dexamethasone. One class of co-regulators, the HDACs, binds GR at canonical GBS- containing sites only when bound by exogenous drug RU486, thus calling into question the physiological significance of these interactions. By contrast, at IR-GBS-containing sites, HDACs interact with GR bound by standard ligands. Below, we briefly consider examples from each functional class.

Structural and enzyme-interacting

pl60 SRC family.—The pl60 family contains three members: SRC-1 (also known as NCoA-1),SRC-2 (also known as NCoA-2, TIF2 and GRIP1) and SRC-3 (also known as NCoA-3, CIP, AIB1, ACTR and TRAM1)[100]. These proteins function as scaffolds and can associate with between six and ten other proteins[116], including other coregulators, such as coiled-coil coactivator (COCOA), cell division cycle and apoptosis regulator 1 (CCAR1), histone acetyltransferases (CREB-binding protein (CBP)/p300 and CBP/p300-associated factor (PCAF; also known as KAT2B)) and histone methyltransferases (coactivator-associated Arg methyltransferase 1 (CARM1; also known as PRMT4) and protein Arg *N*-methyltransferase 1 (PRMT1))[117–121]. Knockdown of individual SRC proteins showed context-specific effects on GR-mediated transcription regulation[122,123].

The SRC proteins contain three functional domains: the N-terminal basic helix-loop-helix-Per/ARNT/Sim (bHLH-PAS) domain, which binds to the AF1 domain of GR, increasing its stability and α-helical content[109]; the central receptor interaction domain (RID), which contains three LXXLL motifs and two transcription activation domains (AD1 and AD2) and associates with the AF2 domain of GR, within the LBD[124,125] (see also FIG. 4C); and the C-terminal activation domains, which interact with HATs and histone methyltransferases[118].

GR interacts preferentially with SRC-2 over other pl60 members[126–128]. SRC-2 can form distinct foci within the nucleus that also contain p300, PCAF and the nuclear receptors GR, AR and ER, as well as others. Dexamethasone-bound, but not RU486-bound, GR was observed to localize to these substructures[129]. SRC-2 can also be phosphorylated in a GR-interaction- dependent manner, and mutations to key phosphorylation sites result in reduced expression from selected target genes[130]. Also functional in repression, SRC-2 was the first co-regulator shown to deploy distinct regulatory domains[131] for up- and downregulation of GR-dependent transcription in different contexts[29,132–134].

Mediator and other structural and enzyme-interacting.—The ~30-protein, 1.2-MDa Mediator complex forms a physical link between TRFs, such as GR, and the general

transcription machinery, and regulates transcription by affecting RNA polymerase II and TFIIH activity[135]. Structural studies suggest that Mediator activity is controlled allosterically[136–139]. For example, interaction with LBDs of nuclear receptors and other TRFs induces conformational changes resulting in the formation of a Mediator pocket domain, which enables Mediator-RNA polymerase II interaction[137,140]. Core Mediator components also stably interact with kinase modules, including CDK8 or CDK19, which in turn recruit other enzymes, such as the HAT GCN5L (also known as KAT2A)[141]. GR binds to two distinct Mediator subunits. Mediator of RNA polymerase II transcription subunit 1 (MED1) interacts with the GR LBD in a ligand-dependent manner via LXXLL motifs, whereas the MED14 mediator subunit, establishes interactions with the AF1 domain of GR independent of ligand binding[142]. GR target gene regulation seems to be differentially dependent on MED1 or MED14 (REF. [143]). Other co-regulators, such as HIC-5, COCOA and CCAR1, also interact either directly or indirectly with GR, contributing to the formation of multi-subunit, context-specific transcription regulatory complexes[112,113,119–121].

Chromatin-remodelling

Human cells contain an extensive family of evolutionarily conserved multi-protein SWI/SNF-related ATPases that catalyse various transformations of the regular nucleosome packaging of chromatin[144,145]. Predominant among them are the closely related Brahma (BRM) and BRG1 (also known as SMARCA4) complexes, which interact context-specifically with GR[146,147], repositioning nucleosomes to facilitate the accessibility of DNA to TRFs and transcriptional machinery[148–150]. The recruitment of SWI/SNF complexes by GR to facilitate tran-scription[151] served as the first published report of the existence of TRF co-regulators. GR has been shown to interact directly with multiple subunits of the BRM and BRG1 complexes[152,153], with binding reported to the DBD, LBD and AF1 domains of GR, probably in a context-specific manner.

Methyltransferases

PRMTs, such as CARM1, and lysine methyltransferases, including G9a (also known as EHMT2), methylate histone and other proteins. They interact with GR directly or with GR-bound co-regulators p160 or p300, and either activate or repress GR target genes, in a context-dependent manner[154,155].

HATs

HATs modify histones and other proteins, forming ε-*N*-acetyllysine at selected Lys residues[156]. Among the various HAT families, CBP, p300 and PCAF (as a component of ADA (Ada acetyltransferase) and SAGA (Spt-Ada-Gcn5-acetyltransferase) complexes) interact with GR directly, through interactions with the AF1 domain, or indirectly, through pl60 co-regulators associated with the AF2 domain of GR, to modulate transcription[100,124,157–159]. HATs target histones as well as non-histone proteins (including nuclear receptors) in a context-specific manner[117,160] and, although they are generally associated with activation of transcription[161], their mechanisms of action and regulatory outcomes seem also to be context-dependent[162].

HDACs

NCoR and SMRT form multi-protein complexes that include HDACs[163]. NCoR and SMRT contain CoRNR boxes, which can interact with the LBD of GR. This binding occurs preferentially when GR is bound to RU-486 over when GR is bound to a standard glucocorticoid[128], as helix 12 of the GR LBD in the RU-486-bound form is optimally positioned to permit CoRNR association[164] (FIG. 4d). Although that GR interaction may not be physiologically significant, NCoR and SMRT co-occupy IR-GBS-containing or NF-κB- or AP- 1-tethered GORs at which standard glucocorticoid-bound GR is sumoylated within the NTD[92,93].

Precision and plasticity via allostery

Because it is expressed ubiquitously, GR provides a dramatic example of perhaps the most critical property of eukaryotic TRFs — their context specificity. That is, GR regulates networks of genes that are precisely determined in a given setting, yet differ dramatically as a function of cell type and physiological state. This context-driven plasticity likely reflects the integration of the four classes of context-specific signals outlined above — hormonal ligands, PTMs, GR-binding DNA sequences and adjacent binding sequence motifs for non-receptor TRFs. The hormones and PTMs provide physiological context information to GR; the two classes of DNA-binding sequences, which together make composite GREs, provide gene context; and the range of TRFs available to occupy non-GR-binding sequences provide cell context.

We and others have shown that certain GR signals, namely hormonal ligands and DNA-binding sequences, are allosteric effectors, conferring specific alterations on GR conformation. Our model assumes that all four classes of signals affecting GR regulatory activity in transcription operate allosterically, and that their effects integrate to produce context-specific patterns of GR surfaces. These patterns of surfaces are recognized and bound by specific co-regulator complexes, which typically possess enzymatic activities that confer structural and/or functional changes on the transcription machinery and/or the chromatin. Co-regulators are generally not cell specific; rather, we propose that they assemble in unique combinations at each GRE, based on the context-specific interactions established at these sites.

A provocative extension of these ideas is that GR and other TRFs may typically lack intrinsic regulatory activities, instead serving merely as molecular scaffolds, patterns of surfaces produced by signalling, to which co-regulators, the actual regulatory machinery, combi- natorially associate (FIG. 6). This idea accounts readily for the common observation that TRFs can activate transcription in one context and repress it in another. In this sense, the activities of GR reflect its molecular conformations, which emerge owing to context-specific cues, whereas the functions of GR are integrated regulatory outcomes of co-regulatory enzyme actions that associate with those various conformations. Thus, we suggest that GR structure, together with its determinants, is the key to understanding both its regulatory precision and plasticity. These ideas probably extend, at least conceptually, to other eukaryotic TRFs[52], all of which face the same precision-plasticity challenge.

Conclusions and perspectives

Biology is marked by astounding specificity of time and place, whether exemplified by butterfly migration, embryo patterning, neurite outgrowth or chromosome segregation. Here, we have considered such specificity from the perspective of a single polypeptide that regulates vertebrate gene expression. GR (which is ~90kDa) associates non-covalently with simple ligands (such as cortisol, which is only 362 Da) and controls transcription within networks comprising thousands of genes, distinct to cell type and condition. What explains such specificity?

We suggest here that the actions of GR are expanded, refined and directed through its interactions with multiple partner TRFs, dozens of signalling pathway-induced PTMs, hundreds of co-regulators, tens of thousands of potential genomic binding sites, hundreds of cell types (each with unique patterns of chromatin structure) and countless physiological states. This culminates in exquisitely complex regulation of gene transcription characterized by remarkable precision in any particlar contextual setting, yet facile plasticity to adapt when that context is altered. We propose that allosteric regulation of GR by these various inputs lies at the basis of the context specificity of gene expression.

This capacity of GR to integrate signalling inputs allosterically to produce distinct transcriptional outputs can be expanded further by the fact that multiple, distinct regulatory complexes established at individual GORs may collaborate to control any single GR-regulated gene. In addition, ligand chemistry and concentration readily alter GR-regulated gene network transcription, opening the door to speculation that endogenous ligands for GR may not be limited to cortisol. With appreciation of how daunting a task it is to understand this complexity sufficiently to render it predictive, what is a strategy to gain detailed insight into signal- and allostery-determined regulation of transcription? Undoubtedly, neither systems nor reductionist approaches alone will suffice[41].

In our view, an essential step in obtaining holistic understanding of the regulation of metazoan gene transcription is to identify ‘causative primary-regulated genes’, which are defined as genes that are directly regulated by a given TRF and whose regulation by that factor is essential for a given phenotypic change. Next, functional response elements for such genes must be identified. Finally, allosteric, compositional and enzymatic changes in their corresponding regulatory complexes upon change of context could reveal properties and mechanisms (such as a distinct pattern of functional, allosterically specified surfaces) that correspond to the particular phenotype. Importantly, the identification of molecular features that can serve as surrogates for complex physiological or pathological outcomes could form a basis for predicting different combinations of contexts or signals that produce or preclude a given outcome, as well as frame a new approach for screening and assaying therapeutic candidates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors thank the members of the Yamamoto laboratory for critical reading of the manuscript, with special note to Elaine Kirschke for insightful discussions, Samantha Cooper, Sheng-Hong Chen and Benjamin Schiller for use of unpublished data, and Kirk Ehmsen for use of unpublished data and assistance with Figure 3. E.R.W. is supported by US National Institutes of Health (NIH) predoctoral National Research Service Award (NRSA) 1 G31 GM 113397–01A1 from the National Institute of General Medical Sciences. M.T.K. is supported by NIH postdoctoral NRSA 5T32HL007731–20 from the National Heart, Lung, and Blood Institute and by NIH grant R01CA020535 from the National Cancer Institute. E.A.O. is supported by NIH grant R01DK095750 from the National Institute of Diabetes and Digestive and Kidney Diseases, by American Heart Association (AHA) grant 14GRNT20460124 and by a W.M. Keck Foundation Medical Research Grant. K.R.Y. is supported by NIH grants R01CA020535 from the National Cancer Institute and R21ES026068 from the National Institute of Environmental Health Sciences, and by grant MCB-161 5826 from the National Science Foundation.

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Transcriptional regulatory factors

(TRFs). A general class of sequence-specific DNA-binding proteins that regulate transcription (for example, glucocorticoid receptor).

Nuclear receptor

A member of a superfamily of potentially ligand-gated DNA-binding transcriptional regulatory factors.

Glucocorticoid

A natural hormone that binds to glucocorticoid receptor, or a synthetic derivative with physiological effects similar to the natural hormone, cortisol.

Dexamethasone

A synthetic glucocorticoid receptor (GR) ligand, developed in 1957, which is OG specific, unlike cortisol (the natural ligand), which also binds to mineralocorticoid receptor with high affinity, Dexamethasone is universally used clinically as an anti-inflammatory agent and immunosuppressant.

Apo-GR

Inactive glucocorticoid receptor (DR) protein in a ligand-unbound state.

Glucocorticoid response elements

(GREs). Genomic DNA segments (typically 0,5–2 kb long) that confer a specific glucocorticoid receptor response in particular contexts *in vivo.* Tire term ‘response element’ is appropriately unbiased with respect to potential activation (‘enhancement’) or repression of target gene transcription.

Allostery

Conformational changes in one region of a molecule (usually a protein) that alter its function and are induced by binding of a modulator to a different, remote site on the target molecule.

GR-binding sequence

(GBS). A short DNA sequence motif bound specifically and with high affinity by glucocorticoid receptor *in vitro.*

Nuclear magnetic resonance

(NMR). A technique that uses the magnetic properties of atomic nuclei to probe chemical environments experienced by atoms, for example, within a small molecule, protein or protein-DNA complex. See Supplementary information S1 (table).

3-Keto steroid receptors

Members of nuclear receptor subfamily 5 (NR3), including the glucocorticoid receptor (encoded by NR3 group C member 1 (*NR3C1*)), mineralocorticoid receptor (encoded by

NR3C2). progesterone receptor (encoded by *NR3C3*) and androgen receptor(encoded by *NR3C4*).

Epistatic mutations

Gene alterations that display a phenotype only in the context of another mutation.

Chromatin immunoprecipitation followed by sequencing

(ChIP-Seq). A technique to identify genomic segments occupied genome-wide *in vivo* by a particular antigen surface, such as a transcriptional regulatory factor epitope. See Supplementary information S1 (table).

GR-occupied regions

(GORs), Genomic DNA segments occupied by glucocorticoid receptor (GR) in particular contexts *in vivo.* COR terminology, typically identified by chromatin immunoprecipitation (ChIp), improves on the previously used GR-binding region (GBR or GRBR) nomenclature, which implies direct DNA binding rather than a broader proximity to DNA, the parameter measured by ChIp.

DNase I-hypersensitive sites

(DHSs), Short genomic regions that are cleaved by brief exposure to low concentrations of DNase I in permeabilized cells or isolated nuclei. See Supplementary information S1 (table).

Negative regulatory DNA sequence

(NRS). A short DNA sequence motif, under-represented at glucocorticoid receptor (GR)-occupied regions within the genome, that interferes with the ability of GR to functionally interact with DNA proximal to the motif.

Molecular dynamics simulations

A computer simulation method to model the physical movements of atoms within a macromolecule that occur over short, fixed time intervals, giving information about dynamics within a macromolecule. See Supplementary information S1 (table).

RU-486

A synthetic glucocorticoid receptor (GR) ligand, developed in 1980, which also has high affinity for progesterone receptor. As, a non-standard ligand, binding of RU-486 results, in both an altered GR conformation and a distinct pattern of transcription regulation compared to binding of standard glucocorticoids, such as, dexarnethasone and cortisol.

Selective GR modulators

(SCRMs). Glucocorticoid receptor (GR) ligands with a regulatory range distinct from that of the standard glucocorticoid ligands cortisol and dexarnethasone.

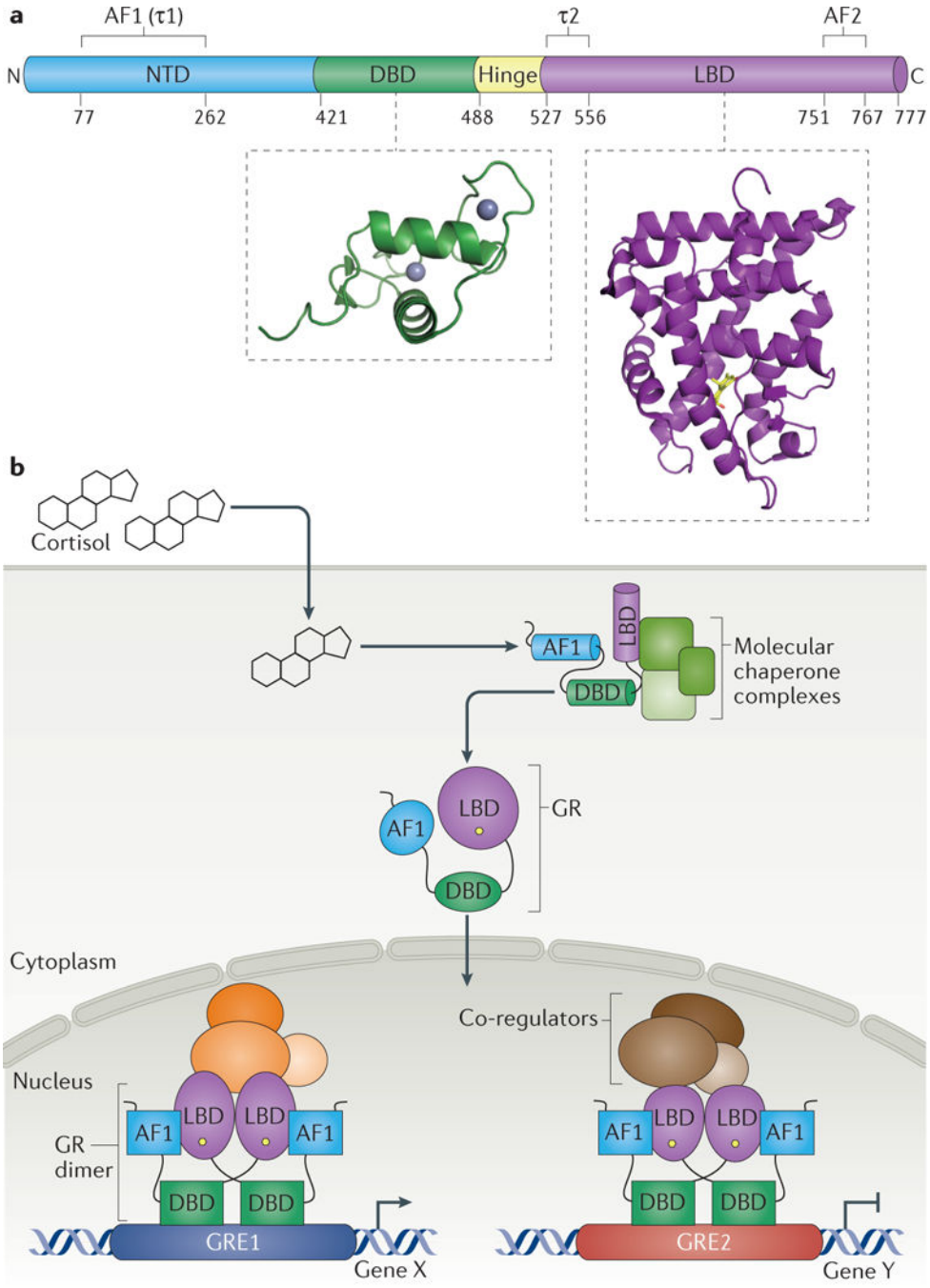

Figure 1 |. GR signalling and DNA binding.

a | Linear domain structure of glucocorticoid receptor (GR). GR comprises: the amino-terminal domain (NTD), DNA-binding domain (DBD), hinge region and ligand-binding domain (LBD). Embedded in these domains are segments that participate, context-specifically, in transcription regulation: activation function domain 1 (AFl,τ1),tau2 (τ2) and AF2. Insets: Crystal structures are shown for a single DBD (green; adapted from RCSB Protein Data Bank identifier (PDB ID): 1R4R[17]), with coordinated zinc ions shown as grey spheres, and for a LBD (purple) liganded with cortisol (yellow) and complexed with steroid

receptor co-regulator 2 (SRC-2) peptide (not shown) (PDB ID: 4P6X[61]). **b** | Overview of signalling mediated by the natural GR ligand, cortisol. Activating ligand interacts with monomeric GR associated with molecular chaperone-containing complexes in the cytosol. This induces local and remote allosteric changes that potentiate nuclear transport and other activities. Within the nucleus, GR nucleates multi-component transcription regulatory complexes containing various other transcriptional regulatory factors (TRFs) and transcriptional co-regulators at different glucocorticoid response elements (GREs) to activate or repress transcript ion of particular target genes. GRE1 and GRE2 represent distinct GREs within the genome, Gene X and Gene Y represent the genes under the control of GRE1 and GRE2, respectively.

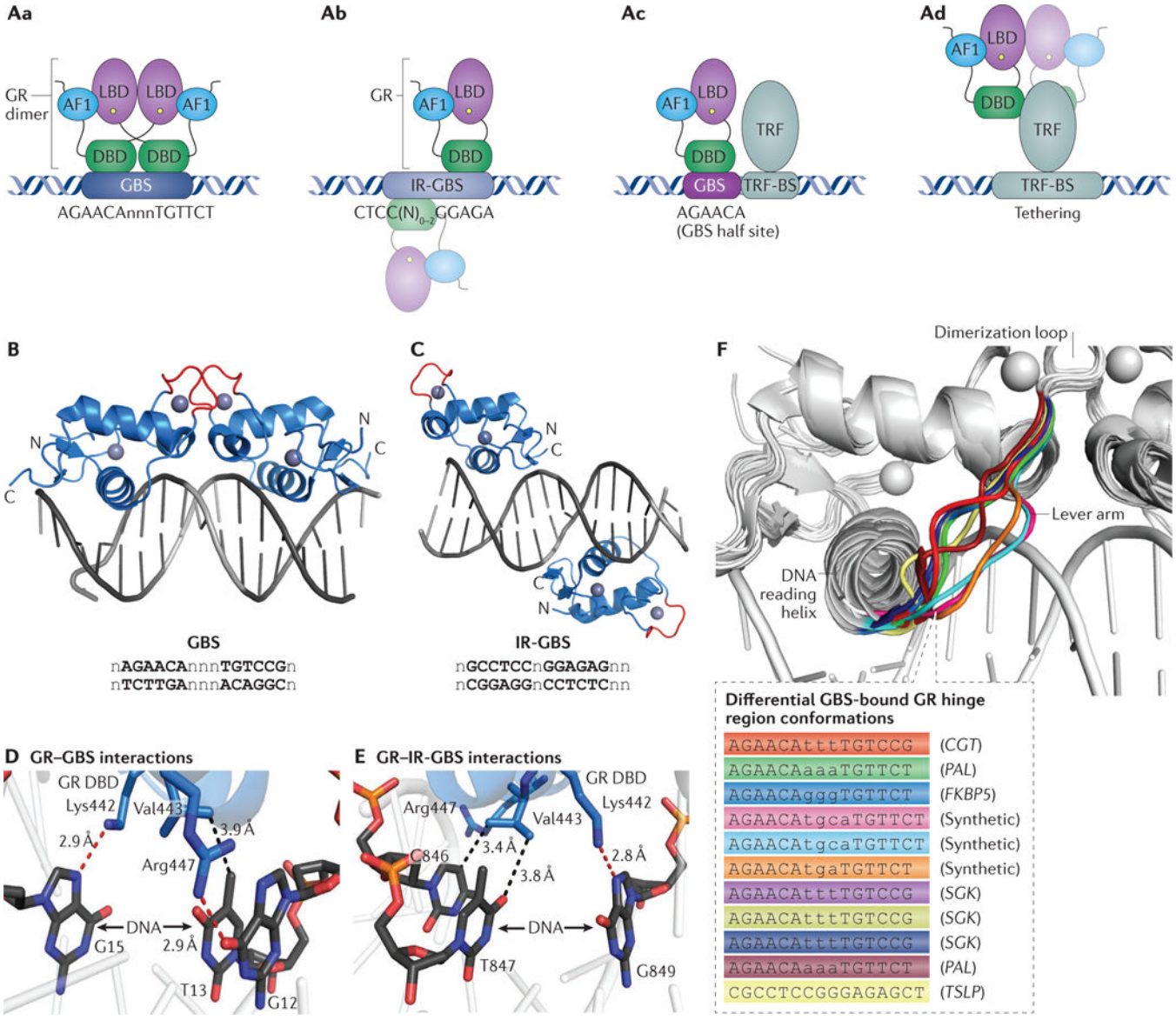

Figure 2 |. Modes of site-specific GR-genome interactions.

A | Glucocorticoid receptor (GR) associates with specific genomic sites in multiple ways. **Aa |** Two GR monomers bind a canonical GR-binding seguence (GBS) present in a glucocorticoid response element (GRE) in a head-to-head fashion; dimerization is achieved through interactions in the sister DNA-binding domains (DBDs). **Ab |** GR bindsto inverted-repeat GBSs (IR-GBSs).The crystal structure of this interaction (see also part **Ac)** shows two GR monomers bound to opposite sides of the DNA in a head-to-tail fashion; however, negative cooperativity argues that GR may bind as a monomer to IR-GBSs *in vivo* (thus, the second GR monomer is shown faded). **Ac |** GR can interact with GBS half sites; these elements typically contain a single hexamer related to the consensus seguence that is palindromic in the full **GBS and** may operate in conjunction with proximal non-GR transcriptional regulatory factors (TRFs), although there is no evidence for enrichment of particular TRF binding site (TRF-BS) motifs contiguous with the GBSs. **Ad |** GR can

interact at specific genomic sites without directly binding to DNA. Here, GR is tethered to a non-GR TRF through protein-protein interactions. The faded GR monomers depict uncertainty of whether monomeric or dimeric GR binds at tethering elements. **B|** Overall crystal structure of the DBD of GR with the canonical GBS(RCSB Protein Data Bank identifier (PDB ID: 3FYL[18]).C| Overall crystal structure of the complex between the DBD of GR and the IR-GBS (PDB ID: 4HN5 (REF. 25)). **D** | Zoom of structure shown in B.The GR DBD makesthree base-specific contacts within the major groove of the DNA at the GBS.This interaction is mediated by hydrogen bonds (red dashed lines) and van der Waals interactions (black dashed lines). **E** | Zoom of structure shown in C. The GR DBD makescontactsto an IR-GBSthat are similartothose made in the case of the canonical GBS, with the exception of Arg447, which does not make any contacts with the DNA. **F** | Superposition of all deposited structures of the interactions between DBD and DNA reveals especially striking conformational differences within the GR lever arm (residues 469–474, a loop connecting the ‘DNA-reading helix’with the dimerization region), demonstrating allosteric modulation of GR by DNA seguence. DNA seguences from each structure are listed and colour coded, the gene the seguence is derived from is listed in parenthesis (PDB IDs listed in the top-down order of the seguences given: 3FYL[18], 3G99 (REF. 18), 3G6U[18], 1GLU[17], 1R4O[17], 1R4R[17], 3G9P[18], 3G9O[18], 3G9M[18], 3G9J[18] and 4HN5 (REF. 25)). CGT, ceramide UDP-galactosyltransferase (also known as UGT8); FKBP5, FK506-binding protein 5 (also known as FKBP1B); LBD, ligand-binding domain; PAL, synthetic palindromic consensus site with AAA spacer; SGK, serum/glucocorticoid-regulated kinase 1; TSLP, thymic stromal lymphopoietin protein receptor (also known as CRLF2).

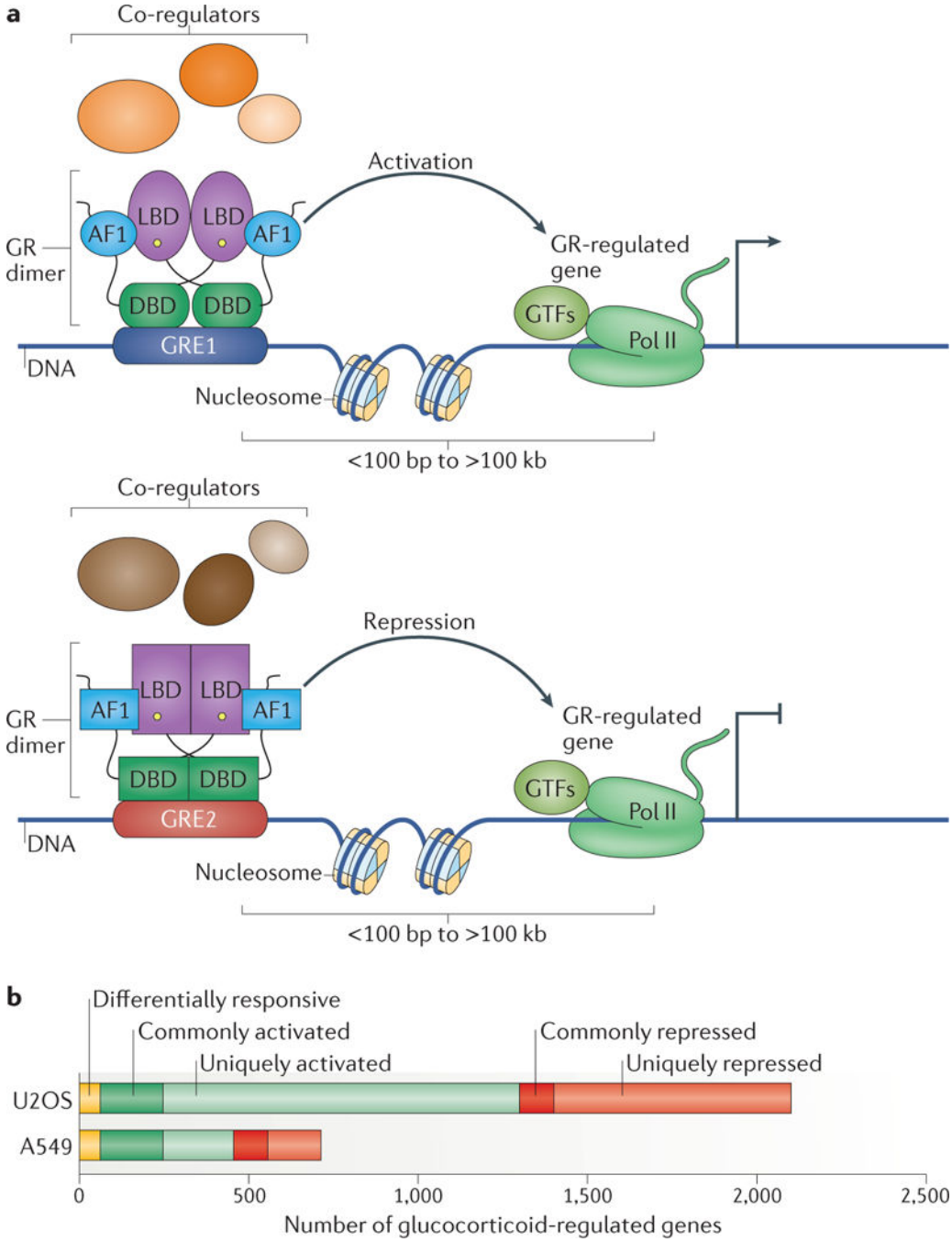

Figure 3 |. Context-specific GR occupancy and gene regulation.

a | Glucocorticoid receptor (GR)-regulated genes are commonly linked to multiple GR-occupied regions (GORs), usually >10 kb from the transcription start site of the regulated gene, one or more of which may be a functional glucocorticoid response element (GRE) for that gene. As shown here, GREs may be near (<100 bp) to or far (>100 kb) from their target genes. **b** | Illumina Human Ref8 beadchip analysis of glucocorticoid-regulated genes. Genes regulated by 4-hour, 100-nM dexamethasone treatment in lung carcinoma (A549) (K.R.Y., S. Cooper, S.-H. Chen and B. Schiller, unpublished observations) and osteosarcoma

(U20S)[21] cells. Differentially responsive genes are upregulated in one cell line and downregulated in the other. Common genes are similarly regulated in both cell lines. Unigue genes are regulated in one cell line and not regulated in the other. The abundance of genes within the unigue and differentially regulated classes demonstrates cell-context-specific regulation by GR. Differentially responsive genes demonstrate distinct mechanisms of regulation of target genes in the two cell types. AF1, activation function domain 1; DBD, DNA-binding domain; GTFs, general transcription factors; LBD, ligand-binding domain; Pol II, RNA polymerase II.

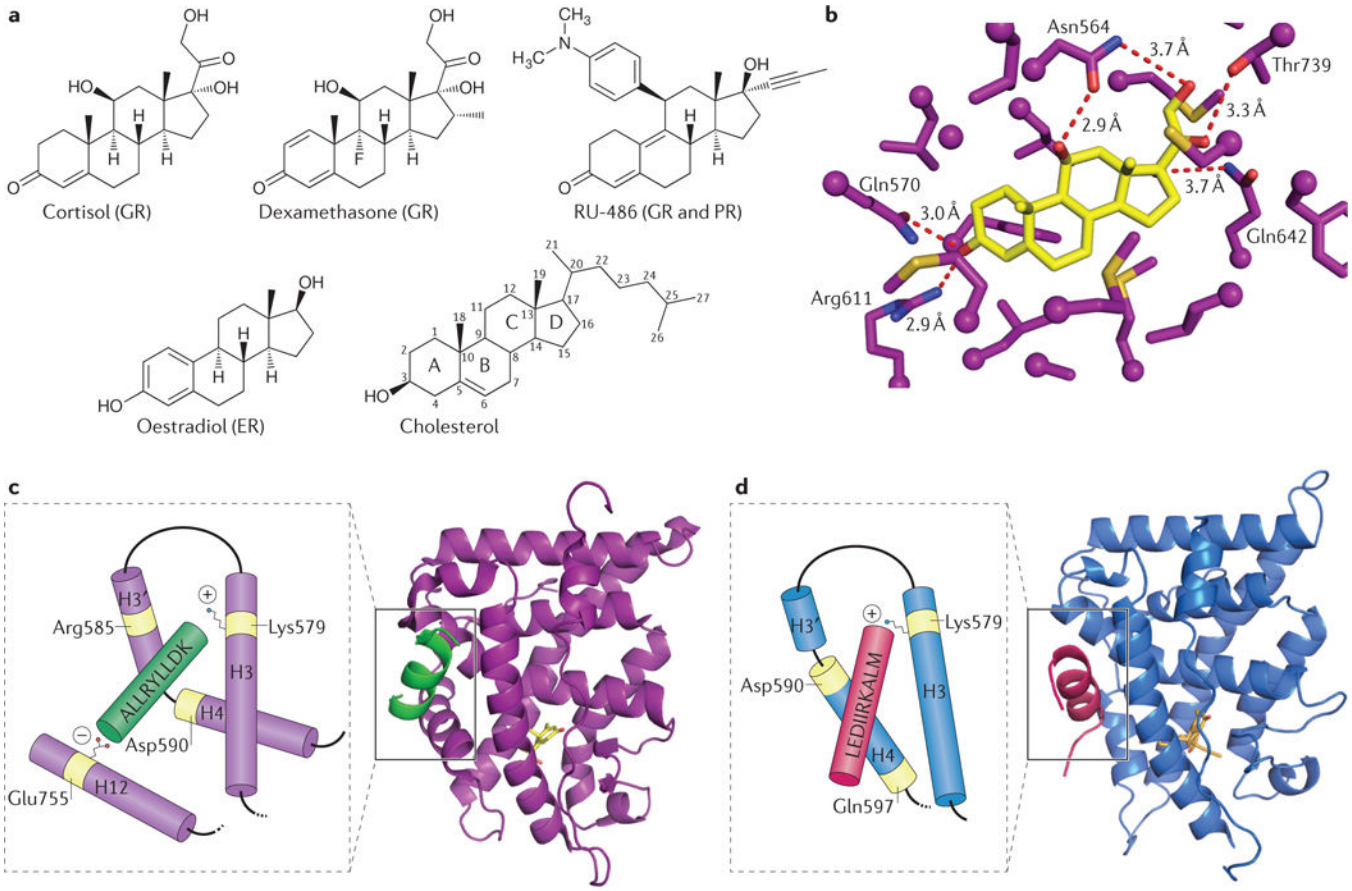

Figure 4 |. GR-ligand interactions.

a | Cortisol, dexamethasone and RU-486 are three glucocorticoid receptor (GR) ligands; RU-486 also binds to progesterone receptor (PR); oestradiol, which binds to oestrogen receptor (ER) but not GR, is shown for comparison; cholesterol is shown to provide the sterol carbon numbering convention. **b** | Ligand-binding pocket (purple) of the ligand-binding domain (LBD) of GR bound to its endogenous ligand, cortisol (yellow). Residues within 4.2 Å are shown, and hydrogen bonds are depicted by dashed red lines. This structure highlights the intricate network of interactions between GR and its ligand, as well as the amount of unoccupied space within the ligand-binding pocket with the ligand bound. This is a zoom of the ligand binding pocket from structure shown in panel **c. c** | Overall structure of the GR LBD (purple) bound to cortisol (yellow) and the steroid receptor co-regulator 2 (SRC-2) peptide (green) (RCSB Protein Data Bank identifier (PDB ID): 4P6X[61]).The inset cartoon represents how the LXXLL motif (the so called nuclear receptor box) of the co-regulator peptide interacts with GR. The peptide is held in place by a conserved charge clamp interaction; the positively charged Lys579 residue (+) and negatively charged Glu755 residue (−) on helix 3 (H3) and helix 12 (H12), respectively, mediate this interaction. There is an additional charge clamp that occurs through the residues Arg585 and Asp590. This is referred to as the active conformation, and is associated with specific co-regulator binding. **d** | Overall structure of the GR LBD bound to RU-486 (yellow) and the nuclear receptor co-repressor (NCoR) co-regulator peptide (pink) (PDB ID: 3H52 (REF. 164)). The inset cartoon represents how the extended co-repressor nuclear receptor (CoRNR) boxes of the co-

regulator peptide interact with GR. The extended peptide makes only one of the conserved interactions, through Lys579, and H12 is displaced. This is considered an inactive form of the LBD. Differences in GR conformations presented in parts **c** and **d** indicate that different ligands can promote the formation of alternative protein surfaces on GR that in turn differentially affect co-regulator binding.

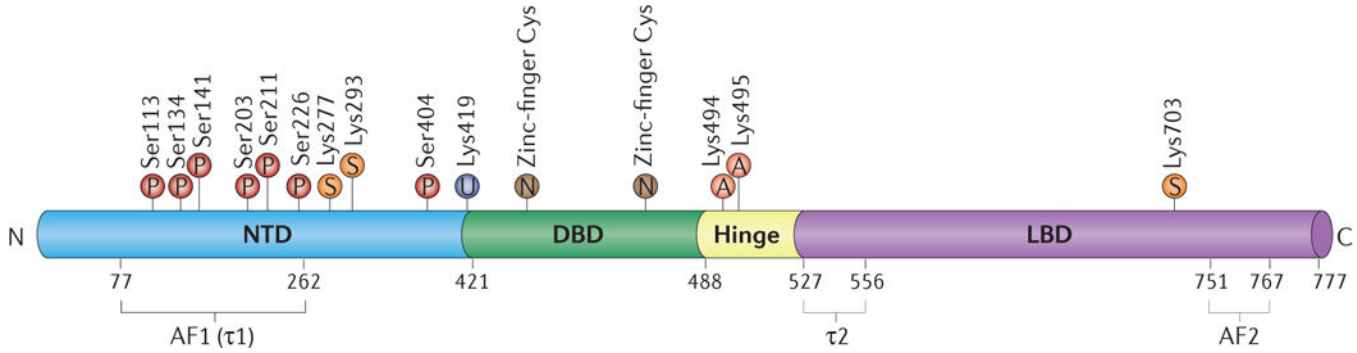

Figure 5 |. Sites of glucocorticoid receptor post-translational modifications.

Major reported modifications, including phosphorylation (P), sumoylation (S), ubiguitylation (U), acetylation (A) and nitrosylation (N), are mapped onto the glucocorticoid receptor domain schematic. AF, activation function domain; DBD, DNA-binding domain; LBD, ligand-binding domain; NTD, amino-terminal domain.

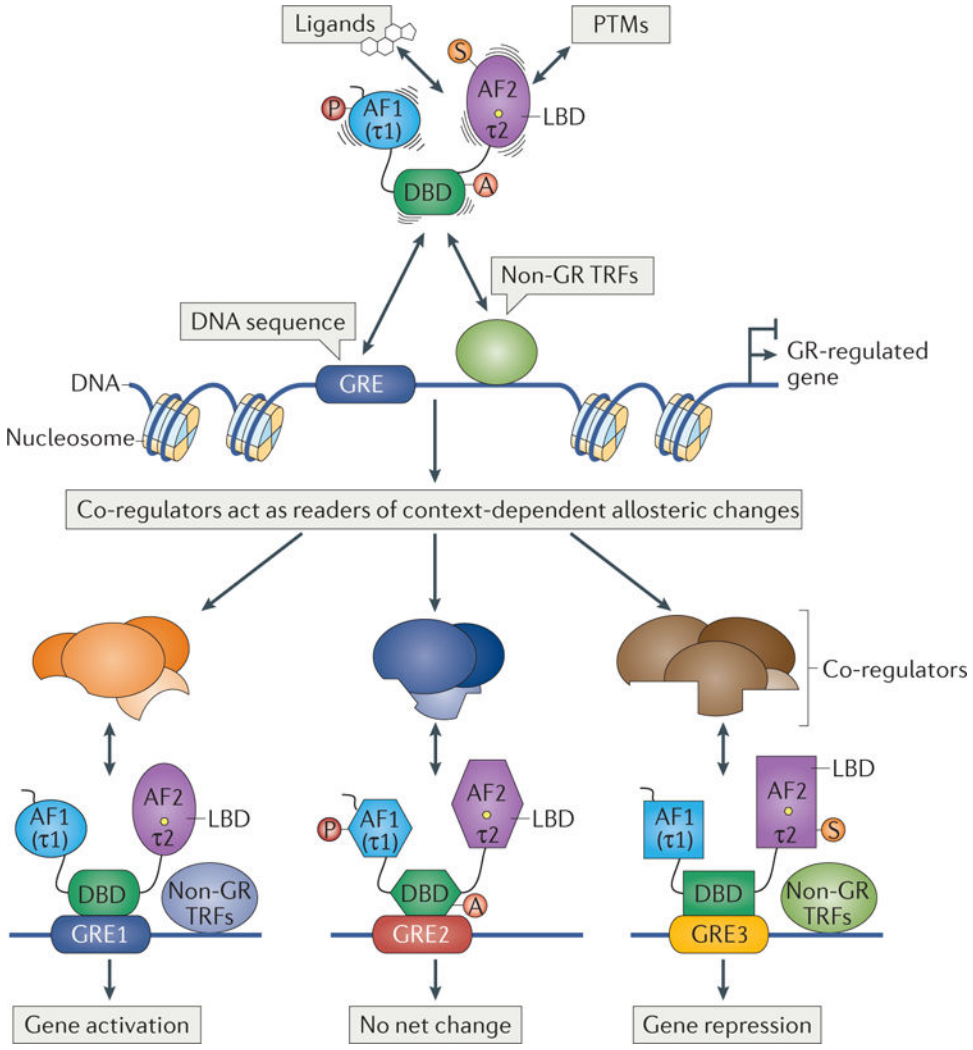

Figure 6 |. A model for transcription regulation: precision and plasticity of TRF function achieved via allostery.

In our model for metazoan transcription regulation, transcriptional regulatory factors (TRFs), such as glucocorticoid receptor (GR), may lack intrinsic regulatory activities, serving instead as molecular scaffolds that can assume different conformations in response to modification by different combinations of specific signalling inputs. Four classes of signalling input are described: ligands and post-translational modifications (PTMs) provide physiological context, DNA-binding seguences provide gene context, and non-GRTRFs provide cell context. Each class of signal confers distinct allosteric effects, and their integrated actions can produce a vast range of GR conformations, which induce, expose or stabilize context-specific proteinsurfacesthat are read by co-regulators. These co-regulators, which are generally large multi-component enzyme-containing complexes, interact in distinct combinations with patterns of cognate GR surfaces and enzymatically modify the general transcriptional machinery and/or surrounding chromatin in and around glucocorticoid response elements (GREs) and target promoters and genes, resulting in the activation or repression of gene expression. Upper panel: lines around GR domains depict direct and allosteric conformational alterations imposed by the signalling inputs. Note that a

lack of net regulation (that is, neither activation nor repression) could reflect balanced actions of one or more GREs acting on a single gene (not shown). AF, activation function domain; DBD, DNA-binding domain; LBD, ligand-binding domain. Circles with letters A, P,S indicate different PTMs.

Table 1

Five functional classes of co-regulators reported to interact with GR

Functional class	Examples	GR-interaction surfaces	Targets and regulatory outcomes	Refs
Structural and enzyme-interacting	pl60 SRC family: • SRC-1 (also known as NCoA-1) • SRC-2 (also known as NCoA-2, TIF2 and GRIP1) • SRC-3 (also known as NCoA-3, CIP, AIB1 and TRAMl)	LXXLL motif interacts with AF2 and bHLH-PAS domain interacts with AF1	• Transcription activation best characterized; but at least SRC-2, at can also repress gene expression in appropriate contexts • Interaction increasesα-helical content of AF1, increasing its structural stability	29,100, 109, 116–134
	Mediator	LXXLL motif within MED1 interacts with the LBD of GR, and MED14 interacts with AF1	• Recruitment and allosteric regulation of transcriptional machinery, including RNA polymerase II and TFIIH • Recruitment of multiple enzymatic activities, including phosphorylation and acetylation (at SerlO and Lys 14, respectively) of histone H3 through associated kinase CDK8 and HATGCN5L	135–138, 140–143
	HIC-5 (also known asTGFB1|1)	HIC-5 binds to the tau2 domain within the GR hinge	• Transcription complex assembly and Mediator recruitment • May block chromatin remodelling • Differently affects DNA binding and transcription regulatory activity of different gene classes	112,113
	COCOA	Binds indirectly via interaction with the amino terminus of pl60	• Acts synergistically with pl60	119,120
	CCAR1	Directly bindsto GR or is indirectly recruited by COCOA	• Recruits Mediator and p160to response elements	121
Chromatin-remodelling	BRG1 (also known as SMARCA4) and BRM SWI/SNF-related ATPases	Multiple BAF subunits interact withthe DBD, LBD and AF1 in different contexts	• ATP-dependent repositioning of nucleosomes • Relieves repressive state of chromatin • Multiple context-specific effects of BRM knockdown on GR-dependent genes	146–153
Met hyltransferases	CARM1 (also known as PRMT4)	Binds indirectly via interaction with carboxy terminus of pl60	• Methylates histone H3 and non-histone proteins *in vitro* • Activates SRC-2-dependent genes	120,154
	Lys methyltransferase G9a (also known as EHMT2)	N-terminal portion of G9a binds to the GR NTD	• Positive regulation by enhanced recruitment of CARM1 and p300to GR target genes • Facilitates methylation of Lys9 on H3, which is associated with transcription repression	155
HATs	CBP/p300	Binds directly to AFl or indirectly through partner pl60-AF2 interations	• Acetylation of histones • Cell-type-specific transcriptional outcomes	100,117, 124,157, 158,160, 165
	PCAF (in ADA and SAGA	Binds directly to AFl or indirectly through either partner pl60-	• Acetylation of histones	100,159, 166

Functional class	Examples	GR-interaction surfaces	Targets and regulatory outcomes	Refs
	complexes)	AF2 or CBP/p300-pl60-AF2 interactions	• Less well defined, but contains TAF subunits, which have been postulated to aid in the recruitment of general transcription factors to DNA	
HDACs	NCoR, SMRT	• (L/I)XX(I/V)I or LXXX(I/L)XXX(I/L) CoRNR motif interacts with AF2 when GR is bound to RU-486 • NCoR and SMRT interact with NTD in GR bound to IR-GBS or tethered GR when GR is bound to a standard ligand	• Transcription repression correlated with histone deacetylation in most cases	24,92,93, 128,163, 164,167, 168

ADA, Ada acetyltransferase; AF,activation function domain; BAF, BRG/BRM-associated factor; bHLH-PAS, basic helix-loop-Fielix-Per/ARNT/Sim; BRM, BraFima; CARM1, coactivator-associated Arg metFiyltransferase 1; CBP, CREB-binding protein; CCAR1, cell division cycle and apoptosis regulator 1; CDK8, cyclin-dependent kinase 8; COCOA, coiled-coil coactivator; CoRNR, co-repressor nuclear receptor; DBD, DNA-binding domain; GBS, GR-binding seguence; GR, glucocorticoid receptor; FIAT, hi stone acetyltansferase; HDAC, histone deacetylase; IR-GBS, in verted-repeat GBS; LBD, ligand-binding domain; MED, Mediator of RNA polymerase II transcription subunit; NCoR, nuclear receptor corepressor; NTD, N-terminal domain; PCAF, p300/CBP-associated factor; SAGA, Spt-Ada-Gcn5-acetyltransferase; SMRT, silencing mediator of retinoic and thyroid receptors; SRC, steroid receptor co-regulator; TAF, TBP-associated factor; TFIIH, transcription factor II human.