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Rev-Erb in Macrophage Gene Expression: A Case Study of Transcriptional Regulation Through Distal Regulatory Elements

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by

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Murphy PA, **Lam MT**, Wu X, Kim TN, Vartanian SM, Bollen AW, Carlson TR, Wang RA (2008). Endothelial Notch4 signaling induces hallmarks of brain arteriovenous malformations in mice. Proc Natl Acad Sci U S A. 105(31):10901-6.

Carlson TR, Yan Y, Wu X, **Lam MT**, Tang GL, Beverly LJ, Messina LM, Capobianco AJ, Werb Z, Wang R (2005). Endothelial expression of a constitutively active Notch4 elicits reversible arteriovenous malformations in adult mice. Proc Natl Acad Sci. Jul12; 102(28):9884-9.

Lam MT and Cheng JF (2003). The Identification of Single Nucleotide Polymorphism in Baboon's Cholesteryl Ester Transfer Protein for Human-Baboon Comparative Analysis. Berkeley Scientific. 7(1):42-47.

Lam MT and Cheng JF (2003). Abstract: The Identification of Single Nucleotide Polymorphism in Baboon's Cholesteryl Ester Transfer Protein for Human-Baboon Comparative Analysis. Journal of Undergraduate Research. 3: 71.

Lam MT and Cheng JF (2002). Abstract: Construction of BAC Resources for Mapping and Sequencing Mammalian Genes. Journal of Undergraduate Research. 2:86.

Abstract of the Dissertation

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This dissertation contains four divisions represented in three chapters. The first chapter introduces transcription factor Rev-Erb, a transcriptional repressor belonging to the superfamily of nuclear receptors. Chapter 1 then continues with the physiological roles and functional mechanisms of enhancers. These are DNA regulatory elements key to orchestrate temporal and spatial expression of genes. Our work on Rev-Erb in macrophages, described in the second chapter, provides evidences of a novel transcription regulatory mechanism at enhancer elements. We identified novel target genes of Rev-Erb, such as *matrix metalloproteases 9 (mmp9)* and *fractalkine receptor (Cx3cr1)*, which are negatively regulated by Rev-Erb from distal enhancer elements. In fact, genome-wide binding analysis shows that most Rev-Erb localizes to

putative macrophage enhancers. A recent discovery demonstrated that RNAs are actively transcribed at enhancers on a genomic-scale. In line with this finding, Rev-Erb represses RNA transcription at enhancers. The mechanism is similar to its action at promoters by inhibition of RNA polymerase II recruitment and deacetylation of histones. Given that both enhancer-RNAs (eRNAs) and mRNAs are negatively regulated by Rev-Erb, we further test whether eRNA is a functional intermediate for target gene regulation. Using *Mmp9* as a model, targeting its eRNA with RNA interference decreases mRNA expression. Furthermore, in Rev-Erb deficient macrophages, both *Mmp9* eRNA and mRNA are derepressed; but targeting eRNA is sufficient to reverse *Mmp9* mRNA derepression. Our finding suggests a functional role for enhancer-RNA. When acting through enhancers, Rev-Erb regulates target genes by repressing transcription at enhancers as a mechanistic intermediate. The last chapter explores ways to utilize the enormousness of genome-scale data from human populations to study functions of transcription regulatory elements relevant to diseases through forward genetics. Understanding mechanisms of transcriptional regulation, including discoveries of functional and targetable intermediates such as enhancer-RNAs, suggests new methods of disease detection and treatment.

Chapter 1 Nuclear Receptor Rev-Erb and Distal Transcriptional Enhancers

A. Rev-Erb and inflammatory implication

Nuclear Receptors are an important class of transcription factors that regulate diverse physiological processes. Generally, nuclear receptors modulate cellular function by transcriptional regulation in response to ligands such as hormones (e.g. glucocorticoid, estrogen, androgen) or cellular metabolites (e.g. fatty acids, oxysterols, etc). Thus nuclear receptors integrate cellular and endocrine signals to maintain homeostasis and proper physiological responses. In inflammatory contexts, nuclear receptors are important in modulating metabolic and inflammatory homeostasis. Upon activation by inflammatory stimuli, nuclear receptor expression undergoes rapid and drastic changes to adjust and adapt to the dynamic inflammatory process [1]. Disrupting nuclear receptor function in macrophages alone can alter inflammatory responses and initiate systemic inflammatory diseases [2-6]. Compounds targeting nuclear receptors have thus been used for therapeutics. Although such compounds are effective, unwanted side effects at therapeutic levels frequently limit their use [7]. A concept is thus developed to achieve desirable therapeutic effects by using a "cocktail" of compounds. The key is to target nuclear receptors that independently regulate biological pathways of interest (i.e. inflammation). This synergistic effect achieves higher therapeutic potency with lower dosages of

each compound, thus reducing compound-specific side effects [8]. Thus expanding the knowledge of nuclear receptors' function, including gene regulation, mechanism of action, and their biological role has promising therapeutic potential.

Rev-Erbs form a unique subclass of nuclear receptors whose function in macrophages remains largely unexplored. In mammals, the Rev-Erb family consists of two members, Rev-Erb α and β, which share high homology in their DNA and ligand binding domains. Both Rev-Erb members were recently found to bind heme, and several studies further indicate that heme is used as a prosthetic group for sensing cellular diatomic gases, such as carbon monoxide and nitric oxide [9-13]. Unlike other nuclear receptor families Rev-Erb lacks helix 12, the AF2 domain which is required for ligand-dependent C-terminus activation; hence, Rev-Erb is considered as a dedicated repressor [14]. Mechanistically, Rev-Erb recruits the Nuclear Receptor Corepressor (NCoR) and Histone Deacetylase 3 (HDAC3) complex to mediate gene repression in response to ligand binding [15]. The NCoR-HDAC3 complex is important for negative regulation of inflammatory programs by other members of the nuclear receptor family [2, 3, 16]. Whether Rev-Erb adopts a similar mechanistic strategy to regulate inflammatory responses in macrophages is not known. One study in human macrophages demonstrates that REV-ERBα negatively regulates LXR-induced expression of Toll-like Receptor 4 (TLR4) [17]. TLR4 is a pathogen recognition receptor crucial to initiate immune responses upon recognizing the gram-negative bacterial cell wall component lipopolysaccharide (LPS); knockout studies show that TLR4 is

important for both acute and chronic inflammatory diseases [18]. This suggests that Rev-Erb is involved in regulating immune response in macrophages. We are interested in understanding the role of Rev-Erb in macrophage – it senses a different class of ligands and "crosstalks" with other nuclear receptors, suggesting that perhaps Rev-Erb and other nuclear receptors integrate different cellular signals into regulating overlapping pathways. Exploring the transcriptional program, mechanism of action, and biological role of Rev-Erb in macrophages is the very first step to understand this interaction.

Rev-Erb is best known in the context of circadian biology. Circadian rhythm is the rhythmic behavioral and physiological processes that are consistently entrained to the 24-hour-day cycles. The central clock, located in the suprachiasmatic nucleus, receives light cues via the visual system and synchronizes "slave oscillators" in peripheral tissues. Peripheral tissues also exhibit their own 24-hour rhythm. This peripheral clock specializes to maintain optimal physiology by driving rhythmic expression of its output genes. The molecular clock is a core of positive and negative feedback loops consisting of the *Bmall/Clock* activator and the *Cry/Per* repressor. *Rev-Erb* is involved in regulating the positive limb of the feedback loop. To drive rhythmic gene expression, the Bmall/Clock heterodimer activates the expression of *Rev-Erb*α, while Rev-Erbα in turn represses *Bmall*, *Clock*, and its own expression through the Rev-Erb/ROR response element (RevRE). Rev-Erbα knockout (KO) resulted in elevated expression of *Bmall* and *Clock*, with rhythmic amplitude severely blunted [19, 20]. In the liver, absence of Rev-Erbα also impairs circadian output

genes in cholesterol metabolism, resulting in abnormal plasma level of lipoproteins [21]. Rev-Erbβ, although less studied in the circadian context, is suggested to play a redundant role [20, 22]. Animal studies in Rev-Erbα and other clock components demonstrate the importance of circadian rhythm in maintaining proper physiological function [19, 21, 23, 24].

The interplay between inflammation and circadian biology is emerging from several lines of investigation. Pathological events such as atherosclerotic complications [25, 26] and death from endotoxic shock [27] are well documented to have circadian regulation. Macrophages also have a circadian clock, which has been implicated in regulating phagocytic activity and cytokine production [28, 29]. Interrogating Rev-Erb's biological function and mechanism allows us to explore the interplay between inflammatory homeostasis and circadian biology in macrophages.

Recent technological advances permit us to investigate the function of Rev-Erb in genome-wide scales. Transcription profiling with microarrays reveals Rev-Erb's gene program and an overall cellular phenotype in macrophages. Chromatin immunoprecipitation coupled with parallel sequencing (ChIP-seq) makes it possible to determine genome-wide localization of Rev-Erb in an unbiased fashion. We will use these methods to address the following fundamental questions about Rev-Erb's role in macrophages: What genes do Rev-Erbs regulated? Through what mechanism(s) do Rev-Erbs mediate gene regulation? Do Rev-Erbs alter macrophage function in-vivo?

B. Functional diversity of distal transcription enhancers

i. The role of transcriptional enhancer in cell type specificity

Division of labor and functional specialization among tissues and cell-types is vital to multicellular organisms. Efficiency can be achieved when each cell type engages its unique biological process, such that when acting in concert, an organism is capable to respond and adapt to external changes. Given that a distinct set of responses comes from a distinct set of instructions, a challenge presents itself – how does complexity of cell-specific functions arise from DNA information that is the same across different cell types? Complexity can be generated by diversifying gene expressions in a spatial and temporal fashion. Circadian regulation of gene expression offers a good example. Genetic clock components – like Bmall, Clock, Rev-Erb, etc – are expressed across cell types. Up to 10% of protein-coding gene transcripts are under circadian regulation in any given tissue. Yet when comparing across tissues, rhythmic genes are very tissue specific [30, 31]. As expected, the set of “clock output” genes are often associated with tissue-specific functions [31]. Similar phenomena are observed in other biological processes such as inflammation. Different cell types respond differently to the same stimulus even when the core signal transduction pathway is shared. This further highlights the importance of transcriptional regulatory components that give rise to cell-type specificity.

An emerging concept in eukaryotic transcription is that promoter-distal enhancers play a predominant role in cell identity and specialized functions.

They provide sequence-specific sites for transcription factor binding. In the genome, active enhancers are typically found in DNAseI-sensitive open chromatin regions that allow transcription factors accessibility. The concept of enhancers as major determinants of cell type specificity arises from observations of genome-wide transcription factor binding patterns. 1) Within the same cell type, different transcription factors tend to co-localize in a genome-wide scale [32, 33], implying the existences of "regulatory islands." 2) In different cell types, the same factor exhibits remarkably distinct binding patterns [34-37]. This suggests that each cell type has its own sets of regulatory "islands." In support of this, genome-wide mapping of DNAseI hypersensitive regions illustrates that open chromatin regions are very cell-type specific. Furthermore, methodical cell-type comparisons of epigenomic marks (e.g. post-translationally modified histone tails) supported the same notion. Cell type specific enhancer elements are demarcated with high enrichment of monomethylation on histone 3 lysine 4 (H3K4me1) and low enrichment for trimethyl H3K4 (H3K4me3). Comparison of two cell lines – K562, a human erythroleukemia, and HeLa, a human cervical carcinoma – resulted in an estimate of 24,000-36,000 $H3K4me1^{hi}$ $H3K4me3^{lo}$ putative enhancers from each line; only an estimated 5,000 regions were found present in both cell types. Cell-type specific enhancer elements strongly correlated to cell-type specific gene expression on a global scale. Furthermore, these enhancers activated transient reporter genes in a cell specific fashion [38]. In combination with H3K4me mapping, association of additional epigenetic marks

(e.g. H3K27Ac) or binding of cofactors (e.g. p300) and transcription factors further increases the accuracy of enhancer activity predictions [39, 40].

Establishment of genome-wide *cis*-acting regulatory elements, or cistrome for short, depends on lineage-determining factors – transcription factors which are required for cell type differentiation. While expression of one lineage-determining factor may often not be limited to a single cell type, interactions between a small set of factors is sufficient to dictate establishment of cell type specific cistromes. This is exemplified by the Ets factor PU.1. PU.1 is required for the generation of both granulocyte-macrophage progenitor and common-lymphoid progenitor cells, which will then differentiate respectively into macrophages and B cells. The latter require PU.1's collaborative interactions with macrophage and B-cell specific transcription factors, respectively. Genome-wide localization analysis of PU.1 shows a distinct binding pattern in promoter-distal enhancer elements of macrophage and B-cells. In macrophages, PU.1-bound regions are enriched for motifs of macrophage lineage-determining factors AP1 and CEBP; in B-cells, E2A, EBF and Oct-2. While PU.1 is required for the binding of these factors, each of these factors can modify the binding of PU.1. This collaborative interaction defines specificity. Subsequent recruitment of nucleosomal remodeling complexes and deposition of epigenetic marks (H3K4me1) by the PU.1 complex finalize the large sets of potential *cis*-regulatory elements in a cell-type specific fashion. Other non-tissue specific transcription factors are then allowed to access these regulatory elements and exert function in a cell-specific fashion [37].

ii. Mechanism of enhancer function

Given the long linear distance from transcriptional start sites, where RNA Polymerase II (RNAPII) and the transcriptional preinitiation complex situate, enhancers have been thought to exert function by direct interaction with target gene promoters through "looping." This "looping model" is supported by numerous studies of nuclear architecture utilizing "chromosome conformation capture" or 3C method. This assay identifies interactions between two genomic regions by crosslinking, restriction enzyme digestion, intermolecular ligation, and PCR analysis of the ligated products. If two genomic loci interact in three dimensions, then we predict a higher detection frequency of ligation between these two loci over more proximal fragments or by random ligation. 3C has been adapted to produce genome-wide maps of locus interaction either between a specific locus made with other regions in the genome ("4C"), or association among multiple sequences with other sequences in the genome ("5C" and "Hi-C") [41-43]. One enhancer can interact with multiple transcription start sites (est. avg. 18), whereas one transcription start site interacts with multiple enhancers (est. avg. 5) [42, 44]. In other words, each gene could be regulated by multiple enhancers, and each enhancer could regulate multiple genes.

Exactly how does an enhancer work? Theoretically, at least three events have to occur in order for an enhancer to function. i) Enhancer elements, or promoters in an equal manner, are mobilized. ii) Enhancer-promoter interaction is stabilized. iii) Regulatory activity is transferred from enhancer to the transcriptional machinery at the promoter. Combining loss of function and 3C

studies, two categories of transcription factors have been defined – those that affect both looping and transcriptional changes, and those that solely affect transcriptional changes [45]. The two classes of findings suggest that transcriptional components have specialized roles in pre- and post-loop formation. Most of these studies utilized the well-characterized locus control region (LCR) of the beta-globin gene cluster. The LCR is defined by six erythroid-specific DNAseI hypersensitive sites and temporally orchestrate globin gene expression throughout development. Transcription factors essential for erythroid differentiation like GATA1, EKLF, and FOG-1 are required for proper looping between the LCR and beta-globin promoter. Similarly, structural proteins (e.g. cohesin complex) or nucleosome remodelers (e.g. Brg1 in the SWI-SNF complex) are also required for looping. On the other hand, knockout of erythroid-specific transcription factor NF-E2 decreases beta-globin expression and promoter RNAPII recruitment without affecting looping. This class has greater affect on RNAPII recruitment rather than looping formation. As most studies revealed, changes in looping go hand-in-hand with changes in expression; the converse is not true. This suggests that looping is a prerequisite for enhancer-mediated transcriptional regulation.

Stability of loop formation must also be modulated – the cohesin complex has been a candidate to partake in this process. Cohesin complex forms a ring structure that is known to mediate proper chromosomal segregation of sister chromatids during cell division. Cohesin can also interact with insulator protein CTCF to define transcriptional looping domain. In human Hep3B liver cells,

interaction of CTCF and cohesin maintain two transcribing loops within the APOA1/C3/A4/A5 cluster – one loop containing the C3 enhancer, APOC3, APOA4, and APOA5; the other containing APOA1 [46]. Depletion of CTCF or RAD21, a component of the cohesin complex, disrupts chromatin looping and alters APOs and nearby gene expressions [46]. Cohesin has also been suggested to stabilize looping between enhancers and promoters through association with the Mediator complex [47]. ChIPseq in embryonic stem cells showed that Mediator, cohesin, and cohesin loading factor nipped B-like protein (NIPBL) co-localized at enhancers and promoters. Knockdown of cohesin, NIPBL, or Mediator led to changes in expression of genes whose regulatory elements were occupied by cohesin and mediator. Moreover, depletion of cohesin component reduced interaction between enhancer and promoter of *Nanog*. These data suggests that Mediator and cohesin physically and functionally connect the enhancers and promoters of active genes [47].

Lastly, transcriptional activity of an enhancer will ultimately change the activity of RNAPII at gene promoters. It has been traditionally thought that enhancers activate transcription by facilitating the recruitment and assembly of the preinitiation complex and RNAPII [Matson 2006]. Recent results suggest that enhancers may also affect downstream processes, including the release of RNAPII from promoter-proximal pausing. This is again exemplified by the beta-globin LCR. Target deletion of the LCR resulted in a drastic decrease in RNAPII activation and elongation with only a mild reduction of RNAPII recruitment at beta-globin gene promoter [48]. Transcription regulation through promoter-

proximal pausing of RNAPII occurs at many eukaryotic genes [49]. This mode of regulation allows rapid and synchronous gene induction, which becomes important in timely regulated events such as development [50]. Upon a signaling event, enhancer can serve as a platform for recruitment of coactivators and transcriptional elongation factors to promote release of RNAPII from promoters [51, 52]. In addition to promoters and gene bodies, RNAPII could also be found at enhancer elements. This led to a "facilitative tracking and transcription" model where RNAPII scans through the intervening sequence from the enhancer to a transcriptionally productive promoter [53]. Additional modifications are needed for this model when considering a context where an intervening sequence is effectively "looped" out.

iii. Active RNA transcription at enhancers

RNA Polymerase II is not only found at promoters or gene bodies, it is also found globally at enhancers [54, 55]. The presence of RNAPII prompts the question whether RNA is being transcribed at enhancers. Using neuronal membrane depolarization as a model, Kim *et al* demonstrated that RNAs are globally transcribed at enhancers, and the expression of these mRNAs is induced upon this strong signaling event [55]. Similarly, De Santa *et al* showed a global change in RNAPII recruitment at enhancers of macrophages upon inflammatory activation by LPS; a selected few of these sites show correlated RNA induction [54]. Expression of enhancer-associated RNA has also been correlated to addition of nuclear receptor ligands [56, 57]. For their association,

these RNA species are collectively termed enhancer-RNA (eRNA). This consists a new class of non-coding RNA that is receiving much attention.

RNA-seq and ChIP-seq studies have defined the following characteristics for eRNA transcripts. (i) eRNAs are produced from regions defined by high enrichment of H3K4me1 and low enrichment of H3K4me3 (ii). These regions are enriched for RNA polymerase II (PolII) and transcriptional co-regulators (e.g. p300 co-activators, CBP, etc). (iii) Transcription of eRNAs initiates from PolII-binding sites and elongates bidirectionally. (iv) DNA sequences encoding eRNAs are evolutionarily conserved. (v) Enhancer-associated RNA transcripts have a short half-life. (vi) RNA transcripts are dynamically regulated upon signaling, and (vii) are positively correlated to expression levels of nearby mRNAs [54-56]. (viii) Induction of eRNA has been suggested as a precise mark for enhancer function (e.g. looping), more so than the enrichments of p300 and Med12 [57]. The last two points are of specific interest; they prompt the question of whether eRNA is functional in transcriptional activation.

Although still speculative, several lines of evidence suggest a functional role for eRNAs as transcriptional activators. Reports of enhancer-transcribed RNAs date back to as early as 1992, when Tuan et al. reported RNA transcripts at the locus control region of the beta-globin locus [58, 59]. In a transient reporter assay using HS2 as the enhancer, insertion of a transcriptional terminator downstream at the intervening sequence was sufficient to decrease RNA expression from HS2 enhancer. This reduced reporter gene activity driven by the epsilon-globin promoter [60]. In another line of experiments, ChIP studies

demonstrated RNAPII recruitment to both HS2 enhancer and adult beta-globin promoter. When its elongation was inhibited pharmacologically, RNAPII enrichment decreased at the beta-globin promoter, but not at the HS2 enhancer [61]. This suggests that promoter recruitment of RNAPII, but not to enhancer's, is dependent on RNA synthesis. One may speculate that RNA synthesis from the enhancer is required for promoter RNAPII recruitment.

Several studies provided functional evidences for RNA in the context of transcription. Using GENCODE database, one recent study identified a class of long non-coding RNAs (lncRNA) that can act as transcriptional activators. Depleting these lncRNA using RNA interference led to gene-specific decrease in mRNA expression; furthermore, DNA segments encoding these RNA were sufficient to induce transient reporter activity. This transcriptional effect was also sequence-specific; sequence substitution with another open reading frame abolished transcriptional effect [62]. The authors of this study, however, emphasized that these non-coding RNAs and eRNA are distinct – the lncRNA are transcribed from H3K4me3 enriched promoter-like elements, and more than 50% of them are spliced [62]. Whether eRNA and these enhancer-like lncRNA are distinct classes requires further systematic comparison.

Mechanistically, lncRNA can regulate gene expression by serving as beacons for transcriptional complexes [63-66]. *HOX* antisense intergenic RNA *(HOTAIR)* is a 2.2kb lncRNA expressed in *HOXC* cluster that regulates *HOXD* cluster *in trans*. The 5' end of *HOTAIR* is bound by Polycomb repressive complex, whereas the 3' end is bound by the LSD1/REST/CoREST complex

(Lysine-specific demethylase 1; RE-1 silencing transcription factor; corepressors for REST). Polycomb complex represses genes by trimethylation of H3K27; for LSD1/REST/CoREST, by removing dimethyl mark of H3K4. In essence, *HOTAIR* enables tethering of two distinct repressive complexes to chromatin for coupled H3K27 methylation and H3K4 demethylation [66]. The same group identified *HOTTIP*, a 3.8kb, spliced and poly-adenylated lncRNA for 'HOXA transcript at distal tip', which regulates *HOXA* cluster genes *in cis*. *HOTTIP* binds and direct WDR5/MLL complexes to HOXA gene cluster for trimethylation on H3K4 and activate gene transcription [63]. Interestingly, although its depletion diminishes gene expression, *HOTTIP* is dispensable for long-range interaction within the *HOXA* cluster where it exerts regulation [63]. Collectively, theses studies demonstrated diverse but specific functions of non-coding RNA in transcriptional regulation.

Although by description lncRNA and eRNA may belong to different classes [67], studies in lncRNA provide valuable insights into possible functionality and mechanisms of eRNAs, especially when RNA attributes important for function – the primary sequence and secondary structure – are fundamental across any RNA species.

C. Conclusion and Future Perspectives

In summary, enhancer elements are likely crucial players in diversifying transcriptional responses for multicellular organism to adapt in a complex environment. Lineage determining factors establish distinct cistromes for each

cell type, allowing general transcription factors to exert cell-type specific responses at appropriate times. Chromatin-remodeling and structural protein complexes establish accessibility and maintain interaction between enhancer and promoters. The resulting proximity of transcriptional complexes modulates activity of the transcriptional machinery at gene promoters. Each gene could be regulated by multiple enhancers. A signaling event can thus activate a selective subset of enhancers, with the docking of transcriptional complexes as a signal transduction relay in modulating proper gene expression.

Advances in sequencing technology have begun to permit elucidation of the significance of regulatory DNA elements – the once unappreciated “junk” of the genome – on biology of complex multicellular organism. Numerous studies demonstrated how widespread transcription factors are situated at enhancer elements. Nuclear receptors are no exception.

The discovery of RNA transcription at enhancers adds a new layer of intricacy to transcriptional regulation. As of now, eRNA expression is a correlation to nearby mRNA expression [55, 56]; eRNA is a marker for enhancer function [57]. Clearly, RNAs as a class of biological molecules are highly functional -- they possess enzymatic activity (e.g. spliceosomes), structural role (e.g. tRNA, ribosomes), and transcriptional function (e.g. microRNA, lncRNA). Many questions arise as more functional evidences for RNA emerge. How are eRNAs regulated? Are they transcriptionally functional? What are their contributions to enhancer function? Are they an integral part of physiological

processes? Addressing these functional and mechanistic questions will improve our understanding of enhancers in controlling gene expression.

If eRNAs indeed play an integral function in gene regulation, then therapeutic potential of targeting eRNA is high. eRNAs, by definition, are cell type specific; they are also targetable products made by cells. The effect of targeting eRNAs will therefore be highly cell- and gene(s) specific. Even if the same gene is expressed in two cell types, it is likely being regulated differentially because of cell-specific enhancers. Incorporating the high specificity of eRNAs could ultimately be used to achieve therapeutic effects with reduced undesired side effects and toxicity.

Chapter 2 Rev-Erb Regulates RNA Transcription at Distal Regulatory Elements of Macrophages

A. Introduction

The recent discovery of active RNA transcription at enhancers opens up the possibility of having RNA as an intermediate of gene regulation. Indeed RNA expression at enhancers is positively correlated with expression of nearby protein coding genes [54-56]. High-throughput sequencing studies of RNA and immunoprecipitated chromatin (ChIPseq) characterize these RNA as follows: 1) they are produced from regions demarcated with high monomethylated H3K4 (H3K4me1) and low trimethylated H3K4 (H3K4me3), the histone signature for putative enhancers [38]. 2) These regions are enriched for RNA polymerase II (RNAPII) and other transcriptional co-regulators. 3) Transcription of these RNAs initiates from RNAPII sites and elongates bidirectionally. 4) They are short-lived and 5) dynamically regulated by signal transduction events. Because these RNAs are found at enhancers, they are collectively termed enhancer-RNAs (eRNAs) [55]. Several studies suggest that enhancer binding transcription factors can activate expression of eRNAs [54, 55]. Furthermore, treatment of ligands for estrogen receptor and androgen receptor induces transcription of eRNAs [56, 57]. Whether these RNAs are transcriptionally functional is still not known.

Rev-Erbα and β are members of the nuclear receptor family of transcription factors. They bind as homodimers to a direct repeat of Rev-Erb

responsive elements with 2-basepair separation (RevRE DR2) [68]. Both Rev-Erbs regulate gene expression by interacting with Nuclear Receptor Corepressor (NCoR) [69], a core component of a co-repressor complex that mediates active repression by unliganded nuclear receptors and other classes of transcriptional repressors. Unique to other nuclear receptor families, Rev-Erbs lack the C' terminal activation function-2 domain (AF2) required to interact with coactivator complexes [68]. The Rev-Erbs have thus been considered as dedicated repressors. Rev-Erbs are best known to repress the circadian clock gene *bmall* and other members of the core circadian molecular machinery [15, 19, 70]. Recent studies also placed Rev-Erbα as a regulator of lipid and glucose metabolism in liver [21, 71, 72]. The interplay between inflammation and circadian biology is emerging from several lines of investigations. Macrophage functions have also been implicated as targets for circadian regulation [28, 29, 73]. However, the roles of Rev-Erbα and β in the inflammatory context are largely unknown.

We explore here the role of Rev-Erbα and β in inflammation by investigating the genome localization of Rev-Erb and analyzing gene expression using genetic approaches in macrophages. We found that Rev-Erbs primarily localize to putative enhancers of macrophages and negatively regulate genes involved in macrophage function, such as *Mmp9* and *Cx3cr1*. Unexpectedly, we also found Rev-Erb as a negative regulator of eRNA expression. Detection of nascent RNA transcripts by Global Run On sequencing (GRO-seq) in Rev-Erbα and Rev-Erbβ deficient (Rev-Erb DKO) primary macrophages demonstrated a

positive correlation between changes in Rev-Erb-bound eRNA expression with changes in expression of nearby protein-coding genes. With *Mmp9* as a model, we showed that production of eRNA temporally preceded production of mRNA. RNAi targeting of *Mmp9* eRNA was sufficient to reduce mRNA expression. Furthermore, targeting eRNA was sufficient to reverse *Mmp9* depression due to Rev-Erb-deficiency. Here we report a mechanism whereby Rev-Erb negatively regulates target gene expression by suppressing transcriptional activity at its enhancer.

B. Results

i. Defining Rev-Erbs binding sites in macrophages

To study function and mechanism of Rev-Erb in gene regulation, we sought to identify Rev-Erb target genes by determining genome-wide binding of Rev-Erbα and Rev-Erbβ in macrophages using chromatin immunoprecipitation couple with deep sequencing (ChIPseq). Because ChIP-grade antibodies for Rev-Erbα and Rev-Erbβ were not readily available, we established RAW264.7 macrophage cell lines stably expressing Rev-Erbα or Rev-Erbβ tagged with biotin-ligase recognition peptide (BLRP). By coexpressing BirA biotin ligase, BLRP-tagged Rev-Erbs are biotinylated within the cell, allowing efficient pull-down of the protein-DNA complex by streptavidin [37].

Analysis of ChIPseq data for Rev-Erbα and Rev-Erbβ shows significant overlapping binding. Of these sites, 2079 peaks are common to both Rev-Erbα and β. These sites have the highest enrichment among all identified Rev-Erb peaks, thus representing the most confident sites (Fig 1). We therefore focused our analysis on these commonly bound regions. Rev-Erb is best known to regulate circadian clock gene bmall. We detect significant enrichment of Rev-Erbα and Rev-Erbβ on the bmall promoter, in accordance with previous studies [15, 19] (Fig 2A). In addition, de novo discovery for transcription factor motifs revealed high enrichment for the Rev-Erb responsive element, Rev-Erb DR2 (Fig 2B). Furthermore, Rev-Erbs binding in RAW264.7 macrophages is significantly different to the profile of Liver X Receptors (LXR) that was determined using the same biotinylation strategy in this model macrophage cell line [37] (Fig 3). These studies therefore define a macrophage-specific genomic binding profile for Rev-Erbs.

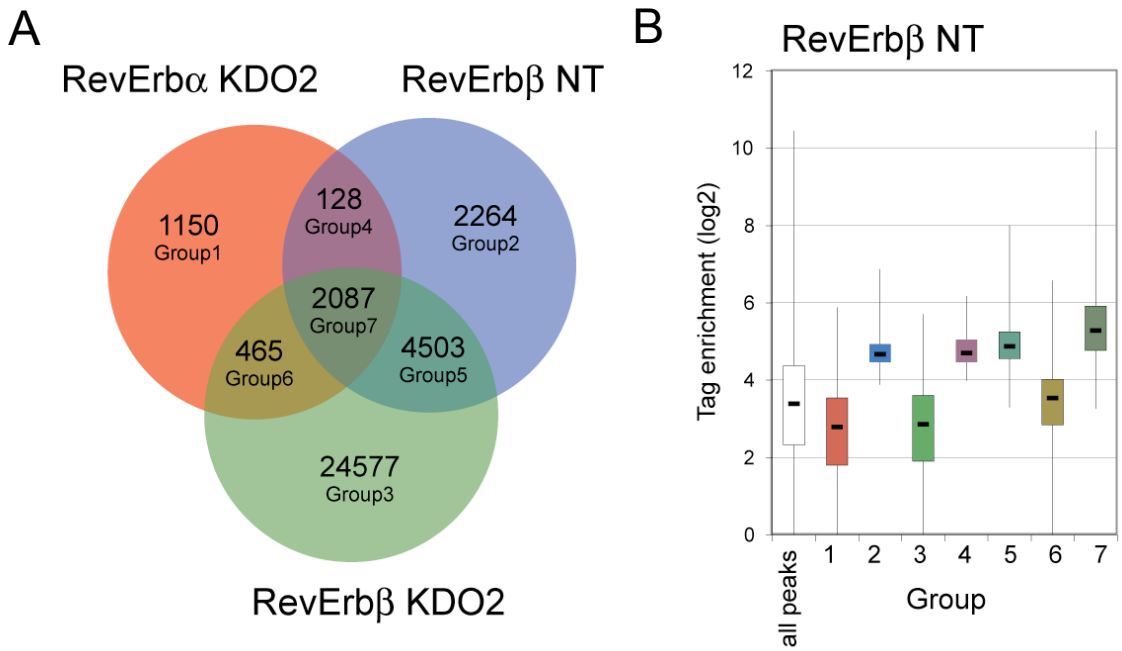
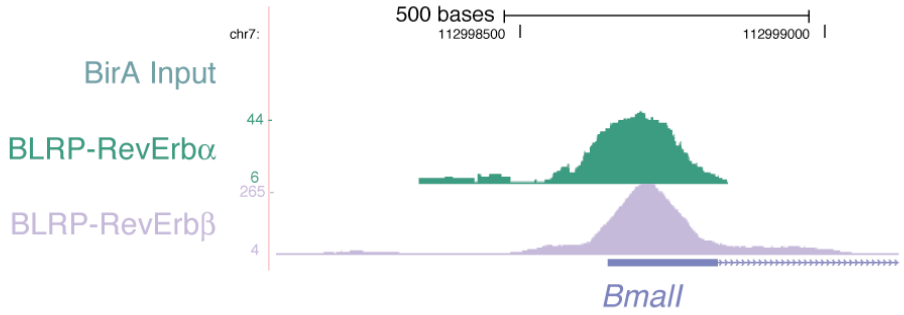


Figure 1. Defining Rev-Erb commonly bound regions for analysis. A) To determine the global localization of Rev-Erbα and Rev-Erbβ, chromatin immunoprecipitation with high throughput sequencing (ChIPseq) was performed using RAW264.7 macrophages stably expressing biotinylated Rev-Erbα or Rev-Erbβ. ChIPseq was performed using RAW264.7 macrophages in steady (non-treated) or activated state by treatment with100ng/mL of KDO2 lipid A, a toll-like receptor 4 ligand, for 1 hour. A) ChIPseq shows distinct but overlapping binding between Rev-Erbα and Rev-Erbβ. B) The distribution of tag enrichments for each peak within each subgroup in (A) is represented in box and whisker plot. The overlapping peaks (i.e. group 7) has the highest binding enrichment, representing the most confident Rev-Erb peaks. These peaks are used for subsequent analyses. The steady state of Rev-Erbβ is shown as a representative example.

A



B

Motif	p-value
RevErb DR2 7.1% (0.29%)	1e-174

Figure 2. Rev-Erbα and Rev-Erbβ ChIPseq show specific binding enrichment. A) ChIPseq experiments show specific Rev-Erbα (middle) and Rev-Erbβ (bottom track) enrichment at *bmall* promoter, a known Rev-Erb target gene. No enrichment was detected in macrophages without expression of tagged Rev-Erb (BirA-input, top track). B) *De novo* transcription factor motif discovery shows high enrichment for Rev-Erb responsive element (RevRE DR2), which is observed in 7.1% of the peaks compared to the expected 0.29% based on genomic background.

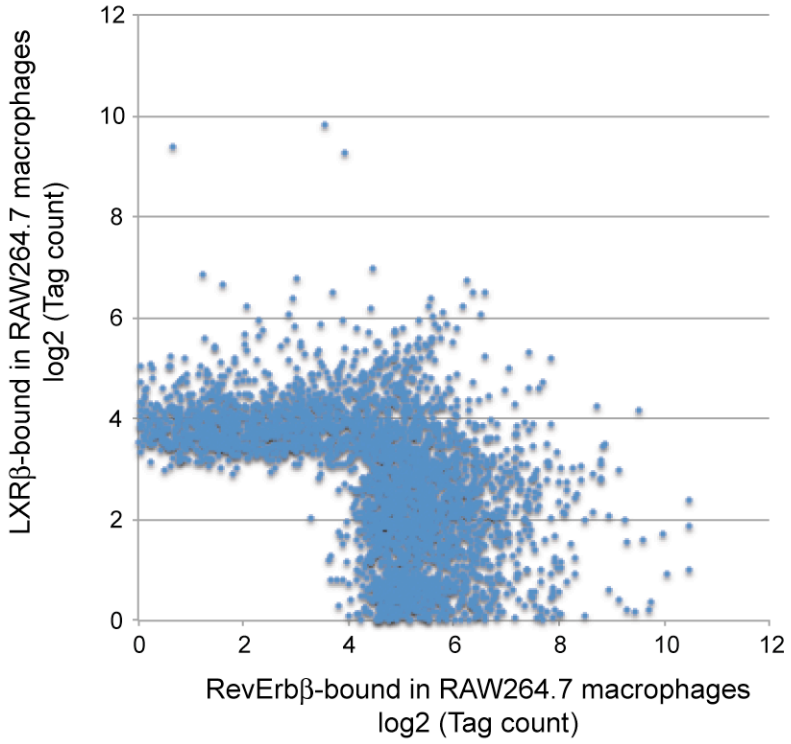


Figure 3. Rev-Erbβ and LXRβ localize to different loci in the macrophage genome. Global localization for RevErbβ and LXRβ [37] were determined using in vivo protein biotinylation approach followed by ChIP-sequencing in RAW264.7 macrophages. LXRβ-bound sites (GW-treated, n = 1,534) were combined with Rev-Erb commonly bound sites (n = 2,079), yielding a total of 3,514 unique sites represented by each dot on the scatter plot. Enrichment for Rev-Erbβ is plotted on x-axis; for LXRβ, y-axis. Site shared between Rev-Erb and LXRβ would lie near the imaginary line x=y. 595 sites (16.9% of total) are shared between RevErbβ and LXRβ, with no more than 2-fold differences in binding enrichment. 2,325 peaks (66.2%) have at least 4-fold binding enrichment differences between RevErbβ and LXRβ, suggesting that most sites are either Rev-Erbβ or LXRβ specific.

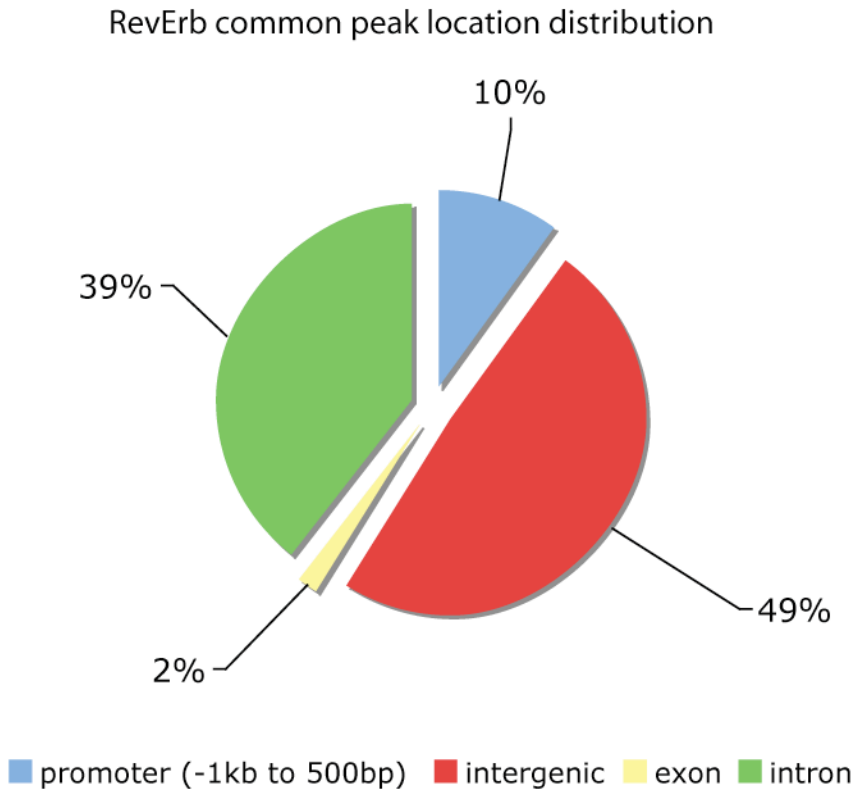


Figure 4. The majority of Rev-Erb bound sites, approximately 90%, reside in promoter-distal regions at least 1kb away from annotated transcription start sites.

ii. Rev-Erbs bind mainly at distal regulatory elements

To date, Rev-Erb had been shown to regulate transcription mainly at promoters [9, 19, 74]. However, our ChIPseq data shows that approximately 10% of Rev-Erb are found in promoters; the majority of Rev-Erb binding sites are in intra- and intergenic regions at least 1kb away from annotated transcription start sites (Fig 4). This suggests that Rev-Erb is binding mainly to enhancer regions of macrophages. Transcriptional enhancers are cell-type specific, and they are demarcated with a histone signature showing high enrichment for monomethylation on histone 3 lysine 4 (H3K4me1) and low enrichment for trimethylation on H3K4 [38]. **70%** of Rev-Erb binding sites reside in H3K4me1^{hi} and H3K4me3^{lo} regions (Fig 5). Furthermore, these sites are co-localized with transcription factor PU.1 (Fig 5). We have shown previously that PU.1 initiates nucleosome remodeling and depositing monomethylation at H3K4 in distal elements of macrophages [37]. When looking at Rev-Erb-bound sites, the H3K4me1 ChIP-Seq signal is much higher in macrophages than in B-cells [75] (Fig 6A), suggesting that these sites are not regulatory elements in B-cells. Furthermore, monomethylation at Rev-Erb-bound sites is dependent on PU.1 (Fig 6B). Consistent with this, *de novo* motif analysis of Rev-Erb-bound sites showed high motif enrichment for PU.1 as well as AP1 (Fig 7), a family of transcription factors required for macrophage differentiation. Collectively, this suggests that majority of Rev-Erb is bound to promoter-distal enhancer elements in macrophages.

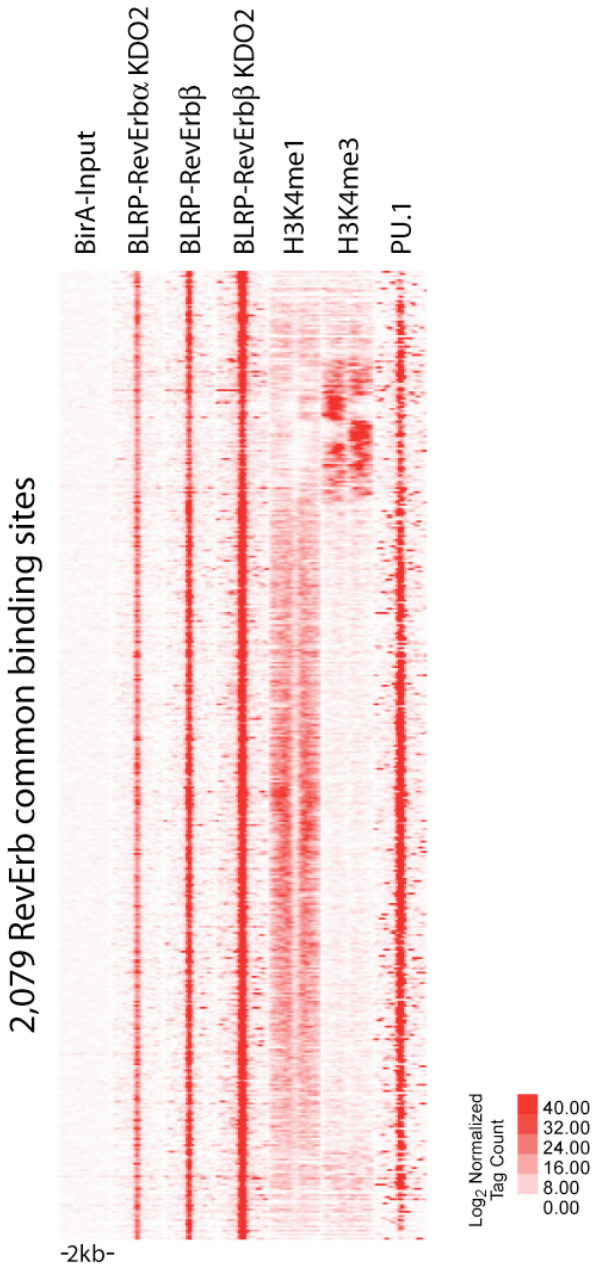


Figure 5. Rev-Erb binds primarily to macrophage putative enhancers. Cluster analysis shows approximately 70% of Rev-Erb reside in regions with enhancer histone signatures -- H3K4me1^{hi} H3K4me3^{lo}. These regions are also highly co-enriched for PU.1. H3K4me1, H3K4me3, and PU.1 ChIP-Seq data are from Heinz et al, 2010 [37].

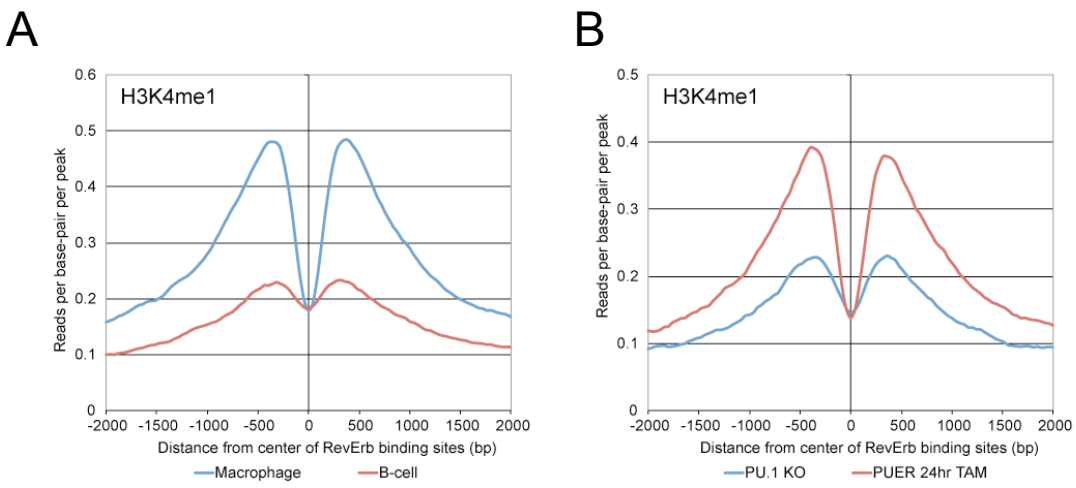


Figure 6. Macrophage-defined Rev-Erb bound promoter-distal sites reside in macrophage specific H3K4me1 regions. 1,388 Rev-Erb-bound sites reside in promoter-distal regions with high enrichment of H3K4me1 and low enrichment of H3K4me3. A) H3K4me1 enrichments at these sites are significantly higher in macrophages than in B cells, suggesting that these sites are macrophage specific. B) Deposition of H3K4me1 at Rev-Erb-bound sites is dependent on PU.1. H3K4me1 enrichment around Rev-Erb-bound promoter-distal elements is low in PU.1 KO cells (blue). Enrichment of H3K4me1 is restored when PU.1 function was induced after 24-hour treatment of tamoxifen in PU.1 KO cells reconstituted with tamoxifen-inducible PU.1 (Red, PUER). H3K4me1 data from macrophages, PUER cells, and B cells are published in Heniz et al, 2010 [37].

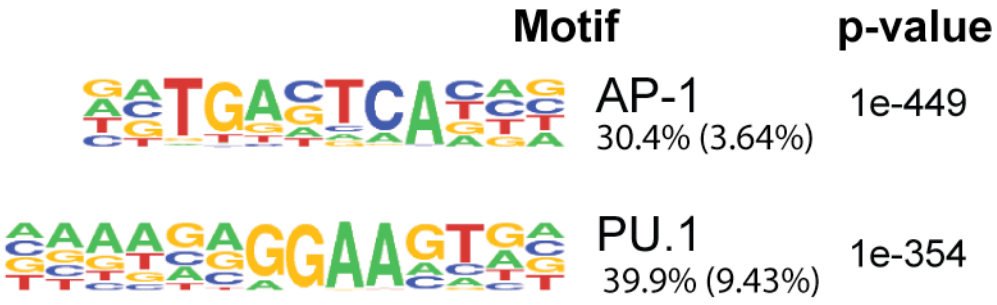


Figure 7. De novo transcription factor motif discovery reveals strong enrichment for macrophage lineage determining factors in Rev-Erb common binding sites. Percentages of observed and expected (parentheses) frequency and p-values of each motif are indicated.

iii. Rev-Erb regulates gene expression from distal binding elements

We started examining genes with nearby strong Rev-Erb enrichment. At the *Mmp9* genomic locus, Rev-Erb binding was detected approximately 5kb upstream of the *Mmp9* transcription start site (Fig 8A). We predicted that, as a repressor, Rev-Erbs should negatively regulate expression of *Mmp9*. To test this hypothesis, we used loss of function and gain of function approaches to examine the consequence of *Mmp9* expression. Primary macrophages differentiated from bone marrows of *Tie2-Cre; Rev-Erb*$\alpha^{flox/flox}$*; Rev-Erb*$\beta^{flox/flox}$ animals (Rev-Erb DKO) and Cre-negative littermates (WT) were generated. *Tie2* is expressed in hematopoietic stem cells [76] so macrophage differentiated from bone marrow of Rev-Erb DKO mice have the majority of Rev-Erbα and Rev-Erbβ alleles excised. In Rev-Erb DKO macrophages, *Mmp9* mRNA expression is de-repressed (Fig 9A); in RAW264.7 macrophage cell lines stably expressing either Rev-Erbα or Rev-Erbβ, *Mmp9* expression is repressed dose dependently to the expression of Rev-Erbs (Fig 9C, E) (r^2 values = -0.39 and -0.36, respectively). We observed a similar functional relationship for *Cx3cr1*, where Rev-Erbs strongly bind at 9kb and 28kb downstream of its transcription start site (Fig 8B, 9B, 9D, 9F).

Rev-Erbs regulate *Mmp9* and *Cx3cr1* by binding only to promoter-distal enhancer elements. We therefore predicted that these distal elements alone are sufficient for gene regulation. Approximately one kilobase (kb) of Rev-Erb-bound region in *Mmp9* was cloned downstream of a luciferase reporter driven by a TATA-like heterologous promoter. The presence of this site, but not a sequence

devoid of Rev-Erbs binding, rendered the reporter gene sensitive to Rev-Erb repression (Fig 10). Like Rev-Erb, members of the ROR nuclear receptor subfamily also recognize Rev-Erb responsive elements; yet RORs activate gene expression [77]. Cotransfection of RORα increases reporter activity in the presence of Rev-Erb-bound Mmp9 distal element. Moreover, this could be antagonized by co-expressing Rev-Erb but not by a Rev-Erb with a mutation in its DNA binding domain (DBD) (Fig 11). We performed similar experiments on six other Rev-Erb-bound distal regions, all of which are sensitive to RORα activation. Four of the six could be antagonized by Rev-Erb co-transfection. These results indicate that Rev-Erb-bound distal elements are sufficient for Rev-Erb-mediated transcriptional regulation.

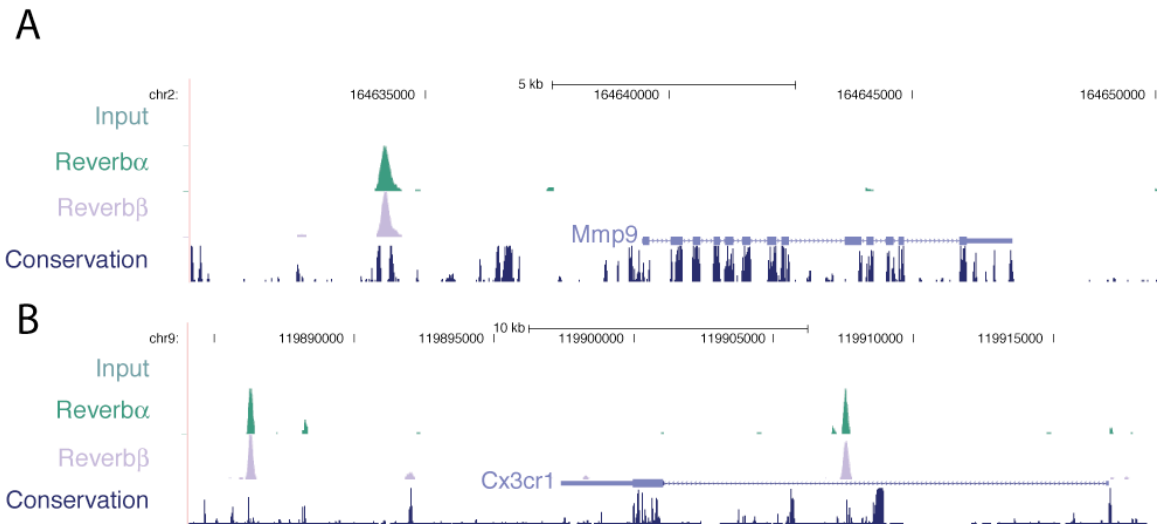


Figure 8. Rev-Erb binds to distal-promoter regions of *Mmp9* and *Cx3cr1* genomic loci. A) At the Mmp9 genomic locus, Rev-Erbα (middle track) and Rev-Erbβ (bottom track) bind to an element 5kb upstream of the transcriptional start sites. B) At *Cx3cr1*, Rev-Erbs bind 9kb and 28kb downstream of *Cx3cr1* transcription start sites.

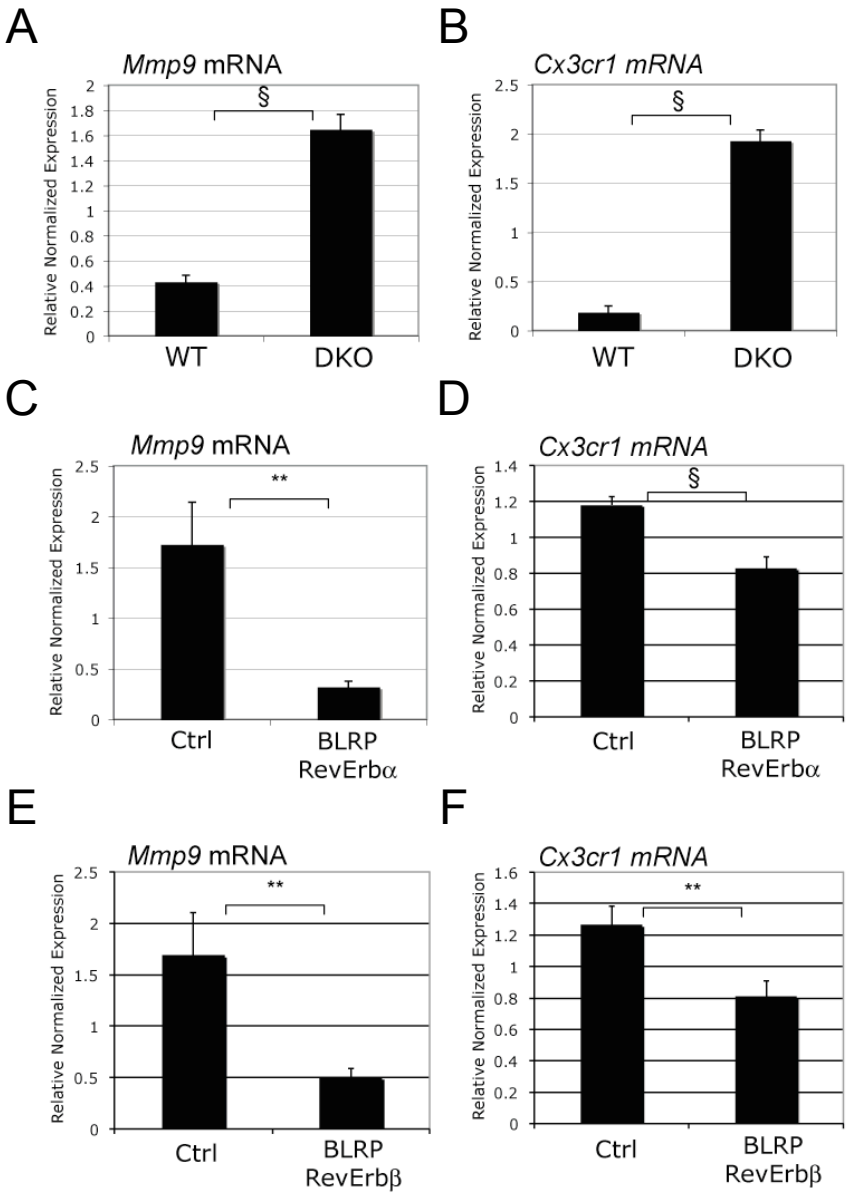


Figure 9. Rev-Erb negatively regulates expression of *Mmp9* and *Cx3cr1*. *Mmp9* (A) and *Cx3cr1* (B) expression are de-repressed in Rev-Erbα and Rev-Erbβ deficient macrophages (DKO). N = 8 and 7 for wild type and DKO respectively. (C-F) Stable expression of either Rev-Erbα (C-D) or Rev-Erbβ (E-F) in RAW264.7 macrophage cell line suppresses *Mmp9* and *Cx3cr1* expression. At least 10 clones from each line were selected for this experiment. Data represent mean ± S.E.M. Two-tails Student's t-test, p-value $^{**} < 0.01$, $^{\S} < 0.005$.

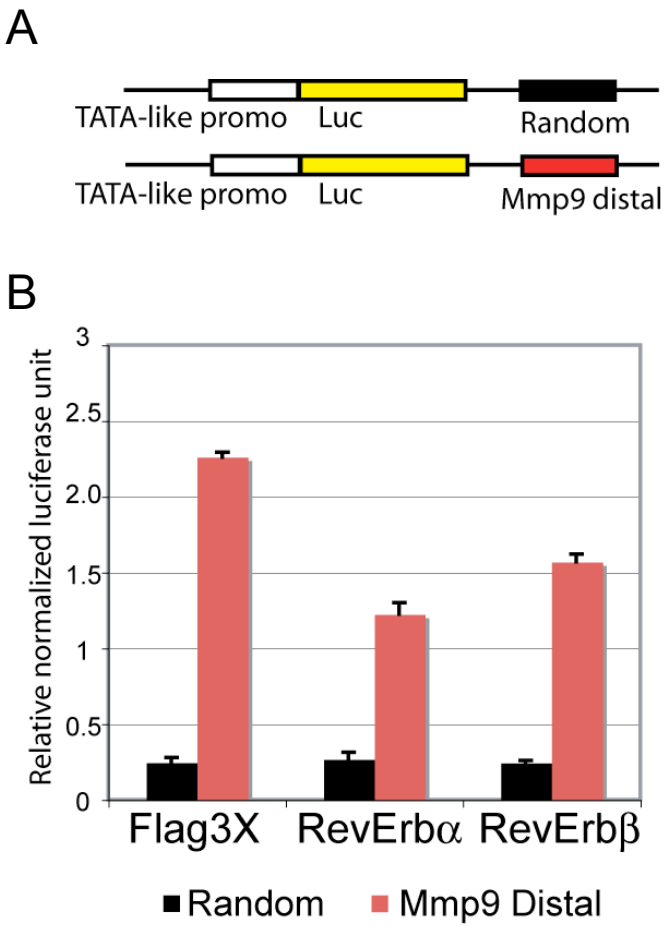


Figure 10. Rev-Erb bound Mmp9 distal element is transcriptionally active and is sufficient for Rev-Erb mediated repression in a transient reporter system.
A) Transient reporter design. One kb of Rev-Erb-bound *Mmp9* distal element was cloned downstream of the luciferase reporter gene (*Luc)* at BamHI and SalI sites in the pGL4.10 plasmid. The reporter gene is driven by a TATA-like promoter. For negative control, a genomic region with similar size and GC content, but devoid of Rev-Erb binding, was cloned into the BamHI and SalI site.
B) The presence of the Mmp9 distal site (red), but not the random genomic region (black), is sufficient to induce luciferase reporter activity. Furthermore, co-transfection of either Rev-Erbα or Rev-Erbβ suppresses luciferase activity.

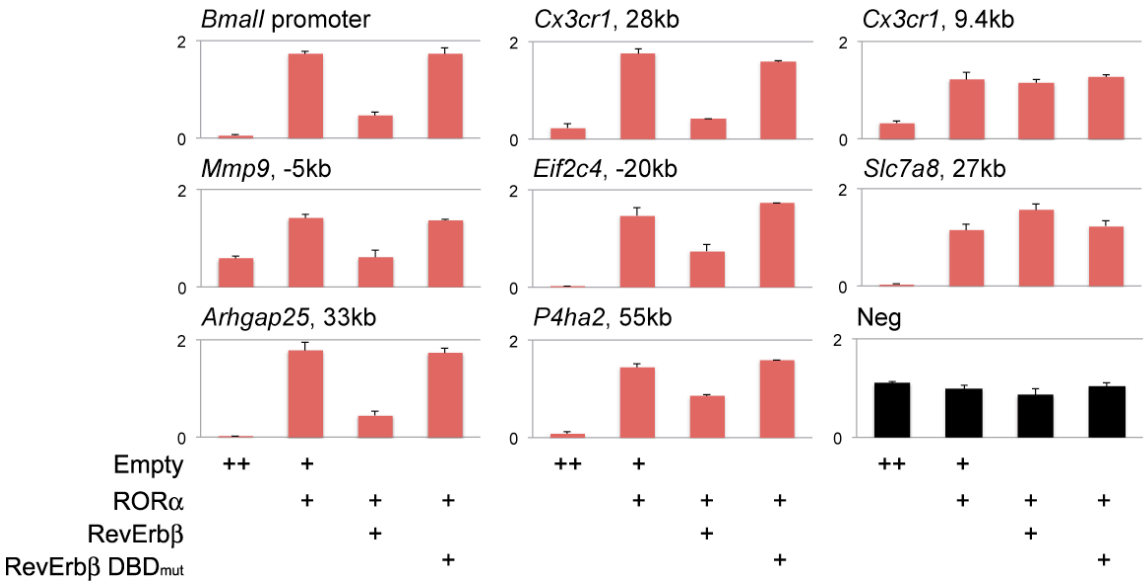


Figure 11. Rev-Erb bound DNA elements are sufficient for Rev-Erb/RORα-mediated transcriptional regulation. Seven Rev-Erb-bound distal DNA elements were cloned into a luciferase reporter as described in figure 9. All seven sites are responsive to RORα induced activation in transient reporter assay. Five sites are sensitive to repression by Rev-Erbβ. Mutation in DNA binding domain abolishes Rev-Erb-mediated repression. For positive control, *bmall* promoter was used; a random genomic region was used as a negative control for enhancer. Data represent mean ± S.E.M from 2-3 independent experiments.

iv. Rev-Erbs regulate enhancer associated RNA expression

We began looking globally at Rev-Erb-bound sites distal to promoters. Beside the preferential localization in H3K4me1^{hi} H3K4me3^{lo} regions, we were surprised to see co-enrichment of the initiated form of RNA Polymerase II (RNAPII with serine 5 phosphorylation) at Rev-Erb-bound sites (Fig 12), prompting us to ask whether RNA are transcribed at these regions. Using primary macrophages we performed global run on sequencing (GROseq), a method to detect transcription of nascent RNA [78]. We detected RNA expression at Rev-Erb-bound -5kb and 28kb distal regulatory elements of *Mmp9* and *Cx3cr1* respectively (Fig 13). Transcription occurs in both directions from Rev-Erb binding sites, with preferential expression in the sense strand in relation to target genes' transcribing direction. Rev-Erbs also bind to *Cx3cr1*'s intron at approximately 9kb downstream of its transcription start site. RNA expression of this distal regulatory element is evident by detection of an antisense transcript. In fact, several antisense transcript "islands" are detected in *Cx3cr1* coding region, and they coincide with binding of PU.1, p300, and DNAse-hypersensitive region [79, 80].

Recent studies have shown RNA transcription at enhancers, and their expressions change upon signal transduction events that correlate to expression of nearby genes [54, 55]. We are interested to test whether Rev-Erbs regulate expression of these transcripts. In Rev-Erb DKO macrophages RNA transcripts at distal elements of *Mmp9* and *Cx3cr1* are derepressed as evident by GROseq

(Fig 13). This is further reconfirmed by RT-PCR at -5kb and 28kb of *Mmp9* and *Cx3cr1* respectively (Fig 14A-B). Consistently, stable expression of Rev-Erbα in RAW264.7 cells is sufficient to decrease expression of enhancer-associated RNAs (Fig 14C-D). We concluded that Rev-Erbs negatively regulate enhancer-associated transcripts of *Mmp9* and *Cx3cr1*.

We further examine the general effect of Rev-Erb regulation on RNA expression at promoter-distal enhancer elements of macrophages. Among 2,079 common Rev-Erb binding sites, 1388 loci are distal to annotated transcription start sites and are marked with enhancer histone signature H3K4me1^{hi} and H3K4me3^{lo}. To further minimize confounding RNA signals from gene-coding regions, distal regulatory elements in introns, exons, and UTRs were excluded. 721 sites were thus identified; 73% of which had detectable RNA expression (defined by detecting at least 6 unique tags within a 1.6kb window). Histogram analysis shows RNA transcripts extending bidirectionally from the center of Rev-Erb-bound regions, with maximum RNA signal at around 300bp on either strand (Fig 15). Expression of these RNAs is not arbitrary - no significant RNA was detected in Rev-Erbα-bound intergenic sites defined in liver [72], suggesting that RNA expression at distal regulatory elements is cell-type specific.

Overall, Rev-Erb DKO macrophage shows on average higher RNA expression at Rev-Erb-bound intergenic regions compared to control macrophages (Fig 16). De-repression is even more pronounced at sites with highest Rev-Erb enrichments (1st quartile Rev-Erb sites based on binding enrichment), suggesting Rev-Erb is the predominant factor repressing RNA

expression at those sites. On the population level, 58% of intergenic enhancers have increased RNA expression (p-value 0.00024, paired Student's t-test).] As an internal control, we examined the changes of RNA expression at H3K4me1^{hi}, H3K4me3^{lo} intergenic sites bound by PU.1 (n = 5587). No significant change was detected (p-value = 0.61, pairwise Student's t-test).

We further explore whether RNA expression is correlate between distal regulatory elements and protein coding genes. We assigned each Rev-Erb intergenic site to its closest expressing gene. RNA de-repression at enhancer RNA co-varies with RNA de-repression of nearby protein coding gene expression (Fig 17, spearman correlation rho = 0.596, p-value < 0.0005). Given that not all distal regulatory sites exert transcriptional effects on their immediate nearby genes, this high correlation suggests a close link between transcription of promoter-distal regulatory elements and protein-coding genes. *Mmp9* and *Cx3cr1* are two such examples.

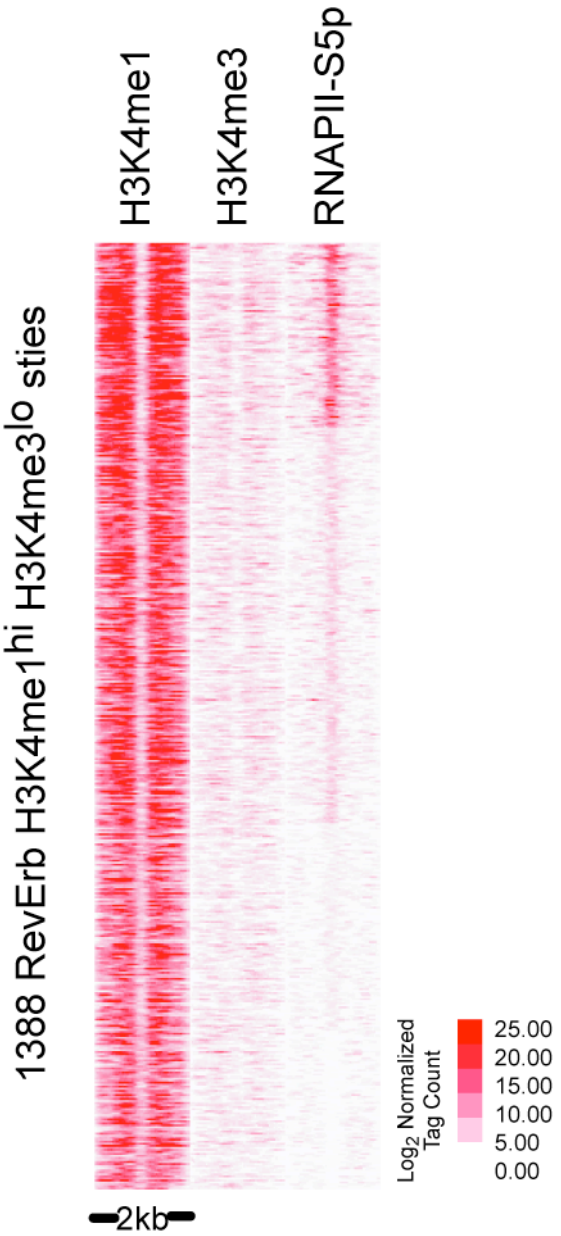


Figure 12. Rev-Erb-bound H3K4me1^{hi} H3K4me3^{lo} regions are enriched for RNAPII-S5p recruitment. Heat map shows ChIPseq of RNAPII-S5p in 1,388 Rev-Erb-bound, H3K4me1^{hi} H3K4me3^{lo} regions.

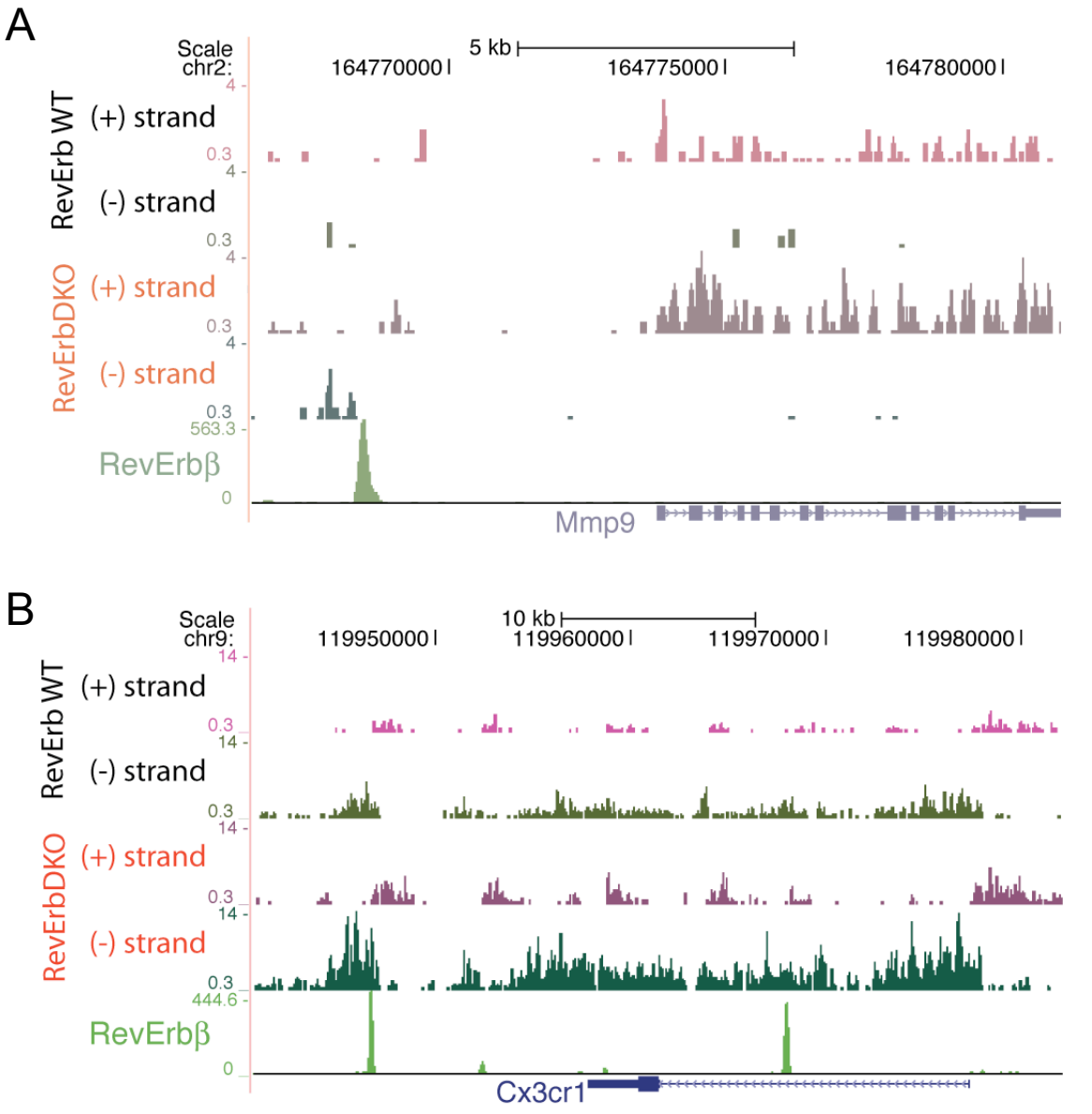


Figure 13. Rev-Erb bound promoter-distal elements in *Mmp9* and *Cx3cr1* are expressing RNA. GROseq shows expression of nascent RNA in *Mmp9* (A) and *Cx3cr1* (B) genomic loci in wildtype control (top 2 tracks) and Rev-Erb DKO macrophages (3rd and 4th track). Bidirectional RNA expressions are evident in RevErb bound -5kb *Mmp9* and 28kb *Cx3cr1* distal regulatory elements. For the RevErb-bound Cx3cr intronic regulatory element, RNA expression is detected from the antisense (plus strand) transcript. Based on GROseq, RNA expressions at these elements are increased in Rev-Erb DKO macrophages compared to controls.

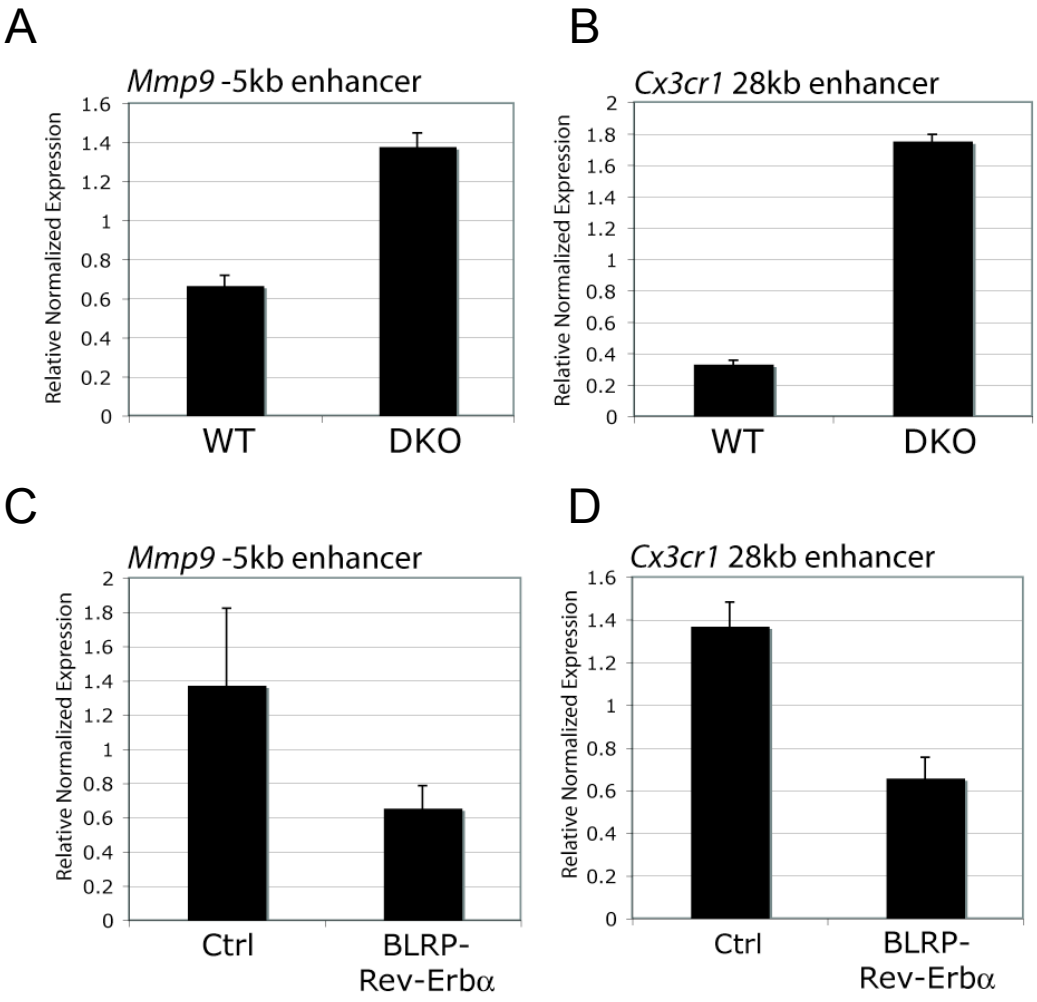


Figure 14. Rev-Erb negatively regulates *Mmp9* and *Cx3cr1* enhancer-associated RNA expression. (A-B) Deficiency of Rev-Erbα and Rev-Erbβ lead to de-repression of *Mmp9* -5kb (A) and *Cx3cr1* +28kb (B) enhancer-RNA (n = 6 and 5 for wild type and DKO, respectively). (C-D) Conversely, stable expression of Rev-Erbα represses these RNAs. At least 10 clones from each line were used for this experiment.

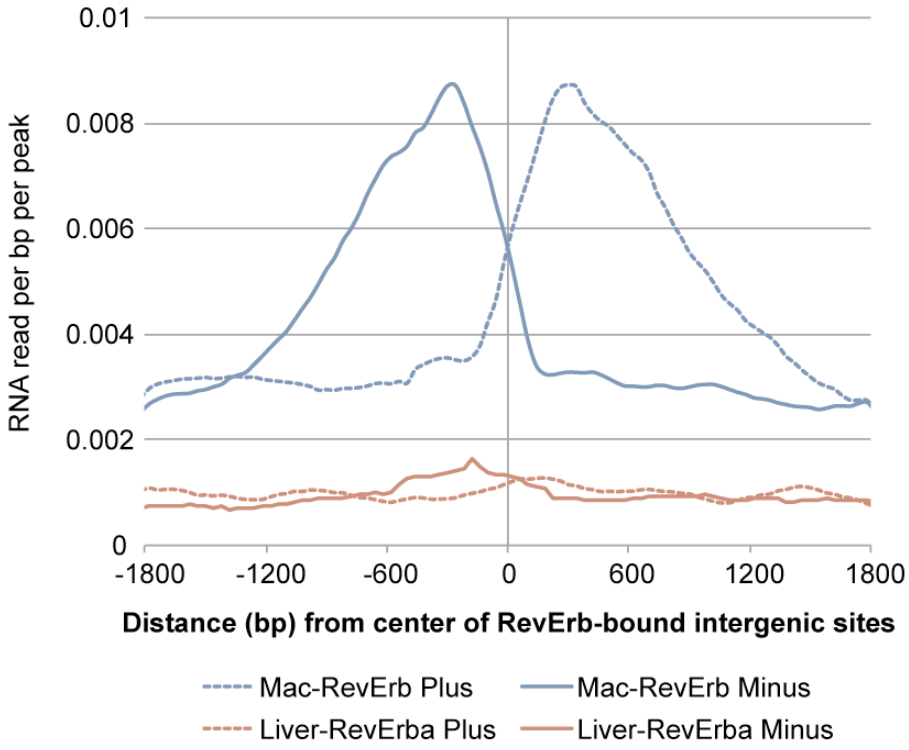


Figure 15. Rev-Erb-bound intergenic sites are expressing RNA. Histogram of nascent RNA expression around 722 Rev-Erb-bound intergenic sites (blue). For control, Rev-Erbα-bound intergenic sites from liver Rev-Erbα-ChIPseq (red) were examined and show absence of RNA signal in macrophage.

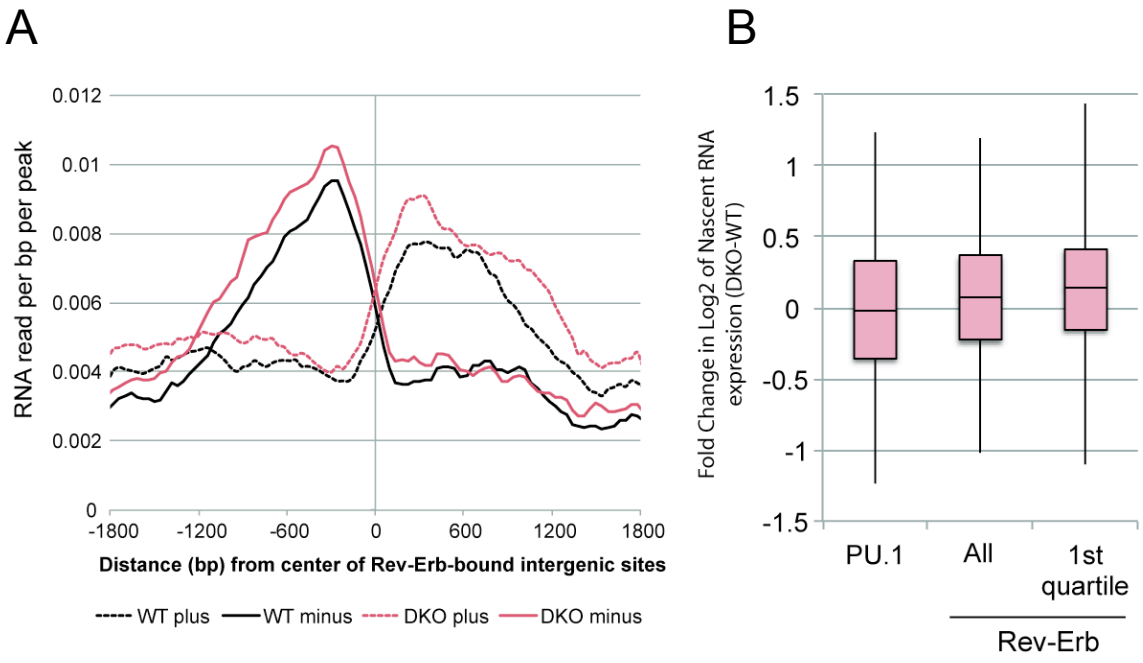


Figure 16. Global de-repression of RNA transcripts at Rev-Erb-bound intergenic enhancers in Rev-Erb deficient macrophages. A) Centering at the 1st quartile Rev-Erb-bound sites, histogram shows increase of nascent RNA expression in DKO (red) compared to WT (black) macrophages. B) Box and whisker plot shows a global de-repression of eRNA expression in all RNA expressing Rev-Erb-bound intergenic sites (n=518) between WT and DKO macrophages. The shift is even more prominent from the first quartile of Rev-Erb binding sites (n = 128). PU.1-bound H3K4me1^{hi} H3K4me3^{lo} regions (n =5587) show no changes. Boxes mark the 1st, 2nd, and 3rd quartile of the distribution. Whiskers represent 2 standard deviations from the population mean.

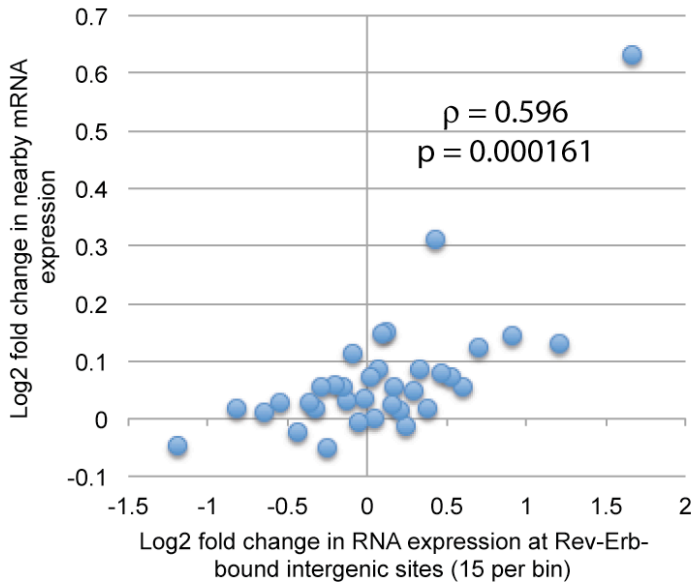


Figure 17. Changes in eRNA levels at Rev-Erb-bound intergenic sites is positively correlated to changes in expression of nearby protein-coding genes in WT versus Rev-Erb deficient macrophages. Each dot represents fold-change average of RNA level at fifteen Rev-Erb-bound intergenic sites and their corresponding nearest protein-coding genes. Spearman correlation coefficient, or r, is 0.596 with p-value = 0.000161.

v. Rev-Erb regulated PolII recruitment at distal regulatory elements

We next questioned whether Rev-Erbs regulate RNAPII recruitment at distal elements in order to mediate repression of enhancer-associated RNA. We examined and detected RNAPII enrichment by ChIP at *Cx3cr1* 28kb distal region. RNAPII recruitment was negatively regulated by Rev-Erbs in DKO macrophages. Enrichment for total RNAPII, Ser5-phospholyated RNAPII, but not Ser2-phosphorylated RNAPII, was increased (Fig 18A-C). In contrast, when Rev-Erbα was overexpressed, both total and Ser5-phosphorylated RNAPII is decreased (Fig 18D-E). Consistent with this, acetylation of H3K9, a histone mark associated with transcriptional activation, is negatively regulated by Rev-Erb at *Cx3cr1* 28kb distal enhancer element (Fig 19). This is consistent with Rev-Erb's interaction with HDAC3/NCoR complex repressing genes expression at gene promoters [15].

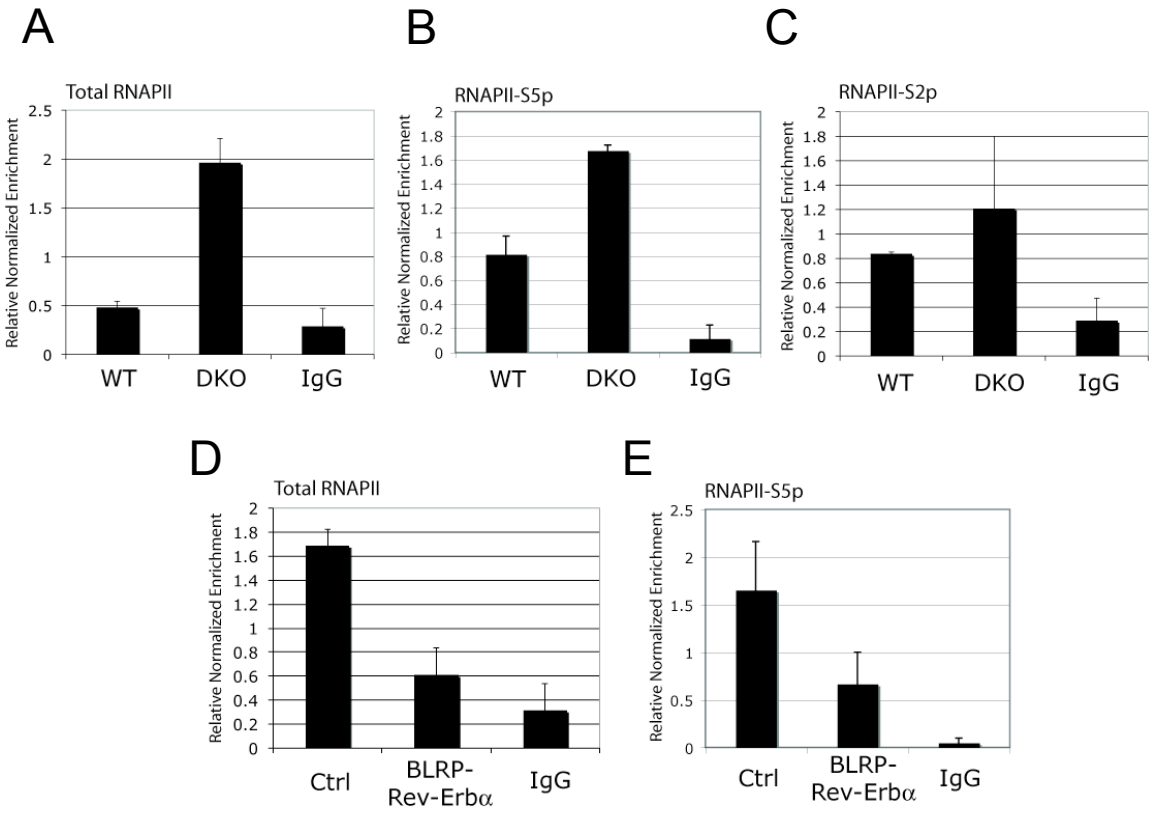


Figure 18. Rev-Erb inhibits RNAPII recruitment at a distal regulatory element of *Cx3cr1*, 28kb downstream of its transcription start site. Recruitment of total, serine-5-phospho (S5p), and serine-2-phospho (S2p) RNAPII were determined by ChIP followed by qPCR. Deficiency of Rev-Erb (DKO) leads to higher enrichment of total (A) and S5p (B), but not S2p (C) RNAPII. Conversely, stable expression of Rev-Erbα in RAW264.7 macrophages decreases total (D) and S5p (E) RNAPII enrichment.

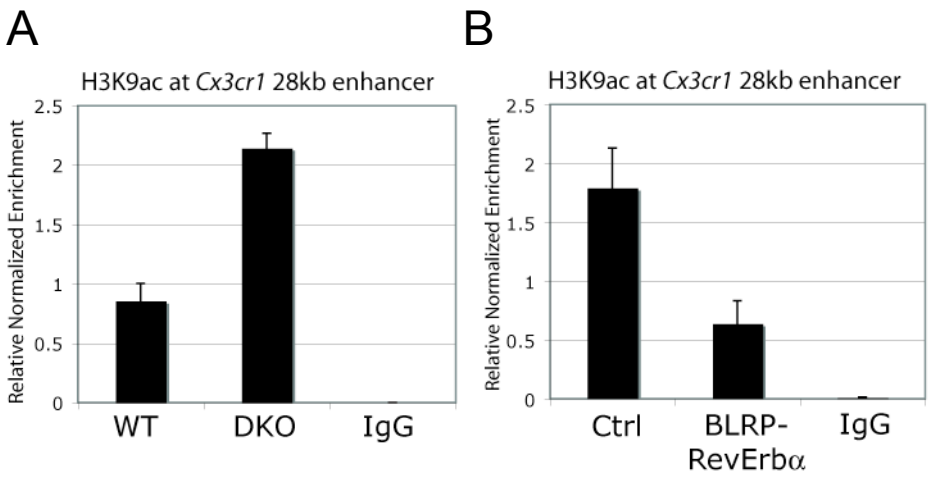


Figure 19. Rev-Erb promotes deacetylation of H3K9ac at *Cx3cr1*'s 28kb distal regulatory element. Enrichment of H3K9ac was determined by ChIP followed by qPCR. A) Rev-Erb deficiency leads to higher enrichment of H3K9ac. (n = 3 for WT and DKO macrophages). B) Stable expression of Rev-Erba in macrophages decreases H3K9ac enrichment. Three different clones (n = 3) for control and BLRP-Rev-Erba were used in this experiment.

vi. Targeting enhancer-associated transcripts is sufficient to modulate target gene expression.

Thus far we have shown that in macrophages, Rev-Erbs bind predominantly at distal regulatory elements. In addition, enhancer-associated RNA and target mRNA expressions are both negatively regulated by Rev-Erbs (Fig 17). This prompted us to ask whether the relationship of enhancer RNA and mRNA is casual or causal. First we determine the temporal relationship between eRNA and mRNA. If eRNA is required for mRNA expression, then induction of eRNA should precede that of mRNA. *Mmp9* expression is under circadian regulation in macrophages [73]. We examine circadian expression of *Mmp9* mRNA and eRNA from splenic $CD11b^+$ monocytes of wildtype mice every four hours for twenty-four hours. Rhythmic expression of *Rev-Erb*α and *Rev-Erb*β are antiphasic to *bmall* expression, suggesting we are detecting circadian expression from splenic monocytes (Fig 20A). *Mmp9* mRNA, eRNA, and primary transcript expression are also rhythmic (Fig 20B). More importantly, *Mmp9* eRNA's circadian peak precedes *Mmp9* mRNA and primary RNA by about four hours. This temporal relationship suggests that *Mmp9* eRNA could be functional.

To further directly address the function of eRNA, we used RNA interference (RNAi) to target enhancer RNA transcripts of *Mmp9*. Four siRNA oligos (A-D) were designed to target the 5kb upstream distal element of Mmp9 where RNA expression is detected (Fig 21A). In addition, in order to minimize potential binding interferences with transcription factors at the *Mmp9* distal enhancer, these siRNA oligos were designed to avoid AP-1, RevErb DR2, and

the two PU.1 motifs. The two closest siRNA oligos are 564bp and 207bp away from AP-1 and PU.1 respectively (Fig 21B). In wild type thioglycolate-ellicited peritoneal macrophages, transfection of siRNA oligo B and C, but not A, D or the scramble control, consistently reduces expression of *Mmp9* mature and primary mRNA (Fig 22A-B). We then hypothesized that, if *Mmp9* eRNA exerts transcriptional regulatory effects, and its level is increased in Rev-Erb-deficient macrophages, then targeting eRNA should reverse the *Mmp9* de-repression phenotype in Rev-Erb DKO macrophages. Transfection of siRNA oligo B and C in Rev-Erb DKO macrophages, but not the scramble control, was sufficient to reduce *Mmp9* mRNA to level similar to wild-type control under the same transfection conditions (Fig 22A-B). In addition, RNAi decreased *Mmp9* eRNA in a strand specific fashion -- siRNA oligo B and C were designed to target the sense-strand of the eRNA (Fig 22C). The antisense eRNA was not significantly affected (Fig 22D). This suggests that phenotype reversal was due to reducing eRNA expression without affecting transcriptional initiation at *Mmp9* enhancer. These data demonstrated that enhancer RNA from *Mmp9* is functional, and de-repression of *Mmp9* expression by Rev-Erb deficiency is partly due to the de-repression of eRNA expression.

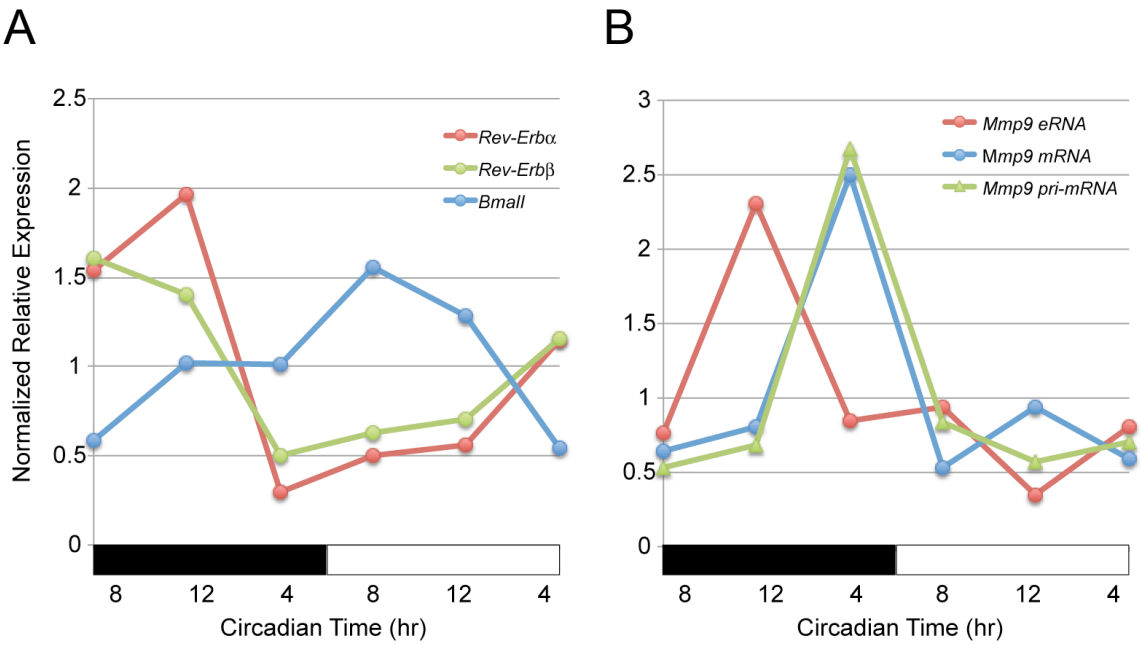


Figure 20. The circadian phase of *Mmp9* eRNA precedes mRNA expression. Monocytes were isolated from spleens of C57Bl/6 mice by sorting using CD11b antibody at 4am, 8am, 11.5am, 3pm, 7pm, and 11pm. Two animals were used for each time point. A) mRNA expression of *Rev-Erbα* (red), *Rev-Erbβ* (green), and *bmall* (blue) are phasic. B) Expression of *Mmp9* eRNA (red), primary (green) and mature mRNA (blue) are also phasic. The peak expression of eRNA precedes that of the primary and mature *Mmp9* mRNA by approximately 4 hours.

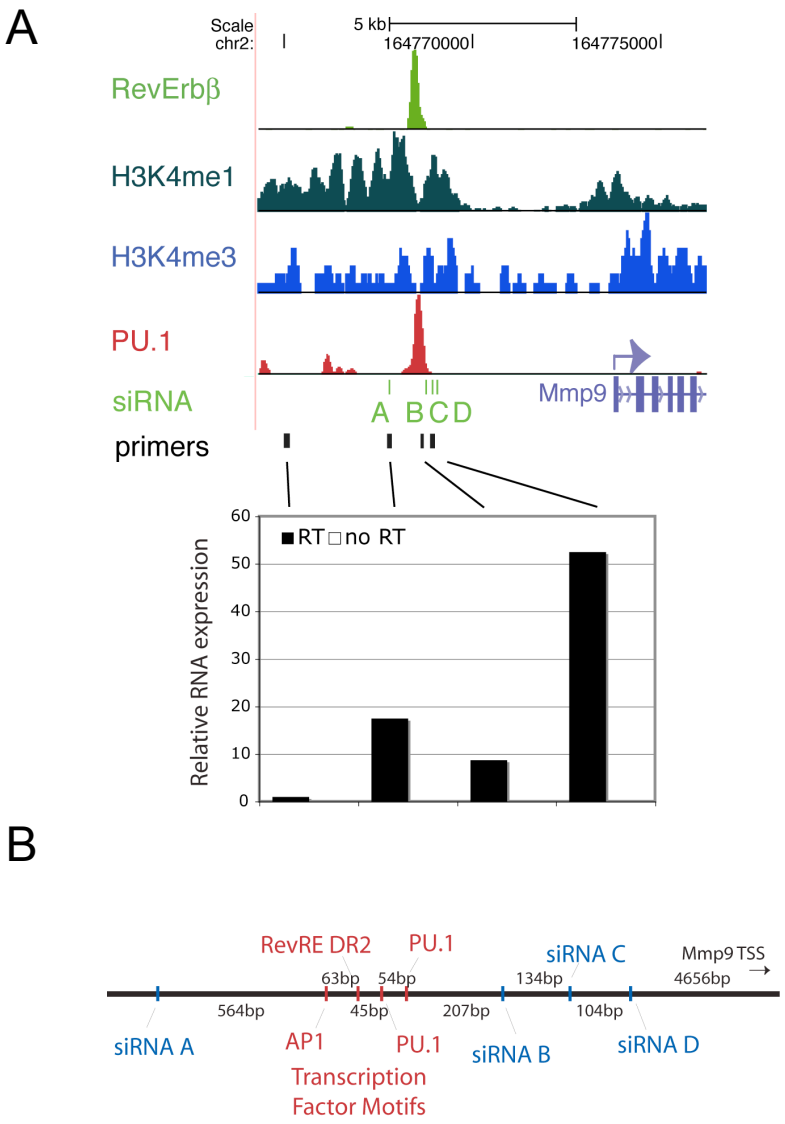


Figure 21. Experimental scheme for testing eRNA function using RNAi. A) siRNA oligos A-D were designed to target eRNA expressed from the Rev-Erb/PU.1-bound *Mmp9* enhancer B) Schematic of Mmp9 enhancer showing locations of siRNA targeting sequence with respect to AP1, PU.1, and Rev-Erb transcription factor motifs. siRNA A targets the distal side of the transcription factor binding elements with respect to the Mmp9's transcription start sites. siRNAs B, C, D target the proximal side.

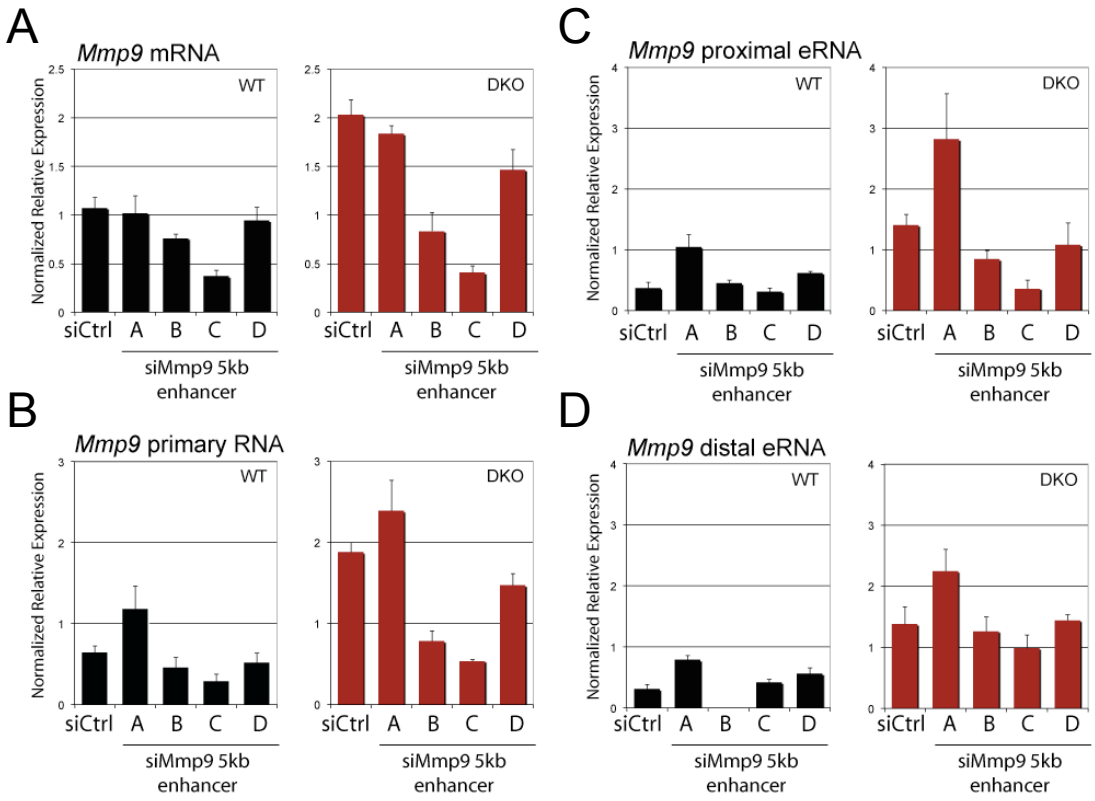


Figure 22. RNAi targeting of *Mmp9* enhancer transcript is sufficient to decrease *Mmp9* mRNA expression and to reverse Rev-Erb DKO de-repression phenotype. A) Transfection of siRNA oligo B and C, but not the scramble control, decreases *Mmp9* mature (A) and primary (B) mRNA expression in wildtype macrophages (black). Furthermore, transfection of siRNA B or C is sufficient to reverse *Mmp9* mRNA level in Rev-Erb DKO macrophage (red) back to the level of wild type macrophages under the same conditions. C) Transfection of siRNA oligo B and C targets and decreases the proximal enhancer RNA transcripts in Rev-Erb DKO macrophages as expected. However, eRNA distal to Mmp9 TSS (see figure 21's schematic) is not affected by transfections of siRNA oligos. Data values represent mean $\pm$ SEM. At least 3 independent experiments were performed.

C. Discussion

We present here a study of Rev-Erb nuclear receptors in macrophages. We first used an *in vivo* protein biotinylation approach to defined genome-wide localization of both Rev-Erbα and Rev-Erbβ in a macrophage cell line. A major portion of Rev-Erbs bind at least 1kb away from annotated transcription start sites. These Rev-Erb-bound distal regions are co-enriched for binding of the macrophage lineage specifying factor PU.1 and are demarcated with the enhancer histone signature H3K4me1^{hi} H3K4me3^{lo}. Combining genetic approaches, we identified novel Rev-Erb target genes such as *Mmp9* and *Cx3cr1*, genes relevant to physiological and pathological inflammatory process such as wound healing and atherosclerosis. Importantly, these genes are bound by Rev-Erb at promoter-distal elements, which function as transcriptional enhancers when cloned into transient reporter genes. Genes nearby Rev-Erb-bound regions are enriched for functional annotations in wound healing, programmed cell death, and angiogenesis; in liver, Rev-Erbα mainly regulates genes in glucose and lipid metabolism [21, 72]. Indeed, localization of Rev-Erb differs vastly between macrophage and liver (Data not shown). The predominant occupancy of Rev-Erb at distal regulatory elements and the differences in transcriptional role of Rev-Erb in macrophage and liver support the notion that Rev-Erb regulates gene expression from cell type-specific enhancers.

A major finding in this study is that Rev-Erbs regulate active RNA transcriptions at Rev-Erb-bound distal regulatory elements. By detection of

nascent RNA transcripts, we show that RNA expressions at Rev-Erb-bound distal regulatory elements are globally de-repressed in Rev-Erb-deficient macrophages. Consistently, over-expressing Rev-Erbα is sufficient to repress transcription at enhancers of *Mmp9* and *Cx3cr1*. Rev-Erbs have been shown to interact with the NCoR and HDAC3 co-repressor complexes at gene promoters to repress transcription. We demonstrated a similar mechanism of repression at distal elements whereby Rev-Erbs promote deacetylation of H3K9 and inhibit RNAPII recruitment. Thus Rev-Erbs act as repressors of RNA expression at distal regulatory elements. In line with this, analysis of ChIPseq data in liver for HDAC3 and NCoR shows binding of this corepressor complex at Rev-Erb-bound distal regulatory elements in liver [72].

Active transcription at distal enhancer elements has been described during cellular responses to robust stimuli such as membrane depolarization in neurons and inflammatory activation in macrophages [54, 55]. These studies focused on transcriptional activation of enhancer RNA. Increasing RNA expression at distal regulatory elements correlates with increasing enrichment of transcriptional activators, as exemplified in the study by Kim *et al*. Our study supports these findings. It also points to the importance of repression as a mechanism of modulating RNA transcription at enhancers. It further shows how genetic manipulation of a single class of transcription factor is sufficient to affect enhancer-associated RNA transcription. In our case, Rev-Erbs regulate enhancer RNAs at enhancers that contain RevErb binding sites. We have previously shown the cistrome of Liver X Receptor in macrophages using the

same biotinylation approach. LXR and Rev-Erb binds to their respective unique set of promoter-distal regulatory elements. We thus speculate that each transcription factor regulates its own set of enhancer based on where they are bound within a given cell type.

Our third major finding suggests enhancer associated RNAs are functional. First, our GROseq experiment shows a significant covariation between de-repression of eRNA with de-repression of nearby protein coding genes. Second, both *Mmp9* eRNA and mRNA are rhythmically expressed in a circadian fashion; the peak of *Mmp9* eRNA temporally precedes mRNA's. Third, RNAi targeting *Mmp9* enhancer transcript was sufficient to reduce *Mmp9* mRNA expression. More so, targeting enhancer RNA was sufficient to reverse Rev-Erb deficiency phenotype. These results suggest a mechanism where Rev-Erbs suppress target gene expression by suppressing enhancer RNA transcription.

Several lines of evidences suggest a functional role for enhancer-associated non-coding RNA as transcription activators. Reports of enhancer-transcribed RNAs date back to as early as 1992, when Tuan *et al* discovered RNA transcripts at the locus control region (LCR) of the beta-globin locus. LCR orchestrates temporal regulation of globin genes throughout development. Cloning of HS2, a DNAseI hypersensitive (HS) region in LCR, is sufficient for enhancer activity in context of a transient reporter. Terminating RNA transcription at HS2 by inserting a transcriptional repressor LacO prevented expression of the reporter gene. Congruously ChIP studies demonstrated RNAPII recruitment to both HS2 enhancer and adult beta-globin promoter.

Inhibition of RNAPII elongation reduces RNAPII enrichment at beta-globin promoter but not at HS2 enhancer [61]. One may speculate how RNA synthesis from HS2 enhancer is required for promoter RNAPII recruitment.

Our work suggests that the sense strand of *Mmp9* eRNA acts as an activator of mRNA expression; yet we do not know how it works mechanistically. Recent studies on other classes of non-coding RNA may shed mechanistic insights. The ultraconserved long intergenic non-coding RNAs (linc-RNA) serve as beacons for transcriptional complex assembly to regulate gene expression in *cis* [63-66, 81, 82]. Similarly a group of short RNAs expressed at promoters of poised genes recruit components of the Polycomb complex. The loss of these RNAs upon cellular responses leads to dissociation of the PcG complex, allowing rapid induction of these poised genes [83]. Future studies should investigate whether eRNAs could bind and modulate transcriptional function of coregulatory complexes.

Collectively, we would like to propose a model where Rev-Erb represses the expression of mRNA target genes by negatively regulating transcriptional activity of enhancers.

Chapter 2, Lam M.T., Cho H., Tanaka-Oishi Y., Heinz S., Benner C., Kaikkonen M., Kosaka M, Lee C., Evans R., Glass C.K., “Rev-Erb regulates RNA Transcription at Distal Regulatory Elements of Macrophages.” will eventually be submitted for publication in a much revised form.

Chapter 3 The Post-Genome Revolution and Transcriptional Regulation

We are experiencing a revolution in genetics. It first began with the uncovering of DNA as the genetic blueprint of life. DNA is why Mendel's defining of genes as the carrier of hereditary traits, its functional identification by Avery-Macleod-McCarty as the genetic material, and its structural elucidation by Watson-Crick as the basis of precision and invariance in replication and transcription. The certainty and significance of the natural selection theory, put forth in Darwin's 1859 *On the Origin of Species*, was established only by those later discoveries. Within a century, genetics was born. The fundamental biological invariant is DNA. But if the components are the same and are synthesized by the same process in all living beings, then what is the source of their physiological diversity? Jacob and Monod's work on the *Lac* operon spawned the second phase of the genetic revolution. It showed that the genome is very much "alive" – it responses to environment through an intricate series of positive and negative feedback loops with its intermediate and final products. Later work further expanded and refined the complexity and versatility of gene regulation. The genome is not simply a code for protein; it dictates when and where genes should be transcribed. Not until the initiation of the Human Genome Project and the subsequent advances in sequencing technology did we become capable of "seeing" and appreciating how widespread transcriptional

regulation is a part of the genome. We can predict – with unprecedented confidence – functional regulatory elements using cross-species conservation, epigenomic marks and open chromatin mapping. In many regards, this is similar to the finding of genetic codons for translation; the non-coding genomic DNA is no longer “junk” as we know it.

We are about able to dive into gene regulation and appreciate its wide-spread significance in human physiology. In the past half-century, our mechanistic and physiological insights on transcriptional regulation generally derived from a protein-centric approach. We studied the transcriptional consequences by genetic or molecular manipulation of transcription factors. More recently, attention has been placed on control of epigenetics, including histone modifications and DNA methylation. What is less understood, primarily due to detection limitation, is how DNA sequences, which constitute functional regulatory elements, affect transcriptional regulation in physiology. A single class of transcription factor is capable of binding to a set of distinct but similar sequences collectively termed “motifs.” Mechanistically, changes in motif sequences can dictate binding affinity of the transcription factor. Moreover, motif sequence exerts allosteric effect on the bound transcriptional complex; single base pair change can alter how the transcription factors interact with other regulators [84]. Before genomic scale sequencing, identifying functional regulatory elements were restricted to promoter regions. It was like finding a needle in a haystack. We now know approximately 10% of the genome corresponds to putative regulatory elements, whereas only 2-3% are protein

coding. Yet our understanding of functional consequences and causality of regulatory elements is very rudimentary. Advances in sequencing technology, both in extending throughput capacity and reducing cost, permit the use of human population genetics and genomics to question functional aspect of the genome in an unbiased fashion. The essence is forward genetics, using human phenotypes to discover causal genotypes. Massive efforts have been taken to map genetic markers, primarily using single base-pair DNA variants known as single nucleotide polymorphisms (SNPs), to represent co-segregating units (i.e. haplotype blocks) at genomic scales. The idea is to localize regions of causal variants by testing associations between the frequencies of the genotypes in affected versus unaffected individuals in a population. Collectively these are known as genome wide association studies (GWAS). The agnostic nature of forward genetics often leads to discovery of new and unexpected pathways. Furthermore, detection of disease-associated variants is not restricted to the nature of the sequence (e.g. protein coding vs. regulatory element). Currently, GWAS revealed more than 200 loci associated with at least 50 diseases that could be replicated across different populations.

Many associations implicate non-protein coding regions. Approximately 5% of the genome is under evolutionary conservation, a scale significantly higher than the number of protein-coding genes [85]. Many disease-associated SNPs locate sufficiently far enough from any protein-coding regions, pointing to regulatory elements as culprits. Human chromosome 9p21 is a good example. A 58kb non-protein coding region located at least 150kb from the nearest genes,

9p21 is in high association with coronary arterial diseases (CAD) and Type 2 Diabetes (T2D). To show function, deletion of an orthologous 70kb region in the mouse genome showed down-regulated expression of its nearest genes, the cell cycle arrest proteins *Cdkn2a* and *Cdkn2b*. Primary cultured aortic smooth muscle cells from mice homozygous for deletion of this locus showed hyperproliferation and diminished senescence, a cellular phenotype consistent with CAD pathogenesis [40]. In humans, systematic functional annotation with epigenomic marks showed a cohort of general and tissue-specific enhancer elements in 9p21. One risk allele resulted in mutation of a STAT1 recognition sequence in a cell-type specific enhancer of 9p21. Consequently, disrupted STAT1 binding altered transcriptional responses to interferon signaling in a cell specific fashion [86]. Other studies revealed similar phenomena. A SNP in 1p13, which created a C/EBP site increasing expression of SORTILIN in a liver-specific fashion, strongly associated to plasma-low-density lipoprotein (LDL-C) and myocardial infarction. Genetic manipulation of Sortilin in mice was sufficient to recapitulate plasmid lipoprotein level as observed in human [87]. Characterization of pancreatic islet cells for open chromatin and putative enhancer marks revealed known T2D-associated SNP that affect gene expression of the pancreas [88].

Identification of causal variants in regulatory elements could reveal novel insights about mechanisms of diseases. First of all, in the cases given above, disease-association variants were found in promoter-distal enhancer regulatory elements. Enhancers are often cell-type specific and may thus explain the

tissue- and disease-specific nature of common susceptibility alleles. It also suggests the likely cell types contributory to the diseases. Secondly, regulatory elements on a global scale, also termed cistromes, could undergo dynamic changes upon different environmental or cellular contexts. Finding causal variants in "context"-specific regulatory elements inspire new testable ideas on how transcriptional regulation may partake in pathogenesis of diseases. Furthermore, localizing the causal variants reveals motif sequence(s) and thus implicates candidate transcription factor(s) in play. The nature of the variants – whether it be single nucleotide polymorphism, copy number variant, insertion, deletion or inversion – suggests how transcription could be altered. Insertion of repeats creates a concatemer, for example. The increased number of binding sites will likely change regional transcriptional output. A SNP can create a new binding site, much like the case of 1p13 that changes regulation of SORTILIN [87].

Functional mapping of the human genome will have immediate impact on understanding of GWAS. The launching of a public research consortium ENCODE, **Enc**yclopedia **O**f **D**NA **E**lements, began the effort of annotation functional part of the genome by characterizing DNAseI hypersensitive regions, transcription factor binding, and comprehensive histone modifications. This enabled investigators to identify whether disease-associated variants are located in regulatory elements and can be quickly tested for functionality and possible causality. Yet most SNPs were selected based on recombination and segregation pattern of the genome. It should be noted that SNPs may not be

functional and act only as proxies to other highly linked DNA variants. Early GWAS were designed for localizing genomic regions of query at a resolution of 10-100kb; deeper sequencing was intended for identifying causal DNA variant(s). The search for causal variants can therefore be strategically focused on functionally annotated regions, regardless if they are protein-coding or regulatory elements. The initial goal of ENCODE focuses on functional mapping in seven commonly used cell lines, in part for practical reasons (i.e. ease of large-scale culturing). Some are transformed cell lines, which may not necessarily be physiologically or pathologically representative. More investigators are now mapping regulatory regions in cell types relevant to their research interests. Availability of these data will be invaluable for diseases involving tissue- or cell-types not represented in the public domain. Finally, unlike the readout of DNA sequences, chromatin structure, epigenetic modifications, and transcription factor localization are highly dynamic. Functional mapping should also encompass various physiological and pathological conditions to comprehensively capture "context" specific regulatory elements.

With identifying disease-associated regulatory elements and their variants, we can now generate hypotheses and devise experiments to test mechanisms and functionality of these elements. First, modification or deletion of the disease-associated regulatory element could be made in animal or cell models for functional testing. Second, physical interactions between the disease-associated regulatory elements with other genomic regions, which are suggestive of direct regulation, should be identified by genome-wide chromatin conformation capture-

based method (i.e. 4C, ChIA-PET). The third and the simplest of all three, is to study the association between genetic variation and gene expression to connect risk variants to their putative target genes or transcripts. Quantitative expression profiling could be done using direct RNA sequencing. Among the many advantages it offers over the hybridization-based microarray, two are especially important. First since the counting of each molecule is done by sequencing, it is possible to discern allelic-specific expression when profiling heterozygous individuals. This becomes a powerful asset to clue in whether the regulatory element(s) is exerting function specifically upon the same strand of DNA. Second, sequencing is not limited to known annotated regions (e.g. protein-coding genes). Hence we can examine expression of all RNAs, including miRNA, long non-coding RNA, and snoRNA, which are all biologically active. RNA transcriptions are also detected at enhancer regulatory elements [54-57]. Through our work and others, these enhancer-associated RNA (eRNA) may directly indicate activity levels of enhancers [57]. We can thus assess the consequential effect of the DNA variants on enhancer functions. DNA variants that influence expression of specific transcripts are referred to as expression quantitative trait loci (eQTLs), which explain a greater proportion of trait variance than is typically seen for risk alleles and clinical traits [89]. We can now incorporate the expression of these non-protein-coding transcripts as molecular phenotyping, further augmenting and improving clinical-trait assessment.

Disease owing to variance in gene regulation suggests different therapeutic strategies. Modulating levels of gene expression may thus prove

more yielding than replacing a defective protein or turning off a gain-of-function allele. Pharmacological reagents could be targeted at different mechanistic steps – 1) the transcription factor(s) exerting function at the regulatory element(s) in query, 2) dysregulated target gene(s) affected by the "casual" regulatory elements, and 3) transcriptional intermediates, such as eRNAs, that modulate enhancer functions. Simultaneous targeting at multiple or all these steps will likely yield synergistic effects, thus reducing the dosages needed for each medication to reach therapeutic potential while minimizing unwanted side-effects due to high dosage from any single reagents. Since we could infer cell-type specificity, delivery methods could also be strategized to increase therapeutic efficiency.

We are in the midst of the second phase of genetic revolution. The goal is to understand the interconnecting regulatory circuits of transcription, which, according to Francois Jacob in *Logic of Life,* "give living systems both their unity and the means of conforming to the laws of thermodynamics." We are witnessing how this has slowly changed the way we understand and approach human diseases. The acquired knowledge will also propel the next revolution frontier – application of transcriptional regulation. In many regards, this stage has already begun. In stem cell biology, the ability to induce pluripotency from differentiated cells is in essence controlling transcription. The Yamanaka factors required for induced-pluripotency are transcription factors that reshape the functional landscape of the genome. The same goes true to directing cell fate differentiation. Understanding transcription goes far for fields like tissue

regenerative engineering, where the function of each cell depends heavily on space and time. We are at the right time with the right tools for this endeavor. The human population is arguably the most ideal genetic cross. With carefully designed experiments, human phenotypes and human diseases will spawn a series of exciting discovery leading to the better understanding of human biology.

Materials and Methods

Cloning and plasmid preparation

For expression vectors of Rev-Erbα (amino acid 1-614), Rev-Erbβ (amino acid 1-576), and RORα (amino acid 1-460), DNA fragments encoding these corresponding coding regions were cloned into p3XFlag-CMV7.1 expression construct (Sigma) at the NotI and BamHI sites. To facilitate cloning, NotI (5'-GCGGCCGC-3') and BamHI (5'-GGATCC-3') recognition sites were added to sense and antisense PCR primers respectively. We amplified the fragments using DNA Phusion polymerase (New England Biolabs). The digested fragments were ligated using T4 DNA ligase (Enzymatics). The following primer sequences were used for amplification. Rev-Erbα: forward 5'-AGCTTGCGGCCGCTATGACGACCCTGGACTCC-3', reverse 5'-ATTACGGATCCTCACTGGGCGTCCACCCG-3'; Rev-Erbβ: forward 5'-AGCTTGCGGCCGCTATGGAGCTGAACGCAGGA-3', reverse 5'-ATTACGGATCCTTAAGGATGAACTTTAA-3'; RORα: forward 5'-AGCTTGCGGCCGCTATGAAAGCTCAAATTGAA-3', reverse 5' ACCCGGGATCCTTACCCATCGATTTGCATG-3'. Mutation of the DNA binding domain of Rev-Erbα and Rev-Erbβ was generated using QuickChange II site directed mutagenesis (Stratagene). The cysteines in the zinc finger domain (Rev-Erbα, amino acid 133 and 136; Rev-Erbβ, amino acid 133 and 136) were mutated to alanines using the following oligos. Rev-Erbα: sense 5'-

GGCATGGTGCTACTGGCTAAGGTGGCTGGGGACGTGGCCTC-3' antisense 5'-GAGGCCACGTCCCCAGCCACCTTAGCCAGTAGCACCATGCC-3'; Rev-Erbβ sense 5'-AGTGGCATGGTTCTACTGGCTAAAGTCGCTGGGGATGTGGCATCAGG-3' antisense, 5'-CCTGATGCCACATCCCCAGCGACTTTAGCCAGTAGAACCATGCCACT-3'.

For construction of Rev-Erb enhancer reporter plasmids Primer3 software [90] was used to design primers amplifying 900-1100 bp of sequence centered on Rev-Erb-bound sites flanked by sequences demarcated by H3K4me1, and cloned into the pGL4-TATA-TK at the BamHI/SalI sites downstream of the luciferase reporter gene. The pGL4-TATA-TK is the pGL4.10 luciferase reporter plasmid (Clontech) modified by inserting the minimal TATA-containing thymidine kinase (TK) promoter (-119/+25) from pTAL 34 bp upstream of the luciferase coding region. For positive control, 551bp of *Bmall* promoter was cloned into XhoI and HindIII sites 5' of *luc* in pGL4.10. Clones were screened for insert by restriction enzyme and sequencing analyses. Large cultures of positive clones were prepared using PureLink HiPure Plasmid Maxiprep Kit (Invitrogen).

Rev-Erb DKO animals.

Mice carrying floxed allels of Rev-Erbα and Rev-Erbβ (*Rev-Erb*$\alpha^{flox/flox}$; *Rev-Erb*$\beta^{flox/flox}$) were crossed with *Tie2-Cre* to obtain hematopoietic specific Rev-Erbs deletion. Mice with genotype *Tie2-Cre; Rev-Erb*$\alpha^{flox/flox}$ *; Rev-Erb*$\beta^{flox/flox}$

(Rev-Erb DKO) were crossed with *Rev-Erb*$\alpha^{flox/flox}$ *; Rev-Erb*$\beta^{flox/flox}$ (Rev-Erb WT) to generate experimental and control groups.

Generation of Biotin-Tagged Rev-Erbα and Rev-Erbβ

Because the lack of antibodies providing sufficient ChIP enrichment of Rev-Erbα and Rev-Erbβ on known target genes in macrophages, we implemented and validated an in vivo biotin tagging strategy [37, 91, 92]. Briefly, to generate biotin-tagged Rev-Erb for ChIP-Seq analysis, Rev-Erbs were fused in frame with amino acid peptide MAGGLNDIFEAQKIEWHEDTGGGGSGGGGSGENLYFQS at the N' terminus. Amino acids 1-20 represent a biotin ligase recognition peptide (BLRP), in which the lysine residue at position 13 is a substrate for the bacterial biotin ligase (BirA). The glycine-rich stretch following the BLRP sequence provides a spacer region, and the ENLYFQS sequence provides a specific cleavage site for TEV protease (Invitrogen). BLRP-Rev-Erb was placed under the control of the CMV early region enhancer/chicken actin promoter in expression plasmid with puromycin resistance gene for stable transfection. BLRP-Rev-Erb expression plasmid was then transfected into RAW264.7 macrophages engineered to stably express BirA (BIRA-RAW264.7 stable cell line). Multiple stable cell lines were isolated and screened for BLRP-Rev-Erb expression and biotinylation via western blot using anti Avi-tag antibody specifically recognizing the BLRP tag (Genscript) or HRP-streptavidin (Jackson Immunoresearch).

Cell culture

Primary cells were isolated from male 6-10 week-old mice. (C57Bl/6, *Rev-Erb$\alpha^{flox/flox}$; Rev-Erb$\beta^{flox/flox}$* mice with or without *Tie2-Cre)*. Peritoneal macrophages were isolated by peritoneal lavage 3 days after intra-peritoneal injection of 3mL thioglycollate (3%). Cells were plated in 10% FBS RPMI for overnight culture and adherence selection. Bone marrow derived macrophages (BMDM) were generated as described with slight modifications (Valledor et al., 2004). Briefly, total bone marrow flushed from tibiae, femurs, and ilia was cultured in 30% L-cell conditioned media for 7 days in RPMI + 20% FBS. BMDM were then switched to 10% FBS RPMI supplemented with 10ng/mL mCSF. For in vivo biotinylation of Rev-Erb, RAW264.7 macrophages were stably transfected and selected for co-expression of bacterial ligase (BirA) and BLRP-empty, -Rev-Erbα or –Rev-Erbβ using G418 (275ng/mL) and puromycin (2.5ug/mL) respectively. Multiple stable clones were generated for each BLRP-construct.

Chromatin Immunoprecipitation

Chromatin Immunoprecipitation (ChIP) was performed as previously described [37]. Briefly, 5-20 million cells were crosslinked in 1% formaldehyde/PBS for 10 minutes at room temperature (RT). Fixation reaction was quenched by adding glycine to a final concentration of 125mM. After washing twice in ice-cold PBS, cells were resuspend in swelling buffer (10 mM HEPES/KOH pH7.9, 85 mM KCl, 1 mM EDTA, 0.5% IGEPAL CA-630, 1x protease inhibitor cocktail (Roche), 1 mM PMSF) for 5 minutes. Cells were spun

down and resuspended in 500uL lysis buffer (50 mM Tris/HCl pH 7.4@20°C, 1% SDS, 0.5% Empigen BB (Fluke), 10 mM EDTA, 1x protease inhibitor cocktail (Roche), 1 mM PMSF)). Chromatin was sheared to an average size of 300-400 bp by sonicating for six 10-second pulses at 13W power output with 30 seconds pause on wet ice using a Misonix 3000 sonicator. Lysate was cleared by centrifugation (10 min, 16000 x g, 4ºC), and 400uL of supernatant was diluted with 600uL dilution buffer (20 mM Tris/HCl pH 7.4@20°C, 100 mM NaCl, 0.5% Triton X-100, 2 mM EDTA, 1x protease inhibitor cocktail (Roche)). Diluted lysates were pre-cleared with 40uL of Protein A-separose beads (GE Health Science) by rotating for 1hr at 4ºC. Protein A beads were washed twice with 0.1% BSA TE buffer, blocked for at least 30 minutes at room temperature with 0.5% BSA TE with 20ug/mL glycogen, wash twice and brought back to original volume with 0.1% BSA TE buffer. After pre-clearing, 1% of the supernatant was removed for input, while the rest was immunoprecipitated at 4ºC rotating overnight with antibodies of interest. On the next day, immunocomplexes were captured using blocked Protein A sepharose beads for 1-2hr at 4ºC with rotation. With supernatant discarded, protein-captured beads were transferred to 0.45um filter cartridges transferred (Millipore, Billerica, MA, USA) and washed twice with 500 µl of each of the following: wash buffer I (WB I) (20 mM Tris/HCl pH 7.4@20°C, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA), WB II (20 mM Tris/HCl pH 7.4@20°C, 500 mM NaCl, 1% Triton X-100, 2 mM EDTA), WB III (10 mM Tris/HCl pH 7.4@20°C, 250 mM LiCl, 1% IGEPAL CA-630, 1% Na-deoxycholate, 1 mM EDTA), and TE. Immunoprecipitated chromatin was eluted

twice with 100ul elution buffer (100 mM $NaHCO_3$, 1% SDS) into fresh tubes for 10 and 1 min, respectively. Samples were reversed crosslinked overnight in a 65ºC hybridization oven. Samples were sequentially incubated at 37°C for 2 hr each with 0.33 mg/ml RNaseA and 0.5 mg/ml proteinase K. DNA was isolated using the QiaQuick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and eluted in 100uL of elution buffer (EB).

Chromatin precipitation of biotinylated Rev-Erbs was performed following standard protocol with the following modifications. To precipitate biotinylated Rev-Erb complex, sonicated lysates were purified with BSA-blocked streptavidin T1 Dynabeads (Invitrogen) overnight at 4ºC with rotation. After washing steps, samples were equilibrated in TEV buffer (50mM Tris/HCl pH 8.0, 100mM NaCl, 0.1% NP-40 (CA630), 0.5mM EDTA) for 5 min at RT. Rev-Erb-DNA complex were specifically eluted after TEV cleavage (5-10U AcTEV, invitrogen) for 1hr at RT, which cleaves at a recognition site engineered in the biotinylation-fusion tag. Eluted samples were reverse crosslinked, RNAseA and Proteinase K treated following standard protocol.

ChIP-sequencing and analysis

DNA from chromatin immunoprecipitation (10-50 ng) was adapter-ligated and PCR amplified according to the manufacturer's protocol (Illumina, San Diego, USA). Briefly, fragmented ChIP DNA was filled-in using T4 DNA pol (0.75U, Enzymatics), Klenow (0.25U, Enzymatics), and T4 PNK (2.5U, Enzymatics) for 30 minutes at 20ºC. Deoxyadenosine (2mM) was added to the 3' of the repaired

DNA fragment using Exo$^-$ Klenow (2.5U, NEB) for ligation with genomic adaptor (Illumina) using Rapid T4 DNA Ligase (600U, Enzymatics). Adaptor ligated DNA fragments were size selected (150-250bp) and PCR amplified for 14-17 cycles. ChIP fragments were sequenced for 36 cycles on an Illumina Genome Analyzer according to the manufacturer's instructions.

ChIPseq peak identification, quality control, and motif analysis were performed using HOMER as described [37, 57]. An in-depth description for HOMER will be published elsewhere (Benner et al, manuscript in preparation). For de novo motif analysis, transcription factor motif finding was performed on ± 100bp relative to the peak center. Background sequences were selectively weight to normalize for G+C content. Peak sequences were compared to randomly selected genomic fragments of the same size and normalized G+C content to identify motifs enriched ChIP-seq versus the genomic background. ChIP-seq heatmaps were generated using Cluster (http://bonsai.hgc.jp/~mdehoon/software/cluster/) and visualized using Java TreeView [93].

RT-PCR

Total RNA was purified with RNeasy columns (Qiagen) according to manufacture's instruction. To reduce DNA contamination, RNA samples were treated with either on-column DNAse set (Qiagen) or Turbo DNAse (Ambion). Total RNA was reverse-transcribed using SuperScript Reverse Transcriptase III (Invitrogen) with either oligo dT or random hexamer to generate cDNA. No-

template controls were generated by excluding reverse-transcriptase from the reaction. Quantitative transcript analysis was performed on an Applied Biosystems 7300 Real-time PCR system or Step One Plus using SYBR GreenER qPCR mastermix (Invitrogen). Values are normalized for 36B4 content. A modified ΔΔCT method, which incorporates PCR efficiencies, was used to determine relative expression of RNA.

Enhancer reporter assay

Reporter constructs were transfected into RAW264.7 macrophages was achieve using Superfect reagent (Qiagen) according to manufacture's recommendation. RAW cells were cotransfected with 300ng of firefly luciferase expression construct driven by various promoters with enhancers and 200ng of Rev-Erbα, Rev-Erbβ, and RORα expression construct. Cells were seeded at 100,000 per well in 24-well tissue culture treated plates (Corning). At 48hr post-transfection, cells were lysed and firefly luciferase activity was measured using a Veritas microplate luminometer (Turner Biosystems) according to manufacturer's protocol. A β-galactosidase expression vector was cotransfected (100 ng/well) as an internal control for transfection efficiency. Relative light units (RLU) were given as transformed ratio of firefly luciferase to β-galactosidase. Values are the mean ratio of at least three independent experiments performed in triplicate.

Global Run-On Sequencing

Global run-on and library preparation for sequencing were done as

described [57]. Briefly, four 10-cm plates of confluent BMDM from control (*Rev-Erb$\alpha^{flox/flox}$; Rev-Erb$\beta^{flox/flox}$*) or Rev-Erb DKO (Tie2-Cre^{+}; *Rev-Erb$\alpha^{flox/flox}$; Rev-Erb$\beta^{flox/flox}$*) were washed three times with cold PBS buffer. Cells were then swelled in swelling buffer (10mM Tris pH 7.5, 2mM $MgCl_2$, 3mM $CaCl_2$, 4U/mL SUPERase-In (Ambion)) for 5 min on ice. Harvested cells were re-suspended in 1 ml of the lysis buffer (swelling buffer with 0.5% IGEPAL and 10% glycerol, 2U/mL SUPERase-In) with gentle vortex and brought to 10 ml with the same buffer for extraction of nuclei. Nuclei were washed with 10ml of lysis buffer and re-suspended in 1 ml of freezing buffer (50mM Tris pH 8.3, 40% glycerol, 5mM $MgCl_2$, 0.1mM EDTA, 2U/mL SUPERase-In), pelleted down again and finally re-suspended in 100ul of freezing buffer.

For run-on assay, re-suspended nuclei were mixed with an equal volume of reaction buffer (10mM Tris pH 8.0, 5mM $MgCl_2$, 1mM DTT, 300mM KCl, 20 units of SUPERase-In, 1% Sarkosyl, 500mM ATP, GTP and Br-UTP, 2mM CTP, 0.6U/mL SUPERase-In) and incubated for 5 min at 30ºC. Nuclei RNA were extracted with TRIzol LS reagent (Invitogen) according to the manufacturer's instructions. RNA was then re-suspended in 20ul of DEPC-water and subjected to base hydrolysis by addition of 5ul of 1M NaOH and incubated on ice for 40min. Then, 25ul of 1M Tris pH 6.8 was added to neutralize the reaction. RNA was purified through a p-30 RNase-free spin column (BioRad), according to the manufacturer's instructions and treated with 6.7ul of DNase buffer and 10ul of RQ1 RNase-free DNase (Promega), and purified again through a p-30 column. A volume of 25ul of purified sample, 1ul of SUPERase-In and 7ul of 5X PNK buffer,

and 2uL of PNK (20U) was added to the run-on RNA and treated for 1 hr at 37ºC. Before proceeding to immunopurification, RNA was heated to 65ºC for 5 min and kept on ice.

Anti-BrdU argarose beads (Santa Cruz Biotech) were blocked in blocking buffer (0.5X SSPE, 1mM EDTA, 0.05% Tween-20, 0.1% PVP, and 1 mg/ml BSA) for 1 hr at 4ºC. Heated run-on RNA (85ul) was added to 60ul beads in 500ul binding buffer (0.5 SSPE, 1mM EDTA, 0.05% Tween-20, 4U/mL SUPERase-In) and allowed to bind for 1 hr at 4 ºC with rotation. After binding, beads were washed once in low salt buffer (0.25X SSPE, 1 mM EDTA, 0.05% Tween-20, 4U/mL SUPERase-In), twice in high salt buffer (0.2X SSPE, 1 mM EDTA, 0.05% Tween-20, 150 mM NaCl, 4U/mL SUPERase-In) and twice in TET buffer (TE pH7.4, 0.05% Tween-20, 4U/mL SUPERase-In). BrU-incorporated RNA was eluted with 4X 100ul elution buffer (20mM DTT, 150uM NaCl, 50mM Tris pH 7.5, 1mM EDTA and 0.1% SDS, 4U/mL SUPERase-In). Glycogen (20ug, Invitrogen) was added to eluted RNA, and the final NaCl concentration was brought to 300mM. RNA was precipitated in ethanol overnight. The precipitated RNA was re-suspended in 5ul ultrapure water with 1U/uL SUPERase-In.

Complementary DNA (cDNA) synthesis was performed as follow. First, RNA was denatured at 65ºC for 3 min and cooled on ice. Denatured RNA fragments were subjected to poly-A tailing reaction in 6.7ul volume containing 0.7ul poly-A polymerase buffer, 0.25ul 4mM ATP, and 0.75 ul E. coli poly(A)-polymerase (3.75U, NEB). The reaction was performed for 30 min at 37ºC. Subsequently, reverse transcription was performed using oNTI223 primer (5’-

pGATCGTCGGACTGTAGAACTCT;CAAGCAGA
AGACGGCATACGATTTTTTTTTTTTTTTTTTTVN-3') where the p indicates 5' phosphorylation, ';' indicates the abasic dSpacer furan and VN indicates degenerate nucleotides.

Tailed RNA (6.7ul) was mixed with 0.9ul dNTP (10mM each) and 0.8ul 10mM oNTI223, heated for 3min at 75ºC and chilled briefly on ice. Then, 0.5ul SUPERase-In, 1.6uL 0.1M DTT, 3uL 25mM $MgCl_2$, 1.6ul 10X reverse transcription buffer and 1ul Superscript III reverse transcriptase (Invitrogen) was added to the tube. The tube was incubated for 30 min at 48ºC. After that, 4ul of Exonuclease I (80U, Fermentas) was added into the reaction and the tube was incubated for 1 hr at 37ºC to eliminate extra oNTI223. Then RNA was hydrolyzed by adding 2uL 1M NaOH and incubated for 20 min at 98ºC. The reaction was neutralized with 2ul of 1M HCl. After running on a 10% polyacrylamide TBE-urea gel, the extended first-strand cDNA product (105-400 nucleotide) was excised and recovered by soaking the grinded gel in DNA gel elution buffer (TE with 0.1% Tween-20) for 2 hours at RT. Eluate was separated from the gel debris by transferring the entire content to Millipore MC column. The Elution step was repeated with 150uL of elution buffer. cDNA was then precipitated with ethanol.

Circularization of first-strand cDNA was performed by re-suspending cDNA in 10uL reaction solution (7.5ul water, 1ul 10X CircLigase buffer, 0.5ml 1mM ATP and 0.5ml 50mM $MnCl_2$) and then adding 0.5ul CircLigase (50U, Epicentre). The reaction went for 1hr at 60ºC and then was heat-inactivated for

10 min at 80ºC. Circularized single-stranded DNA (ssDNA) was relinearized by adding 3.3ul of 4X relinearization supplement (100mM KCl, 2mM DTT) followed by 1ul of ApeI (15U, NEB). The reaction was incubated for 45min at 37ºC. Another 1uL of ApeI was added for 45min at 37ºC. The ssDNA template was amplified by PCR using the Phusion High-Fidelity enzyme (NEB) according to the manufacturer's instructions. The oligonucleotide primers oNTI200 (5'-CAAGCAGAAGACGGCATA-3') and oNTI201 (5'-AATG ATACGGCGACCACCGACAGGTTCAGAGTTCTACAGTCCGACG-3') were used to generate DNA for sequencing. PCR was performed with an initial 30sec denaturation at 98ºC, followed by 14-16 cycles of 10sec denaturation at 98ºC, 15sec annealing at 57ºC and 15sec extension at 72ºC. The PCR product was run on a non-denaturing 8% polyacrylamide TBE gel, and gel corresponding to 140-225bp was excised and recovered as described above, but by eluting in 0.1% Tween-20, 150mM NaCl, TE. Eluted DNA was purified using ChIP DNA clean & Concentrator Kit (Zymo Research). Purified DNA was then sequenced on the Illumina Genome Analyzer II according to the manufacturer's instructions with small RNA sequencing primer 5'-CGACAGG TTCAGAGTTCTACAGTCCGACGATC-3'.

Enhancer-associated RNA analysis

GRO-Seq read densities were analyzed to classify genomic regions into contiguous transcripts using HOMER as described previously [57]. To analyze Rev-Erb regulated enhancer RNA (eRNA), we first defined Rev-Erb sites

localized in putative enhancers based on enrichment of H3K4me1 and H3K4me3. Normalized sequencing tags for BMDM H3K4me1 and H3K4me3 were tabulated within ± 1000bp window from the center of Rev-Erb binding. We defined putative enhancers with the following 2 criteria. 1) Regions have more than 16 tags of H3K4me1 and 2) H3K4me1 tag count is greater than H3K4me3. Less than 15% of annotated promoters have more H3K4me1 than H3K4me3, so the latter criterion is justifiable. With these parameters, we have identified 1,388 Rev-Erb bound putative macrophage enhancers. To quantitatively examine eRNA expression, we further exclude Rev-Erb sites locating within gene bodies in order to minimize reads from coding strand that will confound eRNA expression. This resulted in 721 intergenic sites. Histograms of RNA expression at these sites were generated by tabulating RNA tag counts in resolution of 40bp and a moving average of 400bp within 2kb ± from centers of Rev-Erb peaks. Sites with > 4 tags within ± 800bp of Rev-Erb peak centers were considered as RNA expressing [55].

To determine the correlation between eRNA and nearby protein coding gene expression, each Rev-Erb bound intergenic sites was paired to its nearest transcription start sites that are expressing RNA (at least 20 sequencing tags normalized to the length of the gene body). Averages of differential expression of eRNA between controls (*Rev-Erb*$\alpha^{flox/flox}$*; Rev-Erb*$\beta^{flox/flox}$) and Rev-Erb DKO (Tie2-Cre^{+}; *Rev-Erb*$\alpha^{flox/flox}$*; Rev-Erb*$\beta^{flox/flox}$) macrophage was calculated. The same was performed for mRNA. The DKO:control ratios for eRNA expression were ranked in an ascending order. Both the eRNA and mRNA were then

grouped into 35 bins (i.e. 15 sites per bin) before plotting. Spearmen rank correlation was used to test whether changes in eRNA and mRNA covary.

Table 1. Primers for cloning DNA fragment for enhancer activity assays.

Genes	location/coordinate	Primer sequences	amplicon length (bp)	melting Tm (°C)
Mmp9	-5kb enhancer	gatcGGATCCGTGGCTCAGCATCAGGAAAT	1003	77.1
		gatcGTCGACACTTGGCAGGCAGAGTGAGT		75.9
Cx3cr1	9.4kb intronic enhancer	gatcGGATCCTACACCTGCACAAGCACACA	1016	75.3
		gatcGTCGACAACTGGGCGGAAATTGTAAA		74.2
Cx3cr1	28kb enhancer	gatcGGATCCGACCCTGGGTTGTCAGTAGG	987	76.6
		gatcGTCGACACTTATGGGGGAGGATCTGG		75.2
Arhgap25	33kb enhancer	gatcGTCGACTTTCCATGGGTCCAGAGATG	930	74.8
		gatcGGATCCAGCAGGCTGGGATATGAGTG		76.1
Eif2C4	-20kb enhancer	gatcGTCGACCCCTCAAAGCTAACCATCCA	975	75.4
		gatcGGATCCAAAGTCATGCGAGACCTGAAA		75.5
P4ha2	55kb enhancer	gatcGGATCCGCCACAGCTCTGCTTTATGG	936	77.2
		gatcGTCGACGCTCACTGGCCTTGCTAACT		75.9
Slc7a8	27kb enhancer	gatcGTCGACCTGCATCCCGACTCATACCT	997	75.8
		gatcGGATCCTTCCAGCAAGCACTCTTTCA		74.5
Bmall	promoter 551bp	GATCCTCGAGGCAGAGTCCGCAACGCAGTGG	551	81.8
		GATCAAGCTTCGCACCCGCACTCGGATCCCG		83.6
Negative	chr5:30,278,928-30,279,847	gatcGGATCCAGTTCCATGTCCAGCGAATC	920	76.1
		gatcGTCGACGGAGCAAGGAGGGAGAGAG		76.2

Table 2. Primers for quantitative PCR.

Genes	Application	Primer Sequence	Amplicon (bp)	Tm
Mmp9	mRNA	CATTCGCGTGGATAAGGAGT	112	60.1
		GAAACTCACACGCCAGAAGA		59
Mmp9	primary transcript	AAGCGGACATTGTCATCCA	120	60.1
		CAGGCATAAGAGCGGACAG		59.6
Mmp9	proximal eRNA	AAGATGGGGGAAATGGTAGG	123	60.01
		ACTTGGCAGGCAGAGTGAGT		60.06
Mmp9	distal eRNA	TGGAGTCCCACAAAATCCTC	106	59.9
		TAGCTCAACTGTGGGGTGTG		59.7
Mmp9	ChIP enhancer	TGGGAGCTTGGGATCTAGTG	135	60
		CCCAAATTTGCTGAAGAGGA		60
Cx3cr1	mRNA	AGTTCCCTTCCCATCTGCTC	139	60.6
		AATGTCGCCCAAATAACAGG		59.9
Cx3cr1	28kb eRNA	CTGCCTCAGGGAGAAACAAG	158	59.98
		CTGCAACTCTCAGCAACCAG		59.77
Cx3cr1	28kb ChIP	CTGCCTCAGGGAGAAACAAG	158	59.98
		CTGCAACTCTCAGCAACCAG		59.77
36B4	mRNA	AGGGCGACCTGGAAGTCC	77	62.6
		CCCACAATGAAGCATTTTGGA		63

References

1. Barish, G., et al., *A Nuclear Receptor Atlas: Macrophage Activation.* Molecular Endocrinology, 2005.

2. Pascual, G., et al., *A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR-gamma.* Nature, 2005. **437**(7059): p. 759-63.

3. Ghisletti, S., et al., *Parallel SUMOylation-dependent pathways mediate gene- and signal-specific transrepression by LXRs and PPARgamma.* Mol Cell, 2007. **25**(1): p. 57-70.

4. Hevener, A.L., et al., *Macrophage PPAR γ is required for normal skeletal muscle and hepatic insulin sensitivity and full antidiabetic effects of thiazolidinediones.* Journal of Clinical Investigation, 2007. **117**(6): p. 1658-1669.

5. Odegaard, J.I., et al., *Macrophage-specific PPARgamma controls alternative activation and improves insulin resistance.* Nature, 2007. **447**(7148): p. 1116-20.

6. Saijo, K., et al., *A Nurr1/CoREST Pathway in Microglia and Astrocytes Protects Dopaminergic Neurons from Inflammation-Induced Death.* Cell, 2009. **137**(1): p. 47-59.

7. Li, A.C. and C.K. Glass, *Use of mouse models to evaluate roles of nuclear receptors and their ligands in the pathogenesis and treatment of atherosclerosis.* Current drug targets, 2007. **8**(12): p. 1273-87.

8. Ogawa, S., et al., *Molecular Determinants of Crosstalk between Nuclear Receptors and Toll-like Receptors.* Cell, 2005.

9. Yin, L., et al., *Rev-erbalpha, a heme sensor that coordinates metabolic and circadian pathways.* Science, 2007. **318**(5857): p. 1786-9.

10. Raghuram, S., et al., *Identification of heme as the ligand for the orphan nuclear receptors REV-ERBalpha and REV-ERBbeta.* Nat Struct Mol Biol, 2007. **14**(12): p. 1207-13.

11. Pardee, K.I., et al., *The Structural Basis of Gas-Responsive Transcription by the Human Nuclear Hormone Receptor REV-ERB β.* PLoS Biol, 2009. **7**(2): p. e43.

12. Marvin, K.A., et al., *Nuclear Receptors Homo sapiensRev-erb β and Drosophila melanogasterE75 Are Thiolate-Ligated Heme Proteins Which Undergo Redox-Mediated Ligand Switching and Bind CO and NO.* Biochemistry, 2009. **48**(29): p. 7056-7071.

13. Reinking, J., et al., *The Drosophila nuclear receptor e75 contains heme and is gas responsive.* Cell, 2005. **122**(2): p. 195-207.

14. Fontaine, C. and B. Staels, *The orphan nuclear receptor Rev-erb α : a transcriptional link between circadian rhythmicity and cardiometabolic disease.* Current Opinion in Lipidology, 2007.

15. Yin, L. and M.A. Lazar, *The orphan nuclear receptor Rev-erbalpha recruits the N-CoR/histone deacetylase 3 corepressor to regulate the circadian Bmal1 gene.* Mol Endocrinol, 2005. **19**(6): p. 1452-9.

16. Ghisletti, S., et al., *Cooperative NCoR/SMRT interactions establish a corepressor-based strategy for integration of inflammatory and anti-inflammatory signaling pathways.* Genes & Development, 2009. **23**(6): p. 681-693.

17. Fontaine, C., et al., *THE NUCLEAR RECEPTOR REV-ERB{alpha} IS A LIVER X RECEPTOR (LXR) TARGET GENE DRIVING A NEGATIVE FEEDBACK LOOP ON SELECT LXR-INDUCED PATHWAYS IN HUMAN MACROPHAGES.* Molecular Endocrinology, 2008: p. me.2007-0439v1.

18. Tobias, P.S. and L.K. Curtiss, *Toll-like receptors in atherosclerosis.* Biochem Soc Trans, 2007. **35**(Pt 6): p. 1453-5.

19. Preitner, N., et al., *The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator.* Cell, 2002. **110**(2): p. 251-60.

20. Liu, A.C., et al., *Redundant Function of REV-ERB α and β and Non-Essential Role for Bmal1 Cycling in Transcriptional Regulation of Intracellular Circadian Rhythms.* PLoS Genetics, 2008. **4**(2): p. e1000023.

21. Le Martelot, G., et al., *REV-ERB α Participates in Circadian SREBP Signaling and Bile Acid Homeostasis.* PLoS Biol, 2009. **7**(9): p. e1000181.

22. Baggs, J., et al., *Network features of the mammalian circadian clock.* PLoS Biol, 2009.

23. Turek, F.W., et al., *Obesity and metabolic syndrome in circadian Clock mutant mice.* Science, 2005. **308**(5724): p. 1043-5.

24. Kornmann, B., et al., *System-driven and oscillator-dependent circadian transcription in mice with a conditionally active liver clock.* PLoS Biol, 2007. **5**(2): p. e34.

25. Muller, J., et al., *Circadian variation of cardiovascular disease and sympathetic activity.* J Cardiovasc Pharmacol, 1987. **10**: p. Suppl 2:S104-9; discussion S110-1.

26. Shaw, E. and G.H. Tofler, *Circadian rhythm and cardiovascular disease.* Current atherosclerosis reports, 2009. **11**(4): p. 289-95.

27. HALBERG, F., et al., *Susceptibility rhythm to E. coli endotoxin and bioassay.* Proc Soc Exp Biol Med, 1960. **103**: p. 142-4.

28. Hayashi, M., S. Shimba, and M. Tezuka, *Characterization of the molecular clock in mouse peritoneal macrophages.* Biol Pharm Bull, 2007. **30**(4): p. 621-6.

29. Petrovsky, N. and L.C. Harrison, *Diurnal rhythmicity of human cytokine production: a dynamic disequilibrium in T helper cell type 1/T helper cell type 2 balance?* J Immunol, 1997. **158**(11): p. 5163-8.

30. Miller, B.H., et al., *Circadian and CLOCK-controlled regulation of the mouse transcriptome and cell proliferation.* Proc Natl Acad Sci USA, 2007. **104**(9): p. 3342-7.

31. Yan, J., et al., *Analysis of gene regulatory networks in the Mammalian circadian rhythm.* PLoS Comput Biol, 2008. **4**(10): p. e1000193.

32. Chen, X., et al., *Integration of External Signaling Pathways with the Core Transcriptional Network in Embryonic Stem Cells.* Cell, 2008. **133**(6): p. 1106-1117.

33. MacArthur, S., et al., *Developmental roles of 21 Drosophila transcription factors are determined by quantitative differences in binding to an overlapping set of thousands of genomic regions.* Genome Biol, 2009. **10**(7): p. R80.

34. Lupien, M., et al., *FoxA1 translates epigenetic signatures into enhancer-driven lineage-specific transcription.* Cell, 2008. **132**(6): p. 958-70.

35. Odom, D.T., et al., *Control of pancreas and liver gene expression by HNF transcription factors.* Science, 2004. **303**(5662): p. 1378-81.

36. Sandmann, T., et al., *A temporal map of transcription factor activity: mef2 directly regulates target genes at all stages of muscle development.* Dev Cell, 2006. **10**(6): p. 797-807.

37. Heinz, S., et al., *Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities.* Mol Cell, 2010. **38**(4): p. 576-89.

38. Heintzman, N.D., et al., *Histone modifications at human enhancers reflect global cell-type-specific gene expression.* Nature, 2009. **459**(7243): p. 108-12.

39. Zinzen, R.P., et al., *Combinatorial binding predicts spatio-temporal cis-regulatory activity.* Nature, 2009. **462**(7269): p. 65-70.

40. Visel, A., et al., *ChIP-seq accurately predicts tissue-specific activity of enhancers.* Nature, 2009. **457**(7231): p. 854-8.

41. Simonis, M., J. Kooren, and W.D. Laat, *An evaluation of 3C-based methods to capture DNA interactions.* Nat Methods, 2007. **4**(11): p. 895.

42. Fullwood, M.J., et al., *An oestrogen-receptor-α-bound human chromatin interactome.* Nature, 2009. **461**(7269): p. 58-64.

43. Göndör, A. and R. Ohlsson, *Chromosome crosstalk in three dimensions.* Nature, 2009. **461**(7261): p. 212-217.

44. Lieberman-Aiden, E., et al., *Comprehensive mapping of long-range interactions reveals folding principles of the human genome.* Science, 2009. **326**(5950): p. 289-93.

45. Sexton, T., F. Bantignies, and G. Cavalli, *Genomic interactions: chromatin loops and gene meeting points in transcriptional regulation.* Semin Cell Dev Biol, 2009. **20**(7): p. 849-55.

46. Mishiro, T., et al., *Architectural roles of multiple chromatin insulators at the human apolipoprotein gene cluster.* The EMBO Journal, 2009. **28**(9): p. 1234.

47. Kagey, M.H., et al., *Mediator and cohesin connect gene expression and chromatin architecture.* Nature, 2010. **467**(7314): p. 430.

48. Sawado, T., et al., *The beta -globin locus control region (LCR) functions primarily by enhancing the transition from transcription initiation to elongation.* Genes Dev, 2003. **17**(8): p. 1009-18.

49. Core, L. and J. Lis, *Transcription Regulation Through Promoter-Proximal Pausing of RNA Polymerase II.* Science, 2008. **319**(5871): p. 1791.

50. Levine, M., *Paused RNA Polymerase II as a Developmental Checkpoint.* Cell, 2011. **145**(4): p. 502-11.

51. Zippo, A., et al., *Histone crosstalk between H3S10ph and H4K16ac generates a histone code that mediates transcription elongation.* Cell, 2009. **138**(6): p. 1122-36.

52. Karam, C.S., et al., *14-3-3 mediates histone cross-talk during transcription elongation in Drosophila.* PLoS Genet, 2010. **6**(6): p. e1000975.

53. Zhu, X., et al., *A facilitated tracking and transcription mechanism of long-range enhancer function.* Nucleic Acids Research, 2007: p. gkm595v1.

54. De Santa, F., et al., *A large fraction of extragenic RNA pol II transcription sites overlap enhancers.* PLoS Biol, 2010. **8**(5): p. e1000384.

55. Kim, T.-K., et al., *Widespread transcription at neuronal activity-regulated enhancers.* Nature, 2010. **465**(7295): p. 182.

56. Hah, N., et al., *A rapid, extensive, and transient transcriptional response to estrogen signaling in breast cancer cells.* Cell, 2011. **145**(4): p. 622-34.

57. Wang, D., et al., *Reprogramming transcription by distinct classes of enhancers functionally defined by eRNA.* Nature, 2011.

58. Tuan, D., S. Kong, and K. Hu, *Transcription of the hypersensitive site HS2 enhancer in erythroid cells.* Proc Natl Acad Sci USA, 1992. **89**(23): p. 11219-23.

59. Kong, S., et al., *Transcription of the HS2 enhancer toward a cis-linked gene is independent of the orientation, position, and distance of the enhancer relative to the gene.* Molecular and Cellular Biology, 1997. **17**(7): p. 3955-65.

60. Ling, J., et al., *HS2 enhancer function is blocked by a transcriptional terminator inserted between the enhancer and the promoter.* J Biol Chem, 2004. **279**(49): p. 51704-13.

61. Johnson, K.D., et al., *Highly restricted localization of RNA polymerase II within a locus control region of a tissue-specific chromatin domain.* Molecular and Cellular Biology, 2003. **23**(18): p. 6484-93.

62. Orom, U.A., et al., *Long Noncoding RNAs with Enhancer-like Function in Human Cells.* Cell, 2010. **143**(1): p. 46-58.

63. Wang, K.C., et al., *A long noncoding RNA maintains active chromatin to coordinate homeotic gene expression.* Nature, 2011.

64. Yap, K.L., et al., *Molecular interplay of the noncoding RNA ANRIL and methylated histone H3 lysine 27 by polycomb CBX7 in transcriptional silencing of INK4a.* Molecular Cell, 2010. **38**(5): p. 662-74.

65. Feng, J., et al., *The Evf-2 noncoding RNA is transcribed from the Dlx-5/6 ultraconserved region and functions as a Dlx-2 transcriptional coactivator.* Genes Dev, 2006. **20**(11): p. 1470-84.

66. Tsai, M.-C., et al., *Long noncoding RNA as modular scaffold of histone modification complexes.* Science, 2010. **329**(5992): p. 689-93.

67. Kaikkonen, M.U., M.T.Y. Lam, and C.K. Glass, *Non-coding RNAs as regulators of gene expression and epigenetics.* Cardiovasc Res, 2011. **90**(3): p. 430-40.

68. Harding, H. and M. Lazar, *The monomer-binding orphan receptor Rev-Erb represses transcription as a dimer on a novel direct repeat [published erratum appears in Mol Cell Biol 1995 Nov;15(11):6479].* Mol Cell Biol, 1995. **15**(9): p. 4791.

69. Zamir, I., J. Zhang, and M.A. Lazar, *Stoichiometric and steric principles governing repression by nuclear hormone receptors.* Genes & Development, 1997. **11**(7): p. 835-46.

70. Ukai-Tadenuma, M., et al., *Delay in feedback repression by cryptochrome 1 is required for circadian clock function.* Cell, 2011. **144**(2): p. 268-81.

71. Yin, L., et al., *Nuclear receptor Rev-erbalpha is a critical lithium-sensitive component of the circadian clock.* Science, 2006. **311**(5763): p. 1002-5.

72. Feng, D., et al., *A circadian rhythm orchestrated by histone deacetylase 3 controls hepatic lipid metabolism.* Science, 2011. **331**(6022): p. 1315-9.

73. Keller, M., et al., *A circadian clock in macrophages controls inflammatory immune responses.* Proc Natl Acad Sci USA, 2009.

74. Raspé, E., et al., *Identification of Rev-erbalpha as a physiological repressor of apoC-III gene transcription.* J Lipid Res, 2002. **43**(12): p. 2172-9.

75. Lin, Y.C., et al., *A global network of transcription factors, involving E2A, EBF1 and Foxo1, that orchestrates B cell fate.* Nature Immunology, 2010. **11**(7): p. 635.

76. Schlaeger, T.M., et al., *Tie2Cre-mediated gene ablation defines the stem-cell leukemia gene (SCL/tal1)-dependent window during hematopoietic stem-cell development.* Blood, 2005. **105**(10): p. 3871-4.

77. Giguere, V., et al., *Isoform-specific amino-terminal domains dictate DNA-binding properties of ROR alpha, a novel family of orphan hormone nuclear receptors.* Genes and Development, 1994. **8**(5): p. 538.

78. Core, L.J., J.J. Waterfall, and J.T. Lis, *Nascent RNA sequencing reveals widespread pausing and divergent initiation at human promoters.* Science, 2008. **322**(5909): p. 1845-8.

79. Ghisletti, S., et al., *Identification and characterization of enhancers controlling the inflammatory gene expression program in macrophages.* Immunity, 2010. **32**(3): p. 317-28.

80. Leddin, M., et al., *Two distinct auto-regulatory loops operate at the PU.1 locus in B cells and myeloid cells.* Blood, 2011. **117**(10): p. 2827-38.

81. Zhao, J., et al., *Polycomb Proteins Targeted by a Short Repeat RNA to the Mouse X Chromosome.* Science, 2008. **322**(5902): p. 750-756.

82. Huarte, M., et al., *A Large Intergenic Noncoding RNA Induced by p53 Mediates Global Gene Repression in the p53 Response.* Cell, 2010. **142**(3): p. 409-419.

83. Kanhere, A., et al., *Short RNAs are transcribed from repressed polycomb target genes and interact with polycomb repressive complex-2.* Mol Cell, 2010. **38**(5): p. 675-88.

84. Lefstin, J.A. and K.R. Yamamoto, *Allosteric effects of DNA on transcriptional regulators.* Nature, 1998. **392**(6679): p. 885-8.

85. Consortium, M.G.S., et al., *Initial sequencing and comparative analysis of the mouse genome.* Nature, 2002. **420**(6915): p. 520-62.

86. Harismendy, O., et al., *9p21 DNA variants associated with coronary artery disease impair interferon-γ signalling response.* Nature, 2011. **470**(7333): p. 264-8.

87. Musunuru, K., et al., *From noncoding variant to phenotype via SORT1 at the 1p13 cholesterol locus.* Nature, 2010. **466**(7307): p. 714-9.

88. Stitzel, M.L., et al., *Global Epigenomic Analysis of Primary Human Pancreatic Islets Provides Insights into Type 2 Diabetes Susceptibility Loci.* Cell Metabolism, 2010. **12**(5): p. 443-455.

89. Cheung, V.G. and R.S. Spielman, *Genetics of human gene expression: mapping DNA variants that influence gene expression.* Nature Reviews Genetics, 2009. **10**(9): p. 595-604.

90. Rozen, S. and H. Skaletsky, *Primer3 on the WWW for general users and for biologist programmers.* Methods Mol Biol, 2000. **132**: p. 365-86.

91. de Boer, E., et al., *Efficient biotinylation and single-step purification of tagged transcription factors in mammalian cells and transgenic mice.* Proc Natl Acad Sci USA, 2003. **100**(13): p. 7480-5.

92. Mito, Y., J.G. Henikoff, and S. Henikoff, *Genome-scale profiling of histone H3.3 replacement patterns.* Nat Genet, 2005. **37**(10): p. 1090-1097.

93. Saldanha, A.J., *Java Treeview--extensible visualization of microarray data.* Bioinformatics, 2004. **20**(17): p. 3246-8.