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REVIEW

How to tame your genes: mechanisms of inflammatory gene repression by glucocorticoids

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Glucocorticoids (GCs) are widely used therapeutic agents to treat a broad range of inflammatory conditions. Their functional effects are elicited by binding to the glucocorticoid receptor (GR), which regulates transcription of distinct gene networks in response to ligand. However, the mechanisms governing various aspects of undesired side effects *versus* beneficial immunomodulation upon GR activation remain complex and incompletely understood. In this review, we discuss emerging models of inflammatory gene regulation by GR, highlighting GR's regulatory specificity conferred by context-dependent changes in chromatin architecture and transcription factor or co-regulator dynamics. GR controls both gene activation and repression, with the repression mechanism being central to favourable clinical outcomes. We describe current knowledge about 3D genome organisation and its role in spatiotemporal transcriptional control by GR. Looking beyond, we summarise the evidence for dynamics in gene regulation by GR through cooperative convergence of epigenetic modifications, transcription factor crosstalk, molecular condensate formation and chromatin looping. Further characterising these genomic events will reframe our understanding of mechanisms of transcriptional repression by GR.

Keywords: chromatin; epigenetic; gene repression; glucocorticoid; glucocorticoid receptor; inflammation; macrophage; phase condensates; transcription

In the presence of infection, the body induces a strong and potent local or systemic inflammatory response comprising innate and adaptive immune cells that generate and respond to pro-inflammatory cytokines and chemokines. While initially beneficial, an overactive immune system and a dysregulated hyperinflammatory state can cause more harm than good in the long term, such as in the case of sepsis. Thus, the ability to safely

Abbreviations

AF-1, activation function 1; AF-2, activation function 2; ALI, acute lung injury; AN, Adriamycin-induced nephropathy; ARDS, acute respiratory distress syndrome; ChIA-PET, Chromatin Interaction Analysis by Paired-End Tag Sequencing; ChIP, Chromatin immunoprecipitation; CoRNR-box, corepressor/nuclear receptor box; GC, glucocorticoid; GR, glucocorticoid receptor; GRE, GR response element; HAT, histone acetyl transferase; HDAC, histone deactetylase; Hi-C, chromosome conformation capture; HPA, hypothalamic–pituitary–adrenal; IDR, intrinsically disordered region; IL, interleukin; LCDs, low-complexity domains; LBD, ligand-binding domain; LLPS, liquid–liquid phase separation; LPS, lipopolysaccharide; NHL, non-Hodgkin lymphomas; NRbox, nuclear receptor box; NTD, N-terminal domain; SARS-CoV-2, Severe Acute Respiratory Syndrome-Coronavirus 2; TAD, topologically associating domain; TAT, trans-activator of transcription peptide; Th2, T helper cells 2; TRE, O-Tetradecanoylphorbol-13-acetate response element; TSS, transcription start site; κBRE, κB response element.

regulate and dampen inflammation is of great value, and in clinical settings, glucocorticoids (GCs) are commonly used to treat a variety of immune and inflammatory diseases.

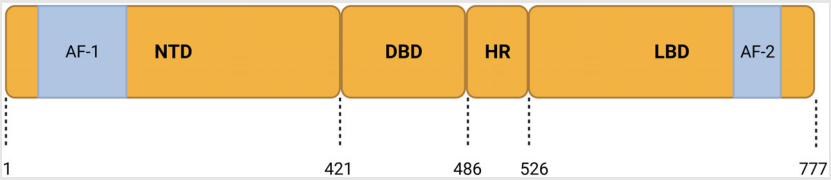
Glucocorticoids, mainly cortisol, belong to a class of evolutionarily conserved steroid hormones secreted in a diurnal and stress-responsive manner from the adrenal cortex upon activation of the hypothalamic–pituitary–adrenal (HPA) axis [1]. Endogenous glucocorticoids, which exhibit potent developmental (e.g. lung maturation), behavioural, metabolic and immunosuppressive effects, are released in different biological and physiological circumstances and affect nearly every part of the body's cellular and molecular network. For that reason, dysregulated secretion can result in diverse pathological outcomes with opposite extremes, such as Cushing's syndrome and Addison's disease, caused by excess or lack of circulating GCs respectively [2,3]. Due to their potent immunomodulatory and anti-inflammatory capacities, GCs have pharmacologically been used to treat many types of cancers, for example, non-Hodgkin lymphomas (NHL), as well as both acute and chronic inflammatory diseases, including bacterial and viral infections, allergies, asthma, rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis and sepsis [4–7]. Moreover, due to their importance in pulmonary development, GCs are routinely used to accelerate lung maturation in pre-term neonates [8].

The main therapeutic use of GCs in infections and inflammation aims at rapidly dampening cell-mediated immunity by inhibiting the expression of pro-inflammatory cytokines and chemokines, while simultaneously activating the expression of anti-inflammatory immune mediators [9–12]. Monocytes and macrophages are key players in the innate immune system, which triggers the onset of an inflammatory response upon recognition of pathogens. Through the integration of inflammatory signals via pattern recognition receptors, for example, Toll-like receptors (TLRs), macrophages activate pro-inflammatory transcription factors such as nuclear factor-kappa B (NF-κB), activator protein 1 (AP-1), and interferon response factors (IRFs) [13]. Additionally, macrophages sense immunological messengers such as interferons or interleukins via their cognate receptors, which in turn activate tyrosine kinases, mainly of the Janus kinase family, that then activate transcription factors such as signal transducer and activator of transcription (STATs) [14,15]. Together these transcription factors drive the expression of inflammatory response genes and, in clinical settings, serve as important targets for GC treatment.

Transcriptional profiles of GC-stimulated monocytes reveal differential expression of hundreds of genes. This includes, in addition to the glucocorticoid receptor (GR) itself (Box 1), the activation of anti-inflammatory factors such as Dual specificity phosphatase 1 (DUSP1), Glucocorticoid-induced leucine-zipper (GILZ), Interleukin-1 receptor associated kinase-M (IRAK-M), TNF induced protein 3 (TNFAIP3), inhibitor of nuclear factor kappa B (IκB) or Krüppel-like factors (KLF) 2 and 4 that can directly interfere with inflammatory signalling [16–21]. Additionally, GC-treatment represses transcriptional activation of pro-inflammatory mediators involved in apoptosis, adhesion and T-cell chemotaxis [22]. In macrophages, the role of GCs in the resolution of inflammation is known to extend beyond the regulation, synthesis and release of short-term inflammatory mediators [23]. By influencing macrophage differentiation and polarisation towards an anti-inflammatory (M2) macrophage phenotype, GCs can indirectly exert immune suppression through downstream factors [24–29]. For instance, in a tail amputation model in zebrafish larva, GC treatment has been shown to inhibit macrophage differentiation towards a pro-inflammatory (M1) phenotype without affecting macrophage migration to the site of injury [28]. Consistently, macrophages from GILZ$^{-/-}$ mice show an increased expression of pro-inflammatory M1 markers such as CD86, Major histocompatibility complex class II, induced nitric oxide synthase, chemokine (C-C motif) ligand 2, interleukin (IL)-6, and tumour necrosis factor alpha (TNFα) upon IFN/ lipopolysaccharide (LPS)-stimulation [25]. Concordantly, therapeutic administration of TAT-GILZ, a GILZ fusion protein containing a trans-activator of transcription peptide (TAT) to allow *in vivo* delivery, promotes resolution of inflammation [25,26]. While neither loss nor gain of GILZ seem to affect the expression of M2 macrophage markers, GILZ-deficiency does reduce efferocytosis (the phagocytic removal of apoptotic cells), a feature commonly associated with the M2 state and the resolution of inflammation [25,26,30]. Potentially, the capacity of GILZ to reduce the activation of M1-like macrophages tips the balance in favour of M2 polarisation and enhanced efferocytosis, further supporting a role for GCs in M1 and M2 macrophage differentiation and polarisation [25]. In addition, through regulation of signal transduction pathways and defined tissue-specific gene expression programmes, GCs not only contribute to the resolution of inflammation but also to the cellular plasticity of macrophages, which shape and regulate inflammatory responses [31]. In purified human monocytes and monocyte-derived macrophages, GC treatment has

Box 1. GR structure and function.

The immunomodulatory effects of GCs are mediated by the glucocorticoid receptor (GR, encoded by the *NR3C1* gene), a ubiquitous intracellular ligand-gated transcription factor. The GR protein structure comprises three distinct domains (Figure Box 1): The N-terminal transactivation domain (NTD) containing the activation function (AF)-1 domain, which mediates interaction with co-regulators; the central DNA-binding domain (DBD); and the C-terminal ligand-binding domain (LBD) containing the AF-2 domain responsible for the ligand-dependent recruitment of co-regulators [57–59]. Moreover, the NTD of GR is known to be an intrinsically disordered region (IDR), allowing for formation of and integration in phase condensates [60]. A short intrinsically disordered hinge region also connects the GR DBD with the LBD. In the absence of ligand, GR is maintained in the cytoplasm as a multi-protein complex including heat shock proteins 70 and 90 (Hsp70 and Hsp90), FKBO Propyl Isomerase 4 and 5 (FKBP4, FKBP5), and calreticulin (CALR) [61,62]. In this complex, the nuclear localisation signal within the GR LBD is masked. Upon GC binding, GR translocates to the nucleus where it regulates the transcription of its target genes. GR homodimers are known to bind to 15 bp palindromic consensus DNA sequences (AGAACANNNTGTTCT) termed glucocorticoid response elements (GREs), which are present in the enhancer and promoter regions of GR target genes.



been shown to drive differentiation-associated expression changes of genes regulating cell-matrix adhesion, MAP kinase signalling, metabolism and immune responses [29,32]. These cell-intrinsic changes are linked to remodelling of histone H3K27 acetylation (H3K27ac) regions, often resulting in increased H3K27ac signals [31,32]. ChIP-seq analyses have found that these H3K27ac signatures are either occurring directly at GR-binding sites or at other GR-bound topologically associating domains (TADs) of neighbouring chromosomal loci, suggesting that TADs may accommodate information from distal GC response elements [31,32]. Furthermore, mature human M2 macrophages, differentiated in the presence of IL4, could be rendered responsive to Transforming growth factor beta (TGF-β) by GC-induced cell-surface expression of TGF-β receptor II (TGF-βRII) [29]. In addition to IL10, M2 macrophages express TGF-β, suggesting that the activation is partially occurring in an autocrine fashion [33]. By regulating the surface expression of TGF-βRII in a time- and dose-dependent manner, TGF-β1-stimulated M2 macrophages activate a multi-step gene expression programme [29]. This expression programme features 'early response' genes involved in transcriptional regulation and signalling, and 'late response' genes involved in (Th2) immune modulation, lipid metabolism and atherosclerosis [29]. These actions ultimately limit the pro-inflammatory function of tissue residential leukocytes and consequently the composition and numbers of infiltrating immune cells, as observed in murine models for acute lung injury (ALI), acute respiratory distress syndrome (ARDS) and chronic kidney disease [Adriamycin-induced nephropathy (AN)] [33–36]. In the case of ALI, methylprednisolone has been shown to ameliorate LPS-induced lung and tissue injury *in vivo* by blocking M1 polarisation and by promoting M2 polarisation [33]. Notably, sorted macrophages had, in co-culture with $CD4^+$ naïve T cells, the capacity to induce regulatory T-cell (Treg) differentiation by secretion of IL-10 and TGF-β [33,37]. Similarly, in AN chronic kidney disease model, adoptive transfer of M2 macrophages limited both renal inflammation and fibrosis by reducing host macrophage and $CD4^+$ and $CD8^+$ T-cell infiltration [36]. Therefore, these effects might constitute a general mechanism by which GC/GR and M2 macrophages regulate or suppress inflammation.

However, the use of GCs is not without cost, as long-term GC exposure induces glucose intolerance and insulin resistance, adipocyte hypertrophy, osteoporosis, muscle and skin atrophy, glaucoma, impaired wound healing, steroid resistance, as well as various psychological side effects such as insomnia and depression [38–44]. Moreover, in the context of infection, GCs not only have the potential of delaying the

clearance of pathogens and impairing lymphocyte proliferation, but have also been associated with reactivated latent human cytomegalovirus, thus increasing the risk of secondary virus-mediated complications [45–48].

These opposing favourable and adverse effects of GC treatment have become central with the emergence of the COVID-19 pandemic, caused by severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) viral infection. COVID-19 presents in various sequelae ranging from mild to severe cases, marked by the massive release of pro-inflammatory cytokines, the development of lung injury and ARDS, and subsequent multi-organ failure [49–53]. Further, in a recent systematic profiling of GR expression in COVID-19 patients with severe disease, it was reported that the expression of GR is downregulated in GR- and IL6-co-expressing alveolar macrophages as well as in non-immune cells [52]. Moreover, the expression of GR in this macrophage population was significantly lower in patients with severe disease compared to those with mild disease [52]. In severe cases, this phenotype may contribute to the steroid resistance observed in some SARS-CoV-2 infected patients [52,54]. Nevertheless, the advantages of using GCs for treating COVID-19 have been firmly established [54–56]. In a recent update from the Randomised Evaluation of COVID-19 Therapy (RECOVERY) trial, the use of the synthetic corticosteroid dexamethasone significantly lowered the mortality among severe COVID-19 patients receiving either invasive mechanical ventilation or oxygen [55]. The benefits of dexamethasone treatment were, however, not observed in patients without respiratory failure [55]. Further, the timing of treatment initiation and duration after symptom onset turned out to be a critical factor [54]. Early initiation or shorter treatment duration has found to be less beneficial and shown to increase the risk of rebound phenomena and refractory COVID-19 [54]. Accordingly, in patients hospitalised with severe COVID-19 and with respiratory difficulties, treatment with dexamethasone or other corticosteroids is now part of the gold standard regimen [54,55].

In this review, we discuss recent insights and emerging models for context-dependent GR-mediated inflammatory gene regulation in macrophages. We further aim to highlight the genomic and epigenomic control of transcription by GR, and to discuss emerging concepts such as three-dimensional (3D) genome organisation, the crosstalk between GR, other transcription factors and co-regulators, and spatial–temporal distribution of co-activators and co-repressors, as well as competition for GR sites.

Mechanisms of transcriptional regulation by GR

Profiling chromatin dynamics in transcription regulation

The transcriptional activation via GREs is extensively studied and includes the recruitment of co-activators and histone acetyl transferases (HATs) followed by production of anti-inflammatory factors [63–67] (Fig. 1A). In general, while the immediate transcriptional repression of inflammatory genes is considered as GR's main mechanism of action, GR also changes the cellular sensitivity for pro-inflammatory stimuli. For example, GR induces the expression of signalling factors or transcriptional regulators, which drive anti-inflammatory states [16,17,19,22,68]. These changes confer a long-lasting immunomodulatory environment by polarisation of immune cells towards an anti-inflammatory state, and by active participation to resolve inflammation. For the purpose of this review, though, here we focus on the mechanisms of primary and immediate repression of inflammatory genes. Globally, the significance of repression by GR has been demonstrated both *in vitro* and *in vivo*, with more than half of all LPS-TLR-induced, NF-κB- and IRF-regulated genes being GC sensitive [69–72]. However, on a mechanistic level GR mediated repression is less well-understood [73–75]. Recent findings have challenged classical models of repression, which featured GR tethering to DNA-bound AP-1 and NF-κB pro-inflammatory transcription factors via protein–protein interactions [17,21,76–82]. Several studies found direct DNA binding of GR to GREs or to composite elements embedded in AP-1 or NF-κB motifs near genes downregulated by GCs [17,83–85].

GR has further been shown to interact with GREs inside inflammatory loci in the absence of active NF-κB and AP-1, and in some scenarios to act synergistically with NF-κB, as exemplified by the activation of *Tnfaip3* and *Nfkbia*, two negative regulators of NF-κB and TNFα-signalling [16,20,85–87]. In the inflammation-responsive cell line HeLa B2, it appears that synergistic activation mainly occurs at sites that are readily accessible to either GR or NF-κB, and that are weakly activated by single binding [87]. These genes correlate with the inactivation of inflammatory signalling pathways and constitute an additional mode for GR-mediated suppression of inflammatory responses [88,89].

Squelching, the competition for common cofactors such as glucocorticoid receptor interacting protein 1 (GRIP1), has been proposed as a potential mechanism of GR-mediated repression of pro-inflammatory

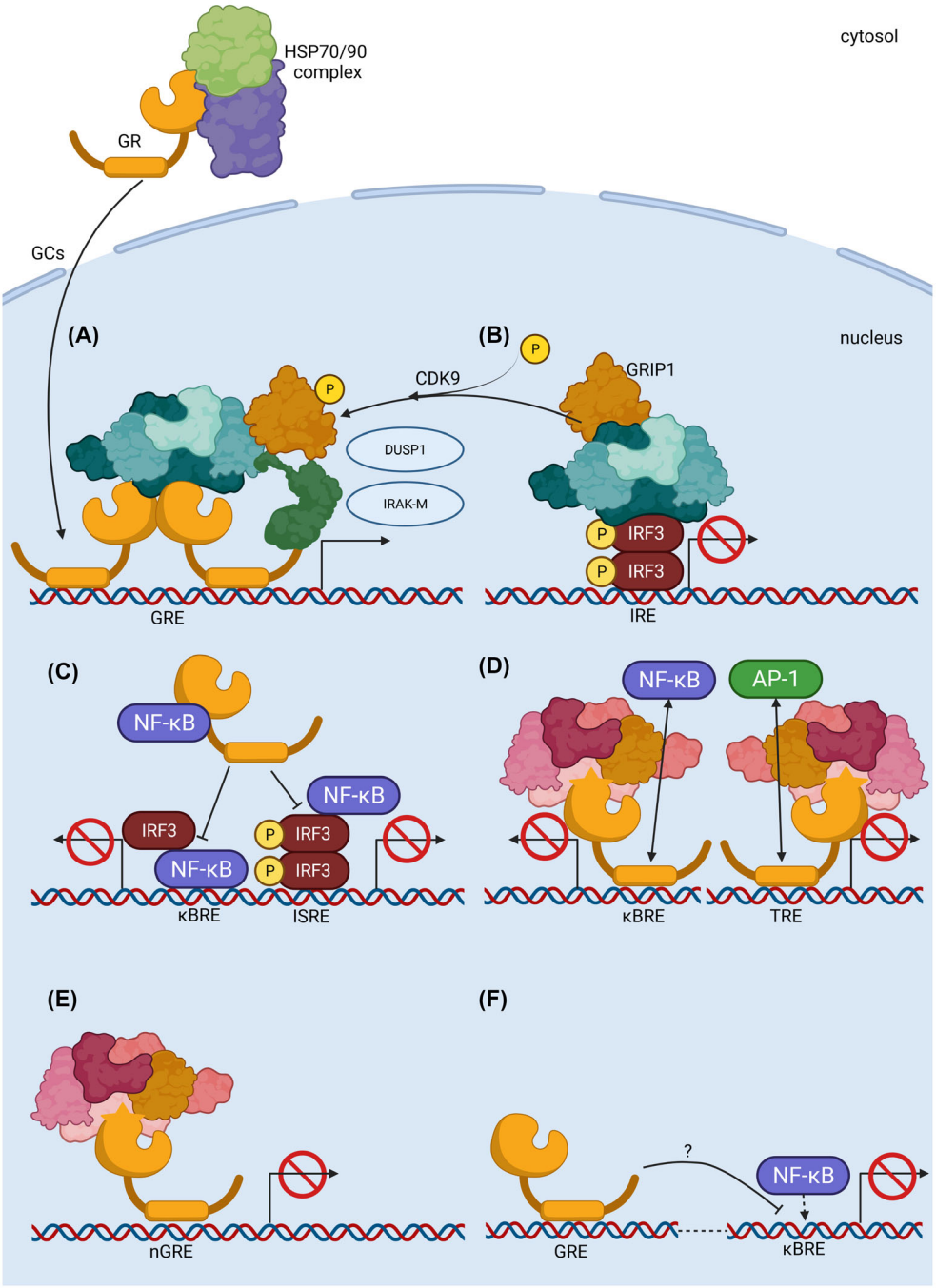


Fig. 1. Mechanisms of GR-mediated inflammatory gene regulation. Upon activation by ligands (GCs), GR translocates to the nucleus to control transcription. (A) GR binds to GREs and assembles a transcriptional complex to activate the expression of anti-inflammatory genes like DUSP-1 or IRAK-M. this complex is dependent on incorporation of GRIP1 phosphorylation. (B) Since IRF3 controlled loci are also dependent on GRIP1, squelching of the cofactor GRIP1 abrogates IRF3 dependent transcription. (C) Additionally, GR can bind NF-κB via protein–protein interactions. This disturbs the pro-inflammatory NF-κB-IRF3 complex and reduces expression of IRF3 and NF-κB target genes. (D) GR binding to cryptic GREs within the NF-κB (κBREs) and AP-1 motifs (TREs) represents competitive binding. While NF-κB and AP-1 binding leads to activation, GR binding represses expression due to conformational changes encoded in the DNA as an allosteric modulator directing co-repressor recruitment. (E) Similarly, nGREs induce DNA binding of the GR but involve a conformation that leads to recruitment of a co-repressor complex. (F) GR activation may reduce NF-κB binding to κBREs via unknown mechanisms.

transcription factors (Fig. 1B). For example, competition with GR for GRIP1 has been shown to antagonise IRF3 activity, identifying the GRIP1:IRF3 interaction as a novel target for glucocorticoid immunosuppression in macrophages [90]. This competition is thought to be actively regulated by the GR, as treatment with GCs leads to altered GRIP1 phosphorylation at specific loci, dependent on Cyclin-dependent kinase 9 (CDK9) [73,91,92]. It appears that this phosphorylation potentiates the coactivator functions of GRIP1, recruiting it specifically to GREs [73,91,92]. Conceivably, this might diminish the pool of IRF3-accessible GRIP1 and impair IRF3 function. Concordantly, GRIP1 overexpression was found to antagonise the repressive effects of GR on IRF3 target genes in murine macrophage cells (RAW264.7) [90].

While tethering between GR and NF-κB has been observed near repressed genes, the protein:protein interaction between GR and NF-κB has been reported to disrupt the interaction between NF-κB and IRF3 [70], abrogating NF-κB/IRF3-dependent transcription in macrophages (Fig. 1C). Notably, this interaction may affect TLR4- and TLR9-, but not TLR3-dependent gene activation and required signalling via MyD88, enabling GR to differentially regulate pathogen-specific gene programmes [70]. Similarly, peroxisome proliferator-activated receptor gamma (PPARγ) and liver X receptor (LXR) were found to cooperate with GR to synergistically repress distinct subsets of TLR4-responsive genes [70].

As another means of GR-mediated repression, it has recently been shown, that the GR can bind to cryptic GREs within NF-κB and AP-1 motifs termed κB response elements (κBREs) and O-Tetradecanoylphorbol-13-acetate response elements (TREs), respectively. Seemingly, these cryptic binding sites are evolutionary conserved and competition between GR and AP-1/NF-κB for these sites leads to transcriptional antagonism of NF-κB and AP-1 action [75,83,84] (Fig. 1D). Moreover, DNA binding at these sites causes allosteric changes in GRs conformation, mainly by shifting the position of a highly flexible loop region within the GRs DBD, referred to as the lever arm [60,93–95]. Altered conformations of the lever arm can also affect the positioning of the remote helix H3 in the LBD, as seen in different GR isoforms [94]. Together with additional helices located in the LBD (H1, H4, H5 and H12), the H3 helix present in the GR and other nuclear receptors recruits co-activators and co-repressors by a 'charge clamp mechanism' that binds both nuclear receptor box (NRbox) and corepressor/nuclear receptor box (CoRNRbox) containing proteins [96–98]. The different conformations of the H3 helix, upon DNA-induced alterations of the lever arm, might thus allow for locus specific interactions with co-regulators. While these insights are derived from experiments in non-myeloid cell lines or *in vitro* biochemical studies, it is conceivable that the DNA sequence itself may function as an allosteric regulator of GR conformation, providing differentially accessible binding surfaces that specify cofactor recruitment in macrophages [93–95].

In addition to classical GREs, GR binding to 'negative GREs' (nGREs with the consensus sequence $CTCC(n)_{0\text{-}2}GGAGA$) has been suggested to contribute to the transcriptional repression of inflammatory genes [99] (Fig. 1E). Subsequent structural analysis observed a shift within the lever arm that might induce conformational changes leading to corepressor recruitment. However, these sequences do not appear in the proximity of pro-inflammatory promoters nor in genome-wide GR ChIP-seq studies, and thus remain insufficient in explaining major transcriptional effects in macrophages [85,100].

Finally, repression of target genes by GCs has been linked to diminished DNA binding of NF-κB in a secondary effect [17]. While a mechanism based on IκB-mediated nuclear export of NF-κB has been postulated, the nuclear abundance of NF-κB and the strength of upstream activating signals were not found to be reduced [17,21]. Conceivably, GR might rather sequester NF-κB by direct protein:protein interactions, by indirectly changing the NF-κB interactome, or by competitive binding at κB response elements (Fig. 1F). Importantly, all these mechanisms are rapid and independent of *de novo* protein synthesis [16,92,101,102].

All these scenarios described above are affected by locus-specific differences in the chromatin microenvironment, ranging from differentially neighbouring transcriptional regulators to altered chromatin accessibility [103,104]. The long-standing notion of steady on-and-off states, with transcription factors binding to DNA in a static manner, assembling the transcriptional machinery and driving gene expression, has been changed by advanced microscopy. It has become evident that binding events are highly dynamic, and happen in the order of seconds at the chromatin level [102]. This high mobility of transcriptional regulators allows for a 'screening' of DNA sequences to find high-affinity binding sites, potentially involving recruitment of chromatin remodelers such as the SWI/SNF complex [105]. By temporarily bringing nucleosomes to a high-energy state, these ATPase containing complexes enable the GR and other transcription factors to bind DNA [105]. This transition state only lasts for a short period of time, and the transcription factors are removed from the DNA once the nucleosome returns to the ground state. This model accounts for

multiple transcription factors facilitating each other's binding, a phenomenon termed assisted loading [106]. Here, binding of one transcription factor would turn the nucleosome into a high-energy state by recruiting chromatin remodelling complexes, which provides space for other transcription factors in close proximity. Indeed, GR has been reported to fulfil the role of a pioneer factor [106,107]. While these insights have not yet been studied in macrophages, ChIP-seq experiments have shown that new loci become accessible for GR in response to LPS, arguing that similar mechanisms may take place in this cellular context [85].

In the nuclei of murine mammary adenocarcinoma cells (3617 cell line), the GR displays two independent populations of reduced mobility [108]. One is the fraction of chromatin-bound GR, whereas the other seems to be a confinement state where GR is localised to a limited space by means of phase separation [108]. The observation that the residence time of GR on chromatin is in the order of seconds, together with the recent observation of a predefined confinement state, could account for the high specificity of GR binding. The limited space predefined by phase condensates reduces the availability of binding sites accessible to GR, allowing highly specific binding events to take place. This will be discussed in more details later.

One final point concerning the dynamics of GR action is the mode of stimulation. While most insights into GR's action have been acquired *in vitro* by continuous stimulation using GR-ligands, a more physiological context of studying GR activation is by creating a pulsatile activation state, simulating the fluctuating hormone levels *in vivo* [109–115]. In murine mammary tumour cells (3134 cell line), it has been shown that pulsatile hormone treatment, compared to continuous GR activation, differently affects the lifetime of accessible chromatin [116]. This establishes rather short-lived interactions with regulatory elements and transient changes in chromatin accessibility [116]. Conversely, continuous treatment increased GR binding and chromatin accessibility and established additional binding sites [116]. Whether these insights can be exploited pharmacologically remains to be investigated, as synthetic glucocorticoids display an enhanced affinity for the GR LBD and cannot be easily depleted upon binding, resulting in prolonged activation of GR [117].

The chromatin microenvironment as a determinant of GR activity

As described above, GR protein conformation and its subsequent interactions with different cofactors can be affected by sequence differences in the GRE motif and in flanking nucleotides [93,118]. This might particularly affect GR dimers, which form phase-separated condensates that potentially concentrate specific co-regulators to activate or repress target genes [60,118,119]. These locus-specific domain requirements were reportedly mirrored by cofactors such as GRIP1 [120]. An additional parameter could be the shape of the DNA strand at the GRE itself [121]. Recently, GR is shown to bind to DNA as a tetramer with increased capacity for both gene activation and repression in non-myeloid cells, raising the possibility of additional DNA-dependent mechanisms of GR function [122,123]. Speculatively, tetrameric GR might itself be involved in DNA looping, with dimers binding two distal elements. With the rise of single-cell omics and advanced imaging methods, future insights into 3D nuclear dynamics will shed new light on transcriptional mechanisms.

Furthermore, neighbouring transcription factors and co-factors might alter the local microenvironment in a cell type- and stimulus-specific manner. In particular, pioneer factors like AP-1 or PU.1, which govern macrophage M1 polarisation, might control the accessibility of particular sites for GR [72,103,104,124–126]. These concepts not only support the notion of lineage specific chromatin accessibility, but also match current phase separation models proposing distinct transcriptional condensates, which are spatially divided by differential solubility.

GR interaction with the core transcription machinery

Negative GR target genes cannot all be lumped together, as they display heterogeneity and require at least a dual classification into initiation-controlled and elongation-controlled groups [71,127]. For initiation-controlled genes, treatment with GR ligand diminishes recruitment of the HAT p300 and RNA Polymerase 2, and consequently histone acetylation. Conversely, elongation-controlled genes recruit negative elongation factors as well as GRIP1, resulting in failure to assemble the Mediator complex [71,127] (Box 2). In addition, histone deacetylases (HDACs) play an instrumental part in GR mediated repression. It was observed, that HDAC1 together with silencing mediator for retinoid or thyroid-hormone receptors (SMRT, alternatively known as nuclear receptor co-repressor (NCoR) 2) is involved in a GR-bound complex, possibly to repress gene transcription, in natural killer (NK) cells [128]. Moreover, other reports showed that HDAC3 seems to be indispensable for gene repression at nGREs. Notably, HDAC3 also seems to be

Box 2. Co-repressors

New techniques have identified a plethora of GR interacting proteins. These include HDACs, N-CoR (NCoR1) and SMRT (NCoR2), GRIP1, BRG1 and other components of the SWI/SNF complex, the COMPASS (complex of proteins associated with Set1) complex and components of the Nucleosome Remodelling Deacetylase (NuRD) complex [69,136]. Some of these are demonstrated to be relevant for GR mediated repression. For example, in a knockdown screen in MCF-7 cells, N-CoR, HDAC1/3 and CBP, but not SMRT, are identified as required for TNFα repression [84]. Sirtuin 1 and 2 are also found to be essential for IL6, but not for TNFα, repression, arguing for locus specific mechanisms [84]. In murine epithelial fibroblasts (MEFs), N-CoR, SMRT and HDAC3 are required for GR target gene repression, which may point towards cell type-specificity [130]. Moreover, it has been proposed that the interaction between GR and N-CoR or SMRT leads to recruitment of HDACs and the Sin3 complex [137]. Interestingly, while HDAC1 and 3 are essential for GR-mediated repression, HDAC2 has also been shown to mediate repression of Granulocyte-macrophage colony-stimulating factor (GM-CSF) expression [84,129,130,132].

Intriguingly, many co-regulators have dual roles and can mediate both activation and repression. For example, GRIP1 can function as a co-activator together with p300/CBP as well as a corepressor together with HDACs [85,120,138].

involved in a complex together with SMRT in COS-1 cells [129,130]. HDAC2 was found to be recruited to the nucleus upon GC treatment in NK cells and is involved in repression of the negative GR target proopiomelanocortin in pituitary tumour cells [128,131]. Further, it seems to control the interaction between GR and NF-κB by catalysing the deacetylation of GR, which is required for this interaction in a human lung cancer cell line (A549) [132]. Nevertheless, HDAC2 has not been shown to be directly involved in GR mediated repression. Together, these findings indicate an involvement of HDACs in GR mediated repression, even though there might be cell type- and locus-specific differences.

In general, chromatin accessibility is mediated by nucleosome remodelling complexes like SWI/SNF, that allow for transient, reversible, and periodic DNA binding by GR and other transcription factors [105]. Notably, while GR may open the chromatin for other transcription factors, these regulators may themselves create new loci accessible to GR in inflammation, arguing for a bidirectional mechanism [85,106,107]. GR was found to differentially affect the residence time of its cofactors in the 3617 cell line. While GRIP1 displayed increased occupancy and prolonged residence time after treatment with dexamethasone, this was not the case for the central SWI/SNF component Brahma related gene-1 (BRG-1) or for AP-1. Conversely, GRIP1 knockdown did not affect GR residence time or fraction of binding [124]. Large-scale chromatin reorganisation and environmental stimuli potentially shape the genomic response to GCs [16,133]. For instance, repression by GR was abolished by hypoxia, suggesting that GC-mediated anti-inflammatory responses may be regulated by cellular metabolism, which is frequently altered at sites of inflammation [134,135].

GR crosstalk with pro- and anti-inflammatory transcription factors

In the context of inflammation, GR does not work in isolation, but talks to other transcription factors, that is, GR and AP-1 that reciprocally diminish each other's genomic actions [101,139,140]. Mutually, repressive effects have also been observed for GR and NF-κB, and other transcription factors such as STATs [70,141–143]. Herein, the underlying mechanisms range from tethering, binding to composite elements and competition for cofactors, to competition for DNA binding and chromatin accessibility.

Interestingly, while STAT5 has been shown to inhibit GR-mediated activation of target genes, GR on the other hand enhanced STAT5 transcriptional activity [144–146]. In hepatocytes, neurons, adipocytes and T-cells, GR and STAT5 have been found to physically interact during target gene repression [144,147–150]. In monocytes, the STAT5-mediated transcription of *Cyclooxygenase-2* is inhibited both directly and indirectly by GR [151]. GR macrophage cistromes are shown to be enriched for co-occurring STAT motifs, raising the possibility of myeloid GR-STAT5 crosstalk [100,136,152,153].

STAT3 tethering to chromatin-bound GR was shown to result in transcriptional synergism, while tethering of GR to residing STAT3 was associated with negative regulation in pituitary tumour cells [154]. GR-STAT3 binding to composite or neighbouring elements has also been linked to reciprocal synergism [154]. Consistently, cooperative binding between GR and STAT3 is reported to drive transcription and cells growth in basal-like triple negative breast cancer [155].

As STAT3 is an important mediator of IL6 and IL10 signalling, a putative crosstalk between GR and STAT3 in macrophages or monocytes might control inflammatory responses. In certain situations, GR may also drive enhanced STAT1 activation in monocytes indirectly [156]. Elucidating the cis-regulatory logic between these interactions and deciphering the mechanistic layers of inflammatory signalling will certainly be active areas of future studies [70,157].

Besides pro-inflammatory transcription factors, GR acts in concert with other nuclear receptors in co-binding scenarios and via formation of heterodimers. For instance, GR and the nuclear receptors PPARγ and LXR display synergistic effects on gene repression [70]. The synergy between GR and the PPAR family is of special interest, as PPARα and PPARγ have been shown to potentiate dexamethasone's anti-inflammatory properties in non-myeloid cells and *in vivo* [70,158,159]. Co-stimulation of GR and PPARα leads to enhanced repression of NF-κB and attenuated cytokine production, while simultaneously inhibiting GR-mediated gene activation. In a murine inflammatory bowel disease model, dexamethasone-induced repression was attenuated in PPARα knockout mice, indicating that PPARα may modulate anti-inflammatory GC effects [160]. In addition, in hepatocytes, GR and PPARα have been shown to co-occupy common binding sites, resulting in cooperative induction of genes controlling lipid/glucose metabolism [161]. Finally, GR interactions with the oestrogen receptor-alpha (ERα) and the androgen receptor (AR) both result in reciprocal antagonism in MCF7 and CV-1 cells respectively [162,163]. Of note, GR was found to repress ERα-activated transcriptional programmes by interacting with ERα-bound enhancers, thus suppressing growth in MCF7 cells [162]. Potentially, if similar GR-nuclear receptor interactions occur in inflammatory settings, one might speculate that the combination of different NR agonists could be clinically relevant [164].

Chromatin architecture and GR specificity

Phase separation in transcriptional regulation

As discussed above, locus-specific transcriptional control requires the coordination of numerous proteins and the formation of protein complexes on *cis*-regulatory elements [136,165–167]. Recent molecular, genetic and biochemical studies have found these regulatory components to be organised in distinct 3D transcription factories [168–171]. Particular protein domains, involved in the transcriptional process, interact with each other to form biomolecular condensates inside the nucleus [172–174]. The spatial organisation and dynamics of key components of the transcription apparatus within these condensates have several implications in gene regulatory mechanisms [175]. Advancements in various imaging techniques present powerful platforms to characterise the behaviour of individual proteins within dense clusters of transcription factors and co-regulators within an individual condensate. Some of these biomolecular condensates form membraneless droplets or nuclear foci via liquid–liquid phase separation (LLPS) [173,176–178]. While phase separation represents an essential component of GR function in different cell types, mechanistic insights into GR condensates in macrophages are still lacking. Hence, here we describe a general view of GR signalling mechanisms by phase separation. The underlying principle of phase separation involves multivalent interactions between domains or motifs in proteins or nucleic acids that are referred to as either low-complexity domains (LCDs) or intrinsically disordered regions (IDRs) [172,175,179,180]. Recent studies have elucidated the interplay of chromatin-associated transcription factors, co-factors, chromatin remodelers and transcription itself in phase separation. The recruitment of IDR-containing transcription factors (e.g. SP1 or GR), co-activators (e.g. Mediator, BRD4, and NCoA3) and RNA Polymerase 2 appears to be driven by phase separation [60,172,181–183]. Further involving specific histone marks, these dynamic IDR:IDR interactions contribute to the formation of discrete sub-compartments around chromatin-binding sites and confer locus-specific chromatin landscapes.

In response to ligand, GR accumulates into a discrete series of nuclear foci with non-homogeneous distribution in 1471.1 and BHK cells, respectively [184,185]. These GR foci exhibit the properties of biomolecular condensates and form a dynamic network of molecular interactions pertaining to phase separation in U2OS cells [186,187]. Reports have described the formation of 1000–2000 nuclear foci in COS-1 cells, each containing approximately 40–50 GR molecules with different subnuclear localisation patterns controlling receptor availability and interaction with specific binding sites [188,189]. Moreover, a comparative analysis of 13 GR ligands displayed a large variation in their abilities to induce foci formation, potentially due to differences in affinity [188]. In addition, GR scans the genome for GREs and its binding is reported to generate low-mobility chromatin domains. Mutation or deletion of the GR-DBD increases receptor mobility and affects the nuclear

distribution, leading to aberrant compartmentalisation of liganded GR [188]. DNase I treatment further disrupted GR foci formation, providing evidence for GR chromatin occupancy as a seed for biomolecular condensate formation in U2OS cells [187]. Moreover, foci formation has been associated with GR oligomer formation. In line with this, the GR mutant (GR^{P481R}) that mimics the structural change upon GRE binding, presents a constitutive tetrameric conformation. This allows for increased GR interactions with target DNA and is linked to enhanced transcription activity [123].

GR multivalency is a key determinant of phase separation in response to ligand [108,190]. The modular architecture of GR is well suited to recruit a wide spectrum of co-regulators. [191,192]. Current models of GR foci describe the genomic DNA as a scaffold to recruit GR and its associated co-regulators to establish a network of interactions forming condensates [119,185]. To unravel the molecular determinants of liquid condensate formation, NTD and LBD deleted GRs have been analysed. GR mutants lacking the NTD retained the capability of foci formation, whereas LBD deleted GR showed homogeneous distribution across the nucleus of U2OS cells [187]. Presumably, GR's IDR region may not be essential for the condensation process, while its LBD appeared to be required for foci formation at specific genomic regions. However, a recent study has uncovered two distinct sub-diffusive states of ligand-bound GR with limited mobility [108]. The most restricted low mobility state is associated with specific GR binding to chromatin, while the IDR interactions between GR and other interacting proteins represented a novel confinement state potentially implicated in GR condensate formation. In the same study, however, analysis of NTD-deleted GR mutants revealed a complete loss of protein:protein interactions associated with IDRs, while retaining the chromatin-binding state [108]. Future studies are needed to clarify how GR precisely controls the assembly and function of biomolecular condensates.

GR co-regulator assembly into dynamic transcriptional condensates may provide an elegant molecular mechanism underlying context-dependent selective recruitment of co-activators or co-repressors. Accordingly, GRE sequences or composite motifs may present a control mechanism for condensate selectivity during gene activation and repression [190,192]. Recently, activating and repressive GREs with varying propensities to coordinate multivalent interactions between GR and its interactors (co-activators and co-repressors) were observed *in vitro*. They may induce differential effects on condensate formation and potentially be responsible for compositional co-regulator bias at their respective genomic sites [60]. Taken together, GR binding to specific DNA elements, the IDR properties of transcription factors, and the recruited co-regulators may jointly control the selective partitioning of GR condensates and thus specify opposing fates of GR responsive genes (Fig. 2A–B).

Three-dimensional (3D) chromatin organisation in transcriptional regulation

Various GR cistromes (ChIP-seq data) have revealed unexpected genomic binding patterns: only a small proportion of GR-binding sites map to the proximal regulatory regions of target genes; while the vast majority are distributed non-uniformly, particularly at distal enhancers [17,136,167]. For example, 50% of GR-binding sites are located at a distance > 10 kb from the transcriptional start-site (TSS) and of GR responsive, upregulated genes [193]. Down-regulated genes presented a median distance of over 100 kb between the GR-binding site and the TSS [193]. A separate study identified corresponding GRE motifs near up- and down-regulated genes in dexamethasone-treated inflammatory macrophages to mostly be located within ±20 kb of the TSS [85]. It seems that GR mostly exerts its actions via distal regulatory regions, which may interact with their target TSS based on the spatial organisation of 3D chromatin folding in the nucleus.

Over the last decade, the advancement in various technical methods in chromatin biology have broadened our understanding of 3D genome architecture and organisation [194–196]. Such 3D structures are partly cell type specific and may constitute epigenetic control of transcription via chromatin looping between enhancers and promoters in terminally differentiated cells [197,198]. Contact matrices obtained by Hi-C (chromosome conformation capture) methods show the interaction frequency for all chromosomal loci at various scales. At multi-megabase scale, chromosomes are partitioned into discrete territories [199] (Fig. 2C). At a resolution between 0.1 to 1 Mb, the 3D genome adopts a hierarchical organisation of chromosomes into compartments, named A and B, which mainly correspond to transcriptionally active and inactive states of chromatin, respectively [200,201]. In A549 cells, dexamethasone treatment accounts for small dynamic changes in compartment associations, that is, the enrichment of mostly dexamethasone-repressed genes in B-like regions with increased interactions with compartment B [202]. On the other hand, the A-like compartment is enriched for dexamethasone-induced

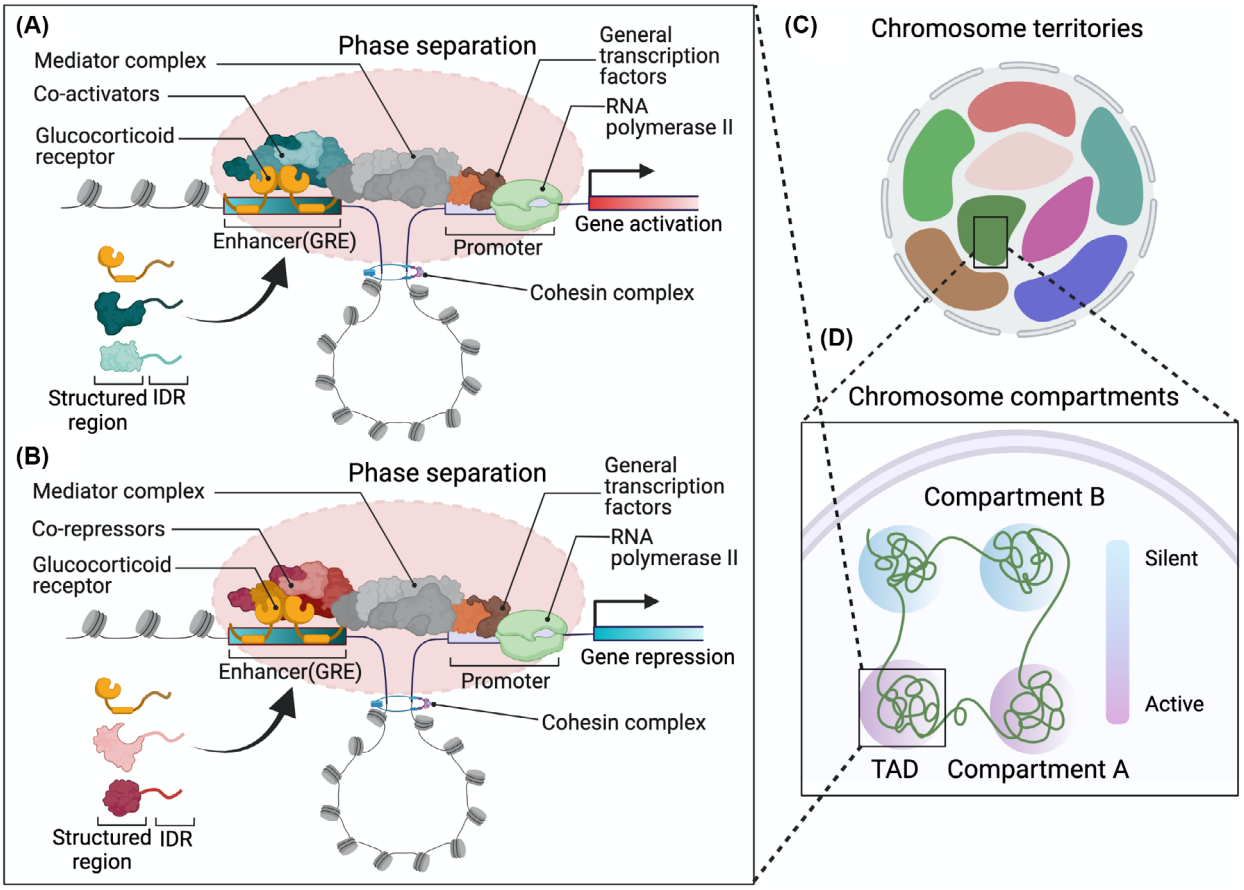


Fig. 2. Phase separation during GR interactions with chromatin and transcriptional complexes. GR binding to DNA may form phase-separated condensates and display selective bias in co-regulator recruitment. (A) Proposed models of transcriptionally active condensates upon GR binding to GREs include the establishment of protein:protein interaction networks by intrinsically disordered region containing co-activators (such as MED1, NCOA3, etc.). (B) Potentially, GR repressive condensates may form as a result of recruitment of co-repressor complexes at glucocorticoid response elements (GREs) via multivalent protein:protein interactions mediated by their intrinsically disordered regions. Chromatin inside cell nuclei is organised at multiple scales. The three-dimensional relationships between GREs and gene promoters might comprise specific areas of active and repressive transcription factories within phase-separated condensates. (C) Chromosome territories represent specific domains inside the nucleus that limit inter-chromosomal interactions. (D) At megabase scale, chromosomes are divided into two compartments classified as 'A' and 'B'. The compartments A and B represent three-dimensional positions of active and inactive genes, respectively. TADs may facilitate specific interactions between GR-binding sites and target transcription start sites that drive either up- or down-regulation.

genes [202] (Fig. 2D). At a resolution of ~ 100 kb, larger chromosome compartments are segmented into smaller domains of high local interactions called TADs [203,204] (Fig. 2D). In general, TAD boundaries are enriched for active histone modifications (e.g. H3K4me3, H3K36me3) and are characterised by binding sites for architectural proteins such as CCCTC-binding Factor (CTCF) or cohesins [194,204–207]. Dexamethasone induced dynamics in chromatin interactions mainly relied on cohesin binding, as CTCF appeared to be depleted at these sites [202,208]. Furthermore, additional cell type-specific loops can substratify the TAD into nested substructures at the subTAD level. These smaller units represent local intrachromosomal contacts and may encompass single gene regulatory units [209,210].

Most of the interactions between enhancers and promoters occur within TADs [211,212]. This level of organisation by TADs favours the regulatory activity on the genes lying within the same domain, independently of the genomic distances that separate them. GR binding and gene regulation are connected, and GR-binding sites that 'loop' to the promoters of genes are more likely to coincide with transcriptional changes than those sites that do not show such interactions [104,202]. Moreover, transcription factor binding

to specific chromatin sites may occur through a combination of diffusion, sliding and linear tracking along the chromatin fibre [213]. The fact that the genome folds readily in a non-random manner may favour such 'hopping' from regions far away on the linear genome, and may direct GR to specific sites [104]. The rewiring of promoter-enhancer contacts upon GC exposure may provide an additional explanation for cell type-specific transcriptional responses. This rewiring is accompanied by concomitant changes in chromatin structure and local TAD restructuration [208]. For instance, in the case of *GILZ* GR-binding site in U2OS cells, cell type-specific genomic organisation could rewire the enhancer contacts with its proximal or distal isoform promoter, producing specific transcript isoforms with potentially different cell type-specific functions [208].

In addition, GR may operate on pre-existing chromatin interactions between GREs or enhancers and promoters, which define the potential transcriptional targets and which allow enhanced activation upon GC stimulation [104,202]. For instance, by applying the Chromatin Interaction Analysis by Paired-End Tag Sequencing (ChIA-PET) approach to p300 in HeLa B2 cells, it has been shown that in response to ligand, GR is mainly recruited to regulatory elements that are pre-bound by p300. These elements were engaged in pre-existing interactions with their target promoters prior to GC exposure [214]. Another report found that GR ligand-activated quantitative changes in the frequencies of pre-established interactions without causing dramatic changes in 3D genome organisation [202]. An increase in chromatin interaction frequency might also reflect the stabilisation or synchronisation of active chromatin states in a large proportion of a bulk of cells, though, to ensure simultaneous regulation of GR target genes [214]. Furthermore, an overall 7% of chromatin interaction frequency changed upon dexamethasone treatment in A549 cells, as determined by *in situ* HiC. As a result, the quantitative increase in enhancer-promoter interactions is widely linked to increased expression of GR target genes [202]. This is further suggestive of cooperativity among multiple transcription factor-binding sites that interact to synergistically activate gene expression. Similarly, GR repressed genes were also associated with increased chromatin interactions between distal binding sites and gene promoters. However, compared to induced genes, GR-binding sites were looped to repressed genes over a larger genomic distance [202].

Interestingly, GR appeared to facilitate *de novo* recruitment of HATs to sites that dynamically interact with promoters and to pre-existing sites for efficient transcriptional changes [215,216]. Moreover, growing evidence also indicates that GR regulated genes often form clusters as specialised nuclear hubs (or transcription factories) [173,217]. Even with shared nuclear positioning of GR induced and repressed regulatory regions, transcription factor and co-regulator composition was predicted to differ significantly between regions [218]. However, on a linear genomic scale, such clustering of GC responsive genes inside nuclear hubs remained invariant upon exposure to ligand, warranting further studies of these dynamics to determine the function of higher level structural genome organisation for GR. Even though the phenomena of 3D genome architecture are well established in various cellular models, how the spatial organisation might impact GR dependent transcriptional regulation in inflammatory macrophages still needs to be investigated.

Conclusions and perspectives

The mechanisms of glucocorticoid-mediated gene repression still represent a fascinating molecular mystery. The models herein proposed may provide numerous opportunities for future research in the context of GR signalling in innate immunity. So far, diverse and complex scenarios of GR-dependent transcriptional repression have been outlined, especially with respect to long-range interactions among target gene promoters (or TSSs) and GR-binding sites of variable affinity. In addition, the dynamic nature of the chromatin environment modulates the DNA sequence availability for GR occupancy, which in turn shapes the transcriptional complex assembly and crosstalk [69,136]. Interactions between GR, NF-κB and AP-1 require the precise assembly of transcriptional repressor complexes at *cis*-regulatory elements to control inflammatory gene expression [85,87]. Additionally, GR engagement with other transcription factors, including PPARs and STATs, might be poised for target gene regulation [70,154]. In summary, neither tethering, nor nGRE binding, nor binding to AP-1 and NF-κB elements, nor recruitment of co-repressors such as GRIP1, can in isolation explain the wide-reaching repressive effects of GCs [17,85]. We therefore propose that these mechanisms might partly contribute to transcriptional repression and sum up to the genomic effects in a locus-specific way. Moreover, several studies have identified a number of silencer elements in various eukaryotic genomes that function in a position- and orientation- independent manner to mediate transcriptional repression [219–221]. Depending on the cellular context, silencers often contain bifunctional regulatory sequences that may also act as activators. Macrophage

cis-regulatory elements bound by GR and specifying repression may thus potentially be categorised as non-classical silencers. Furthermore, the existence of additional mechanisms driven by other means such as altered cellular metabolism might be envisioned.

Detailed genome-wide studies will be necessary to further dissect the interplay between regulatory mechanisms in an inflammatory context. Furthermore, biomolecular condensate formation could possibly enable robust and precise regulation of GR target genes [60]. Continued investigation in this direction will benefit from diverse experimental techniques and super-resolution imaging tools. The implication of spatial 3D genome organisation in communication between distal GR-binding sites and promoters might provide a new framework for negative target gene regulation [214]. Experimental approaches including single-cell analyses or ligation-independent techniques may provide opportunities for in-depth genomic and transcriptomic analyses leading to the discovery of novel molecular mechanisms in macrophages and other cell types. Finally, integrating multi-omics datasets and advancing computational pipelines may deepen our understanding of anti-inflammatory GR actions and open up avenues for the development of safer and more specific immunomodulators.

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