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Towards an Understanding of Inflammation in Macrophages

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Biomedical Sciences by

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2014
This Dissertation of Dawn Xiaobin Zhang is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2014
DEDICATION

To my family

and

the love of my life,

Matthew.
EPIGRAPH

How often have I said to you that when you have eliminated the impossible, whatever remains, however improbable, must be the truth?

-Sherlock Holmes, From The Sign of the Four, Sir Arthur Conan Doyle
**TABLE OF CONTENTS**

Signature Page ........................................................................................................ iii

Dedication ................................................................................................................ iv

Epigraph .................................................................................................................. v

Table of Contents .................................................................................................... vi

List of Figures .......................................................................................................... viii

List of Tables ............................................................................................................ x

Acknowledgements .................................................................................................. xi

Vita ............................................................................................................................. xiii

Abstract of the Dissertation .................................................................................... xv

**Chapter I: Introduction: Towards an Understanding of Cell-Specific Function of Signal-Dependent Transcription Factors** ........................................ 1

A. Abstract .............................................................................................................. 2

B. Introduction ...................................................................................................... 2

C. Setting the stage: roles of lineage determining factors ............................... 6

D. Signal-dependent transcription factors bind in a cell-specific manner .......................................................................................................................... 9

E. Lineage determining factors in other cell types ...................................... 13

F. Histone modifications associated with active and poised regulatory elements ................................................................................................................. 16

G. Signal-dependent control of the epigenetic landscape .......................... 18

H. Functional roles of enhancer RNAs .......................................................... 22

I. Conclusions ..................................................................................................... 23

J. Key questions to be addressed ..................................................................... 24
Chapter II: Rev-Erbs Repress Signal-Dependent Enhancers in Macrophages Vital for Tissue Remodeling and Wound Healing ...... 33

A. Abstract ........................................................................................................ 34
B. Introduction .................................................................................................... 34
C. Results ........................................................................................................... 39
D. Discussion ..................................................................................................... 52

Chapter III: Transcriptional Regulatory Roles of Coronin 2A in Macrophages ........................................................................ 70

A. Introduction .................................................................................................... 71
B. Coro2A ubiquitinylation ................................................................................ 73
C. Coro2A transcriptional properties revealed through loss of function studies ........................................................................ 75
D. Coro2A transcriptional properties revealed through gain of function studies ........................................................................ 78
E. Intersection between Coro2A and NCoR ...................................................... 79
F. Discussion ..................................................................................................... 80

Chapter IV: Conclusions and Future Directions .................................................. 89

A. Rev-Erb as a transcriptional activator ............................................................ 91
B. Genome-wide roles of Coro2A in regulating transcription ...................... 92
C. Physiological studies of Coro2A depletion ............................................... 94
D. A possible intersection of the Coro2A and Rev-Erb transcriptional systems ........................................................................ 96

Materials and Methods ...................................................................................... 98
References ......................................................................................................... 117
# LIST OF FIGURES

## Chapter I: Introduction: Towards an Understanding of Cell-Specific Function of Signal-Dependent Transcription Factors

| Figure 1.1 | Hierarchical organization of transcription factor networks | 30 |
| Figure 1.2 | Histone marks at active and poised promoters and enhancers | 31 |
| Figure 1.3 | Mechanisms for regulating gene expression in macrophages: co-regulator exchange from co-repressor to co-activator | 32 |

## Chapter II: Rev-Erb DKO Repress Signal-Dependent Enhancers in Macrophages Vital for Tissue Remodeling and Wound Healing

| Figure 2.1 | Overall impact of Rev-Erb DKO on signal-dependent gene expression | 59 |
| Figure 2.2 | Rev-Erb DKO macrophages display increased inflammatory responses to damaged tissue | 60 |
| Figure 2.3 | Rev-Erb DKO up-regulated genes intersect with both canonically M1 and M2 macrophage phenotypes | 61 |
| Figure 2.4 | Locus-specific effects of Rev-Erbs and signal-dependent transcription factors | 62 |
| Figure 2.5 | Rev-Erbs repress signal-dependent enhancer transcription | 63 |
| Figure 2.6 | Rev-Erb DKO bone marrow transplanted animals display enhanced wound closure in a full thickness wound healing model | 64 |
| Figure 2.7 | Rev-Erb DKO bone marrow transplanted animals display increased macrophage persistence at the wound site | 65 |
| Figure 2.8 | Rev-Erb double knock-out strategy | 66 |
| Figure 2.9 | Differential gene expression in Rev-Erb DKO macrophages | 67 |
| Figure 2.10 | M1, M2, and de-activated macrophage transcriptomic intersections | 68 |
| Figure 2.11 | Bone marrow transplant efficiencies | 69 |
Chapter III: Transcriptional Regulatory Roles of Coronin 2A in Macrophages

Figure 3.1 NCoR is associated with ubiquitinylated and unmodified Coro2A ................................................................. 82

Figure 3.2 Genome-wide analyses of Coro2A KO macrophages ...... 83

Figure 3.3 Overall impact of Coro2A KO on signal-dependent gene expression ........................................................................ 84

Figure 3.4 Gene expression profiles of Coro2A KO macrophages ...... 85

Figure 3.5 Coro2A KO up-regulated genes are linked to cholesterol biosynthetic processes, down-regulated genes are linked to immune responses ............................................................. 86

Figure 3.6 Coro2A-modulated transcriptional signal emanates from NCoR (AP-1) genomic locations .............................................. 87

Figure 3.7 Similarities between NCoR KO and Coro2A KO macrophages .................................................................................. 88
LIST OF TABLES

Chapter I: Introduction

Table 1.1  Abbreviations and full names of transcription factors cited in this review  ............................................................ 28

Table 1.2  Summary of studies demonstrating signal-dependent cell-specificity in transcription ............................................ 29
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McGillivray SM, Tran DN, Ramadoss NS, Alumasa JN, Okumura CY, Sakoulas G, Vaughn MV, Zhang DX, Keiler KC, Nizet V. Pharmacological inhibition of the ClpXP protease increases bacterial susceptibility to host
cathelicidin antimicrobial peptides and cell-envelope active antibiotics.


*Literary:*


**FIELDS OF STUDY**

Major Field: Biomedical Sciences (Transcriptional Regulation in Immune Cells)

Genome-wide and Physiological Studies of Inflammation

Professor Christopher K. Glass
ABSTRACT OF THE DISSERTATION

Towards an Understanding of Inflammation in Macrophages

by

Dawn Xiaobin Zhang

Doctor of Philosophy in Biomedical Sciences
University of California, San Diego, 2014
Professor Christopher K. Glass, Chair

This dissertation utilizes in vivo and genome-wide approaches to investigate the roles of NCoR, Rev-Erb, and Coro2A in regulating transcription in a signal-dependent manner. Chapter 1 will review our current understanding of transcriptional regulation in mammalian cells, especially how certain cell-
specific enhancers and genes are selected for transcription. Chapter 2 will characterize the new and unexpected role of Rev-Erbs in regulating signal-dependent enhancers during wound healing. Unexpectedly, we found that loss of Rev-Erbs in hematopoietic cells results in accelerated wound healing. Our studies suggest that Rev-Erbs regulate a broad range of enhancers vital for wound healing and that these enhancers are neither pro-inflammatory nor anti-inflammatory. Instead, the ability of Rev-Erbs to select for a subset of “wound healing” enhancers results in the fine-tuning of macrophage-mediated responses to damaged tissue. Chapter 3 describes the generation and analysis of a Coro2A total knock-out mouse. In this chapter, we utilize a genome-wide approach to investigate the role of Coro2A, a novel member of the NCoR complex, in mediating transcriptional regulation. Furthermore, our studies show that Coro2A and NCoR cooperate to regulate the expression of lipid biosynthetic and immune response genes. Finally, Chapter 4 will discuss the broader implications of this research as a whole.
Chapter I:

Introduction:
Towards an Understanding of Cell-Specific Function of Signal-Dependent Transcription Factors
A. Abstract

The ability to regulate gene expression in a cell-specific manner is a feature of many broadly expressed signal-dependent transcription factors, including nuclear hormone receptors and transcription factors that are activated by cell surface receptors for extracellular signals. As the most plastic cells of the hematopoietic system, macrophages are responsive to a wide spectrum of regulatory molecules and provide a robust model system for investigation of the basis for cell-specific transcriptional responses at a genome-wide level. Here, we review evidence suggesting a model in which cell-specific actions of signal-dependent transcription factors are the consequence of priming functions of lineage-determining transcription factors, focusing on recent studies in macrophages. We also discuss recent findings relating lineage-determining and signal-dependent transcription factor activity to alterations in the epigenetic landscape as well as the production and function of enhancer RNAs. These findings have implications for the understanding of how natural genetic variation impacts cell-specific programs of gene expression and suggest new approaches for altering gene expression in vivo.

B. Introduction

A central question in molecular endocrinology is how a single hormone-responsive transcription factor, reading the same DNA template, is capable of regulating different genes in different cell types. For example, activation of the
glucocorticoid receptor by endogenous or synthetic glucocorticoids promotes a
gluconeogenic program of gene expression in hepatocytes; mediates negative
feedback control of adrenocorticotropic hormone secretion from corticotrophs
of the anterior pituitary; and suppresses pro-inflammatory responses of
macrophages. In each case, the same glucocorticoid receptor controls
expression of a distinct set of genes that are crucial to the specific biological
functions of the corresponding cell type. This ability to regulate gene
expression in a cell-specific manner is shared by many broadly expressed
signal-dependent transcription factors, including other nuclear hormone
receptors and members of the diverse families of transcription factors that are
activated by cell surface receptors for extracellular signals (e.g., STAT
transcription factors, NF-κB family members, CREB, etc.). (For a complete
listing of transcription factor names and abbreviations, see Table 1.1). The
development of high-throughput sequencing methods to characterize
transcription factor binding, histone modifications, and nascent RNA
production at the genome-wide level has provided a powerful array of new
tools for investigating the molecular basis for cell-specific transcriptional
responses. These tools have been applied in a number of biologically
important model systems, including models for cellular differentiation (e.g.,
embryonic stem cell differentiation, adipogenesis, and hematopoiesis) and
signal-dependent gene expression (e.g., macrophage activation and hormone-
dependent breast and/or prostate cancers). In this review, we focus on recent
insights into the molecular basis of cell-specific functions of broadly expressed
signal-dependent transcription factors derived from genome-wide studies in macrophages.

Macrophages were discovered in 1884 by Ilya Mechnikov, a Russian bacteriologist who later shared the 1908 Nobel Prize in Physiology or Medicine with Paul Ehrlich for their studies in innate immunity. Classically, macrophages differentiate from bone marrow-derived monocytes; however, recent studies have found that at least some tissue resident macrophages such as microglia, Langerhans cells, and Kupffer cells are derived from hematopoietic stem cells during fetal development. As mediators of innate immunity and normal homeostatic processes, macrophages are essential to the body's ability to control inflammation. Consequently, these immune cells have been implicated in multiple disease processes such as diabetes, rheumatoid arthritis, multiple sclerosis, and atherosclerosis. Primary macrophages are easily obtainable for in vitro studies (as bone marrow-derived macrophages, thioglycollate-elicited macrophages, circulating monocytes, splenic monocytes, etc.) from wild type and genetically modified animals and can also be differentiated from human blood monocytes. The ability to obtain large numbers of these cells makes them a robust model system for contemporary 'omics' technologies – proteomics, lipidomics, and genomics – for investigating signal-dependent mechanisms.

As the most plastic cells of the hematopoietic system, macrophages are responsive to many regulatory molecules, including growth factors, pro- and anti-inflammatory cytokines, pathogen-associated molecular patterns.
(PAMPs), damage-associated molecular patterns (DAMPs) and nuclear receptor ligands. Responses to PAMPs and DAMPs are mediated by pattern recognition receptors that include the family of toll-like receptors (TLRs). Toll-like receptor 4 (TLR4) recognizes lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria. Upon binding LPS, TLR4 signal transduction activates a number of latent transcription factors, which include NF-κB and interferon regulatory factors. These factors subsequently enter the nucleus and regulate the expression of inflammatory response genes, which play essential roles in innate immunity and contribute to the development of adaptive immunity. This transcriptional response is among the most dramatic in biology with respect to both the number of genes regulated (thousands) and the magnitude of change in gene expression (>4 orders of magnitude for the most highly regulated genes). This response is also subject to counter-regulation by anti-inflammatory signaling molecules that include ligands for nuclear hormone receptors, such as the glucocorticoid receptor, PPARs, and LXR5. These properties of the macrophage make it a particularly rich cell type for the application of genome-wide approaches.

Recent studies of signal-dependent transcriptional responses in macrophages and B cells suggest a general model to explain many of the cell-specific functions of broadly expressed signal-dependent transcription factors (SDTFs). In this model, relatively simple combinations of lineage determining transcription factors (LDTFs) are proposed to function collaboratively to select genomic enhancer-like regulatory regions in a cell-
specific manner. The collaborative binding of these factors results in nucleosome remodeling to generate open regions of chromatin that provide access to SDTFs. Thus, signal-dependent transcription factors are directed to the vicinity of target genes in a cell-specific manner as a consequence of the priming functions of the lineage determining factors for that particular cell type.

C. Setting the stage: roles of lineage determining factors

It has long been proposed that the functional output of transcription factors is determined by their competitive binding with nucleosomes. Until nucleosomes are displaced and chromatin regions are open, most transcription factors bind transiently to chromatin in a manner insufficient for efficient transcription \(^28\). For example, during hematopoietic stem cell differentiation, nucleosome remodeling by the ATP-dependent, SWI/SNF chromatin-remodeling complex allows for transcription factor binding at erythroid LDTF binding sites \(^29\). Furthermore, studies utilizing novel high-throughput sequencing methods have been used to define open chromatin regions, which contain important transcriptional regulatory elements that delineate cell-specific identity \(^30\).

Recent studies of the ENCODE consortium estimate that the mammalian genome contains hundreds of thousands of enhancers \(^31\), the majority of which are selected for activity in a cell-specific manner. This process of enhancer selection is proposed to result from collaborative interactions of LDTFs, also referred to as pioneer factors or master regulators.
These factors are sequence-specific DNA-binding proteins with the ability to access their binding sites even when those sites are wrapped in nucleosomes. The factors also represent placeholders that can be replaced by other transcription factors at later stages during development\(^{32}\). For example, in embryonic stem cells, developmental pioneer transcription factors, SOX2 and FOXD3, bind at tissue-specific elements, and are replaced by cell-specific transcription factors after differentiation\(^{33}\). Currently, two prevailing theories exist to explain how pioneer transcription factors function in defining cell-specificity during development. In the passive model, pioneer transcription factor occupancy decreases the number of binding events necessary for transcriptional activation\(^{32}\). In contrast, the active model suggests that pioneer transcription factors function by recruiting chromatin remodeling complexes to activate transcription\(^{32}\).

The roles of LDTFs in priming cell-specific regulatory sites have been well-characterized in cells of the hematopoietic lineage. The LDTF, spleen focus forming virus (SFFV) proviral integration oncogene, SPI1, more commonly referred to as PU.1, provides a particularly instructive example of a LDTF that is required for specification of more than one hematopoietic cell type; genetic deletion of PU.1 results in loss of macrophages, neutrophils, and B cells\(^{34,35}\). In myeloid lineage precursor cells, PU.1 instructs hematopoietic progenitors to up-regulate myeloid-specific cell surface antigens and to down-regulate other cell-specific markers and transcription factors\(^{36}\). Recent studies have shown a dependence on cell cycle lengthening and subsequent PU.1
accumulation, which dictates myeloid differentiation from the common myeloid lymphoid progenitor \(^3\). In lymphoid progenitor cells, PU.1 is required for expression of an alternative set of genes required for progression to mature B cells \(^{13}\). Thus, even a single LDTF can promote distinct programs of gene expression in different cell types.

PU.1 is a member of the E-twenty six (ETS) family of transcription factors, which bind to the canonical ETS-motif, 5’-GGAA-3’. Using genetic and genomic methods, PU.1 was recently shown to select macrophage or B cell-specific enhancers based on co-occurrence of nearby binding sites for either macrophage or B-cell LDTFs, which are selectively expressed in one or the other cell type \(^{13}\). In macrophages, PU.1 binding occurred at enhancer-like regions exhibiting nearby binding sites for other essential myeloid LDTFs, CCAAT/enhancer binding protein alpha and beta (CEBPs) and/or AP-1 transcription factors, a heterodimeric protein composted of Jun proto-oncogene (JUN) and FBJ murine osteosarcoma viral oncogene homolog (FOS) \(^{13}\). In B cells, PU.1 binds at enhancer-like regions containing binding sites for other B cell LDTFs including TCF3, EBF1, and FOXO1 \(^{13}\). Notably, binding of PU.1 and alternative LDTFs was mutually dependent; genetic deletion of one factor resulted in loss of binding of the other at closely spaced sites \(^{13}\). Although generally within 100 bp of each other, no strict spacing relationship was observed between PU.1 and collaborative transcription factor binding sites, suggesting a mechanism other than a ternary complex model for enhanced occupancy \(^{13}\). Gain-of-function studies indicated rapid nucleosome
remodeling following collaborative binding of PU.1 to regions destined to acquire enhancer-like features in myeloid cells based on subsequent H3K4 mono-methylation. Furthermore, PU.1 is required for the maintenance of the macrophage epigenome and its expression in PU.1-negative myeloid progenitors is sufficient to induce nucleosome remodeling and H3K4 mono-methylation at cell-specific sites.

D. Signal-dependent transcription factors bind in a cell-specific manner

Genome-wide studies to date of SDTFs indicate that while binding at promoters is enriched over that expected by chance, the majority of binding sites for these factors are located at inter- and intra-genic locations that exhibit features of enhancers. Initially demonstrated for the estrogen receptor, this pattern of genome-wide binding is also observed for other members of the nuclear receptor family, including the glucocorticoid receptor, LXRα, PPARγ, androgen receptor (AR), estrogen receptor related receptors, and nuclear receptor subfamily 1, group D, members 1 and 2 (NR1D1 and NR1D2), known colloquially as Rev-Erbs. Similarly, studies of transcription factors responsive to extracellular signals also primarily localize to distal enhancer-like elements, including the RELA component of NF-κB, SRF, FOXO1, STAT1, and SMAD3. Although identification of specific enhancer-promoter interactions remain challenging at a genome-wide level, the binding of SDTFs at distal locations is correlated with changes in expression of nearby genes.
In all of the cases in which the genome-wide locations of SDTFs have been examined in macrophages, a large fraction of the DNA binding sites are observed to be in close proximity to binding sites for one or more macrophage LDTFs (PU.1, CEBPs and/or AP-1 factors)\(^{13}\). Because LDTFs select enhancers in a cell-specific manner, the co-localization of SDTFs implies a cell-specific binding pattern. Consistent with this, the genomic locations of PPARG and Rev-Erbs in macrophages are very different from their genomic locations in adipocytes\(^{40}\) and hepatocytes\(^{50}\), respectively.

An important question is the hierarchical relationship between LDTFs and SDTFs, as well as their respective roles in the selection of cell-specific enhancers. Studies of LDTFs and SDTFs in macrophages suggest primary roles of LDTFs, with variable roles of SDTFs that depend on the specific factor analyzed. Genome-wide binding analyses of LXRs indicated that nearly all LXR binding sites were in close proximity to binding sites for macrophage LDTFs (PU.1, AP-1, C/EBPs)\(^ {13}\). Genetic deletion of LXRs had virtually no effect on the overall enhancer atlas in macrophages and did not result in a loss of nearby PU.1 binding\(^ {13}\). In contrast, deletion of PU.1 resulted in loss of nearby LXR binding\(^ {13}\). Thus, in this case, a clear hierarchy was observed in which the binding of LXRs was dependent on the initial selection of enhancers by LDTFs.

A different picture emerged from studies of the RELA subunit of NF-κB, which is strongly activated in macrophages by signaling through TLR4. Following activation and nuclear entry, \(~85\%\) of RELA binding occurred at pre-
existing enhancer-like regions of the genome that were occupied by macrophage LDTFs. However, ~15% of the binding sites for RELA occurred at genomic regions that lacked features of enhancers, but acquired these features subsequent to RELA binding. Intriguingly, these binding sites were highly enriched for nearby recognition motifs for the macrophage LDTFs AP-1 and PU.1. Subsequent experiments demonstrated that RELA binding required PU.1, while PU.1 binding required nuclear entry of RELA. Thus, in this case, RELA acted as both a signal-dependent and collaborating transcription factor to open up regions of chromatin and select new enhancers. The ability of RELA but not LXR to perform this function may relate to relatively higher nuclear concentrations and/or its ability to interact with nucleosome remodeling factors.

Recent studies have utilized natural genetic variation as an in vivo mutagenesis screen to examine the collaborative binding model for LDTFs and the hierarchical relationship with NF-κB. Genome-wide transcription factor binding and gene expression analyses were used to characterize how genetic variations affect the binding of LTDFs (PU.1 and CEBPA) and the associated transcriptional output in macrophages derived from two inbred rodent strains, C57BL/6J and BALB/c. Single nucleotide variations and other non-coding genomic variants were shown to directly perturb LDTF as well as SDTF binding. However, these studies also demonstrated that a variant in a binding motif for a LDTF not only affected the binding of that factor, but also the binding of nearby LDTFs, even though there were no alterations in the
binding motifs for those factors. In the case of differential NF-κB binding in the two mouse strains, mutations in motifs of LTDFs were three times more likely to result in decreased RELA binding than mutations in the binding sites for RELA itself. These findings provided genetic evidence that collaborative binding of LTDFs to variably spaced sequences was essential for enhancer selection and subsequent acquisition of histone modifications associated with enhancer activity. These studies also provided genetic evidence for a hierarchical relationship between LTDFs and the majority of the binding sites for NF-κB.

From these studies, the authors also formulated a genetic definition of a functional binding matrix that could be used to predict whether a specific variant in a LTDF binding motif would elicit a functional consequence. These results hint at the potential power of defining functionally important non-coding regulatory variants and suggest a strategy for prioritizing natural genetic variants that may play roles in human disease.

A similar study detailed the binding profile of three tissue specific transcription factors (HNF4A, CEBPA, and FOXA1) in the livers of six inbred rodents. From these studies, the authors describe that although there are extensive binding occupancy differences in relatively similar species, single nucleotide variation plays only a modest role in these differences. Regions that are bound by combinations of LTDFs are more evolutionarily stable; however, binding by a single LTDF can become de-stabilized through the deletion of one its LTDF co-binding partners.
E. Lineage determining factors in other cell types

The roles of LDTFs in mediating cell-specific and signal dependent activation of transcription have been described in many cell types. Here, we provide examples derived from genome-wide studies in dendritic cells, T cells, and in model systems used to study transforming growth factor beta (TGFβ) mediated and hormone-dependent gene expression (Table 1.2).

Dendritic cells

Dendritic cells and macrophages derive from a common myeloid lineage. In support of a LDTF/SDTF regulatory model, H3K4me3, H3K4me1, H3K27Ac, and a comprehensive panel of myeloid transcription factors were utilized to map the promoter and enhancer landscape in dendritic cells. Like in macrophages, the LDTFs in dendritic cells include PU.1 and CEBPB. In this model, a second tier of LDTFs (JUNB, IRF4, and ATF3) exist, termed “primers”, which co-bind with PU.1 and CEBPB at LPS-inducible genes pre-stimulation. Later, these factors become associated with specific and dynamic activation factors, mediating a signal-specific response to stimulation.

T cells

Three seminal papers have described the cell-specific enhancer landscape in T cells, focusing on variations amongst different subsets of T helper (Th) cells. In one study, the authors sought to define the subset-specific enhancer landscape in regulatory T (Treg) cells. LDTFs in the RUNX
(Runt-related transcription factor) and ETS families, CBFB, ETS1, and ELF1, were shown to predominantly occupy quiescent Th cell enhancers. Surprisingly, the majority of FOXP3 binds to both already established and Treg polarized enhancers. Only 2% of FOXP3 binding sites appear to be Treg-specific and are highly enriched for an AP-1 motif. In some cases, FOXP3 was found to displace its paralog, FOXO1, or other cofactors that served as placeholders for FOXP3 binding. Furthermore, during Treg polarization and TCR activation, the SDTF, FOXP3, is primarily recruited to previously established enhancers likely though AP-1 and nuclear factor of activated T-cells (NFAT) facilitated chromatin remodeling. A second study describing the transcriptional network for Th17 polarization found two previously established LDTFs, BATF and IRF4, to exhibit similar binding patterns between quiescent Th cells and Th17 cells. In the presence of Th17-polarizing cytokines, BATF and IRF4 prime pre-established enhancers for SDTF (RORG, STAT3, and MAF) recruitment. Lastly, to define the active enhancer landscape after Th1 or Th2 polarization, a comprehensive analysis of EP300 binding was conducted in the two subsets. The two closely related cell subsets display common and distinct EP300 binding. Moreover, STAT6 and GATA3 or STAT4/STAT1 and TBX21 were found to activate Th2 or Th1 subset-specific enhancers, respectively, while suppressing those of alternative fates.

*Embryonic Stem Cells, Pro-B cells, Myotubes*
Genome-wide binding analyses were conducted for three unique cell types, embryonic stem cells, myotubes, and pro-B cells, each in the quiescent state and after TGFβ signaling. The authors found that the LDTFs for these respective cell types, POU5F1, MYOD1, and PU.1, directed the TGFβ-induced SDTF, SMAD3, to unique cell-specific enhancer sites. Motif-finding at SMAD3 bound sites showed an enrichment for LDTF motifs specific to the cell type in question, highlighting the importance of LDTFs in directing the binding of SDTFs.

Breast, Prostate, and Osteoblasts

FOXA1 is essential for the development and differentiation of several organs such as liver, kidney, pancreas, lung, prostate, and mammary gland. In breast cancer epithelial (MCF7) cells and prostate cancer (LNCaP) cells, FOXA1 creates a cell-specific enhancer network, which regulates other activating factors such as ESR1 and AR, respectively. In estrogen-responsive breast cancer cells, tamoxifen-mediated ESR1 activity is dependent on LDTF FOXA1. In tamoxifen-resistant cells, ESR1 binding occurs independent of ligand but is still dependent on FOXA1. Another transcription factor implicated in breast cancer oncogenesis, TFAP2C, binds to ESR1-binding sites in a ligand-independent manner, co-localizing with FOXA1, and priming enhancer sequences for ESR1 binding. Furthermore, treatment with estradiol results in a global increase of enhancer RNA (eRNA) transcription at enhancers in the vicinity of 17β-estradiol regulated genes,
which promote specific E2/ESR1/eRNA-induced enhancer-promoter looping

44. In addition, a recent study conducted in a murine mammary epithelial cell line found AP-1 to be an essential LDTF, mediating GR-chromatin interactions and GR-regulated transcription 39.

In U2OS cells, an osteoblast-like cell line, ESR1 binding is preceded by the lineage determining factor, GATA4 58. GATA4 is necessary for H3K4me2 deposition at ESR1 binding sites, suggesting that it is a LDTF for ESR1 recruitment in this cell type 58. Furthermore, these results illustrate the cell-specific nature of priming and activating transcription factors; future studies will help clarify the differences amongst diverse cell types.

F. Histone modifications associated with active and poised regulatory elements

LDTFs and SDTFs bind at both enhancer and promoter elements to regulate transcription. Enhancers and promoters are associated with distinct chromatin signatures - active promoters are marked by high levels of histone H3 lysine 4 tri-methylation (H3K4me3) relative to mono-methylation (H3K4me1), whereas enhancers are marked by high levels of H3K4me1 relative to H3K4me3 59 (Figure 1.2). H3K4me2 occurs at both promoter and enhancer regions 15, which we define here by their distance from a transcription start site (around <2kb to indicate a promoter and >2kb to indicate an enhancer). Studies in yeast, Drosophila, and humans suggest that
the SET domain-containing methyl-transferases are responsible for depositing the majority of H3K4me3 at promoters.\textsuperscript{60-63}

Mono-methylation at enhancers is dynamic and regulated in a cell-specific manner. Currently, methyl-transferases regulating H3K4me1 at mammalian enhancers include MLL1\textsuperscript{64} and MLL1/3\textsuperscript{15}. At enhancer loci, the H3K4me1 mark functions as an active mark; its de-methylation in mouse embryonic stem cells by LSD1 results in enhancer silencing and cell differentiation. The process by which H3K4me1 is lost at enhancers is called enhancer “decommissioning” and further illustrates the fine tuning mediated by cell-specific enhancers.\textsuperscript{65} Not surprisingly, loss of LSD1 is associated with increased H3K4me1 and H3K4me2 at hematopoietic stem cell promoter and enhancer elements as well as subsequent gene de-repression, resulting in altered transcription and compromised differentiation.\textsuperscript{66}

While the presence of H3K4me2 dictates active transcriptional regions, active enhancers are also marked by EP300 (or CBP) histone acetyltransferase\textsuperscript{59,67} (Figure 1.2). EP300/CBP acetylates H3K27\textsuperscript{68-70}, a mark for active transcription, which distinguishes active enhancers from poised or disengaged enhancers\textsuperscript{71-74} in human and mouse embryonic stem cells\textsuperscript{71,73,74}, as well as adult tissues\textsuperscript{71,72,74}.

In contrast to active acetylation marks, the combination of H3K4me3 and H3K27me3 marks promoters that are “poised” for transcription\textsuperscript{75} (Figure 1.2). In mouse and human embryonic stem cells, poised enhancers are marked by H3K4me1, EP300, SMARCA4, and H3K27me3, and are bound by
the Polycomb complex. Unable to drive gene expression, poised enhancers transition into active enhancers during differentiation through the acquisition of H3K27ac and the loss of H3K27me3, as well as the recruitment of tissue-specific transcription factors and RNA polymerase II. Furthermore, poised enhancers in hematopoietic stem cells contain H3K27me1 and H3K9me1 prior to their activation, whereas promoters in embryonic stem cells, hematopoietic stem cells, and adult tissues, all contain H3K27me3 and may contain other repressive marks such as H3K20me3 (Figure 1.2).

G. Signal-dependent control of the epigenetic landscape

Investigation of macrophage differentiation and activation has provided a powerful experimental system for linking the actions of LDTFs and SDTFs to specific histone modifications and subsequent transcriptional output. Macrophage gene expression is stimulus-specific, gene-specific, cell-specific, and macrophage-subset specific. In early genome-wide studies to study the stimulus-specific nature of gene expression, microarrays were used to systematically examine the response of macrophages to various bacterial pathogens that act through TLRs, comparing the shared and differential transcriptional output. These studies were the first to document the very dramatic transcriptional responses of macrophages to these signals, and revealed that these responses were finely tuned to specific pathogens.

Several lines of evidence indicate that the program of macrophage activation is subject to many types of negative regulation that act prior and/or
subsequent to stimulus responses. BCL6 has been shown to co-repress almost a third of the TLR4 cistrome, such that its loss results in hypersensitivity to pro-inflammatory de-repression. The NCOR1/HDAC3 co-repressor complex has been proposed to function as a transcriptional checkpoint for some TLR-responsive genes under basal conditions through recruitment to non-phosphorylated forms of AP-1. The HDAC3 component of this complex contributes to the repressive functions of the NCOR1 complex by removing histone acetylation marks required for transcriptional activity (Figure 1.3). Activation of inflammatory genes necessitates signal-dependent phosphorylation of c-Jun and removal of NCOR1 from AP-1 target genes. Surprisingly, loss of HDAC3 in macrophages results in decreased activation of almost half of the inflammatory program. This phenomenon has been suggested to be the result of decreased Ifnb1 expression and the secondary STAT1-mediated transcriptional response, a pre-requisite for inflammatory induction. In addition to HDAC3, NCOR1 complexes in macrophages also contain the histone methyl-transferase SMYD5, which contributes to repression by catalyzing H4K20 tri-methylation, inhibiting the expression of TLR4 target genes. Signal-dependent de-methylation of H4K20me3 at promoters occurs through the recruitment of the histone de-methylase PHF2 by the RELA component of NF-κB (Figure 1.3).

After co-repressor dismissal from inflammatory genes, transcriptional activation in response to TLR4 signaling occurs in two phases – primary response genes are immediately up-regulated in response to stimuli whereas
secondary response genes require expression of gene products from the initial wave of transcription, such as the type I interferons. CpG islands are usually found at the promoters of primary response genes and associate with nucleosome-destabilizing elements, allowing these genes to be transcribed at low levels in the basal state. This low level of transcription produces nonfunctional transcripts that are rapidly degraded until the recruitment of the positive transcription elongation factor (P-TEFb) complex, which phosphorylates RNA polymerase II and couples transcriptional initiation with elongation \(^{82,83}\). In contrast, promoters with low CpG content are indicative of secondary response genes, which undergo stimulus-dependent H3K4me3 and H3 acetylation, requiring selective nucleosome remodeling \(^{82,83}\). Inflammatory genes in macrophages can be further classified into two categories on the basis of function and regulation – genes capable of being induced after stimulation of tolerant macrophages and those that will not be induced during re-stimulation. Genes capable of being re-stimulated exhibit more H4 acetylation and maintain H3K4me3 after re-stimulation \(^{84}\). Like the genes in naïve macrophages, these genes are capable of recruiting the SMARCA4 and CHD4 chromatin remodeling complexes to their promoters; however, they exhibit different transcriptional requirements than those in naïve macrophages \(^{84}\).

Macrophage activation results in the sequential binding of lineage-determining and stimulus-activated transcription factors to cell-specific regions, enabling the novel deposition of cis-regulatory enhancer marks such
as H3K4me1 and H3K27ac\textsuperscript{12, 15, 27}. In one study, the authors removed the stimuli post-treatment in a “washout” experiment. Surprisingly, H3K4me1 did not revert to its latent state; instead, it persisted and conditioned a faster and stronger response upon re-stimulation, suggesting the existence of epigenetic memory in response to stimuli exposure\textsuperscript{12}.

Recent studies have reported the productive transcription of RNA polymerase II-associated non-coding RNA\textsuperscript{85} (ncRNA) from cis-regulatory enhancers located both intragenically\textsuperscript{86} and intergenically in response to LPS stimulation\textsuperscript{15}. TLR4 signaling regulates macrophage gene expression through both a pre-existing enhancer landscape as well as the induction of ~3000 enhancer RNAs (eRNAs) from \textit{de novo} enhancer regulatory regions (\textit{de novo} eRNAs)\textsuperscript{15} (Figure 1.1). In all, ~2200 \textit{de novo} eRNAs are induced >2 fold in response to TLR4. Regions that show eRNA induction also gain H3K4me2 and are enriched for AP-1, NF-κB, CEBP, IRF/STAT, and PU.1 motifs\textsuperscript{15}. Surprisingly, the inhibition of RNA polymerase II elongation and eRNA synthesis at enhancers resulted in decreased H3K4me1 and H3K4me2 deposition without affecting H4K8ac\textsuperscript{15}. This result suggests that enhancer transcription precedes and may be essential for H3K4me1/2 deposition, at least for the set of enhancers that are established \textit{de novo} following TLR4 activation. Systemic depletion of all known H3K4 methyl-transferases indicated that TLR4-induced H3K4 methylation was dependent upon the histone methyl-transferases, MLL1, MLL2/4, and MLL3\textsuperscript{15}. 
H. Functional roles of enhancer RNAs

It has been proposed that transcription at enhancers maybe due to non-specific interactions of RNA polymerase II with the genome, thus representing noise rather than biological function \(^{87}\). Others have speculated that the functional effects of enhancer transcription are due to the transcription process and machinery or RNA polymerase II moving unobstructed in either direction upon binding DNA. In contrast, studies have shown that in vivo developmental enhancers have highly conserved non-coding elements \(^{88, 89}\), positing that developmental enhancers may have retained their function throughout evolution.

Enhancer RNAs are believed to exert their function through two main mechanisms: in \textit{cis}, the eRNAs may act on the same chromatin fiber or in \textit{trans}, the eRNAs may function at distant sites in the genome. Recently, in macrophages, Rev-Erbs were described to repress key inflammatory genes through direct promoter-mediated mechanisms as well as in \textit{cis} through eRNAs \(^{14}\). Primarily, Rev-Erbs bind at distal elements, which are marked by PU.1 co-binding and H3K4me1 \(^{14}\). The binding of Rev-Erbs at enhancer elements inhibits enhancer mediated transcription at two distal \textit{cis} regulatory elements, subsequently decreasing transcription at the nearby protein coding genes, \textit{Mmp9} and \textit{Cx3cr1} \(^{14}\). Genetic studies depleting either Rev-Erbs from these loci or the eRNAs themselves resulted in the subsequent de-repression of \textit{Mmp9} and \textit{Cx3cr1} mRNA \(^{14}\). Similar studies in multiple cell types have found the transcription of ncRNAs, especially eRNAs, to be essential in
promoting and inhibiting gene expression. Further studies will help elucidate the cell-specific mechanisms of enhancers in regulating diverse facets of transcription.

I. Conclusions

In concert, these studies suggest a general model to explain how genes with a broad distribution of expression can be regulated in a cell-specific manner and how broadly expressed SDTFs are capable of regulating discriminative responses in different cell types. Traditionally, the regulation of transcription is thought to occur primarily at the promoter. The above described studies show that LDTFs collaboratively select and prime distal enhancer regulatory elements in a cell-specific manner, genome-wide. In a signal-dependent manner or during development, LDTFs may remodel and open chromatin at promoter and enhancer regions, broadly defining the regulatory potential of genes in a cell-specific manner. For example, PU.1 binds to more than two-thirds of enhancer-like regions in macrophages, priming target genes for the subsequent recruitment of SDTFs to their vicinity, and their subsequent activation. Thus, although transcription can occur at both promoters and enhancers – transcription at promoters is often ubiquitous amongst different cell-types while that at enhancers tends to be cell-specific.

Furthermore, genetic analyses have shown that mutations of SDTF motifs can only account for a small percentage of the variable gene expression that is the result of genetic variation. Understanding how mutations in
LDTF and SDTF motifs result in direct and indirect effects on enhancer selection and function is essential to defining relevant non-coding variants in the genome and their impact on human disease \(^{51}\). Comprehension of how key lineage determining transcription factors modulate signal dependent mechanisms in different cell types may also have cell-specific therapeutic applications. Many human disease states could benefit from cell-specific alterations in gene expression in a manner that would decrease toxicity and increase efficacy. New technologies such as antisense oligonucleotides and small interfering RNAs can be potentially used to modulate cell-specific eRNA transcripts that are associated with malfunctioning genes \(^{14}\). In principle, this methodology would enable the cell-specific regulation of aberrant gene expression implicated in disease states such as cancer and chronic inflammatory diseases without adversely modulating normal physiological expression in unaffected cell types.

**J. Key questions to be addressed**

The body’s unique ability to control inflammation is essential to the regulation of innate immunity and normal homeostatic processes. A lack of inflammatory response can result in overwhelming infection whereas inflammatory dysregulation can initiate and exacerbate diseases such as diabetes, rheumatoid arthritis, multiple sclerosis, and atherosclerosis \(^{5-7,96}\). Transcriptional co-repressor complexes nuclear receptor co-repressor (NCoR) and silencing mediator of retinoid and thyroid receptor (SMRT) are vital to the
regulation of inflammatory signaling $^{97-100}$. Nuclear receptors exert an additional level of regulation through interactions with co-repressors and other transcriptional regulatory proteins. The following work will build upon prior studies on the NCoR complex and Rev-Erb nuclear receptor family in the Glass laboratory. NCoR is precisely regulated by protein kinases, nuclear receptors, and other modifying enzymes, as well as functions as a histone deacetylase and methyltransferase to establish repression and de-repression of specific pro-inflammatory promoters $^{101}$. Moreover, the Rev-Erb nuclear receptor family consists of two members, Rev-Erbα and Rev-Erbβ (Rev-Erbs), which regulate the expression of genes involved in the control of circadian rhythm $^{102,103}$, metabolism $^{104-107}$, and inflammatory responses $^{108,109}$.

Our laboratory’s unique approach in this field has been guided by the study of the transcriptional basis of inflammatory signaling, more specifically, the effect of nuclear receptors on toll-like receptor (TLR) signaling. We have utilized genome-wide systematic approaches, such as microarrays, poly(A) RNA-Sequencing (RNA-Seq), Global Run-on Sequencing (GRO-Seq)$^{52,53}$, and chromatin immunoprecipitation with parallel DNA sequencing (ChIP-Seq), which have allowed us to recognize that various transcription factors use distinct molecular mechanisms to regulate repression, and that these mechanisms are targeted to both overlapping and distinct sets of genes. One such mechanism of repression is mediated by the NCoR complex, whose de-repression is modulated in both a signal-specific and gene-specific manner $^{100}$. Rev-Erbs and Coronin 2A (Coro2A) are both associates of the NCoR complex
that remain inadequately characterized \(^{110, 111}\). To date, no members of the Coronin family have been characterized in the nucleus \(^{112}\). Studies in our laboratory have shown that Coro2A binds oligomeric actin in the nucleus and its presence is required for de-repression of inflammatory response genes \(^{113}\). In contrast to Coro2A, Rev-Erb\(\alpha\) and Rev-Erb\(\beta\) (Rev-Erb\(\alpha\)s) are sequence specific transcription factors that recruit the NCoR complex to specific target genes thus mediating transcriptional repression. Surprisingly, we found that Rev-Erb\(\alpha\)s bind primarily at distal enhancer elements in macrophages that are highlighted by co-bound spleen focus forming virus (SFFV) proviral integration oncogene (PU.1) and histone 3 lysine 4 mono-methylation \(^{114}\). Subsequently, this distal binding decreases histone 3 lysine 9 acetylation, resulting in decreased \(Mmp9\) and \(Cx3cr1\) expression as well as changes in other genes important for macrophage homeostatic programs.

This dissertation utilizes in vivo and genome-wide approaches to investigate the roles of NCoR, Rev-Erb, and Coro2A in regulating transcription in a signal-dependent manner. Chapter 1 will review our current understanding of transcriptional regulation in mammalian cells, especially how certain cell-specific enhancers and genes are selected for transcription. Chapter 2 will characterize the new and unexpected role of Rev-Erb\(\alpha\)s in regulating signal-dependent enhancers during wound healing. Since Rev-Erb\(\alpha\)s are primarily repressors, loss of Rev-Erb\(\alpha\)s would allow for increased activation of macrophage-specific genes in a signal-dependent manner. Through genomic and in vivo studies, we unexpectedly found that loss of Rev-Erb\(\alpha\)s in
hematopoietic cells results in accelerated wound healing. Furthermore, our studies suggest that Rev-Erbs regulate a broad range of enhancers vital for wound healing and that these enhancers are neither pro-inflammatory nor anti-inflammatory. Instead, the ability of Rev-Erbs to select for a subset of “wound healing” enhancers results in the fine-tuning of macrophage-mediated responses to damaged tissue. Chapter 3 describes the generation and analysis of the Coro2A total knock-out. In this chapter, we utilize a genome-wide approach to investigate the role of Coro2A, a novel member of the NCoR complex, in mediating transcriptional regulation. Furthermore, our studies show that Coro2A and NCoR cooperate to regulate the expression of lipid biosynthetic and immune response genes. Finally, Chapter 4 will discuss the broader implications of this research as a whole.

Chapter I, in part, is a reprint of the publication as it appears in Journal of Molecular Endocrinology, 2013; Zhang, Dawn; Glass, Christopher K., “Towards an understanding of cell-specific functions of signal-dependent transcription factors”. The dissertation author was the primary author of this paper.
Table 1.1 Abbreviations and full names of transcription factors cited in this review.

<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Transcription Factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>PU.1 or SPI1</td>
<td>Spleen focus forming virus (SFFV) proviral integration oncogene SPI1</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1 (heterodimeric protein complex)</td>
</tr>
<tr>
<td>CEBPs (CEBPA and CEBPB)</td>
<td>CCAAT-enhancer-binding proteins (alpha and beta)</td>
</tr>
<tr>
<td>SOX2</td>
<td>SRY (sex determining region Y)-box 2</td>
</tr>
<tr>
<td>FOXD3</td>
<td>Forkhead box D3</td>
</tr>
<tr>
<td>JUN</td>
<td>Jun proto-oncogene</td>
</tr>
<tr>
<td>FOS</td>
<td>FBJ murine osteosarcoma viral oncogene homolog</td>
</tr>
<tr>
<td>TCF3</td>
<td>Transcription factor 3</td>
</tr>
<tr>
<td>EBF1</td>
<td>Early B-cell factor 1</td>
</tr>
<tr>
<td>FOXO1</td>
<td>Forkhead box O1</td>
</tr>
<tr>
<td>LXR</td>
<td>Liver X receptor</td>
</tr>
<tr>
<td>NR1D1 and NR1D2</td>
<td>Nuclear receptor subfamily 1, group D, members 1 and 2 (Rev-Erb alpha and beta)</td>
</tr>
<tr>
<td>RELA</td>
<td>V-rel reticuloendotheliosis viral oncogene homolog A (avian)</td>
</tr>
<tr>
<td>SRF</td>
<td>Serum response factor</td>
</tr>
<tr>
<td>STATs (STAT1, STAT3, etc.)</td>
<td>Signal transducer and activator of transcription (1, 3, etc.)</td>
</tr>
<tr>
<td>SMAD3</td>
<td>Mothers against decapentaplegic homolog 3 or SMAD family member 3</td>
</tr>
<tr>
<td>HNF4A</td>
<td>Hepatocyte nuclear factor 4, alpha</td>
</tr>
<tr>
<td>FOXA1</td>
<td>Forkhead box A1</td>
</tr>
<tr>
<td>JUNB</td>
<td>Jun B proto-oncogene</td>
</tr>
<tr>
<td>IRF4</td>
<td>Interferon regulatory factor 4</td>
</tr>
<tr>
<td>ATF3</td>
<td>Activating transcription factor 3</td>
</tr>
<tr>
<td>RUNX1</td>
<td>Runt-related transcription factor</td>
</tr>
<tr>
<td>CBFB</td>
<td>Core-binding factor, beta subunit</td>
</tr>
<tr>
<td>ETS1</td>
<td>V-ets erythoblastosis virus E26 oncogene homolog 1 (avian)</td>
</tr>
<tr>
<td>ELF1</td>
<td>E74-like factor 1 (ets domain transcription factor)</td>
</tr>
<tr>
<td>FOXP3</td>
<td>Forkhead box P3</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T-cells</td>
</tr>
<tr>
<td>BATF</td>
<td>Basic leucine zipper transcription factor, ATF-like</td>
</tr>
<tr>
<td>RORG</td>
<td>RAR-related orphan receptor C</td>
</tr>
<tr>
<td>MAF</td>
<td>V-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)</td>
</tr>
<tr>
<td>EP300</td>
<td>E1A binding protein p300</td>
</tr>
<tr>
<td>GATAA3 and GATA4</td>
<td>GATA binding protein 3 and 4</td>
</tr>
<tr>
<td>TBX21</td>
<td>T-box 21</td>
</tr>
<tr>
<td>POU5F1 (OCT4)</td>
<td>POU class 5 homeobox 1</td>
</tr>
<tr>
<td>MYOD1</td>
<td>Myogenic differentiation 1</td>
</tr>
<tr>
<td>ESR1</td>
<td>Estrogen receptor 1</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>TFAP2C</td>
<td>Transcription factor AP-2 gamma (activating enhancer binding protein 2 gamma)</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>MLL (1/3, 2/4, 3, etc.)</td>
<td>Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila) (1/3, 2/4, 3, etc.)</td>
</tr>
<tr>
<td>LSD1</td>
<td>Lysine (K)-specific demethylase 1A</td>
</tr>
</tbody>
</table>
**Table 1.2 Summary of studies demonstrating signal-dependent cell-specificity in transcription.**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Lineage-Determining Transcription Factors</th>
<th>Stimulus</th>
<th>Stimulus-Activated Transcription Factors</th>
<th>Cell-specific (LDTF-regulated) Gene Expression</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophages</td>
<td>PU.1, CEBPs, AP-1</td>
<td>LPS, GW3965 (LXR agonist)</td>
<td>LXRs, RELA, BCL6</td>
<td>Csf1r, Cd14, Ccl3, Ccl4, Cxcl2, Abcg1</td>
<td>13, 45</td>
</tr>
<tr>
<td>Dendritic cells</td>
<td>PU.1, CEBPB</td>
<td>LPS</td>
<td>JUNB, IRF4, ATF3</td>
<td>It1a, Il1f1, Il1f2, Il1f3, Tnf, Stat1, Nfkbi</td>
<td>53</td>
</tr>
<tr>
<td>ES cells</td>
<td>POU5F1</td>
<td>TGFB1</td>
<td>SMAD3</td>
<td>Sox2</td>
<td>43</td>
</tr>
<tr>
<td>Pro-B cells</td>
<td>PU.1</td>
<td>TGFB1</td>
<td>SMAD3</td>
<td>Vpreb2</td>
<td>43</td>
</tr>
<tr>
<td>Myotubes</td>
<td>MYOD1</td>
<td>TGFB1</td>
<td>SMAD3</td>
<td>Adora1</td>
<td>43</td>
</tr>
<tr>
<td>T cells</td>
<td>BATF and IRF4</td>
<td>Th17 polarization</td>
<td>RORG, STAT3, MAF</td>
<td>Il17a, Il12rb1</td>
<td>55</td>
</tr>
<tr>
<td>T cells</td>
<td>?</td>
<td>Th1 polarization</td>
<td>STAT4, STAT1, TBX21, STAT6, GATA3</td>
<td>?</td>
<td>48</td>
</tr>
<tr>
<td>T cells</td>
<td>CBFB, ETS1, ELF1</td>
<td>Treg polarization</td>
<td>FOXP3</td>
<td>Junb</td>
<td>54</td>
</tr>
<tr>
<td>MCF7 cells</td>
<td>FOXA1, TFAP2C</td>
<td>Tamoxifen, 17β-estradiol</td>
<td>ESR1</td>
<td>Ret, Xbp1, Tff</td>
<td>38, 43, 44, 57</td>
</tr>
<tr>
<td>LNCaP cells</td>
<td>FOXA1</td>
<td>5α-dihydrotestosterone</td>
<td>AR</td>
<td>Chka</td>
<td>38, 41, 42</td>
</tr>
<tr>
<td>Murine mammary epithelial cells</td>
<td>AP-1</td>
<td>Dexamethasone</td>
<td>Glucocorticoid receptor</td>
<td>Klrb1c</td>
<td>39</td>
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<tr>
<td>U2OS</td>
<td>GATA4</td>
<td>17β-estradiol</td>
<td>ESR1</td>
<td>Faslg, Wnt4, Foxc1, Alpl, Runx2</td>
<td>58</td>
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</tbody>
</table>
Figure 1.1 Hierarchical organization of transcription factor networks.
In the poised or repressed state, closed chromatin at enhancer loci contain motifs for lineage determining transcription factors. The collaborative binding of lineage determining transcription factors such as PU.1 and CEBPs results in nucleosomal remodeling and basal enhancer transcription at sites containing low H3K4me1/2 and low H3/H4 acetylation. Next, in response to various stimuli, signal-dependent transcription factors, such as LXRs, co-bind with PU.1 and C/EBPs to activate enhancer transcription. Finally, activated eRNA transcription precedes the active deposition of H3K4me1/2 and H3/H4 acetylation. LDTF: Lineage Determining Transcription Factor; SDTF: Signal-Dependent Transcription Factor.
Figure 1.2 Histone marks at active and poised promoters and enhancers. Promoters are designated by high levels of H3K4me3, while enhancers contain high levels of H3K4me1/2. In the poised or repressed state, promoters contain histone methylation marks, H3K27me3 and H3K20me3, while enhancers contain H3K27me3/me1 and H3K9me1. Active promoters and enhancers are marked by H3K4me2 and H3K27Ac. GTF: General Transcription Factor; LDTF: Lineage Determining Transcription Factor; SDTF: Signal Dependent Transcription Factor.
Figure 1.3 Mechanisms for regulating gene expression in macrophages: co-regulator exchange from co-repressor to co-activator.
In the poised or repressed state, the NCOR1/HDAC3/SMYD5 complex inhibits active transcription through de-acetylation of histone H3 and H4, as well as tri-methylation of H4K20. After signal dependent recruitment of activating transcription factors such as NF-κB, the NCOR1 complex is exchanged for co-activator complexes that remove repressive marks and acetylate histones H3 and H4, recruiting the P-TEFb complex to activate transcriptional elongation.
Chapter II:

Rev-Erbs Repress Signal-Dependent Enhancers in Macrophages Vital for Tissue Remodeling and Wound Healing
A. Abstract

Rev-Erbα and Rev-Erbβ (Rev-Erbs) are members of the nuclear receptor family of transcription factors that regulate a common program of circadian gene expression throughout the body as well as cell-specific programs of gene expression that control organ-specific functions. Rev-Erbs regulate gene expression by acting as constitutive repressors through the recruitment of NCoR/HDAC3 co-repressor complexes. Recent studies in macrophages have demonstrated that Rev-Erbs inhibit mRNA expression by repressing transcription at distal enhancers, but the biological consequences of this repressive function have not been explored. Here, we provide evidence that Rev-Erbs function in macrophages to repress genes that play essential roles in the response to wounding and tissue remodeling. Rather than reinforcing a specific program of macrophage polarization, Rev-Erbs repress subsets of genes that are activated by TLR ligands, IL4, TGFβ and damage-associated molecular patterns (DAMPS) by suppressing signal-dependent enhancer transcription. Rev-Erbs thereby integrate complex combinations of signals that are likely to be encountered in the wound environment. These findings reveal new biological functions of Rev-Erbs that have implications for understanding and potentially treating diseases characterized by defective wound repair and disordered tissue remodeling.

B. Introduction
Macrophages are myeloid-lineage cells that reside in all tissues of the body and play key roles in homeostasis, immunity, and disease. As immune cells, macrophages serve as sentinels of infection and injury and are active participants in both innate and adaptive immune responses. Detection of pathogens and tissue damage is mediated by a diverse array of pattern recognition receptors for pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs), exemplified by the toll-like receptors (TLRs). Ligation of TLRs initiates profound changes in gene expression that include induction of chemokines, cytokines, anti-microbial peptides and other factors that contribute to the innate immune response and influence adaptive immunity. This response has been extensively characterized in vitro by treating cultured macrophages with specific TLR ligands such as lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria that is a potent activator of TLR4. TLR4 ligation regulates gene expression through signal transduction pathways culminating in the activation of latent signal-dependent transcription factors, which include members of the NFκB, interferon regulatory factor (IRF), and AP-1 families. Upon activation, these factors positively regulate gene expression by binding to DNA recognition elements in the promoters and enhancers of target genes. In macrophages, these factors are primarily directed to macrophage-specific enhancers that are selected by macrophage lineage determining transcription factors, PU.1 and C/EBPs. The macrophage activation phenotype resulting from selective treatment with LPS, or in some cases a combination of LPS and
interferon γ (IFNγ), is commonly referred to as ‘classical’ or ‘M1’ activation\(^\text{115}\), and is considered vital for the host response to bacterial or viral infection.

Macrophages also play important roles in regulating the resolution phase of inflammation as well as the repair of tissue damage. These functions are controlled by alternative signaling pathways that include transforming growth factor β (TGFβ) and interleukin 4 (IL4). TGFβ is generally considered to be an inducer of a “de-activated” macrophage phenotype, although it also acts as a potent chemoattractant for monocytes and can potentiate their transition into activated cells\(^\text{116}\). Macrophages respond to TGFβ in both an autocrine and paracrine manner. For example, phagocytosis of apoptotic cells results in increased macrophage-mediated secretion of TGFβ and subsequent inhibition of inflammatory cytokine production\(^\text{116}\). Conversely, production of TGFβ by neurons and astrocytes in the brain plays an important role in maintaining the deactivated phenotype of microglia, the major CNS-resident macrophages\(^\text{117}\).

In addition to dampening inflammatory responses, secreted TGFβ plays key roles in accelerating wound healing and fibrosis\(^\text{118}\). At the transcriptional level, TGFβ signal transduction pathways function primarily in a SMAD-dependent manner through SMAD2, SMAD3, and SMAD4-mediated activation, as well as SMAD7-mediated inhibition\(^\text{119}\). Like other signal dependent transcription factors, ligation of TGFβ receptors causes the localization of SMAD3 to genomic loci containing lineage-determining transcription factors\(^\text{49}\).
Regulation of macrophage gene expression by IL4 plays roles in containment of parasitic infections and in homeostatic functions of adipose tissue. IL4 acts through the IL4 receptor to activate STAT6\textsuperscript{120}, which positively regulates gene expression upon binding to recognition elements in promoters and enhancers of target genes\textsuperscript{115}. IL4 signaling regulates genes that control tissue remodeling, phagocytosis, scavenging and the arginase pathway. The macrophage activation phenotype resulting from selective treatment with IL4 is commonly referred to as ‘alternative’ or ‘M2’ activation and is considered vital for the role of macrophages in wound repair\textsuperscript{16}.

While M1, M2 and de-activated macrophage phenotypes are clearly distinct in vitro, they result from selective activation of signaling pathways by strongly polarizing ligands. In vivo, macrophages encounter a diversity of signals that change over time as the response to infection and/or tissue damage evolves. The responses of genes downstream of TLR, IL4, TGFβ and other relevant signaling pathways must therefore be integrated to achieve coordination of immune and tissue repair activities. Nuclear receptors have been shown to play multiple immuno-modulatory roles through interactions with co-repressors and transcriptional regulatory proteins. For example, peroxisome proliferator activator receptor γ (PPARγ)\textsuperscript{121}, liver X receptors (LXRs)\textsuperscript{122}, and glucocorticoid receptors (GR)\textsuperscript{123} inhibit inflammatory stimulation through a variety of different mechanisms. Similarly, nuclear receptor related-1 protein (Nurr1)\textsuperscript{124} and estrogen receptor β (ERβ)\textsuperscript{125} have also been shown to play immuno-suppressive roles in myeloid cell types.
The Rev-Erb nuclear receptor family consists of two members, Rev-
Erbα and Rev-Erbβ (Rev-Erbs), which regulate the expression of genes
involved in the control of circadian rhythm \(^{50, 102, 103}\), metabolism \(^{104-107}\), and
inflammation \(^{108, 109}\). Rev-Erbs mediate transcriptional repression through
recruitment of the nuclear co-repressor (NCoR) and histone de-acetylase 3
(HDAC3) complex \(^{126}\). They lack the carboxy-terminal (AF2) transactivation
domain, which is classically associated with recruitment of co-activators \(^{127}\). In
addition, Rev-Erbs have been shown to bind hemin at physiological
concentrations, suggesting that this compound is a natural ligand that can
potentiate Rev-Erb-mediated repression \(^{128}\).

Genome-wide location analysis of Rev-Erbα and Rev-Erbβ in
macrophages revealed thousands of binding sites, the vast majority of which
resided at macrophage-specific enhancer like regions of the genome
established by PU.1 and other macrophage lineage determining factors \(^{129}\).

Gain and loss of function experiments indicated that Rev-Erbs function to
suppress the activities of these enhancers by repressing enhancer-directed
transcription. These studies further demonstrated that the enhancer-derived
RNAs (eRNAs) subject to negative regulation by Rev-Erbs were required for
full enhancer activity. While these studies provided insights into the functional
significance of enhancer transcription, the biological consequences of the
actions of Rev-Erbs at these distal regulatory elements were not explored.

Here, we provide evidence that Rev-Erbs repress the transcription and
function of signal-dependent enhancers that are targets of TLR, IL4, TGFβ
and DAMP signaling. Rather than exerting a pattern of repression that reinforces a particular polarization phenotype, Rev-Erbs regulate subsets of signal responsive genes that span those associated with classical M1 activation, M2 activation, and deactivated phenotypes, enriching for functions highly implicated in wound repair. Consistent with these in vitro observations, deletion of Rev-Erbs from the hematopoietic lineage in vivo results in accelerated wound repair.

C. Results

Rev-Erbs occupy signal-dependent enhancers

To investigate possible biological roles of Rev-Erbs in macrophages, we initially compared the genomic locations of Rev-Erbα and Rev-Erbβ defined by ChIP-Seq in RAW264.7 macrophages with macrophage binding sites for signal dependent transcription factors involved in M1 and M2 polarization. For this analysis, we considered 7889 high confidence binding sites occupied by both Rev-Erbα and Rev-Erbβ. Substantial co-localization was observed with the previously identified genomic locations of the p65 subunit of NFκB following macrophage activation with KDO2 lipid A (KDO2), a highly specific TLR4 ligand (Figure 2.1A). In addition, we performed ChIP-Seq analysis of the cFos and cJun subunits of AP-1 in resting and KDO2-stimulated macrophages. KDO2 treatment greatly increased the number of binding sites for both factors, and in both cases, overlap was observed with a substantial fraction of Rev-Erb binding sites (Figure 2.1A). To investigate
potential regulation of enhancers subject to regulation by M2 polarization, we performed ChIP-Seq analysis of STAT6 after treatment of macrophages with IL4 (Figure 2.1A). Co-localization was also observed with a substantial fraction of Rev-Erb binding sites. These findings suggest the possibility that Rev-Erbs regulate enhancers involved in M1, as well as M2 polarization states.

Since Rev-Erbs bind primarily to distal elements in macrophages, we sought to define the characteristics of these regions by considering 24471 regions delineated by histone 3 lysine 27 acetylation (H3K27Ac), a mark defining the cell-specific presence of an active enhancer, as well as 683 super-enhancers in macrophages. Originally defined as 50kb regions of the genome highly enriched for clusters of enhancers, lineage determining transcription factors, and Mediator, super-enhancers play important roles in maintaining cell-specificity and cellular identity. Recent advances have shown that the presence of significant H3K27Ac enrichment can be used as a surrogate marker for super-enhancers. Since H3K27Ac was found at a substantial number of genomic loci co-bound by Rev-Erb and signal-dependent transcription factors involved in M1 and M2 polarization (Figure 2.1A), we also overlaid these loci with regions delineating macrophage super-enhancers. Surprisingly, almost all of the super-enhancer regions contained genomic loci co-bound by Rev-Erb and M1/M2 signal dependent transcription factors while a minority of sites was enriched for signal-dependent transcription factors alone (Figure 2.1A). This significant co-localization shows
that Rev-Erb-bound enhancers may be considerably involved in conferring differential polarization states to macrophages.

**Deletion of Rev-Erbα and Rev-Erbβ in macrophages results in enhanced signal-dependent transcription at a specific subset of gene loci**

To study the phenotypic contribution of Rev-Erbs to signal-dependent gene expression in macrophages, we performed RNA-Sequencing (RNA-Seq) of poly(A) mRNA isolated from wild type macrophages and those deficient for both Rev-Erbα and Rev-Erbβ double knockout (DKO) macrophages were generated from bone marrow differentiation of Tie2-Cre Rev-Erbα flox/flox Rev-Erbβ flox/flox (Rev-Erb DKO) animals and compared to control macrophages derived from Cre-negative littermates (WT) in the basal state, as well as after polarization to different states. Deletion of Rev-Erbβ exons to generate a non-functional Rev-Erbβ mRNA results in marked de-repression of Rev-Erbα expression and increased expression of a non-functional Rev-Erbα mRNA. Similar effects can be seen following deletion of Rev-Erbα exons with respect to Rev-Erbβ de-repression. Therefore, measurements of Rev-Erb mRNAs are misleading unless they are specifically assayed for the mRNA segments corresponding to deleted exons. To do this, we quantified RNA-Seq tag counts associated with the floxed exons in WT and Rev-Erb DKO macrophages, and calculated the relative Cre-excision efficiency. From these measurements,
reduction of functional Rev-Erb mRNAs averaged 76% for Rev-Erb α and 78% for Rev-Erb β (Figure 2.8).

We observed that ligation of TLR3 with polyinosinic:polycytidylic acid (PolyIC), TLR4 with KDO2, TLR1/2 with Pam3CSK4 (Pam3), as well as co-treatment with KDO2 and interferon γ (IFNγ) induced a characteristic pro-inflammatory gene signature (FDR < 0.05, >1.5-fold change compared to basal state, Figure 2.1B, D; Figure 2.9A) in both WT and Rev-Erb DKO macrophages. In contrast, interleukin 4 (IL4) or transforming growth factor β (TGFβ) stimulation of macrophages resulted in the expected alternatively activated and de-activated gene profiles, respectively (FDR < 0.05, >1.5-fold change compared to basal state, Figure 2.1C, D; Figure 2.9A).

Comparing the gene expression signature from WT and Rev-Erb DKO macrophages, a general trend emerged where genes de-repressed or repressed in Rev-Erb DKO macrophages retained a similar profile under different conditions (Figure 2.1C). However, for the majority of genes, the magnitude of differential expression of WT and DKO macrophages varied depending on the polarization state, in some cases only being observed under basal conditions, and in other cases only observed in response to stimulus (Figure 2.1C). Gene ontology analysis of mRNAs exhibiting differential expression (>1.5-fold, FDR < 0.05) in at least one condition unexpectedly revealed significant enrichment for genes involved in the response to wounding (Figure 2.1E, Figure 2.9B).
This enrichment for functional annotations linked to a wounding response led us to devise a DAMP preparation from a homogenate of skin tissue that would directly mimic damage signals present in a wound. This tissue homogenate was used to stimulate WT and Rev-Erb DKO macrophages for 6 and 24 hours; subsequently, RNA-Seq analysis was performed of the corresponding macrophage mRNA. The response to tissue homogenate overlapped with, but was distinct from, that observed in response to treatment with TLR agonists, IL4, or TGFβ (Figure 2.1B). Notably, although the dynamic range of altered gene expression in WT macrophages treated with tissue homogenate was somewhat less than that observed for saturating concentrations of single polarizing ligands (Figure 2.1C, D), this response resulted in the most differentially expressed genes between WT and DKO macrophages (Figure 2.1D). Including the tissue homogenate treatment, 810 genes were significantly de-repressed at least 1.5-fold in DKO macrophages in one or more conditions, and 753 genes were significantly down-regulated at least 1.5-fold in one or more conditions (FDR < 0.05, Figure 2.1C). Gene ontology analyses of this expanded set of de-repressed or repressed genes in Rev-Erb DKO macrophages further reinforced the functional relationship of differentially expressed genes to the wounding response (Figure 2.1E, Figure 2.9B).

Genes de-repressed in Rev-Erb DKO macrophages treated with tissue homogenate are illustrated in Figure 2.2A. These genes segregate into broad categories with functional annotations that include extracellular matrix,
chemotaxis, pattern binding, cell adhesion, peptidase/protease inhibition, scavenger receptor, fatty acid biosynthetic process, apoptosis, and transcription. Classically, tissue injury of the skin, muscle, or organ systems induces an initial local inflammatory response, which is followed by subsequent regenerative processes involving macrophages and other immune cells, as well as mesenchymal stem cells. To investigate the extent to which responses of macrophages to skin tissue homogenate are observed in the setting of skin wound responses, we performed temporal transcriptomic analysis of biopsied wounds compared to unwounded skin. Although myeloid cells represent only a small fraction of the total cells analyzed in the wound biopsy, 280 genes exhibited concordant changes in expression (p-value < 0.0001, Pearson's Chi-Square Test, Figure 2.2B-C). Gene ontology analysis of the 280 overlapping genes between biopsied wounds and macrophages stimulated with tissue homogenate found enriched biological process terms related to the wounding response, immune response, and leukocyte migration (Figure 2.2D). Unsupervised clustering of these genes indicated the closest relationship of the 6h homogenate treated macrophage transcriptome with day 1 and day 4 wound transcriptomes (Figure 2.2B).

**Rev-Erb DKO macrophages span a M1, M2, and deactivated macrophage phenotype**

We next compared the responses of wild type macrophages to skin tissue homogenate, IL4, TGFβ, or KDO2. As expected, relatively little overlap
in induced mRNAs was observed between macrophages treated with IL4 or KDO2, consistent with their distinct activation states (Figure 2.3A). Of the ~900 genes activated by tissue homogenate, ~300 overlapped with genes activated by KDO2 and/or IL4, with a slight preference for IL4 induced genes (Figure 2.3A). Similarly, relatively little overlap is observed for the mRNAs induced by TGFβ and those induced by IL4 and KDO2 (Figure 2.10A-C), with the response to tissue homogenate capturing a subset of the TGFβ induced genes. Thus, the response to the tissue homogenate DAMP signal contains elements of the responses of macrophages to ligands that selectively promote M1, M2 and de-activated phenotypes.

Conversely, we compared the responses of genes de-repressed in DKO macrophages to tissue homogenate, IL4, KDO2, and TGFβ. Of the 810 genes de-repressed in the basal state and/or post-activation with different stimuli, 165 are induced by KDO2, 136 by IL4, 102 by homogenate, and 82 genes overlap the three distinctive polarization states (Figure 2.3B). Additionally, 123 de-repressed genes are activated by TGFβ (Figure 2.10D-F).

To further evaluate the relationship between Rev-Erb target genes and M1 or M2 polarization, we plotted expression values for the genes de-repressed in the basal state and after treatment with KDO2, IL4, or tissue homogenate on a continuum representing the M1/M2 activation states. This analysis indicated that the pattern of de-repression is largely independent of M1/M2 polarization (Figure 2.3C-F). For example, in the basal state and after KDO2 treatment, the de-repressed genes cluster in the bottom left quadrant, which do not
correspond to either a traditional M1 or M2 phenotype, but rather a mixed phenotype \((p < 0.04\) and \(p < 0.03\), respectively, Pearson’s Chi-Square Test, Figure 2.3C). However, after tissue homogenate stimulation, de-repressed genes cluster more significantly in the top left and right quadrants \((p < 0.0001\) and \(p < 0.003\), respectively, Pearson’s Chi-Square Test, Figure 2.3E). This result suggests that Rev-Erb DKO macrophages respond more readily to a distinctive tissue injury-like inflammatory state.

**Rev-Erb repression is regulated in a signal-dependent manner**

Deletion of Rev-Erbα/β from macrophages resulted in both increased and decreased gene expression (Figure 2.1B, D). As Rev-Erbs are considered to be dedicated repressors, the mechanisms responsible for reduced gene expression are unclear, but most likely result from indirect effects of Rev-Erb deletion. In contrast, previous studies demonstrated that the loss of binding of Rev-Erbs from enhancer like elements throughout the genome was associated with increased enhancer transcription and increased expression of nearby genes \(^{129}\), consistent with their established repression functions. Notably, the patterns of de-repression observed in Rev-Erb DKO macrophages varied under different treatment conditions, suggesting that the repression activities of Rev-Erbs can be over-ridden in a signal-dependent manner.

Examples of context-dependent repression of Rev-Erb target genes are provided by the Cx3cr1, Mmp9, Arg1 and Il1b genes, each of which exhibit
transcriptional responses to one or more polarizing ligands and all of which are
induced by treatment with tissue homogenate at 6h and/or 24h (Figure 2.4A-D). *Cx3cr1* is strongly repressed by traditionally inflammatory stimuli, and
induced by TGFβ (Figure 2.4A), providing an example of a gene induced by
this de-activating signal. *Mmp9* is activated by KDO2 alone, but strongly
repressed by the combination of KDO2 and IFNγ (Figure 2.4B). *Arg1*, a gene
characteristically expressed by M2 macrophages, is strongly induced by IL4
(Figure 2.4C). *Il1b*, a gene that is characteristically expressed by M1 polarized
macrophages, is strongly induced by KDO2 (Figure 2.4D). Each gene exhibits
a nearby enhancer-like region that is occupied by Rev-Erbα and Rev-Erbβ in
concert with their obligate co-repressors NCoR/SMRT. These binding sites
also co-occur with macrophage lineage-determining factors, such as PU.1,
and signal dependent transcription factors. For example, one of the two
enhancer-like regions in the vicinity of *Arg1*, which is induced by IL4, is
occupied by STAT6 in IL4 treated macrophages (Figure 2.4C). Similarly, three
of the enhancer-like regions occupied by Rev-Erbs in the vicinity of the *IL1b*
gene are also occupied by the p65 subunit of NFκB in macrophages treated
with KDO2 (Figure 2.4D).

Notably, comparisons of WT and Rev-Erb DKO macrophages under
different treatment conditions indicate that Rev-Erb repression of each of
these genes is often context dependent. While *Cx3cr1* and *Mmp9* were de-
repressed in Rev-Erb DKO macrophages under all treatment conditions, the
magnitude of de-repression varied. In the case of *Arg1*, marked de-repression
was observed in the setting of activation by tissue homogenate and KDO2/IFN$\gamma$ treatment, but not under maximal stimulation by IL4 treatment (Figure 2.4C). Similarly, Il1b de-repression was observed in the context of treatment with tissue homogenate and KDO2/IFN$\gamma$, but not in the setting of the stronger induction resulting from treatment with Pam3 (Figure 2.4D). In concert, these results suggest that the ability of Rev-Erbs to repress target gene expression is dependent on cellular context and the range of enhancers that respond to the diverse signal dependent transcription factors activated by tissue injury and other stimuli.

**Rev-Erbs repress signal-dependent enhancer transcription**

Prior studies in macrophages suggest that Rev-Erbs primarily inhibit target gene expression by repression of nearby enhancer transcription \(^{129}\). However, these studies were carried out in resting macrophages and the ability of Rev-Erbs to regulate signal-dependent enhancer transcription was not evaluated. To examine this question, we evaluated mechanisms by which Rev-Erbs repress signal-dependent activation of Cx3cr1. Rev-Erbs primarily repress Cx3cr1 expression by binding to an enhancer element located 28kb downstream of the Cx3cr1 transcriptional start site (color coded yellow in Figure 2.4A). This enhancer element produces enhancer RNAs that are required for basal Cx3cr1 expression. A composite of genomic data at the Cx3cr1 28kb enhancer is indicated in Figure 2.5A. Transcriptional start sites of + and – strand eRNAs are illustrated by 5’GRO-Seq reads, which measure the
points of initiation of 5’ capped nascent RNA transcripts. Notably, treatment with TGFβ and the milder tissue injury stimuli induces Cx3cr1 eRNA (Figure 2.5B-C) and mRNA transcription (Figure 2.4A, Figure 2.5C).

To isolate the enhancer contributions to transcriptional regulation at the Cx3cr1 genomic loci (Figure 2.5A), we utilized luciferase constructs containing either the 28kb enhancer element or a random genomic sequence. The Rev-Erb binding site in the Cx3cr1 28kb enhancer is also recognized by RAR-related orphan receptors (such as RORα in macrophages), which are transcriptional activators that frequently function in an antagonistic manner to Rev-Erbα. Consistent with this, expression of RORα substantially increases the basal activity of the 28kb enhancer element (Figure 2.5B). Stimulation with TGFβ or tissue injury further induces Cx3cr1 enhancer activity while over-expression of Rev-Erbα or Rev-Erbβ inhibits luciferase activation. Conversely, over-expression of dominant-negative Rev-Erbα or Rev-Erbβ DNA binding mutants results in the de-repression of the Cx3cr1 enhancer loci. In contrast, the same enhancer-luciferase reporter construct containing a random 1kb segment of DNA in place of the 28kb enhancer element was not influenced by RORα, Rev-Erbα/β, or TGFβ/DAMP treatment. In concert, these observations indicate that the 28kb enhancer responds to TGFβ and DAMPs in a manner that is subject to repression by Rev-Erbα.

To assess whether regulation of the 28kb enhancer by TGFβ is functionally linked to regulation of Cx3cr1, we quantified the effects of TGFβ treatment on the 28kb enhancer eRNAs. Prior studies indicated that the
minus strand eRNA contributes to the activity of this enhancer and is required for normal basal expression of the Cx3cr1 mRNA. TGFβ treatment was found to significantly increase the transcription of the minus strand eRNA coordinately with an increase in the expression of the Cx3cr1 mRNA (Figure 2.5C). Furthermore, in primary macrophages, knock-down of Cx3cr1 eRNA using antisense oligonucleotides in both the basal state and after TGFβ stimulation resulted in decreased expression of Cx3cr1 mRNA (Figure 2.5C). This result strongly supports a direct functional connection between the activity of the 28kb enhancer and TGFβ response of the Cx3cr1 promoter. In addition, these results suggest that Rev-Erbs directly repress transcription of Cx3cr1 eRNAs to inhibit signal dependent activation of Cx3cr1.

**Rev-Erb deficient animals display enhanced wound closure in a full thickness wound healing model**

Many lines of evidence indicate that macrophages are essential during the immune phase of wound healing – helping to secrete pro-inflammatory and growth factors during early phases, and remodeling tissue during later phases of the tissue injury response. Rev-Erb DKO macrophages exhibit increased wound responses to tissue injury, showing selective de-repression of a number of genes that are essential for the wound healing process. For example, M2 and de-activated macrophage genes, Arg1 and Cx3cr1, as well as M1 genes, Il1a and Il1b. Interestingly, loss of these Rev-Erb target genes, for instance, Cx3cr1 and Arg1, have been shown to hinder efficient wound
healing in mice\textsuperscript{134,135}. These observations therefore raised the possibility that Rev-Erb deficient animals might display an accelerated response during wound healing.

In order to test the hypothesis that Rev-Erbs influence wound healing, we utilized a full thickness wound healing model in mice following bone marrow reconstitution with either WT or Rev-Erb DKO bone marrow (Figure 2.11A). Bone marrow transplant efficiencies were ascertained using both flow cytometry of circulating leukocytes and RT-qPCR of bone marrow cells from transplanted mice. By each method, reconstitution efficiency exceeded 95% (Figure 2.11B-C). From three independent experiments (N = 28-132 wounds per genotype, p-value < 0.0005), we consistently found that Rev-Erb deficiency in bone marrow derived hematopoietic cells resulted in accelerated wound closure (Figure 2.6A-B). The difference in wound healing between WT and DKO bone marrow transplanted animals was especially apparent on Days 1-5 post-injury (Figure 2.6A), suggesting that Rev-Erb deficiency results in a faster response during the immune phase of wound injury.

Blinded histological analysis of the samples found that animals transplanted with Rev-Erb DKO bone marrow exhibited enhanced re-epithelialization and increased granulation tissue development (Figure 2.6C-E), characteristics correlated with an accelerated immune response during wound healing. In addition, Rev-Erb DKO bone marrow transplanted mice displayed significantly more macrophages at the wound site on Days 4 and 6 post-injury (Figure 2.7A-C), while neutrophil persistence at the wound site
remained similar between WT and Rev-Erb DKO transplanted mice (Figure 2.7D-E). These analyses correspond with histological observations that wounds from the Rev-Erb DKO mice display greater immune cell infiltration and faster wound healing progression (Figure 2.6C). Moreover, matrigel migration assays show increased extravasation of Rev-Erb DKO macrophages when compared to their WT counterparts (Figure 2.7F), demonstrating that the increased migration of the macrophages may be cell autonomous. Furthermore, matrigel is a complex extracellular matrix that contains many growth factors including TGFβ, a cytokine that potently induces Cx3cr1 in WT and Rev-Erb DKO macrophages (Figure 2.5A-C). Therefore, the increased persistence of Rev-Erb DKO macrophages at the wound site could be mediated by various de-repressed genes involved in tissue extravasation or extracellular matrix remodeling (Figure 2.2A) such as Cx3cr1 (Figure 2.4A) or Mmp9 (Figure 2.4B). Taken together, these results suggest that Rev-Erb DKO bone marrow transplanted animals display enhanced wound closure and increased macrophage persistence at the wound site.

D. Discussion

The present studies have utilized in vivo and genomic approaches to investigate the biological roles of Rev-Erbs in the regulation of macrophage gene expression. Although Rev-Erbs have been established to regulate the promoters of ubiquitously expressed genes such as Bmal that control circadian rhythms, the great majority of Rev-Erb binding sites in macrophages
are located at cell-specific enhancers that are selected by macrophage lineage determining factors such as PU.1. These observations predicted that in addition to cell autonomous regulation of circadian rhythm, Rev-Erbs would also regulate a macrophage-specific program of gene expression. Here, using loss of function and transcriptome analysis, we demonstrate that Rev-Erbs function to repress a network of genes associated with the response to wounding. Consistent with this, loss of Rev-Erb expression in cells derived from the bone marrow compartment results in an accelerated wound healing phenotype.

The proper control of wound healing is an essential homeostatic process mediated by diverse cell types such as epidermal cells, endothelial cells, as well as cells of the hematopoietic lineage, which are recruited by inflammatory mediators. Wound healing can be summarized grossly by four phases; hemostasis, inflammation, proliferation, and remodeling\textsuperscript{136, 137}. Immediately following wounding, thrombocytes immediately aggregate at the site of injury to form a fibrin clot. Injury also induces the secretion of inflammatory cytokines and chemokines, often mediated by injured cells such as keratinocytes and fibroblasts, which recruit immune cells such as macrophages, neutrophils, dendritic cells, and T cells to the injury. Previous studies have described that the recruitment of innate immune cells to wounds can be broadly classified into two stages, the “pro-inflammatory” stage (lasting hours to days) and the “wound healing” stage (lasting days to weeks), which are primarily dictated by the macrophage subtypes recruited into the wounds.
at these time points \(^{135, 138, 139}\). Early on, immune cells such as macrophages and neutrophils phagocytose debris and bacteria \(^{20, 132}\). Later, neutrophils are phagocytosed by macrophages, triggering their differentiation into a more capable “wound healing” macrophage. During the second and third weeks post-injury, the proliferative stage predominates, consisting of collagen deposition, granulation tissue formation, re-epithelialization, and wound contraction \(^{136}\). Macrophages assist this stage of wound healing through the secretion of growth factors and cytokines that activate keratinocyte migration and fibroblast maturation \(^{20, 132, 137}\). The final phase of wound healing, lasting weeks to years, consists of fibroblast secretion of collagen and collagen remodeling by macrophages and other immune cells \(^{136}\). At this point, non-essential cells can be removed by apoptosis and macrophage phagocytosis. Furthermore, our findings suggest that Rev-Erbs regulate genes in macrophages that contribute to many phases of wound repair.

Although the present studies did not directly evaluate a circadian component of Rev-Erb function, \(~8\%\) of the splenic macrophage transcriptome exhibits circadian rhythmicity \(^{140}\). Rev-Erbs themselves exhibit pronounced circadian rhythmicity in macrophages \(^{50}\), predicting that their transcriptional targets would exhibit corresponding cycles of repression and de-repression even within normal tissue environments. Recently, monocytes have also been shown to undergo diurnal oscillations in “anticipation” of possible environmental and physiological changes \(^{141}\). These observations raise the
possibility that tissue remodeling is under continuous circadian control by resident macrophages, in part regulated by repressive functions of Rev-Erbs.

Macrophage-specific actions of Rev-Erbs appear to primarily result from their binding to distal enhancer elements that are targets of distinct signal transduction pathways. Rather than reinforcing a particular in vitro defined pattern of macrophage polarization, the functional response reflects alterations in the expression of subsets of the genes that are activated by ligands that drive M1, M2 and alternative activation states. Consistent with this, Rev-Erbs co-localized with subsets of NFκB and AP-1 factors at enhancers activated by TLR ligands, and with STAT6 at enhancers activated by IL4. It is likely that Rev-Erbs repress the TGFβ dependent activity of the Cx3cr1 enhancer by antagonizing transcriptional functions of a SMAD protein given the presence of a consensus SMAD binding site in this element. Since the response to the tissue homogenate DAMP signal results in a pattern of gene expression that overlaps with but is distinct from genes induced by TLR, IL4, and TGFβ ligands, and that this response is particularly sensitive to Rev-Erb repression, it is likely that there are additional signal-dependent transcription factors that are subject to antagonism by Rev-Erbs at DAMP-responsive enhancers. Overall, our findings suggest that Rev-Erbs act to repress a specific combination of genes downstream of multiple signaling pathways that collectively function in an integrated manner to promote the response to wounding.
An important observation from these studies was that Rev-Erb repression of target genes was context dependent. Many genes exhibiting de-repression in the absence of Rev-Erb expression under one set of conditions showed no evidence of repression under another. At least two general molecular mechanisms could account for this observation. One would be that the pattern of Rev-Erb binding is altered by specific signaling events due to gain and/or loss of binding of collaborating transcription factors at specific enhancers. An alternative, and not mutually exclusive, possibility relates to the mechanisms by which multiple enhancers interact functionally to control a particular gene. The Cx3cr1 gene exhibits de-repression in the Rev-Erb DKO under all treatment conditions examined, suggesting that the 28kb enhancer is the dominant regulatory element for this gene under each of these conditions. However, many genes subject to context-dependent repression by Rev-Erbs exhibit multiple nearby enhancer-like regions that are not occupied by Rev-Erbs. The lack of de-repression observed for such genes in the context of the Rev-Erb DKO suggests that local repressive actions of Rev-Erb at an enhancer do not necessarily result in repression of other enhancer elements that contribute to signal-dependent gene activation. Such a local mode of repression would in some respects resemble the mechanisms that underlie the pattern of stripes of developmental genes in Drosophila embryos, exemplified by Eve\textsuperscript{142}. Substantial further studies will be required to delineate the basis of context dependent Rev-Erb repression in macrophages.
A major challenge in understanding enhancer function is in determining the specific gene targets that a particular enhancer regulates. Chromatin conformation capture assays provide information on enhancer/promoter proximity, but do not necessarily establish functional connections in the case of signal-dependent gene expression. Genetic deletion of enhancers provides an alternative approach that allows direct readouts of transcriptional consequences, but this method can also potentially alter the chromatin landscape in such a way as to affect other regulatory elements. Here, we have used the analysis and targeted reduction of the Cx3cr1 28kb enhancer eRNAs to document a direct functional relationship between the regulation of this enhancer by TGFβ and the corresponding regulation of the Cx3cr1 genes. These findings confirm and extend prior observations made for estrogen receptor regulated genes in breast cancer cells \(^4^4\) and suggest a simple general strategy to define functional connections between enhancers and target genes.

Finally, the present findings may have practical applications based on the development of small molecules that enhance or inhibit Rev-Erb repressive activity \(^1^4^3\). Delayed wound healing is observed in a number of pathological contexts, including in diabetics \(^1^4^4^-^1^4^6\) and in immunocompromised individuals \(^1^4^7,^1^4^8\). In these settings, it is possible that Rev-Erb antagonists could be evaluated as a means of enhancing wound repair. Alternatively, a large number of devastating and largely untreatable diseases are characterized by exaggerated tissue fibrosis, such as idiopathic pulmonary
fibrosis, interstitial renal fibrosis, and liver fibrosis. It will therefore be of interest to evaluate whether defects in Rev-Erb signaling are associated with these diseases and whether Rev-Erb agonists might be of therapeutic benefit.

Chapter II, Zhang, Dawn X.; Lam, Michael T.; Cho, Han; Gosselin, David; Tanaka, Yumiko, Stender, Joshua D.; Lesch, Hanna; Muto, Jun; Gallo, Richard L.; Evans, Ronald M.; Glass, Christopher K., “Rev-Erbs repress signal-dependent enhancers vital for wound healing”; will eventually be submitted for publication in a much revised form. The dissertation author was the primary investigator and author of this manuscript.
Figure 2.1 Overall impact of Rev-Erb DKO on signal-dependent gene expression. A. Venn diagram demonstrating overlap of Rev-Erb and H3K27Ac/super-enhancers with p65, cJun, cFos, or STAT6. B. Heatmap showing genes captured by RNA-Seq as differentially expressed 1.5-fold in the Rev-Erb DKO macrophage compared to WT in the basal (NT), 9 hour TGFβ, 29 hour TGFβ, 24 hour IL4, 6 hour Pam3, 6 hour PolyIC, 6 hour KDO2, 6 hour tissue injury, 24 hour tissue injury, and 24 hour KDO2-IFNγ states. Data is represented as Log2 normalized gene expression. C. Heatmap showing the same genes as above, but depicting the Log2 fold change between DKO and WT. D. Scatterplots showing genes differentially expressed 1.5-fold in the Rev-Erb DKO macrophage compared to WT in the basal, 6 hour tissue injury, 24 hour IL4, and 6 hour KDO2 states. E. Gene ontology analysis of genes demonstrating de-repressed expression in Rev-Erb DKO macrophages by more than 1.5-fold.
Figure 2.2 Rev-Erb DKO macrophages display increased inflammatory responses to damaged tissue. A. Venn diagrams and heatmap showing genes differentially expressed both in the mouse wound and in macrophages after stimulation with tissue injury homogenate. Wound genes from Days 1, 4, 8, and 14 post-injury and macrophage tissue injury genes at 6 and 24 hours post-stimulation were compared to uninjured skin and unstimulated macrophages, respectively. Differentially expressed genes were those induced or repressed 1.5-fold compared to baseline (FDR < 0.05). The heatmap was generated using average linkage unbiased clustering using Cluster 3 and visualized using Java TreeView. The 280 overlapping genes induced in both the mouse wound and in macrophages after stimulation with tissue homogenate were considered significant by Pearson’s Chi-Square Test, p-value < 0.0001. B-C. Bar graphs depicting specific genes de-repressed 1.5-fold in Rev-Erb DKO macrophages after 6 hour tissue injury. Data is represented as the Log2 fold change between DKO and WT (FDR < 0.05); Actb represents a control gene. Genes are clustered based on their gene ontology clusters/matrices, determined using DAVID. D. Summary of gene ontology analysis of overlapping wound healing and homogenate genes shown in (C).
Figure 2.3 Rev-Erb DKO up-regulated genes intersect with both canonically M1 and M2 macrophage phenotypes. A. Venn diagram showing the similarities and differences amongst genes induced more than 1.5-fold after 6 hour KDO2 treatment, 24 hour IL4 treatment, and 6 hour tissue injury. B. Venn diagram depicting all genes de-repressed in Rev-Erb DKO macrophages, and those similarly and differentially de-repressed at least 1.5-fold compared to WT macrophages and also induced by their respective stimuli. C. Scatterplot showing genes de-repressed in Rev-Erb DKO macrophages in the basal state (red) and their relation to all macrophage genes (gray), as well as canonical M1-type genes (left upper quadrant, KDO2-IFNγ) and canonical M2-type genes (right lower quadrant, IL4-induced genes). Pearson’s Chi-Square test, * \( p < 0.04 \). D. Scatterplot as described in (C) showing genes de-repressed in Rev-Erb DKO macrophages after 24 hour IL4 treatment (red). E. Scatterplot as described in (C) showing genes de-repressed in Rev-Erb DKO macrophages after 6 hour tissue injury (red). Pearson’s Chi-Square test, ** \( p < 0.0001 \), * \( p < 0.003 \). F. Scatterplot as described in (C) showing genes de-repressed in Rev-Erb DKO macrophages after 6 hour KDO2 treatment. Pearson’s Chi-Square test, * \( p < 0.03 \).
Figure 2.4 Locus-specific effects of Rev-Erbs and signal-dependent transcription factors. A-D. UCSC genome browser images depicting the genomic region surrounding Rev-Erb target genes, Cx3cr1, Mmp9, Arg1, and Il1b. The images also show the responses of the genes to different signal dependent stimuli, as well as transcription factors binding to surrounding genomic loci.
Figure 2.5 Rev-Erbs repress signal-dependent enhancer transcription. A. Diagram of the Cx3cr1 -28kb enhancer region that was cloned downstream of the luciferase reporter gene driven by a minimum promoter. B. Luciferase activities of the enhancer reporter construct containing either the Cx3cr1 -28kb enhancer region or a random genomic sequence. Luciferase reporter constructs were co-transfected with the indicated Rev-Erb and RORα plasmids. Luciferase reporter gene is driven by a minimum promoter. (N = 5 independent experiments, Data represent mean ± SEM, *p-value < 0.01, **p-value < 0.05, analysis of variance (ANOVA) by Tukey HSD test). C. RT-qPCR analysis of Cx3cr1 messenger and enhancer RNA in the basal and TGFβ treated states after knock-down of enhancer RNA using Cx3cr1 or control (CTL) anti-sense oligonucleotides (N = 3, Data represent mean ± SD, *p-value < 0.01, analysis of variance (ANOVA) by Tukey HSD test).
Figure 2.6 Rev-Erb DKO bone marrow transplanted animals display enhanced wound closure in a full thickness wound healing model. A. Wound healing efficiency calculated as percent of original wound size in WT and Rev-Erb DKO bone marrow transplanted animals (N = 28-132 wounds per genotype, **p-value < 0.0005, Data represent mean ± SEM). B. Macroscopic digital photographs of wound closure in WT and Rev-Erb DKO bone marrow transplanted animals. C. Histological images of wound healing in WT and Rev-Erb DKO bone marrow transplanted animals. Arrow heads show differential re-epithelization between WT and Rev-Erb DKO bone marrow transplanted animals. Abbreviations: g = granulation tissue, d = dermis. D. Rate of wound re-epithelialization measured by determining the distance between epithelial tips in histological sections (N = 4 wounds per genotype, 2 pictures per wound, *p-value < 0.05, Data represent mean ± SEM). E. Ratio granulation tissue to total cross-section area measured in histological sections (N = 4 wounds per genotype, 2 pictures per wound, *p-value < 0.05, Data represent mean ± SEM).
Figure 2.7 Rev-Erb DKO bone marrow transplanted animals display increased macrophage persistence at the wound site. A-B. Blinded histological analysis of F4/80+ cells at the wound site (N = 4-8 wounds per genotype, >3 pictures per wound, **p-value < 0.01, Data represent mean ± SEM). C. Blinded histological analysis of CD68+ cells at the wound site (N = 4 wounds per genotype, >3 pictures per wound, *p-value < 0.05, Data represent mean ± SEM). D-E. Blinded histological analysis of Ly6B.2+ cells at the wound site (N = 4 wounds per genotype, >3 pictures per wound). F. Migration of WT and Rev-Erb DKO macrophages through matrigel extracellular matrix for 24 hours (***p-value < 0.01, Data represent mean ± SD from one of three experiments).
Figure 2.8 Rev-Erb double knock-out strategy. A. Diagram showing the knock-out strategy. B. UCSC genome browser images depicting the exons targeted in Rev-Erbα and Rev-Erbβ genes after various ligand treatments. C. Quantification of the average percent deletion efficiency of the Rev-Erbα and Rev-Erbβ targeted exons in the Cre+ versus the Cre- animals. For each condition, the total number of sequenced tags for the targeted exon(s) was quantified for both Cre+ and Cre- animals, and then the percent deletion was calculated and averaged.
Figure 2.9 Differential gene expression in Rev-Erb DKO macrophages. A. Scatterplots showing genes differentially expressed 1.5X in the Rev-Erb DKO macrophage compared to WT after treatment with 6 hour Pam3, 6 hour PolyIC, 9 hour TGFβ and 29 hour TGFβ. B. Gene ontology analysis of genes demonstrating repressed expression in Rev-Erb DKO macrophages by more than 1.5-fold.
Figure 2.10 M1, M2, and de-activated macrophage transcriptomic phenotypes. A-C. Venn diagrams showing the similarities and differences amongst genes induced more than 1.5-fold after 6 hour KDO2 treatment, 24 hour IL4 treatment, 9 hour TGFβ treatment, and 6 hour tissue injury. D-F. Venn diagram depicting all genes de-repressed in Rev-Erb DKO macrophages, and those similarly and differentially de-repressed at least 1.5-fold compared to WT macrophages and also induced by their respective stimuli.
Figure 2.11 Bone marrow transplant efficiencies. A. Bone marrow harvested from Rev-Erb DKO animals and control littermates were injected retro-orbitally into wild type irradiated congenic (CD45.1) mice. After reconstitution for 6-10 weeks, the wound healing response was followed for 2 weeks. B. Bone marrow reconstitution efficiency was determined by flow cytometry of circulating leukocytes (B) and RT-qPCR (C) of bone marrow after wound healing (N = 7 per genotype, *p < 0.05).
Chapter III:

Transcriptional Regulatory Roles of Coronin 2A in Macrophages
A. Introduction

In 1979, a ground breaking paper by Gerrity and colleagues showed macrophages to be the first inflammatory cells associated with atherosclerosis\textsuperscript{149}. Since then, an assortment of pro-inflammatory cells have been implicated in atherosclerotic lesion development\textsuperscript{150}. Atherosclerosis develops in the chronic inflammatory state when activated macrophages consume modified lipoproteins to become foam cells, which contribute to atherosclerotic lesion progression through the release of chemotactic proteins, inflammatory cytokines (such as $\text{Il6}$, $\text{Il1}$, and $\text{Tnfa}$), and growth factors. Inflammatory dysregulation has been implicated in many disease processes such as diabetes, rheumatoid arthritis, multiple sclerosis, and atherosclerosis\textsuperscript{5-7, 96}. The nuclear co-repressor (NCoR) complex resides on the promoters of a broad set of inflammatory responsive genes under basal conditions and is cleared through various signal-dependent events in response to activation of pro-inflammatory signaling pathways\textsuperscript{80, 151}. SUMOylated nuclear receptors, peroxisome proliferator-activated receptor $\gamma$ (PPAR$\gamma$) and liver X receptors (LXRs), exert an additional layer of regulation through trans-repression\textsuperscript{121, 122}, a mechanism involving inhibition of signal-dependent NCoR removal. As a consequence, NCoR complexes maintain repression of pro-inflammatory genes known to contribute to atherosclerosis and other inflammatory diseases.

NCoR complex
NCoR functions as the core component of a co-repressor complex consisting of histone deacetylase 3 (HDAC3)\textsuperscript{152}, transducin β-like protein 1 (TBL1) and transducin β-like related 1 (TBLR1)\textsuperscript{110}, and has been found to also contain additional factors such as Coro2A\textsuperscript{110, 111}, GPS2\textsuperscript{110, 153}, and TAB2\textsuperscript{110, 152}. In resting macrophages, the NCoR complex resides on inflammatory promoters though interactions with unphosphorylated cJun, mediating basal repression. Inflammatory signaling, such as activation of toll-like receptors, TLR2 and TLR4\textsuperscript{98}, initiates recruitment of actin to Coro2A, a step required for de-repression\textsuperscript{113}, the dissociation of the NCoR complex from inflammatory promoters. Trans-repression is mediated through ligand dependent SUMOylation of nuclear receptors, which inhibit signal-dependent interaction of actin with Coro2A in response to inflammatory stimulation. As a consequence, NCoR complexes remain bound and continue to exert a repressive function.

**Role of Coro2A in the regulation of inflammation**

Coro2A is a member of the Coronin family of actin-binding proteins, the more extensively characterized members have been shown to regulate cytoskeleton and membrane trafficking in the cytoplasm\textsuperscript{112, 154, 155}. Recently, Marshall et al. distinguished Coro2A in the cytoplasm as a regulator of focal-adhesion signal transduction events through the cofilin pathway\textsuperscript{156}. However, Coro2A was initially identified as a component of the NCoR complex in the nuclear extracts of HeLa cells\textsuperscript{110, 111} and has been found to be highly
expressed in the cells of hematopoietic lineage\textsuperscript{157}. Like other proteins in the Coronin family, the N-terminus of Coro2A contains a conserved WD40 region implicated in actin binding, while the C-terminus contains a coiled coil domain responsible for other protein-protein interactions \textsuperscript{112, 155, 158}. In order to define the specific proteins regulating NCoR clearance, we sought to identify the molecular targets of SUMOylated PPAR\textsubscript{\gamma} and LXR\textsubscript{s}, which allow for the inhibition of NCoR clearance. A systematic search of proteins in the NCoR complex for potential SUMO interaction motifs identified an evolutionarily conserved SUMO2/3 interaction motif in the C-terminus of Coro2A \textsuperscript{159}. A recent study suggested that GPS2 is a docking site for SUMOylated LXR\textsubscript{s} in the liver \textsuperscript{160}; however, we have found no evidence for such a role in macrophages. Previous work in our laboratory has confirmed that Coro2A is an active transcriptional component of the NCoR complex in bone marrow derived macrophages.

**B. Coro2A ubiquitinylation**

Actin-dependent Coro2A interaction is required for NCoR clearance and subsequent de-repression of inflammatory response genes; however, the exact signal transduction pathway in which Coro2A mediates de-repression remains unclear. Post-translational modifications play an essential role in many cellular functions. Ubiquitinylation, for example, has been shown to mediate protein interactions, protein localization, and DNA repair, to activate transcription factors, to modulate protein activity, and to target proteins to the
lysosome or the proteasome for degradation as reviewed in \(^{161}\). Ubiquitin is attached to the ε-amino group of Lys residues in substrates and exists in mono-, multi-mono-, and poly-ubiquitinylation forms. Proteins targeted for degradation are often misfolded and/or damaged, and are polyubiquitinated. Polyubiquitinylation is recognized by the 19S proteasome and is subsequently targeted to the 20S proteasome \(^{162}\). The signal-dependent removal of NCoR complexes from inflammatory genes has been found to require recruitment of the 19S proteasome \(^{80,121,151}\). TBL1 and TBLR1 are components of NCoR and/or SMRT complexes \(^{110,163}\) that serve as adaptor proteins, mediating signal-dependent recruitment of UBCH5, an E3 ubiquitin ligase \(^{80,121,151}\). It has been speculated that the removal of the NCoR complex by the 19S proteasome subsequently targets the NCoR complex for degradation by the 20S proteasome \(^{164}\); however the data remains inconclusive partly due to the involvement of the 20S proteasome in TLR signaling upstream of NCoR, more specifically, mediating IκBα degradation that allows for activation of NF-κB and its translocation to the nucleus \(^{165}\). Furthermore, western blot analysis in our laboratory has shown no change in nuclear NCoR in response to lipopolysaccharide (LPS) stimulation \(^{97}\) and ChIP analysis of NCoR at TLR responsive promoters shows NCoR re-recruitment to inflammatory promoters after continued LPS-stimulation for >24 hours. Thus, it is likely that NCoR turnover is mediated not by degradation, but by shuttling to another region of the nucleus.
The original purification of the NCoR complex from HeLa cells showed a higher molecular weight form of Coro2A, resulting in a size shift of >8 kDa. Co-immunoprecipitation (coIP) experiments have shown that NCoR is associated with higher and lower molecular weight Coro2A (Figure 3.1A) and that the higher molecular weight band represented an ubiquitinylated version of the protein (Figure 3.1B). Subsequently, we found that Coro2A is ubiquitinylated in the basal state and becomes de-ubiquitinylated upon LPS-stimulation (Figure 3.1C). Although many of the cytoplasmic Coronin family members were previously shown to be phosphorylated at the serine 2 residue by protein kinase C, thus regulating cell motility and actin polymerization, the de-ubiquitinylation of Coro2A may be the nuclear signal, which allows for actin binding and the subsequent removal of the NCoR complex (Figure 3.1C). To identify the site of ubiquitinylation, we truncated Coro2A into N and C terminal fragments and found the C terminal of Coro2A to be ubiquitinylated. Subsequently, we systematically mutated all of the lysines to arginines in the C terminal and found one mutation, K327R, which decreases and/or eliminates the ubiquitinylation of Coro2A (Figure 3.1D). Furthermore, Coro2A mutants were shown to modify the induction of TNFα-luciferase in the basal state and after 16 hours of LPS induction (Figure 3.1E).

C. Coro2A transcriptional properties revealed through loss of function studies
Coro2A mediates NCoR clearance from a number of inflammatory responsive genes; however, the role of Coro2A on a global scale is not well understood. To study the global transcriptional properties of Coro2A in inflammatory signaling, we used poly(A) RNA sequencing (RNA-Seq) to characterize the change in steady state RNA expression. Due to contradictory previous data generated from RNA-Seq data of macrophages transfected with Coro2A siRNA, we generated Coro2A knockout animals from commercially available ES cells. RNA-Seq was conducted in the basal state, after 7 and 9 hours post-LPS stimulation, 9 hours post-IL4 stimulation, and 9 hours post-TGFβ stimulation (Figure 3.2A-G). After initial analysis showing that Coro2A was adequately depleted in all samples (Figure 3.2A), the data was further analyzed using Homer\textsuperscript{13}, our in-house analysis platform, which uses edgeR for differential expression analysis\textsuperscript{170}, and the Web-based DAVID functional-annotation tool\textsuperscript{171}.

Comparing the gene expression signature from WT and Coro2A KO macrophages, a general trend emerged where repressed or de-repressed genes in the KO, although displaying differential expression after treatment with various signal-dependent stimuli (Figure 3.3A), still retained a similar profile under different conditions (Figure 3.3B). Genes repressed and de-repressed in Coro2A KO macrophages were further stratified depending on whether those genes were de-repressed in more than two (Figure 3.4A), three (Figure 3.5A), or four (Figure 3.4C) conditions, or repressed in more than two (Figure 3.4B), three (Figure 3.4C), or four (Figure 3.5B) conditions. Genes
significantly de-repressed in four or more conditions included Col4a5, Eps8l1, Ptpn, Fdps, Aldoc, Cyp51, Fads2, Fdft1, Dhcr24, Cpan11, Sc5d, Stard4, Fos, Zdhhc2, Idi1, Vwf, Sqle, Hsd17b7, Chi3l3, Ociad2, and Serpinb6b. While
genes significantly repressed in three or more conditions included Emr4, H2-Eb1, Coro2a, Mefv, Ly86, Ppfia4, Abca9, Cmkir1, Adam19, Pla2g7, Mras, Tmcc3, Cd5l, Gpr126, Acp5, Ptpnc1, Atp6v0d2, F10, Mmp27, MglI, Gpr141, Gm13710, Ptg21, Icosl, Vcam1, and Fcgr4.

Gene ontology analysis of targets differentially expressed greater than 1.5-fold in Coro2A KO macrophages found biological process terms for
cholesterol and sterol biosynthesis processes (Figure 3.5A) enriched in de-repressed genes while biological process terms for immune response and
response to wounding were enriched in repressed genes (Figure 3.5B). Not
surprisingly, this phenotype is very similar to that of the NCoR KO
macrophage. UCSC genome browser images of the Nos2 (Figure 3.5C)
and the Scd2 (Figure 3.5D) loci shows NCoR and p65 binding at these sites,
suggesting that these two transcription factors may be important for modifying
expression of these genes. Furthermore, an analysis of motifs associated with
PU.1-bound regions nearest to genes demonstrating de-repressed or
repressed expression in KO macrophages found the expected macrophage-
lineage determining transcription factors as well as ATF4, CTCF, EWS-FLI1,
KLF4, and STAT6 motifs in the enhancer regions of the de-repressed genes,
and CTCF and IRF in the enhancer regions of the repressed genes.
Furthermore, these transcription factors, as well as NCoR and p65 may
contribute to the differential expression of genes in WT and Coro2A KO macrophages.

**D. Coro2A transcriptional properties revealed by gain of function studies**

To complement the loss of function studies in Coro2A KO macrophages, we also conducted 5’ global run-on sequencing (5’ GRO-Seq) in three individual control and Coro2A over-expressing macrophage cell lines. Global Run-On Sequencing, GRO-Seq and 5’ GRO-Seq\(^{42,173}\), are methods that have been recently established\(^{13,42,46}\) to characterize the change in nascent RNA expression, as well as the transcriptional regulation of long non-coding RNAs, which have been only recently described in the literature\(^{174-176}\), reviewed in\(^{177-182}\). Our laboratory has previously shown that transcription factors bind at both promoter and non-promoter elements\(^{13,27,46}\), such as enhancers that regulate the transcription of a non-coding RNA subset called enhancer RNAs (eRNAs). Three individual macrophage cell lines (RAW264.7) were generated over-expressing Flag Coro2A conjugated to a N-terminal biotin ligase receptor peptide (BLRP), a spacer, a tobacco etch virus (TEV) protease cleavage site\(^{183}\), and then an N-terminal Flag tag. BLRP is a 22 amino acid peptide that is a specific substrate for the bacterial biotin ligase (BirA). Co-expression of the tagged protein with BirA results in in vivo biotinylation, enabling high efficiency precipitation with streptavidin-based affinity reagents. After precipitation, the BLRP marker is cleaved off with TEV protease and the enriched protein is used for downstream applications. For
these stable cell lines, Coro2A is found predominantly in nuclear lysates, can be efficiently cleaved with TEV protease, and retains the transcriptional functions of endogenous Coro2A (data not shown).

RNA-Seq and 5’ GRO-Seq in over-expression models were conducted in the basal and 6 hour (RNA-Seq) or 1 hour (5’GRO-Seq) KDO2-stimulated states. Surprisingly, over-expression of the protein resulted in decreased expression of inflammatory genes as well as increased expression of mitotic and cell cycle genes. Furthermore, an analysis of the 5’ GRO-Seq data shows that over-expression of Coro2A results in the repression of many macrophage physiologically significant genes (Figure 3.6A-F). For example, there is significant overlay of the differential Coro2A 5’ GRO-Seq transcriptional signal (transcriptional start sites where over-expression of Coro2A results in decreased 5’ GRO-Seq signal) with the NCoR (Figure 3.6A), cFos (Figure 3.6C), and p65 (Figure 3.6E) ChIP-Seq. Similarly, histograms showing the distribution of averaged 5’ GRO-Seq signal in control and Coro2A over-expressing macrophages reveals that that over-expression of Coro2A results in decreased 5’ GRO-Seq signal from NCoR (Figure 3.6B), cFos (Figure 3.6D), and p65 (Figure 3.6F) genomic locations. Furthermore, these results suggest that Coro2A may function as a transcriptional regulator at NCoR, cFos, and p65 bound genomic locations.

E. Intersection between Coro2A and NCoR
Since the transcriptional phenotype of the Coro2A KO macrophages is strikingly similar to that of the NCoR KO macrophage, we assessed genes captured by poly(A) RNA-Seq as induced or repressed 1.5 fold in the Coro2A KO macrophage compared to WT and compared them to those induced or repressed 1.5 fold in the NCoR KO macrophage compared to WT (Figure 3.7A). Thirty nine genes were similarly de-repressed in NCoR and Coro2A KO macrophages while ninety seven genes were similarly repressed in NCoR and Coro2A KO macrophages (Figure 3.7A). Gene ontology analysis of genes differentially expressed in both Coro2A KO and NCoR KO macrophages found biological process terms for lipid and fatty acid biosynthesis processes enriched in de-repressed genes while biological process terms for immune response and response to wounding were enriched in repressed genes (Figure 3.7B). UCSC genome browser images of the Scd2 (Figure 3.7C) and the Nos2 (Figure 3.7D) loci shows increased and decreased transcriptional signals, respectively, in the NCoR and Coro2A KO macrophages. These results suggest that Coro2A and NCoR may function cooperatively at specific transcriptional loci regulating lipid biosynthetic and immune response genes.

F. Discussion

In concert, these studies describe how Coro2A plays an integral part in transcriptional regulation of multiple pathways that intersect with the NCoR complex. Previous findings in our laboratory showed that Coro2A associates with NCoR on a small number of inflammatory responsive genes; however, the
role of Coro2A on a global scale is not well understood. Moreover, our studies are only beginning to reveal the importance of Coro2A in mediating both NCoR-dependent and NCoR-independent mechanisms. Further experiments are necessary to reveal the extent of this co-regulation as well as the precise molecular details as to how depletion of Coro2A affects transcription.

Previous studies have also elucidated a novel role of Coro2A in actin-binding to facilitate actin-mediated de-repression of inflammatory genes, as well as its role in binding SUMOylated LXR s, which inhibits actin recruitment and de-repression of inflammatory genes. These studies focused predominantly on the Nos2 and Ccl2 promoters, which are co-bound by the NCoR complex. Our current findings suggest that Coro2A may function at promoter and enhancer loci in both a mechanistically NCoR-dependent and independent manner. Therefore, it would be of interest to determine whether Coro2A also regulates these loci through actin-mediated de-repression and inhibition of transcriptional activation by the binding of SUMOylated LXR s.

Furthermore, the surprising finding that NCoR KO macrophages are hypo-responsive to inflammatory signaling due to increased synthesis of insulin-sensitizing omega 3 fatty acids through a LXR-dependent pathway and the similarities between the Coro2A and NCoR KO transcriptional phenotypes lead us to believe that similar in vivo consequences may result from Coro2A depletion. Thus, future experiments should also focus on investigating the functional similarities and differences between NCoR and Coro2A in regulating in vivo homeostatic programs in macrophages.
Biosystems) and normalized to $\beta$ activity was measured using a Veritas microplate luminometer (Turner Biosystems). Lysates from RAW264.7 cells (left) or bone marrow derived macrophages (BMDMs) (right) were immunoprecipitated (IP) with IgG or anti-NCoR as indicated, and immunoblotted (IB) with anti-Coro2A or anti-NCoR. B. Coro2A is ubiquitinylated in the basal state. Lysates from HeLa cells transfected with BLRP-null and BLRP-Flag-Coro2A expression vectors were immunoprecipitated (IP) with streptavidin dynabeads (Invitrogen; left two lanes) or Flag antibody (right two lanes) and immunoblotted (IB) with anti-Ub or anti-Coro2A. C. Coro2A is de-ubiquitinylated in response to LPS stimulation. Lysates from BMDMs (left) or RAW264.7 cells (right) were treated with the indicated antibodies and immunoprecipitated (IP) with anti-Coro2A as indicated, and immunoblotted (IB) with anti-Coro2A or anti-Ub. D. K327R mutation decreases and/or eliminates Coro2A ubiquitinylation. Lysates from HeLa cells transfected with the indicated Coro2A expression vectors were immunoprecipitated (IP) with Flag antibody and immunoblotted (IB) with anti-Ub or anti-Flag. E. Coro2A mutants modify the induction of TNF$\alpha$-luciferase in the basal state and after 16 hours LPS induction. RAW264.7 cells were transfected with the indicated Coro2A expression vectors, TNF$\alpha$-luciferase, and a $\beta$-galactosidase plasmid. Forty-eight hours post-transfection, luciferase activity was measured using a Veritas microplate luminometer (Turner Biosystems) and normalized to $\beta$-galactosidase activity (Applied Biosystem).
Figure 3.2 Genome-wide analyses of Coro2A KO macrophages. A. UCSC genome browser image showing the Coro2A genomic locus. B-G. Scatterplots showing genes differentially expressed 1.5 fold in the Coro2A KO macrophage compared to WT.
Figure 3.3 Overall impact of Coro2A KO on signal-dependent gene expression. A. Heatmap showing genes captured by poly(A) RNA-Seq as differentially expressed 1.5 fold in the Coro2A KO macrophage compared to WT. Data is represented as Log2 normalized gene expression. B. Heatmap showing the same genes as above, but depicting the Log2 fold change between KO and WT.
Figure 3.4 Gene expression profiles of Coro2A KO macrophages. A-C. Heatmap showing genes captured by poly(A) RNA-Seq as induced (A, C) or repressed (B, C) 1.5 fold in the Coro2A KO macrophage compared to WT in two or more (A-B), three or more (C), or four or more conditions (C).
Figure 3.5 Coro2A KO up-regulated genes are linked to cholesterol biosynthetic processes, down-regulated genes are linked to immune responses. A-B. Gene ontology analysis of genes differentially expressed greater than 1.5-fold in Coro2A KO macrophages. C-D. UCSC genome browser images showing the Nos2 and Scd2 loci. E-F. Sequence motifs associated with PU.1-bound regions nearest to genes demonstrating induced or repressed expression in KO macrophages by more than 1.5-fold in >2 experimental conditions.
Figure 3.6 Coro2A-modulated transcriptional signal emanates from NCoR (AP-1) genomic locations. A-F. Overlay of differential Coro2A 5' GRO-Seq transcriptional signal with NCoR (A), cFos (C), and p65 (E) ChIP-Seq. Distribution of averaged 5' GRO-Seq signal from NCoR (B), cFos (D), and p65 (F) bound genomic locations in macrophages.
Figure 3.7 Similarities between NCoR KO and Coro2A KO macrophages. A Heatmap showing genes captured by poly(A) RNA-Seq as induced or repressed 1.5 fold in the Coro2A KO macrophage compared to WT and the NCoR KO macrophage compared to WT. B. Gene ontology analysis of genes differentially expressed in both Coro2A KO and NCoR KO macrophages. C-D. UCSC genome browser images showing the Nos2 and Scd2 loci.
Chapter IV:

Conclusions and Future Directions
On the genome-wide level, transcriptional regulation is a precise and involved process that spans a hierarchy of diverse transcription factors – lineage determining, signal dependent, and collaborating transcription factors. To regulate transcription, NCoR cooperates with a multitude of different transcription factors – of which, Coro2A and Rev-Erb represent a small minority. In comparison to Coro2A, Rev-Erb is a more “traditional” transcriptional repressor; Rev-Erb contains a DNA binding domain that has been documented to bind DNA and regulate transcription. In contrast, Coro2A lacks a conserved DNA binding domain – instead, it contains an actin binding domain as well as a C terminus that traditionally mediates protein-protein interactions. Nevertheless, Coro2A, unlike other members of the Coronin family, is found primarily in the nucleus \(^{113}\), and loss of function as well as gain of function studies have shown its ability to regulate transcription on a genome-wide level.

Furthermore, although both Coro2A and Rev-Erb function as dedicated transcriptional regulators, their roles are extraordinarily complex. Loss of function studies in both Coro2A KO macrophages and Rev-Erb KO macrophages found a surprising number of repressed genes in addition to the expected de-repressed genes, resulting from the deletion of a member of the NCoR complex. These results suggest that both Coro2A and Rev-Erb may function as transcriptional trans-activators as well as transcriptional repressors.
A. Rev-Erb as a transcriptional activator

Since Rev-Erbs do not contain the traditional AF2 domain, which allows for co-repressor/co-activator exchange, repression of genes in Rev-Erb depleted macrophages suggests that Rev-Erbs may function as trans-activators at certain loci. Most of the literature currently focuses on the roles of Rev-Erbs as transcriptional repressors, thus making this finding extraordinarily novel. Hypothetically, one or more of the following three possibilities may explain the trans-activation potential of Rev-Erbs. (1) Rev-Erbs may function as transcriptional activators by cooperating with other transcription factors. (2) Rev-Erbs may repress the expression of certain transcriptional repressors. (3) Rev-Erbs may regulate upstream activities of transcriptional activators/repressors in the nucleus and/or the cytosol.

To determine whether one or more of these possibilities may explain our molecular observations, the enhancers and promoters of genes exhibiting repression in Rev-Erb DKO macrophages can be examined via luciferase assays. Plasmids containing Rev-Erbα, Rev-Erbβ, and RORα, as well as their DNA-binding mutants, can be co-transfected into a luciferase system. Preliminary data shows that over-expression of Rev-Erbs in luciferase assays results in activation of certain enhancers such as the Soc3 +22kb enhancer, the Mt2 -1kb enhancer, and the Id3 -20kb enhancer (data not shown). Furthermore, knock-down of different transcriptional activation complexes using siRNA or shRNA would allow the determination of possible transcriptional activators, which can then be used in co-immunoprecipitation
assays with Rev-Erbα, Rev-Erbβ, as well as RORα. In addition, it would be especially interesting to determine whether Rev-Erbα and Rev-Erbβ can both function as transcriptional trans-activators at these loci or whether this function is more attuned to a specific family member.

**B. Genome-wide roles of Coro2A in regulating transcription**

In the nucleus, Coro2A was initially identified as a component of the NCoR complex in the nuclear extracts of HeLa cells. To this end, at specific genomic loci (especially promoters), the roles of Coro2A in transcriptionally regulating inflammation have been studied. Previous findings have shown that Coro2A regulates co-repressor/co-activator exchange by serving as a binding protein for actin-mediated de-repression of inflammatory genes, as well as a docking site for SUMOylated LXRs to inhibit inflammatory gene expression. What remains is to study the roles of Coro2A on a genome-wide level at both promoters and enhancers. Specifically, to address whether Coro2A regulates transcription at genomic loci in collaboration with or independent of the NCoR complex as well as to study the mechanism by which Coro2A regulates transcription at these loci.

To better understand the transcriptional properties of Coro2A KO macrophages in order to compare them with NCoR KO macrophages, global run-on sequencing and subsequent motif finding should be conducted in Coro2A KO macrophages. Surprisingly, it was recently shown that Coro2A interacts with Tug1, a non-coding RNA important for methylation-dependent
relocation of the Polycomb 2 protein \(^{184}\); thus, GRO-Seq would enable the characterization of Coro2A-dependent regulation of non-coding RNAs. For these experiments, 5’ GRO-Seq and possibly GRO-Seq will be conducted in WT and Coro2A KO primary macrophages in the basal and 1 hour KDO2, TGFβ, and IL4-stimulated states, time points that should correspond with the basal and 7 or 9 hour stimulated states for RNA-Seq. Data analysis will be conducted using the same programs as the RNA-Seq analysis. Afterwards, this data can be analyzed with the Coro2A RNA-Seq data, as well as the NCoR RNA-Seq, ChIP-Seq, and GRO-Seq data that is currently available in our laboratory.

To complement the gene expression data, chromatin immunoprecipitation with parallel high-throughput sequencing for Coro2A should be performed in order to better understand the direct targets regulated by Coro2A in cis and in trans. To conduct these experiments, a ChIP-Seq grade antibody must be generated that recognizes mouse Coro2A. Afterwards, ChIP-Seq should be conducted in WT and Coro2A KO primary macrophages in the basal and 1 hour KDO2, TGFβ, and IL4-stimulated states. Subtraction of the Coro2A KO ChIP-Seq signal from the WT signal would allow a better appreciation of the specific Coro2A genomic targets and eliminate non-specific antibody binding. In parallel, ChIP-Seq for the following transcription factors and histone marks should be performed in the basal and stimulated states in both WT and Coro2A KO macrophages: H3K4me2, H3K27ac, H4K5ac, p65, and LXR. Furthermore, to better characterize the
LXR-Coro2A interaction axis, both LXR and Coro2A ChIP-Seqs should be conducted following KDO2 stimulation with and without LXR ligand co-treatment. Finally, our laboratory has previously generated Coro2A mutants (actin-binding mutant, SUMOylated LXR binding mutant, ubiquitin mutant); lentiviral mediated transduction of Coro2A KO macrophages with these mutants and subsequent molecular analyses will allow us to definitively pinpoint how Coro2A is involved in co-repressor/co-activator exchange on a genome-wide level.

C. Physiological studies of Coro2A depletion

TLR4 activation and its downstream effects have been implicated in animal models of type 2 diabetes \(^{185}\) and atherosclerosis \(^{186}\). Since macrophages have been shown as the primary pathogenic cell types in these diseases, it would be interesting to study whether depletion of Coro2A modulates the macrophage mediated chronic inflammatory states in these diseases. Previous studies have surprisingly shown that NCoR KO macrophages are hypo-responsive to inflammatory signaling due to increased synthesis of insulin-sensitizing omega 3 fatty acids. Thus it would be fascinating to study whether depletion of Coro2A would mirror these results, and to also appreciate whether the hypo-responsive inflammatory state seen in NCoR KO mice would affect the development of atherosclerosis. Since the Coro2A KO mouse is a total body knock-out, Coro2A KO and WT bone marrow must be transplanted into WT congenic mice for diabetes studies.
After transplantation, studies should be conducted with at least fifteen mice per group for both studies. Furthermore, a detailed lipodomics analyses should be performed to study cholesterol, lipids, and/or fatty acids that may be increased in synthesis by Coro2A KO macrophages. In concert, these studies may provide us a better understanding of the breadth and extent of the effect currently exhibited by depleting Coro2A in macrophages.

For atherosclerosis studies, the LDL-R−/− mouse model, in collaboration with the Witztum laboratory, can be utilized wherein LDL-R−/− mice will be transplanted with WT, Coro2A KO, NCoRfl/fl, and NCoR KO bone marrow187. Male LDL-R−/− mice can be irradiated and reconstituted at 8 weeks of age with bone marrow from our mice as described in188,189. After transplant, the animals recover on a standard diet for 4 weeks before being fed a high fat diet, which normally causes LDL-R−/− mice to develop atherosclerotic lesions throughout the arterial tree and a plasma lipid profile similar to human hyperlipidemia190. Atherosclerotic lesion development can be assessed through histological analysis of the lesions in the aortic sinus as well as the descending aorta188. Total plasma cholesterol concentrations and mouse plasma lipoproteins can be measured from blood sampled from the retro-orbital plexus and quantified by enzymatic assays189. Expression of pro-inflammatory genes such as Nos2, Mcp1, and Tnfa can be determined from atherosclerotic lesions and thioglycollate-elicited peritoneal macrophages as described in191.

To study the effect of Coro2A depletion on diabetes, WT and Coro2A
KO bone marrow transplanted mice can be fed a high-fat diet, introduced at 4 weeks of age \(^{192}\). Weekly blood samples can be drawn from the tail vein of non-fasted anesthetized mice. Insulin measurements can be determined via radio-immunochemical analysis and plasma glucose will be determined by the glucose oxidase method. Body weights can also be measured on a weekly basis. Insulin tolerance tests and hyperinsulinemic-euglycemic clamp studies can be conducted as described in \(^{193}\). At the end of each clamp study, muscle, liver, and fat tissues will be harvested for the analysis of pro-inflammatory gene expression. In the event that the Coro2A KO bone marrow transplanted animals exhibit only a minor phenotype in the two inflammatory models proposed above, additional challenge with TLR4 ligands in conjunction with high fat feeding can precipitate a more profound phenotype \(^{194}\).

**D. A possible intersection of the Coro2A and Rev-Erb transcriptional systems**

Coro2A and Rev-Erb represent different arms of the NCoR transcriptional regulatory system. While Rev-Erb is predominantly as repressors, both seem to have possible functions as transcriptional trans-activators and repressors. The next step in these studies would be to elucidate whether Coro2A and Rev-Erb regulate overlapping genes in macrophages. Preliminary analyses of RNA-Seq data have shown overlapping transcriptomes between the Coro2A and Rev-Erb KO macrophages (data not shown). A better characterization of the genes that Coro2A and Rev-Erb co-
regulate can be performed by isolating the promoter and enhancer elements and conducting cell-based assays using an in vitro luciferase system. We can also harness the power of Coro2A and Rev-Erb mutants such as the Coro2A actin-binding, SUMO-binding, and ubiquitin mutants, as well as the Rev-Erb DNA-binding mutants in our cell-based assays. Also, co-immunoprecipitation assays and in vitro GST pull-down assays can be utilized to reveal whether Coro2A and Rev-Erb collaborate to modulate transcription at these sites. If these proteins indeed cooperate, it would be interesting to see whether Coro2A is regulated in a circadian fashion and whether Rev-Erb target genes show actin-dependent mechanisms.

In summary, our findings suggest that Rev-Erbs and Coro2A, two members of the NCoR complex, modulate transcriptional programs in macrophages. While Rev-Erbs regulate a complex enhancer architecture vital for wound healing, Coro2A exhibits both transcriptional activating and repressive effects on inflammatory gene expression. Furthermore, future in vivo experiments using nuclear receptor agonists and antagonists may allow us to modulate chronic inflammatory states such as non-healing diabetic wounds. In this case, the concurrent use of Rev-Erb antagonists and possibly TGFβ agonists may allow quicker and more resilient wound healing in such pathogenic states.
Materials and Methods
**Plasmids**

Rev-Erb$^{129}$ and Coro2A$^{113}$ plasmids were previously described. For Coro2A ubiquitin mutants, site directed mutagenesis was performed using QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies).

**Construction of enhancer reporters and plasmids**

Cx3cr1 enhancer constructs, Rev-Erb plasmids, and RORα plasmids were previously described$^{129}$. For Rev-Erb DNA binding mutants, site directed mutagenesis was performed using QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies) according to manufacturer's instructions.

**Cell culture**

Mouse bone marrow derived macrophages were obtained and cultured as previously described$^{13}$. For cytokine stimulation studies, macrophages were cultured in RPMI 1640 (Invitrogen) supplemented with 0.5% heat-inactivated FBS (Hyclone) for 24 hours and then stimulated with Pam3CSK4 (300 ng/ml, InvivoGen), polyinosinic:polycytidylic acid (50 ng/ml, GE Healthcare Bioscience), KDO2-Lipid A (100 ng/ml, Avanti Polar Lipids), recombinant interferon γ (10 units/ml, R&D Systems), interleukin 4 (20 ng/ml, R&D Systems), or tumor growth factor β (1 ng/ml, Cell Signaling) for the indicated time points.

For tissue injury studies, skin from shaved wild type congenic mice was harvested and homogenized in RPMI 1640 supplemented with 0.5% heat-
inactivated FBS using a Precellys 24 tissue homogenizer (6500 rpm 2x20 seconds) and metal beads (2.8 mm/ 2 mL, Cayman Chemical) according to the manufacturer’s instructions. To stimulate macrophages, the homogenized skin was plated on a 0.4 µm transwell (Corning) or diluted into RPMI 1640 supplemented with 0.5% heat-inactivated FBS. For stimulation studies in 24-well plates, skin homogenate was centrifuged at 4000rpm for 15 minutes at 4°C, and the supernatant was filtered through a 0.2 µm filter (Nalgene, ThermoScientific). Approximately 25 mL of conditioned media was collected per mouse.

**Luciferase assays**

RAW264.7 cells were transfected with the indicated luciferase constructs, expression vectors, and a β-galactosidase plasmid using Superfect (Qiagen), according to manufacturer’s instructions. Twenty-four hours post-transfection, cells were cultured in low serum media (0.5% FBS in RPMI) for 24 hours and then treated with the indicated stimuli. Forty-eight hours post-transfection, luciferase activity was measured using a Veritas microplate luminometer (Turner Biosystems) and normalized to β-galactosidase activity (Applied Biosystem).

**Generation of BLRP-Flag-Coro2A cell lines**

BLRP cell lines were generated as previously described. Briefly, individual macrophage cell lines (RAW264.7) were co-transfected with
plasmids over-expressing bacterial biotin ligase (BirA) and Flag Coro2A conjugated to a N-terminal biotin ligase receptor peptide (BLRP), a spacer, a tobacco etch virus (TEV) protease cleavage site \(^{183}\), and then an N-terminal Flag tag. BLRP is a 22 amino acid peptide that is a specific substrate for the bacterial biotin ligase (BirA). The following PCR primers: 5’XhoI-Flag (5’-CTTCCTCGAGATGGACTACAAAGACCATGACG) and 3’PmeI-Coro2A (5’-GAAGGGTTTAAACTCAGAGCTGCTCTGAGC-3’) were used to amplify the Flag Coro2A plasmid \(^{113}\). The amplification product and BLRP backbone was subjected to restriction digestion by XhoI and PmeI, followed by subsequent ligation into the plasmid backbone. Stable cell lines were generated through stable transfection of the BLRP-Flag-Coro2A plasmid using Superfect (Qiagen) according to manufacturer’s instructions. Antibiotic selection for neomycin (275 ug/mL) and puromycin (2.5 ug/mL) were performed and individual colonies were selected for downstream propagation. Expression of the inserted gene was examined using RT-qPCR and western blot analyses.

**Rev-Erb DKO mice and genotyping**

Rev-Erbα and Rev-Erbβ double floxed mice were generated as previously described \(^{50}\) and crossed with Tie2-Cre \(^{129}\). Breeding and genotyping were performed as previously described \(^{129}\). For experiments, double floxed littermates not expressing the Tie2-Cre transgene were used as WT controls.
**NCoR KO mice and genotyping**

NCoR floxed mice were generated as previously described and crossed with LysM-Cre\textsuperscript{172}. Breeding and genotyping were performed as previously described\textsuperscript{172}. For experiments, floxed littermates not expressing the LysM-Cre transgene were used as WT controls.

**Generation of Coro2A knock-out animals**

Coro2A total knock-out (Coro2A\textsuperscript{tm1(KOMP)Wtsi}) heterozygote animals were purchased from the UC Davis KOMP repository knock-out mouse project wherein the Coro2A gene was replaced by a LacZ knock-in. Heterozygote and knock-out animals were maintained through brother/sister matings. Allele knock-out was determined using polymerase chain reaction. Genotyping primers for the WT allele were Common-F (5'-CCATGTTTCCATGACACAGC-3’) and WT-R (5’-TGGATCCCAAGTCTCACACA-3’) and the knock-out allele were Common-F (5’-CCATGTTTCCATGACACAGC-3’) and KO-R (5’-CCACAACGGGTTCCTCTGT-3’). Experiments were conducting using age-matched WT and knock-out animals from the same ancestry.

**Western blot analysis**

RAW264.7 cells, HeLa cells, or bone marrow derived macrophages were washed two times with ice cold PBS and lysed in lysis buffer (10 mM Tris pH 8, 420 mM NaCl, 1 mM EDTA, 0.5% IGEPAL-CA 630 (Sigma), 1x protease inhibitor cocktail (Roche), 1 mM PMSF). Lysate was collected and sonicated.
using a Misonix 3000 sonicator by administering 10 pulses of 1 second
duration, on wet ice, using 13 watt power output. After sonication, the lysate
was spun at 14000 rpm for 10 minutes at 4°C. The supernatant was saved
and the input was removed for subsequent comparison. Samples were diluted
2 fold with dilution buffer (10 mM Tris pH 8, 1 mM EDTA, 0.5% IGEPAL-CA
630 (Sigma), 1x protease inhibitor cocktail (Roche), 1 mM PMSF) and 2 µg of
normal goat IgG (Santa Cruz Biotechnology, sc-2028) or NCoR (Santa Cruz
Biotechnology, sc-1609) was added overnight with rocking at 4°C. Antigen-
antibody complexes were collected with 40 µL of PAS or PGS beads for 1
hour and washed three times with ice cold wash buffer (10 mM Tris pH 8, 150
mM NaCl, 1 mM EDTA, 0.5% IGEPAL-CA 630 (Sigma), 1x protease inhibitor
cocktail (Roche), 1 mM PMSF). The supernatant was then discarded and the
samples and input were resuspended in 10 µL LDS sample buffer (Invitrogen)
and eluted by heating to 70°C for 10 minutes. The samples were run on a
NuPAGE 4-10% Bis-Tris gel (Invitrogen) or a NuPAGE 3-8% Tris-Acetate gel
(Invitrogen) at 150 volts and the subsequent product was transferred onto
PVDF membranes (0.45 µM pore size, Millipore) using NuPAGE 20X transfer
buffer (Invitrogen) according to manufacturer’s instructions. Membranes were
blocked with 5% bovine serum albumin (BSA) in PBS and immunoblotted with
the following primary antibodies overnight at 4°C in 5% BSA PBS: Coro2A
(Santa Cruz Biotechnology, M-105, 1:500), NCoR (ThermoScientific, PA1-
844A, 1:1000), Flag (Sigma, F3165, 1:1000), and Ubiquitin (Zymed, 13-1600,
1:1000). Afterwards, membranes were washed three times with 0.1% Tween
PBS and immunoblotted with rabbit polyclonal (DakoCytomation, P0448, 1:2000) or mouse polyclonal secondary antibodies (DakoCytomation, P0447, 1:2000) for 1 hour at room temperature. Membranes were developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, 34078) according to manufacturer’s instructions.

For streptavidin or flag immunoprecipitation, lysates from HeLa and RAW264.7 cells were transfected with the indicated expression vectors using Lipofectamine 2000 (Invitrogen) or Superfect Transfection Reagent (Qiagen) according to the manufacturer’s instructions. Immunoprecipitation was conducted with T1 Streptavidin Dynabeads (Invitrogen, 65601) or M2 anti-Flag beads (Sigma, A2220).

**RNA isolation and RT-qPCR**

Total RNA was harvested from tissue and cells using the RNeasy Mini Kit (Qiagen) with in column DNAse digestion performed according to the manufacturer’s instructions. DNAse-treated RNA was used for cDNA synthesis using Superscript III (Invitrogen) according to the manufacturer’s instructions. Quantitative polymerase chain reaction was performed using SYBR GreenER Master Mix (Invitrogen) or SYBR Fast qPCR Master Mix (Kapa Biosystems) on an Applied Biosystems 7300 Real-time PCR system or a Step One Plus PCR System. Relative expression of RNA was determined by normalization to 36B4 through a ΔΔC_{T} method.
For anti-sense oligonucleotide experiments, ASOs were transfected at a final concentration of 40 nM, using Cytofectin (ISIS Pharmaceuticals), according to manufacturer's instructions. The Cx3cr1 eRNA and mRNA primers, as well as the control and Cx3cr1 ASOs have been previously described.129

**RNA-Seq**

Detailed protocols for RNA-Seq experiments have been previously described.195 Briefly, total RNA was treated with the TURBO DNA-free kit (Ambion). Poly(A) selection was performed using the Poly(A)Purist kit (Invitrogen) according to the manufacturer’s instructions. Poly(A) RNA was fragmented using RNA Fragmentation Reagents (Ambion) and subsequently purified by running through P-30 columns (Bio-Rad). Fragmented RNA was 5’ de-capped using tobacco acid pyrophosphatase (Epicentre) and 3’ de-phosphorylated using T4 polynucleotide kinase (New England Biolabs). De-phosphorylated RNA was subsequently 5’ phosphorylated using T4 polynucleotide kinase in order to facilitate subsequent adapter ligations processes. Trizol LS was used to quench the reaction and extract phosphorylated RNA. For indexed library preparation, Illumina Tru-Seq compatible adapters were ligated to the 3’ and 5’ ends of the RNA using a truncated mutant RNA ligase 2 (K227Q) and RNA ligase 1 (New England Biolabs), respectively. Reverse transcription and cDNA isolation were performed, followed by PCR-mediated library amplification was performed for
12 cycles. Ten percent TBE gels (Invitrogen) were used for size selection of final libraries for an insert size of 60-110 base pairs.

5' GRO-Seq

For detailed protocols, please refer to 129. Twenty million cells were utilized for each 5' GRO-Seq experiment. Briefly, cells plated on 15 cm plates were washed three times with PBS (4°C) and were incubated for 5 minutes with 10 mL ice-cold Swelling Buffer (10mM Tris-HCl pH 7.5, 2mM MgCl2, 3mM CaCl2, 2U/mL Superase-In (Ambion)). Cells were scraped into 15 mL conicals, pelleted at 1300 rpm, 4°C for 10 minutes, and resuspended in 500 µL Swelling Buffer/10% glycerol supplemented with 4 U/mL Superase-In (Ambion). Cells were then vortex at 800 rpm (allowing the liquid column to rise 1-2 cm), and 500 µL Lysis Buffer (Swelling Buffer, 10% glycerol, 1% IGEPAL CA-630, 2U/mL Superase-In (Ambion)) was added to the tubes and the lysate was incubated on ice for 5 minutes. Afterwards, 9 mL of Lysis Buffer was added to the samples and the samples were centrifuged at 1550 rpm, 4°C for 5 minutes. Nuclei were subsequently washed with 10 mL Lysis Buffer, centrifuged as before, and resuspended in Freezing Buffer (40% glycerol, 5mM MgCl2, 0.1mM EDTA, 50mM Tris-HCl pH 7.5/8, 2U/ml Superase-In before use) at a concentration of 5 million nuclei per 100 µL. Nuclear run-on buffer (16.5 mM Tris-HCl pH8, 496 mM KCl, 8.25 mM MgCl2, 1.65% Sarkosyl) was preheated to 30°C. To 1 mL nuclear run-on buffer, the following was added: 16.75 µL 0.1M DTT, 33 µL Superase-In, 8.25 µL 100 mM ATP and
GTP, 11.25 µL 0.4 mM CTP, and 27.5 µL 30 mM Biotinylated-UTP. 100 µL of nuclei was incubated with 50 µL of the above supplemented nuclear run-on buffer at 30°C for 5 minutes. Nascent RNA was subsequently isolated using 450 µL of Trizol LS according to manufacturer's instructions. The RNA pellet was resuspended with DNAse, RNAse free water containing Superase-In (1 U/µL). DNAse treatment was conducted using the TURBO DNAse kit (Ambion) according to manufacturer's instructions followed by fragmentation using RNA Fragmentation Reagents (Ambion) according to manufacturer's instructions. Subsequently, the 3' ends of the RNA were dephosphorylated with T4 polynucleotide kinase (25 µL sample, 1 µL Superase-In, 7 µL 5x PNK buffer (0.5 M MES, 50mM MgCl2, 50 mM mercaptoethanol, 1.5 M NaCl), 2 µL PNK (10 U/µL) for 1 hour at 37°C and an additional 1 µL of T4 PNK was added to the sample and incubated for an additional hour. Afterwards, 1 µL 0.5 M EDTA was added to the sample to chelate Mg ions and PNK was inactivated by heating the sample for 5 minutes at 75°C. During RNA dephosphorylation, anti-BrUTP beads (Santa Cruz, sc-32323AC, 60-80 µL per sample) were washed two times with 500 µL Binding Buffer (0.25x SSPE, 0.05% Tween, 37.5 mM NaCl, 1mM EDTA, 2 µL Superase-In/10mL buffer) for 5 minutes with rotation and pelleted at 3000rpm for 2 minutes. Beads were blocked in 2 mL Blocking Buffer (1x Binding Buffer, 0.1% polyvinylpyrrolidone, 0.1% BSA, 4 µL Superase-In) for 1 hour at room temperature, subsequently washed twice with 500 µL Binding Buffer, and resuspended in 500 µL Binding Buffer. Five hundred microliters of resuspended beads in Binding Buffer were
added to each sample and incubated for 1 hour at room temperature. Centrifugations for the following washes were performed at 3000 rpm for 2 minutes. Samples were washed once with 500 µL Binding Buffer and moved to Millipore Ultrafree MC columns (Millipore). Afterwards, samples were washed once with 500 µL Low Salt Buffer (0.2x SSPE, 0.05% Tween, 1mM EDTA, 2 µL Superase-In per 10 mL), once with 500 µL High Salt Buffer (0.25x SSPE, 137.5 mM NaCl, 0.05% Tween, 1 mM EDTA, 2 µL Superase-In per 10 mL), twice with 500 µL 0.1% Tween-20 in TE, and then eluted four times with Elution Buffer (50 mM Tris pH 7.5, 300 mM NaCl, 0.1% SDS, 1mM EDTA, 20mM DTT, 2 µL Superase-In per 10 mL). Eluted samples were precipitated overnight using 2 µL GlycoBlue (Ambion) and 2.5 volumes 100% ethanol at -20°C and washed two times with 80% ethanol before resuspension in 16.5µL water. RNA de-capping was performed using 5 units of tobacco acid pyrophosphatase (Epicentre) for 2 hours at 37°C (1 µL Superase-In, 2 µL 10x TAP buffer, 0.5 µL TAP) and 5’ phosphorylated (10 µL 10x T4 DNA ligase buffer, 66 µL water, 1 µL 10% Tween-20, 1 µL Superase-In, 2 µL PNK) at 37°C for 90 minutes and then placed on ice. 2 µL 0.5M EDTA (10mM final) was added to the sample to chelate Mg ions; afterwards, samples were extracted using 306 uL Trizol LS according to manufacturer’s instructions. Library preparation was performed using the above protocol for RNA libraries. **ChIP-Seq**
Previously published Rev-Erbα and Rev-Erbβ ChIP-Seqs, PU.1 ChIP-Seqs, and NCoR ChIP-Seq, deposited as GSE45914 \(^{129}\), GSE38379 \(^{12}\), and GSE27060 \(^{196}\), respectively, were utilized for analyses. Detailed protocols for ChIP experiments have been previously described \(^{13,15,172,195}\). Antibodies against p65 (Santa Cruz Biotechnology, sc-372), STAT6 (Santa Cruz Biotechnology, sc-981), cFos (Santa Cruz Biotechnology, sc-253), and cJun (Santa Cruz Biotechnology, sc-1694) were utilized for these experiments. Briefly, for p65, cFos, and cJun ChIPs, macrophages were first cross-linked in 2mM disuccinimidyl glutarate (Pierce) in PBS for 30 minutes, followed by subsequent 1% formaldehyde (Sigma) cross-linking in PBS for 10 minutes at room temperature. For STAT6 ChIPs, cells were cross-linked using 1% formaldehyde in PBS for 10 minutes at room temperature. After cross-linking, glycine (Sigma) was added to a final concentration of 0.2625 M to quench the reaction. Subsequently, cross-linked macrophages were centrifuged (5 minutes, 1200 rpm, 4°C), washed twice with PBS, and resuspended in cell lysis buffer (10 mM HEPES/KOH pH 7.9, 85 mM KCl, 1 mM EDTA, 0.5% IGEPAL CA-630 (Sigma), 1x protease inhibitor cocktail (Roche), 1 mM PMSF). After five minutes lysis on ice, cells were centrifuged (5 minutes, 4000 rpm, 4°C), and the supernatant was removed.

For p65 ChIP and STAT6 ChIPs, the pellet was then resuspended in nuclear lysis buffer 1 (50 mM Tris/HCl pH 7.4, 1% SDS, 0.5% Empigen BB (Sigma), 10 mM EDTA, 1x protease inhibitor cocktail (Roche), 1 mM PMSF) and the chromatin was sonicated using a Misonix 3000 sonicator by
administering 6 pulses, each of 10 seconds duration, on wet ice using 13 watt power output with 30 seconds pausing on ice in between pulses. For cFos and cJun ChIPs, the pellet was resuspended in nuclear lysis buffer 2 (10 mM Tris/HCl pH 8.0, 100mM NaCl, 1mM EDTA, 0.5mM EGTA, 0.1% Na-Deoxycholate, 0.5% N-lauroylsarcosine, 1x protease inhibitor cocktail (Roche), 1mM PMSF) and the chromatin was sonicated using a Biorupter (Diagenode) for 15 minutes (30 seconds ON / 30 seconds OFF). An additional 10% volume of 10% Triton X-100 was added to that cFos and cJun lysate. All lysates were cleared by centrifugation (5 minutes, 14000 rpm, 4˚C). ChIPs for p65 and STAT6 were further diluted 2.5 fold using dilution buffer (20 mM Tris/HCl pH 7.4, 100 mM NaCl, 0.5% Triton X-100, 2 mM EDTA, 1x protease inhibitor cocktail (Roche)). Input was then saved for subsequent analysis. Protein A or G dynabeads (Invitrogen) pre-bound with antibody was added to the diluted cell lysate overnight at 4˚C. Immunoprecipitated complexes were washed three times with wash buffer 1 (20 mM Tris/HCl pH 7.4, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA), three times with wash buffer 2 (10 mM Tris/HCl pH 7.4, 250 mM LiCl, 1% IGEPAL CA-630, 0.7% sodium deoxycholate, 1 mM EDTA), and twice with Tris-EDTA plus 0.1% Tween-20 before eluting two times with 100 µL elution buffer (TE, 1% SDS, 30 and 10 minutes, room temperature). Elution buffer was also added to the input. After pooling the eluted samples, their sodium concentration was adjusted to 300 mM and cross-links were reversed overnight at 65°C. Samples were treated with 0.5 mg/ml proteinase K for 1 hour at 55°C and 0.25 mg/ml RNAse A for 1
hour at 37°C before the DNA was isolated using the ChIP DNA Clean and Concentrator (Zymo Research) according to the manufacturer’s instructions. For library preparation, Illumina Genomic Adapters or NEXTflex DNA barcode adaptors (BioO Scientific) were ligated to the genomic DNA. Polymerase chain reaction mediated library amplification was performed and final libraries were size selected on 2% TBE agarose gels.

**High-throughput sequencing and data processing**

RNA-Seq and ChIP-Seq libraries were sequenced for 50 cycles on an Illumina HiSeq 2000 according to the manufacturer’s instructions. Reads were mapped to the mouse mm9 assembly using Bowtie 197, allowing up to two mismatches. Differential splicing was determined using Tophat 198. Mapped reads were visualized using the UCSC genome browser 199 and downstream data processing was performed using HOMER 13.

**Genome-wide gene expression analysis with RNA-Seq**

RNA-Seq analysis of genome-wide transcriptome data was performed using HOMER followed by edgeR 170. Differential expression was determined by edgeR for replicates using FDR < 0.05. For heatmap analysis, genes differentially expressed 1.5 fold in WT vs. Rev-Erb DKO macrophages were clustered using average linkage hierarchical clustering on Cluster 3 200 and visualized using Java TreeView 201. Gene ontology analysis was performed using DAVID Bioinformatics Resources 6.7 171,202.
Enhancer-associated RNA analysis and de novo motif discovery

Peak finding and motif finding were conducted using HOMER as previously described \(^{13,129}\). Transcription factor co-bound peaks were those determined as overlapping within 100 base pairs. For motif analysis using differentially expressed genes determined by RNA-Seq, de novo motif finding was conducted at the nearest PU.1-bound regions. To determine whether Coro2A 5’ GRO-Seq signal overlays with NCoR, cFos, and p65, differential 5’ GRO-Seq transcriptional signal from Coro2A control and over-expressing cell lines were plotted using Venn diagrams and histograms. For Venn diagrams, differential 5’ GRO-Seq transcriptional signal was considered significant if it showed greater than 1.5-fold difference from control cell lines and was within 100 base pairs of cFos/p65/NCoR-bound region. Histograms plotted all differential signal from Coro2A control and over-expressing cell lines emanating from the cFos/p65/NCoR-bound region.

Bone marrow transplantation

Bone marrow harvested from WT and Rev-Erb DKO mice was injected retro-orbitally into lethally irradiated B6.SJL-Ptprca Pepc^b~/BoyJ (CD45.1) wild type congenic mice. Approximately 6-7 million bone marrow cells were injected per mice. Transplanted mice were housed in autoclaved cages (changed every two days) and supplemented with antibiotics the day before irradiation until two weeks post-transplantation. Bone marrow transplantation
efficiency was evaluated by harvesting bone marrow and conducting subsequent flow cytometry and gene expression analyses.

**Flow cytometry**

To evaluate bone marrow transplant efficiency, whole blood from WT and Rev-Erb DKO bone marrow transplanted mice was collected through cardiac puncture into EDTA tubes (Becton Dickinson). 100 µL whole blood was washed once with PBS and resuspended in 2% FBS in PBS. Samples were blocked with 1 µL anti-mouse CD16/32 (eBioscience, 14-0161-82) for 15 minutes at room temperature. The following antibodies were utilized for staining: CD45 (Biolegend, 103122) and CD45.2 (Biolegend, 109813). Samples were incubated with directly labeled antibodies for 40 minutes (4°C in the dark). Stained cells were washed with 0.1% BSA in PBS, pelleted (1200 rpm, 5 minutes, 4°C), and lysed with hemolysin (Beckman Coulter) for 20 seconds. Samples were quenched with 10X PBS, diluted, and gently washed before analysis using a LSR II flow cytometer (BD Bioscience). Unstained and single stained samples were used for setting up compensations and gating. For bone marrow transplant efficiency analysis, events were first gated on forward and side scatter to determine single, unaggregated events, before evaluation of other fluorescent markers.

**Wound healing studies**
Wound healing studies were conducted 6-10 weeks post-transplantation. Briefly, 15.5 mL tert-amyl alcohol was added to 25 grams of 2,2,2 tribromoethanol (Aldrich Chemical) and dissolved overnight in a dark bottle to generate a stock solution. The subsequent solution was diluted with PBS, dissolved overnight, and filtered through a 0.2 µm filter to generate a working solution (20 mg/ml). To achieve anesthesia, 0.4-0.75 mg/g was administered intra-peritoneally. A 3 mm punch biopsy (Miltex) was used to generate four wounds on the dorsal skin of each animal. Wounds were systematically photographed from a fixed distance daily. For macroscopic analysis, the size of the wound was analyzed by Adobe Photosho and normalized to its size on Day 0. Mice were housed singularly throughout the duration of the study.

**Histological analyses**

At the indicated time points, mice were euthanized and wounds were harvested using a 6 mm punch biopsy (Miltex). Harvested wounds were cut along the mid-sagittal plane and paraffin-embedded for subsequent histological analyses. The first section along the mid-sagittal plane was utilized for hematoxylin and eosin staining for re-epithelialization and granulation tissue analyses. Subsequent sections were utilized for immunohistochemical analysis using the following primary antibodies: biotinylated anti-F4/80 (AbD Serotec, MCA4978, 1:50 dilution), IgG (Dako), and rat anti-Ly6B.2 (AbD Serotec, MCA771GA, 1:200), and the following secondary antibodies:
biotinylated anti-rat (1:500, BD Pharmingen), as well as HRP-conjugated streptavidin (1:500, Jackson Laboratory). Briefly, slides were de-paraffinized and washed three times in 0.1% Tween PBS. Blocking was performed sequentially using 3% hydrogen peroxide (10 minutes), 1% BSA in 0.1% Tween PBS (10 minutes), 0.1% avidin (10 minutes), and 0.01% biotin (10 minutes). Three washes were performed between each blocking step using 0.1% Tween PBS. Antigen retrieval was performed using proteinase K (Dako, S3020), followed by three washes and subsequent overnight incubation with the indicated primary antibodies. After three washes, the slides were incubated with the indicated secondary antibodies for 30 minutes and developed using AEC Peroxidase Substrate Kit (Vector Labs, SK-4200) according to the manufacturer’s instructions. Counterstaining was performed using Mayer’s Hematoxylin (Sigma, MHS16), after which samples were mounted in an aqueous gel mount (Vectamount, Vector Labs, H-5501).

**Matrigel migration assays**

Briefly, macrophages were cultured in RPMI 1640 (Invitrogen) supplemented with 0.5% heat-inactivated FBS (Hyclone) for 24 hours and resuspended at a density of 1 million cells per milliliter. 100 µL of macrophages was added to the top chamber of a transwell while 650 µL of media was added to the bottom chamber. For matrigel migration assays, macrophages were allowed to migrate through basement membrane extract (Corning, 3458) for 24 hours. Afterwards, the wells were briefly washed with
PBS, and migrated macrophages were dissociated from the membrane and incubated with Calcein AM. Relative fluorescence was measured using a SpectraMax M3 plate reader and the SoftMax Pro software (485 nm excitation, 520 nm emission). A standard curve was used to convert relative fluorescence to cell numbers.

**Statistical Analyses**

Statistical analyses were performed using excel and figures were prepared using Adobe Photoshop. Unless otherwise stated, data are presented as the mean ± SEM. Paired student’s t-test was utilized to determine significance; p < 0.05 was considered significant.
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