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Glucocorticoid-dependence and independence of the circadian liver transcriptome

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The liver, a key metabolic organ, shows clear day-night variation in gene and protein expression, and in metabolic pathway activity. Liver cells possess molecular circadian clocks, but systemic factors are critical contributors to the circadian rhythmicity of liver gene expression. Glucocorticoid action is reported to be one such factor. Glucocorticoids are essential hormones, with strong circadian oscillations, which control multiple cellular processes via the glucocorticoid receptor (GR). We compare clock factor and GR binding at promoters and enhancers linked to rhythmic genes and find increased clock factor binding at those promoter elements where GR is also present. Genes with GR binding at the promoter are more highly expressed and more likely to be detected as rhythmic. We then test the role of GR directly, by profiling rhythmic gene expression in mice with/without hepatocyte-targeted GR deletion. Notably, we observe only a small effect of GR deletion, with most rhythmic genes showing unchanged rhythmicity, despite evidence for local GR binding. We find a small number of genes showing lost or gained rhythmicity with GR deletion; genes which lose rhythmicity associate with GR binding sites. Contrary to expectations, GR appears redundant for conveying timing information to most of the rhythmic liver transcriptome.

Liver gene expression is robustly rhythmic over the day-night cycle, with studies reporting 16–40% transcripts showing a circadian rhythm of abundance in laboratory mice^{1–4}. In the liver—perhaps more than in any other tissue—signals driven by the master suprachiasmatic nucleus clock, and the action of the local tissue clock converge to regulate gene expression^{5,6}, although their relative contribution remains to be clarified⁷. 24 h variation in liver gene and protein expression, and indeed function, is of major importance to health, given the strong associations between hepatic/metabolic pathology (e.g. MASLD, T2DM) and circadian disruption (shift work, sleep disorders, mistimed meals)^{8–10}. Factors conferring rhythmicity to the hepatic transcriptome have thus attracted considerable research interest.

Here, we examine the contribution of glucocorticoid signalling. Glucocorticoid hormones are essential for life, regulating energy metabolism and the inflammatory response. They are synthesised and secreted by the adrenal cortex in a strongly circadian manner, with superimposed responses

to feeding and stress. The adrenal cortex is regulated both by sympathetic innervation and endocrine control in the form of pituitary ACTH. Circulating levels of glucocorticoid peak just before waking, which is thought to be important for the transition from the resting to the active phase. Glucocorticoid hormones act on the glucocorticoid receptor (GR, NR3C1), of the nuclear hormone receptor superfamily. In the liver, GR signalling is important for metabolism of glucose, lipids and amino acids¹¹, and is directed by hepatic lineage determining factors including C/EBP β ¹² and HNF4A¹³.

Multiple studies report interactions between GR and those transcription factors (TFs) that make up the molecular transcription-translation feedback loop of the circadian clock mechanism. Data suggest that clock TFs can serve to regulate and/or direct GR action. For example, CLOCK, one of the key TFs of the positive limb of the clock, can exert negative control over GR through acetylating its hinge region¹⁴. The Cryptochrome (CRY)

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proteins interact with multiple members of the nuclear receptor family, including GR, and have a repressive effect on GR action¹⁵. There is also evidence for a functional interaction between the circadian repressor REV-ERB α (NR1D1), also a nuclear receptor, and GR^{16,17}. Conversely, GR and glucocorticoid actions can feed into the clock. When the existence of peripheral circadian oscillators was first reported, it became clear that glucocorticoid action was a powerful means to synchronise these oscillators¹⁸. This is thought to be through induction of Period (PER)¹⁸, which then feeds back to inhibit BMAL1/CLOCK, and thereby re-set the oscillator. In vivo, the actions of endogenous glucocorticoids are more subtle, with adrenalectomy serving to increase the rate of entrainment to phase-shifts in the light-dark cycle or feeding schedule^{19,20}. Therefore, endogenous glucocorticoids may contribute to the stability of the central circadian oscillator and render it resistant to environmental change. Clarifying the contribution of glucocorticoid signalling to rhythmic liver function is important for understanding how disrupted adrenal glucocorticoid secretion might impact on liver physiology, and whether supraphysiological, and mistimed glucocorticoid exposure (as occurs with therapeutic glucocorticoid administration) might impact the liver circadian clock, and circadian energy metabolism.

Here, we interrogate the contribution of GR to the orchestration of the rhythmic liver transcriptome in vivo under circadian conditions (with no external light-dark cues). With in silico analyses of published datasets, we find that clock TF binding is greater at gene promoters where there is also GR binding. The majority of rhythmic genes in mouse liver have GR binding at the promoter, and we observe differences in distribution of phase between those rhythmic genes that do and do not have GR binding at the promoter. Genes that are GR-bound at the promoter are more likely to be rhythmic, even when we correct for the effect of expression level on likelihood of rhythmicity. We proceeded to test the contribution of GR by selectively deleting GR in the hepatocytes of adult mice, and profiling the circadian transcriptome. Whilst we find alterations in the expression pattern of a subset of core circadian clock genes, we find, remarkably, that rhythm is unchanged for the majority of cycling transcripts. A minority of transcripts lose or gain rhythmicity with GR knockout, findings which are replicated by a new analysis of existing time series liver gene expression data. Thus, GR is not required for the normal cycling of many circadian transcripts, and despite the high prevalence of GR binding in proximity to rhythmic genes, hepatocyte GR appears to play a minor role in conveying timing information to the liver transcriptome.

Results

GR and clock TF binding co-occur at gene promoters

As previous work identified functional associations between GR and the core circadian transcription factors CLOCK, CRYs, and REV-ERB α ¹⁴⁻¹⁷, we extended these observations to a more complete set of core clock TFs, based on genome binding distribution. We first studied all annotated promoter sequences, defined as -2000 bp, and $+200$ bp relative to the transcription start site (TSS) (Fig. 1A). We integrated liver GR ChIP-seq data¹³, from both vehicle- and dexamethasone-treated mice, with published clock factor ChIP-seq^{21,22} and RNA-seq data²³ obtained from mouse liver under circadian conditions. We chose to use GR ChIP-seq data obtained from both vehicle- and dexamethasone-treated mice in order to identify as many potential GR binding sites as possible, acknowledging that this might identify sites seldom occupied under physiological conditions. GR binding at promoters was associated with increased binding of clock TFs, as indicated by increased mean ChIP-seq read counts over time (Fig. 1B), particularly in the case of CRY1/2 and PER1/2. We also observed that GR promoter-bound genes tended to be more highly expressed, as indicated by increased RNA-seq read counts (Fig. 1B; bottom right panel).

GR promoter binding is associated with higher gene expression and increased likelihood of rhythmicity

We divided genes from the liver circadian RNA-seq series²³ into those that did (JTK cycle; adjusted $p < 0.05$) and did not (adjusted $p > 0.05$) show

rhythmic expression. We found that the majority (63%) of rhythmic genes had GR-bound promoters (Fig. 1C). Interestingly, we observed small differences in the distributions of phase between GR-bound and non-GR bound rhythmic genes, with two peak phases of gene expression evident in non-GR bound rhythmic genes (CT0, CT6), and a peak phase of expression at CT0 in GR-bound rhythmic genes (Fig. 1C). In nocturnal mice, the peak serum concentration of the endogenous GR ligand corticosterone is at the end of the inactive period (CT8-12)²⁴, thus we anticipated that, if GR binding was driving rhythmic expression, we would see peak phase of GR-bound rhythmic genes at this time. By contrast, however, phase of GR-bound rhythmic genes was found to be nearly in antiphase to corticosterone levels, indicating that rhythmic control of these genes must be more complex.

As our counts-based analysis had indicated a difference in RNA expression levels between GR-bound and non-GR-bound genes (Fig. 1B; bottom right panel), we applied unsupervised clustering analysis to the circadian RNA-seq series (Fig. 1D, left panel), and plotted expression across time for each cluster (Fig. 1D, right panel). This approach supported the idea that GR-bound genes were more strongly expressed than non-GR-bound genes, and also raised the possibility that gene expression may be a significant confounder in rhythmicity analysis. Consequently, we analysed circadian rhythmicity as a function of logged gene expression, using a logistic regression model (Fig. 1E, Fig. S1A,B). We did indeed detect a highly significant relationship between mean expression level and likelihood of rhythmicity detection (Fig. 1E), but the odds ratio for rhythmicity for GR-bound genes remained >1 , even when expression level was taken into account, thus indicating that GR binding was marking a set of circadian rhythmic genes irrespective of gene expression. In fact, a higher proportion of GR-bound genes were rhythmic, as compared to non-GR-bound genes, for non-extreme quantiles of expression (Fig. S1A).

GR-bound promoters may have more open chromatin which may in turn lead to increased ChIP-seq signal for other TFs (including our reported clock TFs). To check for this effect, we analysed the chromatin openness at our defined promoters using published DNase-seq data²⁵. We found little difference in chromatin openness between GR-bound promoters and non-GR-bound promoters and still found an effect of GR binding on TF binding after adjusting for chromatin openness (Fig. S1C).

Taken together, these analyses suggest clock factor binding is greater at gene promoters where GR is also bound. Promoter GR binding is associated with a small but significant increase in likelihood of rhythmic gene expression, even when gene expression level is controlled for. Furthermore, rhythmic genes which are GR-bound show a different distribution of phase compared to rhythmic genes which are not GR-bound at the promoter. This supports the idea that, in mouse liver, GR binding at promoters may play a role in conferring rhythmicity to a subset of genes, but without a direct relationship between phase of the endogenous GR ligand, corticosterone, and phase of expression for GR-bound rhythmic genes.

GR binds enhancers linked to rhythmic genes

As GR binds to, and acts through distal regulatory elements, as well as promoters^{26,27}, we extended our observations by studying GR binding at enhancers. We defined potential enhancers in mouse liver by calling peaks on H3K4me1 ChIP-seq data²² (Fig. 2A), a robust epigenetic marker for enhancers²⁸. We excluded regions which overlapped with defined promoters (Fig. 1A). We again used our liver GR ChIP-seq data¹³, and integrated these with the published circadian TF binding atlas²² and liver REV-ERB α binding data²¹. We found that GR-bound and non-GR-bound enhancer elements showed a similar distribution across different types of genomic region (Fig. 2B, left panel), but GR-bound enhancers were more distal from transcription start sites than non-GR-bound enhancers ($p < 0.01$, Fig. 2B, right panel).

We then mapped ChIP-seq reads from the eight analysed clock TFs (BMAL1, CLOCK, CRY1, CRY2, PER1, PER2, NPAS2, and REV-ERB α), as well as the H3K27ac histone mark for transcriptionally-active chromatin, onto these enhancer regions (Fig. 2C), averaging over multiple timepoints. Here, we found evidence for increased binding of core circadian clock TFs at

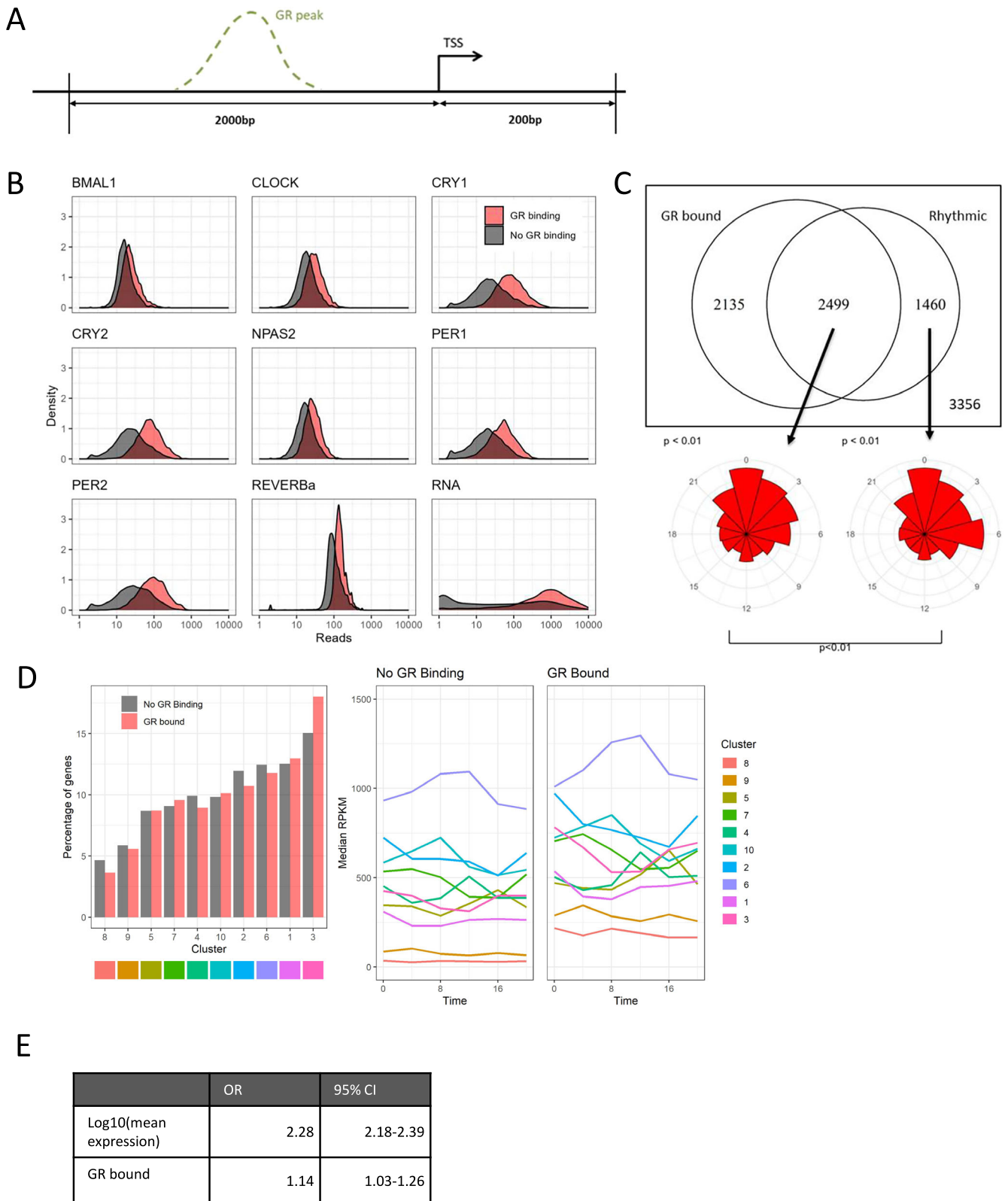
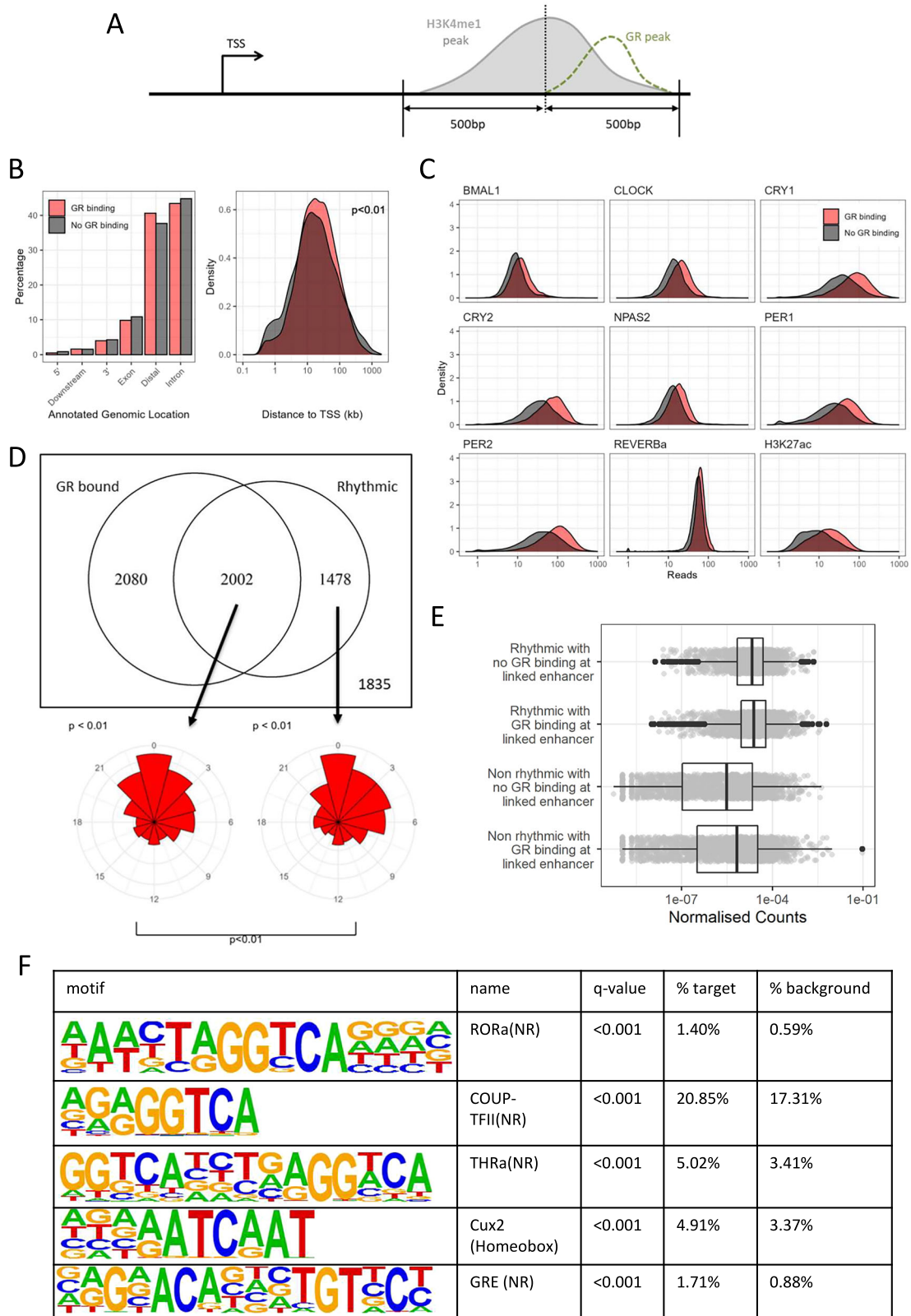


Fig. 1 | GR binding at the promoter is associated with the rhythmicity of gene expression. **A** Clock TF ChIP-seq reads were counted on promoter regions 2000bp downstream/400 bp upstream from the TSS. **B** Density plots of ChIP-seq reads (plus RNA-seq reads in bottom right plot) for GR bound promoters (red) and non-GR bound promoters (black) for clock TFs and gene expression. Clock TF ChIP-seq from Koike et al. (2012) with the exception of REVERBa from Hunter et al. (2020), RNA-seq data from Yang et al. (2016). Data averaged over multiple timepoints with exception of REVERBa where only a single timepoint (ZT8) was available. **C** Top: Venn diagram of genes bound by GR at the promoter and rhythmically expressed

genes (JTK cycle, $p < 0.05$). Bottom: Peak phase of rhythmically called genes, both GR bound (left) and non-GR bound (right), p -values above plot are from a Rayleigh test of uniformity, p -value below the plot is from Watson's test for homogeneity on two samples. **D** Cluster analysis on the dynamic behaviour of gene expression. Left: Percentage of GR and non-GR bound genes assigned to each cluster. Right: Median gene expression per cluster for GR and non-GR bound genes. **E** Odds ratios and 95% confidence interval from logistic regression model of rhythmicity (JTK-cycle defined) with log gene expression and presence of GR binding at promoter as covariates.



GR-bound enhancers. We also observed that the co-bound loci had a higher H3K27ac signature, suggesting a more active chromatin landscape (Fig. 2C).

To investigate potential functional consequences of co-bound enhancers, we linked all identified enhancers to their nearest gene promoter. We analysed rhythmicity of expression (JTK cycle adjusted $p < 0.05$) in genes linked to a GR-bound enhancer, and those linked to a non-GR-bound

enhancer. As with GR-promoter binding, we found that the majority (58%) of circadian rhythmic genes were linked to a GR-bound enhancer (Fig. 2D). We once again observed a difference in phase distribution between rhythmic genes linked to GR-bound enhancers and those linked to a non-GR-bound enhancer (Watson’s test for homogeneity on two samples; $p < 0.01$), with a narrower distribution of phase evident in those rhythmic genes linked

Fig. 2 | GR and clock factor binding co-occur at enhancers. **A** For enhancers defined by H3K4me1 peaks, clock TF ChIP-seq reads were counted on regions 500 bp either side of the consensus H3K4me1 peak. **B** Left: Annotated genomic locations of GR-bound (red) and non-GR-bound (black) enhancers. Right: Density plot of distance (in kb) from enhancer to closest transcription start site (TSS), p-value from a t-test on the logged data. **C** Density plots showing effect of GR binding (red) on clock TF binding (first 7 plots) and H3K27ac (8th plot) from Koike et al. and REVERBa (9th plot) Density plots showing amount of ChIP-seq reads for GR bound promoters (red) and non-GR-bound promoters (black) for clock TFs and H3K27ac. Clock TF and H3K27ac ChIP-seq from Koike et al. (2012) with the exception of REVERBa from Hunter et al. (2020). Data averaged over multiple

timepoints with exception of REVERBa where only a single timepoint (ZT8) was available. **D** Top: Venn diagram of genes with linked enhancers (linked to nearest promoter) bound by GR and rhythmically expressed genes (JTK cycle, $p < 0.05$). Bottom: Peak phase of rhythmically called genes, both GR bound (left) and non-GR bound (right), p-values above plot are from a Rayleigh test of uniformity, p-value below the plot is from Watson's test for homogeneity on two samples. **E** Gene expression (normalised counts) of genes with linked enhancers (each enhancer linked to closest gene TSS). Stratified by rhythmicity of gene and presence of GR binding at enhancer site. **F** Homer known motif analysis showing top 5 enriched motifs in GR-bound rhythmic enhancers as compared to a background of non-GR-bound rhythmic enhancers.

to a GR-bound enhancer, with peak phase of expression for both groups being CT0 (Fig. 2D).

For both rhythmic and non-rhythmic genes, those linked to GR-bound enhancers trended towards being more transcriptionally active compared to those linked to non-GR-bound enhancers, but this was not significant (Fig. 2E, $p = 0.07$). We performed motif analysis on enhancer sequences to identify possible TF binding motifs. Compared to a background of rhythmic enhancers not bound by GR, motif analysis of rhythmic GR-bound enhancers showed enrichment of GR binding sites (GRE), as well as other nuclear receptors which do not share similar binding sequences (ROR, COUP-TF, THRA) (Fig. 2F).

We again found a positive relationship between the likelihood of rhythmicity detection and expression level, but, in contrast to promoters, did not find increased likelihood of rhythmicity detection with enhancer GR binding (Fig. S2A–C), with the confidence interval for the odds ratio spanning 1.

We again considered the effect of chromatin openness on TF binding. Using published DNase-seq data²⁵, we analysed openness at our defined enhancers. We found a slight increase in chromatin openness in GR-bound enhancers compared to non-GR-bound enhancers (Fig. S2D left). After adjusting for chromatin openness, we found that the increased clock TF binding in GR-bound enhancers was lost, suggesting that this was purely due to open chromatin effects (Fig. S2D right). Thus taken together, in contrast to what we observed at promoters, a contribution of GR-enhancer binding to rhythmicity is not clear.

As linking enhancers to the nearest gene does not confirm a functional link, we extended our analyses using published mouse liver promoter capture Hi-C data (pC-HiC)²⁹ to link enhancers to genes. We found that 84% of the emergent genes were within the set we previously identified (Fig. S3A). Whilst the pC-HiC analyses were biased towards genes with higher expression (Fig. S3B) and linked enhancers tended to be more distal (Fig. S3C), it revealed a similar division by GR binding status, and rhythmicity detection (Fig. S3D) as our previous approach. After controlling for gene expression, we found that GR binding conferred a slight reduction in rhythmicity detection (Fig. S4A–C), potentially down to the bias towards genes with higher expression.

Deletion of hepatocyte GR in adulthood remodels the liver transcriptome

Having found some associations in silico between GR binding, clock TF binding profiles, and rhythmic gene expression, we sought to test directly the contribution of GR to rhythmic liver gene expression by inducing GR deletion postnatally in the liver. We used the inducible, hepatocyte-targeted *Gr* (*Nr3c1*) knockout mice (*Nr3c1*^{fllox/fllox}*Alb*^{CreERT2}; GR-LKO) and littermate controls (*Nr3c1*^{fllox/fllox}; WT) (Fig. S5A). We confirmed loss of liver GR protein expression with Western blot (Fig. S5B), following Cre induction with tamoxifen as previously described^{21,30}.

We collected liver tissue from these mice every 2 h for 48 h, under circadian conditions (continuous dark; DD), and assessed liver gene expression by RNA-seq (Fig. 3A). As expected, GR gene expression was significantly reduced at all timepoints (Fig. 3B; EMM1 > 1, see “Methods”).

Differential gene expression was performed comparing WT mice against GR-LKO in all timepoints combined. We detected that 403 genes were differentially expressed; 197 up and 206 down in GR-LKO (Fig. S5C). In order to examine relationships between the GR cistrome, and altered gene expression we turned to peaks-to-genes analysis (PEGS³¹), using our GR ChIP-seq data¹³, and applying that to the differentially expressed genes. This analysis revealed that there was only statistically significant enrichment between GR binding and genes whose expression was reduced in the absence of GR (Fig. S5D), and therefore likely to be those genes positively regulated by glucocorticoids. In contrast, genes which were upregulated in the absence of GR only showed weak association with GR binding sites, supporting a distinctly different mechanism of gene regulation, and certainly compatible with an indirect mechanism of negative regulation requiring upregulation of an intermediary gene^{13,32,33}.

Rhythmic expression of many liver genes is independent of hepatocyte GR

We then proceeded to analyse differences in circadian rhythmicity of gene expression in the GR-LKO mice compared to WT. For this, we used the compareRhythms R package³⁴. This is a rigorous approach for comparing circadian rhythmicity between conditions, and is an improvement over previous approaches which led to significant misclassification³⁴. First, we filtered out genes which were not expressed at any timepoint in either WT or GR-LKO, or not expressed in both. With compareRhythms, genes were then classified as arrhythmic in both WT and GR-LKO (10,773 genes), showing the same rhythm in both WT and GR-LKO (4370 genes), showing a gain of rhythm in GR-LKO (349 genes), showing a loss of rhythm in GR-LKO (330 genes) or showing altered rhythm in GR-LKO (26 genes) (Fig. 3A). Additionally, 3836 genes did not meet the criteria for classification, indicating that rhythmic status could not be confidently ascribed.

The Period genes are highly glucocorticoid responsive, so are candidate mediators of GR actions on rhythmic liver gene expression. Therefore, in both the WT and GR-LKO samples, we assessed gene expression over time of the core circadian clock genes (*Bmal1*, *Clock*, *Cry1/2*, *Per1/2*, *Rev-erb α/β*) (Fig. 3C). Using a Gaussian process regression-based method³⁵, we performed differential time-series analysis on the core clock genes. As expected, we saw changes in *Per1*, and *Per2* expression through time, and in *Cry1* at CT14–22 and again in the second cycle at CT40–46. However, analysis of the core circadian clock genes suggests the clock remained oscillatory in the GR-LKO livers, with all the components continuing to cycle, but with small differences in amplitude.

To investigate whether there might be a relationship between the phase of rhythmic gene expression and GR signalling, we plotted circadian time of peak expression (phase) for the rhythmic transcripts (Fig. 3D). Transcripts showing the same rhythm in WT and GR-LKO, and transcripts gaining rhythm in GR-LKO had clear patterns of phase distribution (Fig. 3D), but those transcripts losing rhythmicity in GR-LKO showed a much broader distribution, this was robust to the method of phase detection (replicated using JTK-cycle, Fig. S5E). This again was unexpected, as we might expect glucocorticoid-responsive genes to oscillate in phase with the endogenous ligand, corticosterone, but echoes what we observed with promoter GR-bound genes in our in silico analysis.

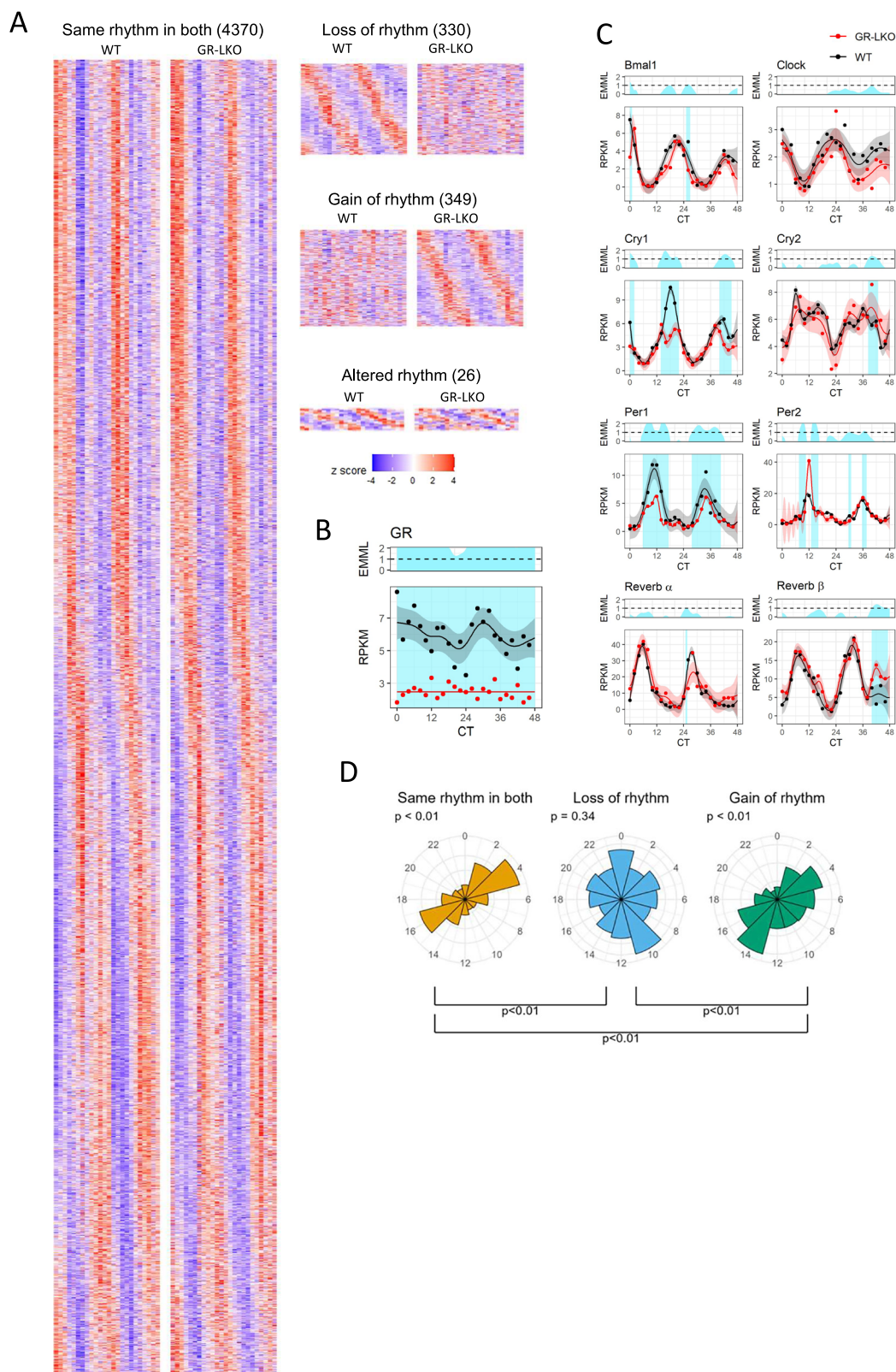


Fig. 3 | Hepatocyte GR deletion has a limited impact on the rhythmic liver transcriptome. **A** Heatmaps showing z-score normalised gene expression in the 4 rhythmic groups. Ordered by phase of gene (in relevant condition and WT in altered rhythm). **B** Time series of gene expression of GR (Nr3c1) for WT (black) and GR-LKO (red). Light blue shading shows regions of differential expression (EMML > 1). **C** Time series of gene expression of core clock genes for WT (black) and GR-LKO

(red). Light blue shading shows regions of differential expression (EMML > 1). **D** Peak phase of genes by rhythmic group; same rhythm in both (phase of shared signal), gain of rhythm in GR-LKO (phase in GR-LKO) and loss of rhythm in GR-LKO (phase in WT), p -values above plot are from a Rayleigh test of uniformity, p -values below the plot are from Watson's test for homogeneity on two samples.

To investigate how the magnitude of gene expression impacted our rhythmicity analysis, we considered the “same rhythm in both” group, and compared amplitude with expression (Fig. S5F). As expected amplitude was highly correlated with expression in both WT and GR-LKO, but the relationship did not seem to differ between the two and differences between WT and GR-LKO amplitude and expression were also linearly correlated.

Taken together, our data show only modest differences in rhythmicity of liver gene expression between GR-LKO mice and WT. The vast majority of classified transcripts were arrhythmic in both genotypes, or showed the same rhythm in both genotypes (10,773 and 4370 genes, respectively), with only 705 genes showing some change in rhythmic expression (loss, gain, or alteration in rhythmicity). Phrased another way, 86% classified rhythmic genes showed the same rhythm in WT and GR-LKO samples. Overall, these observations support a limited role of hepatocyte GR in contributing to circadian rhythmicity of the liver transcriptome.

Previously published work on rhythmic gene expression in the liver, and the role of GR, suggested a much greater impact than we observed³⁶. However, there are important differences between the studies, with the previous work conducted in the presence of light cycles (that is, not under circadian conditions, with light known to regulate the HPA axis and glucocorticoid production³⁷), and employing different methods to compare rhythmicity between conditions. We took the opportunity to re-analyse the gene expression data from this earlier work using our pipeline, to permit direct comparisons between the studies (Fig. S6). In our new analysis of the published data, we observed that most rhythmic genes were unaffected by loss of the GR (Fig. S6A,B). With our approach, we noted a marked difference in the number of genes reported to lose rhythmicity with GR knockout compared to the original report (2980 vs 793). This reduced number is still greater than the 330 that we observed under strictly circadian conditions. We also saw a difference in the number of genes reported to show an increase in rhythmicity between the studies (fewer in our study). The circadian phase relationship also differed between the studies, possibly reflecting the effects of light cycles, with the largest group of rhythmic genes, those that remained rhythmic with and without GR, showing a peak phase at ZT 0-5. (Fig. S6B). Overall, when data from the two studies are analysed using a rigorous method for directly comparing rhythmicity, conclusions are similar. The majority of rhythmic genes show unchanged rhythmicity with loss of GR, with the larger numbers of genes that do change possibly reflecting the input of light cycles. For direct comparison, we also analysed gain and loss of rhythmicity using the JTK cycle method as used in the earlier publications¹⁰ (Fig. S6C). Here, a substantial difference in genes with ascribed changes in rhythmicity is seen (e.g. loss of rhythm with GR deletion = 933 genes with compareRhythms, and 2980 genes with JTK cycle analysis).

Hepatocyte GR is required for the rhythmicity of a minor portion of the circadian transcriptome

To gain insights into why certain genes did lose or gain rhythmicity with hepatocyte GR deletion, we examined these gene lists in greater detail. Pathway enrichment analysis (KEGG 2019 Mouse, WikiPathways 2019 Mouse, via Enrichr web portal) did not find any pathways to be significantly over-represented (adjusted $p < 0.05$) in any of the groups (data not shown). We did observe an enrichment of genes which lost rhythmicity in the GR-LKO mice in proximity to GR binding sites (PEGS analysis, Fig. 4A), which was not seen for genes which gained rhythmicity in GR-LKO. This suggests that GR does play a role in the direct regulation of these genes. However, enrichment was also seen, strongly, for those genes with the same rhythmicity of expression in GR-LKO and WT (Fig. 4A), further indicating that GR binding appears redundant for rhythmic expression of a large number of genes.

We performed upstream regulator analysis (IPA) for genes with the same, lost, or gained rhythmicity in GR-LKO (genes with altered rhythm were not further analysed, due to the very small number). Echoing the PEGS analysis, GR (NR3C1) was revealed as a putative upstream regulator for both genes in the same and loss of rhythm groups, but not for genes in the gain of

rhythm group (Fig. 4B). The list of putative upstream regulators for the same rhythm group was notable for including established, broad-acting transcription factors such as HNF4A, TP53, MYC; HNF4A is of particular interest as it has been linked to definition of tissue circadian rhythms through BMAL1::CLOCK^{38,39}. The emergence of MLXIP1 (ChREBP) in this group, which is a nutrient-sensitive transcription factor, also indicates a mechanism through which timing of food intake (a potent zeitgeber to the liver^{40,41}) could direct rhythmicity of gene expression. Thus, we can speculate that GR may play a role in regulating expression of this group of genes, but our circadian RNA-seq data suggests that it is redundant for the delivery of timing information, for which other TFs are dominant. For the loss of rhythm group, GR (NR3C1) was found as a putative upstream regulator alongside, interestingly, clock factors RORA and RORC. Although phase distribution was broad for this group of genes, peak phase of expression was CT10-12 (Fig. 3D), coinciding with the zenith of endogenous glucocorticoid levels. Therefore, GR likely does confer timing information to a proportion of the hepatic transcriptome, but what distinguishes this proportion is unclear based on current data.

For those genes which gain rhythmicity when GR is deleted, we observed the emergence of STAT1 and STAT5A as putative upstream regulators (Fig. 4B). There is a body of literature reporting interactions between the glucocorticoid receptor and both STAT1 and STAT5; for example, a direct interaction between GR and STAT5 in hepatocytes has been shown to regulate gene expression in mice⁴². Thus we can speculate that an outcome of GR-STAT interactions is that a proportion of genes are normally not rhythmic, but with GR removal, rhythmicity of expression emerges.

Discussion

Glucocorticoid action in the liver has been strongly linked to the actions of the circadian clock, especially with regards to regulation of energy metabolism. Secretion of glucocorticoid hormones is both circadian and fasting-regulated. Excess glucocorticoid induces diabetes mellitus⁴³. Previous work has identified functional crosstalk between the GR and core clock proteins, including CLOCK, CRY, and REV-ERBa¹⁴⁻¹⁷, and glucocorticoid actions in the liver are gated by circadian phase¹⁷. We set out to investigate GR-clock interaction further, first with in silico analysis of existing data, and then by testing directly the role of GR, in a postnatal, conditional, hepatocyte-targeted GR knockout mouse model.

We found that GR-bound promoters are significantly more likely to also be bound by core circadian clock transcription factors, including CLOCK, CRYs, REV-ERBa, and PERs. Our analysis also revealed that GR-promoter-bound genes are highly expressed in the liver, and we also found high gene expression to be an important confounder for the detection of rhythmicity. This is a predicted outcome and relates to the confidence of detecting a rhythmic signal over background noise increasing with the level of gene expression^{22,34,44}. It also highlights the need to control for gene expression in comparing rhythmicity, to avoid confounding by expression level. Importantly, when we control for gene expression we still see significant association between promoter GR binding and circadian rhythmicity, with the majority of circadian rhythmic genes having GR-bound promoters. Interestingly, we discovered a significant difference in peak phase between GR-bound, and non-GR-bound promoters, implying that GR binding may be driving a distinct set of circadian targets.

We extended the analysis of GR and gene expression rhythmicity to enhancer elements, defined using the histone H3K4me1 mark. Again, most rhythmic genes had GR bound at a linked enhancer. The distribution of peak phase differed depending on whether GR was bound at the enhancer or not, but this effect was more subtle than observed in the promoter analysis. We also saw co-recruitment of a subset of circadian clock transcription factors, but this effect was lost once we controlled for chromatin openness in our analysis. Furthermore, when gene expression levels were taken into account, we did not see an increased likelihood of rhythmicity detection for genes with GR binding at linked enhancers. Therefore, we do not have

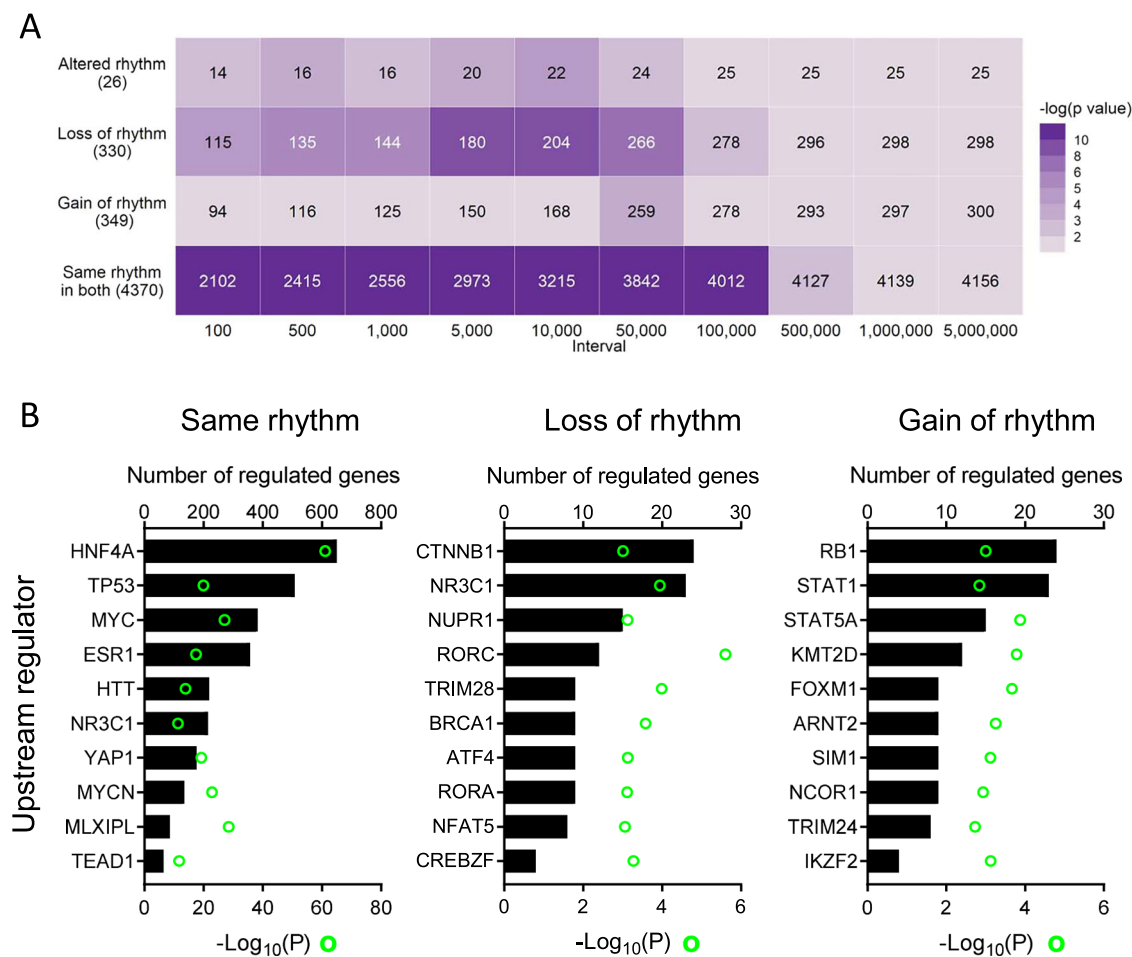


Fig. 4 | Direct regulation by GR is limited to a subset of the rhythmic liver transcriptome. A Peaks to genes analysis of enrichment of GR Chip-seq peaks in relation to distance from genes in rhythmic groups from compareRhythms analysis

in WT and GR-LKO. **B** IPA upstream regulator analysis (transcription factors and nuclear receptors) for groups of rhythmic genes (altered rhythm group not analysed due to small size).

evidence supporting a clear role for GR binding at enhancers in conferring rhythmicity to gene expression.

To investigate the causal role for GR in conferring rhythmicity to the liver we deleted GR in hepatocytes postnatally using an inducible Cre-Lox system. We conducted an experiment with frequent animal sampling over a full 48 h under true circadian conditions, constant darkness. We identified a high-confidence set of GR-dependent genes and mapped these to the known GR cisome. As predicted, we found enrichment of the down-regulated genes for GR binding, but no relation between GR binding and the genes that were induced by loss of GR. This fits with the more robust and consistent mechanism of GR action as a transactivator. The genes which showed an increase in expression with GR loss may be regulated by a range of mechanisms, likely explaining the lack of a clear relationship with GR binding, a finding seen in other systems^{13,32,33,45}.

We analysed gene expression through time, and compared between genotypes using compareRhythms. This approach directly compares rhythmicity between conditions and avoids misclassification resulting from categorisation of rhythmicity per condition, and the assumption that rhythmic changes can be inferred from differences in individual rhythmic status^{34,46}. Interestingly, we found that similar numbers of genes gained and lost rhythms in the absence of GR, but that the majority of rhythmic genes showed unchanged rhythmicity. A gain or loss in rhythm maybe down to a complete loss/gain of rhythmicity or a change in the amplitude of the rhythm bringing it past the signal-to-noise ratio threshold needed to be detected.

We did observe small changes in circadian expression of several of the core clock genes, but this was clearly insufficient to affect the rhythmic status

of the vast majority of rhythmic genes in intact animals. Here the preserved rhythmicity of the *Per* genes in the absence of GR, despite being glucocorticoid-responsive genes, likely reflects the sensitivity of *Per1/2* to other systemic cues (e.g. feeding time, insulin action^{47,48}). The phase plots of the genes rhythmic in both genotypes showed clear peaks at CT4 and CT16. In contrast, the phase plots of genes which lost rhythm in the absence of GR had a broad distribution through the circadian cycle. This suggests that although GR is important for rhythmicity, it is not the primary signal conveying phase timing information, and are other zeitgebers which dominate, or can compensate for the loss of the GR signal. This finding resonates with earlier work looking at rhythmic changes in inflammatory status in lung which requires the presence of glucocorticoid, but does not rely on the glucocorticoid for timing information²⁴. Similar findings are reported in intact zebrafish⁴⁹.

Previous work had suggested that GR function in the liver may be important for setting the circadian landscape, with a more major impact on rhythmicity noted in liver GR null animals³⁶. However, our work differs in some significant experimental, and analytical ways. We explored this further by re-analysing the previously published data using compareRhythms, and found our re-analysis to be far closer to our findings than in the original report, suggesting that some of the changes in rhythmic status previously reported may have resulted from categorisation issues. This is now a recognised issue with non-comparative rhythmicity analysis approaches (compareRhythms, and dryR). The re-analysed data did reveal interesting changes in phase, with those genes losing rhythm having a peak phase at ZT18, which does not align with the serum peak in corticosterone concentration (typically ZT10-12)²⁴.

Our study focused on the convergence of GR and circadian clock action in the liver. Despite finding that most circadian genes were bound by GR, there was a surprisingly small impact on the circadian repertoire resulting from GR loss. The relatively small impact of GR loss on liver rhythmicity may be partly explained by the entraining influences present in an intact animal, including sympathetic innervation, and rhythmic feeding driven by the central clock. However, changes in liver gene rhythmicity are sensitive to physiological changes, and the enrichment of GR binding at rhythmic gene promoters suggested a functional role. Importantly, the genes that lost rhythmicity in the GR null animals were enriched in relation to GR binding sites, suggesting that these are genes which require GR action for rhythmicity, through a GR binding-dependent mechanism.

There are limitations to our work. We performed the computational analysis on previously published datasets, some of which were obtained years ago. The quality of data collected by different labs over time, and assembled for analysis may be variable, causing confounding, or bias, despite our best efforts. The observed strong association between GR binding and gene expression raised an important issue. This may have increased the risk of detecting spurious co-binding with clock TFs as a result of open and permissive chromatin, as indeed appeared to be the case at enhancers. We used arbitrary, but accepted, annotations to define promoters and enhancers in the early stages of the analysis using published data^{21,22,50}. The hypotheses arising were then tested in conditional liver GR knockout animals. We defined circadian rhythmicity using well-accepted analytical algorithms, but we are well-aware of the risks of misclassification. We were careful to use a specific method to compare rhythmicity between conditions^{4,34}. Our in-vivo analysis persisted through 48 h under circadian conditions. There was a relatively weak overlap between the identity of rhythmic genes in our analysis and those defined as rhythmic in earlier work. One issue is that the earlier work was not conducted under circadian conditions, and was over a single day-night cycle. However, analysis of both experimental datasets using the same method did, in both cases, detect that GR loss had a small effect on the proportion of rhythmic genes. Our upstream regulator analysis is computational, and brings in TFs which may not be relevant to the liver, but restricting analysis to only liver factors risks artificially constraining the analysis, and generating false negative results.

Taken together, our new data and analysis support a major presence of GR binding in the promoters. GR binding also is a major marker of high gene expression in the liver. However, under direct experimentation, the functional importance for GR in determining the circadian repertoire of the liver was clearly defined, but limited in scope. Therefore, in the liver, the actions of the GR are determined by the circadian clock to a greater extent than the actions of the circadian system are determined by the GR. This conclusion is strengthened by previous work examining the impact of adrenalectomy on circadian rhythmicity²⁰. In this paper adrenalectomy had minimal impact on the orchestration of circadian rhythmicity. However, adrenalectomized animals re-entrained after feeding schedule phase shifts, implying a long feedback loop involving rhythmic corticosterone may help stabilise the circadian clock in physiological settings, and render it resistance to shifting in response to environmental change.

It remains unclear what the functional importance of GR recruitment to highly expressed, and rhythmic genes in the liver is. It may be that more actively expressed genes, with open chromatin, are permissive for GR binding resulting in non-productive GR occupancy, or that the GR binding is rendered redundant by other TF recruitment. It is also possible that the role of the GR is unmasked under conditions of stress when GR is activated by ligand, although we did sample through two complete circadian cycles, and so through two cycles of circulating corticosterone oscillation, without seeing much effect. It may be that only a more severe insult to drive much higher than physiological corticosterone would be needed to see an effect, but this is would only be relevant under pathological conditions. As the importance of circadian rhythmicity becomes more widely acknowledged, it is important that studies adhere to robust analytical methodology to avoid false discovery⁵¹. In summary, in the liver, GR binding is a dominant feature

of the circadian genome. However, we define only a small subset of rhythmic genes to be reliant on the GR for their circadian oscillation.

Methods

All data were analysed in R (v4.0.2; R Core Team, 2020) using Rstudio (v2024.04.2-764⁵²), unless otherwise mentioned, and plotted using ggplot2 (v3.5.1⁵³) and patchwork (v1.2.0⁵⁴).

Animal experiments

The study protocol was approved by the University of Manchester Animal Welfare and Ethical Review Body, and carried out under project licence 70 8558 (Professor David A. Bechtold), according to the UK Animals (Scientific Procedures) Act 1986. Mice had ad libitum access to standard laboratory chow and water, and were group housed on 12 h:12 h light:dark (LD) cycles unless otherwise stated. Adult male and female mice were used in the study.

The *Nr3c1*^{fllox/fllox}*Alb*^{CreERT2} line was created by crossing mice with the *Nr3c1*^{tm2Gsc} transgene⁵⁵, kindly donated by Professor Jan Tuckermann (Universität Ulm, Germany), and mice with the *Alb*^{tm1(creERT2)Mtz} transgene³⁰ kindly donated by Professors Daniel Metzger and Pierre Chambon (IGBMC, Strasbourg, France), both on a C57BL/6 background.

Cre-mediated recombination was induced by injecting mice with 0.1 mg tamoxifen i.p. (T5648, Sigma-Aldrich) each day for 5 days, as previously described²¹. 14 days after the first injection, at ZT0, all mice were transferred to light-tight cabinets and maintained in constant darkness. Such a move from LD to constant dark conditions does not impair plasma corticosterone rhythms⁵⁶ or the circadian rhythm of GR nuclear translocation⁵⁷. After a further 24 h (CT0), tissue collection was commenced, with one Cre- (“WT”) and one Cre+ (“GR-LKO”) mouse being euthanised (by cervical dislocation) every 2 h for 48 h. As far as possible, the sex of the mice euthanised was alternated at each time point (e.g. males at CT0, CT4, CT8; females at CT2, CT6, CT10). Anaesthesia was not used.

RNA extraction and sequencing

Total RNA was extracted from flash-frozen liver tissue using the ReliaPrep™ RNA Tissue Miniprep System (Promega), as per manufacturer instructions. Library preparation and RNA-sequencing using the HiSeq 4000 platform (Illumina) was carried out by the University of Manchester Genomic Technologies Core Facility as previously described²¹.

Assessment of liver GR deletion

Liver GR deletion in tamoxifen-treated mice was confirmed by Western blot, using methods previously described²¹. The primary antibodies used were anti-GR (M-20) sc-1004 (Santa Cruz; RRID:AB_2155786), and anti-GAPDH 10494-1-AP (ProteinTech; RRID:AB_2263076), with DyLight 800 anti-rabbit secondary antibody 5151 P (Cell Signaling; RRID:AB_10697505). The blot was imaged using the LI-COR Odyssey system.

Published clock TF and histone marker ChIP-seq data

Raw ChIP-seq data from ref. 22 (BMAL1, CLOCK, CRY1&2, NPAS2, PER1&2, H3K27ac and H3K4me1 from GEO accession number GSE39977) and ref. 21 (REV-ERBa from Array Express accession number E-MTAB-8413) was aligned to the mm10 genome using bowtie (v1.1.0⁵⁸;) and sorted and indexed using Samtools (v1.2^{59,60}).

Promoters were defined from the transcription start sites (TSS) (obtained from the R package TxDb.Mmusculus.UCSC.mm10.knownGene; v3.10.0⁶¹) with a promoter region being defined as –2000bp, and +200 bp relative to the TSS⁵⁰.

Enhancers were defined by first calling peaks on H3K4me1 data using MACS (v2.1.1⁶²). These peaks were removed if they intersected with the mm10 blacklist. Peaks from the five samples were then merged using DiffBind (v2.16.2⁶³) and re-centred 500 bp either side of the median (across samples) summit; overlapping peaks were merged.

DiffBind was then used to count clock TFs and histone marks on these defined promoter and enhancer regions. Enhancers were linked to genes by

finding the closest defined promoter. For clock TFs and H3K27ac from ref. 22 multiple timepoints (12 across 48 h) were counted onto promoter/enhancer regions separately and then means were taken. REV-ERBa data was only from ZT8.

Enhancer genomic locations were annotated using the annotatePeak function from the ChIPseeker R package (v1.24.0⁶⁴) aligning to TxDb.Mmusculus.UCSC.mm10.knownGene (v3.10.0⁶¹).

Motif analysis was performed on GR-bound rhythmic enhancer regions as compared to a background of non-GR-bound rhythmic enhancers regions using Homer (v4.11.1⁶⁵).

Published circadian RNA-seq data

Normalised RNA-seq data from WT mice housed under LD conditions were obtained from ref. 23 (GEO accession number GSE70499).

Expression data were further normalised by read depth and then detrended across each gene. Rhythm detection was performed using JTK-cycle (MetaCycle R package v1.2.0⁶⁶). Rayleigh test of uniformity, used to determine if phases of genes were distributed uniformly, and Watson's two-sample test of homogeneity, used to determine if two phase distributions were the same, were performed using the R package circular (v0.5-0⁶⁷).

Gaussian mixture modelling was utilised to perform clustering on the dynamics of the gene expression data. First, the data was z-scored across time and then clustered using the R package Mclust (v6.1.1⁶⁸).

To test for effect of GR binding on rhythmicity, whilst controlling for gene expression, JTK-rhythmicity classifications were modelled using a logistic regression model with log gene expression and presence of GR binding as covariates; glm function from stats R package (v4.0.2⁶⁹). This was done using both continuous log gene expression and log gene expression binned into deciles.

Published DNase-seq data

Normalised DNase-seq data from WT mouse liver housed under LD conditions were obtained from ref. 25 (GEO accession number GSE60430).

Data was lifted over from mm9 to mm10 using R package easyLift (v0.2.1⁷⁰), before being counted onto defined promoter/enhancer regions using the R package PopSV (v.1.1⁷¹). The average value across time points (ZT2, 6, 10, 14, 18, 22) was taken.

To test for the effect of GR binding on core circadian clock TF binding, whilst controlling for chromatin openness, clock TF ChIP-seq reads were modelled by linear regression with DNase-seq reads for the same region and presence of GR binding as covariates. The R package effectsize (v1.0.1⁷²); was used to calculate standardised effect sizes and 95% confidence intervals for effect of GR binding.

Published promoter-capture Hi-C data

Normalised promoter-capture Hi-C data from WT mouse liver housed under LD conditions was obtained from²⁹ (https://github.com/mandok/circadian_3Dchrom).

Data was lifted over from mm9 to mm10 using R package easyLift (v0.2.1⁷⁰). Overlaps between interacting fragments and our defined enhancer regions were found using findOverlaps from the GenomicRanges R package (v1.58.0⁵⁰).

GR ChIP-seq

Mouse liver GR ChIP-seq was previously performed in C57BL/6 mice acutely treated with dexamethasone or cyclodextrin vehicle, with tissue collected one hour after drug administration¹³. That study utilised tissue collected at ZT6 (Zeitgeber Time 6; 6 h after lights on); for this study, we used both the ZT6 data and GR ChIP-seq data from tissue collected at ZT18 (18 hours after lights on) in the same experiment, to capture the GR cis-trome across both day and night (ZT18 raw data available at GEO accession number GSE280340). The GR ChIP was performed as described in ref. 13, with ChIP libraries sequenced (paired-end) on the Illumina HiSeq 4000 platform. Reads were aligned to the mm10 genome using bowtie (v1.1.0⁵⁸) and sorted and indexed using Samtools (v1.2^{59,60}). Peaks were called across

ZT6 and ZT18 samples, separately for dexamethasone- and vehicle-treated, using MACS (v2.1.1⁶²), peaks were removed if they intersected with the mm10 blacklist.

Promoters/enhancers were called as "GR bound" if a GR peak overlapped with the promoter/enhancer region as defined above.

WT and GR-LKO RNA-seq data

Adaptors were removed and ends trimmed using Trimmomatic (v0.36⁷³), then reads were mapped against the mouse genome (mm10/GRCm38) using STAR (v2.5.3⁷⁴). Reads were counted, normalised and annotated using the Rsubread (v1.28.1⁷⁵), edgeR (v3.30.3⁷⁶) and biomaRt (v2.44.0⁷⁷) packages, respectively.

Differential expression analysis was run in R using edgeR (v3.30.3⁷⁶). Genes were considered to be differentially expressed (DE) if the false discovery rate (FDR) was less than 0.05. Peak set enrichment of gene sets was done using PEGS (v0.3.0³¹). Differential rhythmicity analysis was performed on the RPKMs (calculated via edgeR) using the compareRhythms R package (v0.99.0^{34,78}). A model selection approach was used with genes being assigned to either arrhythmic, differentially rhythmic (gain, loss or change of rhythm) or the same rhythm in both. A probability of being in a category of at least 0.6 was required for assignment. The "differentially rhythmic" category was then assigned gain, loss or change in rhythm based on which model had the highest probability to avoid losing genes that met the threshold for differential rhythmicity, but were unable to be distinguished between gain, loss or change of rhythm. Rayleigh test of uniformity, used to determine if phases of genes were distributed uniformly, and Watson's two-sample test of homogeneity, used to determine if two phase distributions were the same, were performed using the R package circular (v0.5-0⁶⁷). Differential time series analysis of the clock genes was performed using the R package nsgp (v1.0.5³⁵). Upstream regulator analysis on rhythmic groups was done using QIAGEN IPA (QIAGEN Inc., <https://digitalinsights.qiagen.com/IPA>) (v111725566)⁷⁹. Raw and processed RNA-seq data is available at GEO accession number GSE280339. The researcher conducting this data analysis was not blinded to the experimental groups, due to the nature of the analysis required.

For comparison, GR-LKO and WT RNA-seq data in LD mice from ref. 36 is analysed in the same way as our own data and presented.

Data availability

All data are available to suitably qualified applicants. Genomic data are deposited for open access on GEO (RNA-seq at GEO accession number GSE280339 and ChIP-seq at GEO accession number GSE280340).

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Author contributions

R.J.M., A.L.H., M.R., A.S.I.L. and D.W.R. conceived the analysis and planned the study. A.L.H., N.B. and M.B. performed the animal experiments. R.J.M. and A.L.H. analysed the data sets. R.J.M., A.L.H., M.R. and D.W.R. wrote the main manuscript text. R.J.M. and A.L.H. prepared the figures. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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