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Review

Glucocorticoid receptor signaling: intricacies and therapeutic opportunities

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The glucocorticoid receptor (GR) is a major nuclear receptor (NR) drug target for the treatment of inflammatory disorders and several cancers. Despite the effectiveness of GR ligands, their systemic action triggers a plethora of side effects, limiting long-term use. Here, we discuss new concepts of and insights into GR mechanisms of action to assist in the identification of routes toward enhanced therapeutic benefits. We zoom in on the communication between different GR domains and how this is influenced by different ligands. We detail findings on the interaction between GR and chromatin, and highlight how condensate formation and coregulator confinement can perturb GR transcriptional responses. Last, we discuss the potential of novel ligands and the therapeutic exploitation of crosstalk with other NRs.

Highlights

By integrating technologies that study chromatin conformation, accessibility, modification, and coregulator recruitment, the molecular basis of the cell-type-specific actions of glucocorticoids (GCs) can be increasingly understood.

Therapeutic exploitation of crosstalk between nuclear receptors is gaining traction and has offered strategies to sensitize cells to GCs.

Conformational analyses of the glucocorticoid receptor (GR) [via hydrogen–deuterium exchange mass spectrometry (HDX-MS)] are offering a biophysical basis for ligand-binding domain (LBD)–DNA-binding domain (DBD) interdomain communication, the degree of which differs depending on the ligand.

Chromatin remodeling complexes are essential to finetune GR activity, not only for transcriptional activation but also for transcriptional repression.

Condensate formation is emerging as an important determinant of GR complex assembly on chromatin.

The intricacies of GR signaling

Endogenous glucocorticoids (GCs), such as cortisol in humans and corticosterone in rodents, are stress hormones that regulate many physiological processes including metabolism, development, mental health, cognition, and immune and inflammatory responses [1]. Following their discovery in the 1940s, synthetic variants such as dexamethasone (Dex) and prednisolone were developed with improved potency and half-lives, and are still a mainstay in the treatment of inflammatory and autoimmune disorders and various cancers [2].

GCs activate GR, a transcription factor (TF), and NR (Box 1) [3]. Apo-GR is mainly cytoplasmic and is associated with a chaperone complex, which exhibits a cyclical pattern in composition and determines the receptor's ligand-receptive state [4–8]. Following ligand binding, this chaperone cycle culminates in the nuclear translocation of GR, where the receptor fulfills two major genomic actions (Figure 1, top) [1]. First, GR can activate target genes containing a GC response element (GRE) in their regulatory regions by binding these GREs as a homodimer (Figure 1, middle left). This mechanism drives the cell-type-specific expression of anti-inflammatory and/or proapoptotic genes. Unfortunately, genes driving gluconeogenesis or lipogenesis pathways are also induced, causing metabolic side effects [9]. Second, GR can interact with proinflammatory or prosurvival TFs (NF-κB, AP-1, STAT) to repress their transcriptional activity, originally proposed to be mediated by GR monomers [10,11], while recent evidence supports a role for GR dimers (Figure 1, bottom left) [12,13]. In addition, GR may repress transcription by binding of GR monomers to inverted repeat negative GREs (IR-nGREs) (Figure 1, middle right) [14]. Nonetheless, GR's oligomerization status remains controversial. GR monomers were claimed to have no physiological role [15], while higher-order oligomers, such as GR tetramers, would be predominant [16,17], although the latter model awaits evidence in an endogenous context. Analogously, there is also ongoing debate on whether GR needs to contact DNA directly to perturb the activity of other TFs [18–20], or whether binding to other DNA-bound TFs, known as tethering, is predominant [21]. In support of the former, cryptic GREs within NF-κB or AP-1 response elements were identified as a crucial mechanism for

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Box 1. Glucocorticoid receptor domains and isoforms

The GR is a ubiquitously expressed member of the NR superfamily. GR is encoded by *NR3C1* and is most closely related to the MR, AR, ER, and PR, which also belong to the steroid receptor family [3]. Alternative splicing of *NR3C1* leads to the expression of five different GR isoforms (GRα, GRβ, GRγ, GR-P, and GR-A), of which GRα is the predominant isoform and mediates the majority of GC actions. Exon 2 contains eight highly conserved AUG start codons, which results in the existence of eight translation initiation variants of GRα (also known as N-terminal proteoforms), termed GRα-A, -B, -C1, -C2, -C3, -D1, -D2, and -D3 [2].

Most members of the NR family share a similar domain organization that consists of an NTD, a central DBD, a flexible hinge region (HR), and a C-terminal LBD [1,3]. The NTD is intrinsically disordered and is the least conserved among the different NRs. It contains the first activation function (AF-1) and is crucial for (mostly ligand-independent) recruitment of coregulators and proteins of the basal transcriptional machinery [1]. Most known PTM sites of GR are located in this domain. The central DBD is highly conserved and contains two zinc fingers, separated by a flexible lever arm. The first zinc finger is responsible for DNA-binding via the proximal box (P-box), which interacts with the major groove of the DNA. The second zinc finger is responsible for dimerization via the distal box (D-box), by which homodimers and heterodimers can be formed [3]. The lever arm allows structural flexibility and can communicate conformational input between the DNA-binding and dimerization regions of NRs [1]. The DBD also contains the first nuclear localization signal (NLS1), a nuclear retention signal (NRS), and a nuclear export signal (NES), which all affect GR shuttling between the cytosol and the nucleus [1]. The most C-terminal domain is the LBD, which is separated from the DBD by a flexible HR and is also highly conserved among the NRs. It harbors a hydrophobic ligand-binding pocket (LBP) and is responsible for the ligand-dependent recruitment of NR coregulators. This is coordinated by the most C-terminal helix of the LBD (helix 12), which comprises the second activation function (AF-2). GR's LBD also contains a second dimerization interface, which is unique within the NR superfamily [30], and NLS2.

GR-mediated gene repression of inflammatory genes and were also proposed for STATs (Figure 1, middle right) [22].

Regardless of the mechanism, prolonged use of exogenous GCs is associated with many side effects and therapy resistance, exemplifying the need for alternative GR-based targeting strategies. To identify routes towards enhanced therapeutic benefit, we need to better understand and consider all factors that contribute to the transcriptional activity and functional effects mediated by GR. To this end, five emerging concepts are discussed in this review: (i) the impact of the ligand on domain communication and GR conformation [23]; (ii) the intricacies related to chromatin binding of GR; (iii) the role of coregulators and **condensate formation** (see Glossary) [24,25]; (iv) the importance of crosstalk between GR and other NRs, and the impact on therapy responsiveness [26,27]; and (v) the potential of selective GR ligands and GC–antibody drug conjugates as novel GC-based therapeutic strategies.

How GR domains dimerize and allosterically communicate

Over the past 5 years, new structural insights have emerged from studying GR dimerization or oligomerization mechanisms and the binding of structurally different ligands and coregulators (Figure 2 and Box 2). From the theoretical analysis of all available GR ligand-binding domain (LBD) crystal structures, one dimer, in which the α-helix (H)9 assembles in an antiparallel way (the apH9 dimer), has emerged as the most stable architecture with highly conserved residues at the interface [28]. Remarkably, none of the dimerization surfaces of GR LBD crystals resembled the extensive, stable, and conserved 'butterfly-like' head-to-head dimer seen in NRs [28]. Moreover, antagonist binding resulted in completely different GR LBD dimers compared to agonist binding. Additionally, the team of Estébanez-Perpiñá concluded that GR exhibits complex multimerization behavior due to the existence of 20 topologically different GR LBD homodimers [29]. In 2023, the first high-resolution multidomain structures combining GR [DNA-binding domain (DBD)–hinge–LBD] bound to the nonsteroidal selective modulator velsecorat or the steroidal agonist fluticasone furoate, with the GRE of the target gene *SGK1* and the coregulator peptide PGC1$\alpha_{134-154}$, were assembled and revealed a head-to-tail LBD dimer placed asymmetrically

Glossary

AlphaFold2: an artificial intelligence-based approach to predict 3D protein structure starting from the amino acid sequence.
BRG1 (SMARCA4) and BRM (SMARCA2): the two possible catalytic subunits of the SWI/SNF chromatin remodeling complex, which assists in nucleosome shifts and exchanges. Activity of the SWI/SNF complex is a crucial factor in determining which chromatin regions can be bound by other TFs.
COMPASS complex: a family of histone lysine methyltransferases, also known as the SET1/MLL or the KMT2 complexes, that catalyze methylation of histone H3K4. These complexes contain one of six catalytic subunits, each with their own substrate specificity: MLL1/2 (KMT2A/2B), MLL3/4 (KMT2C/2D), or SETD1A/SETD1B (KMT2F/2G). Depending on the catalytic subunit, the composition of the complex is different.
Glucocorticoid binding sequence (GBS): an overarching term for all types of GR DNA recognition sites, typically revealed via ChIP sequencing technology. This includes canonical GREs (consensus sequence: AGAACAnnnTGTTCT), IR-nGREs [CTCC(N)$_{0-2}$GGAGA], cryptic GREs, and half-site GREs.
Hydrogen–deuterium exchange coupled to mass spectrometry (HDX-MS): a technique that allows the study of protein conformational changes that are not accounted for or observable in crystal structures, by analyzing the rate of hydrogen–deuterium exchange under different conditions (ligands, peptides, and mutations).
Nuclear receptor crosstalk: the interplay between different NRs that can result from an indirect mode of regulation, such as competitive binding of DNA or coregulators, or a direct physical interaction and the formation of hetero(di)mers, resulting in a unique signaling and gene expression profile.
Polyamidoamine (PAMAM): polyamidoamine dendrimers are hyperbranched polymers that can function as nanocarriers to deliver drugs or other carriers.
Protein condensate formation: a biophysical phenomenon that results from multivalent interactions between proteins, increasing local protein concentrations and driving the assembly of the protein complex [24]. The presumed underlying mechanism is

on the head-to-head DBD dimer (Figure 2, middle) [30]. This LBD dimer shows a structurally distinct arrangement in comparison to previously reported dimers of sole GR LBD described earlier.

To study the physiological consequences of GR dimerization status, the Libert team generated a mouse model with a dimerization-defective double mutant (A458T/I628A in humans; A465T/I634A in mice; also called GRmon). This model showed a complete loss of function due to insufficient ligand binding, reduced DNA binding, and reduced gene expression, shedding light on the pivotal role of I628 in both GR dimerization and ligand binding [31]. In search of the potential impact of GR dimerization state on its turnover rate, a lack of dimerization was found to slow down GR turnover [32].

As well as the influence of dimerization on GR structure, the binding of ligands and coregulators gives rise to dynamic changes and allosteric effects. Computational analysis followed by comparison of protein cavities revealed two clusters of active pockets within GR's LBD, one corresponding to the canonical pocket bound by steroidal ligands, and one that binds larger nonsteroidal **selective GR agonists and modulators (SEGRAMs)** by enlarging the pocket's dimension via the repositioning of R611 (Figure 2, top) [33]. In addition, the efficacy of the ligands correlated with the strength of the allosteric communication between the ligand-binding and coregulator-binding pockets, with the Dex-bound system showing the highest efficacy-related conformational changes [34,35]. Among a class of ligands that share a common pharmacophore, the allosteric transmission pathway is maintained, although minor modifications in ligand structure can affect the allosteric free energy. By contrast, different ligand classes alter this allosteric communication pathway [36]. Intriguingly, a study using **hydrogen–deuterium exchange mass spectrometry** (**HDX-MS**) and fluorescence polarization (FP) revealed ligand-specific (velsecorat, fluticasone furoate, or Dex) communication between GR LBD and DBD domains even in the absence of DNA (Figure 2, top) [30]. DNA itself can also act as an allosteric ligand of GR, exemplifying another layer of control over target gene transcription [37,38]. Ortlund's team uncovered that both DNA-bound and DNA-free GR DBDs share a similar structure while displaying differences in the lever arm and D-loop. DNA binding and dimerization lead to sequence-specific reductions in the number of lever arm conformations [38]. Additionally, the coregulator peptide sequence was identified as an allosteric regulator of the receptor's response to ligand binding (Figure 2, top). The coregulator peptides of nuclear receptor coactivator 2 (NCOA2) and PGC1α affect the dynamics and stability of LBD helix 12 differently, which is important in transmitting the allosteric signal. The PGC1α peptide stabilizes the agonist-like conformation of helix 12 more efficiently than the NCOA2 peptide, which contributes to our understanding of the bidirectional allosteric crosstalk between the ligand-binding and coregulator-binding pockets [35,36,39]. Altogether, these insights impact and aid the design of novel ligands with different activities.

Deciphering GR chromatin code

While the consensus sequences of **glucocorticoid binding sequences (GBSs)** such as GREs and IR-nGREs are well characterized [1], knowledge about the assembly of GR-associated transcriptional complexes and the coregulators involved is still incomplete. Since GR primarily binds enhancers (Figure 3A) [40,41], it requires the formation of a chromatin loop to mediate transcriptional regulation (Box 2). Hi-C experiments in A549 lung epithelial carcinoma cells showed that Dex treatment does not evoke *de novo* chromatin interactions but rather changes the frequency of pre-existing ones [42], which was observed for both GR-induced and GR-repressed genes (Figure 3) and coincided with increased cohesin complex occupancy [43]. Groups of GR-activated or GR-repressed genes also exhibit spatial separation into **topologically associated chromosomal domains** (**TADs**) [44]. In addition, GR was initially thought to solely bind nucleosome-depleted regions previously occupied by **BRG1**, one of the catalytic subunits of the switch/sucrose nonfermentable (SWI/SNF) complex (Box 2). However, recent studies have

liquid–liquid phase separation (LLPS) driven by IDRs within proteins, although this is heavily debated [105–107].

Selective GR agonists and modulators (SEGRAMs): GR ligands displaying strong therapeutic effects (e.g., anti-inflammatory or anticancer) and an improved side effect burden. The agonists have a steroidal scaffold, while the modulators have a nonsteroidal nature. SEGRAMs can be further divided into SEDIGRAMs and SEMOGRAMs (selective dimerizing or monomerizing GR agonists and modulators) that may be useful in acute or chronic inflammatory conditions, respectively, as they are hypothesized to differentially steer the dimerization status of GR compared to classic GCs.

Super enhancers: a large cluster of regulatory regions with high levels of TF binding.

Topologically associated chromosomal domains (TADs): self-interacting domains in the genome. Regions within a TAD are expected to interact with each other more than regions outside the TAD, and have been proposed as structural scaffold for regulatory landscapes.

Triple-negative breast cancer (TNBC): breast cancers that do not express ER, PR, and human epidermal growth factor receptor 2 (HER2).

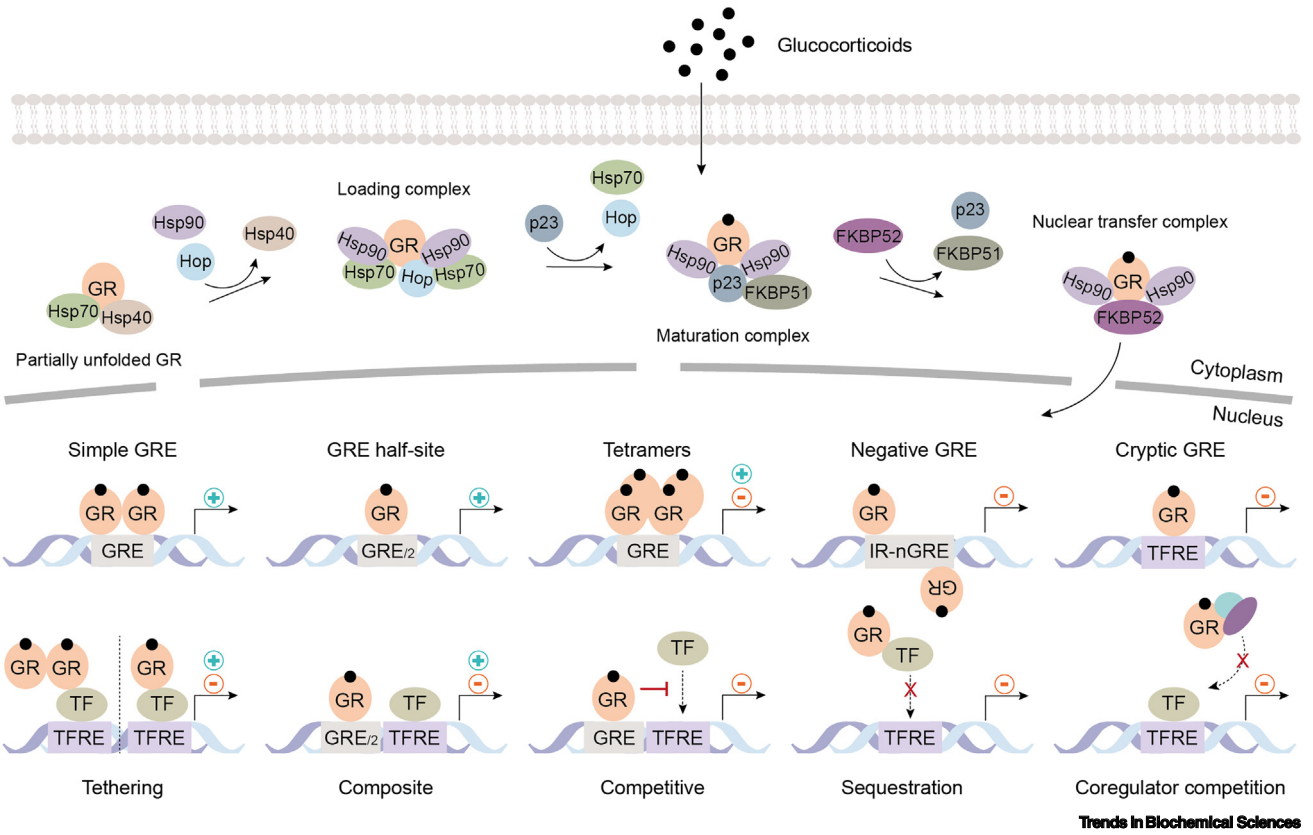

Figure 1. Diverse action mechanisms of the glucocorticoid receptor (GR). In the ligand-unbound state, GR mainly resides in the cytoplasm, where the receptor is partially unfolded by a Hsp70–Hsp40 chaperone complex, thus preventing ligand binding. Hsp70 then instigates the assembly of an inactive GR–Hsp70–Hsp90–Hop loading complex, followed by reshuffling to a GR–Hsp90–p23 maturation complex, in which GR adopts a folded, ligand-receptive conformation stabilized by Hsp90 and p23. Following ligand binding, release of p23 and an interchange of the cochaperone FKBP51, which likely binds Hsp90 in the maturation complex, with FKBP52, finally allows the formation of an Hsp90–GR–FKBP52 nuclear transfer complex. The chaperone cycle culminates in nuclear translocation of GR. Being a transcription factor (TF), GR can mediate gene activation or gene repression, the latter by interacting with other TFs such as NF-κB and AP-1, thereby inhibiting their activity. A plethora of underlying mechanisms underpinning activation and repression of GR have been resolved so far. Some require direct DNA binding of GR, while in others, GR tethers to another TF that contacts DNA or even sequesters the TF. Abbreviations: GRE, glucocorticoid response element; GRE/2, GRE half-site; TFRE, transcription factor response element.

indicated that Dex-activated GR also binds a subset of GBSs located in nucleosomal chromatin prior to BRG1 recruitment (Figure 3A) [40,41,45]. Although GR-mediated BRG1 recruitment occurred exclusively at GR-activated genes, the inhibition of BRG1 activity reduced both GR-mediated transcriptional activation and repression [40,45]. Taken together, these findings show that the spatial organization of the genome contributes to the direction of GR's transcriptional response.

Deciphering the dynamic behavior of GR's interaction with chromatin is another determinant in understanding GR action and was pioneered by Hager's team using single-molecule tracking [46,47]. In their recent work, repetitive switching between two states of lower mobility was identified for TFs including GR [48,49]. The states with the lowest mobility corresponded to specific DNA-binding events, while the intermediate, slow diffusion state was dependent on the N-terminal transactivation domain (NTD), which may hint at a role in GR condensate formation [48]. The Schaaf group confirmed the dynamic behavior of GC-bound GRs in the nucleus, transitioning between a high-mobility state, a slow diffusion state allowing for brief nonspecific DNA interactions, and an immobile state where GR specifically binds to target gene DNA [50].

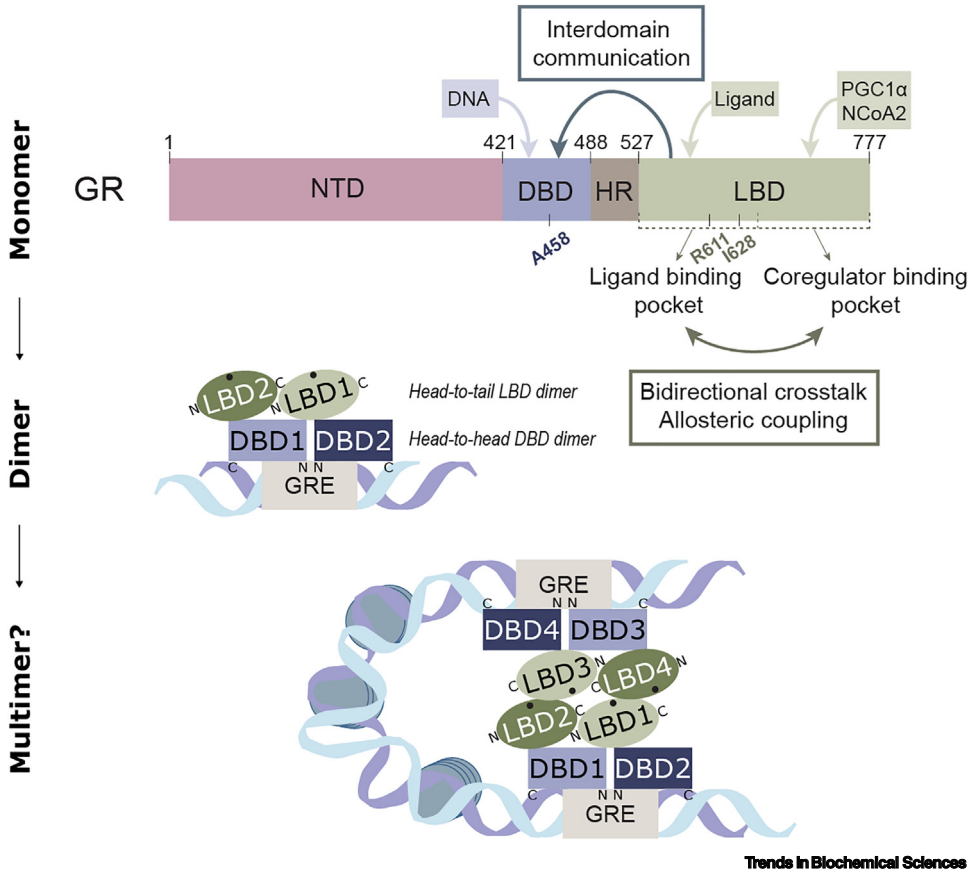

Figure 2. Allosteric communication within the GR structure and its new dimer interface. GR consists of four domains: the NTD, DBD, HR, and LBD. DNA is proposed as an allosteric modulator of DBD conformation. Analogously, binding of different ligands and coregulators alters the conformation of the LBD in different ways. In the LBD, there is a ligand- and a coregulator-binding pocket that exhibit bidirectional crosstalk with each other. In addition, interdomain communication emerges from the LBD towards the DBD upon ligand binding. The repositioning of R611 generates enlarged pocket dimensions, allowing larger compounds to bind. The residues A458 (DBD) and I628 (LBD) are essential in the dimer interface, where a double mutant leads to complete loss of function. The new proposed LBD dimer shows a head-to-tail architecture. Higher oligomeric states of GR are still being questioned. However, a plausible explanation is the formation of tetramers (or higher orders) that bring distal GBSs together. Abbreviations: DBD, DNA-binding domain; GBSs, glucocorticoid binding sequences; GR, glucocorticoid receptor; LBD, ligand-binding domain; NTD, N-terminal domain; HR, hinge region.

Similar mobility states were uncovered for the androgen receptor (AR) [51]. The repetitive switching of GR between DNA-binding modes thus underpins an efficient target searching strategy.

The transcriptional activity of GR is also highly cell-specific, with minimal overlap in GR-occupied GBSs between cell types [52,53]. Lock and colleagues found that in Dex-treated GC-sensitive acute lymphoblastic leukemia (ALL) cells, GR cooperates with CTCF, a crucial factor in shaping chromatin conformation. CTCF enabled interactions between promoters and lymphocyte-specific enhancers by triggering DNA looping and consequently enabled recruitment of the transcriptional machinery. By contrast, in GC-resistant ALL cells and cells of nonlymphoid origin, these enhancers were condensed and inaccessible [54]. A similar role was revealed for CTCF in skeletal muscle, where it controls tissue-specific gene programs by facilitating chromatin looping, allowing contacts between the gene promoters and GR, and MYOD1's co-occupied enhancers [55]. In A549 cells, however, CTCF was not involved in increasing the chromatin looping frequency upon Dex treatment

Box 2. Coregulators: masters of transcription

Chromatin is organized into nucleosomes, which consist of 145–147 base pairs wrapped around a histone octamer [108]. The cell-type-specific organization of chromatin is a substantial driver of the different functional effects of GCs, as condensed chromatin is generally inaccessible for the binding of the GR. The local organization of chromatin can be altered by chromatin remodelers such as the switch/sucrose non-fermentable (SWI/SNF) complex [also known as the **BRG1**/**BRM**-associated factor (BAF) complex], which hydrolyzes ATP to reposition nucleosomes across the DNA [109]. Additionally, chromatin modifiers such as HATs and KMTs catalyze histone PTMs to modulate the strength of histone–DNA interactions. Histone PTMs also serve as recognition sites for other proteins [108]. H3K4 methylation and H3K27 acetylation, catalyzed, respectively, by KMT2 complexes and p300/CBP, are two hallmarks of transcriptionally active promoters and enhancers, and are therefore associated with transcriptional activation [56,110]. The interactions of TFs with histone deacetylases (HDACs) are generally associated with transcriptional repression [111], while the transcriptional effect of histone lysine demethylases (KDMs) depends instead on the histone marker under study.

Chromatin-remodeling and -modifying complexes are well-known GR coregulators, as they assist in GR-mediated regulation of DNA transcription. Other well-known GR coregulators are the NCOA1, -2, and -3 (SRC1, -2, and -3) coactivators, which interact with GRs via an LxxLL motif. They serve as platforms for the recruitment of HATs [112]. By contrast, binding to inverted repeat negative GREs (IR-nGREs) or tethering sites leads to the recruitment of corepressors such as NCOR1 and -2 (NCOR and SMRT), which serve as platforms for HDAC recruitment [111]. Various coregulators such as NCOA2 can act as either activators or repressors, depending on the cellular context, the PTM status, the engaged TF, or the chromatin context.

GR DNA binding and coactivator recruitment lead to the recruitment and assembly of the RNA polymerase II (Pol II) preinitiation complex (PIC) near the TSS of the downstream target gene [113]. In the case of enhancer-based transcriptional regulation, PIC recruitment is facilitated by the Mediator complex, which acts as a physical bridge between the TF-bound enhancer and the TSS [114]. The PIC catalyzes the phosphorylation of Pol II Ser5, leading to transcriptional activation. Next, transcription elongation is dependent on the phosphorylation of Pol II Ser2 by p-TEFb. Consequently, transcriptional repression is either established via the inhibition of PIC assembly or by the inhibition of assembly of the p-TEFb, which leads to recruitment of NELF and paused transcriptional elongation [113].

[42]. This specificity for cell types is thus at least partially mediated by cell-type-specific chromatin organization.

Besides chromatin remodelers, chromatin modifiers also modulate GR transcriptional output (Box 2). In line with H3K4 methylation of histones being a hallmark of transcriptionally active promoters and enhancers (Box 2) [56], GR interacts with the histone lysine methyltransferase 2 (KMT2) **COMPASS complex** in bone-marrow-derived macrophages treated with Dex and LPS [57]. Of the six possible catalytic subunits of the KMT2 complexes, Uhlenhaut's team identified SETD1A (KMT2F) and SETD1B (KMT2G) as GR's most prominent interaction partners [57]. TurboID studies performed in A549 cells treated with Dex in the absence of an inflammatory stimulus pointed instead to MLL3 (KMT2C) and MLL4 (KMT2D) as the most significantly enriched [58]. While MLL3/4 mostly catalyzes H3K4 monomethylation at enhancers, SETD1A/1B shows a preference for H3K4 trimethylation at promoters [56], indicating how GR cell type and inflammatory context can affect GR's interactome. Interestingly, another recent study using murine mammary epithelial adenocarcinoma (3134) cells showed that the GBSs of GR-repressed genes are located further from the transcription start site (TSS) than the GBSs of GR-activated genes (38 kb versus 4 kb, respectively) [44]. More research will be needed to understand how these findings align. Another histone marker, H3K9 methylation, is mediated by G9a (KMT1C) and is generally associated with transcriptional repression [59]. Two recent studies however, demonstrated that G9a and G9a-like protein (GLP) activation, by increasing its methylation or decreasing its phosphorylation status, led to increased GC sensitivity in B cell ALL (B-ALL) [60,61]. Other studies concur with G9a functioning as a GR coactivator, likely as a platform for histone acetyltransferase (HAT) recruitment [62,63]. The studies in B-ALL also indicated that identifying and targeting Dex-induced GR coregulator interactions involved in triggering pro-apoptotic genes constitutes a promising approach to overcoming GC resistance.

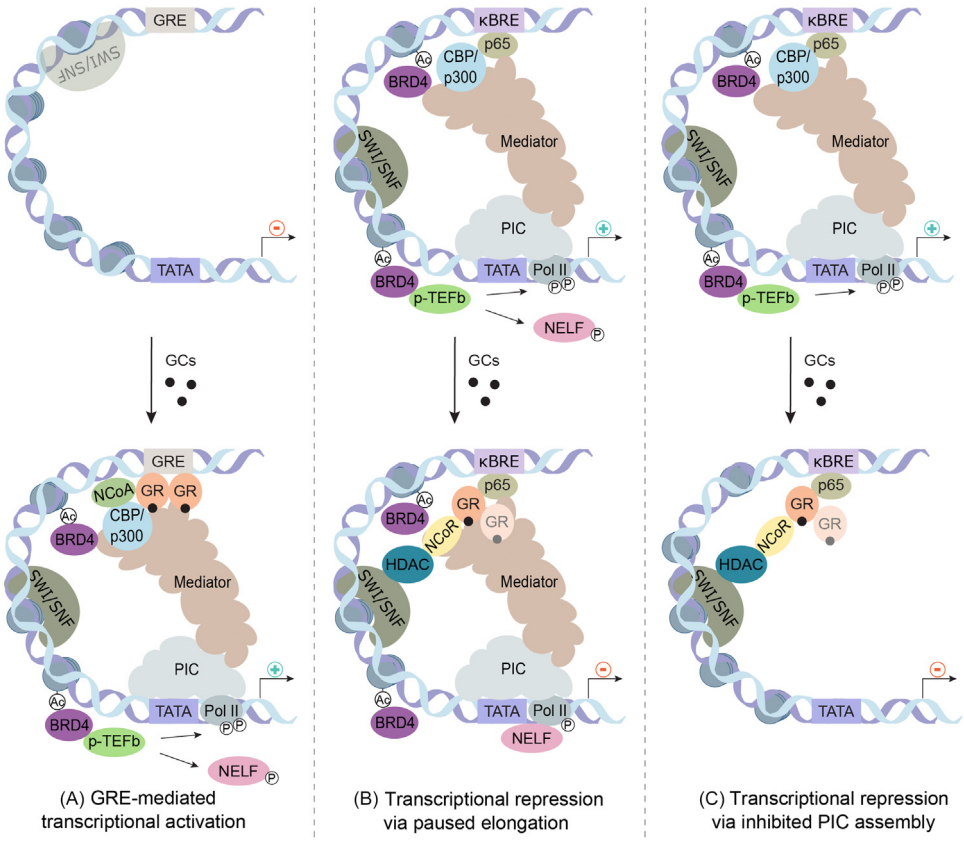

Figure 3. GR complex assembly. (A) Under basal conditions, the regulatory regions of a subset of GR-regulated target genes are pre-bound by the SWI/SNF complex containing BRG1. Following GR activation with GCs, GR binds GREs as a homodimer, followed by recruitment of NCOAs, HATs such as CBP and p300, and, for a subset of target genes, SWI/SNF. Chromatin remodeling and modification leads to recruitment of BRD4 (recognizes and binds acetylated histones), Mediator, and the RNA polymerase II pre-initiation complex (PIC). Polymerase II phosphorylation by Mediator and p-TEFb, respectively, facilitate transcription initiation and elongation of the downstream target gene. (B,C) Under inflammatory conditions, the p65 subunit of NF-κB is bound to its response elements (κBREs) and stimulates transcription of the downstream target gene. Following GC treatment, GR (monomer or dimer) tethering of p65 leads to recruitment of NCOR and HDAC proteins. (B) GR tethering of p65 disrupts p-TEFb binding, inducing recruitment of NELF and subsequently pausing transcription elongation. (C) GR interaction with HDAC proteins causes chromatin deacetylation, which consequently inhibits BRD4 recruitment, PIC assembly, and transcription initiation. Abbreviations: BRD4, bromodomain-containing protein 4; CBP, CREB-binding protein; HDAC, histone deacetylase complex; κBRE, nuclear factor kappa B response element; NCOA, nuclear receptor coactivator; NCOR, nuclear receptor corepressor; NELF, negative elongation factor; Pol II, RNA polymerase II; p-TEFb, positive transcription elongation factor b; SWI/SNF, Switch/sucrose non-fermentable; TATA, TATA box.

Histone acetylation, for instance by the HATs CBP and p300, is known for its role in GR-induced transcriptional activation (Figure 3A). Nonetheless, recent research revealed p300 HAT involvement in GR-mediated transcriptional repression. The Rogatsky team pioneered this switch in gene-regulatory roles for the coregulator NCOA2 [64], and went on to finetune p300's role in GR-mediated transcriptional repression, distinguishing two mechanisms: (i) paused transcriptional elongation, associated with negative elongation factor (NELF) recruitment instead of positive transcription elongation factor b (p-TEFb) recruitment (Figure 3B), and (ii) inhibited RNA polymerase II (Pol II) recruitment and preinitiation complex (PIC) assembly (Box 2 and Figure 3C), linked to attenuated p300 recruitment and histone acetylation [21]. Another study inferred p300 sequestering from non-GBS sites of GR-repressed genes to GR-occupied GBSs of GR-activated genes, thus

explaining rapid GR-mediated gene repression [44]. Although silencing experiments confirmed the role of p300 disassembly in GR-mediated gene repression, the upregulation of GR-induced genes was only mildly affected [44]. This contrasts with a study wherein p300 knockdown reduced the direct transcriptional output of GR, and p300 overexpression reverted tumor necrosis factor (TNF)-induced GC resistance, overall supporting a coregulator reshuffle model [13]. According to these studies, transcriptional complex composition likely depends on the stimulus (ligand, inflammatory context, or duration) and the cell type, paving the way for studying coregulator-targeting molecules.

GR complex assembly and condensate formation: the plot thickens

Recent evidence points to the role of **protein condensate formation** in GR complex assembly and transcriptional output. GR condensates were detected in *in vitro* studies using full-length GR (with an ancestral LBD) and required the NTD, hinge, and LBD [65]. GR condensate formation is modulated by the nature of the GBS, as it was reduced with a canonical GRE compared to an IR-nGRE or a cryptic GRE [65]. In line with this, GR interactions with most of its GBSs were abrogated upon deletion of the NTD and fell back to canonical GREs [47]. This indicates that GR might rely less on condensate formation when binding canonical GREs.

In cellulo, GR nuclear foci formation increased with ligand and appeared DNA- and dimerization-dependent [66]. The coregulator NCOA2, which forms nuclear bodies under basal conditions, redistributed into smaller foci upon Dex treatment and displayed increased colocalization with GR foci over time. The SEGRAM 21-OH or the GRmon mutant (see earlier) were unable to trigger this redistribution of NCOA2 [66]. Cross-correlation analysis revealed that NCOA2 is recruited secondary to the formation of DNA-dependent GR foci [66]. Particular TFs can further modulate GR nuclear organization and coregulator interactions, as recently shown for SOX2 but not for the closely related OCTF4 [67]. Indeed, overexpression of SOX2 impaired ligand-induced condensate formation of GR [and of the mineralocorticoid receptor (MR), progesterone receptor (PR), AR, and GRtetra, a point-mutant showing constitutive tetramerization [17]]. Mechanistically, SOX2 did not interact with GR but modulated the intranuclear compartmentalization and dynamics of NCOA2 in a Dex-independent way, impairing NCOA2 interaction with GR [67]. This resulted in altered GR target gene expression, although not necessarily in an inhibitory way, illustrating the complexity of the SOX2-GR crosstalk [67].

The recruitment of coregulators in GR condensates also depends on the coregulator's identity. While GR interactions with the coregulator NCOA3 largely depend on the classic LxxLL motifs within NCOA3, GR interaction with the coregulator MED1, a subunit of the Mediator complex, rather depends on condensate formation [65], with MED1 having a very large intrinsically disordered region (IDR). The GBS is again decisive, as MED1 was mostly recruited to GR-occupied GRE motifs and less to IR-nGREs or cryptic GREs. In general, MED1 was also found to form condensates with Pol II [68] at **super enhancers** [69,70].

In addition to GR, condensate formation was also demonstrated for PR [71], estrogen receptor (ER) [72], and AR [51,73,74]. As with GR, the AR NTD was found to be crucial for the formation of AR condensates and its transcriptional activity *in cellulo* [74]. Increasing or decreasing AR condensate formation, led to reduced transcriptional activity, indicating there might be an optimal level of condensate formation [74]. Besides the NTD, Zhang and colleagues found that AR's LBD also contributed to the formation of AR-enriched foci in prostate cancer cells [73]. AR foci formation was stimulated by androgen treatment, involved co-localization of MED1, and correlated with AR transcriptional activity [73].

Overall, these studies demonstrated that the condensation of NRs and coregulators plays an important role in transcriptional regulation, and although significant advances have been made by (quantitative) studies in living cells, the field seems to await validation in an endogenous context.

GR talks to other NRs

The intricacies of GR-centered **NR crosstalk** regarding chromatin binding mode and dynamics, and the gene and cell type-specificity thereof are unraveling [75,76]. In breast cancer, GR crosstalk with PR and ER is particularly relevant [77–79], with ER and PR expression determining whether GR confers anti- or proproliferative effects. In ER-positive MCF-7 and T-47D breast cancer cells, coactivation of ER with estradiol, and GR with Dex or the SEGRAMs CORT125134 or CORT118335, blunted the expression of proliferative genes, with GR reducing ER binding to several enhancers compared with estradiol alone [79]. In MDA-MB-231 **triple-negative breast cancer** (**TNBC**) cells, however, GR activation induced genes related to cancer cell survival and invasion, which was reversed by using a GR antagonist (CORT108297 or mifepristone) (Figure 4). Compared to the expression of GR itself, a GR signature comprising a set of 74 genes associated with relapse-free survival was found to be a better predictor for identifying ER-negative, GR-positive breast cancer patients at an increased risk of an early relapse despite adjuvant chemotherapy [78]. In PR-positive breast cancer, apo-GR and, to a greater extent, Dex-activated GR halted PR-mediated breast cancer cells proliferation by modulating the expression of PR target genes, possibly mediated by GR–PR interactions. Following PR agonist (R5020) treatment, GR was also recruited to novel genomic regions not occupied by Dex-activated GR, which were rich in motifs for REL and FOXH1 TFs and located nearby genes coding for chromatin remodelers [77]. As such, a novel mechanism by which GR inhibits the PR signaling pathway surfaces, providing novel therapeutic entry points. Although PR has so far only been used as

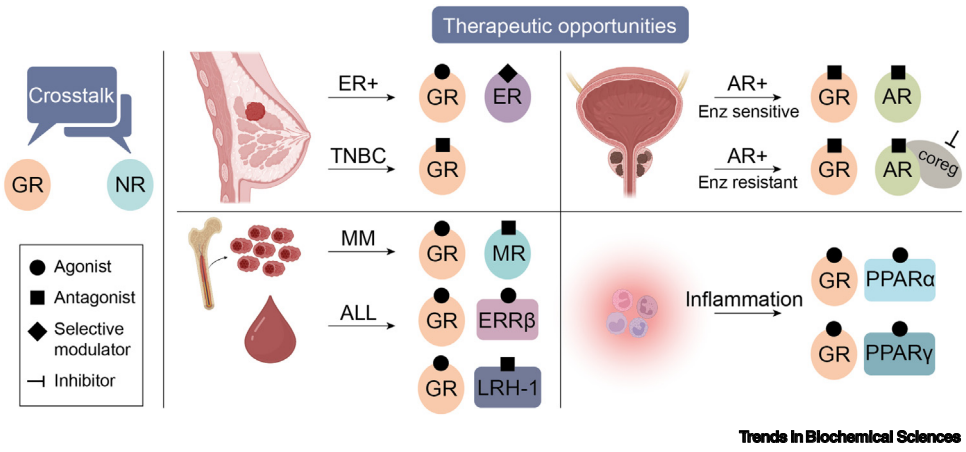

Figure 4. Therapeutic opportunities arising from GR crosstalk. In ER-positive breast cancer, targeting GR–ER crosstalk using GR agonists on top of selective ER modulators may improve treatment responses. In TNBC, GR antagonists may rather be warranted, as in ER's absence, GR may confer proproliferative effects. In AR-driven prostate cancer sensitive to the AR antagonist enzalutamide (Enz), GR antagonists may prevent GR from substituting for AR regarding gene expression. In an Enz-resistant setting, inhibition of specific coregulators may sensitize prostate cancer cells towards Enz. In multiple myeloma, crosstalk between GR and MR may be therapeutically exploited by combining GR agonists with MR antagonists. In ALL, ERRβ agonists or LRH-1 antagonists may sensitize ALL cells to GR agonist-induced cell killing. Finally, the anti-inflammatory activity of GR agonists may be enhanced upon combination with PPARα or PPARγ agonists. Note that the therapeutic opportunities that exploit crosstalk go beyond the strategies mentioned in the main text. Abbreviations: ALL, acute lymphoblastic leukemia; AR, androgen receptor; coreg, coregulator; Enz, enzalutamide; ER, estrogen receptor; ERRβ, estrogen-related receptor β; GC, glucocorticoid; GR, glucocorticoid receptor; LRH-1, liver receptor homolog-1; MM, multiple myeloma; MR, mineralocorticoid receptor; NR, nuclear receptor; PPAR, peroxisome proliferator-activated receptor; TNBC, triple negative breast cancer.

a prognostic marker, a clinical trial found support for the use of the PR/GR antagonist mifepristone in luminal breast cancer patients with high ratios of PR-A/PR-B isoforms [80].

In AR-driven prostate cancer, GR levels are decisive for responsiveness to antiandrogen therapy. Compared with normal tissue, the expression of GR is reduced in primary prostate cancer [81], which originates from AR binding to a prostate-specific enhancer [82] in regulatory regions of *NR3C1* (GR) [82,83]. Upon treatment with antiandrogens such as enzalutamide (Enz), GR levels rise again [81], enabling GR to substitute for AR in terms of gene expression. Coregulators and other members of the transcription machinery also impact this process [82–84]. Indeed, knockout of the AR corepressor TLE3 triggered resistance to antiandrogens, which was mediated by increased expression of GR following treatment with Enz [84]. In line with this study, pharmacological inhibition of the pioneer TF GATA2 inhibited the transcription of a set of six Enz-induced oncogenic genes (including *NR3C1*) by decreasing recruitment of MED1, MED14, and RNA Pol II to these genes, which sufficed to sensitize prostate cancer cells to Enz [83]. Altogether, these studies support the rationale behind a Phase 1/2 trial for castration-resistant prostate cancer (CRPC) in which the GR antagonist mifepristone was combined with Enz [85]. This combination was well tolerated and safe, although the primary endpoint was not met, as prostate specific antigen (PSA)-based progression-free survival was not delayed compared with Enz alone [85]. Nonetheless, studies combining Enz with more selective GR antagonists are warranted (Figure 4).

GR–MR crosstalk, another paradigm, was reported in different tissues. In multiple myeloma cells, MR holds promise as a therapeutic target, as inhibiting MR with spironolactone promoted Dex-induced myeloma cell killing [86] (Figure 4). Although this study is illustrative of MR's potential to impede the transcriptional response of ligand-activated GRs, MR was also found to cooperate with GR in different contexts [87–89]. For example, in the presence of GR, aldosterone-mediated MR-dependent gene transcription was clearly enhanced in mammary carcinoma cells [89]. Mechanistically, whether GR and MR perturb or enforce each other's transcriptional activity by forming heterodimers or higher-order oligomers [90,91] will most likely depend on the target gene [76] and perhaps even the cell type. In addition, while the ligand codictates the binding kinetics of GR and MR to target genes [92], the lifespan of the respective homo- or heterodimers might explain which dimer contributes most to ligand-induced transcriptional activity [76].

Besides steroid receptors, more distantly related NRs, such as peroxisome proliferator-activated receptor (PPAR) α and PPARγ, were also reported to crosstalk with GR, where coactivation provided a means of enhancing anti-inflammatory activity [93,94] (Figure 4), which may be underpinned, at least for GR–PPARα, by a direct interaction. The interplay between orphan NRs and GR is also gaining attention in hematological malignancies [95,96]. A direct interaction between GR and liver receptor homolog-1 (LRH-1) may even be relevant in the context of resistance to GC in T cell ALL (T-ALL). Indeed, Dex combined with the LRH-1 antagonist 3d2, which is hypothesized to decrease this GR–LRH-1 interaction, sensitized previously GC-unresponsive T-ALL cells towards GR transcriptional activity and ultimately cell death [95]. In ALL, estrogen-related receptor β (ERRβ) also cooperated with GR, as the ERRβ agonist GSK4716 enhanced Dex-induced killing of ALL cells [96] (Figure 4). Altogether, these studies highlight the essential role of NR crosstalk for understanding and improving GR-based action.

GCs 2.0: from selective ligands to formulations and antibody–drug conjugates

Developing novel, more selective GR ligands has been a first-line approach to tackle the problem of side effects for many years. Targeting GR with SEGRAMs was hypothesized to deliver enhanced therapeutic benefit by at least partial dissociation of therapeutic and side effects but their clinical translation has so far largely been unsuccessful [97]. Assays with improved predictive

power that capture the complexity of GR signaling seem to be required at minimum to identify successful SEGRAMs [98]. As illustrated earlier, the simultaneous targeting of two receptors, namely GR and a crosstalk partner, using combination treatments has increasingly been explored (Figure 4) [86,96]. Alternatively, **polyamidoamine** (**PAMAM**) dendrimer-linked GCs [99], (PEGylated) liposomal formulations of GCs [100,101], and, more recently, also antibody–drug conjugates (ADCs) [102–104] are increasingly being developed as strategies aimed at minimizing systemic exposure and side effects by delivering the payload to the diseased cells. For instance, in an advanced multiple myeloma mouse model, liposomal encapsulation of Dex showed strong antitumor activity, while an equivalent dose of free Dex was ineffective [101]. In a randomized Phase 3 trial in rheumatoid arthritis patients, intravenous PEGylated liposomal prednisolone sodium phosphate also had superior efficacy compared to the standard of care to treat flare-ups, (i.e., intramuscular methylprednisolone acetate), with both regimens having comparable safety profiles [100]. In the context of ADCs, a proof-of-concept Phase 2a trial in rheumatoid arthritis with the anti-TNF monoclonal antibody adalimumab chemically linked to a nonsteroidal SEGRAM demonstrated greater efficacy and a similar safety profile to adalimumab alone [104]. Together, these studies illustrate that GC-based formulations or ADCs may improve the therapeutic index of GCs.

Concluding remarks

We have discussed several emerging concepts regarding the molecular events driving GR functional effects, knowledge which is key for identifying novel therapeutic avenues. We have highlighted the bidirectional communication between the ligand-binding and coregulator-binding pockets and zoomed in on the relevance of noncanonical LBD dimerization modes of GR, altogether offering alternative targeting interfaces for drug design. We have also discussed the importance of condensate formation in GR's transcriptional activity and how this is influenced by the nature of the GBS and post-translational modifications (PTMs). The next hurdle is going beyond overexpression studies to establish the relevance of condensates in an endogenous context (see Outstanding questions). A similar reasoning holds for studying GR (hetero)oligomerization using imaging-based approaches. Working with inducible systems to finetune receptor levels or CRISPR-based (fluorophore) tagging of endogenous GRs may overcome some of these limitations. We have further highlighted that cell-type-specific actions of GR are governed by chromatin organization and coregulator recruitment. Identifying and targeting GR's coregulator (interactions) therefore seems to be a promising therapeutic strategy. Furthermore, we have illustrated that NR crosstalk is a crucial determinant of GR-based actions. We propose that GR–NR heterodimers may constitute novel drug targets for the development of innovative therapeutics that have enhanced therapeutic benefit and may even combat resistance to GC. A challenge in this regard is that designing such (bivalent) ligands may benefit from a crystal structure of (minimally) the LBD heterodimer, although homology modeling or **AlphaFold2**-based predictions may in part overcome this hurdle. Finally, we have illustrated the potential of ADCs in the context of inflammation and project that their potential for application will expand to the field of cancer. Altogether, our review has illustrated that a full understanding of GR signaling requires an interdisciplinary approach and a collaborative effort by structural and molecular biologists and medicinal chemists.

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Declaration of interests

The authors have no interests to declare.

Outstanding questions

Can pharmacological targeting of coregulators, GR–coregulator interactions, or NR heterodimers with novel ligands provide a means of improving therapeutic benefit?

Is there a predominant NR hetero(di)mer mode within a specific cell type, or is this always gene-dependent; as an extension, is there a preference for mutual DNA-binding or tethering modes?

Do receptor expression levels determine which NR is the predominant crosstalk partner for GR in a given cell type? Or is there a hierarchy between receptors and, if so, what are the determinants?

Can GC resistance be postponed or even circumvented by the simultaneous targeting of GRs and (orphan) NRs or steroid receptors in different pathophysiological contexts?

Does GR–ER and GR–AR crosstalk result from a direct interaction in the context of breast and prostate cancer, respectively?

To what extent is tissue-specificity in terms of GR actions determined by different 3D chromatin organization? Does tissue-specific coregulator expression also play a role?

Is condensate formation physiologically relevant? Can published findings be confirmed in endogenous contexts?

How does the N-terminal domain of GR facilitate or influence interdomain communication, and what molecular mechanisms or structural features determine GR function?

Is (multi)tetramerization of GR an endogenous phenomenon and would it be primarily driven by assembly of (multiple) tetramers on one GBS, or by dimer binding on two (or multiple) distant GBSs? What are the functional implications of these distinct mechanisms for GC-mediated gene regulation?

Will the application potential of GC-based antibody–drug conjugates expand towards the treatment of (hematological) cancers?

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