C/EBP maintains chromatin accessibility in liver and facilitates glucocorticoid receptor recruitment to steroid response elements

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Mechanisms regulating transcription factor interaction with chromatin in intact mammalian tissues are poorly understood. Exploiting an adrenalectomized mouse model with depleted endogenous glucocorticoids, we monitor changes of the chromatin landscape in intact liver tissue following glucocorticoid injection. Upon activation of the glucocorticoid receptor (GR), proximal regions of activated and repressed genes are remodelled, and these remodelling events correlate with RNA polymerase II occupancy of regulated genes. GR is exclusively associated with accessible chromatin and 62% percent of GR-binding sites are occupied by C/EBP β . At the majority of these sites, chromatin is preaccessible suggesting a priming function of C/EBP β for GR recruitment. Disruption of C/EBP β binding to chromatin results in attenuation of pre-programmed chromatin accessibility, GR recruitment and GR-induced chromatin remodelling specifically at sites co-occupied by GR and C/EBP β . Collectively, we demonstrate a highly cooperative mechanism by which C/EBP β regulates selective GR binding to the genome in liver tissue. We suggest that selective targeting of GR in other tissues is likely mediated by the combined action of cell-specific priming proteins and chromatin remodellers.

The EMBO Journal (2013) **32**, 1568–1583. doi:10.1038/emboj.2013.106; Published online 10 May 2013

Subject Categories: chromatin & transcription

Keywords: C/EBP; Chromatin; DNase-seq; GR; Liver tissue

Introduction

Glucocorticoids (GCs) are useful therapeutic tools for the management of auto-inflammatory diseases and for inhibiting cell growth of various cancers (Rhen and Cidlowski, 2005). GCs, synthesized and secreted by the adrenal glands,

are the end product of the hypothalamic-adrenal axis. In basal conditions, secretion follows circadian and ultradian rhythms (Lightman and Conway-Campbell, 2010). The actions of GCs are primarily mediated by binding to the glucocorticoid receptor (GR), which is expressed in most tissues (Heitzer *et al.*, 2007). In liver, GCs control a range of processes such as gluconeogenesis, glycogenolysis, fatty acid metabolism, bile acid synthesis, neonatal growth and adaptation to the acute phase response (Rose *et al.*, 2010).

Following binding to GCs, GR rapidly translocates from the cytoplasm to the nucleus to interact with genomic target sequences within the chromatin (Stavreva *et al.*, 2012). The chromatin, comprised of genomic DNA wrapped around nucleosomes and non-histone factors, is highly organized; this architecture controls selective recruitment of transcription factors (Bell *et al.*, 2011) including GR (Biddie *et al.*, 2010). This facilitates genome-specific association of transcriptional co-regulators that in turn change transcription rates for nearby genes in the nuclear three-dimensional space (Hakim *et al.*, 2011). GR interaction with chromatin is highly dynamic, with GR cycling between bound and unbound states in a few seconds (McNally *et al.*, 2000). During this process, GR recruits additional transcription factors and chromatin remodellers ‘assisted loading’ (Voss *et al.*, 2011); continuous association and dissociation of factors results in a highly dynamic chromatin structure at enhancer/promoter regions (Nagaich *et al.*, 2004; Johnson *et al.*, 2008). Any intervention of these dynamic processes—either gain or loss of factors at a given enhancer—potentially changes this equilibrium and consequently alters enhancer/promoter accessibility (John *et al.*, 2008; Biddie *et al.*, 2011; Voss *et al.*, 2011).

Genome-wide transitions in chromatin structure are efficiently probed by partial DNase I digestion of chromatin combined with massive parallel sequencing (DNase-seq) (Hesselberth *et al.*, 2009). Elaborate studies in multiple cell lines show a high correlation between accessibility and transcription factor occupancy (Thurman *et al.*, 2012). For example, GR is almost exclusively associated with accessible chromatin (John *et al.*, 2011). Strikingly, GR is predominantly recruited to preaccessible chromatin, emphasizing that factors presetting chromatin accessibility are important regulators of GR recruitment to chromatin and subsequently GR-regulated gene expression (John *et al.*, 2011). Accordingly, GR regulates gene expression through direct and/or indirect interaction with a range of transcription factors such as AP1, Oct, NF1, C/EBP, NF-kB, STATs, LXR, PPARs and COUP-TFII, depending on promoter and cellular context (Lee and Archer, 1994; De Martino *et al.*, 2004; Biddie *et al.*, 2011; Patel *et al.*, 2011; Rao *et al.*, 2011; Siersbaek *et al.*, 2011; Langlais *et al.*, 2012). Although a substantial amount of data has addressed how GR cooperates with other transcription factors genome wide in cells grown in culture

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(Biddie *et al*, 2011; Rao *et al*, 2011; Langlais *et al*, 2012; Uhlenhaut *et al*, 2012), little is known about global transcription factor cooperation in mammalian tissue, and how chromatin regulates transcription factor occupancy.

We identify here mechanisms that control GR recruitment to chromatin in intact liver tissue, and correlate binding events with RNA polymerase II (RNAPII) recruitment to GR-regulated promoters. We isolated liver tissue from dexamethasone (dex)-injected adrenalectomized mice and determined RNAPII occupancy of genes and GR recruitment to chromatin by ChIP-seq and chromatin remodelling events by DNase-seq. We report that a high frequency of dex-induced genes has nearby occupancy of GR at pre-programmed and *de novo* remodelled chromatin. In contrast, repressed genes have nearby GR binding to pre-programmed chromatin and loss of specific non-GR bound accessible regions in response to dex. Remarkably, 62% of total GR-binding sites in liver are associated with C/EBP β occupancy. At a subset of sites, C/EBP β recruitment is assisted by GR-induced chromatin remodelling. Disruption of C/EBP β binding to chromatin results in decreased GR recruitment to chromatin at both pre-programmed and *de novo* remodelled GR-binding sites occupied by C/EBP β . We conclude that C/EBP β is a central factor for genome-wide GR recruitment to chromatin in mouse liver tissue.

Results

Dexamethasone controlled gene transcription is associated with chromatin remodelling of nearby regulatory elements

To mimic situations of low GR activity and thus achieve a baseline of gene transcription and chromatin accessibility independent of GR, we removed endogenous GCs by adrenalectomy in mice. GR was activated by intraperitoneal injection of cyclodextrine encapsulated dex, 1 h prior to isolation of intact liver. Control mice received an injection of vehicle (veh), cyclodextrin. To measure direct short-term effects on gene transcription, we mapped RNAPII recruitment to genes using ChIP-seq (Figure 1A and B and Supplementary Figure S1). We identified 266 genes significantly ($P < 0.001$) induced by dex (Figure 1C) and 353 genes repressed by dex (Figure 1D).

To determine the extent of which dex mediated changes in gene transcription correlates with changes in chromatin structure, we mapped chromatin accessibility genome wide using DNase-seq (Supplementary Table S1). We identified more than 71 000 replicate concordant DNase hypersensitive sites (DHS) in liver, where the majority of DHS are shared between veh- and dex-treated mice (Supplementary Table S2). DHS are primarily found in promoters, distal regions and within introns (Supplementary Figure S2A and B) and the mean tag density of DHS is higher at promoters and exons relative to introns, distal regions and downstream regions (Supplementary Figure S2C and D). Injection of dex results in remodelling of chromatin at specific regions of the genome in liver. More than 23 000 sites are *de novo* reprogrammed as a consequence of dex, where 2/3 display increased accessibility (Supplementary Table S2). Notably, the remodelled sites are more abundant in distal and intronic regions compared to the pre-programmed sites (Supplementary Figure S3). Interestingly, in our previous studies, we did not

observe robust reduction of chromatin accessibility in cell lines treated with dex (John *et al*, 2011), suggesting that this phenomenon may be unique for tissue. Moreover, we noticed that dex-induced genes are often associated with regions of induced chromatin accessibility (red arrows, Figure 1A and Supplementary Figure S1A–C) and repressed genes are associated with regions of decreased accessibility (red arrows, Figure 1B and Supplementary Figure S1A, D and E). To determine if this is a general phenomenon for dex-regulated genes in liver, we scored all DHS 50 kb up- and downstream of the transcription start sites (TSS) of dex-regulated genes and divided the DHS in two bins according to association with induced and repressed genes. We next identified differentially dex-regulated DHS within 50 kb of TSS (Supplementary Figure S4A and B) and correlated those with changes in RNAPII occupancy of nearby genes (Figure 1E). In induced genes, 36% of the DHS have increased accessibility in response to dex and 1% of DHS have decreased accessibility (Figure 1E). In contrast, in repressed genes, 4% of DHS have induced accessibility and 19% have decreased accessibility (Figure 1E). Note that the accessibility of most DHS is not changed (Figure 1E), demonstrating that chromatin-remodelling events promoted by dex are specific to a subset of DHS. Moreover, a higher frequency of induced genes than repressed genes has nearby DHS with increased accessibility and vice versa for repressed genes (Supplementary Figures S4C and D). Also, the average ratio of accessibility after dex treatment is higher for DHS in induced genes compared to repressed genes (Supplementary Figure S4E). *De novo* motif analysis of regions with increased and decreased accessibility suggests that direct GR binding to DNA is associated with induced chromatin remodelling (Figure 1F). In contrast, DHS with reduced chromatin accessibility have low frequency of GR motifs but are enriched for DNA motifs likely to bind HNF4 (Figure 1F). Thus, increased chromatin remodelling near dex-induced genes is primarily promoted by direct interaction of GR with DNA, whereas decreased accessibility of DHS near repressed genes may be through indirect binding of GR to DNA or through sequestration of transcriptional co-regulators at DHS leading to reduced accessibility.

GR exclusively occupies accessible chromatin and genes regulated by dex are associated with regions bound by GR

To correlate dex-mediated changes of chromatin accessibility with GR occupancy, we mapped GR-binding genome wide using ChIP-seq (Supplementary Table S1). Dex injection results in marked recruitment of GR to the genome as exemplified at the Pck1 locus (Figure 1A) and other genes regulated by dex (Supplementary Figure S1). The frequency of dex-induced accessible sites (within 50 kb of TSS) with GR peaks is significantly higher than DHS with dex-promoted reduced accessibility (Figure 1G), supporting the *de novo* DNA motif analysis (Figure 1F) and suggesting that repression of accessibility by dex is not mediated through interaction of GR with the remodelled site. Genome wide, we identified more than 11 000 GR ChIP-seq peaks in liver, where GR peaks are primarily located at intergenic distal and intronic regions (Supplementary Figure S5A) and the mean sequence tag density of peaks does not differ significantly between the regions bound by GR (Supplementary

Figure 1 Correlation of RNAPII recruitment to TSS with changes in chromatin accessibility at nearby regulatory elements. (A) Example of DNase accessibility, RNAPII and GR occupancy at the *Pck1* locus, where dex treatment results in induced RNAPII recruitment to the gene body and at *Osgin1* locus (B) where dex injection leads to repressed RNAPII recruitment. Black arrows mark GR-binding sites at pre-programmed chromatin and red arrows mark remodelled chromatin. (C) Activated and (D) repressed genes were identified using RNAPII ChIP-seq. Heatmaps illustrate RNAPII occupancy -2.5 kb to +7.5 kb relative to TSS of genes with significant change ($P < 0.001$) of RNAPII occupancy after dex injection. (E) DHS 50 kb up- and downstream of dex induced ($n = 2555$) and repressed ($n = 3165$) genes were selected. DHS with significant ($P < 0.05$) change of accessibility were identified (see Supplementary Figure S4). (F) *De novo* motif analysis (using HOMER) of differentially regulated DHS within 50 kb of dex-regulated genes. The most significant motif for each group is shown. (G) Frequency of GR peaks at increased and decreased DHS within 50 kb of dex-regulated genes. (H) Venn diagram illustrating amount of DHS unique to livers in veh- and dex-treated animals. Green represents GR peaks overlapping the accessible chromatin landscape. (I) Frequency of dex-induced and (J) repressed genes with nearby GR occupancy ranging from 1 kb to 100 kb up and downstream of TSS.

Figure S5B). When mapped to the accessible chromatin landscape, we observed GR binding primarily at pre-accessible parts of the genome (82%, GR pre-programmed binding sites) (Figure 1H), exemplified at the *Pck1* and *Osgin1* loci (black arrows, Figure 1A and B) and other GR

targets (Supplementary Figure S1). Genome wide, 17% of GR peaks are found at sites where chromatin is *de novo* remodelled (*de novo* GR-binding sites) and none are found at sites where accessibility is depleted (Figure 1H). Moreover, only a fraction (1%) of GR peaks is found at inaccessible regions

(Figure 1H). When the different sets of GR peaks are compared with dex-regulated genes, we observe a similar frequency of dex-induced and repressed genes with nearby GR peaks at pre-programmed DHS (Figure 1I). In contrast, significantly more dex-activated than repressed genes are nearby GR peaks at *de novo* remodelled chromatin (Figure 1J).

Overall, we report that GR-binding events in adult liver tissue are strongly associated with accessible chromatin and GR is primarily recruited to pre-programmed chromatin. Thus, in tissue as well as in cell lines, GR recruitment to chromatin relies mostly on the preset accessible chromatin environment. Therefore, we next sought to identify mechanisms that selectively regulate GR accessibility.

Selective recruitment of GR to the genome in liver is associated with chromatin accessibility and nucleosome occupancy

First, we performed an elaborate analysis of GR binding to the genome across different cell types using the GR ChIP-seq data presented here and previously published GR ChIP-seq data from cell lines generated from mouse mammary adenocarcinoma (3134) (John *et al*, 2011), pituitary gland tumour (AtT20) (John *et al*, 2011), preadipocyte embryonic fibroblast (3T3L1) (Steger *et al*, 2010) and myoblasts (C2C12) (Kuo *et al*, 2012). When we conducted a pairwise comparison of GR binding to the five different cell types, we observed between 5–40% of shared GR-binding sites (Figure 2A). Overall, the mammary adenocarcinoma cell line, myoblasts and the preadipocyte fibroblast cell line demonstrated the highest degree of overlap. Most noticeably, only 0.5% of the 11 000 binding sites in liver are shared between the four other cell types and 83% are unique to liver tissue (Figure 2B). For example, GR binding at the *Per1* locus is conserved among all the cell types, whereas GR occupancy at the *Cyp27a1* locus is liver specific and GR recruitment to the *Cav1* promoter is specific to 3T3L1 and 3134 (Figure 2C). Interestingly, whereas the canonical GR-binding sequence is present at all sites shared between the five cell types, less than 60% of the liver unique sites harbour a canonical GR-binding sequence (Figure 2D). This emphasizes a strong cell-type-specific determinant for GR binding to the genome that relies less on a canonical GR-binding sequence in DNA.

When we extracted cell-specific GR-binding sites from each cell type and probed for level of chromatin accessibility in liver tissue, we observed that chromatin accessibility is strictly associated with liver-specific GR-binding sites and GR-binding sites used in other cell types are inaccessible in liver (Figure 2E). Interestingly when we measured micrococcal nuclease (MNase) accessibility at these cell-specific GR-binding sites using previously published MNase-seq data from mouse liver tissue (Li *et al*, 2011), we observed a clear increase in MNase-seq tag density at the inaccessible GR-binding sites compared to the liver-specific accessible binding sites (Figure 2F), suggesting that highly positioned nucleosomes at inaccessible GR-binding sites protects DNA from MNase digestion in contrast to GR-binding sites that are DNase I hypersensitive. Thus, specific nucleosome positioning may constitute a chromatin environment that occludes GR access to binding sites and this maintains cell-selective GR occupancy. Notably, however, if GR peaks identified in liver were binned into binding sites at preaccessible

chromatin (pre-programmed sites) and binding sites at *de novo* remodelled chromatin (*de novo* sites), a high nucleosome positioning was also observed at *de novo* binding sites (Figure 2G). However, at these sites, GR can bind and remodel chromatin in liver, emphasizing that additional factors determine if nucleosomes are primed for remodelling by GR, specifically in liver.

Several potential mechanisms—such as rotational positioning of the GR response element, DNA methylation, occupancy of initiation/pioneering factors, cooperative binding of other TFs and specific histone modifications—may determine remodelling of specific nucleosomes by GR in a particular cellular setting. To initially address the latter, we employed previously published ChIP-seq data from H3K4 monomethylation, H3K4 trimethylation and H3K27 methylation in liver (Hoffman *et al*, 2010). We observed an enrichment of H3K4me1 tags immediately adjacent to the centre of pre-programmed GR-binding sites (Figure 2H) and, in agreement with the MNase-seq data (Figure 2F and G), the H3K4me1 signal is depleted at the centre of the peak. Interestingly, at *de novo* GR-binding sites, H3K4me1 is enriched at the centre of the binding sites compared to adjacent regions. Moreover, H3K4me1 signal is low at GR-binding sites used in other cell types supporting previous reports of H3K4me1 as a mark of putative enhancers (Wang *et al*, 2008). Thus, H3K4me1 may mark nucleosomes for downstream remodelling by GR. In contrast, H3K4 trimethylation is absent at *de novo*-binding sites but is abundant adjacent to pre-programmed sites (Figure 2I). Moreover, we observed no enrichment of the repressive H3K27 methylation modification at *de novo* or pre-programmed sites. Rather, this modification is more enriched at inaccessible GR-binding sites used in other cell types compared to GR-binding sites used in liver (Figure 2J), emphasizing that inactive GR-binding sites in liver are positioned in inaccessible heterochromatin.

Identification of transcription factors co-occupying chromatin with GR

Transcription factors occupying pre-accessible chromatin and initiation factors bound to inaccessible chromatin that co-operate with GR to remodel chromatin are likely to play a significant role in GR recruitment to chromatin in liver. To identify such factors, we searched for over-represented DNA sequences in GR ChIP-seq peaks using two independent *de novo* DNA motif analysis algorithms MEME (Multiple Em for Motif Elicitation) (Bailey *et al*, 2009) and HOMER (Hypergeometric Optimization of Motif EnRichment) (Heinz *et al*, 2010). Both methods individually identified the canonical GR response element as the most significantly enriched motif, supporting the quality and integrity of the GR ChIP-seq (Figure 3A and Supplementary Figure S6C). More than 60% of the identified GR peaks contained the identified GR-binding motif depending on the stringency of the motif search (Supplementary Figure S6B). In addition to the GR motif, both methods identified highly enriched C/EBP, HNF4 and FOXA1 like motifs (Figure 3A and Supplementary Figure S6A). Remarkably, in contrast to previous reports from 3134 cells, we found relatively low enrichment of AP1-binding sequences at GR-binding sites in liver (Supplementary Figure S6B). However, like 3134, the AP1 motif is highly enriched at GR-binding sites in 3T3L1 and C2C12 (Supplementary Figure S6D). These three cell types

Figure 2 Chromatin accessibility is strongly associated with selective recruitment of GR to the genome. (A) Pairwise percentage overlap of GR-binding sites (GBS) identified in the genome of five different cell types; Adult liver tissue, 3134 (mammary), AtT20 (pituitary), 3T3L1 (embryonic fibroblast) and C2C12 (myotube). (B) Amount of GBS in liver shared between all five cell types (red), present in liver and at least in one other cell type (green) or unique to liver tissue (grey). (C) Frequency of canonical GR-binding sequences at shared and unique GBS (FIMO at $P < 0.001$). (D) Example of GBS shared between all five cell types (Per1), liver-specific GBS (Cyp27a1) and 3T3L1/3134 unique GBS (Cav2). (E) DNase accessibility of chromatin in liver at GBS uniquely used in liver and GBS used in 3134, AtT20, 3T3L1 and C2C12. (F) Nucleosomal density in liver at liver GBS and GBS used in 3134, AtT20, 3T3L1 and C2C12. (G) Nucleosomal density in liver at *de novo* and pre-programmed GBS in liver. (H) Level of H3K4me1, (I) H3K4me3 and (J) H3K27me in liver at GBS used in liver and GBS used in 3134, AtT20, 3T3L1 and C2C12.

Figure 3 Identification of C/EBPβ-binding sites in adult liver in mice injected with veh or dex. (A) *De novo* motif analysis using MEME of the top 5000 most intense identified GR peaks in liver. (B) Frequency of GR and C/EBP motifs identified by HOMER (Supplementary Figure S6) at 400 bp up and downstream of the centre of *de novo* (red) and pre-programmed (black) GR-binding sites. (C) Three examples of C/EBPβ binding determined by ChIP-seq. Black arrows indicate pre-programmed GR-binding sites occupied by C/EBPβ. Red arrows show *de novo* GR-binding sites where C/EBPβ loading is assisted by GR. (D) Correlation of the tag density of all replicate concordant C/EBPβ-binding sites identified by C/EBPβ ChIP-seq in mice injected with veh or dex. (E) Venn diagram illustrating the overlap between the total number of replicate concordant C/EBPβ-binding sites after veh (22 715) or dex (25 393) injection and total replicate concordant GR ChIP-seq peaks (11 666) after dex injection. Sixty-two percent of the total GR-binding sites is associated with occupancy of C/EBPβ. (F) Correlation between dex-affected C/EBPβ occupancy and DNase accessibility. Pearson correlation ($r=0.77$) was performed on all GR-binding sites with C/EBPβ occupancy.

also show the highest degree of genome-wide GR-binding overlap (Figure 2A), emphasizing that transcription factors active in specific cell lines are likely determinants for selective GR recruitment to chromatin. Interestingly, analysis of GR motif distribution at pre-programmed versus *de novo* GR-binding sites demonstrates that canonical GR motifs are

relatively more enriched at *de novo* binding sites compared to pre-programmed sites (Figure 3B, left). Furthermore, the identified C/EBP motif is enriched at the centre of the pre-programmed GR peaks (Figure 3B, right). In contrast, at *de novo* sites the C/EBP motif is less abundant at the centre of GR peaks compared to sequences immediately adjacent.

Identification of C/EBP β -binding sites in liver tissue

C/EBP α and C/EBP β are the most abundant C/EBP subtypes in liver (Ramji and Foka, 2002). C/EBP α binding to the genome in adult mouse liver tissue has previously been mapped by ChIP-seq (Schmidt *et al*, 2010). To complement this analysis and to examine any effects of GR activation on C/EBP recruitment to chromatin, we mapped C/EBP β in liver tissue in the presence and absence of dex. Integration with DHS and GR ChIP-seq data identified several different types of C/EBP β interaction with chromatin in a GR-centric manner. In the *Orm1* locus, GR is primarily recruited to pre-programmed chromatin preoccupied by C/EBP β (Figure 3C, black arrows). Likewise, several GR pre-programmed binding sites surrounding the *Tat* gene are preoccupied by C/EBP β (Figure 3C, black arrows). In addition, dex-induced C/EBP β occupancy is also observed, which is associated with remodelled chromatin (Figure 3C, red arrows). In the *Fkbp5* promoter, multiple *de novo* GR-binding sites are observed and C/EBP β occupancy is strictly dependent on GR-induced chromatin remodelling.

Genome wide, we identified ~22 000 replicate concordant C/EBP β peaks in the absence of dex and ~25 000 replicate concordant peaks in the presence of dex (Figure 3D and E). In both cases, at least 84% of the C/EBP β binding fell into the accessible genome (Supplementary Figure S7A), demonstrating that the majority of C/EBP β -binding events are coupled with accessible chromatin. Direct correlation with the previously published C/EBP α ChIP-seq data demonstrates that C/EBP α and C/EBP β occupy largely the same genomic regions in adult liver ($r^2 = 0.79$, Supplementary Figure S7B). The majority of C/EBP β -binding sites are not changed as a consequence of dex injection (Figure 3D and E), but we do observe a considerable number of dex-induced and repressed C/EBP β binding. Sixty-two percent of identified GR peaks overlap with C/EBP β and occupancy of the majority of these sites (5800) is not changed by dex treatment (Figure 3E). Moreover, almost all GR-regulated genes (activated and repressed) in liver have nearby occupancy of C/EBP β (Supplementary Figure S7C). Close to 1400 GR-binding sites overlap with dex-induced C/EBP β binding (Figure 3E) and the overall dex-altered recruitment of C/EBP β correlates well with dex-mediated changes in chromatin accessibility (Pearson correlation, $r = 0.77$, Figure 3F) and the frequency of GR-induced genes with nearby C/EBP β occupancy is increased (Supplementary Figure S7C). This demonstrates that GR assists loading of C/EBP β at a subset of sites in the genome through mechanisms involving chromatin remodelling. Interestingly, overlapping the C/EBP β -binding profile in liver with the previously published C/EBP β -binding profile in differentiating 3T3L1 fibroblasts shows that like GR, C/EBP β occupies the genome in a highly cell type selective manner (Supplementary Figure S7D). Also, like liver, GR and C/EBP β occupy a significant number of sites in the genome of 3T3-L1 (Steger *et al*, 2010), collectively suggesting that C/EBP β together with additional transcription factors (e.g., HNF4 and FOXA in liver and AP1 in adipocytes) may be involved in genomic programming of cell-type-specific binding of GR.

Correlation of GR, C/EBP β and DHS identifies specific groups of GR genome occupancy in liver

Integration of GR and C/EBP β ChIP-seq profiles with DHS-seq data identifies six unique groups of genome-wide GR-binding

patterns, where group one to three represents pre-programmed and four to six exhibits *de novo* GR-binding sites, respectively (Figure 4A). Figure 4B illustrates representative ChIP-seq peaks and DNase-seq hotspots from each group. Group one and four represent GR-binding sites not bound by C/EBP β and correspond to 31% of the pre-programmed and 72% of the *de novo* GR-binding sites, respectively. In agreement with low C/EBP β occupancy, the genomic sequences from group one and four have low abundance of the canonical C/EBP-binding sequence (Figure 4C).

Interestingly, GR-dependent binding of C/EBP β to chromatin falls into two distinct groups (group two and five), where group two represents GR-assisted loading of C/EBP β at pre-programmed chromatin (10% of pre-programmed GR-binding sites) and group five are assisted C/EBP β loading to *de novo* remodelled chromatin (20% of *de novo* GR-binding sites). This demonstrates at least two different mechanisms for assisted loading of factors to chromatin by GR. Interestingly, in group two, the C/EBP β motif is enriched less compared to the other groups where C/EBP β occupies chromatin (Figure 4C). This suggests that C/EBP β binds these regions in a GR-dependent manner through mechanisms that do not solely involve C/EBP β binding to the canonical C/EBP motif. In contrast, the genomic regions of group five GR-binding sites have relatively high enrichment of C/EBP motifs, implying that C/EBP β is unable to bind these sites directly, unless GR remodels the relatively high positioned nucleosomes (Figure 2G) and thus promote accessibility to the otherwise occluded C/EBP-binding sequence. In both groups, the tag density of the GR peaks is higher compared to the other groups (Supplementary Figure S8A), suggesting an overall stronger binding of GR to chromatin when GR and C/EBP β are recruited together.

Group three and six correspond to GR-binding sites occupied by C/EBP β prior to GR activation (Figure 4A), where group three represents 59% of the pre-programmed GR-binding sites. The C/EBP β peaks within group three are on average more intense compared to the other groups (Supplementary Figure S8A) and C/EBP motifs are present at a relatively high frequency (Figure 4C). In contrast, the GR-binding sequence is present at a relatively low level, suggesting that GR binding to this compartment of the genome is in part through mechanisms that do not involve the classical dimeric binding of GR to canonical GR-binding sequences. Group six, which only constitutes 1% of the total GR-binding sites and 8% of *de novo* binding sites, represents GR-binding sites where C/EBP β occupies DNase inaccessible chromatin prior to GR activation and where GR activation results in *de novo* chromatin remodelling. Group six binding sites are highly enriched for GR and C/EBP composite binding sites, indicating that C/EBP β is bound to inaccessible chromatin though direct interactions with DNA, but at these sites occupancy does not result in robust chromatin accessibility. Only upon GR recruitment will these sites become remodelled.

Interestingly, a higher frequency of dex-induced genes than repressed genes have nearby GR-binding sites with assisted loading of C/EBP β (Figure 4D, group two and five), suggesting that assisted loading of transcription factors such as C/EBP β is involved in transcriptional activation rather than repression. In contrast, a similar frequency of dex-induced

Figure 4 Correlation of GR binding, DNase accessibility and C/EBP β occupancy identifies six groups of GR-binding sites. (A) GR-binding sites were grouped according to overlap with DNase accessibility and C/EBP β occupancy. Tag densities were visualized as heatmaps. Group 1 ($n = 2957$) represents pre-programmed GBS without occupancy of C/EBP β . Group 2 ($n = 988$) are pre-programmed GBS where C/EBP β recruitment is assisted by GR. GBS in group 3 ($n = 5693$) are pre-programmed and preoccupied by C/EBP β . Group 4 ($n = 1434$) are *de novo* remodelled with no occupancy of C/EBP β . Group 5 ($n = 402$) represents sites where GR assists loading of C/EBP β through *de novo* remodelling of chromatin. Group 6 ($n = 155$) are GR-binding sites where C/EBP β preoccupies closed chromatin and GR recruitment results in chromatin remodelling. (B) Representative examples of GR, DHS and C/EBP β peaks belonging to the different groups. (C) Relative distribution canonical GR and C/EBP-binding sequences (FIMO at $P < 0.001$). (D) Frequency of dex-induced and -repressed genes with different modes of nearby GR occupancy ranging from 1 kb to 100 kb up and downstream of TSS.

and repressed genes have nearby GR binding to sites pre-programmed by C/EBP β (Figure 4D, group three), suggesting that GR-mediated transactivation and transrepression is associated with GR recruitment to accessible chromatin preoccupied by C/EBP β . Thus a combination of GR recruitment to pre-programmed and remodelled chromatin in conjunction with GR-assisted loading of factors is correlated with gene activation, whereas GR recruitment to pre-programmed chromatin alone is associated with gene repression.

Accessible chromatin and presence of GR half-sites specifies GR recruitment to a subset of C/EBP β -binding sites

The low abundance of canonical GR motifs at pre-programmed GR-binding sites occupied by C/EBP β suggests that GR may be tethered to many of these sites through interaction with C/EBP β . This is supported by previous observations of direct interaction between GR and C/EBP β (Boruk *et al*, 1998). However, this raises a central question of

Figure 5 Selective recognition of C/EBPβ occupied chromatin by GR. All C/EBPβ – binding sites without canonical GR motifs were ranked according to GR ChIP-seq tag density. (A) Heat map illustrating C/EBPβ and GR tag density. The top and bottom 20% percent of C/EBPβ-binding sites were selected for downstream analysis. (B) *De novo* motif analysis was performed on the top and bottom 20% of C/EBPβ – binding sites using HOMER. (C) Distribution of identified GR half-sites relative to the centre of C/EBPβ peaks. (D) Frequency of GR half-sites at GR peaks grouped according to accessibility and C/EBPβ occupancy (see Figure 4). (E) Level of H3K4me1 and H3K27ac and DNase accessibility at the top and bottom 20% of C/EBPβ-binding sites.

specificity. Why is only a subset of C/EBPβ-binding sites occupied by GR? To address this, we isolated all C/EBPβ-binding sites that do not harbour the canonical GR motif (i.e., potential tethering sites) and ranked the sites according to the density of GR ChIP-seq tags (Figure 5A). The top 20% (i.e., bound by GR) and bottom 20% (i.e., no GR recruitment) were selected for further analysis. *De novo* motif analysis of the GR-bound C/EBPβ-binding sites not only identifies enrichment of DNA-binding motifs for liver-enriched transcription factors (e.g., HNF4 and HNF6) but also demonstrates high enrichment of GR half-sites (Figure 5B). In contrast, C/EBPβ-binding sites not bound by GR are enriched for CTCF motifs. Interestingly, identified GR half-sites are enriched adjacent to the centre of the C/EBPβ peaks occupied by GR and depleted at C/EBPβ peaks not bound by GR (Figure 5C). Moreover, the frequency of GR half-sites and/or canonical GR motifs is between 78–98% of GR peaks grouped according to accessibility and C/EBPβ occupancy (Figures 4 and 5D). Presence of GR half-sites at pre-programmed GR-binding sites occupied by C/EBPβ does not specify the transcriptional state of nearby genes (Supplementary Figure S8B). However, dex-induced genes more frequently have pre-programmed GR-binding sites (with C/EBPβ, group 3) with canonical GR motifs than repressed genes (Supplementary Figure S8B). Collectively, this suggests that most non-canonical GR

recruitment to C/EBPβ pre-programmed GR response elements may not necessarily be through tethering but rather through interaction with GR half-sites. GR has been reported to bind GR half-sites (Eriksson and Wrangé, 1990) and importantly, presence of GR half-sites likely provides specificity for GR recruitment where canonical GR motifs are absent. Additionally, the level of chromatin accessibility and abundance of H3K4 monomethylation and H3K27 acetylation is significantly lower at C/EBPβ sites inaccessible to GR (Figure 5E). Thus, a combination of chromatin accessibility, histone modification and enrichment of direct GR-binding sites specifies GR recruitment to a subset of pre-programmed C/EBPβ-binding sites.

Dominant negative C/EBP specifically perturbs GR recruitment to chromatin at shared GR and C/EBP-binding sites

To analyse the functional role of C/EBP for genome-wide GR recruitment to chromatin, we sought to disrupt C/EBPβ binding to DNA. Since C/EBPα and C/EBPβ potentially can occupy the same accessible regions in the genome of liver tissue (Supplementary Figure S7B), we used a general dominant negative C/EBP (A-C/EBP (Vinson *et al*, 2002), here referred to as DN-C/EBP) in order to diminish potential compensatory occupancy by C/EBP subtypes.

The DN-C/EBP was delivered by adenoviral vectors injected into the tail vein of mice 5 days prior dex injection. As a control, we injected another group of mice with adenovirus-expressing GFP. Using this method, we were able to reproducibly express DN-C/EBP in liver without a significant change of GR protein expression compared to control (Figure 6A). Importantly, DN-C/EBP reduced C/EBP α and C/EBP β occupancy at selective sites determined by ChIP-qPCR (Figure 6B) and reduced C/EBP β occupancy of

chromatin genome wide determined by ChIP-seq (Figure 6C and Supplementary Figure S9A). To examine any potential effects of DN-C/EBP expression on GR recruitment to chromatin, we first analysed GR recruitment to specific sites, where C/EBP β occupies pre-programmed chromatin (Figure 6D, sites 4–8). As a control, we analysed GR recruitment to sites where C/EBP β is absent (Figure 6D, sites 1–3). We observed that DN-C/EBP specifically reduce GR recruitment to sites occupied by C/EBP β and GR recruitment

Figure 6 Disruption of C/EBP occupancy reduces GR recruitment to chromatin occupied by C/EBP β . (A) Protein expression of GFP, DN-C/EBP and GR in livers injected with indicated adenovirus. (B) ChIP against C/EBP β and C/EBP α in livers from mice injected with adenovirus expressing GFP (black bars) and DN-C/EBP (red bars). qPCR was performed on GR-binding sites in absence (site 1–3) and presence (site 4–8) of C/EBP β occupancy. Error bars indicate standard error of the mean (SEM) with $n = 4$ in each group. * P < 0.05, ** P < 0.01, *** P < 0.001, t -test. (C) C/EBP β ChIP-seq was performed in the presence and absence of DN-C/EBP. Sequenced tags were counted at C/EBP β –binding sites identified in presence of dex ($n = 25\,393$) and the ratio of tag density in presence and absence of DN-C/EBP was calculated and plotted as a frequency histogram. The broken line indicates equal binding of C/EBP β in presence and absence of DN-C/EBP. (D) ChIP against GR in livers from mice injected with adenovirus expressing GFP (black bars) and DN-C/EBP (red bars). (E) Genome-wide effects of DN-C/EBP on GR occupancy. The ratio of GR ChIP-seq tag density in the presence and absence of DN-C/EBP was graphed as a boxplot. The broken line indicates equal occupancy of GR in presence and absence of DN-C/EBP. (F) Relative mRNA expression of GR target genes in presence (red) and absence (black) of DN-C/EBP. (G) GR target genes where DN-C/EBP does not affect expression. Expression levels were measured by RT-qPCR and normalized to expression of b-actin. Error bars indicate SEM with $n = 3$ in each group. * P < 0.05, ** P < 0.01, t -test.

to control sites is unchanged. We next sought to determine the genome-wide effects of DN-C/EBP expression on GR recruitment to chromatin in liver. At GR-binding sites absent of C/EBP β (group one and four), DN-C/EBP does not suppress GR recruitment to chromatin (Figure 6E). In contrast, DN-C/EBP reduces GR recruitment to pre-programmed as well as *de novo* remodelled GR-binding sites occupied by C/EBP β (Figure 6E). This effect is most pronounced at sites where C/EBP β is bound before GR recruitment (Figure 6E, group three and six), demonstrating that C/EBP β regulates GR recruitment to pre-programmed chromatin and C/EBP β is required for GR recruitment to chromatin at selective *de novo* GR-binding sites.

To examine a functional role of C/EBP β on GR-regulated gene expression, we analysed the expression of a range of dex-regulated genes in the presence or absence of DN-C/EBP (Figures 6F and G and Supplementary Figure S9C). Expression of genes such as *Orm1*, *Tp53*, *Dusp1*, *Cyp2b10*, *Hp* and *Tat* is significantly impaired by DN-C/EBP expression, demonstrating the importance of C/EBP β for regulation of GR target genes. However, we also observed that DN-C/EBP did not affect expression of GR target genes such as *Fkbp5* and *Per1* (Figure 6G) even though GR and C/EBP β occupy multiple sites near TSS. Thus co-occupancy of GR and C/EBP β near genes does not predict transcriptional co-regulation by GR and C/EBP β . As shown in Figure 4D, most GR-regulated genes are covered with nearby GR-binding sites in the presence and absence of C/EBP β and currently we have insufficient experimental data to determine specifically which of the GR-binding sites are important for expression of a given gene. Hence, some genes may primarily be regulated by enhancers occupied by GR and C/EBP β , whereas other genes are sufficiently regulated by enhancers where GR operates independently of C/EBP β .

Dominant negative C/EBP reduces chromatin accessibility at C/EBP-binding sites and specifically disrupts GR recruitment

Disruption of GR recruitment to chromatin by DN-C/EBP at pre-accessible C/EBP β pre-programmed sites may be explained by a change in chromatin accessibility as a consequence of reduced C/EBP β recruitment. To test genome-wide changes in chromatin accessibility as a consequence of disrupted C/EBP occupancy, we performed DNase-seq in the presence and absence of DN-C/EBP. We first counted sequenced tags at each C/EBP β -binding site in the presence and absence of DN-C/EBP and computed the ratio of tag density, where a value of one represents no effect of DN-C/EBP on chromatin accessibility. We observed that DN-C/EBP disrupted overall chromatin accessibility at C/EBP β -binding sites by a mean of 30% (Supplementary Figure S9B), demonstrating that C/EBP β is an important factor for maintenance of chromatin accessibility in adult liver tissue. In the *Orm1* locus, reduced occupancy of C/EBP β results in decreased chromatin accessibility and impaired GR recruitment to GR-binding sites (Figure 7A, black arrows). Reduction in C/EBP β occupancy and DNase I accessibility is also seen at C/EBP β -binding sites in the *Efh2* gene (Figure 7B, black arrows), but GR binding and chromatin accessibility is not changed at a nearby GR-binding site, where GR occupies chromatin in absence of C/EBP β (Figure 7B, red arrow). Genome-wide, DN-C/EBP expression results in reduced

DNase I accessibility at shared GR and C/EBP β -binding sites compared to sites occupied by GR in absence of C/EBP β (Figure 7C). Accessibility of GR-binding sites pre-programmed by C/EBP β is affected more than at sites where C/EBP β recruitment is assisted by GR (Figure 7C). The same pattern is observed for the DN-C/EBP effect on GR recruitment (Figure 6E), suggesting a correlation between GR binding and DNase accessibility at sites occupied by C/EBP β and GR. We confirmed this by plotting the effect of DN-C/EBP on GR occupancy and chromatin accessibility at all GR-binding sites occupied by C/EBP β (Figure 7D, $r = 0.41$). This suggests that C/EBP β maintains chromatin accessibility at specific regions of the genome and GR recruitment to these regions is dependent on maintained accessibility. Interestingly, we also observe a good correlation when we selectively analysed *de novo* remodelled GR-binding sites, where C/EBP β recruitment is assisted by GR and where C/EBP β occupies inaccessible chromatin prior to GR activation (Figure 7E, $r = 0.35$), suggesting that cooperative interaction between GR and C/EBP β is important for full remodelling of chromatin initiated by GR recruitment.

Distinct modes of GR interaction with chromatin in liver

Collectively, we find three general modes of potential GR interaction with chromatin in liver, summarized in Figure 8. One type of sites harbours putative, inaccessible GR-binding sites used specifically in non-liver cell types (Figure 8A). A second type of sites efficiently recruits GR in liver (Figure 8B). These sites are pre-programmed, occupied by highly mobile nucleosomes and flanked by nucleosomes with H3K4me1 and H3K4m3 modifications (Figure 8B). Remarkably, 59% of these sites are preoccupied by C/EBP β (and C/EBP α , Supplementary Figures S7B and S10) and disruption of C/EBP occupancy results in reduced accessibility and GR recruitment. In addition to C/EBPs, other factors such as HNF4 (Schmidt *et al*, 2010), RXR and its interaction partners such as LXR (Patel *et al*, 2011; Boergesen *et al*, 2012) may be important for liver-specific GR binding to pre-programmed chromatin (Supplementary Figure S10). Chromatin accessibility is maintained via recruitment of chromatin-remodeler complexes such as SWI/SNF and ISWI (Kowenz-Leutz and Leutz, 1999; Steinberg *et al*, 2012). A third set of binding sites are *de novo* remodelled as a result of dex treatment (Figure 8C). GR binding to and remodelling of these sites relies on cooperative recruitment of transcription factors such as C/EBP β and presence (e.g., H3K4me1) and/or absence (e.g., H3K27me) of certain histone modifications.

Discussion

GCs control important biological processes in most tissues of mammals in a highly tissue-selective manner. Identifying mechanisms that selectively control GC signalling in different tissues is central to fully understand the beneficial and non-beneficial effects of clinically used synthetic GCs, such as dex. To gain insight into the mechanism regulating GR-mediated gene regulation in tissue, we injected adrenalectomized mice with dex and analysed RNAPII occupancy of genes, GR occupancy of regulatory elements and changes in chromatin accessibility.

Figure 7 Expression of dominant negative C/EBP results in reduced chromatin accessibility at C/EBP-binding sites. (**A**, **B**) Aligned tags from GR and C/EBPβ ChIP-seqs and DNase-seq in presence and absence of DN-C/EBP at the Orm1 gene and Efhd2 gene. Black arrows indicate sites bound by C/EBPβ, where DN-C/EBP leads to disruption of accessibility. Red arrow shows GR binding with no C/EBPβ occupancy and DN-C/EBP does not lead to disrupted accessibility. (**C**) Sequenced tags from DNase-seq in liver of mice expressing DN-C/EBP or GFP were counted at GR-binding sites and grouped accordingly to overlap with DHS and C/EBPβ co-occupancy (see Figure 4). The ratio of tag density in the presence and absence of DN-C/EBP was graphed as a boxplot. The broken line indicates equal accessibility in presence and absence of DN-C/EBP. (**D**) Correlation between GR occupancy and DNase accessibility at shared GR and C/EBP-binding sites in presence of DN-C/EBP. Pearson correlation, $r=0.41$. (**E**) Correlation between GR occupancy and DNase accessibility at GR-binding sites, where GR assists loading of C/EBPβ (red, group 5) and where C/EBPβ functions as an initiation factor for GR recruitment (green, group 6). Pearson correlation, $r=0.35$.

Figure 8 Different modes of GR interaction with chromatin in liver. (A) GR-binding sites not used in liver, but occupied by GR in other cell types, are inaccessible to GR in liver due to highly positioned nucleosomes and H3K27me. (B) The majority of GR-binding sites in liver are pre-accessible and pre-programmed by C/EBPs and dominant negative C/EBP (DN-C/EBP) reduce chromatin accessibility and impair GR occupancy. At pre-programmed sites, the nucleosomes are continuously remodelled by multiple chromatin remodeling complexes such as SWI/SNF and ISWI. (C) A fraction of the GR-binding sites in liver are *de novo* remodelled in response to GR activation by agonists such as dex and recruitment of chromatin remodellers. Like the non-liver GR-binding sites, the *de novo* remodelled sites have high nucleosomal density at the GR-binding site prior to GR activation. However, in contrast to the inaccessible GR-binding sites in liver, these sites are recognized by GR and recruitment leads to remodelling and increased accessibility. Several mechanisms may account for selective binding and remodelling such as the presence of pre-programmed (1) initiation/pioneering factors, (2) histone modifications (e.g., H3K4me1), (3) DNA methylation and (4) cooperation with other transcription factors active in liver such as C/EBP.

In general, we find that the majority of GR recruitment to the genome is associated with pre-programmed chromatin. This observation is similar to findings in cell lines (Biddie *et al*, 2011; John *et al*, 2011), and thus indicates that preset chromatin accessibility is a general mechanism that controls selective GR recruitment to chromatin in tissue as well as cell lines. Recent reports correlating DNase I accessibility with AR and ER occupancy in prostate and breast cancer cell lines, respectively, show similar trends (He *et al*, 2012), suggesting that this may be a general phenomenon for steroid receptors. Based on single-cell studies, we have proposed that transcription factors act dynamically at a given site in the genome to remodel chromatin, thereby creating a transient state of chromatin accessibility that facilitates binding of secondary factors ('assisted loading' (Voss *et al*, 2011)). This phenomenon may be a major mechanism for pathophysiological deregulation of steroid responses in specific tissues. In support of this concept, activation of AP1, NF- κ B and STAT3 in cell lines has been shown to reprogram GR binding to sites of the genome otherwise not occupied by GR (Rao *et al*, 2011; Langlais *et al*, 2012; Uhlenhaut *et al*, 2012). Also, FOXA1 and GATA3 can

reprogram ER binding to the genome in primary breast tumours (Hurtado *et al*, 2010; Theodorou *et al*, 2012). Disruption of FOXA1 leads to dramatic genome-wide loss of ER occupancy in human breast cancer cells (Hurtado *et al*, 2010), while similar disruption of AP1 leads to genome-wide reduced GR recruitment in mouse mammary adenocarcinoma-derived cells (Biddie *et al*, 2011).

In liver, we show that more than half of GR-binding sites are preoccupied by C/EBP β and disruption of C/EBP β binding results in decreased chromatin accessibility and reduced GR recruitment to the pre-programmed genome occupied by C/EBP β . This suggests a mechanism where C/EBP β maintains chromatin accessibility prior to GR recruitment through recruitment of chromatin remodellers. In agreement, C/EBPs has been reported to interact with components of the SWI/SNF (BRG1) (Kowenz-Leutz and Leutz, 1999; Pedersen *et al*, 2001) and ISWI (SNF2H) (Steinberg *et al*, 2012) chromatin remodellers and disrupted recruitment may lead to reduced chromatin remodelling and decreased accessibility. Additionally, reduced C/EBP β occupancy may also result in disrupted GR recruitment through mechanisms involving direct protein-protein interaction (Williams *et al*, 1991; Boruk *et al*, 1998; Rudiger *et al*, 2002; Wiper-Bergeron *et al*, 2003; Yang *et al*, 2007; Sun *et al*, 2008). However, we find little evidence for high frequency of GR recruitment to chromatin through a tethering mechanism. Rather, GR is recruited specifically to a subset of C/EBP β -bound sites through presence of GR half-sites. Collectively, we suggest that any change in C/EBP expression and/or activity will potentially co-regulate GR signalling in liver. In agreement, genetic studies have shown that GR and C/EBPs regulate processes such as glucose and glycogen metabolism in liver (Wang *et al*, 1995; Lee *et al*, 1997; Liu *et al*, 1999; Opherke *et al*, 2004). Interestingly, C/EBP activity is regulated at multiple levels that may impact chromatin structure at bound sites (Nerlov, 2008). For example, C/EBP β interaction with SWI/SNF is regulated by MAPK phosphorylation and CARM1 methylation of C/EBP β (Kowenz-Leutz *et al*, 2010). Moreover C/EBP β is actively acetylated by p300, PCAF and GCN5 and repressed by G9a (Wiper-Bergeron *et al*, 2007; Pless *et al*, 2008). Thus, recruitment of specific co-regulators to C/EBP β -bound sites may impact nearby gene regulation by GR.

In liver, we show that activated as well as repressed genes have nearby recruitment of GR to pre-accessible C/EBP β -occupied chromatin. However, when we integrate information on changes in chromatin structure (DNase-seq data), we demonstrate that induced chromatin accessibility at GR-binding sites is specifically prevalent near activated genes compared to repressed genes. Also, assisted loading of C/EBP β through transition in chromatin structure is more frequently observed near activated genes. In contrast, repressed genes are associated with nearby regions of decreased chromatin accessibility. Thus, considering GR binding together with transcription factors such as C/EBP β alone is not indicative of up- or downregulation of nearby genes; however, integration with chromatin conformation assays such as DNase-seq provides functional insights to GR recruitment to chromatin. In agreement, a recent report suggests that the combination of DNA motifs and bound transcription factors is not indicative of nearby activation versus repression of genes by GR in primary macrophages (Uhlenhaut *et al*, 2012).

Rather, recruitment of specific co-regulators and altered histone modifications are indicative of the transcriptional output. Thus, in order to discriminate between functional and opportunistic binding of transcription factors, it is important to integrate transcription factor ChIP-seq analysis with chromatin configuration assays.

In conclusion, GR occupancy of chromatin in intact liver tissue is strongly associated with accessible chromatin and liver-specific GR binding is obtained through cell-selective chromatin accessibility. The majority of GR-binding sites associated with glucocorticoid-regulated genes are pre-programmed and occupied by C/EBP β . Disruption of C/EBP β binding results in reduced GR recruitment to pre-programmed sites bound by C/EBP β . Moreover, a number of *de novo* sites remodelled by GR require C/EBP β for full recruitment of GR to chromatin and subsequent remodelling. We conclude that C/EBP β plays a major role for GR recruitment to chromatin in intact adult mouse liver tissue.

Materials and methods

Animal procedures

Male C57BL/6 mice, 8 weeks old, purchased from Harlan Sprague Dawley (Frederick, MD, USA), were maintained according to the NIH guidelines with a 14-h light, 10-h dark cycle and free access to food and water. Bilateral adrenalectomy was carried out via a dorsal approach under ketamine/xylazine anaesthesia as previously described (Ma and Aguilera, 1999). Mice were given intraperitoneal injection of dex-cyclodextrine complex, containing 65 mg/g of dex (1 mg/kg), or veh (1 mg/kg cyclodextrine) in a total volume of 300 μ l of sterile saline. All animal procedures were approved by the Animal Users and Care Committee, the National Institute of Child Health and Human Development and the National Cancer Institute, National Institutes of Health.

Adenovirus

DN-C/EBP was previously described in (Greenwel *et al*, 2000; Bonovich *et al*, 2002). A total of 2×10^9 infectious units per mouse was injected in the tail vein in a volume of 100 μ l sterile saline, 5 days before dex injection.

RNA expression analysis

Livers were snap frozen in liquid N₂ immediately after isolation and stored at -80°C . RNA was extracted using RNA extraction columns (QIAGEN) according to manufactures protocol. cDNA was synthesized using iScript (Biorad) and qPCR was performed using iQ SYBR green supermix (Bio-Rad). Statistical significant changes of expression were identified using a *t*-test.

Western blotting

Snap frozen livers (50 mg) were homogenized in RIPA buffer. Lysate was cleared by centrifugation and 50 μ g total protein was separated on a SDS-PAGE gel and immunoblotted with antibodies against GR (M-20, Santa Cruz), GFP (Invitrogen) and HA (Roche).

DNase-seq

DNase-seq was essentially performed as previously described (Grøntved *et al*, 2012). In short, isolated nuclei from fresh livers were treated with DNase I (Sigma) and DNA fragments of 100 to 500 bp from a chromatin digestion with 60 U/ml DNase I were purified using sucrose gradients. DNA was precipitated and dissolved in nuclease free H₂O (Ambion).

Chromatin IP

Snap frozen livers (300 mg/ChIP-seq, 50 mg/ChIP-qPCR) were homogenized using a tissueuptor (QIAGEN) with a disposable probe (QIAGEN) in PBS with 1% formaldehyde and crosslinked for 10 min at room temperature. ChIP was performed with antibodies against GR (Cocktail of three antibodies (John *et al*, 2011), C/EBP β (Santa Cruz, sc-150), C/EBP α (Santa Cruz, sc-9314)

and RNAPII (Abcam, ab5131). For ChIP-qPCR statistical significant binding was identified using a *t*-test.

Sequencing and data analysis

DNA was sequenced using Illumina GA2x sequencer at the Advanced Technology Center (ATC), National Cancer Institute (NCI) (Rockville, MD, USA). Replicate concordant ($n=2$) ChIP-seq peaks and DHS were identified as previously described (John *et al*, 2011; Siersbaek *et al*, 2011; Baek *et al*, 2012) using a FDR of 0%. For GR and C/EBP β , ChIP-seq peaks a threshold of 30 tags per peak was used. Downstream bioinformatic analysis was performed using HOMER (Heinz *et al*, 2010) and Galaxy (Goecks *et al*, 2010). Differentially regulated DHS were identified using DESeq at $P < 0.05$ ($n=2$) (Anders and Huber, 2010). Heatmaps were generated using MeV, a part of the TM4 microarray software suite (Saeed *et al*, 2006). Sequencing data are summarized in Supplementary Table S1 and are available in GEO: GSE46047.

RNAPII ChIP-seq analysis

RNAPII ChIP-seq tags were counted at all ref-seq genes from the TSS to the end of the gene if the gene is shorter than 5000 bp or from TSS to 5000 bp into the gene if longer than 5000 bp. Genes with a significant change of RNAPII occupancy as a result of dex treatment were identified using DESeq at $P < 0.001$ ($n=2$) (Anders and Huber, 2010).

De novo motif analysis

Motif analysis was performed using MEME (Bailey *et al*, 2009) or HOMER (Heinz *et al*, 2010). HOMER and/or TOMTOM (Gupta *et al*, 2007) in combination with the JASPAR database were used to identify potential factors that bind to the enriched motifs. Frequency of identified motifs was calculated using FIMO at $P < 0.001$ (Grant *et al*, 2011) and HOMER.

Statistical analysis

Significant enriched genomic regions identified by ChIP-seq and DNase-seq were identified by tools described in (Baek *et al*, 2012). Differentially regulated DHS and RNAPII occupancy were found using DESeq (Anders and Huber, 2010). Significant change of RNA expression and ChIP-qPCR was analysed using a *t*-test. Correlation between occupancy and chromatin accessibility was analysed by Pearson correlation.

Previously published sequencing data used in the analysis

MNase-seq tag library (GEO: GSM717558); H3K4me1 and H3K4me3 ChIP-seq tag libraries (SRA008281) and H3K27me2 ChIP-seq tag libraries (GEO: GSM751034). HNF4 and C/EBP α ChIP-seq tag libraries (GEO: GSE22078). H3K27ac ChIP-seq libraries (GEO: GSM1000140). GR peaks from 3134 and Att20 (John *et al*, 2011). GR and C/EBP β peaks from 3T3L1 (Steger *et al*, 2010). GR peaks from C2C12 (Kuo *et al*, 2012).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We thank Tina Miranda, Michael Guertin, Diego Presman and members Vinson lab for critical reading of the manuscript. LG was supported by a research grant from the Lundbeck Foundation. This work was supported by the Intramural Research Program of the National Institutes of Health (NIH), National Cancer Institute (NCI), Center for Cancer Research (CCR).

Author contributions: LG performed the experiments with help from JRB (adenoviral injections), YL (animal work) and GA (animal work and adrenalectomy). CV engineered the DN-C/EBP and provided adenovirus expressing DN-C/EBP. LG performed the data analysis with help from SB and SJ. LG, SJ and GLH designed the experiments and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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