Title
A High-throughput Screening Approach Identifies Selective Regulators of Il12b Transcription

Author
Doty, Kevin Richard

Publication Date
2012

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

A High-throughput Screening Approach Identifies Selective Regulators of *Il12b* Transcription

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular Biology

by

Kevin Richard Doty

2012

ABSTRACT OF THE DISSERTATION

A High-throughput Screening
Approach Identifies Selective Regulators
of *Il12b* Transcription

by

Kevin Richard Doty
Doctor of Philosophy in Molecular Biology
University of California, Los Angeles, 2012
Professor Stephen Smale, Chair

Detection of pathogens by cells of the innate immune system lead to the expression of a subset of inflammatory mediators that will support the clearance of the pathogen present. Pro-inflammatory gene products display a very distinct and controlled pattern of expression. It is hypothesized that there are signaling pathways that, singly or in combination, are responsible for the selective expression of pro-inflammatory factors seen in different physiological settings. It is critical that factors are only induced in the context of an active assault by microbial pathogens and that resolution of this response is complete. Many human pathologies are thought to be caused or exacerbated by the presence of inflammatory mediators expressed outside of a biological need. Two of

these tightly regulated inflammatory mediators that play key roles in inflammation, inflammatory auto-immune disease, and cancer are the cytokines interleukin 12 (IL-12) and interleukin 23 (IL-23). These cytokines share a common subunit, IL12p40, which is encoded by the *Il12b* gene. Transcription of *Il12b* can be induced in macrophages through recognition of pathogen associated molecules by Toll-like Receptors (TLRs). To better understand the signaling pathways and factors involved in the selective regulation of *Il12b* transcription, two cell based, high-throughput screens were performed. These screens were designed to test the hypothesis that treatments capable of modulating expression of a simple promoter reporter plasmid will generally target pathways that regulate proinflammatory gene expression in a non-selective manner, such as the NF-κB and AP-1 pathways; in contrast, treatments that modulate *Il12b* transcription only in the context of native chromatin will target pathways involved in the selective regulation of *Il12b*. To test this hypothesis, we have generated macrophage lines containing an *Il12b* bacterial artificial chromosome (BAC) transgene driving expression of an EGFP reporter. A basic *Il12b* enhancer-promoter-reporter plasmid driving expression of a DsRed gene was stably integrated into the same line. The LPS induced expression of the BAC reporter was directly compared to the expression of the basic DsRed reporter after exposure to the small molecule or siRNA libraries. The hypothesis is we will be able to enrich the pool of hits with treatments likely to be selective for *Il12b* by selecting hits that are selective for the chromatinized BAC. Here we present results from both the small molecule screen and the siRNA screen. One hit from the small molecule screen, β_2-adrenergic agonists, is further established as a selective inhibitor of *Il12b* transcription, dependent on the transcription factor NFIL3.

The dissertation of Kevin Richard Doty is approved.

Douglas L. Black

Linda G. Baum

John J. Colicelli

Lily Wu

Stephen Smale, Committee Chair

University of California, Los Angeles

2012

DEDICATION

This work is dedicated to my wife, Julia and daughter, Adelaide.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

FIGURES

Chapter 1

Chapter 2

Chapter 3

Appendix A

TABLES

Chapter 1

Chapter 2

Chapter 3

Acknowledgements

I would like to thank my mentor, Steve Smale, for his encouragement, guidance, and the chance to work on such an exciting project. I would like to thank my committee, Doug Black, Linda Baum, John Colicelli, and Lily Wu for their helpful discussions. I would like to acknowledge the contributions of the personnel in the Molecular Screening Shared Resource at UCLA where the screens were performed: Ken Bradley, Robert Damoiseaux, Jong Lee, and Winnie Hwong. They were a valuable asset in both design and execution of the screen. Many members of the Smale lab, both past and present, have helped me greatly throughout my training. I would like to acknowledge the contributions of Tirth Patel, Jeremy Flores, and Xin Liu in numerous experiments, including those shown in Figure 3-1 and Figure 3-2. I would also like to acknowledge the contributions of Scott Plevy and Tasuku Kitada at the University of North Carolina for assistance with then NFIL3 knockout experiments.

I would like to thank my wife, Julia, and daughter, Adelaide, for without them I could not accomplish anything. They are a constant supply of love and support.

Appendix A is reprinted from *Cell*, Vol 138/1, by V. R. Ramirez-Carrozzi, D. Braas, D. M. Bhatt, C. S. Cheng, C. Hong, K. R. Doty, J. C. Black, A. Hoffmann, M. Carey, and S. T. Smale, A unifying model for the selective regulation of inducible transcription by CpG islands and nucleosome remodeling, Pages 114-128, Copyright (2009) with permission from Elsevier. Figure 1-1 and Table 1-1 is reprinted from *Cell*, Vol 140/6, by Osamu Takeuchi and Shizuo Akira, Pattern recognition receptors and inflammation, Pages 805-820, Copyright (2010), with permission from Elsevier. Figure 1-2 is reprinted from *Genes & Development,* Vol 20, by V. R. Ramirez-Carrozzi, A. A.

Nazarian, C. C. Li, S. L. Gore, R. Sridharan, A. N. Imbalzano, and S. T. Smale, Selective and antagonistic functions of SWI/SNF and Mi-2beta nucleosome remodeling complexes during an inflammatory response, Pages 282–296, Copyright (2006), www.genesdev.org.

This work was partially funded by the NIH Ruth L. Kirschstein National Research Service Award GM07185.

VITA

2002	B. S., Aquatic Biology University of California Santa Barbara, California
2002	Audrey Lynn Copland Memorial Award University of California Santa Barbara, California
2002-2003	Marine Sciences Institute University of California Santa Barbara, California
2003-2005	Microbiology, Immunology, and Molecular Genetics University of California Los Angeles, California
2006-2009	Cellular and Molecular Biology Training Grant University of California Los Angeles, California
2007-2008	Teaching Assistant Department of Microbiology, Immunology, and Molecular Genetics University of California Los Angeles, California

PUBLICATIONS AND PRESENTATIONS

Ramirez-Carrozzi, V.R., Braas, D., Bhatt, D.M., Cheng, C.S., Hong, C., **Doty, K.R.**, Black, J.C., Hoffmann, A., Carey, M., Smale, S.T. (2009) A unifying model for the selective regulation of inducible transcription by CpG islands and nucleosome remodeling. *Cell* 138(1), 114-128

Kobayashi, T., Matsuoka, K., Sheikh, S.Z., Elloumi, H.Z., Kamada, N., Hisamatsu, T., Hansen, J.J., **Doty, K.R.**, Pope, S.D., Smale, S.T., Hibi, T., Rothman, P.B., Kashiwada, M., Plevy, S.E. (2011) NFIL3 Is a regulator of IL-12 p40 in macrophages and mucosal immunity. *J Immunol* 186:4649-4655

Doty, K.R., Pope, S.D., Smale, S.T. "Using A High Throughput Small Molecule Screen to Find Selective Regulators of *Il12b* Transcription" The Macrophage: Intersection of Pathogenic and Protective Inflammation, Keystone Symposium. Banff, Alberta, Canada, February, 2010. Poster Presentation

Doty, K.R., Pope, S.D., Smale, S.T. "Using A High Throughput Small Molecule Screen to Find Selective Regulators of *Il12b* Transcription." Immunology LA, Malibu, CA. April 22, 2011. Poster Presentation

Chapter 1

Introduction

Selective Regulation of

Pro-Inflammatory Gene Transcription:

IL-12 and IL-23 Cytokines

A. Immune System: Layered Innate and Adaptive Responses

The mammalian immune system has evolved into a complex and layered response to assault from microbial pathogens and tissue injury (Medzhitov, 2008). One of these layers, the adaptive immune system, evolved most recently and is conserved in all investigated jawed vertebrates (Litman et al., 2010). Adaptive immunity is driven by the population of lymphocytes and includes the hallmark processes of clonal selection, somatic hypermutation, class-switch recombination, and immunological memory. These lymphocyte developmental and maturation steps occur in the thymus for T cells and the bone marrow for B cells (Takahama, 2006; Nagasawa, 2006). Through a number of complex steps the organism can produce a diverse array of antigen specific receptor genes, that will be clonally selected to expand if utilized and will persist to provide the organism with a powerful antigen specific immunological memory.

While very potent, and specific, this clonally produced, adaptive system seems to have evolved in the presence of an earlier form of immunity (Janeway, 1989). This ancestral form of immunity, which can be found in organisms as distinct as flies and humans, centers around germ-line encoded pattern recognition receptors (PRRs) that can detect pathogen-associated molecular patters (PAMPs) (Medzhitov and Janeway, 1997). These PAMPs represent molecular structures of the pathogen that are essential for its survival and therefore evolutionarily conserved with little variability among large groups of microbes (Medzhitov, 2001). PRRs can be classified into three different groups: secreted, transmembrane, and cytosolic. Secreted PRRs, such as collectins, ficolins, and pentraxins, bind to microbial cell surfaces. From there, they can activate classical and lectin pathways of the complement system and mark pathogens for

efficient removal (Zipfel and Skerka, 2009). Cellular PRRs were first described in *Drosophila*, where mutations in the Toll signaling pathway dramatically reduces the ability of the organism to combat fungal infection (Lemaitre et al., 1996). Today, a number of transmembrane and cytosolic receptors have been described and each have an affinity to a particular group of PAMPs, thus providing the cell in which it resides with the ability to detect a specific class of pathogen.

B. Pattern Recognition Receptors and Innate Immunity

Cellular PRRs are expressed by key effector cells of the innate immune system. These range from professional antigen presenting cells (APCs) such as macrophages, dendritic cells, and B cells, to cells most likely to encounter pathogens, such as those lining the epithelium . Macrophages are a heterogeneous family of mononuclear phagocytic cells derived from a common myloid progenitor in the bone marrow (Gordon, 2003). They enter circulation and are distributed throughout the organism, remaining in circulation or becoming a resident population in a specific tissue where they adapt to the local environment (Kupffer cells in the liver, alveolar macrophages in the lung, langerhans cells in the skin, osteoclasts in the bone, and microglia in the central nervous system). Two of the major roles which macrophages play involve their ability to phagocytize material in their environment, self or non-self, and secret factors that signal to cells in the immediate tissue and beyond. This provides the immune system with two main functions: first, to remove pathogens or damaged cells and second, to identify the pathogen and mount an appropriate inflammatory response.

Transmembrane and cytosolic cellular PRRs can be classified into four different groups: Toll-like receptors (TLRs), nucleotide-binding domain and leucine-rich repeat containing receptors (NLRs), Rig-I-like receptors (RLRs), and the C-type lectin family (CLRs) (Table 1-1) (Heine, 2011; Palm and Medzhitov, 2009). NLRs are a family of cytosolic receptors characterized by three functional domains: C-terminal leucine-rich repeats (LRR), a central NACHT nucleotide-binding (NB) domain, and a N-terminal effector domain. The known ligands for these receptors are bacterial products (peptidoglycan and flagellin). Activation of these receptors leads to the formation of a multi-protein complex termed the inflammasome, which cleaves and activates proinflammatory caspase-1. The RLRs are named after the founding member, retinoic acid inducible gene I (RIG-I) and are cytosolic viral RNA and DNA sensors (Yoneyama et al., 2004). These PRRs were shown to be important in the virally induced type I interferon response. The C-type lectin family of transmembrane PRRs include Dectin-1 and Dectin-2, that recognize β-glucans and mannan from the cell walls of fungal pathogens, respectively (Brown, 2006; Robinson et al., 2009). The fourth and most studied family of the cellular PRRs are the TLRs.

B1. Toll-like Receptors

The Toll-like Receptor family comprise a group of transmembrane receptors, characterized by an extracellular leucine-rich repeat (LRR) domain and an intracellular Toll/Il1 receptor (TIR) domain (Medzhitov, 2001). To date, 13 mammalian TLRs have been identified and they have two distinct cellular localizations (Uematsu and Akira, 2008; Barton and Kagan, 2009). TLRs that recognize nucleic acids can be found in the

endosomal compartment, and those that do not are found on the cell surface. The ligands of TLRs on the cellular outer-membrane are lipopolysaccharide (LPS) of Gram-negative bacteria (TLR4), lipoteichoic acids of Gram-positive bacteria and bacterial lipoproteins (TLR1/TLR2 and TLR2/TLR6), and flagellin (TLR5). In the endosomal compartment, TLRs mainly detect microbial nucleic acids, such as double-stranded RNA (dsRNA) (TLR3), single-stranded RNA (ssRNA) (TLR7), and dsDNA (TLR9) (Table 1-1) (Iwasaki and Medzhitov, 2004).

B2. Toll-like Receptor Signaling Pathway

Binding of a PAMP by a TLR dimer induces a signaling cascade and transcriptional upregulation of a number of distinct genes based on the TLR family member and the cell type involved (Takeuchi and Akira, 2010a). In each case a TIR domain adaptor protein is recruited to the cytoplasmic TIR domain of the activated TLR dimer. There are five TIR domain-containing adaptors: MyD88, TIR domain-containing adaptor inducing IFN-β (TRIF; also known as TICAM-1), TIRAP/Mal, TRIF-related adaptor molecule (TRAM), and Sterile-alpha and Armadillo motif-containing protein (SARM). TLR signaling has been roughly divided into two distinct arms, namely, the MyD88 and the TRIF-dependent pathways (Figure 1-1).

The MyD88-dependent pathway is used by a diverse set of TLRs, with the notable exception of TLR3. MyD88 contains a TIR domain and a death domain (DD). As shown in Figure 1-1, TIRAP/Mal serves as an adaptor between the TIR domain of TLR2 and TLR4 to MyD88 (Takeuchi and Akira, 2010a). This complex interacts with IL-R-associated kinase 4 (IRAK-4), which in turn activates other IRAK family members,

IRAK-1 and IRAK-2. The IRAKs can then dissociate from MyD88 and interact with the E3 ubiquitin protein ligase TNFR-associated factor 6 (TRAF6). Ubiquitination products of the TRAF6 complex induce the formation of a complex of TGF-β-activated kinase 1 (TAK1), TAK1-binding protein 1 (TAB1), TAB2, and TAB3. This complex is responsible for the phosphorylation of IκB kinase (IKK)-β and MAP kinase kinase 6. This activates the NF-κB and the MAPK pathways. NF-κB is activated when the IKK complex phosphorylates IκBα, leading to its ubiquitination and degradation, freeing NF-κB dimers to translocate to the nucleus and induce the transcription of many target pro-inflammatory genes. Similarly, activation of MKK6 triggers a kinase cascade that is responsible for the activation of the Activator protein 1 (AP-1) transcription factor complex, which targets its own set of pro-inflammatory genes.

The TRIF-dependent pathway is utilized by TLR3 dimers without the need for known adaptors and TLR4 dimers with the TIR adaptor TRAM (Takeuchi and Akira, 2010a). TRIF associates with TRAF3 and TRAF6 through N-terminal TRAF-binding motifs. TRAF3 can activate two IKK-related kinases, TANK-binding kinase 1 (TBK1) and IKK-ε. These kinases can phosphorylate IRF3 and IRF7, leading to dimerization and translocation into the nucleus where they induce the expression of type I IFNs and interferon-inducible genes. In addition to activating IRFs, IKK-ε also phosphorylates STAT1 which in turn supports induction of IFN-inducible genes.

B3. TLR Transcriptional Responses

TLR and other PRR signaling pathways induce the activation of multiple post-translationally modified and/or translocation dependent transcription factors. These

factors are key components of the transcription of many pro-inflammatory genes. As previously mentioned, MyD88-dependent TLR signaling results in the activation of the NF-κB family of transcription factors. This family is characterized by a DNA-binding motif known as the Rel homology domain (RHD). In mammals there are five family members: p65 (RelA), c-Rel, RelB, p100/p50, and p105/p52 (Hoffmann and Baltimore, 2006). These factors are present in the cell as homodimers or heterodimers, sequestered in the cytoplasm by IκB molecules and upon stimulation (as described above) are released to translocate into the nucleus and initiate a transcriptional response (Figure 1-1). The inhibition of NF-κB signaling has broad effects on inflammatory gene expression and results in the inhibition of tens to hundreds of genes, however the dependence of any particular gene on specific dimers is still unclear.

The second key family of transcription factors are the Interferon Response Factors (IRFs). The mammalian IRF family comprises 9 members: IRF1, IRF2, IRF2, IRF4, IRF5, IRF6, IRF7, IRF8 (or ICSBP), and IRF9 (or ISGF3γ). Each IRF contains a well conserved, N-terminal DNA binding domain that recognizes the IFN-stimulated response element (ISRE) consensus sequence found in the promoters of genes responsive to type I IFNs, type I IFNs themselves, and many other genes involved in immunity and oncogenesis (Honda and Taniguchi, 2006). In addition to IRF3 activation by the TRIF dependent pathway stated above, TLR signaling can also activate IRF7 and IRF5 family members. More over, RLRs and type I IFNs induce multiple IRFs and crosstalk between the pathways adds to the complexity of regulation.

Beyond NF-κB and IRF transcription factors, there are a number of other transcription factors that are known to be induced by inflammatory stimuli and play key

roles in pro-inflammatory transcriptional regulation. Mentioned briefly above, activity of AP-1 can be induced not only by the MAPK signaling pathway, but also ERK and JNK signaling pathways that are induced in response to microbial products, cytokines, and growth factors to name a few (Karin, 1995). The AP-1 class of transcription factors function as a dimer of basic leucine zipper proteins from the Jun and Fos families. Activation of these factors requires phosphorylation and dimerization in a number of heterodimer and homodimer configurations that then become competent to bind DNA control regions and regulate transcription.

A fourth class of transcriptional activators that respond to inflammatory stimuli are the nuclear factor in activated T cells (NFAT) family of proteins (Rao et al., 1997). Calcienurin, a calcium/calmodulin-dependent phosphatase, controls the translocation of NFAT family members into the nucleus where it can bind to the control regions of many genes that are induced by microbial stimuli. Interestingly NFAT has been show to bind cooperatively with AP-1 family members, increasing the complexity of the possible targets of NFAT activation.

Finally, a fifth broadly acting, post-translationally modified transcription factor that is important in the inflammatory response is the cyclic-AMP (cAMP) response element-binding protein (CREB). Phosphorylation of CREB by one of several kinases, including cAMP-dependent protein kinase A (PKA), protein kinase C (PKC), or calmodulin kinases (CaMKs), allows for its interaction with the coactivator protein CREB-binding protein (CBP) or p300, a cofactor that stimulates chromatin modifications (Wen et al., 2010). The broad range of transcriptional response pathways induced by microbial products, the majority of which activate most, if not all of the large inflammatory

transcription factor families mentioned above, poses an interesting problem. It is likely that many genes are expressed by a unique combination of multiple members from multiple families. The fact that many of these factors behave as both homodimers and heterodimers further complicates the regulatory landscape. Selectivity of expression is seen and therefore there must be a logic for how these signals are sorted that can be exploited to benefit human health. It then becomes the aim for any research program interested in describing selective transcriptional regulation to attempt to define individual family members required for robust expression at the endogenous gene of interest.

C. TLR4: Model of Complexity

TLR4 is the sole TLR that is known to have both MyD88-dependent and TRIF-dependent activity. Upon LPS stimulation, TRL4 signaling in macrophages induce the rapid transcription of a number of genes in minutes. Regulation of these immediate and other more delayed genes display regulatory requirements that illustrates the complexity of the innate immune response (Smale, 2010). Inducible gene products vary in their biological roles and show different patterns of expression in physiological settings. For instance, antimicrobial peptides and complement factors that directly target pathogenic microorganisms display expression limited to the presence of these microbes. Many chemokines drive the activation of neighboring endothelial cells resulting in recruitment of inflammatory effector cells to the tissue which could be damaging to healthy tissue. Many cytokines that show selective expression signal to the adaptive immune response, tailoring it to the pathogen detected. Finally, induced gene products can have systemic effects, including fever and the acute phase response in the liver. Tight regulation of

each of these responses must occur in healthy individuals and is often altered in human disease.

C1. Approaching a Logic for Inflammation

Many of the genes that are part of the inflammatory program can be classified into a number of groups with similar properties. At the most basic level they can be divided in to primary and secondary response genes (Smale, 2010). Primary response genes are mostly activated rapidly, without the requirement of de novo protein synthesis. This means that transcription of these genes only requires constitutively active factors or factors that only require post-translational modifications to induce transcription. Secondary response genes are induced more slowly on average and require new protein synthesis. These factors can be transcription factors, signaling molecules needed to activate the required transcription factors, or cytokines and chemokines that act in an autocrine fashion for robust expression. Halting translation in activated macrophages would block expression of secondary genes by blocking the production of necessary primary genes.

C2. Epigenetic Control of Inflammation

While having all required factors expressed is a necessary step, another fundamental level of eukaryotic gene regulation is chromatin structure. The basic repeating unit of chromatin is the nucleosome, consisting of 147 base pairs of DNA wrapped around a histone octomer containing two of each of the core histones H2A, H2B, H3, and H4. In addition to the core histone octomer, chromatin structure includes

contributions by linker histones, histone variants, and many nonhistone proteins. Histones undergo a series of regulatory post-translational modifications including methylation, acetylation, phosphorylation, ubiquitination, sumolation, and ADP-ribosylation (Li et al., 2007). This protein matrix can exist in different levels of compaction and regulating the chromatin compaction and nucleosome positioning is a central step in regulated gene transcription. Adjusting the compaction of chromatin and the position of nucleosomes in response to regulatory signals and histone modifications is performed by a number of ATP-dependent nucleosome remodeling complexes. These factors use ATP hydrolysis to break the histone-DNA contacts resulting in unwinding of DNA from the histone octamer (Li et al., 2007). Whether it causes nucleosome eviction, DNA looping, or nucleosome sliding, the net result of nucleosome remodeling activity is the increased accessibility of the DNA to transcription factor binding.

Studies in LPS treated mouse macrophages revealed that some, but not all, induced genes showed a requirement for the SWI/SNF nucleosome remodeling complex (Ramirez-Carrozzi et al., 2006). This study was performed by depleting the BRG1 and BRM ATPase subunits of the mammalian SWI/SNF complex with short hairpin RNAs (shRNAs) in macrophages followed by stimulation with LPS. Analysis of induced genes revealed that most primary response genes were induced in a SWI/SNF-independent manner, while almost all secondary response genes surveyed exhibited SWI/SNF dependence. In addition, SWI/SNF dependence was seen in a subset of primary response genes with a delayed kinetics (Figure 1-2). Some of the remodeling seen in secondary genes requires new protein synthesis. The model these data suggest

is one where a subset of primary response genes contain promoters which require a cellular signal resulting in inducible chromatin remodeling, while other primary genes contain promoters that are accessible to transcriptional activators and the general transcription factors. In addition there are secondary genes that require additional factors to be synthesized and/or secondary signals to be propagated to cause the promoters of the these secondary genes to become accessible to additional transcription activators. This layered regulation, requiring first the synthesis of specific factors, and second, the regulated remodeling of promoters could only serve to regulate expression in a tighter, more specific manner.

D. IL-12 and IL-23 Cytokines

One potently induced cytokine gene which falls into the class of SWI/SNF dependent, secondary response genes is *Il12b*. The protein encoded by the *Il12b* gene is IL12p40, which can be found as a covalently linked heterodimer in two different cytokines, IL-12 and IL-23. When it is in complex with IL12p35, it is the heterodimeric IL-12 cytokine and the founding member of the IL-12 family of cytokines. When it is paired with IL23p19, encoded by the *Il23a* gene, it is IL-23. These two cytokines have very different biological rolls in the inflammatory response.

D1. IL-12

IL-12 was first reported as a novel factor that was able to activate NK cells, induce the production of IFN-γ, and enhance T-cell responses to mitogens (Kobayashi, 1989). It has since been recognized to be a potent inducer of T helper 1 responses, to have a direct proliferative effect on pre-activated T cells and NK cells, and to induce T cells and NK cells to produce several cytokines in addition to IFN-γ, including TNF-α and GM-CSF (Trinchieri, 2003). T helper 1 cells (Th1) are a functional subset of CD4+ T cells that produce type-1 cytokines and are necessary to clear intracellular pathogens via a cell mediated response. Th1 cell products include IL-2, IFN-γ, and other cytokines that support macrophage activation, the generation of cytotoxic T cells, and the production of opsonizing antibodies. This is in contrast to T helper 2 cells (Th2), which IL-12 and IFN-γ oppose, that produce type-2 cytokines (IL-4, IL-5, IL-13, and other cytokines that support B-cell activation, the production of non-opsonizing antibodies, allergic reactions and the expulsion of extracellular parasites). Through the ability of IL-12 to induce a cellular immune response in Th1 and NK cells, it is a powerful mediator of pathogen clearance and tumor surveillance.

D2. IL-23

A second binding partner for IL12p40 was discovered as a result of a bioinformatic structural homology screen for IL-6 family members (Oppmann et al., 2000). Termed IL23p19, it was shown to covalently dimerize with Il12p40 and to interact with a unique high affinity cell surface receptor found on T cells and NK cells, with the receptor inducible upon T-cell activation (Hunter, 2005). This heterodimer, named IL-23,

was found to not affect the population of Th1 cells, but rather to promote the expansion of a pathogenic CD4+ T cell population (termed Th17 cells), characterized by the production of strongly proinflammatory cytokines, in particular IL-17, IL-17F, IL-6, and TNF-α (Langrish et al., 2005). Multiple investigations into the role of IL-12 and IL-23 in disease models has provided evidence of the effect these cytokines may have on the pathogenesis of human disease.

D3. Mouse Models of IL-12/IL-23 in Disease

Development of germline knockout mice for the three gene encoding the two cytokines IL-12 and IL-23 (*Il12a, Il12b, Il23a*) has provided important tools for the development of models of the activities of these cytokines in a number of disease states. A mouse models of inflammatory autoimmune disease in the central nervous system, similar to multiple sclerosis, referred to as experimental autoimmune encephalitis (EAE) reveal a worse phenotype for the *Il12a* knockout while the *Il12b* and the *Il23a* knockouts were not susceptible to the disease (Cua et al., 2003). A similar study was conducted in the mouse collagen induced arthritis model, where mice that could not make IL-23, did not develop the disease (Murphy et al., 2003). This supports a model where Th1 cell types, the production of IFN-γ, and the mounting of a cell mediated response is necessary to reduce the severity of an inflammatory autoimmune disease state, while the expression of IL-23, followed by the development of a Th17 response and the production of IL-17 supports advance of the autoimmune state (Figure 1-3). This model of IL-23 and IL-17 driven inflammatory autoimmune pathologies is also supported by disease models of inflammatory bowl disease and rheumatoid arthritis

(Murphy et al., 2003; Becker et al., 2006). In addition to mouse models of autoimmune diseases, an IL-12/IL-23 axis has been show to be important in the development and severity of tumors. Mice deficient in IL23p19 revealed a higher tumor incidence and load in carcinogen-treated skin, IL12p35 knockout mice faired far worse then wild-type, and IL12p40 knockout has a intermediate phenotype (Langowski et al., 2006). These data support a model where, similar to autoimmune pathologies, the presence of an inflammatory state, driven and/or supported by the presence of Th17 cells and their inflammatory products, exacerbates tumor incidence and growth, while a Th1 population of cells can provide tumor surveillance and protection from malignancies.

D4. IL-12/IL-23 in Human Disease

In addition to mouse models, there is a significant body of research that supports a similar mechanism in human patients. Human genetic studies have identified *Il23r* polymorphisms associated with susceptibility to a number of autoimmune diseases: Crohn's disease, psoriasis, psoriatic arthritis, multiple sclerosis, autoimmune thyroid disease, spondyloarthropathy, acquired aplastic anemia, and Behcet's disease (Ghoreschi et al., 2011). Further more, IL-23 and/or IL-17 mRNA and protein has been elevated in the cerebrospinal fluid of multiple sclerosis patients, in the sera and synovia of rheumatoid arthritis patients, in the sera and colon of patients with inflammatory bowel disorders, as well as the affected skin of patients with psoriasis (Annunziato et al., 2007). The evidence has been convincing enough that the IL-23/IL-17 axis was targeted for therapeutic intervention, with a few notable successes. There is now an FDA approved monoclonal antibody treatment for psoriasis, known as uskekinumab

(brand name Stelara, Centocor Ortho Biotech), that targets the IL12p40 subunit of IL-12 and IL-23 (Reddy et al., 2007; Leonardi et al., 2008). In clinical trials, this treatments was found to be more effective then treatments targeting TNF-α in patients with moderate to severe psoriasis (Griffiths et al., 2010). Ustekinumab is currently being investigated for effectiveness in treating patients with Crohn's disease, psoriatic arthritis, and multiple sclerosis (Sandborn et al., 2008; Gottlieb et al., 2009; Segal et al., 2008). The progress for Crohn's disease and psoriatic arthritis has been very positive, however the attempts to treat relapsing multiple sclerosis have not been successful (Steinman, 2010).

D5. Transcriptional Regulation of IL-12/IL-23

Understanding the regulation of the IL-12 family of cytokines is central to deciphering both a functioning innate immune response and a dysregulated state resulting in an inflammatory autoimmune disease. Transcriptional regulation of these genes has been addressed in many studies. Regulation of the transcription of the *Il12a* gene which encodes the p35 subunit of IL-12 has been a difficult question to approach, complicated by the low level of inducible expression and often the presence of unproductive mRNA in mixed cell types. However, it appears that full length mRNA that is competent for the production of p35 and inducible by microbial stimuli contains SP1, IFN-γ-response element (γ-IRE), PU.1, and C/EBP binding sites in its promoter. The expression of *Il12a* is severely impaired in C/EBPβ deficient mice (Gorgoni et al., 2002). In CD8+ dendritic cells, there is a requirement for the NF-κB family member cRel that is not seen in macrophages (Grumont, 2001). In human dendritic cells, in response to

TLR4 or TLR3 stimulation, IRF-3 is recruited to the promoter of *Il12a*, while in mouse macrophages, IRF-1 and IRF-8 was shown to be recruited (Goriely et al., 2006; Kollet and Petro, 2006; Liu et al., 2004, 2003). The cell type specific requirements in the individual studies illustrates a complex regulation that can be at least partially explained by species differences, cell type specificities, and the promiscuous activity of IL12p35, which is present in both the IL-12 and IL-35 heterodimeric cytokines (Collison et al., 2012).

The transcriptional regulation of *Il23a* encoding IL23p19, the other binding partner of IL12p40, also has been investigated by a number of groups. *Il23a* is a primary response gene that does not require nucleosome remodeling by the SWI/SNF remodeling complex (Ramirez-Carrozzi et al., 2009). Transcription of the *Il23a* gene is dependent on activation of the NF-κB and AP-1 pathways. Robust induction requires the binding of both cRel and RelA, with cRel knockout dendritic cells showing severely reduced mRNA levels (Carmody et al., 2007; Sheikh et al., 2011). The promoter also contains an AP-1 site the becomes inducibly occupied by c-Jun and c-Fos in response to TLR stimulation of MAPK pathways (Liu et al., 2009). In addition, it is strongly inhibited in response to IFN-γ, through competition of IRF1 and NF-κB at the promoter (Sheikh et al., 2010, 2011).

D5.1 Transcriptional Regulation of Il12b

The transcriptional regulation of *Il12b* has been studied in detail by many groups and much is known about its regulators. To continue with the classification made earlier, *Il12b* is a secondary gene, that shows a de novo proteins synthesis requirement for both

its expression and the remodeling of its promoter and enhancer by SWI/SNF nucleosome remodeling complex (Ramirez-Carrozzi et al., 2006; Weinmann et al., 2001). *Il12b* control regions that have been mapped include the proximal promoter and a conserved, nuclease hypersensitive enhancer 10 kb upstream of the TSS (Zhou et al., 2007). The enhancer contains Oct and C/EBP consensus sites that play functional roles in *Il12b* expression. The enhancer also contains CpG dinucleotides that remain demethylated in embryonic stem cells (ESC) and show expansion of this demethylated window when stem cells differentiate to macrophages (Xu et al., 2007, 2009). It is hypothesized that pioneer factors are marking the *Il12b* enhancer in ESCs, preventing further silencing by CpG methylation, and allowing for competence in the expression of *Il12b* in differentiated macrophages, however the sequences and factors required have not been described. The promoter of *Il12b* contains multiple conserved transcription factor binding sites that have been described as required for robust transcriptional induction. It is a NF-κB dependent gene containing multiple binding sites within the promoter and a strong dependence for c-Rel containing dimers (Sanjabi et al., 2005, 2000). Remodeling of the positioned nucleosome proximal to the TSS exposes a conserved binding site for C/EBP family members and binding is required for expression of *Il12b* (Bradley et al., 2003; Plevy et al., 1997). Mutations in the AP-1 binding site of the promoter, which interacts with AP-1 family member c-Jun, have been shown to decrease inducible expression (Zhu et al., 2001). Functional and cooperative binding of the *Il12b* promoter has been shown between NFAT and IRF8 (ICSBP) (Zhu et al., 2003). A conserved ISRE sequence has been reported to positively affect expression and interacts with both IRF8 and IRF1, with expression dysregulated in both IRF1 and

IRF8 knockout mice (Wang et al., 2000; Salkowski et al., 1999). Finally a conserved Ets site, which may interact with Ets-2 and/or PU.1, seems to be important for robust transcriptional induction (Trinchieri, 2003).

In addition to the many known transcription factors that positively regulate *Il12b* transcription, there are some known inhibitory pathways as well. Broad acting anti-inflammatory treatments inhibit *Il12b* expression. Glucocorticoids for example inhibit a broad range of inflammatory genes by activating the gucocorticoid receptor and causing tethering of the activated GR to AP-1 and NF-κB family members, resulting in inhibition of the target genes of these factors (Flammer and Rogatsky, 2011). The main biological pathway that is a potent inhibitor of *Il12b* expression is IL-10. Macrophages and dendritic cells express high levels of the IL-10 receptor and in response to IL-10 inhibit the transcription of a subset of TLR stimulated genes in a STAT3 dependent manner (Murray, 2006). STAT3 is expected to act indirectly on the targets of IL-10 signaling, but it is unclear the particular mechanism or STAT3 induced targets that are responsible for the anti-inflammatory affect. The subset of LPS induced genes that IL-10 inhibits has been reported as 15-20%, therefore it is not a broad anti-inflammatory and further studies of mechanism would address a large lack of understanding in the field (Lang et al., 2002). Until recently, there was a little known of downstream effectors for IL-10 signaling. Two different groups have now reported that NFIL3, a member of the basic leucine zipper family (bZIP) of transcription factors, was shown to be induced by IL-10 signaling in macrophages and to play an inhibitory role on *Il12b* expression (Kobayashi et al., 2011; Smith et al., 2011). An additional signaling pathway that has been reported to block expression of *Il12b* are GPCR signaling with GPCR ligands as varied as

prostaglandin, alpha adrenergic agonists, and beta-adrenergic agonists. These are generally dependent on signaling through cAMP and result in the inhibition of *Il12b* in an IL-10 independent manner (Trinchieri, 2003).

E. Future Directions

It is clear from both studies in mice and humans, modulation of the IL-12 family of cytokines would have broad implications in human health, inflammatory autoimmune pathologies, and cancer. It could be argued that specific inhibition of IL-23 by inhibiting expression or activity of IL23p19 or IL23R, would provide the most clinical benefit. This approach would reduce the Th17 population of cells and the ability of Th17 cells to secrete a large amount of pro-inflammatory cytokines such as IL-17 would be hindered. However, much less is known regarding the regulation of *Il23a* in comparison to *Il12b.* Clinical work targeting IL23p19 and IL23R activity is generally by monoclonal antibody approaches, with mixed success. Treatments that inhibit the expression or activity of IL12p40 would prevent activity of both IL-12 and IL-23, therefore inhibiting the activity of Th17 cells and cellular immunity driven by Th1 cells, cytotoxic T cells, and the production of IFN-γ. This would reduce the patients ability to clear intracellular pathogens and have a robust tumor surveillance response. While this case is not ideal, in an active autoimmune disease state, there are still significant health benefits to targeting IL12p40. This is clear from the use of the IL12p40 specific antibody ustekinumab in treating moderate to severe plaque psoriasis and Crohn's disease.

Despite the large amount of data on *Il12b*, there are still many questions regarding specific signals that regulate the transcriptional regulation of *Il12b*.

Specifically, it is unclear what signals determine selective expression and inhibition of *Il12b* over the majority of pro-inflammatory genes. In a physiological setting, selective expression is seen. In particular, different PRRs, despite working through many of the same signaling cascades (such as NF-κB, MAPK, and IRFs), often show very different phenotypes in the induction of pro-inflammatory cytokines, including *IL12b*. It is also unclear which of these signaling cascades and which DNA-binding or histone binding factors are responsible for the SWI/SNF dependent nucleosome remodeling at the enhancer and promoter of *Il12b*. It is our hypothesis that these steps are highly regulated and very few proinflammatory genes will share the same requirements. The ability to understand the logic of the innate immune response and selective signals driving induced transcription will provide important targets for medical intervention in human disease.

The specificity that an antibody treatment has can be informative on the benefit of targeting that particular protein. However, due to the expense and the need for it to be injected as an outpatient procedure, there is much interest in finding small molecule treatments for the same targets that can be produced more cheaply and taken at home. It is therefore the major goal of the pharmaceutical industry to discover and develop small molecule modulators of disease pathway that are specific as possible. Often a high-throughput screening approach is considered as a first step to find possible drug candidates.

E1. High-throughput Will Play a Role

In the modern, genomic era, the information available has made unbiased, brute force, high-throughput approaches much more attractive and rewarding. Data handling capabilities of vast amounts of information is possible for most academic labs. Deep sequencing technologies have provided the opportunity to ask and answer questions on the level of genomes, transcriptomes, or epigenomes. Revealing the specificity of a response is now more possible than ever. Even high-throughput small molecule and RNAi screens have moved from an industry specific endeavor into the academic lab. Screens, designed to test tens of thousands to hundreds of thousands of treatments in a short time frame, provide the opportunity to identify novel biological mechanisms. Combining a well designed and controlled screen (small molecule and/or RNAi) and intelligent use of RNA and DNA sequencing technologies provides a very attractive route to teasing out the logic of complex biological responses.

Figure Legends

Table 1-1: Patterns Recognition Receptors and Their Ligands

Pattern recognition receptors are listed by family and include Toll-like receptors (TLR), RIG-I-like receptors (RLR), NOD-like receptors (NLR), and C-lectin-type receptors (CLR). TLRs and NLRs are transmembrane receptors, while RLRs and NLRs are cytoplasmic. Included for each receptor is the specific pathogen-associated molecular pattern (PAMP) that acts as its cogant ligand.

Figure 1-1: Toll-like Receptor Signaling Pathway

TLR2, TLR3, and TLR4 signaling pathways are represented to summarize MyD88-dependent and TRIF-dependent pathways. A detailed description can be found in text and elsewhere (Takeuchi and Akira, 2010b). For the MyD88-dependent pathway, a heterodimer of TLR1/6 and TLR2 or a homodimer of TLR4 bind to lipoproteins or LPS, respectively, and initiate signaling. TIRAP and MyD88 are recruited and a complex of IRAKs and TRAF6 is formed. TRAF6 catalyzes the generation of an unconjugated polyubiquitin chain, in addition to a K63-linked polyubiquitin chain on itself. Activation of a TAK1, TAB1, and TAB2/3 complex results in the phosphorylation of NEMO, the activation of an IKK complex, phosphorylation and degradation of IκB, and NF-κB activation. In parallel, TAK1 activates MAPK cascades resulting in the activation of AP-1. For the TRIF-dependent pathway, LPS induces delayed TLR4 signaling upon transitioning to the endosome in a TRAM dependent step. TLR3 and TLR4 activate NF-

κB through a TRAF6 and RIP1 dependent pathway, while IRF3 is activated via phosphorylation by TBK1/IKK-*i* in response to TRAF3 activation.

Figure 1-2: SWI/SNF Nucleosome Remodeling Is Only Required for a Subset of LPS Inducible Genes

Depicted is a summary of the model describing the contribution of nucleosome remodeling by SWI/SNF during pro-inflammatory gene induction. Model is based on a study that identified reduced expression of LPS inducible genes after shRNA depletion of BRG1 and BRM in macrophages. There is a selective requirement for SWI/SNF nucleosome remodeling at secondary and primary genes with delayed kinetics (late primary response genes). Early primary response genes do not seem to be regulated by SWI/SNF complexes. Genes listed for each category are representative and not exhaustive. For a more detailed list refer to Appendix A (Figure 1) or publication (Ramirez-Carrozzi et al., 2006).

Figure 1-3: IL-12 and IL-23 in Chronic Inflammation

Macrophages produce IL-12 and IL-23 cytokines that play central and competing roles in the development of chronic inflammation (Iwakura and Ishigame, 2006). IL-23 promotes the expansion of Th17 cells and the production of IL-17. IL-17 triggers potent inflammatory responses from a number of different cell types that can persist in a feed-forward chronic state. IL-23 also acts on macrophages in an autocrine/paracrine manner resulting in the generation of proinflammatory cytokines, such as IL-1, IL-6, and TNF-α. IL-12 promotes the development of Th1 cells and the secretion of IFN-γ that can suppress the development of Th17 response.

Table 1-1: Pattern Recognition Receptors and Their Ligands

(Takeuchi and Akira, 2010)

PRRs	Localization	Ligand	Origin of the Ligand
TLR			
TLR1	Plasma membrane	Triacyl lipoprotein	Bacteria
TLR2	Plasma membrane	Lipoprotein	Bacteria, viruses, parasites, self
TLR3	Endolysosome	dsRNA	Virus
TLR4	Plasma membrane	LPS	Bacteria, viruses, self
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6	Plasma membrane	Diacyl lipoprotein	Bacteria, viruses
TLR7 (human TLR8)	Endolysosome	ssRNA	Virus, bacteria, self
TLR9	Endolysosome	CpG-DNA	Virus, bacteria, protozoa, self
TLR10	Endolysosome	Unknown	Unknown
TLR11	Plasma membrane	Profilin-like molecule	Protozoa
RLR			
RIG-I	Cytoplasm	Short dsRNA, 5′triphosphate dsRNA	RNA viruses, DNA virus
MDA5	Cytoplasm	Long dsRNA	RNA viruses (Picornaviridae)
LGP2	Cytoplasm	Unknown	RNA viruses
NLR			
NOD1	Cytoplasm	iE-DAP	Bacteria
NOD2	Cytoplasm	MDP	Bacteria
CLR			
Dectin-1	Plasma membrane	β-Glucan	Fungi
Dectin-2	Plasma membrane	β-Glucan	Fungi
MINCLE	Plasma membrane	SAP130	Self, fungi

Figure 1-1: Toll-like Receptor Signaling Pathway
(Takeuchi and Akira, 2010)

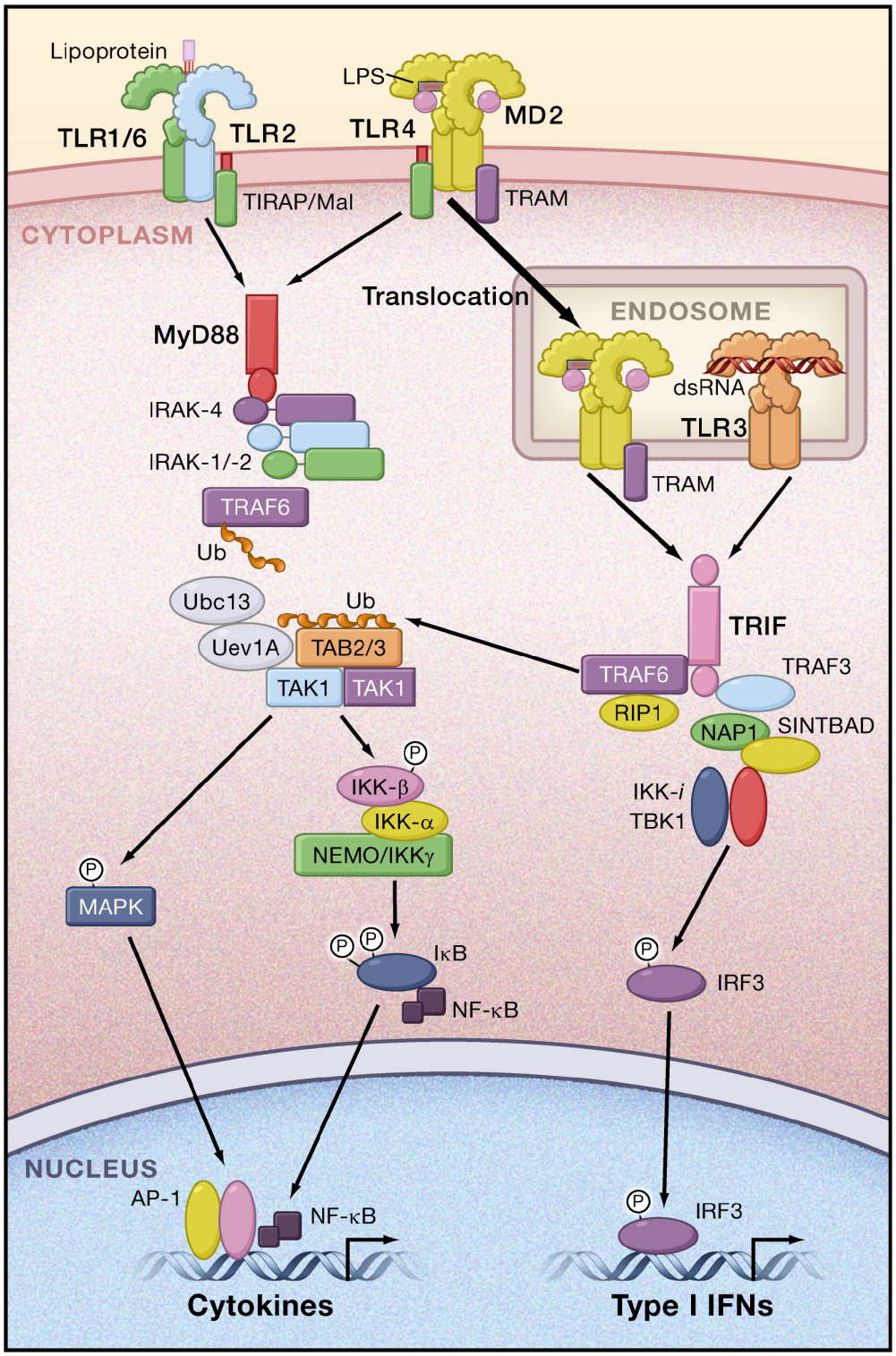


Figure 1-2: SWI/SNF Nucleosome Remodeling Is Required for Only a Subset of LPS Inducible Genes

(Ramirez-Carrozzi et al., 2006)

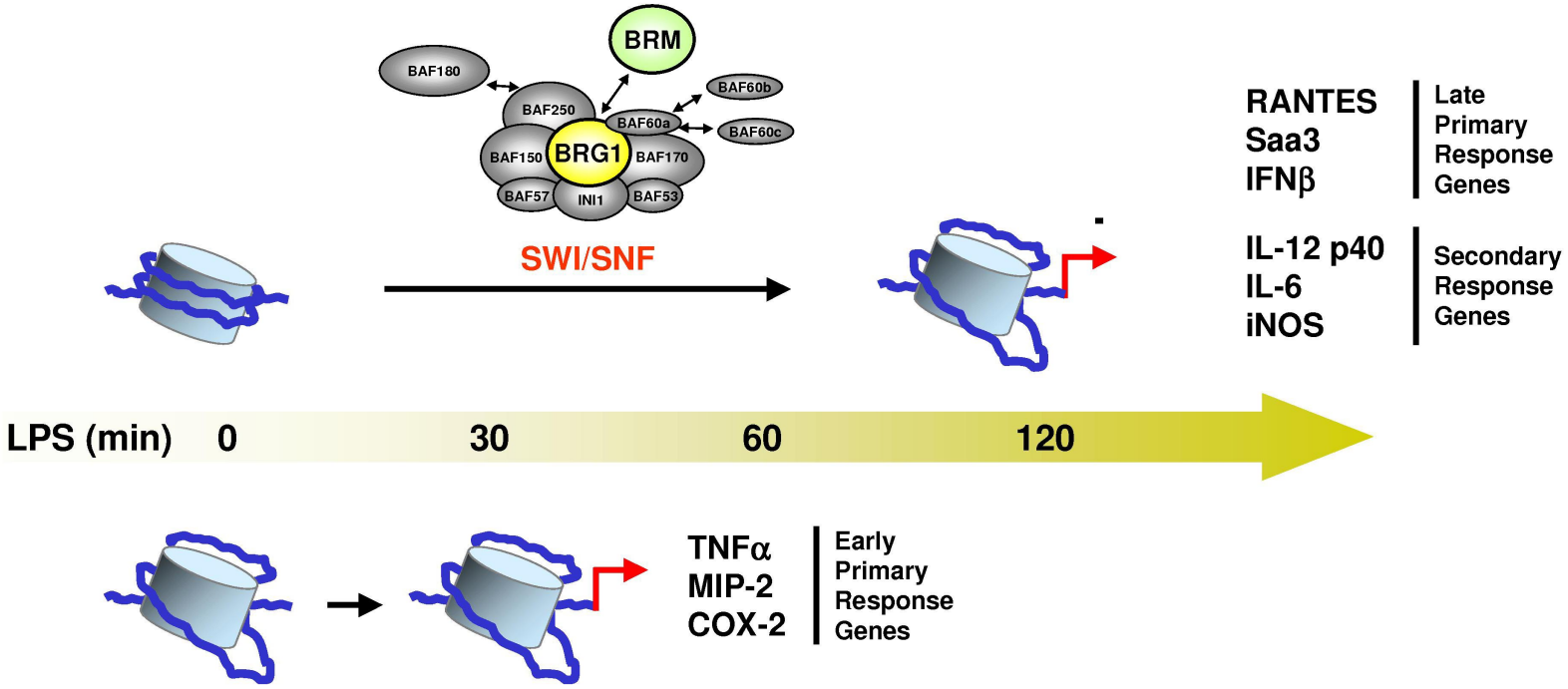
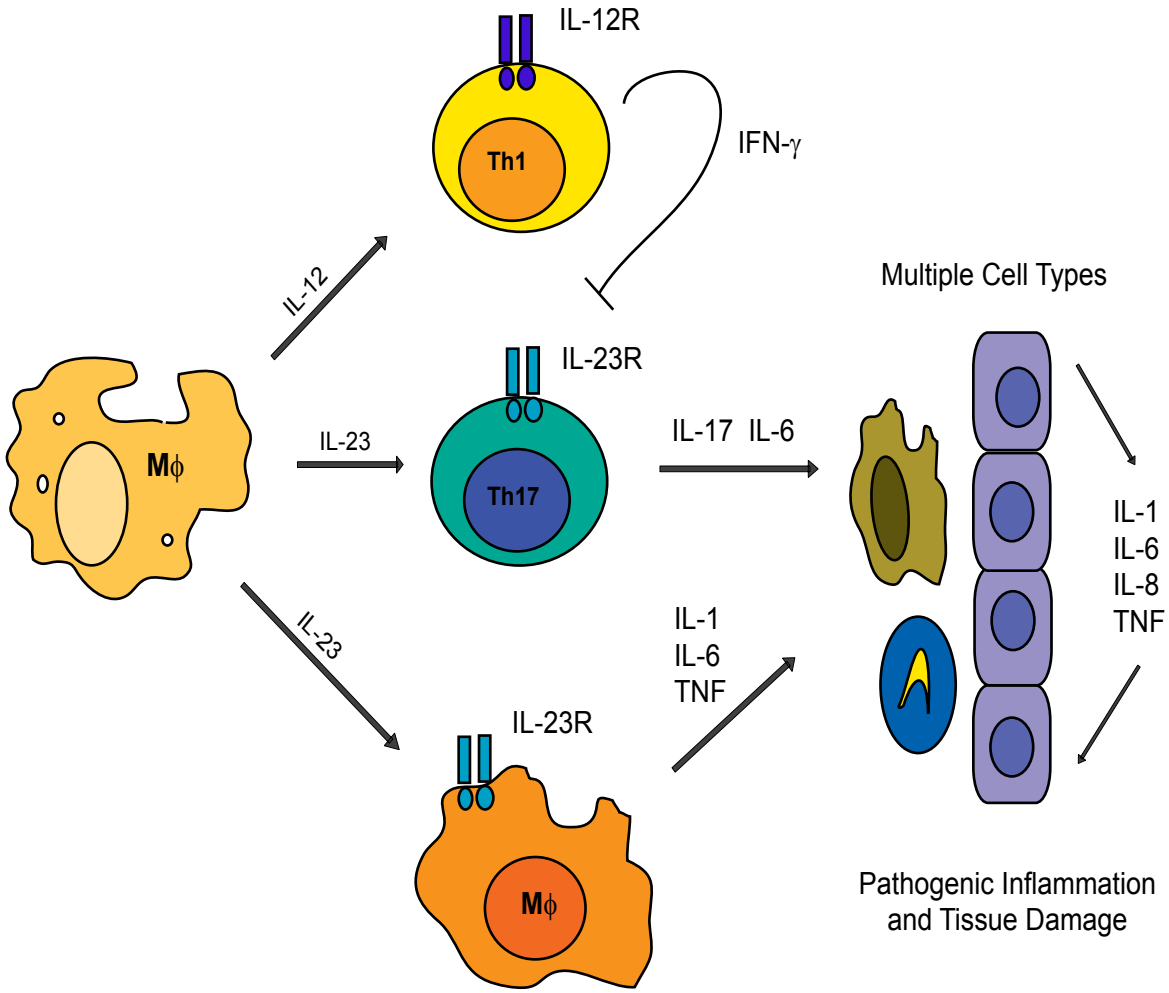


Figure 1-3: IL-12 and IL-23 in Chronic Inflammation

(Adapted from Iwakura & Ishigame, 2006)



Works Cited

Annunziato, F., Cosmi, L., Santarlasci, V., Maggi, L., Liotta, F., Mazzinghi, B., Parente, E., Filì, L., Ferri, S., Frosali, F., et al. (2007). Phenotypic and functional features of human Th17 cells. The Journal of experimental medicine *204*, 1849–1861.

Barton, G. M., and Kagan, J. C. (2009). A cell biological view of Toll-like receptor function: regulation through compartmentalization. Nature reviews. Immunology *9*, 535–542.

Becker, C., Dornhoff, H., Neufert, C., Fantini, M. C., Wirtz, S., Huebner, S., Nikolaev, A., Lehr, H.-A., Murphy, A. J., Valenzuela, D. M., et al. (2006). Cutting Edge: IL-23 Cross-Regulates IL-12 Production in T Cell-Dependent Experimental Colitis. J. Immunol. *177*, 2760–2764.

Bradley, M. N., Zhou, L., and Smale, S. T. (2003). C/EBP Regulation in Lipopolysaccharide-Stimulated Macrophages. Molecular and Cellular Biology *23*, 4841–4858.

Brown, G. D. (2006). Dectin-1: a signalling non-TLR pattern-recognition receptor. Nature reviews. Immunology *6*, 33–43.

Carmody, R. J., Ruan, Q., Liou, H.-C., and Chen, Y. H. (2007). Essential roles of c-Rel in TLR-induced IL-23 p19 gene expression in dendritic cells. Journal of immunology (Baltimore, Md. : 1950) *178*, 186–191.

Collison, L. W., Delgoffe, G. M., Guy, C. S., Vignali, K. M., Chaturvedi, V., Fairweather, D., Satoskar, A. R., Garcia, K. C., Hunter, C. A., Drake, C. G., et al. (2012). The composition and signaling of the IL-35 receptor are unconventional. Nature immunology *13*, 290–299.

Cua, D. J., Sherlock, J., Chen, Y., Murphy, C. A., Joyce, B., Seymour, B., Lucian, L., To, W., Kwan, S., Churakova, T., et al. (2003). Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. Nature *421*, 744–748.

Flammer, J. R., and Rogatsky, I. (2011). Minireview: Glucocorticoids in autoimmunity: unexpected targets and mechanisms. Molecular endocrinology (Baltimore, Md.) *25*, 1075–1086.

Ghoreschi, K., Laurence, A., Yang, X.-P., Hirahara, K., and O'Shea, J. J. (2011). T helper 17 cell heterogeneity and pathogenicity in autoimmune disease. Trends in immunology *32*, 395–401.

Gordon, S. (2003). Alternative activation of macrophages. Nature reviews. Immunology *3*, 23–35. 2012].

Gorgoni, B., Maritano, D., Marthyn, P., Righi, M., and Poli, V. (2002). C/EBP{beta} Gene Inactivation Causes Both Impaired and Enhanced Gene Expression and Inverse Regulation of IL-12 p40 and p35 mRNAs in Macrophages. J. Immunol. *168*, 4055–4062.

Goriely, S., Molle, C., Nguyen, M., Albarani, V., Haddou, N. O., Lin, R., De Wit, D., Flamand, V., Willems, F., and Goldman, M. (2006). Interferon regulatory factor 3 is involved in Toll-like receptor 4 (TLR4)- and TLR3-induced IL-12p35 gene activation. Blood *107*, 1078–1084.

Gottlieb, A., Menter, A., Mendelsohn, A., Shen, Y.-K., Li, S., Guzzo, C., Fretzin, S., Kunynetz, R., and Kavanaugh, A. (2009). Ustekinumab, a human interleukin 12/23 monoclonal antibody, for psoriatic arthritis: randomised, double-blind, placebo-controlled, crossover trial. Lancet *373*, 633–640.

Griffiths, C. E. M., Strober, B. E., van de Kerkhof, P., Ho, V., Fidelus-Gort, R., Yeilding, N., Guzzo, C., Xia, Y., Zhou, B., Li, S., et al. (2010). Comparison of ustekinumab and etanercept for moderate-to-severe psoriasis. The New England journal of medicine *362*, 118–128.

Grumont, R. (2001). c-Rel Regulates Interleukin 12 p70 Expression in CD8+ Dendritic Cells by Specifically Inducing p35 Gene Transcription. Journal of Experimental Medicine *194*, 1021–1032.

Heine, H. (2011). TLRs, NLRs and RLRs: innate sensors and their impact on allergic diseases--a current view. Immunology letters *139*, 14–24.

Hoffmann, A., and Baltimore, D. (2006). Circuitry of nuclear factor kappaB signaling. Immunological reviews *210*, 171–186.

Honda, K., and Taniguchi, T. (2006). IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. Nature reviews. Immunology *6*, 644–658.

Hunter, C. A. (2005). New IL-12-family members: IL-23 and IL-27, cytokines with divergent functions. Nature reviews. Immunology *5*, 521–531.

Iwakura, Y., and Ishigame, H. (2006). The IL-23/IL-17 axis in inflammation. The Journal of clinical investigation *116*, 1218–1222.

Iwasaki, A., and Medzhitov, R. (2004). Toll-like receptor control of the adaptive immune responses. Nature immunology *5*, 987–995.

Janeway, C. A. (1989). Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harbor symposia on quantitative biology *54 Pt 1*, 1–13.

Karin, M. (1995). The Regulation of AP-1 Activity by Mitogen-activated Protein Kinases. J. Biol. Chem. *270*, 16483–16486.

Kobayashi, M. (1989). Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. Journal of Experimental Medicine *170*, 827–845.

Kobayashi, T., Matsuoka, K., Sheikh, S. Z., Elloumi, H. Z., Kamada, N., Hisamatsu, T., Hansen, J. J., Doty, K. R., Pope, S. D., Smale, S. T., et al. (2011). NFIL3 Is a Regulator of IL-12 p40 in Macrophages and Mucosal Immunity. The Journal of Immunology *186*, 4649–4655.

Kollet, J. I., and Petro, T. M. (2006). IRF-1 and NF-kappaB p50/cRel bind to distinct regions of the proximal murine IL-12 p35 promoter during costimulation with IFN-gamma and LPS. Molecular immunology *43*, 623–633..

Lang, R., Patel, D., Morris, J. J., Rutschman, R. L., and Murray, P. J. (2002). Shaping Gene Expression in Activated and Resting Primary Macrophages by IL-10. J. Immunol. *169*, 2253–2263.

Langowski, J. L., Zhang, X., Wu, L., Mattson, J. D., Chen, T., Smith, K., Basham, B., McClanahan, T., Kastelein, R. A., and Oft, M. (2006). IL-23 promotes tumour incidence and growth. Nature *442*, 461–465.

Langrish, C. L., Chen, Y., Blumenschein, W. M., Mattson, J., Basham, B., Sedgwick, J. D., McClanahan, T., Kastelein, R. A., and Cua, D. J. (2005). IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. The Journal of experimental medicine *201*, 233–240.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J., and Hoffman, J. (1996). The Dorsoventral Regulatory Gene Cassette Controls the Potent Antifungal Response in Drosophila Adults. Cell *86*, 973–983.

Leonardi, C. L., Kimball, A. B., Papp, K. A., Yeilding, N., Guzzo, C., Wang, Y., Li, S., Dooley, L. T., and Gordon, K. B. (2008). Efficacy and safety of ustekinumab, a human interleukin-12/23 monoclonal antibody, in patients with psoriasis: 76-week results from a randomised, double-blind, placebo-controlled trial (PHOENIX 1). Lancet *371*, 1665–1674.

Li, B., Carey, M., and Workman, J. L. (2007). The role of chromatin during transcription. Cell *128*, 707–719.

Litman, G. W., Rast, J. P., and Fugmann, S. D. (2010). The origins of vertebrate adaptive immunity. Nature reviews. Immunology *10*, 543–553.

Liu, J., Cao, S., Herman, L. M., and Ma, X. (2003). Differential regulation of interleukin (IL)-12 p35 and p40 gene expression and interferon (IFN)-gamma-primed IL-12 production by IFN regulatory factor 1. The Journal of experimental medicine *198*, 1265–1276.

Liu, J., Guan, X., Tamura, T., Ozato, K., and Ma, X. (2004). Synergistic activation of interleukin-12 p35 gene transcription by interferon regulatory factor-1 and interferon consensus sequence-binding protein. The Journal of biological chemistry *279*, 55609–55617.

Liu, W., Ouyang, X., Yang, J., Liu, J., Li, Q., Gu, Y., Fukata, M., Lin, T., He, J. C., Abreu, M., et al. (2009). AP-1 activated by toll-like receptors regulates expression of IL-23 p19. The Journal of biological chemistry *284*, 24006–24016.

Medzhitov, R. (2008). Origin and physiological roles of inflammation. Nature *454*, 428–435.

Medzhitov, R. (2001). Toll-like receptors and innate immunity. Nature reviews. Immunology *1*, 135–145.

Medzhitov, R., and Janeway, C. A. (1997). Innate Immunity: The Virtues of a Nonclonal System of Recognition. Cell *91*, 295–298.

Murphy, C. A., Langrish, C. L., Chen, Y., Blumenschein, W., McClanahan, T., Kastelein, R. A., Sedgwick, J. D., and Cua, D. J. (2003). Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. The Journal of experimental medicine *198*, 1951–1957.

Murray, P. J. (2006). Understanding and exploiting the endogenous interleukin-10/STAT3-mediated anti-inflammatory response. Current opinion in pharmacology *6*, 379–386.

Nagasawa, T. (2006). Microenvironmental niches in the bone marrow required for B-cell development. Nature reviews. Immunology *6*, 107–116.

Oppmann, B., Lesley, R., Blom, B., Timans, J. C., Xu, Y., Hunte, B., Vega, F., Yu, N., Wang, J., Singh, K., et al. (2000). Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. Immunity *13*, 715–725.

Palm, N. W., and Medzhitov, R. (2009). Pattern recognition receptors and control of adaptive immunity. Immunological reviews *227*, 221–233.

Plevy, S. E., Gemberling, J. H., Hsu, S., Dorner, A. J., and Smale, S. T. (1997). Multiple control elements mediate activation of the murine and human interleukin 12 p40 promoters: evidence of functional synergy between C/EBP and Rel proteins. Molecular and cellular biology *17*, 4572–4588.

Ramirez-Carrozzi, V. R., Braas, D., Bhatt, D. M., Cheng, C. S., Hong, C., Doty, K. R., Black, J. C., Hoffmann, A., Carey, M., and Smale, S. T. (2009). A Unifying Model for the Selective Regulation of Inducible Transcription by CpG Islands and Nucleosome Remodeling. Cell *138*, 114–128.

Ramirez-Carrozzi, V. R., Nazarian, A. A., Li, C. C., Gore, S. L., Sridharan, R., Imbalzano, A. N., and Smale, S. T. (2006). Selective and antagonistic functions of SWI/SNF and Mi-2beta nucleosome remodeling complexes during an inflammatory response. Genes & Development *20*, 282–296.

Rao, A., Luo, C., and Hogan, P. G. (1997). Transcription factors of the NFAT family: regulation and function. Annual review of immunology *15*, 707–747.

Reddy, M., Davis, C., Wong, J., Marsters, P., Pendley, C., and Prabhakar, U. (2007). Modulation of CLA, IL-12R, CD40L, and IL-2Ralpha expression and inhibition of IL-12- and IL-23-induced cytokine secretion by CNTO 1275. Cellular immunology *247*, 1–11.

Robinson, M. J., Osorio, F., Rosas, M., Freitas, R. P., Schweighoffer, E., Gross, O., Verbeek, J. S., Ruland, J., Tybulewicz, V., Brown, G. D., et al. (2009). Dectin-2 is a Syk-coupled pattern recognition receptor crucial for Th17 responses to fungal infection. The Journal of experimental medicine *206*, 2037–2051.

Salkowski, C. A., Kopydlowski, K., Blanco, J., Cody, M. J., McNally, R., and Vogel, S. N. (1999). IL-12 is dysregulated in macrophages from IRF-1 and IRF-2 knockout mice. Journal of immunology (Baltimore, Md. : 1950) *163*, 1529–1536.

Sandborn, W. J., Feagan, B. G., Fedorak, R. N., Scherl, E., Fleisher, M. R., Katz, S., Johanns, J., Blank, M., and Rutgeerts, P. (2008). A randomized trial of Ustekinumab, a human interleukin-12/23 monoclonal antibody, in patients with moderate-to-severe Crohn's disease. Gastroenterology *135*, 1130–1141.

Sanjabi, S., Hoffmann, A., Liou, H. C., Baltimore, D., and Smale, S. T. (2000). Selective requirement for c-Rel during IL-12 P40 gene induction in macrophages. Proceedings of the National Academy of Sciences of the United States of America *97*, 12705–12710.

Sanjabi, S., Williams, K. J., Saccani, S., Zhou, L., Hoffmann, A., Ghosh, G., Gerondakis, S., Natoli, G., and Smale, S. T. (2005). A c-Rel subdomain responsible for enhanced DNA-binding affinity and selective gene activation. Genes & development *19*, 2138–2151.

Segal, B. M., Constantinescu, C. S., Raychaudhuri, A., Kim, L., Fidelus-Gort, R., and Kasper, L. H. (2008). Repeated subcutaneous injections of IL12/23 p40 neutralising antibody, ustekinumab, in patients with relapsing-remitting multiple sclerosis: a phase II, double-blind, placebo-controlled, randomised, dose-ranging study. Lancet neurology *7*, 796–804.

Sheikh, S. Z., Kobayashi, T., Matsuoka, K., Onyiah, J. C., and Plevy, S. E. (2011). Characterization of an interferon-stimulated response element (ISRE) in the Il23a promoter. The Journal of biological chemistry *286*, 1174–1180.

Sheikh, S. Z., Matsuoka, K., Kobayashi, T., Li, F., Rubinas, T., and Plevy, S. E. (2010). Cutting edge: IFN-gamma is a negative regulator of IL-23 in murine macrophages and experimental colitis. Journal of immunology (Baltimore, Md. : 1950) *184*, 4069–4073.

Smale, S. T. (2010). Selective transcription in response to an inflammatory stimulus. Cell *140*, 833–844.

Smith, A. M., Qualls, J. E., O'Brien, K., Balouzian, L., Johnson, P. F., Schultz-Cherry, S., Smale, S. T., and Murray, P. J. (2011). A distal enhancer in Il12b is the target of transcriptional repression by the STAT3 pathway and requires the basic leucine zipper (B-ZIP) protein NFIL3. The Journal of biological chemistry *286*, 23582–23590.

Steinman, L. (2010). Mixed results with modulation of TH-17 cells in human autoimmune diseases. Nature immunology *11*, 41–44.

Takahama, Y. (2006). Journey through the thymus: stromal guides for T-cell development and selection. Nature reviews. Immunology *6*, 127–135.

Takeuchi, O., and Akira, S. (2010a). Pattern recognition receptors and inflammation. Cell *140*, 805–820.

Takeuchi, O., and Akira, S. (2010b). Pattern recognition receptors and inflammation. Cell *140*, 805–820.

Trinchieri, G. (2003). Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nature reviews. Immunology *3*, 133–146.

Uematsu, S., and Akira, S. (2008). Toll-Like receptors (TLRs) and their ligands. Handbook of experimental pharmacology, 1–20.

Wang, I. M., Contursi, C., Masumi, A., Ma, X., Trinchieri, G., and Ozato, K. (2000). An IFN-gamma-inducible transcription factor, IFN consensus sequence binding protein (ICSBP), stimulates IL-12 p40 expression in macrophages. Journal of immunology (Baltimore, Md. : 1950) *165*, 271–279.

Weinmann, A. S., Mitchell, D. M., Sanjabi, S., Bradley, M. N., Hoffmann, A., Liou, H. C., and Smale, S. T. (2001). Nucleosome remodeling at the IL-12 p40 promoter is a TLR-dependent, Rel-independent event. Nature immunology *2*, 51–57.

Wen, A. Y., Sakamoto, K. M., and Miller, L. S. (2010). The role of the transcription factor CREB in immune function. Journal of immunology (Baltimore, Md. : 1950) *185*, 6413–6419.

Xu, J., Pope, S. D., Jazirehi, A. R., Attema, J. L., Papathanasiou, P., Watts, J. A., Zaret, K. S., Weissman, I. L., and Smale, S. T. (2007). Pioneer factor interactions and unmethylated CpG dinucleotides mark silent tissue-specific enhancers in embryonic stem cells. Proceedings of the National Academy of Sciences of the United States of America *104*, 12377–12382.

Xu, J., Watts, J. A., Pope, S. D., Gadue, P., Kamps, M., Plath, K., Zaret, K. S., and Smale, S. T. (2009). Transcriptional competence and the active marking of tissue-specific enhancers by defined transcription factors in embryonic and induced pluripotent stem cells. Genes & development *23*, 2824–2838.

Yoneyama, M., Kikuchi, M., Natsukawa, T., Shinobu, N., Imaizumi, T., Miyagishi, M., Taira, K., Akira, S., and Fujita, T. (2004). The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nature immunology *5*, 730–737.

Zhou, L., Nazarian, A. A., Xu, J., Tantin, D., Corcoran, L. M., and Smale, S. T. (2007). An inducible enhancer required for Il12b promoter activity in an insulated chromatin environment. Molecular and cellular biology *27*, 2698–2712.

Zhu, C., Gagnidze, K., Gemberling, J. H., and Plevy, S. E. (2001). Characterization of an activation protein-1-binding site in the murine interleukin-12 p40 promoter. Demonstration of novel functional elements by a reductionist approach. The Journal of biological chemistry *276*, 18519–18528.

Zhu, C., Rao, K., Xiong, H., Gagnidze, K., Li, F., Horvath, C., and Plevy, S. (2003). Activation of the murine interleukin-12 p40 promoter by functional interactions between NFAT and ICSBP. The Journal of biological chemistry *278*, 39372–39382.

Zipfel, P. F., and Skerka, C. (2009). Complement regulators and inhibitory proteins. Nature reviews. Immunology *9*, 729–740.

Chapter 2

Use of a Bacterial Artificial Chromosome Transgene Reporter in High Throughput Small Molecule Screen Identifies a β_2-Adrenergic/NFIL3 Axis as a Specific *Il12b* Transcriptional Inhibitory Pathway

Abstract

The availability of high-throughput technologies for the academic researcher, including automated screens with small molecules, RNAi, and next generation sequencing, have provided an attractive unbiased, brute force approach previously only available to industry. This provides a new avenue to approach questions that have been difficult to address with other methodologies. The finding that many autoimmune pathologies and cancers can be established and/or exacerbated by misregulated inflammatory cytokines have established cytokine targets for medical intervention. Cytokines, part of both the innate and adaptive immune response, have complex expression requirements that remain to be completely mapped, however selectivity is seen in most physiological settings. IL12p40, encoded by the *Il12b* gene, is one such protein that displays complex and selective regulation, requiring a series of regulator actions, involving a number of DNA binding transcription factors, histone modifications, and nucleosome remodeling. It is clear that selectivity is dependent on a properly chromatinized locus and therefore we performed a high-throughput small molecule screen looking for differential modulators of a chromatinized BAC transgene reporter against a non chromatinized minimal enhancer/promoter plasmid. It is our hypothesis that this novel approach will enrich the hit pool with small molecules that act selectively to inhibit the transcription of *Il12b*. This screen has established β_2-adrenergic agonists as a selectively strong inhibitor of *Il12b* transcriptional induction whose major outcome is dependent on the transcription factor NFIL3.

Introduction

High-throughput screening (HTS) has largely been a tool unique to the pharmaceutical industry. This has mostly been due to both the start-up cost associated with screening hardware and robotics, and the continued cost to develop and maintain large chemical libraries. A notable step in providing these types of services to the academic researcher occurred in 2004 when the NIH developed the Molecular Libraries Initiative (MLI) (Austin et al., 2004). This initiative resulted in the establishment of chemical screening programs at many universities in the US, originally under the Molecular Libraries Screening Center Network (MLSCN), and then replaced in 2008 by the Molecular Libraries Production Center Network (MLPCN) (Silber, 2010). Now a number of vendors provide chemical libraries of various sizes and academic screening facilities are assembling in-house libraries, providing large sets that can cover much of the bioactive chemical landscape. These screening centers provide a centralized core where tens to hundreds of labs share the cost providing an excellent HTS resource.

Academic screening facilities are continually growing more accessible and more capable and with these services academic labs can approach many difficult questions in an unbiased, brute force manner. These researchers now have affordable access to not only the screening equipment and libraries, but also the computational power required to analyze the large data sets generated by screens. One advance in computational power is the development of software tools with approachable interfaces. Script based tools which require specialized knowledge and training to use successfully, have been replaced with pre-package tools with less flexibility, but more suited for new users (Thomas, 2010). These software packages allow for rigorous and meaningful

conclusions to be draw from screen data by individual researchers who are very knowledgeable about the biology of the system and less so regarding computational screening methodologies. One computationally demanding method of screening that has matured over the last few years and is being used to answer basic biological questions is high content screening.

High-content screening (HCS) is a type of cell based assay which allows for the collection and analysis of multiparameter datasets in a high-throughput screening environment (Thomas, 2010). HCS relies on automated, high-speed fluorescent microscopes to collect images of the cells undergoing screening. The format requires that these screens utilize fluorescent reporter genes, cell compartment specific dyes, immunofluorescent approaches to label specific proteins, or cellular censors engineered to have a fluorescent response. Advances in microscope technology, including automated laser focusing, allow for image capture of each non-overlapping emission channel in a very short time frame (Zanella et al., 2010). With this method, screens can be performed on complex systems, extracting multiple parameters of data and providing a detailed quantification of how the system is manipulated by standard HTS treatments, including small molecule, siRNA, or cDNA libraries. This technology represents an efficient approach for a large-scale research program directed at basic cell biology, providing the context benefit of cell based screens. Even with HCS, in order for a screen to be successful, the most important step is assay design and any good design must start with the biology.

Macrophages, cells central to the induction of the innate immune response, have the ability to detect multiple classes of pathogens through germ-line encoded pattern

recognition receptors (PRRs). These receptors detect conserved pathogen associated molecules, initiate signaling, and trigger an appropriate inflammatory response (Uematsu and Akira, 2008). One family of transmembrane PRRs, the Toll-like receptors (TLRs), are found on the cell surface or in endosomal compartments. Many TLR agonists including lipopolysaccharide (TLR-4), CpG-DNA (TLR-9), ssRNA (TLR-7), and PolyI:C (TLR-3) induce a strong inflammatory response, in which *Il12b* is a highly up-regulated gene. IL12p40, encoded by the *Il12b* gene, is secreted when covalently associated with one of two other proteins (IL12p35 or IL23p19) to form the heterodimers IL-12 and IL-23, respectively (Trinchieri, 2003). The cytokine IL-12 is produced by activated macrophages and dendritic cells and plays a central role in the differentiation of naive CD4+ T cells into T helper 1 cells that produce IFN-γ and drive a cell-mediated response. IL-23 is central in supporting the expansion of the T helper 17 population, a potent proinflammatory cell type. Th17 cells produce IL-17A, IL-17F, IL-21 and IL-22 in addition to other inflammatory cytokines and is likely needed for anti-microbial activity at mucosal and epithelial barriers against extracellular bacterial (Ouyang et al., 2008). More importantly Th17 cells are a driver of multiple inflammatory-autoimmune pathologies (Hunter, 2005).

The screen was designed to monitor transcriptional regulation of the *Il12b* gene in macrophages upon lipopolysaccharide (LPS) stimulation. The first issue when designing this screen was to define the assay parameters. Due to that fact that IL12p40 is secreted, directly measuring protein by immuno-fluorescence by blocking export is not practical because it would require extreme manipulation of the system. Enzyme-linked immunosorbent assays (ELISA), which would measure IL12p40 in the

supernatant, could also provide an attractive assay, however the availability, quality, and cost of antibodies, followed by the difficulty of efficient quantification on the screen scale are major limitations. Therefore, the ideal approach for determining modulators of *Il12b* transcription for the screen was determined to be HCS with a fluorescent transcriptional reporter. When looking to artificial reporters as a measure of endogenous gene transcription, design of the reporters is crucial. It is our hypothesis that ignoring the regulation of gene transcription by distant control elements and endogenous nucleosome occupancy by using a transiently transfected or stably transfected promoter-reporter plasmids leads to many false or misleading hits. The induction of this plasmid reporter will be biased towards activation of a few key, dominate factors, such as NF-κB and AP-1, therefore missing regulation by less dominate factors that are just as necessary at the endogenous loci. Therefore, to detect relevant endogenous signals, one must design and implement a high-throughput screen using a chromatinized reporter. The need for this is clear from previous studies of *Il12b* transcription.

Transcriptional regulation of *Il12b* has been studied for many years and much is known about its activation. There are many conserved transcription factor binding sites in the proximal promoter that have been shown to be required for expression in transfection experiments and have shown inducible recruitment upon microbial stimulation, such as NF-κB, AP-1, C/EBP, NFAT, and IRF family members (Trinchieri, 2003). Specifically, *Il12b* induction shows a strong dependence on NF-κB dimers containing cRel and other specific factors including C/EBPβ, IRF1, IRF8, and NFATc1 (Sanjabi et al., 2000; Plevy et al., 1997; Zhu et al., 2003). An enhancer site with inducible nuclease hypersensitivity approximately 10 kilobases upstream from the TSS

has been identified (Zhou et al., 2007). Both the promoter and the enhancer exhibit inducible nucleosome remodeling of positioned nucleosomes at these sites that is performed by the SWI/SNF nucleosome remodeling complex (Ramirez-Carrozzi et al., 2006). Despite all that is known about the transcriptional induction of *Il12b*, signals and pathways that are selective for the transcriptional induction of *Il12b* over broad pro-inflammatory pathways that regulate large numbers of genes are unclear.

The benefits of specifically targeting IL12p40 on human health are clear in the success of the anti-IL12p40 monoclonal antibody treatment ustekinumab, which is FDA approved to treat moderate to severe plaque psoriasis (Leonardi et al., 2008; Laws and Warren, 2011). Psoriasis is a chronic inflammatory skin disease that affects approximately 2% of the worlds population and is characterized by lesions containing both Th1 and Th17 T cell phenotypes (Schön and Boehncke, 2005; Schlaak et al., 1994; Miossec, 2009). Targeting IL12p40 has been shown to be more successful than targeting TNF-α (etanercept) regardless of dosing regimen and preferred over non-selective immunosuppressive agents (Griffiths et al., 2010). While this approach has proven successful, anti-body treatments are very expensive and they need to be delivered by injection in the clinic. These biological agents can have problematic effects as demonstrated by the withdrawal of another IL12p40 treatment (briakinumab) to perform further tests and clinical trials. (Kurzeja et al., 2011). Therefore moving away from biological agents towards small molecules with the ability to inhibit IL12p40 production in a pill or cream form would provide a very attractive route for autoimmune treatment. The ability to inhibit *Il12b* transcription should also prove to be a benefit for Crohn's disease, multiple sclerosis, and rheumatoid arthritis.

The goal of this study was to develop an assay competent for high-throughput, high-content screening that was capable of including and enriching hits for selective modulators *Il12b* expression. Since our hypothesis is that selective inducible transcription only occurs in the context of native chromatin, we established a transgenic mouse with an *Il12b* bacterial artificial chromosome (BAC) transgene modified to include an EGFP reporter cassette. This reporter, which should be expressed at all levels the same as the endogenous gene, was directly compared to an internal counter selectable reporter. This second reporter was a minimal enhancer-promoter reporter plasmid, driving the expression of a DsRed cDNA and stably integrated into the macrophages which contained the IL12bBAC-EGFP reporter. We feel that this dual reporter cell line was an ideal start as a proof of principle study on the validity of chromatinized reporters, as well as the best way to enrich our hit pool with more specific inhibitors of *Il12b* transcription.

An HCS small molecule screen was performed and several selective hits were identified. Selective hits were defined as small molecules that inhibited the expression of EGFP from the chromatinized BAC transgene, without significantly inhibiting the expression of DsRed being driven by the naked *Il12b* enhancer/promoter plasmid. One group of selective inhibitors of the IL12bBAC-EGFP over the minIL12b-DsRed reporter were β_2-adrenergic agonists. Further analysis of β_2-adrendergic agonists activity with high-throughput mRNA sequencing in activated macrophages revealed a unique modulation of *Il12b*, where treatment inhibited *Il12b* far more than other LPS inducible genes. This activity has been directly attributed to the transcription factor NFIL3, which

is potently induced by β_2-adrendergic agonists and appears to be specific at inhibiting *Il12b* mRNA production in this system.

Materials and Methods

BAC modification

BACs were modified using the RecA method to insert EGFP as described except for instead of selecting out the co-integrate, BACs were maintained in liquid culture throughout the co-integrate processes (Gong and Yang, 2005). BACs were also modified using recombineering as described except for using a targeting construct instead of a long oligo in the second recombineering step (Warming et al., 2005). Mutations made with recombineering altered ATG to GTG for sites upstream of the EGFP insertion site (+4035 bp). BAC integrity was determined using pulsed-field gel electrophoresis and BAC fingerprinting. Pulsed-field gel electrophoresis was performed using a CHEF Mapper XA (Bio-rad), the program run was auto-algorithm with 10 kb for the low and 200 kb for the high. BAC finger printing was performed by overnight cleavage with the BamHI, EcoRI and PstI on BAC-miniprepped DNA.

Transgenic mice

BACs were linearized with PI-SceI (NEB) and purified as described using the Alternate Protocol: Preparation of BAC DNA by Alkaline Lysis and Sepharose CL-4B Chromatography in the following paper (Gong and Yang, 2005). BACs were injected at the UCLA transgenic core. Founders were analyzed by southern blot, traditional PCR and RT-qPCR. Offspring were screened by traditional PCR and RT-qPCR.

Cell Line Construction

Stable transformed reporter macrophage cell lines were constructed as previously described (Blasi et al., 1989). Briefly, bone marrow from two transgenic mice was collected from femurs and tibias and resuspended in phosphate-buffered saline (PBS, Cellgro) with 2% fetal bovine serum (FBS, Omega Scientific). Mononuclear cells were isolated by centrifugation (1500xg at 22°C for 20 minutes) over a ficoll gradient (Fico/Lite-LM [mouse], Atlanta Biologicals). Cells from the interface were washed with PBS with 2% FBS and plated in 10 cm tissue culture coated plates in sterile filtered conditioned media containing murine J2 retrovirus supernatant supplemented with 5% FBS, 5 µg/mL polybreen (stock: 8 mg/ml in 20 mM HEPES, pH 6.2), and 10% conditioned CMG media (M-CSF conditioned media). After 24 hours (37°C, 7% CO_2), the adherent cells were separated from the non-adherent cells and the media was replaced with 20% J2 virus media, 10% FBS, RPMI 1640 (Cellgro), 10% conditioned CMG media, and 50 ng/mL polybreen. Cells were left undisturbed for 7 days at 37°C, 7% CO_2. After 7 days adherent cells were washed with PBS, harvested by scraping, and split into two fractions, cultured with and without conditioned CMG media, until cell lines that continued to divide cytokine free were obtained. The base media was RPMI 1640 supplemented with L-Glutamine (Omega Scientific), 5% FBS, penicillin/streptomycin (Omega Scientific), and HEPES (Gibco). Cell lines were tested for the expression of EGFP and other cytokines upon LPS stimulation by RT-qPCR and FACS analysis.

Minimal Construct

The minimal *Il12b* enhancer-promoter plasmid was designed similarly to previously described (Zhou et al., 2007). Briefly, sequences corresponding to -9981 to -9436 base pairs from the TSS (Enhancer) and corresponding to -386 to -11 base pairs from the TSS (Promoter) were cloned into the pDsRed-Express1 plasmid (Clonetec). HS4 insulators were cloned 5' of the enhancer sequence and 3' of the DsRed poly(A) tail. This plasmid was linearized with the restriction enzyme ApaL1 (NEB) and stably transfected into immortalized macrophage cell lines using electroporation. Clonal cell populations were obtained and tested for robust expression of the reporters

Screen

All screens were performed at the Molecular Screening Shared Resource (MSSR) facility at UCLA. The stable dual reporter cell line (Il12bBAC-EGFP/minIl12b-DsRed) was maintained in RPMI1640 without phenol red (Gibco), supplemented with HEPES, PenStrep, and L-glutamine (base media) supplemented with Geneticin at 255 µg/mL (Invitrogen). Base media (30 µL per well) was plated into 384 well Cellstar optical glass bottom, black sided tissue culture plates (Greiner Bio-one) using a Multidrop 384 (Thermo Labsystems). See Table 2-1 for a list of libraries tested. Small molecule libraries were transferred (0.5 µL per well) using the pin tool on the Biomek FX liquid handling system (Beckman Coulter). Cells were washed with PBS, collected using TrypLE (Gibco), diluted to $1.2x10^6$ cells per mL in base media, and plated using Multidrop 384 (15 µL per well). Plates were centrifuged at 300 rpm for 1 minute to mix wells and maintained in an incubator, 37°C and 5% CO_2. After 4 hours, cells were

stimulated with LPS (10 µL per well, final concentration 100 ng/mL) and incubated overnight. After 14 to 18 hours cells were stained with 25ul of RPMI media containing Hoechst 33342 dye (Invitrogen) at a concentration of 15 ng/ml to obtain a final concentration of 5ng/ml in each well. After a minimum of 30 minutes, images of plates were collected using Image-Xpress high content microscope (Molecular Devices) and analyzed with MetaXpress (Molecular Devices) software. Analysis of signal quality per plate during optimization and screening by Z' and Z-factor calculation was regularly performed (Zhang, 1999)

Screen Analysis and Score Calculations

Raw data collected included total number, EGFP positive, and DsRed positive cells per well. Wells with low cell number (out of focus or toxic compounds) were removed from further calculations. Each well was normalized to plate negative controls and scores (z-score, MAD, robust z-score) were calculated on whole the screen. MAD (median absolute deviation) calculations are shown in Figure 2-2. Prior to moving forward with replicates, each well was visually inspected to rule out other possible confounding phenotypes, such as toxicity that was not reflected in the cell number or fluorescent compounds.

Replicates, Validation, and Serial Dilutions of Small Molecule Hits

After the primary screen, a number of hits were selected for replication. New source plates were created and screening in the dual line was performed as above. For serial dilutions, a source 384 well plate was created with three replicates of each small

molecule at each concentration and a two fold dilution series was completed. The dilution source plates were screen in the dual line as above. Additional low-throughput validation stimulation experiments were performed in the dual line, with mRNA analyzed by RT-qPCR and fluorescence observed by FACs or fluorescent microscopy.

Primary Macrophage Validation of Small Molecule Hits

Macrophages were prepared from four week-old wild-type C57BL/6 or knockout mice. Bone marrow was harvested and cultured in DMEM (Gibco) base media containing 20% FBS (Omega Scientific), 1x PenStrep (Gibco), L-glutamine (Gibco), and 10% conditioned media containing murine M-CSF. Media was changed on day three of differentiation, and cells were scraped and replated on day five at a density of 10^6 cells/mL. Macrophages were activated on day six with LPS (100ng/mL)(Sigma). Small molecules were dissolved in water or DMSO (Sigma) as per the vendors recommendations.

RT-PCR and quantitative PCR

RNA was extracted using TRI-reagent (Molecular Research Center), treated with RNase-free DNaseI, and purified using an RNeasy kit (Qiagen). Quantified RNA (2 µg) was reverse-transcribed using Omniscript RT Kit (Qiagen) and random hexamer primers. cDNA fragments were analyzed by real-time PCR using SensiMixPlus Sybr & Fluorescein (Bioline) and the 7900HT Fast Real-time PCR System (Applied Biosystems). The PCR amplification conditions were 95°C (3 min) and 45 cycles of 95°C (15 sec), 60°C (30 sec), and 72°C (30 sec). Primer pairs were designed

to amplify 80–120 bp mRNA-specific fragments, and unique products were tested by melt-curve analysis.

ELISA

ELISA was performed according to the manufacture's protocols for IL12p40 and TNF-α on supernatants from treated bone-marrow derived macrophages (ELISA Ready-SET-Go!, eBioscience).

cDNA Library Preparation and RNA Sequencing

Whole cell RNA was purified as above. Library preparation was performed with the Tru-Seq RNA Preparation Kit (Illumina) with a modified protocol on 1 μg of total RNA. Poly-A RNA selection and first strand synthesis was performed according to the manufacturer's protocol. Second strand synthesis was performed on samples after AmpureXP bead clean up (Agencourt) according to strand specific dUTP protocol (Parkhomchuk et al., 2009; Levin et al., 2010). Double stranded DNA products were barcoded with Illumina adaptors and USER (NEB) digestion was performed prior to PCR amplification. All samples were run multiplexed, four per lane, on the HiSeq 2000 platform with a single end sequencing length of 50 nucleotides.

Sequence Mapping and Analysis

Reads were aligned to the mouse mm9 reference genome with Tophat, restricting the alignment to only uniquely mapping reads, with 3 possible mismatches permitted (Trapnell et al., 2010, 2012). Quantification by RPKM (reads per kilobase per

million reads) was performed for the NCBIM37 build, mm9 Refseq genes using Seqmonk (http://www.bioinformatics.bbsrc.ac.uk/projects/seqmonk/). Differential expression data was generated using the DESeq package in R (Anders and Huber, 2010).

Nucleosome remodeling

Nucleosome remodeling was performed as described (Ramirez-Carrozzi et al., 2006). Briefly, after stimulation, cells were harvested and nuclei isolated using hypotonic lysis and NP-40. A limiting digest with SpeI (NEB) was performed with the intact nuclei and DNA was purified. Control cleavages with SphI (NEB) and KpnI (NEB) were digested overnight and the southern blot was performed. The membrane was probed with a ^{32}PdCTP labeled probe labeled using the Prime-IT kit (Stratagene). Membranes were exposed to a phosphoimager screen and data collected using a Typhoon Phosophoimager (Amersham Biosciences) and analyzed using the ImageQuant (GE Life Sciences) software.

Results

Design of a Chromatinized Reporter for High-throughput Screening

To obtain a reporter system that more genuinely mimics that of the endogenous *Il12b* gene, a bacterial artificial chromosome (BAC) which contains 191 kb of sequence of chromosome 11 from C57BL/6J mice surrounding the *Il12b* gene was modified into a reporter (Xu et al., 2007). An EGFP reporter cDNA with a poly(A) was inserted into the second exon of *Il12b* (Figure 2-1A). In addition, the upstream ATG codons were

mutated so upon translation of the reporter mRNA, only EGFP will be translated. This BAC was injected into mouse oocytes and a transgenic mouse line, which contains macrophages that were competent for induction of EGFP upon activation was derived. The IL12bBAC-EGFP is highly inducible and appears to assemble into near native chromatin structure (data not shown).

In order obtain a macrophage cell line for screening, a retroviral mediated approach was used to create transformed macrophage lines from the bone marrow of these transgenic mice (see Methods for additional details). The number of copies of the IL12bBAC-EGFP transgene is estimated at 10 (data not shown) and in the immortalized macrophages from a number of isolated clonal populations, the BAC behaves similar to the endogenous gene as measured by RT-qPCR (Figure 2-1C). Specifically, the EGFP is induced upon LPS stimulation, more highly induced with IFN-γ pre-incubation, and can be inhibited with IL-10. Clonal populations were screened for macrophage surface markers, robust expression of a number of induced cytokines and chemokines upon activation, and for efficient remodeling of the *Il12b* gene (data not shown). A clone was selected for further manipulation.

The dual reporter cell line was constructed from these immortalized transgenic macrophages. As the counter-selectable marker, a minimal *Il12b* enhancer-promoter reporter driving expression of a DsRed cDNA was stably transfected under selection into this line (Figure 2-1B). The *Il12b* enhancer-promoter construct, as previously reported, was flanked by HS4 insulator sequences to prevent the influence for sequences and chromatin at the site of intergration on the reporter (Zhou et al., 2007). A number of clonal populations were screened for robust induction of both reporters upon

activation and one was selected for screening (data not shown). This dual reporter cell line inducibly expresses DsRed from the plasmid and EGFP from the BAC upon LPS stimulation (Figure 2-1D). A broad inhibitor of NF-κB signaling, Bay11, inhibits the expression of both reporters. This cell line was then optimized for automated plating and stimulation in 384 well tissue culture plates in a high-throughput format, with analysis of images completed as described (See Methods). In agreement with the low-throughput experiments, efficient induction of both reporters and inhibition by Bay11 can be seen (Figure 2-1E).

Small Molecule Screen Identifies Both Selective and Nonselective Hits

In order to get maximum coverage, the screen was designed to have only one replicate of each of the 62,700 compounds in the primary screen (Table 2-1) (Coma et al., 2009; Macarron et al., 2011). Several methods for calculating significance of inhibition and hit selection were used, including MAD score over the whole screen (Figure 2-2), MAD score by plate, z-score by screen or plate, and robust z-score by screen and plate (data not shown). Wells were removed from data set for low cell numbers or other artifacts. Most wells with inhibition of either reporter were visually inspected to increase the efficacy of hit selection. Selective hits, those that inhibit the Il12bBAC-EGFP reporter without inhibiting the minIl12b-DsRed reporter can be found in the red box (Figure 2-2). Non-selective hit, those that inhibition both reporters, can be found in the blue box. Very few minIl12b-DsRed specific hits were identified and can be found in the green box. Approximately 500 compounds were selected for replicates and re-screened in a high-throughput format. Small molecules that maintained the ability to

inhibit both reporters or were selective for inhibiting the BAC reporter without inhibiting the minimal reporter were indentified and considered for further study.

β_2-Adrenergic Agonists Identified as a Group of BAC Selective Inhibitors

A number of small molecules tested had known targets. Limiting analysis to libraries that contained mostly drugs with known activities, three different compounds with β_2-adrenergic agonist properties had MAD scores that defined them as selective inhibitors of the Il12bBAC-EGFP reporter (Figure 2-3A). Upon additional replicate treatments, this phenotype was preserved (Figure 2-3B). Two β_2-adrenergic agonists, metaproterenol (MPH) and salbutamol (SAL) were further analyzed for inhibitory activities. In a high-throughput format, MPH was serially diluted and the ability to inhibit the BAC selectively in the dual cell line was measured. Over a large range of concentrations, MPH was able to inhibit expression of the EGFP without inhibiting the expression of the DsRed reporter as measured by image analysis after 18 hours of stimulation (Figure 2-3C). At the same time, Bay11, a non-selective inhibitor, showed no significant separation between the two reporters over a range of concentrations (Figure 2-3C).

β_2-Adrenergic Agonists Selectively Inhibit *Il12b* Expression in Macrophages

The selective β_2-aderenergic agonists metaproterenol (MPH) and salbutamol (SAL) were selected for further study in primary cells. In primary bone marrow derived macrophages (BMDMs), both MPH and SAL were able to inhibit transcription of *Il12b* in a dose dependent fashion (Figure 2-4A and data not shown, respectively). The

inhibition of IL12p40 expression and release from the activated macrophage can be reversed in a dose dependent manner by the non-selective β-adrenergic antagonist propranolol (Figure 2-4B). In addition, this inhibition was seen regardless of the TLR stimulus used. The β_2-agonist MPH prevented expression of IL12p40 in response to PolyI:C (TLR-3), LPS (TLR-4), CLO97 (TLR-7), and CpG (TLR9) (Figure 2-4C). This inhibition is seen at the transcriptional level as well (Figure 2-4D). It is unclear if weak inhibition of *Il12b* mRNA by MPH in PolyI:C treated cells is a result of lower expression or represents a biologically significant response. When 62 LPS inducible mRNA transcripts were investigated over an early response time course, inhibition of *Il12b* expression was the most strongly inhibited gene tested (Figure 2-5). While very few of these genes showed more than a 3 fold inhibition, *Il12b* regularly showed a 10 fold inhibition at peak expression. These experiments were conducted with a concentration of 30 μM of MPH, much higher than the effective dose of 1 μM needed for the inhibition of *Il12b*. This is in contrast to Bay11 treated BMDMs that show many more genes inhibited in the 33% - 15% and < 15% groups (Figure 2-5).

β_2-Adrenergic Inhibition of *Il12b* is cAMP and PKA Dependent

The β_2-adrenergic pathway in BMDMs was then investigated for other small molecules that could inhibit *Il12b* transcription. The major signaling outcome in response to β_2-adrenergic activation is activation of Gα, which stimulates adenylyl cyclase to catalyze the production of cyclic adenosine monophosphate (cAMP). The second messenger cAMP has the ability to activate a number of kinases including PKA and EPAC until it is degraded by a phosphodiesterase (PDE). PKA and EPAC

propagate the signal through the activity of their substrates including a number of signaling molecules and transcription factors. Two molecules that have been shown to inhibit transcription of *Il12b* in this study and others are the Gα-protein activator cholera toxin and the adenylyl cyclase activator forskolin (data not shown, Braun et al., 1999; Panina-Bordignon et al., 1997). Multiple cAMP mimics have been developed that are cell permeable, stable, and capable of activating downstream kinases. In BMDMs, non-specific cAMP mimics dibutyryl-cAMP and 8Br-cAMP can efficiently inhibit LPS induced *Il12b* expression, however the EPAC specific cAMP mimic, 8-CPT-2'-O-Me-cAMP, that cannot activate PKA, is not able to inhibit *Il12b* expression (data not shown). Also in support of a role for PKA in β_2-adrenergic agonist inhibition of *Il12b*, H-89, a small molecule inhibitor of PKA, can reverse the effect of MPH on *Il12b* (Figure 2-7C). Lastly, PDE inhibitors were investigated and when LPS stimulated BMDMs are pre-treated with a non-specific or specific PDE inhibitor, IBMX and rolipram (PDE4) respectively, there is no inhibition of *Il12b* expression. However, when added in conjunction with MPH or SAL, there is more inhibition than with β_2-agonist alone (Figure 2-7E and data not shown).

mRNA sequencing of the Transcriptome Reveals Broad Selectivity of *Il12b* Inhibition

In order to define the global selectivity of the anti-inflammatory properties of β_2-agonists on LPS stimulated macrophages, strand-specific mRNA high-throughput sequencing was performed. A group of LPS induced genes was determined by read counts for three control time courses (0, 60, and 120 minutes). Using the DESeq

package in R, 636 genes were determined to be induced at either the 60 minute or 120 minute timepoints (pval < 0.01) (Figure 2-6A) (Anders and Huber, 2010). For this subset of genes, reads per-kilobase per-million reads were calculated (RPKM) for each gene in control and MPH treated samples for the same time course of LPS stimulation. Analysis of two independent experiments comparing control RPMK to MPH treated RPKM reveals *Il12b* consistently located below most other genes on the diagonal and therefore strongly inhibited (Figure 2-6B). Averaging the strength of inhibition from these two independent experiments results in *Il12b* ranked as the most strongly inhibited gene (at approximately 10 fold down) with most genes unchanged (Figure 2-6C). A couple of other notable genes that are inhibited includes *Tnf, Ccl2,* and *Ifnb1* (approximately 2-2.5, fold inhibited).

Il12b Inhibition Does Not Require IL-10 or IFN-β Secondary Signaling

Treatment of BMDMs with β_2-adrenergic agonists and LPS results in a strong increase in the amount to *Il10* mRNA transcribed (Figure 2-6C). IL-10 is a potent inhibitor of *Il12b* transcription. To test the hypothesis that β_2-agonist inhibition of *Il12b* is a secondary effect and the result of the increase in IL-10 signaling, stimulations were performed in IL-10 $^{-/-}$ mouse BMDMs. Although IL-10 deficient macrophages make more *Il12b* than wild type macrophages, MPH is still able to robustly inhibit transcription, similar to wild type (Figure 2-7A).

In addition to *Il12b* inhibition, RNA-seq revealed a number of genes that are partially inhibited. Transcription factor binding site analysis of promoters of these genes shows an enrichment for the interferon stimulated response element (ISRE) (data not

shown). This observation, combined with the moderated inhibition of *Ifnb1,* resulted in the hypothesis that some of the inhibition is due to decreased secondary type-1 interferon signaling. To test this, IFNAR $^{-/-}$ macrophages that cannot respond to type-1 interferon were stimulated with LPS with or without MPH. At the early timepoints, there was no difference in the transcriptional inhibition caused by the β_2-agonist (Figure 2-7B).

Modest Differences in Remodeling at *Il12b* Enhancer and Promoter by β_2-agonist

One of the benefits from using a chromatinized reporter in the small molecule screen is the ability to detech and to enrich the hit pool with small molecules that modulate chromatin dynamics. To test if β_2-agonists represent molecules that inhibit *Il12b* expression at the chromatin level, nucleosome remodeling in β_2-agonist treated BMDMs was assayed at both the *Il12b* enhancer and promoter. Inhibition of nucleosome remodeling at the enhancer, indentified by a decrease in the intensity of the smaller band, is weak and cannot explain the robust inhibition of *Il12b* mRNA (Figure 2-7C). Inhibition of remodeling at the promoter is inhibited more strongly than at the enhancer, this effect is PKA dependent, and each treatment closely correlates with mRNA levels (Figure 2-7C).

***Nfil3* is Induced by β_2-Agonists and Responsible for *Il12b* Inhibition**

After eliminating the possibility that IL-10 or IFN-β were contributing to the inhibition of *Il12b*, we next looked at the kinetics of inhibition. It was observed that pre-treatment of macrophages with β_2-agonists was required to achieve the maximum

inhibition of *Il12b* (data not shown). This suggested that the inhibition was due to an induced gene prior to LPS stimulation. Analysis of the RNA sequencing data for transcripts that were differentially expressed in the MPH treated macrophages revealed only 21 genes were induced by more than 3 fold with MPH treatment alone (Table 2-2). The largest group of these were transcription factors, which contained eight genes. One transcription factor that was much more highly induced by pre-treatment than by LPS alone was *Nfil3*, which has been recently reported as downstream of IL-10 and an inhibitor of *Il12b* (Kobayashi et al., 2011; Smith et al., 2011). *Nfil3* is induced upon addition of β_2-agonists and it reaches a level of expression much higher and with earlier kinetics than that induced by LPS alone (Figure 2-8A). When BMDMs from NFIL3 deficient mice were stimulated with LPS in the presence of β_2-agonists, the knockout macrophages showed much less inhibition than wild type macrophages (Figure 2-8B). The absence of *Nfil3* therefore resulted in the loss of the inhibitory effect of the β_2-agonist on *Il12b* transcription.

RNAseq of *Nfil3* KO Macrophages Reveals Specificity for *Il12b* Inhibition

In order to determine the other possible targets of *Nfil3* anti-inflammatory activity, stranded mRNA high-throughput sequencing was performed on LPS stimulated *Nfil3* deficient macrophages, with and without MPH pre-treatment. RPKM was calculated for the set of 636 LPS induced genes determined earlier. Comparing MPH treated samples in wild type and *Nfil3*$^{-/-}$ macrophages reveals a sharp change in the *Il12b* expression with the knockout showing substantially higher expression (Figure 2-8C). Ranking of the fold change between the MPH treated and untreated *Nfil3*$^{-/-}$ samples after 120 minutes

of LPS stimulation reveals a strong contrast to the same plot in the wild type macrophages (Figure 2-8D). Specifically, *Il12b* mRNA expression has gone from being ranked first in inhibition to 150th, while *Tnf* and *Ccl2* have remained relatively unchanged and *Ifnb1* is more strongly inhibited and ranked higher.

Discussion

The ability to obtain a pool of meaningful hits from a high-throughput screen is the central goal of any screening strategy. Presented here are results from a novel small molecule screening strategy in which the major goal was to enrich the hit pool with small molecules likely to be specific for transcriptional induction of a pro-inflammatory cytokine while eliminating small molecules with broad immunosuppressive effects. Using a promoter-centric approach in contrast to a chromatinized transgenic approach in the same cell and screened in a high-content platform has provided some very interesting conclusions. First, it is clear that by using inhibition of the BAC reporter as the critical selection criteria, we have doubled the number of hits from the primary screen (Figure 2-2, green and blue box vs blue and red box). Replication of these small molecules in both high and low-throughput protocols have continually shown the ability of many to inhibit the EGFP expression selectively. These data reveal that selective versus non-selective phenotypes is more than just a probabilistic occurrence and relates directly to the biology of the small molecule in the system. These robust hits were completely missed by the minimal enhancer/promoter reporter construct. Further analysis of many of these small molecules in primary macrophages, beyond the β_2-agonists presented here, have provided a high rate of endogenous *Il12b* transcriptional

inhibition. It appears as though the screen benefited greatly from the increase of information provided by the BAC reporter and the ability to select against inhibitors of the plasmid reporter.

A second conclusion taken from the screening method was the benefit provided by using an HCS approach. The ability to quantify stained nuclei to obtain a cell number parameter, combined with the option to visually inspect the image of each well, provided an invaluable metric in hit selection. During hit selection, obviously toxic compound could be removed if cell numbers fell far below the rest of the plate or visual inspection revealed unhealthy phenotypes. Low cell numbers also correlated with lower stimulation magnitudes, most likely due to population effects, not to true inhibitory behaviors and multiple approaches were taken to minimize this bias. In addition, small molecules that often, by reporter analysis alone, appeared to be strong inducers of reporter expression, were visually inspected. Repeatedly, images revealed artifacts such as small molecules that appeared to be autofluorescent, that appeared to be intercalating with DNA and fluorescing, that appeared to be restricted to cellular compartments and fluorescing, or that appeared to precipitate forming crystals that interfered with analysis. Being able to indentify toxic compounds or artifacts led to the removal of these compounds from further study, therefore avoiding many compounds unlikely to validate down the pipeline.

Most importantly, the screening strategy we have developed has the potential to work for other inducible genes. In our experience, there are a few aspects of our screen that helped to make it successful. The strategy of using a transgene rather than a knock-in reporter in the gene locus had two basic benefits. First, the reporter was at a

higher copy number and expression of EGFP upon macrophage activation was easily detectable. Endogenous knock-in reporter genes can show diminished responses and can be difficult to observe by fluorescence (data not shown, S.D Pope and P.K. Purbey personal correspondence). Secondly, transgenic founder mice are much easier to produce than knock-in ES cell lines. Another consideration is that *Il12b* is located in a gene poor genomic region (Figure 2-1A). This allowed for minimal modifications of the BAC. For example, no modifications were needed to terminate the transcription of neighboring genes in the BAC so that the additional gene copy number would not lead to over-expression and add additional variables to the system. Finally, selection of the cell type to screen should be the most biologically relevant cell type. For macrophage studies, and possibly other hematopoietic cell types, a good model for stable cell line creation seems to be the J2 retro-viral system (Blasi et al., 1989).

Although reducing the hit pool to small molecule libraries with known targets removed some of the strongest selective hits from the screen, the decision to focus on these hits proved fruitful. Drugs that have been FDA approved and effectively used in patients not only provide extra information by having at least one known activity against a molecule or pathway, but also have proven to be bioavailable and safe in clinical trials. In addition, further analysis in macrophages may lead to new uses for this small molecule, also known as drug repositioning, that is an important aspect of modern drug discovery (Ashburn and Thor, 2004). Grouping the selectivity of the small molecules by their respective activities was informative. Although many drug classes exhibited some compounds that were selectively inhibiting the BAC, the percentage of selective hits from the β_2-adrenergic agonist group was much higher than dopamine receptor

(agonists or antagonists), serotonin receptor (agonist or antagonist), GABA receptor (agonists), or progesterone receptor (agonists) classes. The anti-inflammatory properties of β-agonists and cAMP inducing treatments has been previously reported, however much of this attention was paid to TNF-α inhibition in addition to IL-12 (Kizaki et al., 2008; Panina-Bordignon et al., 1997; Malfait et al., 1999; Donnelly et al., 2010; la Sala et al., 2009). Throughout these studies, there seems to be no clear consensus on mechanism. To our knowledge, this activity has not be investigated on a global level, and this analysis has provide some interesting findings including a strong selective inhibition of *Il12b* transcription. If not for the selective phenotype of the hit in our screen, we would not have pursued this pathway for selective inhibition of *Il12b*.

On the transcriptome scale, similar to previously published studies, upon β_2-adrenergic agonist pre-treatment of LPS stimulated macrophages, we observe transcriptional inhibition of some pro-inflammatory genes. The genes that were consistently inhibited include *Tnf, Ccl2*, *Ccl3*, *Csf2,* and *Ifit2*. Many of these genes share a consensus ISRE sequence in their promoters and some exhibit direct IRF3 dependence (Ramirez-Carrozzi et al., 2009). However, we find it difficult to explain the inhibition we see on reduced IRF activity. A secondary response through activation of the IFNAR receptor seems an unlikely source (Figure 2-7B) and not all IRF3 dependent genes are equally inhibited. The strength of inhibition of these transcripts, 2 to 3 fold, was weak compared with the strength of *Il12b* inhibition, often 10 to 20 fold. This imbalanced response has been underappreciated by previous studies and supports the use of the β_2-adrenergic signaling pathway for investigations into selective regulation of *Il12b*. It also provides a clear correlation to the observation that clinical use of β-

adrenergic blockers can aggravate psoriasis in patients (O'Brien and Koo, 2006). It is interesting to hypothesize why such a response would evolve. The endogenous ligands for β-adrenergic receptors are catecholamines, including epinephrine and norepinephrine. These mediators of the symphathetic nervous system's "fight or flight" response are present at times of high physiological stress. Inhibition of adaptive immune responses, including Th1 and Th17 responses, through the inhibition of *Il12b* production could serve to redirect vital resources while maintaining some ability to combat microbial pathogens.

High-throughput mRNA sequencing was able to capture the selectivity of the β_2-adrenergic response that had previously been unappreciated in the field. It was also a vital tool for the start of determining a mechanism for this selective inhibition. RNA sequencing was able to identify a small set of β_2-agonist induced genes (Table 2-2). Twenty-one genes that were induced three fold or more reflects selectivity in induced expression as well as inhibition after TLR stimulation. Surprisingly these genes are only a small subset of the much larger set of dibutyryl-cAMP induced genes which we expect to see induced (data not shown, reflective of one RNA sequencing time course replicate). Some of this difference could be explained by dosage as the dibutyryl-cAMP was used at a rather high dose, but the selectivity is still striking. Recent structure-activity relationship studies of β_2-adrenergic receptors bound to different ligands has begun to illustrate the molecular mechanism behind classification of ligands as full, partial, reverse, or β-arrestin-biased agonists (Liu et al., 2012). Although it has not been explored here, an important future direction is to determine if the selective inhibition of *Il12b* is specific to a class of agonist (SAL and MPH are considered partial agonists).

Central to this will be the determination of the role of β-arrestin in downstream signaling events.

As stated above, one of the transcription factors induced by β_2-agonists that was shown to be required for the inhibition of *Il12b* transcription was *Nfil3*. Regulation of *Nfil3*, including molecular mechanisms of transcriptional induction and mechanisms of inhibition of *Il12b,* remain unclear. *Nfil3* transcription appears to be PKA dependent but it could be as a result of the activity of many of PKA downstream effectors, such as CREB, CREM, CBP, or ELK1. Activity of NFIL3 on the *Il12b* promoter or enhancer has yet to be conclusively determined. One hypothesis would be that, due to the similarity of their consensus binding sites, NFIL3 competes with C/EBP for binding at the conserved C/EBP site in the enhancer or promoter. This could then result in chromatin modifications, recruitment of additional factors, or prevention of recruitment of factors that inhibit efficient transcription of the *Il12b* gene even upon C/EBP activation. One puzzling observation from this study is that the inhibition of remodeling at the enhancer is not consistent with the magnitude of inhibition of expression. Therefore our hypothesis is that NFIL3 activity is downstream of remodeling at the enhancer, but at the promoter this is less clear. It does seem to mimic expression, namely, reduced expression of *Il12b* correlates with reduced accessibility, but it cannot yet be determined if this is due to NFIL3 occupancy.

HTS techniques were expected to revolutionize drug discovery. With the ability to test hundreds of thousands of small molecules in a system in one study, surely no disease was safe. Over the last decade though, the number of drugs approved from humble HTS beginnings has not been a dominant force (Thomas, 2010). Some of this is

due to the long delay from screen to clinic, but some is probably due to screening methodologies lagging behind the technologies. We propose that our chromatinized reporter approach may be a step forward for screening methodologies, providing a higher percentage of biologically informative hits as a result of including regulation at the chromatin level in the assay.

Figure Legends

Figure 2-1: Reporter and Cell Line Optimization

(A) Schematic of the *Il12b*-BAC-EGFP reporter. BAC: RP23-417P8 was the backbone for the reporter and modified by inserting an EGFP reporter gene into the second exon of the *Il12b* coding region. The EGFP contained a polyadenylation site (yellow bar) and upstream ATG codon after the TSS in exon 2 was mutated to GTG (red bar). **(B)** Schematic of the minimal reporter (Zhou et al., 2007). **(C)** Immortalized macrophage cell line from *Il12b* BAC EGFP transgenic mice were treated as described. IFN-γ (10 U) was added 4 hours pre-stimulation, IL-10 was added with LPS. Expression was analyzed by RT-qPCR. **(D)** FACs analysis of dual reporter cell line with the described treatments after 8 hours of LPS (100 ng/mL) stimulation. BAY11 was used at 10 μM and added 30 minutes prior to LPS. **(E)** Image analysis of dual reporter cell line in high-throughput format. Cells were plated and stimulated with automation as described in Methods in 384 well plates. BAY11 was used at 10 μM. After 18 hours of LPS (100 ng/mL) stimulation, cells were stained with Hoechst 33342 dye and images were collected and analyzed as described in Methods. Each treatment was repeated in 64 wells.

Table 2-1: Small Molecule Libraries

Small molecule libraries screened are presented here with descriptions. When limiting analysis to molecules with known activities, only BioMol, Prestwick Chemical Library, MicroSource Spectrum Collection, and NIH Clinical Collection were considered.

Figure 2-2: Small Molecule Screen Results

Significance of inhibition of the Il12bBAC-EGFP reporter (x-axis) against the minimal DsRed reporter (y-axis) for all wells in the screen determined by MAD score (mean absolute deviation). Especially toxic compounds (those wells with < 300 cells per image) have been removed. Box and whisker plots on the axis summarize the sample data with controls removed. Controls that received DMSO and LPS are in yellow. Controls that are unstimulated or Bay11 treated are in blue. Phenotypic regions are in boxes and include Il12bBAC-EGFP specific hits (red), minIl12b-DsRed specific hits (green) and non-specific hits (blue).

Figure 2-3: β_2 Adrenergic Agonists are Selective Inhibitors of the BAC in Cell Line

(A) Limiting the data to libraries that contain small molecules with known targets, MAD scores for BAC and minimal reporters are represented. β_2 agonists (red dots) are selective inhibitors of the BAC reporter while glucocorticoids (purple) are non-selective hits. Box and whisker plots on the axis summarize the sample data with controls removed. Controls that received DMSO and LPS are in yellow. Controls that are unstimulated or Bay11 treated are in blue. **(B)** Replication in a high-throughput format of three β_2 agonists (red) and four glucocorticoids (purple). Controls from these plates are included for reference. All quantitation is expression normalized to the negative controls on the plate. **(C)** Serial dilution of β_2 agonist (MPH: metaproterenol) in high-throughput format in cell line. Each treatment was replicated 9 times and values are reported as relative to in-plate DMSO and LPS stimulated controls (n=16). Each dilution step was 4 fold with high concentration of 125 μM. LPS was used at 100 ng/mL. Serial dilution of

BAY11 in high-throughput format in cell line. Each treatments was replicated 9 times and values are reported as relative to in-plate DMSO and LPS stimulated controls (n=16). Each dilution step was 2 fold with high concentration of 10 μM. LPS was used at 100 ng/mL.

Figure 2-4: β_2-Adrenergic Agonist Inhibit TLR Induced *Il12b* Transcription in Primary Macrophages

(A) Serial dilution of MPH in primary mouse (C57BL/6) bone marrow derived macrophages (BMDMs). Expression was measured with RT-qPCR. LPS was used at 100 ng/mL and MPH concentration range was 1 μM to 0.1 nM with 5 fold dilution between treatments. **(B)** Expression of IL12p40 in BMDMs as measured by ELISA after 18 hours under the treatments as described. β_2-agonist used was MPH at 1 μM. β antagonist used was propranolol in a range of 10^{-4} M to 10^{-6} M. **(C)** Expression of IL12p40 in BMDMs as measured by ELISA after 18 hours of TLR stimulation. β_2-agonist (MPH) and corticosteroid (CBP: clobetasol propionate) were used at 10 μM and were added one hour prior to TLR ligands. Stimulation was PolyI:C (TLR-3), LPS (TLR-4), CL097 (TLR-7/8), and CpG (TLR-9). **(D)** Primary BMDMs treated as described in C. Cells were collect at 120 minutes and expression was analyzed by RT-qPCR. Each treatment is normalized to own maximum stimulation DMSO control.

Figure 2-5: Expression Profiles of 62 LPS Induced Genes Show High Selectivity for *Il12b* by β_2-Agonist

Primary BMDMs were treated with MPH at 30 μM, DMSO (0.1%), or BAY11 at 10 μM and stimulated with LPS at 100 ng/mL for the given timepoints. Percent calculation is base on LPS stimulated cells without DMSO at the given timepoint where each gene reaches 100% at max expression. Max Peak values refer to the highest expression of the transcript regardless of time-point for the small molecule treated macrophages. 62 LPS inducible genes were investigated and averaged over 3 experiments.

Figure 2-6: RNA sequencing of Primary Macrophages Reveals Specific Inhibition of *Il12b* by β_2 Agonist

(A) DESeq analysis of mRNA sequencing data from three separate control stimulation time-course experiments in BMDMs. Points represent the average strength of expression against the average fold change over the unstimulated control for a specific Refseq gene ID. Red dots represent gene IDs with a significant fold change (p-value < 0.01) at 120 minutes of LPS stimulation (100 ng/mL) as determined by negative binomial test provided by DESeq analysis. **(B)** Strength of expression of 636 refseq IDs determined to be significantly induced (p-value < 0.01, see above) plotted as reads per kilo-base per million reads (RPKM). RPKM of control, LPS stimulated BMDMs at 120 minutes plotted against RPKM of MPH (1 μM) treated BMDMs. Data from two independent experiments are overlaid (Experiment 1 in blue, Experiment 2 in red). **(C)** Fold change of the average expression of the 636 induced genes over two experiments due to MPH (1 μM) treatment. Genes have been ranked and plotted consecutively.

Figure 2-7: β_2-Agonist Inhibition of *Il12b* Is Not Dependent on IL-10 Induction or IFN-β Inhibition and Results in Only Weak Inhibition of Nucleosome Remodeling at the *Il12b* Enhancer and Promoter

(A) Primary mouse BMDMs from wild type (C57BL/6) or *Il10*$^{-/-}$ were stimulated for 60 minutes as described. Rolipram (ROL) was used at 30 μM and added 15 minutes prior to MPH, used at 1 μM and added 120 minutes prior to LPS, 100 ng/mL. Expression was measured with RT-qPCR. **(B)** Primary mouse BMDMs from wild type (C57BL/6) or IFNAR$^{-/-}$ were stimulated for 120 and 360 minutes with 120 minute pre-incubation with MPH or DMSO as described. Expression was measured with RT-qPCR. **(C)** Restriction enzyme accessibility was performed on BMDMs at the *Il12b* enhancer and promoter (Ramirez-Carrozzi et al., 2006). One representative experiment is presented with expression and southern blot assay. BMDMs were treated as described in Methods and stimulated for 120 minutes prior to collection and processing. Concentrations used: MPH 1 μM, ROL 30 μM, H89 25 μM. Schematics show the location of the SpeI restriction enzyme site which shows LPS-inducible accessibility. The larger DNA fragment (*) results from cleavage of purified genomic DNA by SphI and KpnI, which cleaved sites flanking the promoter and enhancer. The smaller fragment (<) was generated with SpeI, which was added to isolated nuclei and cleaved within the promoter or enhancer. Finally, quantification of the band intensities from 4 separate REA experiments using ImageQuant (GE Life Sciences).

Table 2-2: β_2-Agonist Induced Genes

Listed are genes organized by ontology that displayed an average of three fold induction or more from two RNA sequencing experiments after 120 minutes of MPH treatment. Gene ontology and descriptions provided by http://www.ncbi.nlm.nih.gov and pantherdb.org.

Figure 2-8: Induction of NFIL3 is Responsible for the Selective Inhibition of *Il12b* by β_2-Agonist

(A) *Nfil3* mRNA expression as determined by RT-qPCR for treatment of BMDMs as described. LPS was used at 100 ng/ml. MPH and SAL were used at 1 μM. Data represent a average of 4 separate experiments. **(B)** *Il12b* mRNA expression as determined by RT-qPCR for treatment of BMDMs from C57BL/6 or NFIL3 $^{-/-}$ mice as described. Data represent the average expression from 3 separate experiments and is plotted as log transformed fold change over unstimulated controls. **(C)** Strength of expression for 636 LPS induced genes (with p-value < 0.1 as determined by DESeq) in C57BL/6 and NFIL3$^{-/-}$ BMDMs treated with MPH at 1 μM and reported as RPKM at 120 minutes after LPS stimulation (100 ng/mL). **(D)** Fold change of the expression of the 636 induced genes after MPH treatment in NFIL3$^{-/-}$ LPS stimulated BMDMs . Genes have been ranked and plotted consecutively.

Figure 2-1: Reporter and Cell Line Optimization

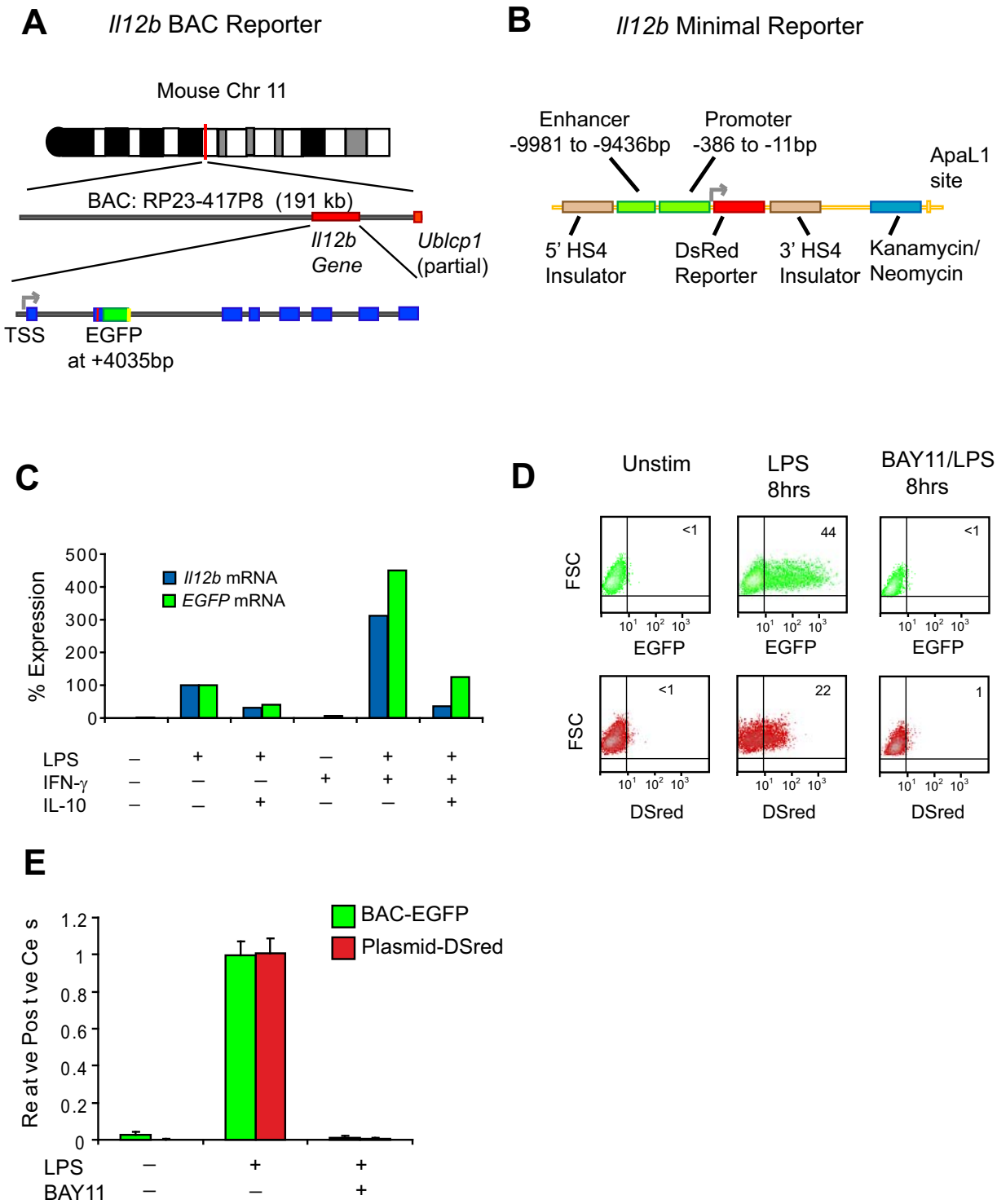


Table 2-1: Small Molecule Libraries

Library Name	Number of Compounds	Description
BioMol	500	Bioactive lipids and small molecules with endo-cannabinoid, ion channel, enzyme, phosphatase and kinase activities, orphan ligands
Prestwick Chemical Library	1,120	FDA approved drugs
MicroSource Spectrum Collection	2,000	50% Known Drugs 30% Natural Products 20% Other Bioactive Molecules
NIH Clinical Collection	600	Compounds have drug-likeness properties and have processed into Phase I-III clinical trials.
ChemBridge Diver Set	30,000	Stringent drug-like and desirable chemical group diversity
Combichem Diverse Library	20,000	A custom set of compounds which have low cellular toxicity and excellent coverage of the chemical space
Druggable Compound Set	8,000	Custom set of compounds targeted at kinase, protease, ion channels, and GPCRs

Figure 2-2: Small Molecule Screen Results

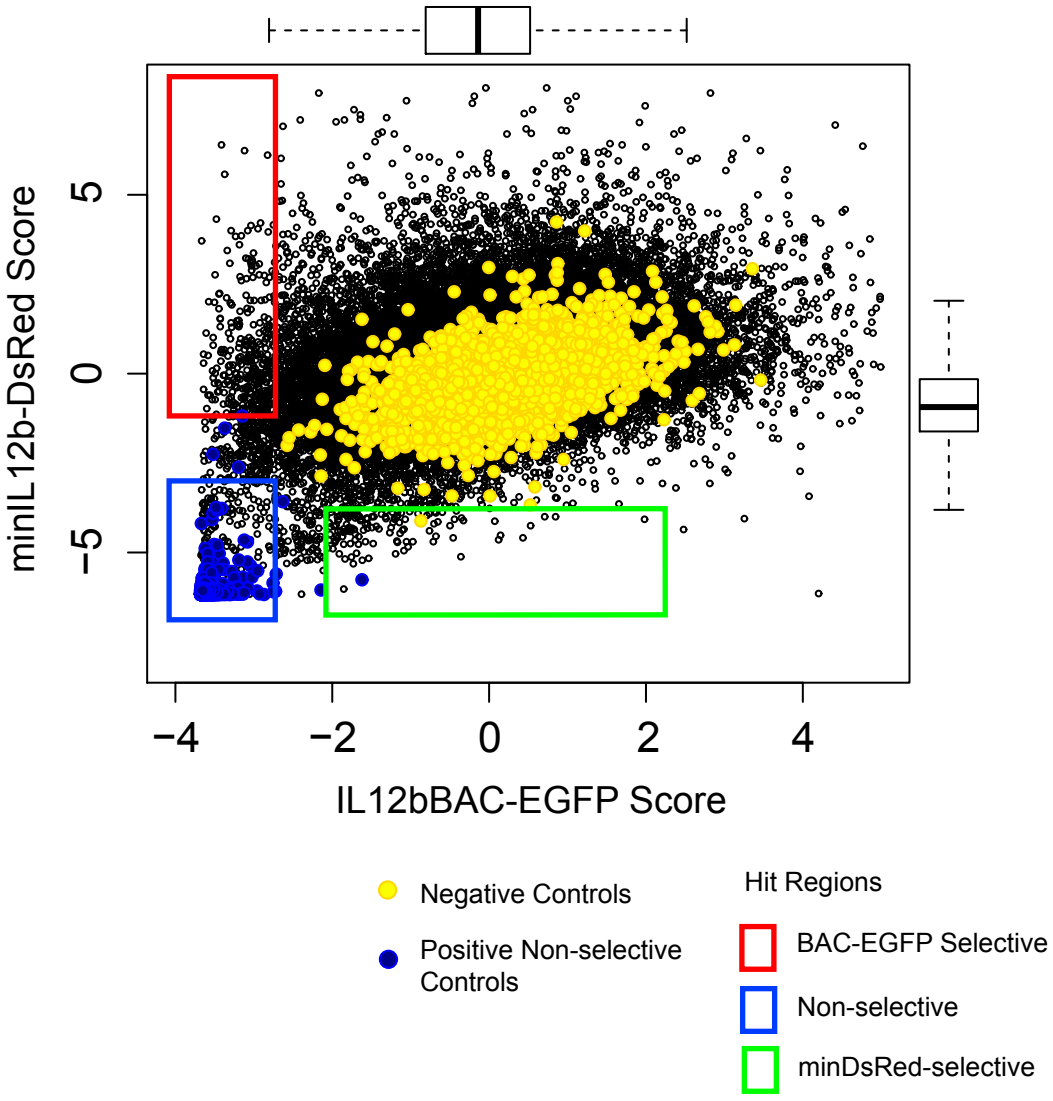


Figure 2-3: β2 Adrenergic Agonists Are Selective Inhibitors of the BAC in Cell Line

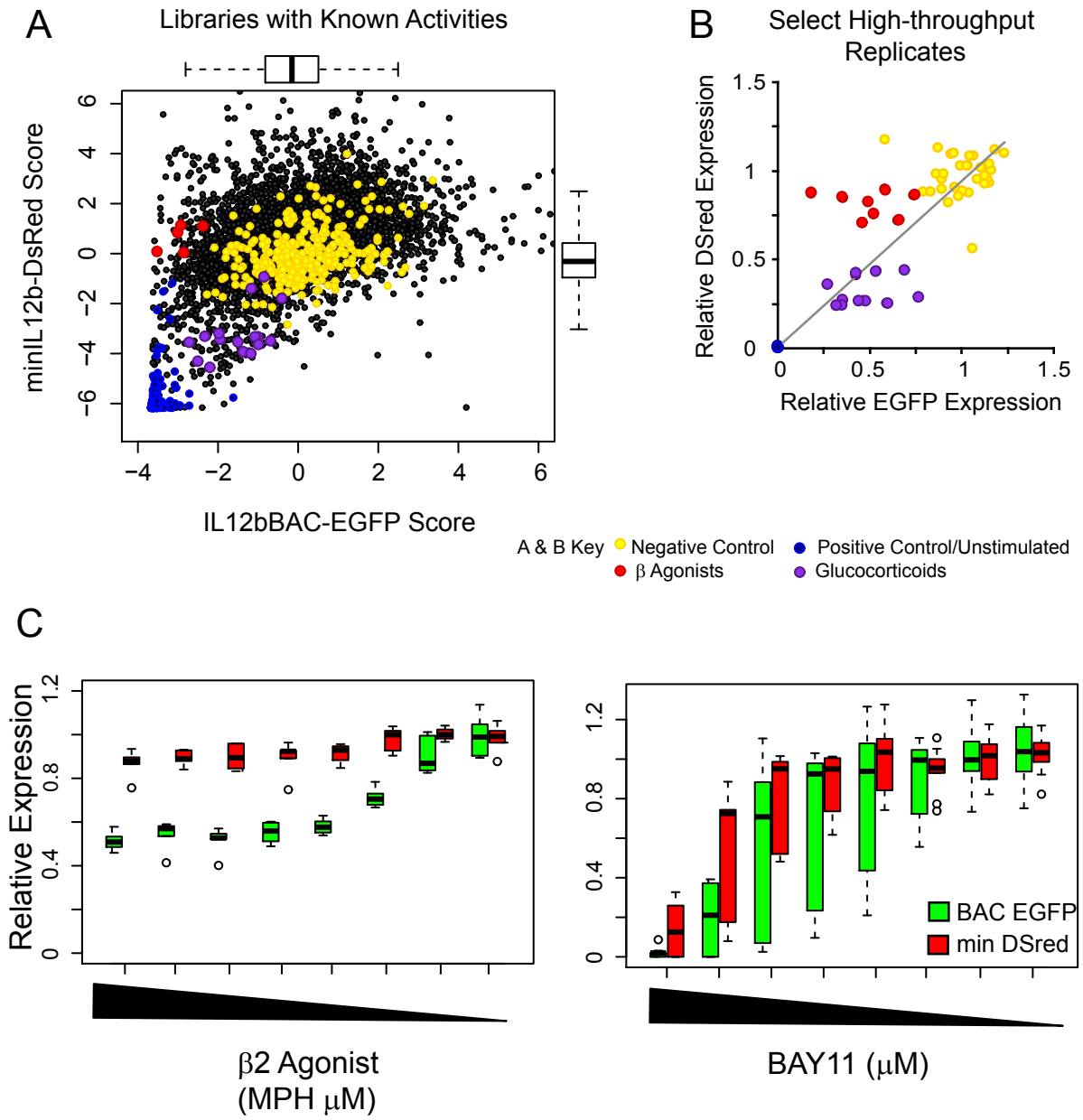


Figure 2-4: β2 Adrenergic Agonist Inhibit TLR Induced *Il12b* Transcription In Primary Macrophages

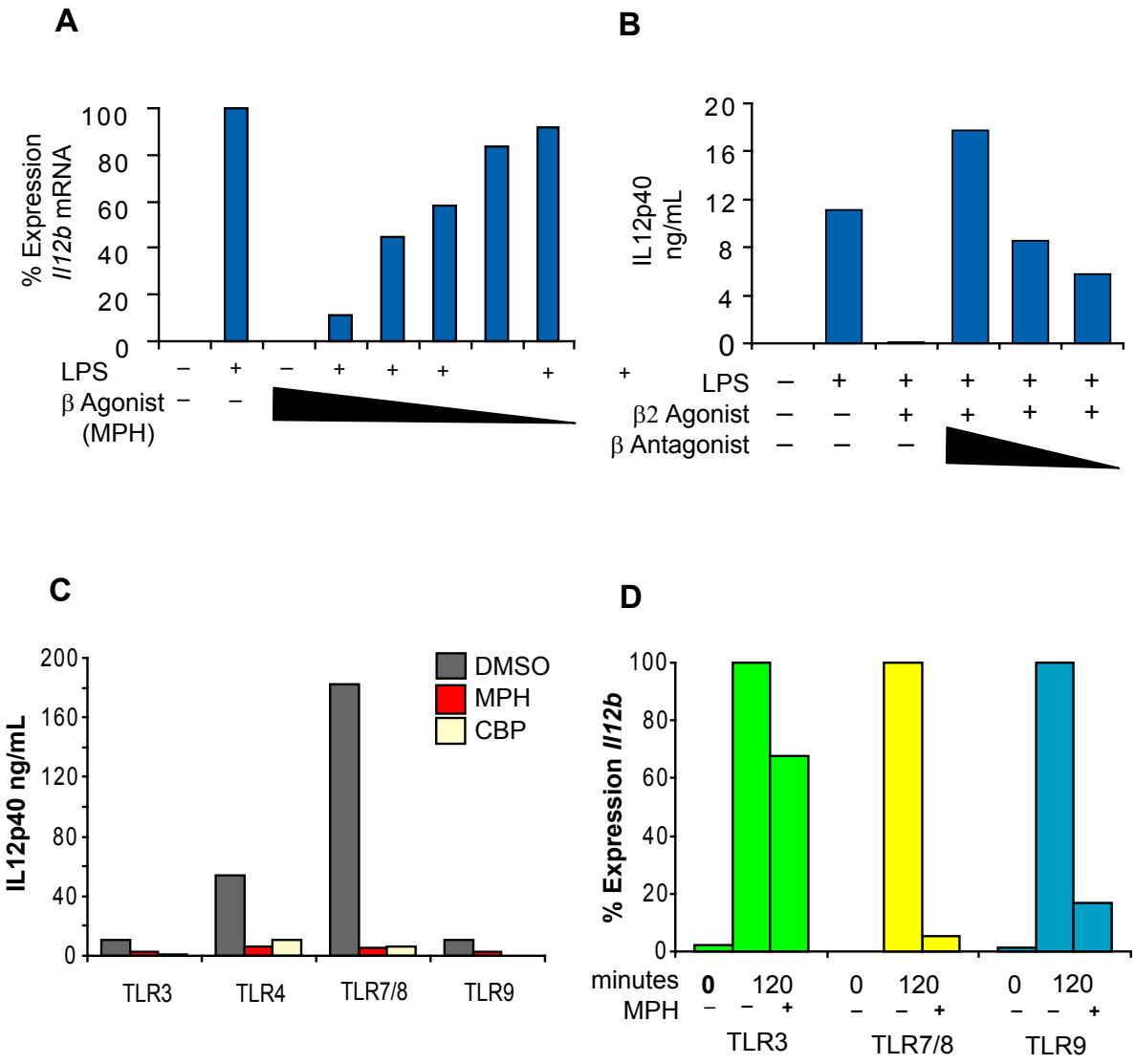


Figure 2-5: Expression Profiles of 62 LPS Induced Genes Show High Selectivity for *Il12b* by β2 Agonist

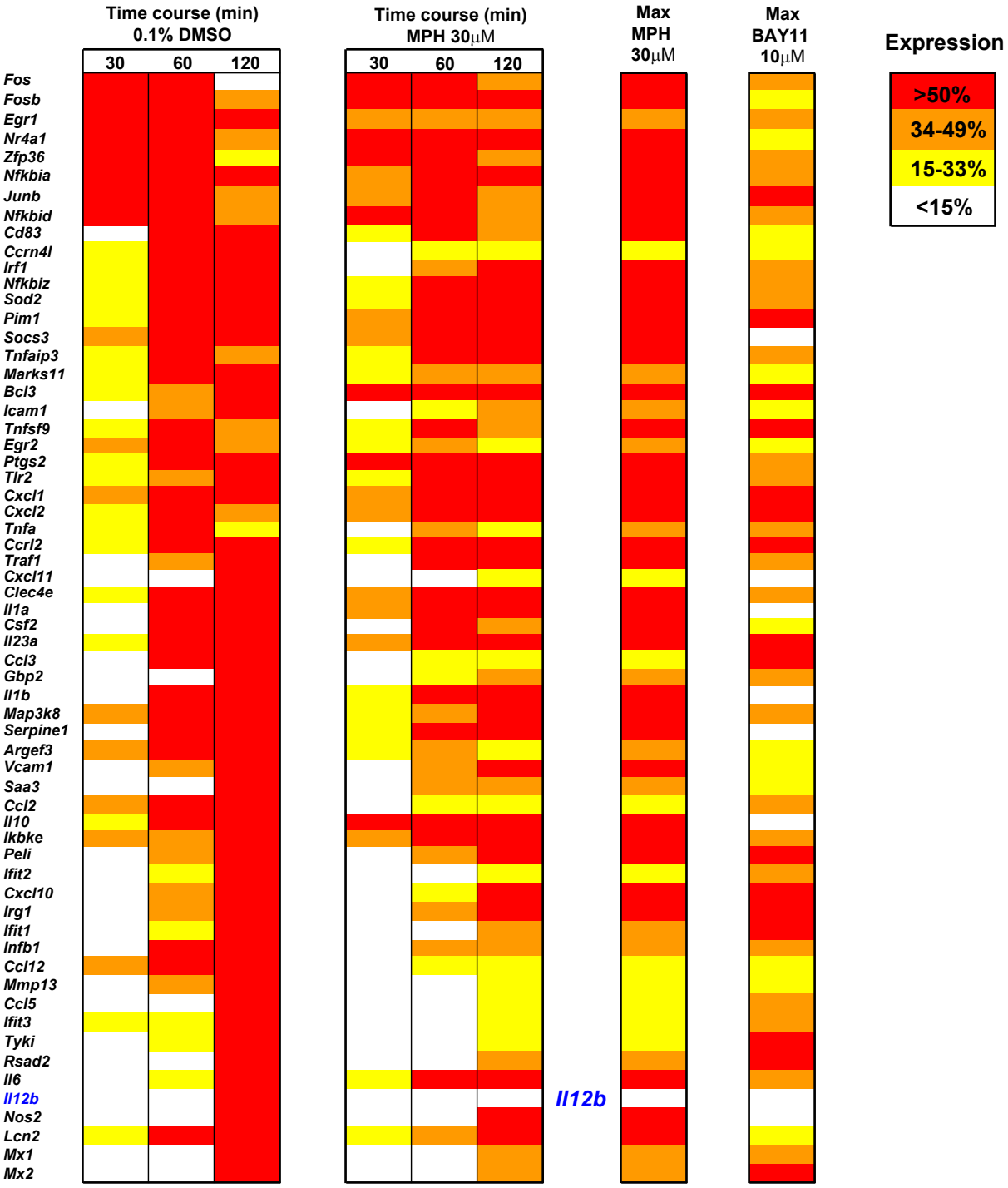


Figure 2-6: RNA sequencing of Primary Macrophages Reveals Specific Inhibition of *Il12b* by β2-Agonists

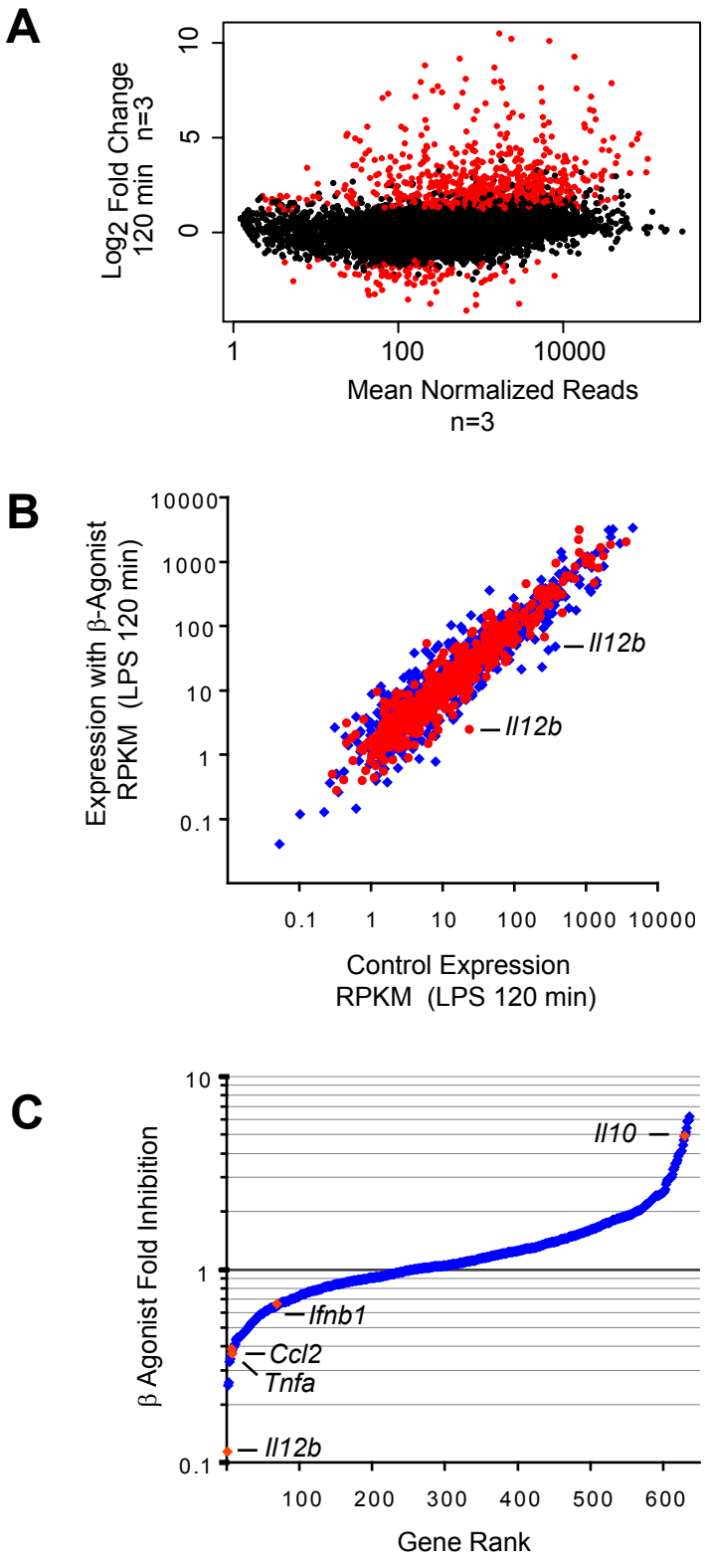


Figure 2-7: β2-Agonist Inhibition Of *Il12b* Is Not Dependent on IL-10 Induction or IFNβ Inhibition and Results in Only Weak Inhibition of Nucleosome Remodeling at the *Il12b* Enhancer

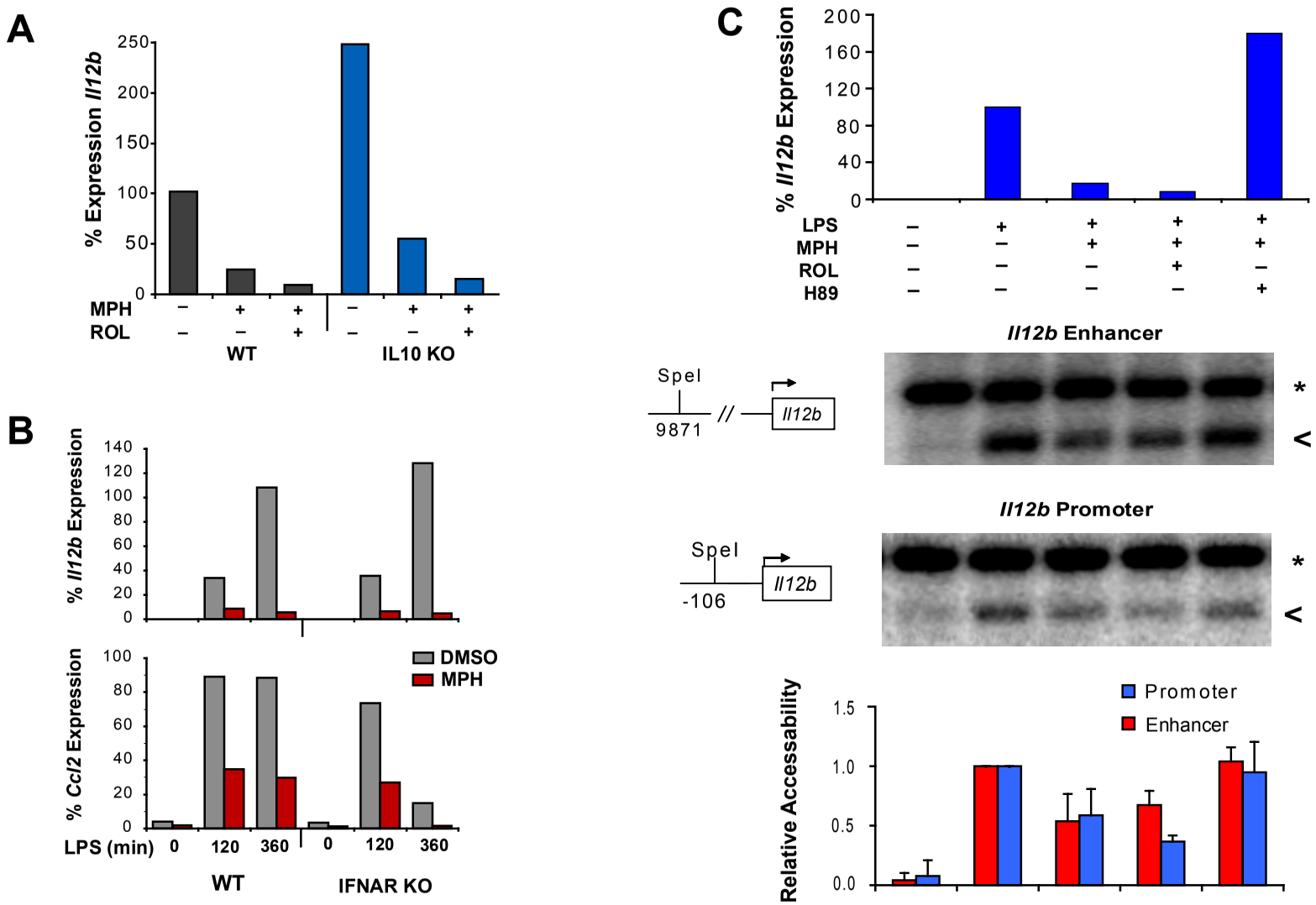
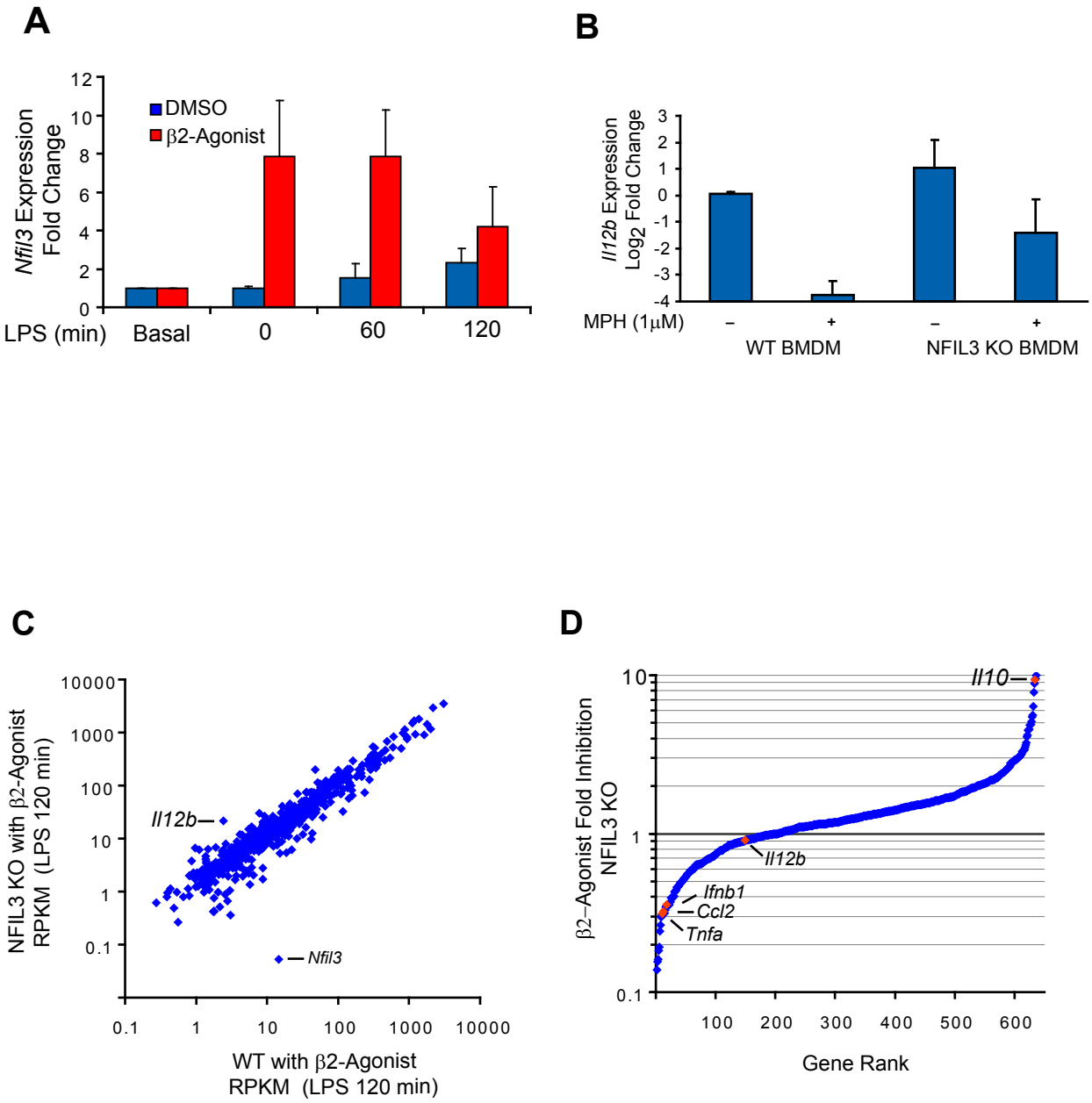


Table 2-2: β2-Agonist Induced Genes by RNA Sequencing

Gene Ontology	Gene Name	Ave Fold Induction
Transcription Factor	*Crem*	58.0
	Nr4a1	11.8
	Fosl2	11.5
	Nfil3	7.9
	Runx3	4.3
	Zfp703	3.6
Receptor Binding	*Thbs1*	71.7
	Osm	10.9
	Hig2	8.7
	Apbb3	3.3
Growth Factor	*Areg*	26.9
	Vegfa	6.0
Signal Transduction	*Arrdc3*	7.9
	Gab1	4.8
Receptor Activity	*Thbd*	12.5
	Trem1	6.7
Cell Adhesion	*Cytip*	39.3
Phosphatase	*Dusp5*	12.6
Protein Kinase Activator	*Rgc32*	4.5
rRNA Processing	*Rrp1b*	4.2
Kinase	*Nuak2*	3.5

Figure 2-8: Induction of NFIL3 is Responsible for the Selective Inhibition of *Il12b* by β2-Agonist



Work Cited

Anders, S., and Huber, W. (2010). Differential expression analysis for sequence count data. Genome biology *11*, R106.

Ashburn, T. T., and Thor, K. B. (2004). Drug repositioning: identifying and developing new uses for existing drugs. Nature reviews. Drug discovery *3*, 673–683.

Austin, C. P., Brady, L. S., Insel, T. R., and Collins, F. S. (2004). NIH Molecular Libraries Initiative. Science (New York, N.Y.) *306*, 1138–1139.

Blasi, E., Radzioch, D., Merletti, L., and Varesio, L. (1989). Generation of macrophage cell line from fresh bone marrow cells with a myc/raf recombinant retrovirus. Cancer biochemistry biophysics *10*, 303–317.

Braun, M. C., He, J., Wu, C. Y., and Kelsall, B. L. (1999). Cholera toxin suppresses interleukin (IL)-12 production and IL-12 receptor beta1 and beta2 chain expression. The Journal of experimental medicine *189*, 541–552.

Coma, I., Clark, L., Diez, E., Harper, G., Herranz, J., Hofmann, G., Lennon, M., Richmond, N., Valmaseda, M., and Macarron, R. (2009). Process validation and screen reproducibility in high-throughput screening. Journal of biomolecular screening *14*, 66–76.

Donnelly, L. E., Tudhope, S. J., Fenwick, P. S., and Barnes, P. J. (2010). Effects of formoterol and salmeterol on cytokine release from monocyte-derived macrophages. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology *36*, 178–186.

Gong, S., and Yang, X. W. (2005). Modification of bacterial artificial chromosomes (BACs) and preparation of intact BAC DNA for generation of transgenic mice. Current protocols in neuroscience / editorial board, Jacqueline N. Crawley ... [et al.] *Chapter 5*, Unit 5.21.

Griffiths, C. E. M., Strober, B. E., van de Kerkhof, P., Ho, V., Fidelus-Gort, R., Yeilding, N., Guzzo, C., Xia, Y., Zhou, B., Li, S., et al. (2010). Comparison of ustekinumab and etanercept for moderate-to-severe psoriasis. The New England journal of medicine *362*, 118–128.

Hunter, C. A. (2005). New IL-12-family members: IL-23 and IL-27, cytokines with divergent functions. Nature reviews. Immunology *5*, 521–531.

Kizaki, T., Izawa, T., Sakurai, T., Haga, S., Taniguchi, N., Tajiri, H., Watanabe, K., Day, N. K., Toba, K., and Ohno, H. (2008). Beta2-adrenergic receptor regulates Toll-like

receptor-4-induced nuclear factor-kappaB activation through beta-arrestin 2. Immunology *124*, 348–356.

Kobayashi, T., Matsuoka, K., Sheikh, S. Z., Elloumi, H. Z., Kamada, N., Hisamatsu, T., Hansen, J. J., Doty, K. R., Pope, S. D., Smale, S. T., et al. (2011). NFIL3 Is a Regulator of IL-12 p40 in Macrophages and Mucosal Immunity. The Journal of Immunology *186*, 4649–4655.

Kurzeja, M., Rudnicka, L., and Olszewska, M. (2011). New interleukin-23 pathway inhibitors in dermatology: ustekinumab, briakinumab, and secukinumab. American journal of clinical dermatology *12*, 113–125.

Laws, P. M., and Warren, R. B. (2011). Ustekinumab for the treatment of psoriasis. Expert review of clinical immunology *7*, 155–164.

Leonardi, C. L., Kimball, A. B., Papp, K. A., Yeilding, N., Guzzo, C., Wang, Y., Li, S., Dooley, L. T., and Gordon, K. B. (2008). Efficacy and safety of ustekinumab, a human interleukin-12/23 monoclonal antibody, in patients with psoriasis: 76-week results from a randomised, double-blind, placebo-controlled trial (PHOENIX 1). Lancet *371*, 1665–1674.

Levin, J. Z., Yassour, M., Adiconis, X., Nusbaum, C., Thompson, D. A., Friedman, N., Gnirke, A., and Regev, A. (2010). Comprehensive comparative analysis of strand-specific RNA sequencing methods. Nature methods *7*, 709–715.

Liu, J. J., Horst, R., Katritch, V., Stevens, R. C., and Wüthrich, K. (2012). Biased signaling pathways in β2-adrenergic receptor characterized by 19F-NMR. Science (New York, N.Y.) *335*, 1106–1110.

Macarron, R., Banks, M. N., Bojanic, D., Burns, D. J., Cirovic, D. A., Garyantes, T., Green, D. V. S., Hertzberg, R. P., Janzen, W. P., Paslay, J. W., et al. (2011). Impact of high-throughput screening in biomedical research. Nature reviews. Drug discovery *10*, 188–195.

Malfait, A.-M., Malik, A. S., Marinova-Mutafchieva, L., Butler, D. M., Maini, R. N., and Feldmann, M. (1999). The {beta}2-Adrenergic Agonist Salbutamol Is a Potent Suppressor of Established Collagen-Induced Arthritis: Mechanisms of Action. J. Immunol. *162*, 6278–6283.

Miossec, P. (2009). IL-17 and Th17 cells in human inflammatory diseases. Microbes and infection / Institut Pasteur *11*, 625–630.

Ouyang, W., Kolls, J. K., and Zheng, Y. (2008). The biological functions of T helper 17 cell effector cytokines in inflammation. Immunity *28*, 454–467.

O'Brien, M., and Koo, J. (2006). The mechanism of lithium and beta-blocking agents in inducing and exacerbating psoriasis. Journal of drugs in dermatology : JDD *5*, 426–432.

Panina-Bordignon, P., Mazzeo, D., Lucia, P. D., D'Ambrosio, D., Lang, R., Fabbri, L., Self, C., and Sinigaglia, F. (1997). Beta2-agonists prevent Th1 development by selective inhibition of interleukin 12. The Journal of clinical investigation *100*, 1513–1519.

Parkhomchuk, D., Borodina, T., Amstislavskiy, V., Banaru, M., Hallen, L., Krobitsch, S., Lehrach, H., and Soldatov, A. (2009). Transcriptome analysis by strand-specific sequencing of complementary DNA. Nucleic acids research *37*, e123.

Plevy, S. E., Gemberling, J. H., Hsu, S., Dorner, A. J., and Smale, S. T. (1997). Multiple control elements mediate activation of the murine and human interleukin 12 p40 promoters: evidence of functional synergy between C/EBP and Rel proteins. Molecular and cellular biology *17*, 4572–4588.

Ramirez-Carrozzi, V. R., Braas, D., Bhatt, D. M., Cheng, C. S., Hong, C., Doty, K. R., Black, J. C., Hoffmann, A., Carey, M., and Smale, S. T. (2009). A Unifying Model for the Selective Regulation of Inducible Transcription by CpG Islands and Nucleosome Remodeling. Cell *138*, 114–128.

Ramirez-Carrozzi, V. R., Nazarian, A. A., Li, C. C., Gore, S. L., Sridharan, R., Imbalzano, A. N., and Smale, S. T. (2006). Selective and antagonistic functions of SWI/SNF and Mi-2beta nucleosome remodeling complexes during an inflammatory response. Genes & Development *20*, 282–296.

la Sala, A., He, J., Laricchia-Robbio, L., Gorini, S., Iwasaki, A., Braun, M., Yap, G. S., Sher, A., Ozato, K., and Kelsall, B. (2009). Cholera toxin inhibits IL-12 production and CD8alpha+ dendritic cell differentiation by cAMP-mediated inhibition of IRF8 function. The Journal of experimental medicine *206*, 1227–1235.

Sanjabi, S., Hoffmann, A., Liou, H. C., Baltimore, D., and Smale, S. T. (2000). Selective requirement for c-Rel during IL-12 P40 gene induction in macrophages. Proceedings of the National Academy of Sciences of the United States of America *97*, 12705–12710.

Schlaak, J. F., Buslau, M., Jochum, W., Hermann, E., Girndt, M., Gallati, H., Meyer zum Büschenfelde, K. H., and Fleischer, B. (1994). T cells involved in psoriasis vulgaris belong to the Th1 subset. The Journal of investigative dermatology *102*, 145–149.

Schön, M. P., and Boehncke, W.-H. (2005). Psoriasis. The New England journal of medicine *352*, 1899–1912.

Silber, B. M. (2010). Driving drug discovery: the fundamental role of academic labs. Science translational medicine *2*, 30cm16.

Smith, A. M., Qualls, J. E., O'Brien, K., Balouzian, L., Johnson, P. F., Schultz-Cherry, S., Smale, S. T., and Murray, P. J. (2011). A distal enhancer in Il12b is the target of transcriptional repression by the STAT3 pathway and requires the basic leucine zipper (B-ZIP) protein NFIL3. The Journal of biological chemistry *286*, 23582–23590.

Thomas, N. (2010). High-content screening: a decade of evolution. Journal of biomolecular screening *15*, 1–9.

Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D. R., Pimentel, H., Salzberg, S. L., Rinn, J. L., and Pachter, L. (2012). Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nature Protocols *7*, 562–578.

Trapnell, C., Williams, B. A., Pertea, G., Mortazavi, A., Kwan, G., van Baren, M. J., Salzberg, S. L., Wold, B. J., and Pachter, L. (2010). Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nature biotechnology *28*, 511–515.

Trinchieri, G. (2003). Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nature reviews. Immunology *3*, 133–146.

Uematsu, S., and Akira, S. (2008). Toll-Like receptors (TLRs) and their ligands. Handbook of experimental pharmacology, 1–20.

Warming, S., Costantino, N., Court, D. L., Jenkins, N. A., and Copeland, N. G. (2005). Simple and highly efficient BAC recombineering using galK selection. Nucleic acids research *33*, e36.

Weinmann, A. S., Mitchell, D. M., Sanjabi, S., Bradley, M. N., Hoffmann, A., Liou, H. C., and Smale, S. T. (2001). Nucleosome remodeling at the IL-12 p40 promoter is a TLR-dependent, Rel-independent event. Nature immunology *2*, 51–57.

Xu, J., Pope, S. D., Jazirehi, A. R., Attema, J. L., Papathanasiou, P., Watts, J. A., Zaret, K. S., Weissman, I. L., and Smale, S. T. (2007). Pioneer factor interactions and unmethylated CpG dinucleotides mark silent tissue-specific enhancers in embryonic stem cells. Proceedings of the National Academy of Sciences of the United States of America *104*, 12377–12382.

Zanella, F., Lorens, J. B., and Link, W. (2010). High content screening: seeing is believing. Trends in biotechnology *28*, 237–245.

Zhang, J.-H. (1999). A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. Journal of Biomolecular Screening *4*, 67–73.

Zhou, L., Nazarian, A. A., Xu, J., Tantin, D., Corcoran, L. M., and Smale, S. T. (2007). An inducible enhancer required for Il12b promoter activity in an insulated chromatin environment. Molecular and cellular biology *27*, 2698–2712.

Zhou, L., Nazarian, A. A., and Smale, S. T. (2004). Interleukin-10 Inhibits Interleukin-12 p40 Gene Transcription by Targeting a Late Event in the Activation Pathway. Molecular and Cellular Biology *24*, 2385–2396.

Zhu, C., Rao, K., Xiong, H., Gagnidze, K., Li, F., Horvath, C., and Plevy, S. (2003). Activation of the murine interleukin-12 p40 promoter by functional interactions between NFAT and ICSBP. The Journal of biological chemistry *278*, 39372–39382.

Chapter 3

Future Directions and Concluding Remarks:

Small Molecule Screen and siRNA Screen Have Provided Numerous Avenues for Investigation Into Novel Anti-inflammatories

Abstract

We have reported a novel screening strategy in which modulation of a chromatinized reporter is directly compared to a minimal promoter reporter plasmid to identify selective modulators of *Il12b* transcription. Hits from this small molecule screen can be expected to inhibit expression of *Il12b* at many possible stages that lead to robust transcriptional activation. Combinatorial libraries of drug-like molecules have provided many validated hits, but determining the mode of action of these novel molecules provides a significant hurdle. Future directions for exploring the regulation of *Il12b* through the use of novel validated small molecule inhibitors includes the possibility of developing new therapeutics for inflammatory autoimmune diseases and reagents for studying inflammatory signaling. A conceptually much more direct screen is to use siRNA libraries, which should provide information on direct pathways and proteins that are required for efficient gene expression. An siRNA screen was performed on the IL12bBAC-EGFP and the minIL12b-DsRed containing dual reporter macrophage cell line. However, siRNA hit validation has proved to be an expensive and timely process, which is in need of more work. Future directions for exploring the regulation of *Il12b* transcriptional induction will include pursuing promising hits from the siRNA screen which remain to be validated.

Introduction

High-throughput small molecules screens can be a double edged sword. A well designed and controlled cell-based screen can sift through hundreds of thousands of small molecules looking for activity in an unbiased, brute force approach. The black box of cell-based screening can yield a number of lead hits with the desired phenotype. These hits will have been shown to be bioavailable and respond with the desired phenotype in the context of an entire cell. However, if the strongest hits come from combinatorial libraries of drug-like compounds, none of which have any known function, it is a very long road to trudge. It is estimated that for the pharmaceutical industry it takes 15 years from lead compound identification to market for the development of novel drugs (Silber, 2010). While an academic lab is not going to proceed in house with an entire drug development program, analysis of lead hits and deciphering even a partial mechanism of action can lead to a very fruitful tool for research and possible downstream collaborative efforts.

Cell-based screening does not end when you run out of new drug-like compounds to throw at the system. For the academic research program, RNAi has provided a new avenue of large scale pathway analysis. Gene silencing by RNA interference (RNAi) provides a genetic screening approach, in contrast to a chemical screen, which has recently been successfully adapted for large scale studies. If the screening system is robust for siRNA transfections or shRNA viral infections, then the technology of RNAi screening is an attractive pursuit. RNAi libraries have been developed by several companies or can be custom designed to cover small groups of targets up to the entire transcriptome. With these libraries, individual genes are targeted

for silencing, ideally removing one or two proteins (depending on the library layout) from the system per well. In theory, validated hits from this type of screen can provide a direct connection to the biological process resulting in the phenotype in question.

Development of RNAi libraries has matured as knowledge about the biological process has increased. Gene silencing by RNAi is a fundamental process in eukaryotic cells by which transcriptionally produced double stranded RNA is cleaved in the cytoplasm by the enzyme Dicer into smaller siRNA fragments (Whitehead et al., 2009). These 21-23 nucleotide long fragments are loaded into the RNA-induced silencing complex (RISC) where Ago2 unwinds the duplex, then cleaves and removes the sense strand, and activates RISC (Reynolds et al., 2004). Active RISC selectively seeks out and degrades mRNA that is complimentary to the antisense strand it contains. Chemically synthesized siRNAs can be delivered into the cell directly removing the need for Dicer in this pathway and bypassing detection by intrinsic factors which remove larger foreign RNA. The technology associated with siRNA delivery has advanced to a point where at least one approach will work for most cell types, however siRNA sequences that are both potent and specific have been a major obstacle.

Early on it was found that not all sequences were able to silence genes to the same efficiency. Several groups have put a substantial effort into identifying sequence parameters that promote potent gene silencing (Reynolds et al., 2004; Birmingham et al., 2006). Design algorithms have been constructed that synthesize this information providing a great tool for investigators. However, off-target effects remain one of the largest concerns for siRNA users. Off-targeting results in a modest inhibition of several to hundreds of unintended genes with as little as 15 base pairs of complementarity that

can induce a measurable phenotype (Birmingham et al., 2006). Many approaches and modifications to library design, screen design, and data analysis have been tested by vendors and researchers with the goal to minimize off-targetting bias. In library design, vendors have reduced off-targetting by (1) validating the individual siRNAs for efficiency while selecting against strong off-target effects, (2) modifying the sense strand to prevent the loading of this strand into the RISC complex, and (3) including more than one validated siRNA per target in the library. For screen design, primary screens are generally performed with replicates. This approach, in combination with libraries that contain more than one siRNA sequence per gene target, provides multiple scores on which to base hit calls. Finally methods for analysis have been developed to distil multiple scores per gene into a rational, statistically sound quantitation designed to minimize sensitivity to off-targetting and outliers (Chiang et al., 2007; Chung et al., 2008).

The main goal of this study was to identify specific modulators of *Il12b* transcription in macrophages. For this purpose both small molecule and RNAi high-throughput screens were performed. Importantly, a novel screening strategy was developed with the explicit purpose of enriching the pool of hits for treatments (small molecules or siRNAs) that selectively modulate the expression of a chromatinized *Il12b* reporter (Il12bBAC-EGFP), while having no effect on a chromatin free enhancer-promoter *Il12b* plasmid (minIl12b-DsRed). A thorough explanation of this approach can be found in the previous chapter.

For the small molecule screen, libraries that contained molecules with at least some known activities were investigated. However, the strongest and most selective

hits came from combinatorial libraries comprised of molecules with unknown properties. Data describing selectivity in the screen for a number of these hits as well as initial stages of validation for one of these hits are presented here. A second high-throughput screen was performed with an siRNA library. Analysis of the screen results with possible avenues to pursue are discussed.

Material and Methods

Small Molecule Screen

See detailed description in Chapter 2.

Small Molecule Validation

After the primary screen, a number of hits were selected for replication. New source plates were created and screening in the Il12bBAC-EGFP/minIl12b-DsRed line was performed as above. For serial dilutions, a source 384 well plate was created with three replicates of each small molecule at each concentration and a two fold dilution series was completed. The dilution source plates were screen in Il12bBAC-EGFP/minIl12b-DsRed line as in Chapter 2.

RT and quantitative PCR and ELISA

RNA was extracted using TRI-reagent (Molecular Research Center), treated with RNase-free DNaseI, and purified using an RNeasy kit (Qiagen). Quantified RNA (2 μg) was reverse-transcribed using Omniscript RT Kit (Qiagen) and random hexamer primers. cDNA fragments were analyzed by real-time PCR using

SensiMixPlus Sybr & Fluorescein (Bioline) and the 7900HT Fast Real-time PCR System (Applied Biosystems). The PCR amplification conditions were 95°C (3 min) and 45 cycles of 95°C (15 sec), 60°C (30 sec), and 72°C (30 sec). Primer pairs were designed to amplify 80–120 bp mRNA-specific fragments, and unique products were tested by melt-curve analysis. ELISA was performed according to the manufacture's protocols for IL12p40 and TNF-α on supernatants from treated bone-marrow derived macrophages (ELISA Ready-SET-Go!, eBioscience).

siRNA Screen

The MSSR (Molecular Screening Shared Resource) at UCLA maintains three Dharmacon siRNA libraries for mouse. These three libraries (Kinase, GPCR, and Druggable Genome) have four different siRNAs per target and are screened in a non-pooled format with two replicates. Libraries were pre-plated, 2 pMoles per well, in 384 well Cellstar optical glass bottom black sided tissue culture plates (Greiner Bio-One) and stored at -80 °C. The stable dual reporter cell line (Il12bBAC-EGFP/minIl12b-DsRed) was maintained in RPMI1640 without phenol red (Gibco), supplemented with HEPES, PenStrep, and L-glutamine (base media) supplemented with Geneticin (Invitrogen). Plates were removed from storage and allowed to thaw at room temperature. The transfection control, siRNA targeting EGFP (Thermo/Dharmacon), was plated with replicates on each plate in screen (2 pMoles). OptiMEM serum free media (Gibco) was added to plate (15 µL per well) using a Multidrop 384 (Thermo Labsystems) to resuspend siRNAs. Then Multidrop 384 was used to deliver 15 µL of 66.7 nL/µL DharmaFECT1 (Dharmacon) diluted in OptiMEM to each well (0.1 µL per

well). After 20 minutes of incubation at room temperature, dual reporter cells were plated (15 µL per well) using a Multidrop 384. These cells were previously washed with PBS, collected using TrypLE (Gibco), diluted to $1.2x10^6$ cells per mL in base media with 3x FBS. Plates were centrifuged at 300 rpm for 1 minute to mix wells and maintained in an incubator, 37°C 5% CO_2. After 48 hours, cells were stimulated with LPS (10 µL per well, final concentration 100 ng/mL) and incubated overnight. After 14 to 18 hours cells were stained with 25ul of RPMI media containing Hoechst 33342 dye (Invitrogen) at a concentration of 15 ng/ml to obtain a final concentration of 5ng/ml in each well. After a minimum of 30 minutes, images of plates were collected using Image-Xpress high content microscope (Molecular Devices) and analyzed with MetaXpress (Molecular Devices) software.

siRNA Screen Analysis

For each well of the screen, raw data included cell number and percent EGFP positive and DsRed positive cells. For score and p-value calculations redundant siRNA analysis (RSA) was performed (König et al., 2007). All p-value calculations shown in this chapter represent RSA approach using weighted and normalized score values without scaling of these scores. Scores and approaches used for this analysis but not shown here include RSA with scaling and use of the cellHTS2 package in R (Boutros et al., 2006).

Results

Novel Anti-Inflammatory Small Molecules

As with the previous analysis completed on screens conducted with small molecule libraries containing drugs with known activities, here MAD scores are shown for libraries containing combinatorial chemicals with no known activity (Figure 3-1A). A number of small molecules that had phenotypes that fell into the red box, Il12bBAC-EGFP specific inhibitor, or the blue box, non-specific inhibitors, were selected for high-throughput replication. Five of these molecules are shown (UK14, UK15, UK16, UK17, and UK20) (Figure 3-1B). Each of these compounds had a strongly selective phenotype upon replicate testing. Interestingly, UK17, which was not selective in the primary screen, was selective in the replicate screen.

Upon maintaining a selective phenotype, small molecules from these unknown libraries were tested further in the dual reporter cell line and primary bone marrow derived macrophages (BMDMs). A complete validation is presented for UK17 in Figure 3-2 and described below. For the remaining four small molecules presented here, each were able to inhibit *Il12b* transcription at early timepoints (data not shown) and IL12p40 accumulation in the supernatant at 18 hours (Figure 3-2C). The ability to inhibit IL12p40 is directly compared with the ability to inhibit TNF-α over a range of small molecule concentrations. While UK15 and UK20 are able to inhibit both cytokines similarly, there is a large concentration range where UK14 and UK16 inhibit IL12p40 without inhibiting TNF-α.

UK17 Shows Selective Inhibition in Dual Reporter and Primary Macrophages

Several small molecules that maintained a selective phenotype after high-throughput replication have undergone initial validation in the dual reporter line and primary macrophages. One of these studies is presented here. UK17 has a particularly selective inhibitory profile. In a high-throughput format, UK17 was serially diluted and over a large range of concentrations, was able to inhibit expression of the Il12bBAC-EGFP without inhibiting the expression of the minIl12b-DsRed reporter as measured by image analysis after 18 hours of stimulation (Figure 3-2A). In BMDMs, UK17 was investigated for the ability to inhibit LPS induced expression of IL12p40 and TNF-α over a range of concentrations (Figure 3-2B). At the highest concentration, 100 μM, both IL12p40 and TNF-α are strongly inhibited. However, at 20 μM, when IL12p40 remains undetectable in the supernatant, TNF-α is expressed similar to controls. Concentration dependent inhibition of IL12p40 is seen with IL12p40 recovery to 50% expression of untreated LPS stimulated macrophages at 0.8 μM.

Finally transcriptional inhibition of 47 LPS inducible genes by UK17 (20 μM) was measured in BMDMs at 120 minutes post stimulation (Figure 3-2C). The expression for each gene, as measured by RT-qPCR, in UK17 treated samples is normalized to the DMSO treated controls at 120 minutes of LPS stimulation. Values are given for the unstimulated samples and demonstrate the fold of induction for these genes. Genes are further classified by their dependence on new protein synthesis or SWI/SNF nucleosome remodeling for robust expression (Ramirez-Carrozzi et al., 2006).

siRNA Screen

The siRNA screen attempted to test the ability to modulate the expression of the reporters in the dual reporter macrophage cell line by silencing one target gene per well. The library consisted of 4 different siRNA sequences targeting 6363 genes. Unfortunately, 13 plates had to be removed from analysis due to faulty source plates. The raw data from the remaining 5180 target genes have been analyzed with a probability-based approach termed Redundant siRNA Analysis (RSA) that intends to limit the impact of off-target effects on score quantitation (König et al., 2007). P-values reveal that for both reporters, there are target genes that have significant inhibition phenotypes (Figure 3-3). About half of the siRNA targets that significantly inhibit the Il12bBAC-EGFP reporter also inhibit the minIl12b-DSred reporter and half are selective for the BAC.

Although a particular siRNA has the ability to inhibit reporter expression, this may be due to off-target effects if the gene is not expressed in macrophages. To verify that a particular gene target is expressed in macrophages, the RNA sequencing data set from Chapter 2 was consulted. Several non-selective hits from the siRNA screen target genes in the TLR signaling pathway (including TLR4), NF-κB pathway, AP-1 family, PI3K signaling, and protein translation initiation (Table 3-1). In Table 3-2, selective hits that seem the most promising to pursue for further validation have been listed. Included with the selective hits was *Smarc4a* (also known as *Brg1*), the ATPase subunit of the mammalian SWI/SNF nucleasome remodeling complex, a known protein required for nucleasome remodeling at the *Il12b* locus (Ramirez-Carrozzi et al., 2006).

Discussion

After screening more than 58,000 small molecules, all of which have no known biological activities, we have isolated approximately 20 that have our desired selective phenotype in the reporter cell line. This small set is comprised of structurally distinct small molecules and represents a substantial amount of future effort to decipher the mechanism of action for any one of them. Many of them have already been shown to inhibit *Il12b* transcription in primary macrophages, but the specificity of inhibition among a large number of proinflammatory genes varies and for most it is not clear. Similar to the previous study in which we identified the selectivity of β_2-agonists, high-throughput RNA sequencing is likely to play a central role in future studies.

The small molecule we have denoted as UK17 has a particularly interesting inhibitory profile. Although it was not a selective hit in the primary screen, subsequent experiments have revealed that it has little effect on the minimal DsRed reporter. Even more striking is the selectivity of inhibition of genes after two hours of LPS stimulation (Figure 3-2C). Very few genes reach the magnitude of inhibition that we see for *Il12b*. There appears to be a trend towards inhibition of remodeling dependent genes more than remodeling independent genes. We would hypothesize that the inhibition of *Il12b* is not occurring due to cAMP signaling because a number of hallmarks for this pathway, namely *Tnf* inhibition and *Il10* induction, are reversed. A number of detailed studies will need to be initiated for each of these small molecules, identifying which signaling cascades and cellular signals are being modified. The central role of IL12p40 in many models of inflammatory autoimmune disease also allows for many options in testing any of these novel small molecules in mouse models.

In addition to the large amount of work still required to take full advantage of the small molecule screen, the data from the siRNA screen is just a peek at the possible pathways to explore. A thorough analysis of the screen data has provided some interesting possibilities. A few hits are informative as to the quality of the screen. One expected non-selective hit ,*Tlr4,* was one of the strongest hits of the subset of targets that were highly expressed in macrophages. Other non-selective hits in the NF-κB pathway supports the hypothesis that expression of the minimal reporter is dominated by the activity of broad proinflammatory transcription factor families. For the selective hits, one hit that can be considered a proof of principle results was *Brg1.* This reveals that the chromatinized BAC is behaving like the endogenous *Il12b* gene, where expression is dependent on the activity of SWI/SNF nucleosome remodeling complex. Targeted silencing of *Brg1* with shRNA resulted in inhibition of endonuclease accessibility at the *Il12b* promoter and enhancer and inhibition of inducible expression (Ramirez-Carrozzi et al., 2006). These expected hits for both selective and non-selective inhibition of the reporters found in an unbiased way give us confidence for pursuing other significant hits.

A few identified hits represent promising selective pathways (Table 3-2). It has been demonstrated that *Il12b*, along with very few other genes, has a selective requirement for the NF-κB family member cRel (Sanjabi et al., 2000). In the field, no signal or pathway has been identified that specifically activates cRel containing dimers. A few selective hits have been connected to NF-κB and therefore should be validated for their role in *Il12b* transcriptional activation by cRel specific pathways: these include *Zmynd11* (also known as *Bs69* or *Bram1*), *Tnfrsf19*, and *Map3k7* (also known as *Tak1*).

The MAPK signaling pathway also plays a central role in innate immunity. In addition to possibly playing a selective role in NF-κB activation, it can be hypothesized that *Map3k7* and *Zmynd11*, along with *Ywhaz*, selectively control *Il12b* expression through a p38/MAPK pathway. Previous publications have demonstrated a connection of these two genes for each of these pathways and therefore, if validated, activity in both pathways will be considered for selective regulatory roles.

A second group of related selective hits fall into the BMP and TGFβ signaling pathway. This pathway includes the hits *Smad1*, *Smad4*, and *Smad9*. BMP and TGFβ signaling are important for a large number of cellular and developmental processes. Interestingly, *Map3k7*, another selective hit, can be recruited to the BMP receptors and contribute to signaling. Also SMAD1 and SMAD4 can bind DNA and recruit p300/CBP coactivators with intrinsic histone acetyltransferase activity, relaxing the chromatin at gene promoters and allowing for transcription initiation (EP300 is also a weak selective hit in the screen, data not shown). Therefore an interesting model for selective *Il12b* expression to test upon validating the SMAD proteins as hits could be that a signal, possibly through Map3k7, can result in SMAD1/SMAD4 recruitment of p300 to the *Il12b* promoter and/or enhancer.

The third group of targets that remain to be validated are expressed at high levels in macrophages and are interesting selective hits to test, despite not having several selective hits that could be directly linked to a common activity. As a stand alone hit, *Sp2* is a transcription factor that has a strong selective phenotype. SP transcription factor binding sites are ubiquitous in many gene families including house keeping genes and inflammatory genes (Philipsen and Suske, 1999; Bouwman and Philipsen, 2002).

Interestingly, *Sp2* is the most divergent member with the most divergent binding site and may play a different biological role then other SP family members. Similar to the dominant signaling pathways mentioned earlier, selective hits were found in the AKT pathway, with *Pdpk1* as a central kinase, and the ERK pathway, with *Spry2* as an inhibitory factor. It is difficult to imagine that *Pdpk1* will maintain a selective phenotype upon validation, however not much is known about *Spry2* and validation could lead to some interesting roles for ERK signaling in selective immune modulation. The lysine methytransferase *Smyd2* has a strong selective screen phenotype, however, like *Brg1*, this might just serve as another proof of principle hit. A chromatinized template would require proper histone methylation and modulation of this regulatory mechanism would only inhibit the BAC reporter, even if that factor regulated the methylation of many endogenous genes. Lastly, the receptor *Trpm2* is a Ca^{2+}-permeable cation channel that is activated by oxidative stress. Interestingly, the *Trpm2* knock-out mice has several inflammatory phenotypes which have informed a model where *Trpm2* controls ROS-induced signaling cascades and chemokine production (Yamamoto et al., 2008). Individually, each of these selective targets seems interesting to test and they may prove to be connected to other pathways above, however the first step is to validate hits in replicate experiments and primary cells.

Concluding Remarks

Over two decades of research by many accomplished scientists has centered on the regulation of *Il12b*. The importance of this protein in proper immune regulation, pathogen clearance, tumor surveillance, and in the pathogenesis of inflammatory auto-

immune pathologies is remarkable. The attention the scientific community pays to this cytokine has not diminished. It is therefore surprising that we as a community are still commenting that not enough is known about the regulation of *Il12b* transcriptional induction.

The studies presented here represent an unbiased, high-throughput approach to attempt to answer questions regarding the innate immune response and identify factors that lead to the regulation of *Il12b* transcription that are not shared by a large number of other proinflammatory genes. The screen methodology was informed by years of study of the transcriptional regulation of *Il12b* in the laboratory of Stephen Smale and others. It is known that in different physiological settings, selective regulation of a number of cytokines, including *Il12b*, is seen. It is therefore valid to expect that there are a number of signaling pathways that have not been thoroughly described responsible for these observations. Our novel screening strategy was developed to test the hypothesis that small molecules capable of modulating expression of a simple promoter reporter plasmid will generally target pathways that regulate proinflammatory gene expression in a non-selective manner, such as the NF-κB and AP-1 pathways; in contrast, small molecules that modulate *Il12b* transcription only in the context of native chromatin will target pathways involved in the selective regulation of *Il12b*. The screening approach described here has identified many small molecules that selectively inhibit the chromatinized reporter and therefore we feel it was validated as an effective HTS methodology.

The attempt to perform two screens during a doctoral training period was a highly ambitious undertaking. Extracting meaningful conclusions from these screen data

involved a sharp learning curve, but informative decisions were made. Identification of the selectivity of β_2-adrenergic agonists on the inhibition of *Il12b* in a *Nfil3* dependent manner represents one important finding resulting from the screen. We are confident that other lead small molecules and siRNA hits will result in the identification of additional selective transcriptional signals allowing significant contributions to the evolving model of innate immune regulation.

Figure Legends

Figure 3-1: Novel Anti-Inflammatory Small Molecules

(A) Significance of inhibition of the Il12bBAC-EGFP reporter (x-axis) against the minimal DsRed reporter (y-axis) for all wells in a subset of screen libraries determined by MAD score (mean absolute deviation). Libraries represented are ChemBridge Diver Set, Combichem Diverse Library, and Druggable Compound set (Table 2-1). Especially toxic compounds (those wells with < 300 cells) have been removed. Box and whisker plots on the axis summarize the sample data with controls removed. Controls that received DMSO and LPS are in yellow. Controls that are unstimulated or Bay11 treated are in blue. Phenotypic regions are in boxes and include Il12bBAC-EGFP specific hits (red) and non-specific hits (blue). Scores for five select hits are identified (see key). **(B)** Replication in a high-throughput format 5 select hits are represented. Controls from these plates are included for reference. All quantitation is expression normalized to the negative controls on the plate. **(C)** Expression of IL12p40 and TNF-α in BMDMs as measured by ELISA after 18 hours under the treatments as described. Serial dilution of each compound was performed and working concentrations are 100 μM, 20 μM, 4 μM, and 0.8 μM.

Figure 3-2: UK17 Shows Selective Inhibition in Dual Reporter and Primary Macrophages

(A) Serial dilution of UK17 in high-throughput format in cell line. Each treatment was replicated 9 times and values are reported as relative to in-plate DMSO and LPS

stimulated controls (n=16). Each dilution step was 2 fold with high concentration of 50 μM. LPS was used at 100 ng/mL. **(B)** Expression of IL12p40 and TNF-α in BMDMs as measured by ELISA after 18 hours under the treatments as described. Serial dilution UK17 was performed and working concentrations are 100 μM, 20 μM, 4 μM, and 0.8 μM. **(C)** BMDMs were stimulated with LPS (100 ng/mL) with or without one hour of pre-stimulation with UK17 (20 μM). Expression of 47 LPS inducible genes were monitored at 120 minutes post stimulation by RT-qPCR. mRNA levels are presented at a percentage of that obtained by the DMSO control at 120 minutes post LPS treatment (not shown). Unstimulated values are shown for reference to determine the magnitude of each genes induction in this experiment.

Figure 3-3: siRNA Screen Reporter Inhibition Significance per Target Gene

P-values for the significance of inhibition for each gene target based on two replicates of four different siRNAs were calculated using RSA (see Methods). The two p-values (Log_{10} transformed) for each gene target, specifically the significance of inhibition for EGFP (x-axis) and for DsRed (y-axis), are plotted. Summaries of the data for each reporter are provided by the box and whisker plot on the corresponding axis. Genes with notable functions have been colored orange for non-selective and green for selective, can be found in Table 3-1 and 3-2, and are discussed in the text.

Table 3-1: Non-selective Inhibitory siRNA Screen Hits

Non-selective targets, defined by inhibition of both the Il12bBAC-EGFP and the minIl12b-DsRed reporters, are shown. Gene ontology and descriptions provided by http://www.ncbi.nlm.nih.gov and pantherdb.org.

Table 3-2: Il12bBAC-EGFP Selective Inhibitory siRNA Screen Hits

Selective targets, defined by inhibition of the Il12bBAC-EGFP without significant inhibition of the minIl12b-DsRed reporters, are shown. Gene ontology and descriptions provided by http://www.ncbi.nlm.nih.gov and pantherdb.org.

Figure 3-1: Early Validation of Novel Ant-Inflammatory Molecules

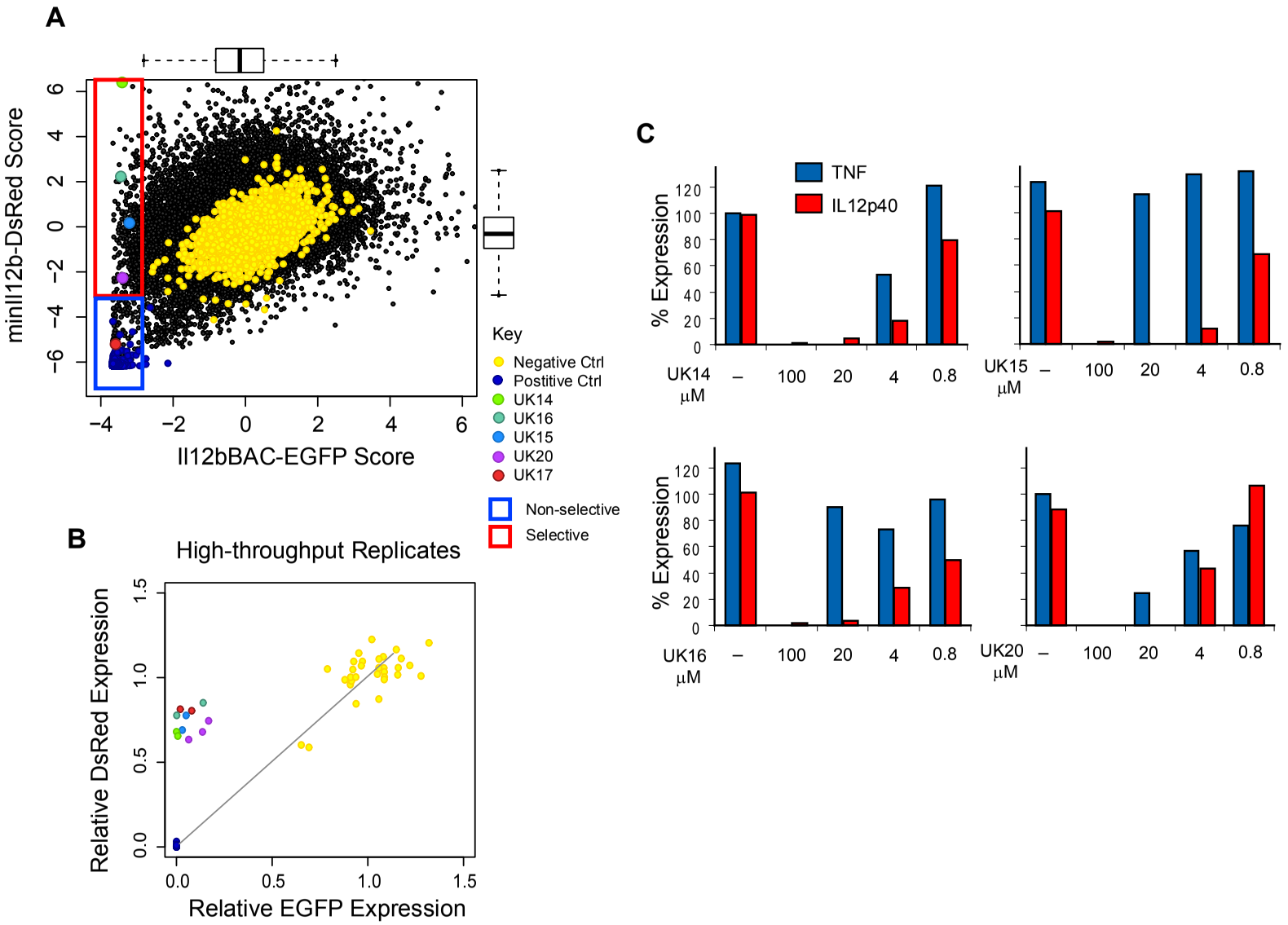


Figure 3-2: UK17 Shows Selective Inhibition in Dual Reporter Cell Line and Primary Macrophages

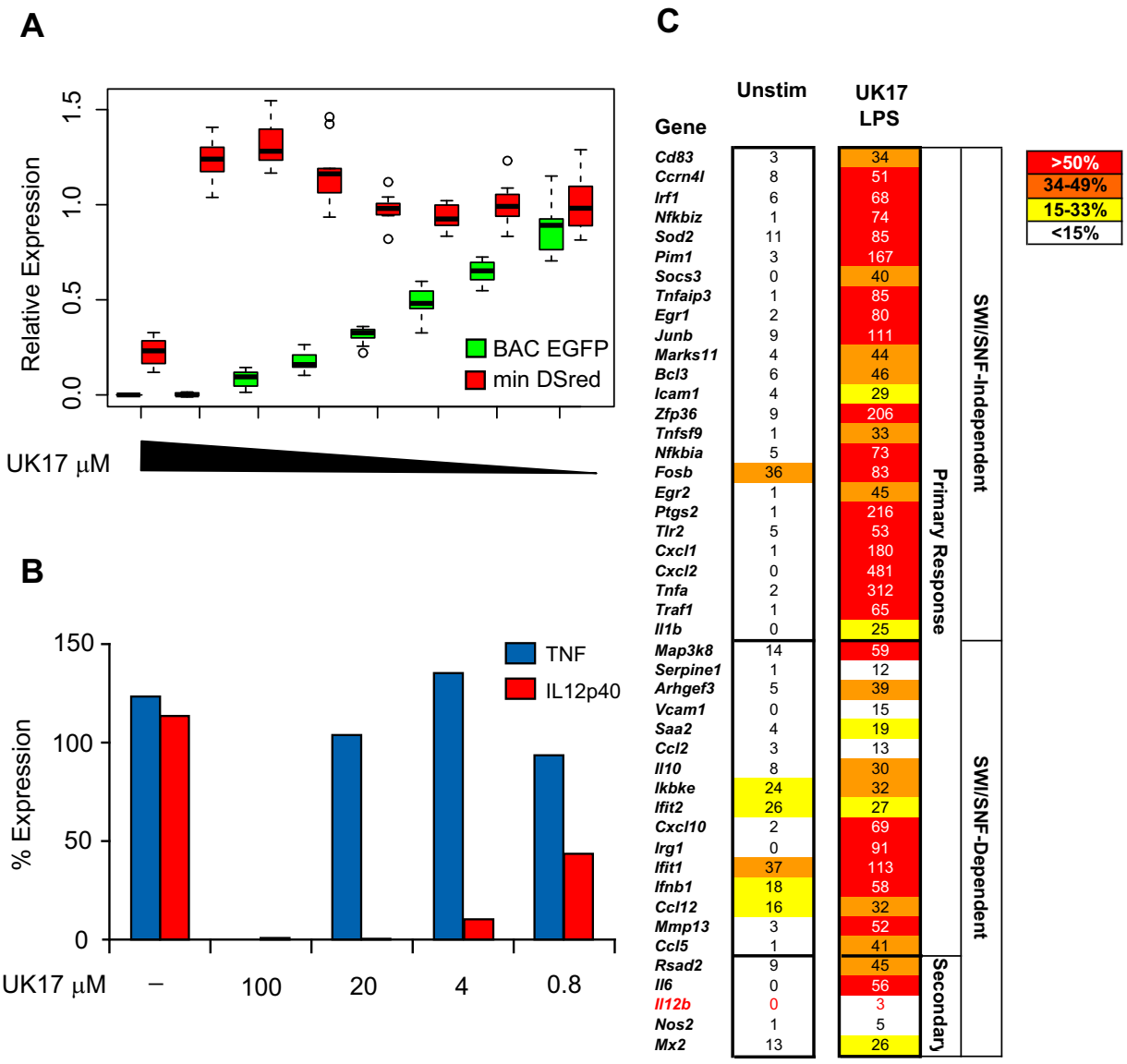


Figure 3-3: siRNA Screen Reporter Inhibtion Significance per Target Gene

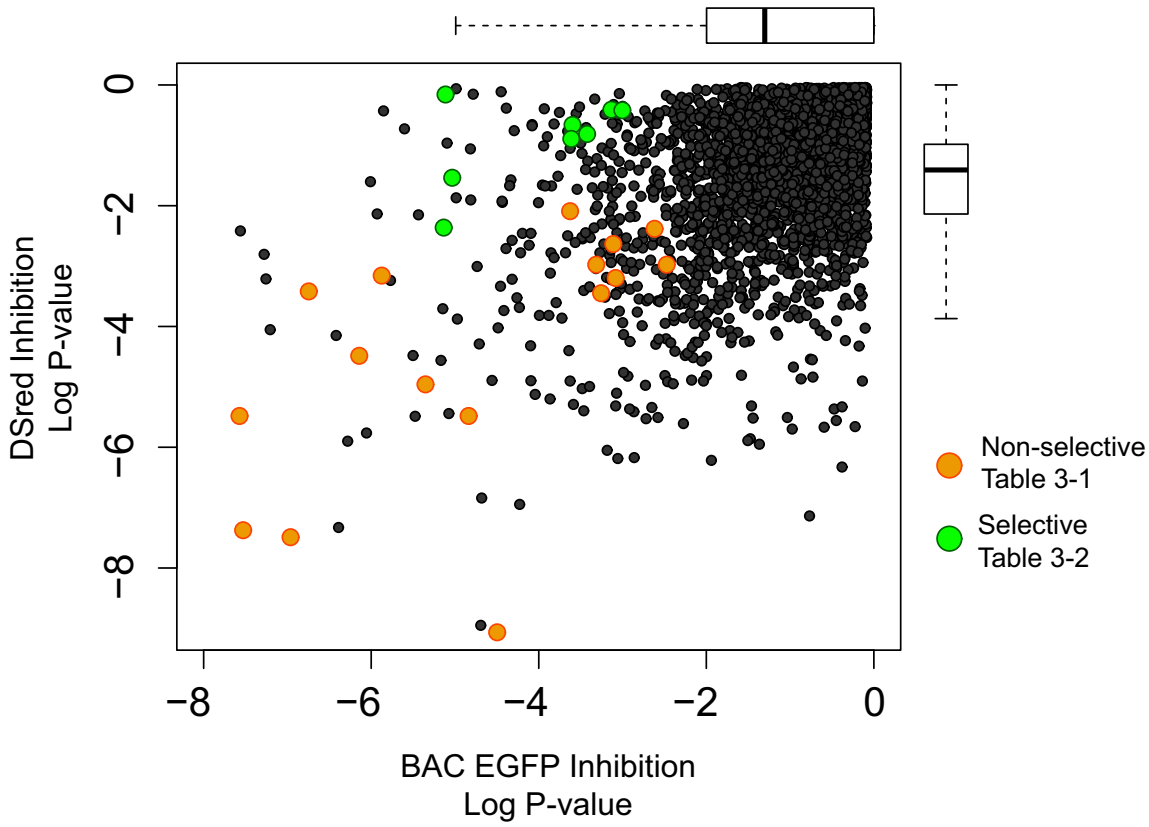


Table 3-1:

Non-selective siRNA Screen Hits

Pathway	siRNA Target	EGFP Inhibition Log Pval	Dsred Inhibition Log Pval	Description
TLR4	*Tlr4*	-4.84	-5.48	LPS Receptor
	Ripk1	-6.75	-3.42	Kinase, transduces signal from PRR to NF-κB pathway
NF-κB	*Psma1*	-7.57	-5.48	All PSM are subunits of the 26S proteasome. Proteasome activity is required for NF-κB activation.
	Psmb4	-6.97	-7.49	
	Psmd2	-6.15	-4.48	
	Psmd14	-5.87	-3.15	
	Psma2	-3.08	-3.20	
	Psmb6	-3.26	-3.45	
	Psmd8	-3.31	-2.98	
AP-1	*Atf4*	-2.61	-2.39	Transcription factor
PI3K	*Gab2*	-7.53	-7.37	Adapter protein downstream of several receptors
Translation	*Eif3j*	-5.35	-4.96	Components of the eukaryotic translation initiation factor complex are required for initiation of protein synthesis
	Eif3i	-3.11	-2.64	
	Eif3f	-4.49	-9.07	
	Eif3e	-3.63	-2.09	
	Eif3b	-2.48	-2.98	

Table 3-2:

BAC-EGFP Selective Hits

Class	siRNA Target	EGFP Inhibition Log Pval	Dsred Inhibition Log Pval	Pathways or Known Interactions	Description (alias)
Expected	*Smarca4*	-3.13	-0.41	SWI/SNF	(Brg1) ATPase subunit of SWI/SNF
NF-κB	*Zmynd11*	-6.01	-1.60	E1A & NCOR1	(BS69, BRAM1) transcriptional repressor
	Tnfrsf19	-3.00	-0.42	JNK & NF-κB	TNF-receptor superfamily
	Map3k7	-3.60	-0.66	NF-κB & p38/MAPK	(TAK1) Ser/Thr protein kinase, mediates TGFβ & BMP sig
MAPK	*Map3k7*	-3.60	-0.66	NF-κB & p38/MAPK	(TAK1) Ser/Thr protein kinase, mediates TGFβ & BMP sig
	Zmynd11	-6.01	-1.60	E1A & NCOR1	(BS69, BRAM1) transcriptional repressor
	Ywhaz	-3.61	-0.89	AKT & MAPK	(14-3-3ζ) adapter protein
BMP	*Smad1*	-4.88	1.00	BMP sig & CBP/P300	Dimerizes with SMAD4 to activate transcription
	Smad4	-5.13	-2.36	BMP sig & CBP/P300	Dimerizes with SMAD1 to activate transcription
	Smad9	-3.07	1.00	TGFβ signaling	
AKT	*Pdpk1*	-5.03	-1.53	AKT signaling	(PDK1) Ser/Thr protein kinase
	Ywhaz	-3.61	-0.89	AKT& MAPK sig	(14-3-3ζ) adapter protein
TF	*Sp2*	-5.12	-0.16		Most divergent of SP family of transcription factors
Histone Modifier	*Smyd2*	-4.06	1.00		Lysine methltransferase
Receptor	*Trpm2*	-2.95	-0.86		Ca-permeable cation channel, regulated by free intracellular ADP-ribose
ERK	*Spry2*	-3.42	-0.82	Inhibits ERK signaling	Negative regulators of RTKs, growth factors

Work Cited

Birmingham, A., Anderson, E. M., Reynolds, A., Ilsley-Tyree, D., Leake, D., Fedorov, Y., Baskerville, S., Maksimova, E., Robinson, K., Karpilow, J., et al. (2006). 3' UTR seed matches, but not overall identity, are associated with RNAi off-targets. Nature methods *3*, 199–204.

Boutros, M., Brás, L. P., and Huber, W. (2006). Analysis of cell-based RNAi screens. Genome biology *7*, R66.

Bouwman, P., and Philipsen, S. (2002). Regulation of the activity of Sp1-related transcription factors. Molecular and cellular endocrinology *195*, 27–38.

Chiang, C.-yuan, Tu, B. P., Ko, R., Yan, S. F., Dejesus, P. D., Romero, A., Bergauer, T., Orth, A., Krueger, U., Zhou, Y., et al. (2007). A probability-based approach for the analysis of large-scale RNAi screens. Nature Methods *4*, 847–849.

Chung, N., Zhang, X. D., Kreamer, A., Locco, L., Kuan, P.-F., Bartz, S., Linsley, P. S., Ferrer, M., and Strulovici, B. (2008). Median absolute deviation to improve hit selection for genome-scale RNAi screens. Journal of biomolecular screening *13*, 149–158.

König, R., Chiang, C.-yuan, Tu, B. P., Yan, S. F., DeJesus, P. D., Romero, A., Bergauer, T., Orth, A., Krueger, U., Zhou, Y., et al. (2007). A probability-based approach for the analysis of large-scale RNAi screens. Nature methods *4*, 847–849.

Philipsen, S., and Suske, G. (1999). A tale of three fingers: the family of mammalian Sp/XKLF transcription factors. Nucleic acids research *27*, 2991–3000.

Ramirez-Carrozzi, V. R., Nazarian, A. A., Li, C. C., Gore, S. L., Sridharan, R., Imbalzano, A. N., and Smale, S. T. (2006). Selective and antagonistic functions of SWI/SNF and Mi-2beta nucleosome remodeling complexes during an inflammatory response. Genes & Development *20*, 282–296.

Reynolds, A., Leake, D., Boese, Q., Scaringe, S., Marshall, W. S., and Khvorova, A. (2004). Rational siRNA design for RNA interference. Nature biotechnology *22*, 326–330.

Sanjabi, S., Hoffmann, A., Liou, H. C., Baltimore, D., and Smale, S. T. (2000). Selective requirement for c-Rel during IL-12 P40 gene induction in macrophages. Proceedings of the National Academy of Sciences of the United States of America *97*, 12705–12710.

Silber, B. M. (2010). Driving drug discovery: the fundamental role of academic labs. Science translational medicine *2*, 30cm16.

Whitehead, K. A., Langer, R., and Anderson, D. G. (2009). Knocking down barriers: advances in siRNA delivery. Nature reviews. Drug discovery *8*, 129–138.

Yamamoto, S., Shimizu, S., Kiyonaka, S., Takahashi, N., Wajima, T., Hara, Y., Negoro, T., Hiroi, T., Kiuchi, Y., Okada, T., et al. (2008). TRPM2-mediated Ca2+influx induces chemokine production in monocytes that aggravates inflammatory neutrophil infiltration. Nature medicine *14*, 738–747.

Appendix A

A Unifying Model for the Selective Regulation of Inducible Transcription by CpG Islands and Nucleosome Remodeling

Cell

A Unifying Model for the Selective Regulation of Inducible Transcription by CpG Islands and Nucleosome Remodeling

Vladimir R. Ramirez-Carrozzi,[1,2,5] Daniel Braas,[1,2,5] Dev M. Bhatt,[1,2,5] Christine S. Cheng,[4] Christine Hong,[1,2] Kevin R. Doty,[1,2] Joshua C. Black,[2,3] Alexander Hoffmann,[4] Michael Carey,[2,3] and Stephen T. Smale[1,2,*]
[1]Department of Microbiology, Immunology, and Molecular Genetics
[2]Molecular Biology Institute
[3]Department of Biological Chemistry
University of California, Los Angeles, CA 90095, USA
[4]Department of Chemistry and Biochemistry, University of California, San Diego, CA 92093, USA
[5]These authors contributed equally to this work
*Correspondence: smale@mednet.ucla.edu
DOI 10.1016/j.cell.2009.04.020

SUMMARY

We describe a broad mechanistic framework for the transcriptional induction of mammalian primary response genes by Toll-like receptors and other stimuli. One major class of primary response genes is characterized by CpG-island promoters, which facilitate promiscuous induction from constitutively active chromatin without a requirement for SWI/SNF nucleosome remodeling complexes. The low nucleosome occupancy at promoters in this class can be attributed to the assembly of CpG islands into unstable nucleosomes, which may lead to SWI/SNF independence. Another major class consists of non-CpG-island promoters that assemble into stable nucleosomes, resulting in SWI/SNF dependence and a requirement for transcription factors that promote selective nucleosome remodeling. Some stimuli, including serum and tumor necrosis factor-α, exhibit a strong bias toward activation of SWI/SNF-independent CpG-island genes. In contrast, interferon-β is strongly biased toward SWI/SNF-dependent non-CpG-island genes. By activating a diverse set of transcription factors, Toll-like receptors induce both classes and others for an optimal response to microbial pathogens.

INTRODUCTION

The availability of complete genome sequences for numerous species has enhanced interest in the organization and regulation of promoters, enhancers, and other DNA regions that control gene transcription in a physiological context. In mammals, promoters can be divided at their most basic level into the approximately 70% that contain CpG islands and the remaining 30% that lack CpG islands (Davuluri et al., 2001; Saxonov et al., 2006). CpG-island promoters are associated with most ''housekeeping'' genes and many regulated genes. Although CpG dinucleotides are substrates for DNA methyltransferases, most CpG islands are constitutively unmethylated in normal cells (Suzuki and Bird, 2008).

Another common property of promoters in mammals and other eukaryotes appears to be low nucleosome occupancy. In yeast, approximately 95% of promoters exhibit nucleosome deficits (Yuan et al., 2005; Mavrich et al., 2008b). Bioinformatic analyses suggest that reduced nucleosome stability due to a prevalence of rigid poly (dA:dT) sequences is responsible for this deficit (Iyer and Struhl, 1995; Anderson and Widom, 2001; Sekinger et al., 2005; Mavrich et al., 2008b), with regions flanking the promoters enriched in periodic AA/TT dinucleotides that favor stable nucleosome formation (Drew and Travers, 1985; Satchwell et al., 1986; Segal et al., 2006; Mavrich et al., 2008b). Yeast promoters that possess higher nucleosome occupancy are generally found in genes that exhibit greater plasticity of expression (Tirosh and Barkai, 2008; Mavrich et al., 2008b). Genome-wide studies have suggested that *Drosophila* and human promoters also exhibit reduced nucleosome occupancy (Heintzman et al., 2007; Ozsolak et al., 2007; Mavrich et al., 2008a; Schones et al., 2008), but the relevance of the nucleosome deficit in these organisms has not been examined.

A third common property of promoters is the preassociation of RNA polymerase II with inactive genes. Initial evidence of preassociation emerged from studies of *Drosophila* heat-shock promoters, the HIV-1 long-terminal repeat, and the c-Myc promoter (Gilmour and Lis, 1986; Kao et al., 1987; Krumm et al., 1992). More recent studies have suggested that polymerase molecules are associated with a high percentage of genes that are generally considered to be inactive (Guenther et al., 2007).

Although some inducible promoters are associated with RNA polymerase prior to activation, other inducible model promoters assemble into stable nucleosomes. For example, at the *S. cerevisiae* PHO5 promoter, activation requires remodeling of

promoter-associated nucleosomes by ATP-dependent remodeling complexes (Williams and Tyler, 2007; Boeger et al., 2008 and references therein). At the human *IFNB* promoter, the SWI/SNF remodeling complex catalyzes the sliding of a nucleosome spanning the TATA box and start site to a location further downstream, allowing preinitiation complex assembly and transcription (Agalioti et al., 2000). At the inducible *Il12b* promoter, SWI/SNF-dependent remodeling coincides with increased accessibility of the promoter DNA, although a positioned nucleosome at the promoter does not slide and does not appear to be evicted (Weinmann et al., 1999; Ramirez-Carrozzi et al., 2006).

Although studies of model genes have revealed diverse mechanisms by which inducible transcription can be regulated in a chromatin context, general principles have remained elusive. For example, it is not known why CpG islands are found at some regulated genes but more generally are associated with constitutively expressed genes. Moreover, the mechanistic and biological distinctions between inducible genes containing a preassociated polymerase and those assembled into stable nucleosomes prior to activation have not been established.

As an initial step toward an understanding of the diverse strategies used to regulate inducible transcription in mammalian cells, we previously used retroviral short-hairpin RNAs (shRNA) to simultaneously knock down expression of Brg1 and Brm, the catalytic subunits of mammalian SWI/SNF remodeling complexes (Ramirez-Carrozzi et al., 2006). Brg1/Brm knockdown in murine macrophages followed by stimulation with lipopolysaccharide (LPS) through Toll-like receptor 4 (TLR4) revealed that only a subset of TLR4-induced genes require SWI/SNF complexes for activation. Almost all secondary response genes (i.e., genes requiring new protein synthesis for activation) exhibited strong SWI/SNF dependence, whereas primary response genes (i.e., genes activated in the absence of new protein synthesis) could be divided into SWI/SNF-dependent and -independent classes. The promoters of representative SWI/SNF-independent genes exhibited constitutively high accessibility to nucleases, whereas SWI/SNF-dependent promoters exhibited inducible accessibility and inducible association of Brg1. However, in this initial analysis, we were unable to identify features of the promoters that could explain why a specific subset could be activated in a SWI/SNF-independent manner.

To better understand the distinctions between SWI/SNF-dependent and SWI/SNF-independent inducible genes, we used microarrays to identify and quantitative RT-PCR (qRT-PCR) to validate a much larger set of genes that are strongly induced by TLR4 in murine macrophages. We mainly focused on primary response genes because of the expectation that secondary response genes would be regulated by a more diverse array of mechanisms. By identifying and characterizing defining features of different promoter classes, we obtained insight into the functional and mechanistic distinctions between inducible CpG-island and non-CpG-island promoters, SWI/SNF-independent and SWI/SNF-dependent promoters, and promiscuous and tightly regulated inducible genes. The resulting model explains the variable properties of mammalian genes induced by a wide range of stimuli.

RESULTS

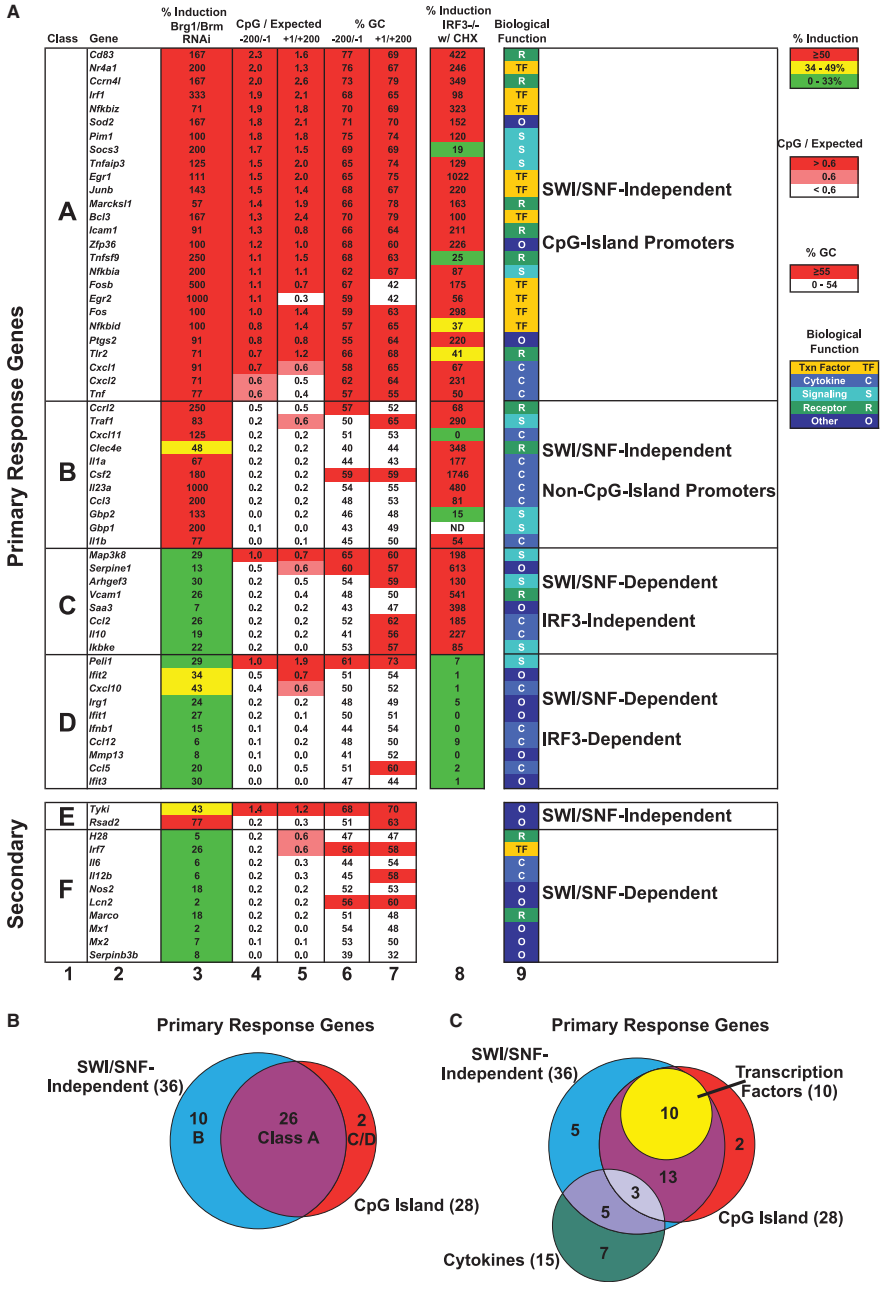
Prevalence of CpG-Island Promoters at SWI/SNF-Independent Primary Response Genes

To understand the distinctions between SWI/SNF-independent and -dependent genes, we used microarrays to expand our set of TLR4-induced genes, with an emphasis on primary response genes. Fifty-five primary response genes were validated using qRT-PCR with mRNA from mouse bone marrow-derived macrophages stimulated with LPS in the presence and absence of the protein synthesis inhibitor cycloheximide (CHX) (Figure 1A and data not shown). Twelve secondary response genes were also included in our analyses. qRT-PCR analyses of these 67 genes rather than microarrays were used for all subsequent expression studies.

The SWI/SNF dependence of each of the 67 genes was determined by simultaneous Brg1/Brm knockdown in LPS-stimulated J774 macrophages as previously described (Ramirez-Carrozzi et al., 2006), using retroviral delivery of an shRNA that targets a conserved region of the Brg1 and Brm mRNAs (see Figure S1 available online). qRT-PCR revealed that the effect of Brg1/Brm knockdown on mRNA levels was highly variable (Figure 1A, column 3). mRNA levels of 16 of the 55 primary response genes (29%) were reduced by at least 3-fold (Figure 1A, column 3, green). We refer to these genes as SWI/SNF-dependent. mRNA levels for 36 others (65%) were reduced by less than 2-fold or were increased relative to the control (Figure 1A, column 3, red). We refer to these genes as SWI/SNF-independent. The mRNA levels for the remaining three genes were reduced by more than 2-fold and less than 3-fold (Figure 1A, column 3, yellow). The moderate effects make these genes difficult to classify. Among the 12 secondary response genes, 10 were SWI/SNF dependent, one was SWI/SNF independent, and one was in the intermediate group (Figure 1A, classes E and F).

It is noteworthy that, in our previous study, SWI/SNF-independent genes were generally induced more rapidly than SWI/SNF-dependent genes (Ramirez-Carrozzi et al., 2006). A similar trend was observed with this larger set of genes (see Figure 5A), but we no longer include activation kinetics in our classification scheme because several exceptions were observed and because the precise activation kinetics for some genes varied from experiment to experiment.

The sequences of the SWI/SNF-dependent and -independent promoters were compared to identify distinguishing features. Remarkably, 26 of the 36 SWI/SNF-independent primary response genes (72%, including only the 36 primary response genes in red in Figure 1A, column 3) contain CpG islands between −1 and −200 relative to the major start site (Figure 1A, column 4; see also Figure 1B). In contrast, CpG islands were observed in only 2 of the 16 (12.5%) SWI/SNF-dependent primary response genes. Figure 1A, columns 3 and 4 show the CpG content for the regions from −200 to −1 and from +1 to +200 (relative to the major start site reported in the DBTSS database). CpG content is indicated as the ratio of observed CpGs to the CpGs expected if this dinucleotide were randomly represented in the genome. Because CpG dinucleotides have been depleted from mammalian genomes, this ratio is generally low (0.1–0.2). CpG islands have been defined as regions containing



Cell *138*, 114–128, July 10, 2009 ©2009 Elsevier Inc.

ratios greater than or equal to 0.55, 0.60, or 0.65 (Gardiner-Garden and Frommer, 1987; Davuluri et al., 2001; Takai and Jones, 2002; Saxonov et al., 2006). For this analysis, we used the intermediate value. The overall percentage of GC bps is also shown in Figure 1A (columns 6 and 7).

Because CpG-island promoters are often found in housekeeping genes, we asked whether TLR4-induced genes containing CpG islands might be transcribed at a higher level than non-CpG-island genes in unstimulated cells. Precursor transcript levels for 30 genes were monitored by qRT-PCR in unstimulated and LPS-stimulated bone marrow-derived macrophages, using primer pairs in which one primer annealed to exonic sequences and the other to an intronic sequence. Precursor transcript levels are thought to reflect transcription rates more accurately than mRNA levels. After normalization of the RT-PCR efficiency for each gene using genomic DNA, a wide range of precursor transcript levels was observed in two independent experiments in unstimulated macrophages, with transcript levels spanning approximately four orders of magnitude (Figure S2). The number of primary transcripts was, on average, slightly higher for CpG-island genes than for non-CpG-island genes, raising the possibility that the higher basal transcription levels may contribute to the SWI/SNF-independent induction of CpG-island genes or, alternatively, may be a consequence of their capacity for SWI/SNF-independent induction. However, no consistent trend was observed, as some SWI/SNF-dependent non-CpG-island genes exhibited basal transcript levels comparable to those observed at SWI/SNF-independent CpG-island genes. Importantly, precursor transcript levels increased two orders of magnitude or more upon LPS stimulation for most of the genes in both classes, with no consistent differences between the two classes (Figure S2). Thus, the existence of basal transcripts and basal transcript levels cannot explain the distinction between SWI/SNF independence and dependence.

Assembly of CpG-Island Promoters into Constitutively Active Chromatin

To understand why TLR4 target genes containing CpG islands are almost always SWI/SNF independent, chromatin immunoprecipitation (ChIP) was used to analyze chromatin at TLR4 target genes in unstimulated bone marrow-derived macrophages. To compare ChIP signals at the various promoters, primer amplification efficiencies were normalized using genomic DNA. Two housekeeping genes, *Actb* and *Gapd*, were included for the purpose of comparison. When examining total histone H3 levels, a significant but imperfect trend toward lower histone occupancy at CpG-island promoters was observed (Figure 2, top; $p < 0.002$). Despite the reduced histone H3 levels at a large fraction of CpG-island promoters, a striking trend toward higher histone H3K9/K14 acetylation and H3K4 trimethylation levels was observed at these promoters (Figure 2). Thus, inducible CpG-island promoters appear to be assembled into chromatin containing modifications characteristic of active genes.

Most CpG-island promoters also exhibited higher levels of RNA polymerase II and TATA-binding protein (TBP) in unstimulated macrophages. Although association of RNA polymerase II with the inducible promoters in unstimulated cells is consistent with the existence of basal transcripts, after LPS stimulation, RNA polymerase II levels did not increase or increased to only a modest extent at several of the CpG-island promoters (Figure S3), despite increases in precursor transcript levels often exceeding 100-fold. These properties are reminiscent of those observed at *Drosophila* heat-shock promoters (Gilmour and Lis, 1986). It is important to emphasize, however, that the existence of significant basal transcription suggests that polymerases at the CpG-island promoters are not retained in the rigidly poised, preinitiated state observed at *Drosophila* heat-shock promoters. Nevertheless, our findings suggest that LPS induction leads to greatly enhanced initiation and/or elongation by polymerase molecules that can readily associate with many of the CpG-island promoters in unstimulated cells. Further studies are needed to determine the precise mechanisms by which initiation and elongation are regulated at these genes.

The trend toward lower histone H3 levels at CpG-island promoters is interesting to consider in light of previous genome-wide studies that suggested that low nucleosome occupancy characterizes active and sometimes inactive promoters in mammalian cells (see Introduction). To determine whether similarly low histone H3 levels are found at both CpG-island and non-CpG-island promoters when they are active, ChIP experiments were performed with macrophages after LPS stimulation for 30 or 120 min. Significant decreases in ChIP signals were observed at some genes after stimulation, but histone H3 levels at several of the non-CpG-island promoters remained high (Figure S4). This finding is consistent with our previous evidence that a positioned nucleosome at the non-CpG-island *Il12b* promoter becomes more accessible to nuclease cleavage, but is not evicted, upon transcriptional activation (Weinmann et al., 1999; Ramirez-Carrozzi et al., 2006). The results suggest that reduced nucleosome occupancy may primarily characterize CpG-island promoters and a limited subset of active

Figure 1. Classification of LPS-Induced Primary and Secondary Response Genes

(A) Sixty seven genes that are potently induced by LPS in mouse bone marrow derived macrophages are shown. Classes A D are primary response genes (resistant to CHX) and classes E and F are secondary response genes (sensitive to CHX). Column 3 shows the effect of Brg1/Brm knockdown on LPS induced mRNA levels as a percentage of the mRNA level observed in control cells (set at 100% for each gene), as determined by qRT PCR. Column 8 shows mRNA levels in IRF3$^{-/-}$ macrophages stimulated with LPS in the presence of CHX as a percentage of mRNA levels in LPS stimulated wild type C57BL/6 macrophages, as determined by qRT PCR. In columns 3 and 8, percentages represent the average of three independent experiments. Columns 4 and 5 show the ratio of the number of observed CpGs to the number expected if CpGs were randomly distributed, for the regions from −200 to −1 (column 4) and +1 to +200 (column 5) relative to the start site indicated in the DBTSS database. Columns 6 and 7 show percentages of GC bps in these same regions. Column 9 shows the established or predicted functions of the 67 genes. Color coded legends for columns 3 through 9 are shown at the right.

(B) A Venn diagram shows that 26 of 28 primary response genes containing CpG island promoters are induced in a SWI/SNF independent manner.

(C) A Venn diagram shows that all 10 primary response genes encoding transcription factors are contained within class A, whereas only 3 of 15 cytokine genes are found in this class.

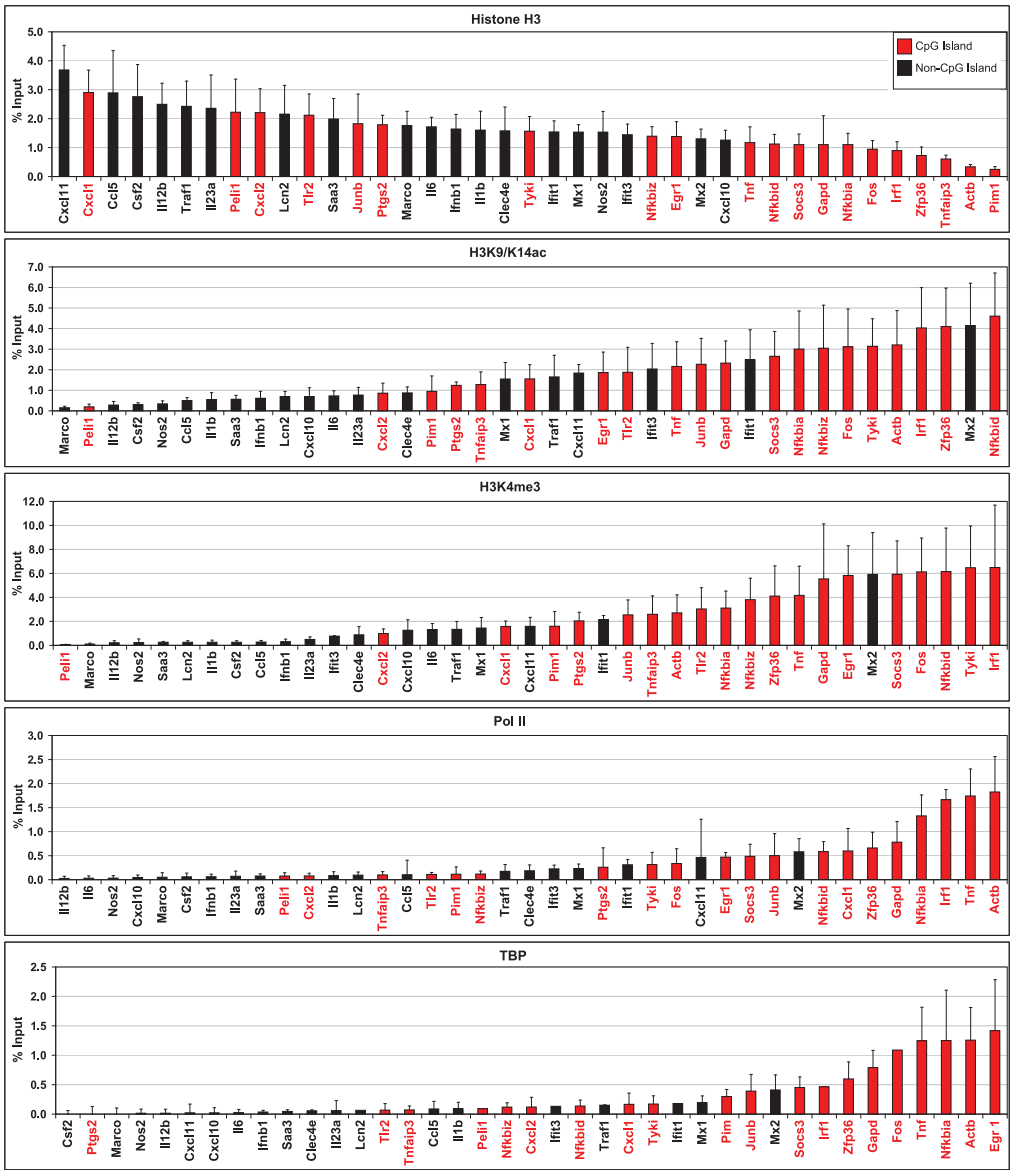


Figure 2. Constitutively Active Chromatin Is Preferentially Found at LPS-Induced CpG-Island Promoters

ChIP was used to monitor chromatin structure at 37 LPS induced genes and 2 housekeeping genes (*Gapd* and *Actb*) in unstimulated bone marrow derived macrophages. Genes containing CpG island and non CpG island promoters are in red and black, respectively. Antibodies against unmodified histone H3, H3K9/K14ac, H3K4me3, RNA polymerase II, and TBP were examined. PCR primer pairs were normalized using genomic DNA. Normalized results are shown as a percentage of input values. Higher values were obtained with the modified histone antibodies than with the unmodified histone antibodies due to different

non-CpG-island promoters from which nucleosomes have been evicted. Furthermore, the continuum of histone H3 levels observed in our analysis (Figure 2) suggests that CpG-island promoters possess nucleosome densities that are reduced to variable degrees.

Strong Constitutive DNase I Hypersensitivity at Inducible CpG-Island Promoters in Human CD4$^+$ T Cells

To understand why CpG-island promoters often exhibit lower histone H3 levels than non-CpG-island promoters, we first hypothesized that the binding of a specific transcription factor, such as Sp1, is responsible for nucleosome loss. Indeed, constitutive Sp1 binding is detectable at many of the class A promoters in ChIP experiments (data not shown; Hargreaves et al., 2009 [this issue of *Cell*]). However, consensus Sp1 sites are also found in some of the non-CpG-island promoters that exhibit high nucleosome occupancy (data not shown). This observation led us to consider the possibility that the full CpG-island sequence, rather than isolated transcription factor-binding sites, might be responsible for the low nucleosome occupancy, analogous to the role of poly (dA:dT) tracts at yeast promoters (see Introduction). Initial support for this hypothesis was provided by previous studies that defined sequences that favor or disfavor nucleosome assembly (e.g., Drew and Travers, 1985; Satchwell et al., 1986; Segal et al., 2006; Mavrich et al., 2008b). In fact, using the computational tools of Segal et al. (2006), virtually all CpG islands in our promoter set are predicted to be devoid of stable nucleosomes (data not shown). Although CpG islands contain the GC-rich sequences whose minor grooves are often located at the exposed surfaces of stable nucleosomes, the periodic AA/TT dinucleotides that favor DNA bending and stable nucleosome assembly are usually absent.

Although the rules defined by Segal et al. (2006) predict that CpG-island promoters are incompatible with stable nucleosome assembly, the ChIP results (Figure 2) suggest that nucleosome occupancy is variable, despite a significant trend toward low occupancy at CpG-island promoters. One possibility is that nucleosome instability does not always lead to a nucleosome deficit. As an independent strategy for comparing the physical state of nucleosomes at inducible CpG-island versus non-CpG-island promoters in vivo, we examined published data that identified DNase I hypersensitive sites at a genome-wide level in quiescent human CD4$^+$ T cells (Boyle et al., 2008).

Strikingly, the human homologs of 18 of our 26 (69%) class A genes exhibited high hypersensitivity scores in resting T cells, whereas only 3 of the 35 (9%) non-CpG-island genes in the other classes exhibited comparable hypersensitivity scores (Figure S5). Furthermore, none of the 7 most strongly induced non-CpG-island genes in T cells exhibited high hypersensitivity scores, and only 2 of these 7 genes exhibited detectable hypersensitivity (Figure S5). Published expression profiles from human CD4$^+$ T cells revealed that at least 9 of the class A genes are induced in CD4$^+$ T cells by CD3 and CD28 antibodies (Figure S5); this number almost certainly represents an underestimate because induction was monitored only at relatively late time points. In sum, these results, obtained with a different cell type and using a different assay, provide further evidence that nucleosomes associated with inducible CpG-island promoters are structurally different than nucleosomes associated with non-CpG-island promoters in unstimulated cells.

Reduced Assembly of CpG-Island Promoters into Nucleosomes In Vitro

Although the above results suggest that nucleosomes at CpG-island promoters may be unstable, perhaps contributing to their SWI/SNF-independent activation, in vivo studies cannot distinguish between intrinsic instability due to nucleotide content and reduced nucleosome occupancy due to the activities of constitutively associated transcription factors. Therefore, we compared intrinsic nucleosome stabilities at CpG-island and non-CpG-island promoters using an in vitro nucleosome assembly/solution competition assay that makes use of purified recombinant histone octamers from *Xenopus laevis* (Figure 3A). Pools of 300 bp DNA fragments spanning 27 CpG-island and non-CpG-island promoters were mixed and assembled into nucleosomes using limiting concentrations of recombinant histone octamers. High-affinity promoters were isolated from the nucleosomal band obtained with reactions in which 10% of the promoter fragments were assembled; low-affinity fragments were isolated from the "free" band obtained in reactions in which 80%–90% of the fragments were assembled (Figure 3A). The fragments were PCR amplified using common primers and were again subjected to nucleosome assembly and EMSA. After each round of assembly, EMSA, and fragment elution, the fraction of each DNA fragment present in the assembled and free DNA pools was quantified by qPCR.

After four rounds of selection, a clear difference in the competition for nucleosome assembly was observed, with non-CpG-island sequences competing much more successfully than CpG-island sequences (Figure 3B). It is important to note that a DNA sequence referred to as 601 was used as a control in this experiment. This sequence was previously selected on the basis of its ability to assemble into unusually stable nucleosomes (Lowary and Widom, 1998). Consistent with the previous data, the 601 sequence exhibited greater enrichment in the nucleosomal fraction than any of the native promoters. Interestingly, the 601 sequence conforms to the definition of a CpG island. However, unlike the native CpG-island promoters, it contains properly phased AT-bps to promote the assembly of stable nucleosomes (Lowary and Widom, 1998).

These results provide strong support for a model in which the reduced nucleosome occupancy and enhanced accessibility observed at CpG-island promoters in vivo are largely due to the reduced stability of nucleosomes at these promoters, as a direct result of their nucleotide content. We hypothesize that the reduced nucleosome stability is responsible, at least in part, for the

antibody qualities. The results are averages of three independent experiments performed with independent chromatin preparations, with standard deviations shown as error bars. p values for the differences between CpG island and non CpG island promoters were as follows: histone H3, $p < 0.002$; H3K9/14ac, $p < 0.001$; H3K4me3, $p < 0.00004$; RNA polymerase II, $p < 0.002$; and TBP, $p < 0.001$.

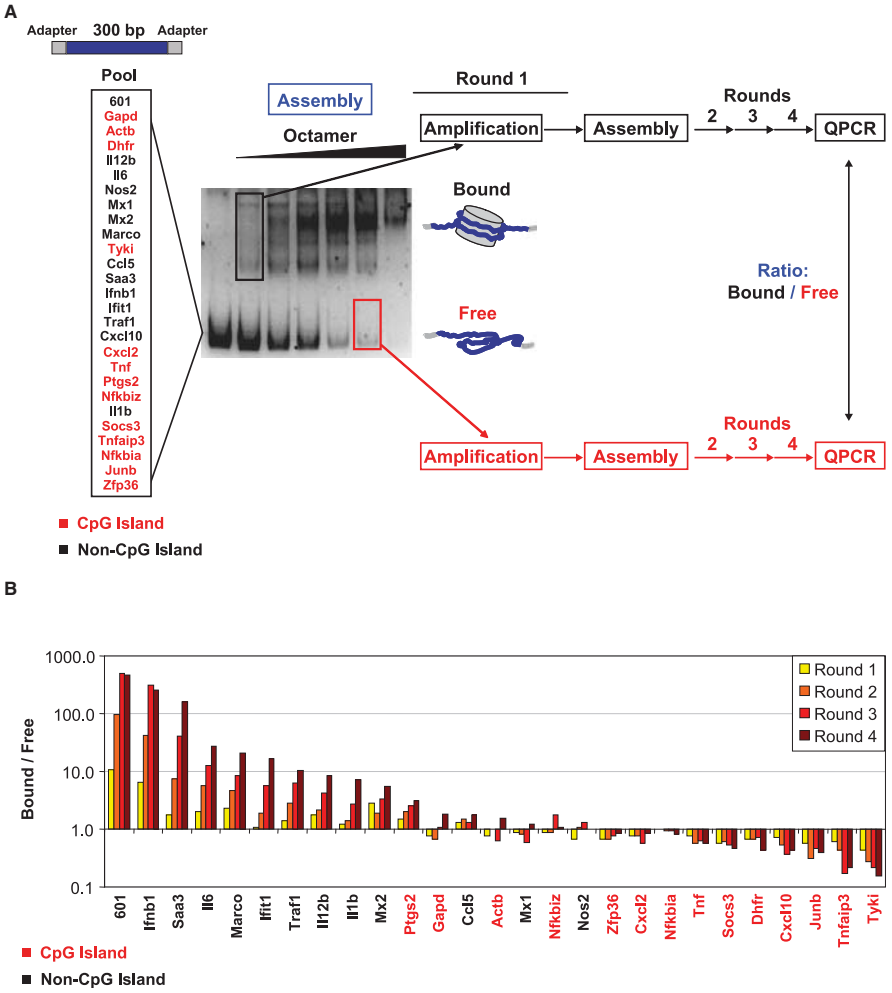


Figure 3. CpG-Island Promoters Compete Less Effectively than Non-CpG-Island Promoters for Nucleosome Assembly In Vitro

(A) A sequential assembly and amplification assay was used to compare the stabilities of nucleosomes assembled on CpG island and non CpG island promoters. 300 bp DNA fragments were pooled from 23 LPS induced promoters, 3 housekeeping promoters (*Gapd*, *Actb*, and *Dhfr*), and a synthetic DNA fragment previously shown to assemble into unusually stable nucleosomes (601; Lowary and Widom, 1998). After assembly into nucleosomes with recombinant histones and separation of nucleosomal fragments from free fragments by gel shift, the nucleosomal and free fragments were isolated. A portion of each resulting pool was reassembled, with another portion used for qPCR to determine the relative amount of each DNA fragment in each pool. Four rounds of assembly, elution, and amplification were performed.

(B) The ratio of each promoter fragment found in the nucleosomal (bound) band to the free band in the gel shift experiments after each assembly and elution cycle is shown. CpG island promoters are in red and non CpG island promoters in black. The *Cxcl10* fragment used for this analysis is depicted as a CpG island, although the *Cxcl10* promoter from −1 to −200 contains an observed:expected CpG ratio of only 0.4 (Figure 1). The reason for this difference is that the 300 bp fragment used for in vitro assembly extends into the CpG rich transcribed region (−161/+139) and, with the adaptor, possesses a CpG ratio of 0.7. The p value for the difference between CpG island and non CpG island promoters is $p < 0.01$.

SWI/SNF-independent activation of these genes. Importantly, this hypothesis is consistent with well-established evidence that nucleosome destabilization in *S. cerevisiae* Sin mutants can result in SWI/SNF-independent activation of genes that normally are SWI/SNF dependent (Muthurajan et al., 2004 and references therein).

It is important to note that, although assembly into unstable nucleosomes may play a major role in the reduced nucleosome occupancy, constitutive DNase I hypersensitivity, and SWI/SNF independence of inducible CpG-island promoters, instrinsic nucleosome instability is unlikely to be sufficient for constitutive histone acetylation and H3K4 trimethylation at these promoters. Most likely, the active chromatin state that characterizes CpG-island promoters benefits from both intrinsic nucleosome instability and the preassociation of transcription factors like Sp1.

Class B Promoters Exhibit SWI/SNF Independence without a CpG Island

Although 26 of 36 LPS-induced, SWI/SNF-independent primary response genes contain CpG-island promoters (Figure 1A, class A), the remaining 10 do not have a high CpG content between −1 and −200. These SWI/SNF-independent, non-CpG-island genes were placed in class B, along with a gene with an ambiguous SWI/SNF dependence (Figure 1A). ChIP data for four class B genes (*Traf1*, *Csf2*, *Il23a*, and *Il1b*) are included in Figure 2, revealing an absence of constitutively active chromatin. Furthermore, stable nucleosomes readily assembled in vitro at the two class B promoters examined (Figure 3; *Il1b* and *Traf1*). This finding is consistent with the prediction that stable nucleosomes can readily assemble on all class B promoters using the computation tools of Segal et al. (2006). Thus, the reason class B genes are activated in a SWI/SNF-independent manner will require further investigation (see Discussion).

Most Primary Response Genes that Require IRF3 for Activation are SWI/SNF Dependent

Although most LPS-induced primary response genes were SWI/SNF independent, 29% (16 of 55) exhibited substantial SWI/SNF dependence, with all but 2 of these genes lacking CpG-island promoters. Notably, several of these genes are known to require interferon regulatory factor 3 (IRF3) for activation in LPS-stimulated macrophages (Doyle et al., 2002). IRF3 activity is induced by a select subset of TLRs, including TLR4, in contrast to NF-κB and AP-1, whose activities are induced by all TLRs (Kawai and Akira, 2007). An analysis of mRNA levels of all 67 genes in LPS-stimulated macrophages from IRF3$^{-/-}$ mice (in the presence of CHX to eliminate redundancy due to factors like IRF7 that are newly synthesized in response to LPS) revealed strong IRF3-dependent expression of 50% (8 of 16) of the SWI/SNF-dependent primary response genes (Figure 1A, column 8). These genes were placed in class D, along with two additional genes (*Ifit2* and *Cxcl10*) that exhibited intermediate SWI/SNF dependence (Figure 1A). Importantly, mRNA levels for only 4 of the 36 SWI/SNF-independent primary response genes were reduced by more than 3-fold in IRF3$^{-/-}$ macrophages, and 3 of these 4 genes remained strongly induced (Figure 1A and data not shown). Thus, genes that are dependent on IRF3 activity for expression in LPS-stimulated macrophages are generally SWI/SNF dependent.

The strong IRF3 dependence in the presence of CHX suggests that the class D genes are direct targets of IRF3. Consistent with this hypothesis, consensus IRF3-binding sites were readily observed in 6 of the 10 class D promoters but in only 6 of the 57 promoters in the remaining classes (Figure S6). In addition, ChIP experiments confirmed that IRF3 can directly associate with the promoters of representative class D genes (Figure S6).

Biological Classification of SWI/SNF-Dependent and -Independent Genes

The finding that IRF3-dependent primary response genes generally contain non-CpG-island promoters and are SWI/SNF dependent suggests that these promoter properties are primarily used to restrict transcriptional activation of genes that require tight regulation. In contrast, genes that are induced by a wide range of stimuli may be more compatible with CpG-island promoters and SWI/SNF independence.

An examination of the biological functions of our set of LPS-induced genes provides additional support for this model. All 10 genes that encode transcriptional regulators among the 55 primary response genes are found within class A (Figure 1A, column 9; Figure 1C). Most of these transcription-factor genes, including *Egr1*, *Egr2*, *Junb*, *Fos*, *Fosb*, and *Bcl3*, are known to be induced by diverse stimuli (Herschman, 1991). In contrast, only 3 of the 15 genes encoding cytokines, which are induced more selectively, are found in class A (Figures 1A and 1C). These findings suggest that CpG-island SWI/SNF-independent promoters are often associated with promiscuous activation, and that non-CpG-island SWI/SNF-dependent promoters correlate with selective activation. It is noteworthy that class B consists primarily of cytokine genes that require selective regulation, despite the SWI/SNF independence of this class.

IRF3 Is Required for Nucleosome Remodeling at IRF3-Dependent Genes

To explore the relationship between SWI/SNF dependence and IRF3, a restriction enzyme accessibility/Southern blot assay was used to monitor nucleosome remodeling at two IRF3-dependent genes, *Ccl5* and *Ifit1*. Like the mRNA analysis (Figures 1A and 4A), this analysis was performed in cells stimulated with LPS in the presence of CHX, which eliminates the secondary activation of the interferon pathway that partially compensates for the loss of IRF3. In wild-type macrophages, a strong increase in restriction enzyme cleavage was observed in stimulated cells at both the *Ccl5* and *Ifit1* promoters (Figures 4B and 4C, lanes 1 and 2). This inducible cleavage was greatly reduced in IRF3$^{-/-}$ macrophages (Figures 4B and 4C, lanes 3 and 4). The strong dependence of nuclease accessibility on IRF3 supports the notion that the assembly of these promoters into stable nucleosomes confers a requirement for remodeling by SWI/SNF complexes, with remodeling dependent on a specialized TLR4-activated factor, IRF3.

A fourth class of primary response genes, class C, includes SWI/SNF-dependent genes that do not require IRF3 for expression (Figure 1A). We hypothesize that one or more specialized LPS-induced transcription factors other than IRF3 promote nucleosome remodeling at promoters within this class, contributing to their selective activation.

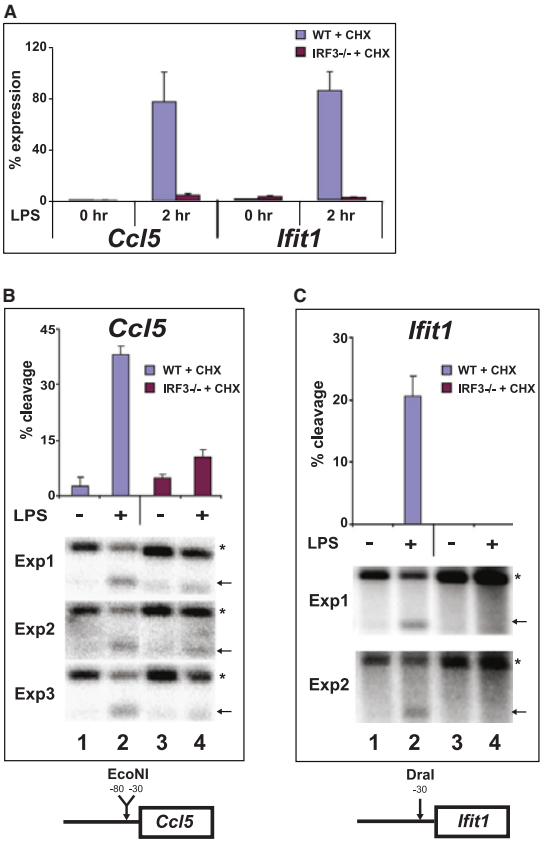


Figure 4. IRF3 Is Required for Nucleosome Remodeling at Class D Promoters

(A) Macrophages from C57BL/6 mice and IRF3$^{-/-}$ mice were stimulated with LPS in the presence of CHX. mRNA levels for the *Ccl5* and *Ifit1* genes were strongly reduced in the IRF3$^{-/-}$ cells. Bar graph shows averages of three independent experiments with standard deviations.

(B) Restriction enzyme accessibility at the *Ccl5* promoter was monitored using a Southern blot assay. Results are shown from three independent experiments, with the average percentage of alleles cleaved in the nuclei shown in the bar graph. The larger DNA fragment (*) results from cleavage of the purified genomic DNA by EcoRI and HindIII, which are restriction enzymes that cleave the DNA at sites flanking the *Ccl5* promoter. The smaller fragment (arrow) was generated when EcoNI, which was added to the isolated nuclei, cleaved within the *Ccl5* promoter. Bar graph shows averages of three independent experiments with standard deviations.

(C) Restriction enzyme accessibility was monitored at the *Ifit1* promoter, as described above for the *Ccl5* promoter. Results from two independent experiments are shown. DraIII was used for digestion of purified DNA at sites flanking the *Ifit1* promoter, with DraI used for digestion of nuclear DNA within the *Ifit1* promoter.

Preferential Activation of SWI/SNF-Dependent versus SWI/SNF-Independent Genes by Other Stimuli

To examine the broader significance of the distinction between SWI/SNF-independent CpG-island and SWI/SNF-dependent non-CpG-island primary response genes, we analyzed the 67 genes after stimulating bone marrow-derived macrophages with other inducers, including peptidoglycan (TLR2), poly I.C (TLR3), interferon-β (IFN-β), and tumor necrosis factor-α (TNF-α). The mRNA levels for each gene at three different time points in response to each stimulus are presented as a percentage of the maximum level of induction by any of the stimuli (100%) (Figure 5; see also Figure S7).

Striking differences were found in the preferences of some stimuli for SWI/SNF-independent versus SWI/SNF-dependent genes. Of particular relevance, TNF-α induction was strongly biased toward class A genes. TNF-α stimulated 23 of the 24 class A genes to a level that was at least 15% of the maximum induction (Figure 5A). However, only 9 of the remaining 37 genes were activated to this level, with these 9 genes scattered among the other classes (Figures 5A–5C). This finding is consistent with the fact that TNF-α signaling does not induce IRF3 and suggests that TNF-α may not directly induce any other transcription factors that can promote efficient nucleosome remodeling in macrophages, thereby restricting strong activation to SWI/SNF-independent primary response genes. We cannot exclude the possibility that TNF-α activates a distinct set of SWI/SNF-dependent non-CpG-island primary response genes via transcription factors that differ from those induced by LPS. However, independent microarray studies of fetal-liver-derived macrophages activated with TNF-α failed to reveal a compelling set of non-CpG-island primary response genes (C.S.C. and A.H., unpublished data).

In striking contrast to the preferential induction of class A genes by TNF-α, IFN-β exhibited a strong preference for SWI/SNF-dependent genes in classes C and D (Figures 5A–5C). This finding is consistent with the view that IFN-β induces transcription via IRF proteins and STAT proteins; both of these protein families have been suggested to promote nucleosome remodeling by SWI/SNF complexes (see Figure 4 and Liu et al., 2002; Huang et al., 2002; Cui et al., 2004). Therefore, IFN-induced factors appear to be well-suited for the selective activation of SWI/SNF-dependent genes assembled into stable nucleosomes, with no need for constitutively active chromatin or a CpG island.

Although TNF-α and IFN-β exhibited strong preferences, TLR2 and TLR3 signaling resulted in the induction of nearly all genes induced by TLR4. The only clear difference was that TLR2 signaling failed to induce the IRF3-dependent genes in class D, as well as some secondary response genes dependent on IFN signaling, consistent with knowledge that TLR2 signaling does not activate IRF3 (Kawai and Akira, 2007).

Further support for the hypothesis that some stimuli preferentially induce SWI/SNF-independent CpG-island genes during a

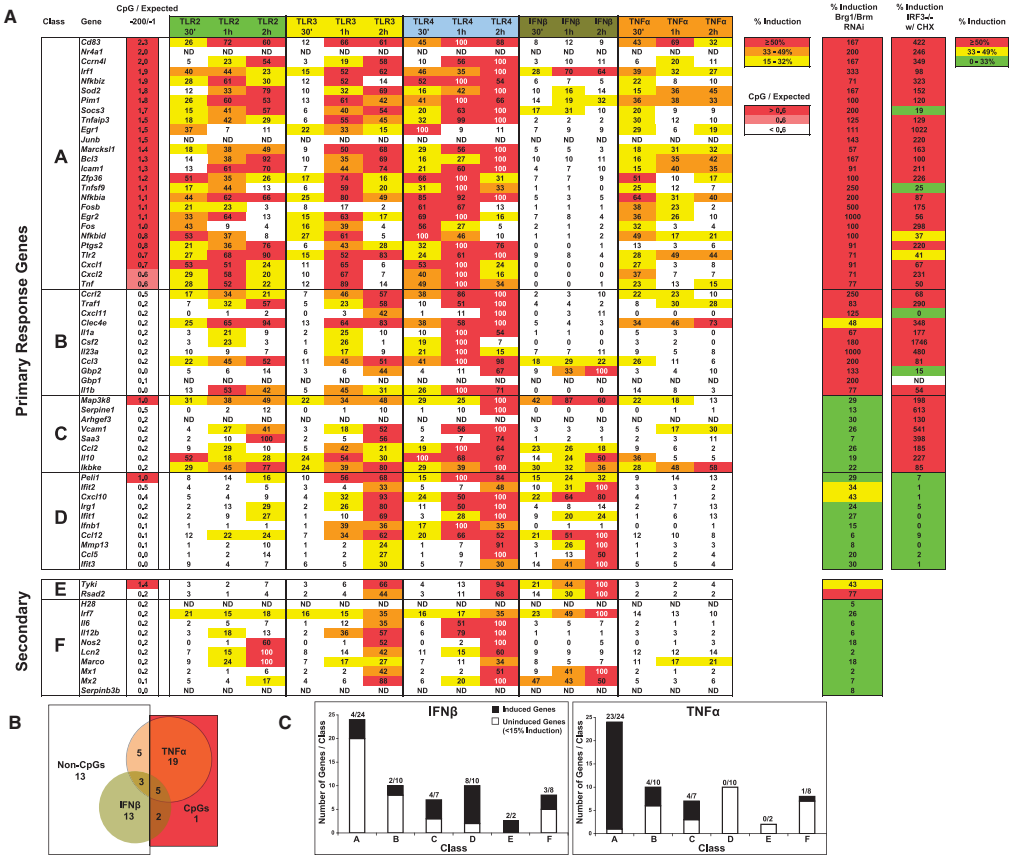


Figure 5. Preferential Activation of CpG-Island and Non-CpG-Island Genes by TNF-α and IFN-β

(A) Bone marrow derived macrophages were left unstimulated or were stimulated for 30 min, 1 hr, or 2 hr with stimuli for TLR2, TLR3, or TLR4 or with IFN β or TNF α. mRNA levels for 61 of the 67 genes shown in Figure 1 were monitored by qRT PCR. mRNA levels are presented as a percentage of the highest level observed at any of the time points by any of the stimuli (set at 100%). Values represent an average of three independent experiments (i.e., independent stimulations of independent macrophage preparations). mRNA levels of at least 15% of the maximum were colored red (>50%), orange (33% 49%), or yellow (15% 32%). CpG numbers, Brg1/Brm dependence, and IRF3 dependence were derived from Figure 1.

(B) A Venn diagram shows that TNF α preferentially induced a high percentage of CpG island genes (mostly in class A), whereas IFN β preferentially induced non CpG island genes (mostly in classes C and D).

(C) The number of genes within each of the six classes that were induced or were not induced by IFN β and TNF α are depicted in a bar graph. Uninduced genes were defined as those induced to a level below 15% of the maximum induction by any of the five stimuli shown in (A).

primary response, perhaps due to the inability of these stimuli to activate transcription factors capable of promoting nucleosome remodeling, was provided from a literature analysis of well-documented primary response genes induced by serum and the tumor promoter TPA. Collections of bona fide primary response genes induced by these stimuli were compiled by Herschman (1991) before promoter sequences for most genes were available. Remarkably, every serum- and TPA-induced gene compiled by Herschman (1991) contains a CpG-island promoter (Figures 6A and 6B). Independent microarray experiments failed to uncover any non-CpG-island genes that are potently induced during the primary response to serum in serum-starved NIH 3T3 cells (data not shown). In contrast, 74% of primary response genes induced by IFN-β by at least 5-fold in real-time RT-PCR experiments lacked CpG islands between −200 and −1 (Figure 6C).

A Serum-Induced Primary Response Genes

Gene	CpGs/Expected -200 / -1	CpGs/Expected +1 / +200	%GC -200 / -1	%GC +1 / +200
Jun	0.8	1.0	58	60
Fosb	1.1	0.7	67	42
Egr2	1.1	0.3	59	42
Cyr61	1.1	1.9	58	70
Fosl1	1.1	1.6	60	71
Srf	1.1	2.1	60	82
Tpm1	1.2	1.4	66	61
Id3	1.3	1.4	64	60
Junb	1.5	1.4	68	67
Egr1	1.5	2.0	65	75
Fn1	1.8	0.9	70	65
Nr4a1	2.0	1.3	76	67
Fosl2	2.0	1.6	68	68
Cdkn1b	2.1	1.4	69	63

B TPA-Induced Primary Response Genes

Gene	CpGs/Expected -200 / -1	CpGs/Expected +1 / +200	%GC -200 / -1	%GC +1 / +200
Ier2	0.7	1.1	52	55
Ptgs2	0.8	0.8	55	64
Fos	1.0	1.4	59	63
Fosb	1.1	0.7	67	42
Cyr61	1.1	1.9	58	70
Fosl1	1.1	1.6	60	71
Zfp36	1.2	1.0	68	60
Myc	1.3	1.4	63	66
Ifrd1	1.4	1.7	68	63
Btg2	2.0	1.3	76	61
Nr4a1	2.0	1.3	76	67
Fosl2	2.0	1.6	68	68

C IFNβ-Induced Primary Response Genes

Gene	CpGs/Expected -200 / -1	CpGs/Expected +1 / +200	%GC -200 / -1	%GC +1 / +200
Ifit3	0.0	0.0	47	44
Gbp2	0.0	0.2	46	48
Ccl5	0.0	0.5	51	60
Mmp13	0.1	0.0	41	52
Mx2	0.1	0.1	53	50
Ccl12	0.1	0.2	48	50
Ifit1	0.2	0.1	50	51
Irg1	0.2	0.2	48	49
Ccl3	0.2	0.2	48	53
Cxcl11	0.2	0.2	51	53
Mx1	0.2	0.0	54	48
Nos2	0.2	0.2	52	53
Ccl2	0.2	0.2	52	62
Il10	0.2	0.2	41	56
Il6	0.2	0.3	44	54
Rsad2	0.2	0.3	51	63
Irf7	0.2	0.6	58	58
Cxcl10	0.4	0.6	50	52
Ccrl2	0.5	0.5	57	52
Ifit2	0.5	0.7	51	54
Ptgs2	0.8	0.8	55	64
Map3k8	1.0	0.7	65	60
Tyki	1.4	1.2	68	70
Socs3	1.7	1.5	69	69
Pim1	1.8	1.8	75	74
Irf1	1.9	2.1	68	65
Ccrn4l	2.0	2.6	73	79

CpG Island		
≤ 0.5	0.6	≥ 0.7

CpG Island		
≤ 54%	55-57%	≥ 58%

Herschman H.R. *Annu Rev Biochem* **60:281 (1991)**

Figure 6. Differential Induction of CpG-Island versus Non-CpG-Island Genes

(A) A collection of well characterized primary response genes induced by serum is shown, along with the CpG content and GC content of their promoters. The list includes every serum induced gene described in Herschman (1991).

(B) A collection of well characterized primary response genes induced by TPA is shown, along with the CpG content and GC content of their promoters. Every TPA induced gene described in Herschman (1991) is included.

(C) A set of primary response genes induced by IFN β in mouse bone marrow derived macrophages is shown. The list includes all genes from the set of 67 LPS induced genes that were induced by IFN β by at least 5 fold in qRT PCR experiments.

Cell-Type-Specific Classification of an LPS-Induced Gene

Finally, an analysis of gene induction in primary mouse embryonic fibroblasts (MEFs) demonstrated that genes induced by a given stimulus can be assigned to different classes in different cell types. This fundamental property was revealed through an analysis of the *Il6* gene. In LPS-stimulated macrophages, *Il6* is a SWI/SNF-dependent secondary response gene (see Figures 7A and 1 and Ramirez-Carrozzi et al., 2006). In contrast, *Il6* was induced in a protein synthesis-independent, SWI/SNF-independent manner in primary MEFs (Figures 7A and 7C). Interestingly, a restriction enzyme accessibility analysis revealed that the *Il6* promoter is highly accessible in unstimulated MEFs, with little change following stimulation, in contrast to its inducible accessibility in macrophages (Figure 7B). Thus, despite the assignment of *Il6* to secondary response class F in macrophages, its properties are more appropriate for primary response class B in MEFs. This dramatic change appears to be unusual, as none of the other class F secondary response genes exhibited properties of a primary response gene in MEFs (data not shown; see Discussion).

DISCUSSION

We have provided a framework for understanding the relationship between CpG islands, nucleosome remodeling, and nucleosome stability during inducible gene transcription. CpG-island promoters were generally associated with primary response genes induced by a broad range of stimuli in a SWI/SNF-independent manner. The high CpG content appeared to be responsible for promoter assembly into unstable nucleosomes, which may directly contribute to the SWI/SNF independence, analogous to the relationship between nucleosome instability

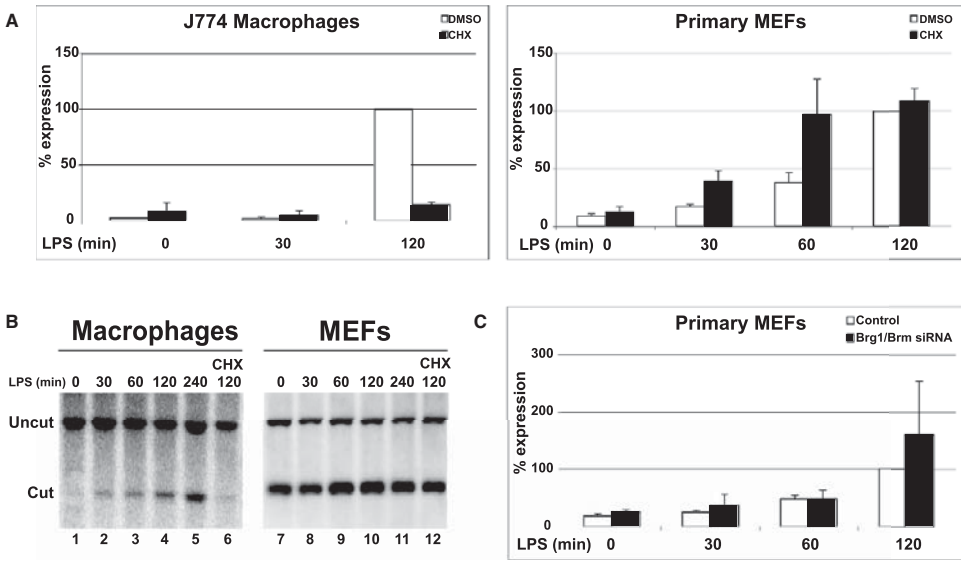


Figure 7. *Il6* Is SWI/SNF Independent in LPS-Stimulated MEFs

(A) *Il6* mRNA levels were monitored by qRT PCR in J774 macrophages or primary MEFs following stimulation with LPS in the presence of CHX or in the presence of the DMSO solvent. Results shown are averages of three independent experiments, with standard deviations. The CHX sensitivity observed in the J774 line was also observed in primary bone marrow derived macrophages (Ramirez Carrozzi et al., 2006).

(B) Restriction enzyme accessibility at the *Il6* promoter was examined in J774 macrophages and primary MEFs as described (Ramirez Carrozzi et al., 2006). Cells were left unstimulated or were stimulated for different time periods. Cells were also stimulated for 120 min in the presence of CHX.

(C) An shRNA that simultaneously targets the Brg1 and Brm mRNAs for degradation was introduced into primary MEFs using a retroviral vector (Ramirez Carrozzi et al., 2006). Efficient knockdown of Brg1 and Brm was monitored by western blot (data not shown). Cells were stimulated with LPS and *Il6* mRNA levels were monitored by qRT PCR. Results represent averages of three independent experiments, with standard deviations.

and SWI/SNF independence in *S. cerevisiae* Sin mutants (Muthurajan et al., 2004). In striking contrast, SWI/SNF-dependent genes lacked CpG-island promoters and assembled into stable nucleosomes. Assembly into stable nucleosomes conferred the capacity for tight regulation, with activation dependent on specialized transcription factors that promote nucleosome remodeling.

We hypothesize that, during the evolution of some genomes, CpG islands provided an attractive platform for promoters of constitutive and broadly induced genes for two reasons. First, the instability of nucleosomes assembled on CpG islands facilitated constitutive expression and rapid induction without an energy requirement for nucleosome remodeling or a requirement for factors that can promote remodeling. Second, CpG-island promoters contained binding sites for ubiquitous factors like Sp1, which are likely to facilitate the establishment of constitutively active chromatin. This dual benefit may have provided selective pressure that contributed to the maintenance of CpG-island promoters through evolution.

The striking differences in the properties of promoters induced by different stimuli have broad biological relevance. Many CpG-island SWI/SNF-independent genes are activated by "generic" signaling pathways, such as NF-κB and MAP kinase pathways, which are targeted by a large number of growth factors, cytokines, and microbial stimuli. The transcription factors induced by these pathways may not readily promote nucleosome remodeling and may be well-suited for the activation of promiscuously induced genes. In contrast, IFN-β, which is known to activate genes with highly specialized functions, preferentially targets non-CpG-island SWI/SNF-dependent genes. The activation of these genes is restricted by the assembly of their promoters into stable nucleosomes.

In addition to facilitating highly selective activation, a second potential benefit of promoter assembly into stable nucleosomes may be to help minimize basal transcription, thereby preventing synthesis of gene products that may be detrimental to the cell when constitutively present at low levels. The higher basal transcription levels observed with some CpG-island genes may be less detrimental and perhaps of some benefit. However, some of these genes are likely to be regulated at the level of mRNA stability (data not shown), allowing little expression of their gene products in quiescent macrophages, despite substantial precursor transcript levels.

It is noteworthy that the SWI/SNF-independent activation of many genes suggests that these genes do not contain distant enhancers that require SWI/SNF-dependent remodeling.

Perhaps, SWI/SNF-independent primary response genes do not require distant enhancers at all for their activation. Alternatively, the enhancers for these genes may be constitutively active. It is also important to consider the possibility that other ATP-dependent nucleosome remodeling complexes may contribute to remodeling at enhancers for these genes.

Previous studies have suggested that reduced nucleosome occupancy may be a general property of mammalian promoters (Heintzman et al., 2007; Ozsolak et al., 2007; Schones et al., 2008). We propose that nucleosome occupancy is reduced to variable degrees at CpG-island promoters as a result of the destabilizing effect of the CpG-island sequence, with nucleosomes evicted from a subset of non-CpG-island promoters during transcriptional activation. The role of CpG islands in generating a nucleosome deficit appears analogous to the role of poly (dA:dT) tracts at *S. cerevisiae* promoters (Iyer and Struhl, 1995; Mavrich et al., 2008b). However, the precise role of CpG-island-induced nucleosome instability in conferring SWI/SNF independence awaits studies to determine whether a SWI/SNF-dependent promoter can be converted to a SWI/SNF-independent promoter by destabilizing nucleosomes through changes in the DNA sequence. Thus far, our efforts to achieve this goal have been unsuccessful, due to the challenge of altering promoter sequences to a sufficient extent to destabilize nucleosomes without disrupting or introducing binding sites for specific transcription factors.

Although the assembly of CpG-island promoters into unstable nucleosomes may contribute to their SWI/SNF independence, these promoters possess other features of transcriptionally active chromatin in unstimulated cells. Unstable nucleosomes may be intrinsically susceptible to acetylation and methylation in the absence of transcription factor targeting. However, a more likely scenario is that constitutively expressed transcription factors play a role in targeting histone modifications. Although CpG-island promoters do not exhibit a functional requirement for SWI/SNF complexes during their activation, we previously found that these promoters are constitutively associated with Brg1 (Ramirez-Carrozzi et al., 2006). We favor the view that constitutive association results from nonspecific binding of SWI/SNF complexes to genomic regions assembled into relatively open chromatin structures. However, we cannot exclude the possibility that SWI/SNF complexes play a role in establishing a constitutively open chromatin structure at CpG-island promoters that is sufficiently stable to permit activation following Brg1/Brm knockdown. We also must consider the possibility that noncatalytic subunits of the SWI/SNF complexes play roles that have not yet been revealed.

Although our current characterization provides considerable insight into the regulation of class A and class D promoters, promoters in classes B and C remain poorly understood. A different nucleosome remodeling complex may be responsible for the SWI/SNF-independent activation of class B promoters. Alternatively, the binding of specific transcription factors to class B promoters in unstimulated cells may facilitate their assembly into constitutively open chromatin, allowing transcriptional activation in the absence of inducible nucleosome remodeling.

The evidence that the *Il6* gene can switch from class F to class B reveals that genes are not fixed in their classification. *Il6* was the only class F gene in macrophages converted to a class B gene in MEFs, which may be related to the need for unusually versatile regulation of *Il6* expression because of its diverse biological functions (Kishimoto, 2006). We hypothesize that the constitutive expression of a factor in MEFs that is inducibly expressed in macrophages is responsible for this switch. Although this hypothetical factor remains to be identified, the classification scheme and mechanistic insights provided by this analysis provide a consistent framework toward a global understanding of the diverse mechanisms responsible for inducible gene transcription, and of the biological necessity for this diversity.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents

Bone marrow derived macrophages were prepared from C57BL/6 and IRF3$^{-/-}$ mice. MEFs were from D13.5 14.5 C57BL/6 embryos and were maintained in DMEM with 10% FBS and 0.05 mM β mecaptoethanol. Macrophages were activated on day 6 with *S. aureus* peptidoglycan (Sigma Aldrich) (PGN) (20 μg/ml), poly I:C (1 μg/ml), *S. typhosa* LPS (Sigma Aldrich) (10 μg/ml), IFN β (PBL Biomedical Laboratories) (250 U/ml), or TNF α (BD PharMingen) (10 ng/ml). MEFs were activated at passage 4. When indicated, cells were preincubated for 15 min with CHX (10 μg/ml).

RT-PCR, Real-Time PCR, and RNAi

RNA was extracted using TRI reagent (Molecular Research Center), treated with RNase free DNaseI, and purified using an RNeasy kit (QIAGEN). Quantified RNA (2 μg) was reverse transcribed using Omniscript RT Kit (QIAGEN) and random hexamer primers. cDNA fragments were analyzed by qPCR using SensiMix *Plus* (Quantace) and the iCycler System (Bio Rad) or a 7900HT (Applied Biosystems). PCR amplification conditions were 95°C (3 min) and 45 cycles of 95°C (15 s), 60°C (30 s), and 72°C (30 s). Primer pairs (see Table S1A) were designed to amplify 80 150 bp mRNA specific fragments, and unique products were tested by melt curve analysis.

The Brg1/Brm shRNA was expressed from a retroviral vector as described (Ramirez Carrozzi et al., 2006). The efficiency of Brg1 and Brm knockdown was monitored by western blot as described (Ramirez Carrozzi et al., 2006). Transduced J774 cells and MEFs were stimulated 5 and 3 days after infection, respectively.

Restriction Enzyme Accessibility and ChIP

Restriction enzyme accessibility was performed as described (Ramirez Carrozzi et al., 2006). Cell nuclei were incubated with restriction enzyme (100 U) (EcoNI for *Ccl5* and DraI for *Ifit1*) for 15 min at 37°C. Purified DNA (10 15 μg) was then digested to completion to generate reference cleavage products using EcoRI and HindIII for *Ccl5* and DraIII for *Ifit1*. Samples were analyzed by Southern blot with ^{32}P labeled probes corresponding to the following regions: *Ccl5* promoter (−297 to −667) and *Ifit1* promoter (−822 to −471).

ChIP experiments were performed as described (Ramirez Carrozzi et al., 2006) with anti H3 (Abcam ab1791), anti trimethyl H3K4 (Abcam ab8580), anti Acetyl H3 (Milipore 06 599), anti RNA Pol II (Santa Cruz sc 899), and anti TBP (Santa Cruz sc 204). Primer sequences are shown in Table S1B. p values were calculated by two tailed Student's t test, using average values for each gene within each group.

Nucleosome Affinity Measurements

300 bp promoter fragments were cloned into pUC19. DNA fragments for nucleosome assembly were generated from these plasmids by PCR using vector specific primers. PCR products were gel purified using Gel Extraction Kit (QIAGEN). Equivalent amounts of each promoter fragment were pooled and 100 ng of the pool was assembled into nucleosomes by incubating with recombinant *Xenopus laevis* histones (Luger et al., 1997; Thåström et al., 2004) at 37°C for 30 min in 10 μl of a 1 M NaCl reaction containing 100 ng BSA. Low salt buffer (20 mM Tris, pH 7.6, 0.1%Triton X 100, 100 μg/ml BSA, 1 mM EDTA, 0.5 mM PMSF, 5 mM DTT) was slowly added in volumes of 5, 10, 15, 30, and 30 μl, with 10 min incubations at room temperature after

each addition. Samples were then run on a 6% 0.5X TBE native polyacrylamide gel and subsequently stained with 5× SYBR Green (Invitrogen). Free DNA and nucleosomal DNA bands were excised and electroeluted into 1× TE. Recov ered DNA fragments were PCR amplified for 18 20 cycles. After determining the DNA concentration by OD analysis, the fragments were either reassembled into nucleosomes or analyzed by qPCR using promoter specific primers. p values were calculated by two tailed Student's t test.

SUPPLEMENTAL DATA

Supplemental Data include seven figures and one table and can be found with this article online at http://www.cell.com/supplemental/S0092 8674(09) 00445 0.

ACKNOWLEDGMENTS

We thank Genhong Cheng, Diana Hargreaves, Harvey Herschman, Siavash Kurdistani, Ruslan Medzhitov, Amy Weinmann, Jonathan Widom, and Michael Zhang for helpful discussions. V.R.R.C. was supported by a Giannini Family Foundation Fellowship, D.B. by the German Academic Exchange Service (Deutscher Akademischer Austausch Dienst, DAAD) and a Jonsson Cancer Center Foundation Fellowship, D.M.B. by NIH T32AI007126, K.R.D. by NIH T32GM07185, and S.T.S. by NIH R01GM086372, R01CA127279, and P50CA092131.

Received: November 10, 2008
Revised: February 6, 2009
Accepted: April 8, 2009
Published: July 9, 2009

REFERENCES

Agalioti, T., Lomvardas, S., Parekh, B., Yie, J., Maniatis, T., and Thanos, D. (2000). Ordered recruitment of chromatin modifying and general transcription factors to the IFN beta promoter. Cell *103*, 667 678.

Anderson, J.D., and Widom, J. (2001). Poly(dA dT) promoter elements increase the equilibrium accessibility of nucleosomal DNA target sites. Mol. Cell. Biol. *21*, 3830 3839.

Boeger, H., Griesenbeck, J., and Kornberg, R.D. (2008). Nucleosome retention and the stochastic nature of promoter chromatin remodeling for transcription. Cell *133*, 716 726.

Boyle, A.P., Davis, S., Shulha, H.P., Meltzer, P., Margulies, E.H., Weng, Z., Furey, T.S., and Crawford, G.E. (2008). High resolution mapping and charac terization of open chromatin across the genome. Cell *132*, 311 322.

Cui, K., Tailor, P., Liu, H., Chen, X., Ozato, K., and Zhao, K. (2004). The chro matin remodeling BAF complex mediates cellular antiviral activities by promoter priming. Mol. Cell. Biol. *24*, 4476 4486.

Davuluri, R.V., Grosse, I., and Zhang, M.Q. (2001). Computational identification of promoters and first exons in the human genome. Nat. Genet. *29*, 412 417.

Doyle, S., Vaidya, S., O'Connell, R., Dadgostar, H., Dempsey, P., Wu, T., Rao, G., Sun, R., Haberland, M., Modlin, R., and Cheng, G. (2002). IRF3 mediates a TLR3/TLR4 specific antiviral gene program. Immunity *17*, 251 263.

Drew, H.R., and Travers, A.A. (1985). DNA bending and its relation to nucleo some positioning. J. Mol. Biol. *186*, 773 790.

Gardiner Garden, M., and Frommer, M. (1987). CpG islands in vertebrate genomes. J. Mol. Biol. *196*, 261 282.

Gilmour, D.S., and Lis, J.T. (1986). RNA polymerase II interacts with the promoter region of the noninduced hsp70 gene in Drosophila melanogaster cells. Mol. Cell. Biol. *6*, 3984 3989.

Guenther, M.G., Levine, S.S., Boyer, L.A., Jaenisch, R., and Young, R.A. (2007). A chromatin landmark and transcription initiation at most promoters in human cells. Cell *130*, 77 88.

Hargreaves, D.C., Horng, T., and Medzhitov, R. (2009). Control of inducible gene expression by signal dependent transcriptional elongation. Cell *138*, this issue, 129 145.

Heintzman, N.D., Stuart, R.K., Hon, G., Fu, Y., Ching, C.W., Hawkins, R.D., Barrera, L.O., Van Calcar, S., Qu, C., Ching, K.A., et al. (2007). Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome. Nat. Genet. *39*, 311 318.

Herschman, H.R. (1991). Primary response genes induced by growth factors and tumor promoters. Annu. Rev. Biochem. *60*, 281 319.

Huang, M., Qian, F., Hu, Y., Ang, C., Li, Z., and Wen, Z. (2002). Chromatin remodelling factor BRG1 selectively activates a subset of interferon alpha inducible genes. Nat. Cell Biol. *4*, 774 781.

Iyer, V., and Struhl, K. (1995). Poly(dA:dT), a ubiquitous promoter element that stimulates transcription via its intrinsic DNA structure. EMBO J. *14*, 2570 2579.

Kao, S.Y., Calman, A.F., Luciw, P.A., and Peterlin, B.M. (1987). Anti termina tion of transcription within the long terminal repeat of HIV 1 by tat gene product. Nature *330*, 489 493.

Kawai, T., and Akira, S. (2007). TLR signaling. Semin. Immunol. *19*, 24 32.

Kishimoto, T. (2006). Interleukin 6: discovery of a pleiotropic cytokine. Arthritis Res. Ther. *8* (*Suppl. 2*), S2.

Krumm, A., Meulia, T., Brunvand, M., and Groudine, M. (1992). The block to transcriptional elongation within the human c myc gene is determined in the promoter proximal region. Genes Dev. *6*, 2201 2213.

Liu, H., Kang, H., Liu, R., Chen, X., and Zhao, K. (2002). Maximal induction of a subset of interferon target genes requires the chromatin remodeling activity of the BAF complex. Mol. Cell. Biol. *22*, 6471 6479.

Lowary, P.T., and Widom, J. (1998). New DNA sequence rules for high affinity binding to histone octamer and sequence directed nucleosome positioning. J. Mol. Biol. *276*, 19 42.

Luger, K., Rechsteiner, T.J., Flaus, A.J., Waye, M.M., and Richmond, T.J. (1997). Characterization of nucleosome core particles containing histone proteins made in bacteria. J. Mol. Biol. *272*, 301 311.

Mavrich, T.N., Jiang, C., Ioshikhes, I.P., Li, X., Venters, B.J., Zanton, S.J., Tomsho, L.P., Qi, J., Glaser, R.L., Schuster, S.C., et al. (2008a). Nucleosome organization in the Drosophila genome. Nature *453*, 358 362.

Mavrich, T.N., Ioshikhes, I.P., Venters, B.J., Jiang, C., Tomsho, L.P., Qi, J., Schuster, S.C., Albert, I., and Pugh, B.F. (2008b). A barrier nucleosome model for statistical positioning of nucleosomes throughout the yeast genome. Genome Res. *18*, 1073 1083.

Muthurajan, U.M., Bao, Y., Forsberg, L.J., Edayathumangalam, R.S., Dyer, P.N., White, C.L., and Luger, K. (2004). Crystal structures of histone Sin mutant nucleosomes reveal altered protein DNA interactions. EMBO J. *23*, 260 271.

Ozsolak, F., Song, J.S., Liu, X.S., and Fisher, D.E. (2007). High throughput mapping of the chromatin structure of human promoters. Nat. Biotechnol. *25*, 244 248.

Ramirez Carrozzi, V.R., Nazarian, A.A., Li, C.C., Gore, S.L., Sridharan, R., Imbalzano, A.N., and Smale, S.T. (2006). Selective and antagonistic functions of SWI/SNF and Mi 2beta nucleosome remodeling complexes during an inflammatory response. Genes Dev. *20*, 282 296.

Satchwell, S.C., Drew, H.R., and Travers, A.A. (1986). Sequence periodicities in chicken nucleosome core DNA. J. Mol. Biol. *191*, 659 675.

Saxonov, S., Berg, P., and Brutlag, D.L. (2006). A genome wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters. Proc. Natl. Acad. Sci. USA *103*, 1412 1417.

Schones, D.E., Cui, K., Cuddapah, S., Roh, T.Y., Barski, A., Wang, Z., Wei, G., and Zhao, K. (2008). Dynamic regulation of nucleosome positioning in the human genome. Cell *132*, 887 898.

Segal, E., Fondufe Mittendorf, Y., Chen, L., Thåström, A., Field, Y., Moore, I.K., Wang, J.P., and Widom, J. (2006). A genomic code for nucleosome posi tioning. Nature *442*, 772 778.

Sekinger, E.A., Moqtaderi, Z., and Struhl, K. (2005). Intrinsic histone-DNA interactions and low nucleosome density are important for preferential accessibility of promoter regions in yeast. Mol. Cell *18*, 735–748.

Suzuki, M.M., and Bird, A. (2008). DNA methylation landscapes: provocative insights from epigenomics. Nat. Rev. Genet. *9*, 465–476.

Takai, D., and Jones, P.A. (2002). Comprehensive analysis of CpG islands in human chromosomes 21 and 22. Proc. Natl. Acad. Sci. USA *99*, 3740–3745.

Thåström, A., Gottesfeld, J.M., Luger, K., and Widom, J. (2004). Histone-DNA binding free energy cannot be measured in dilution-driven dissociation experiments. Biochemistry *43*, 736–741.

Tirosh, I., and Barkai, N. (2008). Two strategies for gene regulation by promoter nucleosomes. Genome Res. *18*, 1084–1091.

Weinmann, A.S., Plevy, S.E., and Smale, S.T. (1999). Rapid and selective remodeling of a positioned nucleosome during the induction of IL-12 p40 transcription. Immunity *11*, 665–675.

Williams, S.K., and Tyler, J.K. (2007). Transcriptional regulation by chromatin disassembly and reassembly. Curr. Opin. Genet. Dev. *17*, 88–93.

Yuan, G.C., Liu, Y.J., Dion, M.F., Slack, M.D., Wu, L.F., Altschuler, S.J., and Rando, O.J. (2005). Genome-scale identification of nucleosome positions in S. cerevisiae. Science *309*, 626–630.

Cell *138*, 114–128, July 10, 2009