Identification of Novel Regulators of Nuclear Factor Kappa B Repression by the Glucocorticoid Receptor

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Identification of Novel Regulators of Nuclear Factor Kappa B Repression
by the Glucocorticoid Receptor

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

in

Biology

by

Samantha Harriet Murphy

Committee in charge:

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Professor Webster K. Cavenee
Professor Ronald M. Evans
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Professor Tracy L. Johnson

2011
The dissertation of Samantha Harriet Murphy is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2011
DEDICATION

I dedicate this work to my biggest fans in life. To my parents, Tim and Shirley Serey, who have shown me continual love and encouragement, who instilled in me a belief that I could one day accomplish great things, and who have supported me in both my successes and failures along the way. To my husband and best friend, Timothy Murphy, who has brought me endless laughter and happiness. Thank you for taking care of me and for helping me make it through this process.
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<th>Definition</th>
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<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>AF-1/2</td>
<td>activation function 1/2</td>
</tr>
<tr>
<td>ALPK3</td>
<td>alpha-kinase 3</td>
</tr>
<tr>
<td>BMDM</td>
<td>bone marrow derived macrophage</td>
</tr>
<tr>
<td>Cox2</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>CDKN2D</td>
<td>cyclin-dependent kinase inhibitor 2D</td>
</tr>
<tr>
<td>CRH</td>
<td>corticotropin-releasing hormone</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA binding domain</td>
</tr>
<tr>
<td>Dex</td>
<td>dexamethasone</td>
</tr>
<tr>
<td>Fkbp5</td>
<td>FK506 binding protein 5</td>
</tr>
<tr>
<td>GILZ</td>
<td>glucocorticoid-induced leucine zipper</td>
</tr>
<tr>
<td>GR</td>
<td>glucocorticoid receptor</td>
</tr>
<tr>
<td>GRE</td>
<td>glucocorticoid response element</td>
</tr>
<tr>
<td>HPA</td>
<td>hypothalamus-pituitary-adrenal</td>
</tr>
<tr>
<td>IP</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>LBD</td>
<td>ligand binding domain</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemotactic protein-1</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse embryonic fibroblast</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Mt2</td>
<td>metallothionein 2</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa B or nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NRBP1</td>
<td>nuclear receptor binding protein 1</td>
</tr>
<tr>
<td>nGRE</td>
<td>negative glucocorticoid response element</td>
</tr>
<tr>
<td>oligo</td>
<td>oligonucleotide</td>
</tr>
<tr>
<td>PME-1</td>
<td>protein phosphatase methylesterase 1</td>
</tr>
<tr>
<td>PTPN23</td>
<td>protein tyrosine phosphatase, non receptor type 23</td>
</tr>
<tr>
<td>PVN</td>
<td>paraventricular nucleus</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RSA</td>
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</tr>
<tr>
<td>SETDB1</td>
<td>set domain, bifurcated 1</td>
</tr>
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<td>serum/glucocorticoid regulated kinase 2</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>XYLB</td>
<td>xylulose kinase</td>
</tr>
</tbody>
</table>
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plate processing machinery with our assay protocol. In addition, I am grateful to Narayana Yeddula who began collaborating with me on this project after identification of the 24 novel genes of interest by reconfirmation screening. He performed immunoblot experiments on nuclear and cytoplasmic extracts to examine the effect of each gene of interest on p65 translocation and is also responsible for the phosphorylation studies.

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ABSTRACT OF THE DISSERTATION

Identification of Novel Regulators of Nuclear Factor Kappa B Repression by the Glucocorticoid Receptor

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Inder M. Verma, Chair

Nuclear factor kappa B (NF-κB) is a family of transcription factors that has an essential regulatory function in inflammation, the immune response, cell proliferation, and apoptosis. Constitutive activation of the NF-κB pathway is often associated with cancer and chronic inflammatory diseases such as multiple sclerosis, inflammatory bowel disease, rheumatoid arthritis, and asthma. Glucocorticoids, which bind to the glucocorticoid receptor (GR), are among the strongest anti-inflammatory agents and one of the most common forms of treatment to suppress inflammation. GR is a transcription factor that
plays an important role in a variety of cellular processes including reproduction, development, differentiation, and metabolism, and it is fundamental to the counteraction of immune and inflammatory responses by acting as a key repressor of NF-κB. Proposed mechanisms of NF-κB repression by GR are complex and varied, and a universal mechanism of repression has yet to be elucidated. By discovering novel genes that play a role in GR repression of NF-κB, potential therapeutic targets and their molecular mechanisms can be identified to specifically improve the use of glucocorticoids in clinical applications. We performed high throughput screens using siRNA oligonucleotide library collections to identify novel genes that affect glucocorticoid repression of NF-κB activity after activation of both the NF-κB and GR pathways. The work presented in this thesis provides evidence that p53 is involved in glucocorticoid receptor repression of NF-κB. We initially establish p53 as a gene of interest by high throughput screening, and validate this finding using luciferase assays in physiologically relevant cell lines and by qPCR. We also confirm its biological significance in vivo in a mouse model of LPS shock. Additionally, we demonstrated that p53 does not play a role in upstream NF-κB or GR signaling cascades, that p53 loss impairs glucocorticoid repression of NF-κB target gene transcription, and that p53 loss impairs GR target gene transcription. We conclude that p53 is an important gene involved in GR repression of NF-κB, a finding that may explain why glucocorticoid treatment is often an ineffective therapy in the repression of
inflammation associated with the tumor microenvironment and why NF-κB is often up-regulated in many human cancers. Additionally, we have identified 24 other novel genes that play a role in NF-κB repression by GR, and have created a survey outlining which of these genes affect aspects of certain NF-κB signaling components. Ultimately, we believe that agonist development targeting one of these genes may have the potential to lead to better anti-inflammatory therapies.
Chapter 1. Introduction

Inflammation, the immune response, and Nuclear Factor kappa B

Immunity and inflammation are important physiological processes that enable us to fight off infection and disease. Upon initial notification of a threatening agent or an invading organism, the host can activate its defense system to eliminate the pathogen in a non-specific manner, a process generally referred to as innate immunity. When a pathogen persists or evades the innate immune system, adaptive immunity then takes over to generate a more effective and specific response to destroy the pathogen. Excessive activation of these responses however, can cause cell damage, tissue damage, or death, and it is therefore fundamental to maintain immune homeostasis through both pro and anti-inflammatory mechanisms in order for an organism to survive.

The inflammation process was first described by Cornelius Celsus, a Roman encyclopedist who recorded redness, swelling, heat and pain as the cardinal signs of inflammation (1). In 1986, an important link between inflammation, the immune response, and their regulation was established when David Baltimore and colleagues discovered Nuclear Factor kappa B (or nuclear factor kappa-light-chain-enhancer of activated B cells, NF-κB), a protein complex which interacts with a defined site in the enhancer of kappa
immunoglobulin molecules (proteins which are cell surface and soluble antibodies that recognize and neutralize foreign, invasive objects) (2). They then went on to show that pro-inflammatory signals powerfully induce NF-κB activation (3), and numerous molecular and biochemical studies since then have revealed a critical role for NF-κB in the regulation of immunity and inflammation.

Nuclear factor kappa B (NF-κB) is a family of transcription factors that has an essential regulatory function in inflammation, the immune response, cell proliferation, and apoptosis (4). NF-κB activates hundreds of target genes which play a role in these processes, including immunoregulatory cytokines, cytokine receptors, chemotactic proteins, adhesion molecules, and other transcription factors. Although crucial for the induction of genes involved in inflammation, constitutive activation of the NF-κB pathway is often associated with cancer and chronic inflammatory diseases such as multiple sclerosis, inflammatory bowel disease, rheumatoid arthritis, and asthma (5-8).

NF-κB family members include five mammalian Rel proteins, p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), RelB, and c-Rel, and these subunits bind to form homo- and heterodimerized complexes (9, 10). All five proteins share a N-terminal Rel homology domain, a conserved stretch of 300 amino acids which mediates dimerization, DNA binding, nuclear localization, and interaction with IκBα, an inhibitory molecule of NF-κB. p50 and p52 are
synthesized from precursor molecules, p105 and p100, which contain ankyrin repeats (30 to 33-amino acid residue motifs that mediate protein-protein interactions) in their C-terminal regions. Processing of p105 and p100 by proteolytic cleavage is required to generate the mature transcription factors. In addition, p65, c-Rel, and RelB, but not p50 or p52, contain C-terminal transactivation domains. Schematic diagrams depicting the modular domains of each NF-κB family member and summarizing which dimerization pairs among family members are transcriptionally active or inactive are shown in **Fig. 1-1** and **Fig. 1-2**.

In the classical NF-κB signaling pathway, the p65 and p50 subunits heterodimerize and are sequestered in an inactive complex in the cytoplasm bound to IκBα (11, 12). Upon activation by pro-inflammatory stimuli such as tumor necrosis factor-alpha (TNF) or lipopolysaccharide (LPS), the IκB kinase (IKK) complex, which is composed of two functionally nonredundant kinases, IKK1 and IKK2 (13-15), as well as regulatory subunits NEMO and ELKS (16, 17), phosphorylates IκBα at Serine 32 and 36 (18), targeting it for ubiquitination and proteasomal degradation (19). This allows the p65/50 complex to translocate to the nucleus where p65 can then bind to the promoter regions of its target genes. A schematic diagram of NF-κB signaling and nuclear translocation is shown in **Figure 1-3**. p65 phosphorylation by PKA or MSK-1 at Serine 276 (20, 21) and also by ζPKC at Serine 311 (22) then aids
Figure 1-1. Overview of NF-κB family members. Schematic representation depicting modular domains of the five NF-κB family members, which can homodimerize or heterodimerize to form transcriptionally inactive or active complexes.
Figure 1-2. Diagram of NF-κB family member complex formations. Schematic representation depicting homodimerized or heterodimerized NF-κB family member complexes and summarizing which forms can bind DNA and which are transcriptionally inactive or active.
Figure 1-3. Schematic representation depicting canonical NF-κB signaling and nuclear translocation. NF-κB protein family members, p65 and p50, are located in the cytoplasm in an inhibitory complex associated with IκBα. Upon pro-inflammatory signals such as TNFα or LPS, the IKK complex phosphorylates IκBα, targeting it for ubiquitination and degradation by the proteasome, allowing the p65/p50 complex to translocate to the nucleus where p65 can bind in the promoter regions of NF-κB target genes to activate transcription.
in the recruitment of transcriptional activation machinery cofactor CREB-binding protein (CBP) and enables subsequent transcription of NF-κB target genes (23). One of these target genes is itself IκBα, and NF-κB mediated transcription of IκBα acts as an autoregulatory feedback loop, which can then repress NF-κB activity (24).

**Glucocorticoids and the glucocorticoid receptor**

The glucocorticoid receptor (GR) is a transcription factor that plays a fundamental role in metabolism, development, reproduction, and homeostasis, and is essential for the counteraction of pro-inflammatory and immune response activation by NF-κB. GR is a steroid hormone activated upon binding to its ligand, glucocorticoids, which are powerful anti-inflammatory agents used to control acute and chronic inflammation, and are among the most important and widely used drugs currently available. Dexamethasone (Dex), a synthetic glucocorticoid that activates GR upon binding, is a commonly prescribed anti-inflammatory drug used to relieve inflammation, infection, and immune diseases associated with NF-κB up-regulation (25-33).

Although dexamethasone and other synthetic glucocorticoids are among the strongest anti-inflammatory agents, there are unfortunately a number of negative side effects associated with long-term steroid use. Patients receiving continuous glucocorticoid treatment may show symptoms of
obesity, diabetes, hypertension, peptic ulcers, metabolic disorders, skin or muscle atrophy, or bone mineralization loss, and may ultimately exhibit signs of drug resistance (34-43). Until an alternative method of potent anti-inflammatory therapy is developed that minimizes these negative events, glucocorticoids remain the most effective and preferred form of treatment in many ailments related to over-activation of the NF-κB pathway.

Steroid hormone research began in the late 1800s and greatly expanded in the twentieth century. In 1950, Edward C. Kendall, Tadeus Reichstein, and Philip S. Hench were awarded the Nobel Prize in Medicine and Physiology for their discoveries on the structure and biological effects of glucocorticoids. Kendall and Reichstein independently isolated and determined the chemical structure of cortisol, the natural glucocorticoid, and Hench demonstrated its use as a therapeutic agent by showing the dramatic effects of cortisol administered to patients with arthritis (44, 45). In 1966, Allan Munck identified the receptor for glucocorticoids, and in 1985 (46, 47), Ron Evans and colleagues cloned the glucocorticoid receptor (GR), leading the way for thousands of molecular studies to determine how it functions (48).

Glucocorticoid synthesis is a tightly controlled process regulated via the hypothalamus-pituitary-adrenal (HPA) axis (49). Corticotropin-releasing hormone (CRH) is first secreted from parvocellular neurons in the paraventricular nucleus (PVN) of the hypothalamus during stress and in a
circadian way (50, 51). Elevated CRH levels stimulate adrenocorticotropic hormone (ACTH) production in, and secretion from, the anterior lobe of the pituitary gland. Increased ACTH expression then induces synthesis of cortisol (the natural glucocorticoid) from the adrenal cortex (located along the perimeter within the adrenal gland), where it is finally released into the bloodstream (52). Upon accumulation of elevated glucocorticoid levels in the blood, CRH and ACTH secretion and transcription is repressed, acting as a negative feedback loop leading to glucocorticoid homeostasis. A schematic representation of HPA axis regulation of glucocorticoid synthesis and release is shown in Fig. 1-4.

The glucocorticoid receptor (GR) is a member of the nuclear hormone receptor superfamily and is classified more specifically as a steroid receptor because of its ability to homodimerize and require ligand binding for its transcriptional activation (Fig. 1-5, bottom) (53). Other nuclear hormone steroid receptor family members include the mineralocorticoid receptor (MR), progesterone receptor (PR), estrogen receptor (ER), and the androgen receptor (AR) (53). These receptors share a similar modular structure including an unconserved N-terminal region, a highly conserved, central DNA binding domain (DBD), and a moderately conserved ligand binding domain (LBD) in the C-terminal region (Fig. 1-5, top) (54, 55).
Figure 1-4. Overview of glucocorticoid synthesis and release. Schematic representation depicting glucocorticoid regulation by the hypothalamic-pituitary-adrenal (HPA) axis. Stress and the circadian clock signal CRH release from the hypothalamus, which leads to ACTH secretion from the pituitary gland, which then allows production of glucocorticoids in the adrenal cortex. Glucocorticoid is released into the bloodstream, and accumulation of excess glucocorticoids suppresses ACTH and CRH transcription and secretion, creating a negative feedback loop.
Figure 1-5. Nuclear hormone receptor structure and classification. Schematic representations showing the modular domains common to nuclear hormone receptors (top) and how they are classified (bottom).
The N-terminal region is variable among receptors, with the exception of the activation function 1 (AF-1) domain, a region rich in negatively charged acidic amino acids (56, 57). AF-1 is a ligand-independent activation function required for transcriptional enhancement through the recruitment of co-factors and association with basal transcriptional machinery, and disruption of AF-1 decreases reporter gene expression (58). The central region consists of the DNA binding domain (DBD), which contains two zinc fingers, eight cysteine residues tetrahedrally organized around two zinc atoms. This configuration creates a three dimensional structure in which the N-terminal zinc finger recognizes DNA response elements, allowing receptor binding to DNA (Fig. 1-5, top). The C-terminal zinc finger is required for receptor dimerization (59). A variable hinge region is also located near the DBD, which typically contains a nuclear localization signal and enables receptor conformational changes (60). The C-terminal ligand-binding domain (LBD) is essential for high affinity binding of glucocorticoids (61). The LBD also contains the activation function 2 (AF-2) domain, which is exposed after a ligand binding-induced conformational change. Exposed AF-2 mediates interaction with co-activators, co-repressors, and can act cooperatively with AF-1 to mediate transcriptional activity (62, 63).

In the absence of ligand, GR is sequestered in the cytoplasm in an inactivating complex associated with chaperone molecules, heat shock protein 90 (HSP90) and heat shock protein 70 (HSP70) (64-66). GR action is further
regulated by co-chaperones including HSP56, HSP40, a low-molecular weight protein (p23), immunophilins FKBP51 or FKBP52, dynein, and BAG-1 (64-68). Upon passive diffusion of glucocorticoids through the plasma membrane, ligand binds and activates GR, causing hyperphosphorylation and initiating substitution of one immunophilin (FKBP51) for another (FKBP52), allowing GR to dissociate from its inhibitory complex (68). Recruitment of dynein, a transport protein, then enables GR to translocate to the nucleus through the nuclear pore via the microtubule network, where GR can then homodimerize and activate its target genes (65, 69). More recent studies have shown that co-chaperones can also affect GR nuclear activity by aiding in dynamic transcriptional regulatory complex disassembly (70-72). A schematic diagram of GR nuclear translocation is shown in Figure 1-6.

Transcriptional activation is initiated by GR homodimerization and binding to glucocorticoid response elements (GREs) in the promoter regions of GR target genes (73). GRE consensus sites consist of a palindromic sequence of two six base pair half-sites separated by a three base pair spacer. However, natural gene sequences recognized by GR at GREs can deviate from this consensus sequence by maintaining important contacts with the receptor through specific functional groups on critical nucleotides within each half site. Upon binding to GRE sites, GR then acts as a scaffold for the assembly of coactivator proteins, chromatin remodeling factors, and other proteins that
Figure 1-6. Schematic representation depicting GR signaling and nuclear translocation. GR is located in the cytoplasm in an inhibitory complex associated with heat shock protein 90 (HSP90) and other co-chaperones. Glucocorticoids undergo passive diffusion and upon binding to GR, induce substitution of FKBP51 for FKBP52, which enables dissociation of GR from HSP90 and other proteins located within the inhibitory complex. GR can then transport to the nucleus through the nuclear pore, where it can homodimerize and bind to GRE sites to activate gene transcription or bind as a monomer to nGRE sites and inhibit gene transcription.
directly or indirectly recruit the transcriptional activation machinery (74-76). Transcriptional repression by GR can also occur upon GR binding as a monomer to negative glucocorticoid response elements (nGREs), sites distinct from GRE consensus sequences (77-79). GR binding at nGREs, located within the promoter regions of genes, inhibits transcription by preventing binding and recruitment of other transcription factors, cofactors, and transcriptional activation machinery (80).

**Glucocorticoid receptor repression of NF-κB**

There are a number of proposed mechanisms of GR-mediated NF-κB repression, however these mechanisms are largely cell-type specific or context specific, cannot fully explain glucocorticoid’s anti-inflammatory effects, and a universal mechanism of repression has yet to be elucidated. Two leading hypotheses, referred to as transactivation and transrepression, have emerged to explain how glucocorticoids suppress inflammation. Transactivation, in general, refers to an increased rate of gene transcription. As a term used to describe glucocorticoid suppression of NF-κB, transactivation suggests that GR can directly target anti-inflammatory genes for transcription, and these genes then directly or indirectly modulate NF-κB activity and function. It is thought that transactivation is also responsible for the negative side effects associated with long-term steroid use. In contrast,
transrepression is a process where one protein impairs the activity of another protein through protein/protein interactions, and this model proposes that GR can repress NF-κB activity through protein/protein interactions of the two transcription factors. Many scientists believe that glucocorticoids repress NF-κB through both mechanisms. Recently however, there has been a shift toward the transrepression model, and an increasing number of reports imply that it may be a more predominant mechanism of NF-κB suppression by GR.

It has been hypothesized that GR can directly target anti-inflammatory genes for transcription, which can then repress the activation of NF-κB. Specifically, back-to-back papers in *Science* from Dr. Albert Baldwin, Jr. and Dr. Michael Karin’s groups reported that GR could directly induce transcription of IκBα, a known repressor of NF-κB (81, 82). As described earlier, an increase in IκBα synthesis leads to IκBα-mediated sequestering of NF-κB in the cytoplasm, thus preventing NF-κB translocation to the nucleus and activation of its target genes. In addition, elevated IκBα levels in vascular endothelial tissue from patients suffering from Crohn’s disease (a specific type of inflammatory bowel disease) were observed after glucocorticoid treatment (83). However, a number of conflicting studies since then have provided evidence that GR is not able to directly induce IκBα transcription (84, 85), suggesting that this proposed mechanism (IκBα transcription by GR) is cell-type specific and/or context specific. Additional studies have shown that
glucocorticoids also target heat shock proteins, ras chaperone proteins, caspases, and proteasomes, and these may then regulate IκBα levels (86-89).

A number of studies provide evidence of NF-κB repression by GR via transrepression. Physical association and functional antagonism has been shown in vitro between the p65 subunit of NF-κB and the glucocorticoid receptor (90-93). Endogenous GR and p65 interaction in the nucleus has also been shown, where GR tethers NF-κB, thereby inhibiting transcriptional activation of its target genes (94). In support of this model, a dimerization-defective mutant GR, which does not bind DNA nor activate transcription of GR target genes, is still able to repress NF-κB activity (95). In addition, these results have been validated in a dimerization-defective mouse model, which shows that the DNA binding is dispensable for Go’s anti-inflammatory activity (96-99). Together, these studies indicate that GR repression of NF-κB persists in the absence of transactivation.

Besides transactivation and transrepression, there are other proposed mechanisms of NF-κB repression by GR. It was discovered that GR could bind as a monomer to nGRE sites in the promoter regions of genes, thus inhibiting their transcription by preventing recruitment of transcriptional activation machinery and other cofactors (100, 101). This finding subsequently led to another potential mechanism of repression, that GR could bind as a monomer to nGRE sites in the promoter regions of NF-κB target genes, thus inhibiting
NF-κB binding and subsequent transcriptional activation. However, nGRE consensus sites are not detected in the promoter regions of the majority of NFκB-regulated pro-inflammatory genes, indicating that GR inhibition of NF-κB via binding in the promoter regions of NF-κB target genes is likely not an integral or prevailing mechanism of repression. In addition, it has also been hypothesized that GR and NF-κB can compete for cofactors CBP and p300, which aid in recruitment of the transcriptional activation machinery (102-105). However, p65 association with CBP was not disrupted upon addition of liganded GR, and a NF-κB mutant deficient in CBP recruitment was repressed as well as the WT molecule (106), implying that competition for cofactors is likely not a critical underlying mechanism of repression.

Other proposed mechanisms of NF-κB repression by glucocorticoids hypothesize a role for GR in post-translational modifications including acetylation/deacetylation, phosphorylation, and methylation. It has been proposed that GR can recruit histone deacetylase complexes (HDAC) to the promoter regions of NF-κB target genes. One study describes GR association with HDAC2 in vivo, and presents evidence that GR antagonist was able to block this interaction (107). In support of this hypothesis, disruption of HDAC2 activity by cigarette smoke in alveolar macrophages was found to increase cytokine expression and block GR transrepression (108). It is also possible that GR could inhibit p65 phosphorylation or increase its methylation.
Recently, GR was shown to counteract MSK-1 phosphorylation of p65 by causing a re-localization of MSK-1 to the cytoplasm, thus impairing transcription of NF-κB target genes (109), and GR has also been linked to alterations in DNA methylation status (110, 111). The role of GR in post-translational modification regulation of NF-κB, as well as in transrepression, transactivation, and co-factor competition and recruitment, continues to be explored. Though no universal mechanisms have yet been described, further insight into NF-κB repression by glucocorticoids will ultimately aid in the development of more effective forms of therapy to treat disorders and diseases associated with NF-κB up-regulation.

To enhance our understanding of glucocorticoid’s anti-inflammatory action, it will be important to identify novel genes that play a role in GR repression of NF-κB and to elucidate their mechanisms. The work presented in this thesis provides evidence of 25 novel regulators of GR-mediated NF-κB repression. In the following chapters of this dissertation, we report identification of these novel genes by RNAi screening and validate our findings in physiologically relevant cell lines by performing in vitro and in vivo assays using the mouse as a model system. Our studies reveal potential therapeutic targets which may lead to more effective anti-inflammatory therapies, indicate novel mechanisms of NF-κB repression by GR, and have implications for the study of NF-κB repression by glucocorticoids in the tumor microenvironment.
Chapter 2. Identification of p53 as a novel regulator of GR mediated NF-κB repression by high throughput RNAi screening

Background

Although a number of proposed mechanisms of repression have been described to explain how GR represses NF-κB, many are cell-type specific or context specific, and universal mechanisms of repression have yet to be clearly established. Discovery of novel genes involved in NF-κB repression by GR, elucidation of their mechanisms, and identification of universal mechanisms, will be critical for a more comprehensive understanding of how GR inhibits NF-κB, and will ultimately be important for improving glucocorticoids’ use in clinical applications. To address these issues, we used a RNAi-based screening approach to identify novel genes that regulate GR repression of NF-κB.

In this chapter, we describe optimization and development of our high throughput screening assay and analyze its potential to discover novel genes involved in NF-κB repression by GR. We report our list of candidate genes that may play a role in GR repression of NF-κB, and identify p53 as the number one gene of interest when genes of interest are ranked in order of significance by logP value. We also performed a number of bioinformatics analyses to further characterize our potential genes of interest, revealing that an over-
represented number of candidate genes play a role in p53-related pathways, reactions, and processes. These results suggest that p53, as well as the other genes in our list of candidate genes, may play a role in GR repression of NF-κB.

Results

High throughput siRNA oligonucleotide assay development and confirmation of screen efficacy to identify potential genes of interest in GR repression of NF-κB

Assay optimization is an essential component in the development of effective high throughput screening assays. Our first step was to find a cell line that gave maximum siRNA oligonucleotide (oligo) transfection efficiency. In initial optimization experiments, we tested seven different human cell lines, including U-937 and THP-1 cells, two monocytic cell lines, SW-480 cells, a colon cancer cell line, HUVEC cells, a type of endothelial cell line, U2-OS cells, an osteosarcoma cell line, and 293 and 293T cells, two embryonic kidney cell lines, for 80% or higher siRNA oligo transfection efficiency. We used varying amounts of Lipofectamine 2000, Lipofectamine LTX, Lipofectamine RNAiMAX, Targefect, HiPerFect, Fugene6, FugeneHD, and calcium phosphate transfection reagents and adjusted the time allowed for maximum siRNA oligo transfection efficiency (24, 48, 72, or 96 hours). Of all
cell lines tested using varying concentrations of transfection reagents, 293T cells were the only cells to give ≥80% transfection efficiency.

We then performed a NF-κB luciferase experiment in 293T cells to determine if we could measure GR repression of NF-κB activity in an assay, which could potentially be converted to a high throughput screening format. 293T cells were stably transfected with a NF-κB luciferase reporter, treated with 10ng/ml TNF +/- 10uM Dex, and luciferase activity was measured. As hoped, Dex addition suppresses TNF-induced NF-κB luciferase activity in this cell line (Fig. 2-1A), demonstrating that this assay has a potential for use in a high throughput screen.

The next step was then to develop screening parameters in 384-well plate format, which would enhance Dex repression of NF-κB luciferase activity, while still maintaining high siRNA oligo transfection efficiency. We tested cell number per well (250, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, or 6000 cells per well) to determine what condition gave us the highest luciferase values while still maintaining 80% transfection efficiency. Densities of both 2,000 and 2,500 cells per well exhibited high luciferase values while still maintaining 80% transfection efficiency (data not shown). We performed dose responses and varied the time of TNF +/- Dex stimulation to determine what concentrations gave maximum repression of NF-κB luciferase activity upon Dex addition. Cells treated for either 24 or 48 hours with Dex
Figure 2-1. Dex repression of TNF-induced NF-κB luciferase activity and predicted trends of siRNA controls observed in 293T cells (A) 293T cells stably transfected with a NF-κB luciferase reporter were treated with 10ng/ml TNF +/- 1μM Dex for 24 hours and subsequently read for luciferase activity. (B) 293T cells stably transfected with a NF-κB luciferase reporter were transfected with siGR, sip65, and si luciferase and treated for 24 hours in 384-well plate format.
exhibited a >2-fold repression in NF-κB luciferase activity (data not shown), and we chose a 24-hour time point to minimize off-target effects. We also confirmed that changes in luciferase activity upon TNF or TNF + Dex treatment were not due to cytotoxicity by performing Annexin V staining and FACS analysis (Fig. 2-2). Before proceeding with high throughput screening, we also performed final luciferase analyses using our optimized parameters in 384-well format and transfected siGR, sip65, and si luciferase as controls to validate our screen design (Fig. 2-1B). Control cells exhibited a >2-fold decrease upon Dex addition, and this repression was impaired in cells with a knockdown in GR. Knockdown of p65 and luciferase decreased NF-κB luciferase activity in both the TNF and TNF + Dex conditions (Fig. 2-1B), confirming that our positive and negative controls worked as expected in our screen design for 384-well format.

After this extensive optimization, we then performed our high throughput luciferase assay using four human siRNA oligo library collections to search for potential genes that play a role in NF-κB repression by the glucocorticoid receptor. The number of genes represented and characteristics of each of two kinase libraries and two druggable genome libraries we used for screening are summarized in Table 2-1. siRNA oligos from each of the libraries were pre-arrayed into 384-well plates. 293T cells stably transfected with a NF-κB luciferase reporter were plated at a density of 2,000 cells per
Figure 2-2. Neither TNF nor Dex-mediated changes in NF-κB luciferase activity are due to cytotoxicity. 293T cells stably transfected with a NF-κB luciferase reporter were treated as indicated for 24 hours. Cells were then resuspended in PBS + 2% FBS, incubated with Annexin V-APC antibody for 45 minutes, washed, stained with 7-AAD, and analyzed by FACS.
Table 2-1. Summary of siRNA library characteristics used in high-throughput screening. Library collection names are listed (left column) and information about the number of genes represented, the number of siRNA oligos targeting each gene, and the number of plates per condition in each siRNA library collection are provided.

<table>
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well, and reverse transfected for 48 hours using Lipofectamine 2000 (Fig. 2-3). Cells were then treated for 24 hours with 10ng/ml TNF or 10ng/ml TNF + 1uM Dex in duplicate and subsequently read for luciferase activity (Fig. 2-3). Raw values of every well in each 384-well plate were normalized to that plate’s average luciferase activity value.

Upon completion of the high throughput RNAi screen, we next wanted to assess its ability to successfully identify genes of interest. Screen efficacy was determined by luciferase analysis of siGR, sip65, si luciferase, and mock controls added to each of the 384-well plates. In the two kinase libraries, we see an average of >3-fold decrease in NF-κB luciferase activity upon Dex addition in mock treated cells (Fig. 2-4A). As expected, knockdown of GR impaired Dex repression of NF-κB luciferase activity, and knockdown of either p65 or luciferase, significantly impaired levels of NF-κB luciferase activity in both the TNF and TNF + Dex conditions (Fig. 2-4A).

Similar results were seen using the druggable genome library collections. siGR, sip65, si luciferase, and mock control luciferase values are depicted as the number of standard deviations away from that plate’s average luciferase value. As expected, cells with siGR exhibited an increase in NF-κB activity in the TNF condition and an even greater increase in the TNF + Dex condition (an average of >3 standard deviations higher than the plate average in this condition), and cells with p65 or luciferase knockdown exhibited a
Figure 2-3. Schematic representation of screen design. siRNA oligos, pre-arrayed into 384-well plates, were reverse transfected into 293T cell stably transfected with a NF-κB luciferase reporter for 48 hours using Lipofectamine 2000. Cells were then treated with 10ng/ml TNF or 10ng/ml TNF + 1μM Dex in duplicate for 24 hours and luciferase activity was measured upon addition of Bright Glo.
Figure 2-4. Screen efficacy analysis of controls confirms potential to identify genes of interest. (A) Average normalized NF-κB luciferase activity of sip65, siGR, si luciferase, and mock controls located in each 384-well plate of the Invitrogen kinase library (left) and the IDT kinase library (right). Average normalized values for controls in both conditions are shown. (B) Average normalized NF-κB luciferase activity of sip65, siGR, si luciferase, and mock controls located in each 384-well plate of the GNF druggable genome library (top) and Qiagen druggable genome library (bottom). Average normalized values for controls in each condition are shown, and the number of standard deviations from the average luciferase value of each condition is indicated. TNF condition (left) and TNF + Dex condition (right).
decrease in NF-κB luciferase activity in both conditions (Fig. 2-4B). Together, these results indicate that predicted changes in NF-κB luciferase activity were seen upon knockdown of GR, p65, and luciferase in all four siRNA oligo libraries, and provide validity that the screen could be used to successfully identify potential genes of interest (Fig. 2-4A and 2-4B).

Screen efficacy was also determined by replicate well analysis (Fig. 2-5). Since conditions in each screen were performed in duplicate, replicate normalized raw luciferase values (raw luciferase read-out number of a particular well on a plate was normalized to that plate’s raw luciferase read-out average as a whole) for each well were plotted against each other to visualize discrepancy among replicate wells. Quality scores were then assigned to the TNF and TNF + Dex condition for each of the siRNA library collections based on the number of replicate wells with dissimilarity between their raw normalized values and also by the extent of these differences.

In general, quality scores to determine screen efficacy based on replicate well value dissimilarity were good, indicating that results from each of these screens could successfully identify potential genes of interest (Fig. 2-5). More specifically, quality scores of conditions in the IDT kinase screen were very good (Fig. 2-5A). Quality scores in the Invitrogen kinase screen, the GNF druggable genome screen, and in the TNF + Dex condition of the Qiagen druggable genome screen were good (Fig. 2-5B, 2-5C, and 2-5D). A high
Figure 2-5. Screen efficacy analysis of replicate wells confirms potential to identify genes of interest. Replicate values of each well in each condition of the (A) IDT kinase library, (B) Invitrogen kinase library, (C) GNF druggable genome library, and (D) Qiagen druggable genome library are plotted to interpret the extent of dissimilarity between duplicate wells. Quality scores were assigned based on the number of divergent replicate values and by the extent of their differences.
quality score was assigned to the TNF condition of the Qiagen druggable screen, but this can be explained due to an error in the automated dispersion of media + cells into 8 plates in the first step of the assay (Fig. 2-5D, left). To adjust for this discrepancy, results from those eight plates were essentially “thrown out” and raw normalized values for those particular genes were only analyzed based on the unaffected replicate plates (no duplicate values for these genes). We conclude that our results from analysis of the relative behavior of controls, as well as the analysis of replicate well values in each condition, are sufficient to predict that we will be able to successfully identify potential genes of interest in GR repression of NF-κB.

**siRNA screen analysis identifies p53 as a gene of interest and reveals a number of p53-related pathways and processes over-represented in our list of candidate genes**

Redundant siRNA activity (RSA) analysis is a convincing statistical method to interpret data from large-scale RNAi screens while minimizing off-target effects (112). Of the 8,515 genes tested, we identified 290 genes as genes of interest by performing 3 RSA analyses. 99 genes of interest were identified by RSA analysis using all 4 siRNA libraries (Table 2-2), 85 genes of interest were identified using data from the two kinase libraries (Table 2-3), and 106 genes of interest were identified by RSA analysis using data from the
Table 2-2. RSA analysis using all 4 siRNA library collections. RSA analysis using all 4 library collections generated a list of 99 genes of interest specific to the TNF + Dex condition. Genes are ranked in order of significance by log \( P \)-value.

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Table 2-3. RSA analysis using the 2 kinase library collections. RSA analysis using the Invitrogen kinase library collection and the IDT kinase library collection generated a list of 85 genes of interest specific to the TNF + Dex condition. Genes are ranked in order of significance by log $P$-value.

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two druggable genome libraries (Table 2-4). Genes in each list are rank ordered according to log P-value, and each gene shown is specific to the TNF + Dex condition and did not significantly affect luciferase activity in the TNF alone condition. In addition, we created heat maps of the three RSA analyses to provide a graphical representation of the number of candidate genes specific to the TNF + Dex condition, as opposed to genes that were common to both conditions or specific to the TNF condition (Fig. 2-6).

Interestingly, the number one gene of interest based on log P-value by RSA analysis using all four library collections was p53 (logP < -27), a tumor suppressor gene that plays a critical role in cell cycle regulation and apoptosis, and is mutated in over 50% of human cancers (Table 2-2). 16 wells containing a siRNA oligo targeting p53 were included as a control in the Invitrogen kinase library collection. Unexpectedly, 14 of the 16 wells were significant outliers when comparing the average normalized raw values between the TNF and TNF and Dex conditions. Of all siRNA oligos tested in the Invitrogen kinase library (636 total genes represented), 10 wells containing a siRNA targeting p53 were in fact the greatest outliers (Fig. 2-7). This suggests that p53 may play a role in GR repression of NF-κB.

We then performed a number of analyses to identify pathways, reactions, and biological and metabolic processes over-represented in our list of candidate genes. Kyoto Encyclopedia of Genes and Genomes (KEGG)
Table 2-4. RSA analysis using the 2 druggable genome library collections. RSA analysis using the Qiagen druggable library collection and the GNF druggable library collection generated a list of 106 genes of interest specific to the TNF + Dex condition. Genes are ranked in order of significance by log $P$-value.

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Figure 2-6. Heat maps of RSA analyses depicting TNF + Dex specific genes of interest. (A) Heat map of hits specific to the TNF + Dex condition, specific to the TNF condition, and common to both conditions upon RSA analysis of all 4 siRNA oligo libraries. * indicates 99 candidate genes specific to the TNF + Dex condition. (B) Heat map of hits specific to the TNF + Dex condition, specific to the TNF condition, and common to both conditions upon RSA analysis of screens using the 2 kinase library collections. * indicates 85 candidate genes specific to the TNF + Dex condition. (C) Heat map of hits specific to the TNF + Dex condition, specific to the TNF condition, and common to both conditions upon RSA analysis of the 2 druggable genome library collections. * indicates 106 candidate genes specific to the TNF + Dex condition. Heat maps were generated based on power ranking.
Figure 2-7. p53 identified as a gene of interest in Invitrogen kinase library. Graphical representation of average normalized raw luciferase values of individual wells in the Invitrogen kinase library treated with TNF (y-axis) and TNF + Dex (x-axis). Red data points indicate 10 wells containing sip53, which were the largest outliers of all genes represented.
pathway analysis showed enrichment for 29 pathways with \( P \)-values < 0.05, including MAPK signaling (a pathway with known p53 and GR interactions), apoptosis, cell cycle, and 13 cancer-related pathways (Fig. 2-8A). Gene ontology (GO) analysis of the 290 genes showed enrichment for 114 biological process, cellular component, or molecular function categories with \( P \)-values < 0.001. Enrichment for a number of homeostasis, developmental, and metabolic processes, as well as apoptotic, cell cycle, and kinase activity categories were among the list of over-represented pathways (Fig. 2-8B).

We also completed Reactome analysis to determine which pathways were over-represented in our list of 290 candidate genes and to confirm results from KEGG pathway analysis. 181 genes, of the 290 analyzed, were not represented in the database, suggesting that a number of uncharacterized and/or unstudied genes may be acting to aid in GR repression of NF-\( \kappa \)B. Reactome analysis revealed that 109 events with \( P \)-values < 0.05 were over-represented, including enrichment for a number of events related to metabolism and MAPK signaling, as well as NF-\( \kappa \)B related signaling events (Fig. 2-9), confirming results obtained by KEGG pathway analysis (Fig. 2-8A).

Through the STRING database, a functional protein association network that depicts known and predicted protein-protein interactions, we analyzed protein-protein interactions for candidate genes located in MAPK signaling and cancer, two over-represented pathways identified by KEGG
Figure 2-8. KEGG and GO analysis of TNF + Dex specific genes of interest. (A) KEGG analysis. 290 genes of interest were analyzed to determine reactions and/or pathways that were statistically over-represented. 16 of 29 over-represented categories with $P$-value < 0.05 are shown. (B) GO analysis. Statistically significant over-representation of biological process, cellular component, and molecular function categories based upon gene ontology mapping of 290 potential genes of interest. 40 of 114 over-represented pathways with $P$-value < 0.001 are shown.
Figure 2-9. Reactome analysis of TNF + Dex specific genes of interest. Reactome analysis. Statistically significant reactions and pathways that are over-represented in the list of 290 potential genes of interest based upon Reactome mapping. 32 of 109 over-represented pathways with \( P \)-value < 0.05 are shown.
analysis of our 290 genes of interest (Fig. 2-10). We included GR in each STRING analysis to map known interactions with GR and the genes of interest shown in each pathway. p53, shown in red, was found in both the MAPK signaling pathway (Fig. 2-10, top) and the cancer pathways (Fig. 2-10, bottom).

Discussion

In this chapter, we reveal a list of 290 genes that may play a role in GR repression of NF-κB and identify a number of pathways, biological processes, and reactions that are over-represented among our genes of interest. We describe optimization of our high throughput screening assay using 4 siRNA library collections and development of screening parameters to maximize NF-κB luciferase repression by Dex while maintaining high transfection efficiency. Importantly, we confirm that our screen could be used to successfully identify genes of interest by showing that there were few replicate wells in each condition with divergent luciferase activity (Fig. 2-5) and by determining that cells with siGR, sip65, or si luciferase controls included in each 384-well plate exhibited predicted changes in NF-κB luciferase activity (Fig. 2-4).

Our high throughput screen utilizes a reporter system specific to NF-κB transcriptional activation. 293T cells stably expressed a reporter that contains a 3x-NFκB consensus sequence, sites which NF-κB uniquely will bind to.
Figure 2-10. STRING analysis of genes in over-represented pathways identified by KEGG analysis. STRING analysis depicting known protein-protein interactions among genes of interest in over-represented pathways, MAPK signaling (top schematic) and cancer pathways (bottom schematic). p53 protein is shown in red and GR is shown in green. Darker lines indicate greater evidence of interaction between two proteins.
NF-κB binding at these sites then leads to transcription of luciferase, which we can measure in a luciferase assay. This experimental setup allows us to identify genes specifically involved in NF-κB activity. Other studies examining changes in NF-κB target gene expression levels by qPCR or by FACS, while informative, can often not distinguish between NF-κB activation of genes vs. activation by other transcription factors (such as AP-1, another gene which regulates inflammatory and immune responses). In this way, we have developed a system, which can identify candidates unique to NF-κB transcriptional activity. When identifying potential genes involved in GR repression of NF-κB, it was also important to distinguish between genes that specifically affected Dex repression of NF-κB and those that affected luciferase activity in both the TNF and TNF + Dex conditions. Genes that significantly altered TNF-induced NF-κB luciferase activity were not included in our list of candidate genes, resulting in a list exclusive to GR repression of NF-κB.

The mechanisms explaining GR repression of NF-κB are complex and varied. Our RNAi screen results may point toward novel mechanisms, which can further elucidate GR’s anti-inflammatory role. Over half of the genes we identified as potential genes of interest were unrepresented in the STRING and/or Reactome databases, suggesting that a number of uncharacterized or unstudied genes may play a role in GR repression of NF-κB. This possibility is
interesting, as these novel genes may represent a number of entirely new therapeutic targets and reveal unknown mechanisms of GR repression. We also discovered that an over-represented number of genes in the MAPK signaling pathways, and in apoptosis and cell cycle regulatory processes (Fig. 2-8 and 2-9), may be involved in GR repression of NF-κB. Further studies to validate these genes of interest and to elucidate how they play a role in GR repression of NF-κB, will be important to enhance our understanding of glucocorticoid’s anti-inflammatory functions. Our studies to confirm and characterize a number of these candidate genes will be discussed in detail in chapter 4 of this dissertation.

Interestingly, p53 was identified as our number one gene of interest when candidates were ranked in order of significance by logP value (Table 2-2 and Fig. 2-7). To our knowledge, there are no published studies that explore or characterize a role for p53 in GR repression of NF-κB. It would be important to confirm p53 as a novel regulator of NF-κB repression by GR, and this finding, if validated, would have clear implications for the repression of the pro-inflammatory microenvironment associated with cancer and in tumors. Our studies to validate, characterize, and explore the role of p53 in GR repression of NF-κB are described in detail in chapter 3.

Although our data suggest that a number of genes may play a role in NF-κB repression by GR, we must also take note of our assay limitations. Our
candidate genes were identified by high throughput screening using 4 siRNA oligo library collections which were unfortunately, not validated. Although each library was created using algorithms which designed each siRNA oligo sequence to specifically target a individual gene, studies have not been performed to confirm that each siRNA targeting each gene is specific to that gene and that gene activity is knocked down at the mRNA and protein levels. In this way, we might be underestimating our list of genes of interest, because genes involved in NF-κB repression by GR may not have appeared to play a role because the siRNAs targeting them were not efficient in knocking down expression at the mRNA or protein level. Future studies on any of our genes of interest would need to include confirmation that the siRNA is knocking down that gene’s activity at the mRNA or protein level.

It is also possible that addition of a siRNA could elicit nonspecific effects. On occasion, a cell may mount an immune response when a specific siRNA sequence is mistaken as a viral by-product. A specific siRNA sequence might also mimic a structurally related microRNA, which could then unintentionally modulate gene expression and cause off-target effects. Future studies on any of our genes of interest would need to include assays utilizing other knockdown methods to confirm that a gene’s role in GR repression of NF-κB is not caused by a siRNA off-target effect.
Another limitation of our original high throughput screen is that the assay did not include luciferase activity normalization to LacZ. We are not able to differentiate whether luciferase activity changes were due to gene knockdown or whether they were because of siRNA toxicity and subsequent cell death. We cannot account for changes in cell number or determine the number of cells that were transfected. Further studies on any of our genes of interest in 293T cells should include transfection of a LacZ construct so that we can normalize our luciferase activity to the number of cells transfected.

Finally, our assay was performed in 293T cells, a cell line isolated from human embryonic kidney cells which expresses SV40 Large T-antigen and viral proteins. Although these characteristics allow for better cell growth and easier transfection efficiency, we would have ideally liked to perform our high throughput screen in a more physiologically relevant cell line. However, initial optimization studies in more physiologically relevant settings showed that siRNA transfection efficiency was not sufficient for high throughput screening in these cell types. Experiments to validate genes of interest should be performed in cells other than 293T cells in order to determine that their roles in GR repression of NF-κB are not due to SV40 Large T-antigen or viral protein over-expression.
Materials and Methods

Initial optimization for high throughput screening

A number of parameters were tested in 384-well format to maximize Dex repression of NF-κB luciferase activity while maintaining high siRNA oligo transfection efficiency. Optimization was performed by testing:

- seven different cell types including U-937 and THP-1 cells, two monocytic cell lines, SW-480 cells, a colon cancer cell line, HUVEC cells, a type of endothelial cell line, U2-OS cells, an osteosarcoma cell line, and 293 and 293T cells, two embryonic kidney cell lines. All cell lines were purchased from American Type Culture Collection.

- varying concentrations of eight different transfection reagents including of Lipofectamine 2000 (Invitrogen), Lipofectamine LTX (Invitrogen), Lipofectamine RNAiMAX (Invitrogen), Targefect (Targeting Systems), HiPerFect (Qiagen), Fugene6 (Roche), FugeneHD (Roche), and calcium phosphate.

- cell dispersion at plating densities of 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, or 6000 cells per well.

- TNF concentrations of 2ng/ml, 4ng/ml, 6ng/ml, 8ng/ml, 10ng/ml, 12ng/ml, 14ng/ml, 16ng/ml, 18ng/ml, and 20ng/ml.

- Dex concentrations of 0.5uM, 1uM 1.5uM, 2uM, and 2.5uM.
- TNF and TNF + Dex treatment lengths of 16 hours, 24 hours, or 48 hours.

**Annexin V Staining**

293T cells stably transfected with a NF-κB luciferase reporter were treated for 24 hours with 10ng/ml TNF, 10ng/ml TNF + 1uM Dex, 1uM Dex, 10uM Dex, or 100uM Dex. Cells were pelleted at 3,000 g for 5 minutes at room temperature, resuspended in 50ul DMEM + 10% FBS, and placed on ice. 5μl AnnexinV-APC antibody (Pharmingen) was added to each sample, and cells were mixed and incubated on ice for 20 minutes. Cells were washed in 1x PBS with 2% FBS and pelleted at 3,000 g for 5 minutes. Cells from each sample were then resuspended in 300ul cold PBS + 2% FBS, and 3ul 7-Aminoactinomycin D (7-AAD, 0.5mg/ml) was added. Cells were mixed and subsequently analyzed by FACS analysis.

**High throughput luciferase assay screening**

293T cells stably transfected with a 3x-NFκB-luciferase reporter were plated at a density of 2,000 cells per well in 384-well format. Cells were grown in 10% FBS in DMEM. 1-2 siRNAs per well were reverse transfected using Lipofectamine 2000 (Invitrogen) and cells were grown for 48 hours to allow for gene knockdown. After 48 hours, cells were treated with 10ng/ml TNF (Calbiochem) or 10ng/ml TNF + 1uM Dex (Sigma) for 24 hours and
subsequently read for luciferase activity using Bright Glo (Promega) reagent. Each condition was performed in duplicate.

**High throughput screen analysis**

3 lists of potential genes of interest were generated by RSA analysis performed on results from the two kinase screens, from the two druggable genome screens, and from data in all four screens combined. Genes that also significantly altered the TNF condition were excluded from our list of candidate genes. This generated a list of 290 genes of interest, which was then used in gene ontology (GO), Kyoto encyclopedia of genes and genomes (KEGG), Reactome, and STRING analysis to discover pathways, reactions, and biological processes over-represented in our data set.

**Acknowledgements**

We are grateful to our collaborators at the Genomics Institute of the Novartis Research Foundation (GNF), Gen Welch, Loren Miraglia, and Dr. Anthony Orth, as well as our collaborators at The Sanford-Burnham Medical Research Institute, Dr. Sumit Chanda and Paul De Jesus. They provided generous gifts of reagents for high throughput screening, gave us access to the 4 siRNA oligo library collections used (3 of which are commercially available and one which was developed exclusively at GNF), and enabled us to perform our assay using GNF’s automated plate processing, high
throughput screening core facility. In addition, Loren Miraglia performed RSA analysis and rank ordered genes in order of significance by logP value, and Gen Welch programmed the automated liquid dispersion and plate processing machinery with our assay protocol.

Chapter 2, in part, is currently being prepared for submission for publication of the material. Murphy, Samantha H; Suzuki, Kotaro; Downes, Michael; Welch, Genevieve L; De Jesus, Paul; Miraglia, Loren; Orth, Anthony P; Chanda, Sumit K; Evans, Ron M; and Verma, Inder M. RNAi screen reveals p53 as a novel regulator of NF-κB repression by the glucocorticoid receptor. The dissertation author was the primary investigator and author of this material.
Chapter 3. Validation and characterization of p53’s role in GR repression of NF-κB

Background

In chapter 2 of this dissertation, we developed a high throughput screen using 4 siRNA oligo library collections to discover novel genes that regulate NF-κB repression by GR. We then presented a list of candidate genes that may play a role in GR repression of NF-κB, and of the 8,515 genes tested, identify p53 as the number one gene of interest when candidate genes are ranked in order of significance by log$P$ value. Upon completion of bioinformatics analyses to further characterize our potential genes of interest, we also found that an over-represented number of candidate genes play a role in p53-related pathways, reactions, and processes. These results suggest that p53 may be a novel regulator of NF-κB repression by GR.

Considered to be one of the most widely studies proteins, p53 is a tumor suppressor that maintains the integrity of the genome by inducing apoptosis or cell cycle arrest upon cellular stress signals (113, 114). In response to DNA damage, hypoxia, oxidative or ribosomal stress, oncogene activation, or telomere erosion, p53 can activate genes that induce cell cycle regulation, DNA repair, senescence, or cell death (115, 116). p53 plays a critical role in the prevention of tumorigenesis, more than half of human
cancers contain mutations that inactivate p53, and loss of p53 function gives cancer cells a selective growth advantage.

p53 is a transcription factor that binds to its target genes as a homotetramer. The protein consists of two N-terminal transactivation domains (TAD1 and TAD2), followed by a proline rich region important for apoptotic activity. A DNA binding domain (DBD) is found in the central region of the protein, and an oligomerization domain, nuclear localization signals, and a regulatory domain comprise the C-terminal region (reviewed in 117 and 118). Upon nuclear accumulation, p53 can bind to its target genes at two decameric half-sites, spaced anywhere from 0 to 13 base pairs apart (119, 120), and transcriptionally activates genes that regulate cell cycle, DNA repair, and apoptosis (121).

p53 is tightly regulated by two oncoproteins, MDM2 and MDMX. MDM2 is an E3 ubiquitin ligase that targets p53 for proteasomal degradation (122-124), and both MDM2 and MDMX bind to the amino terminal transactivation domain of p53 to block its transcriptional activity (125-128). In response to cellular stress and DNA damage, various kinases including ataxia telangiectasia (ATM), ataxia telangiectasia and Rad3 related (ATR), checkpoint kinase 2 (Chk2), and DNA-dependent protein kinase (DNA-PK) can phosphorylate MDM2, MDMX, and p53 (129-138). This modulation releases p53 from its inhibitory MDM2 complex, enables p53 stabilization, and
allows p53 to activate its target genes for transcription (139). A negative feedback loop, in which p53 activation induces synthesis of MDM2, then negatively regulates p53 by targeting it for proteasomal degradation (140).

To our knowledge, a role for p53 in glucocorticoid repression of NF-κB has yet to be described, and because p53 mutations play such an important part in the development of human cancers, confirming p53 regulation of NF-κB suppression by GR would have interesting implications for treatment of pro-inflammatory microenvironments often linked to tumorigenesis. In this chapter, we validate p53 as a novel regulator of GR-mediated NF-κB repression. We confirm p53’s role by luciferase assays in physiologically relevant cell lines and reveal that p53 loss impairs GR repression of NF-κB target gene transcription, while upstream NF-κB signaling remains intact. We then demonstrate that p53 loss inhibits transcription of GR target genes, but does not alter GR nuclear translocation. Importantly, our studies show biological relevance in a mouse model of LPS shock, where p53 loss significantly impaired glucocorticoid rescue of mice from LPS shock-induced death.

Results

p53 validated as a novel mediator of NF-κB repression by GR

To confirm initial screening results, we transfected 293T cells stably expressing a NFκB-luciferase reporter with a RSV-LacZ construct and either
sip53, siGR, sip65, si luciferase, or a siRNA negative control, and treated cells with 10ng/ml TNF or 10ng/ml TNF + 1uM Dex. p53 knockdown was confirmed in this cell line by quantitative polymerase chain reaction (qPCR) (Fig. A-2A). Untransfected cells and cells transfected with a universal siRNA negative control exhibit a 1.9-fold and 1.6-fold respective decrease in NF-κB luciferase activity upon Dex addition (Fig. 3-1, first two conditions). In contrast, cells transfected with sip53 or siGR exhibit only a 1.1 and 1.2 respective decrease in NF-κB luciferase repression upon Dex addition (Fig. 3-1, third and fourth conditions). As expected, a decrease in NF-κB luciferase activity is seen in both the TNF and TNF + Dex conditions upon knockdown of either p65 or luciferase. We conclude that a decrease in p53 expression impairs GR-mediated repression of NF-κB luciferase activity in these cells.

To validate p53’s effect on glucocorticoid repression of NF-κB in a more physiologically relevant cell line, we transduced human monocytic THP-1 cells with a lentiviral 5x-NFκB luciferase reporter construct (Fig. A-1A) and either a lentiviral shp53-GFP vector or a lentiviral GFP vector as control (transduction efficiency shown in Fig. A-1B), and treated cells with 10ng/ml TNF or 10ng/ml TNF + 1uM Dex. GFP control cells showed more than a 3.5-fold decrease in NF-κB luciferase activity upon Dex addition (Fig. 3-2A, left). However, this repression upon Dex addition is lost in cells expressing shp53 (Fig. 3-2A, right). p53 knockdown was confirmed by qPCR (Fig. A-2B).
Figure 3-1. p53 confirmed as gene of interest in 293T cells. NF-κB luciferase assay in 293T cells transfected with sip53, siGR, si luciferase, si p65, or a siRNA negative control for 48 hours and treated with 10ng/ml TNF or 10ng/ml TNF + 1uM Dex for 24 hours.
Figure 3-2. p53 confirmed as gene of interest in physiologically relevant cell lines. (A) NF-κB luciferase assay in THP-1 cells transduced with a GFP control (left) or shp53-GFP (right). Cells were treated for 6 hours with 10ng/ml TNF +/- 1uM Dex. *** = $P$-value < 0.001. (B) NF-κB luciferase assay in WT (left) and p53KO MEF cells (right) following 24 hour treatment with 10ng/ml TNF +/- 1uM Dex. ** = $P$-value < 0.01. (C) NF-κB luciferase assay in p53KO MEF cells (left) and in p53KO MEF cells reconstituted with WT p53 (right) following a 6 hour treatment with 10ng/ml TNF +/- 1uM Dex. *** = $P$-value < 0.001.
To determine the effect of p53 loss on NF-κB repression by GR, we transduced WT and p53KO MEF cells with our lentiviral NF-κB luciferase reporter (Fig. A-1A) and treated cells with 10ng/ml TNF or 10ng/ml TNF + 1uM Dex. WT cells show more than a 1.3-fold decrease in NF-κB luciferase activity upon Dex addition (Fig. 3-2B, left). In contrast, NF-κB activity in p53KO MEF cells is restored in the presence of Dex (Fig. 3-2B, right). p53 loss was confirmed in these cells by qPCR (Fig. A-2C). Based on these results, we conclude that knockdown or loss of p53 impairs GR repression of NF-κB.

To determine if we could rescue Dex repression, we transduced p53KO MEF cells with a lentiviral-WT p53 construct and treated cells with 10ng/ml TNF or 10ng/ml TNF + 1uM Dex. p53KO MEF cells co-transfected with a RSV-LacZ construct and a lentiviral vector containing WT p53, show restored repression of NF-κB luciferase activity upon treatment with Dex (Fig. 3-2C).

p53 activation is often regulated by complex formation with murine double minute-2 (MDM2), an E3 ubiquitin ligase that inhibits p53 transcriptional activity (126). Nutlin-3 is a protease inhibitor that was found to specifically disrupt the MDM2/p53 interaction, consequently stabilizing p53 (141). To determine the effect of p53 stabilization on GR repression of NF-κB, THP-1 cells stably expressing an NF-κB luciferase reporter were treated for 24 hours with 10uM Nutlin-3, then treated for an additional 24 hours with 10ng/ml
TNF or 10ng/ml TNF + 1uM Dex. Cells that were not treated with Nutlin had an average of less than 1.7-fold decrease in NF-κB luciferase activity upon Dex treatment (Fig. 3-3A and 3-3B, left). In contrast, cells treated with Nutlin exhibited an average of more than a 2.3-fold decrease in NF-κB luciferase activity upon Dex treatment (Fig. 3-3A and 3-3B, right). These results suggest that stabilization of p53 enhances GR repression of NF-κB.

To further investigate the role of p53 in GR repression of NF-κB, we isolated bone marrow derived macrophages (BMDMs) from WT and p53KO mice, treated cells with 10ng/ml TNF +/- 1uM Dex or 10ng/ml LPS +/- 1uM Dex for 2 hours, and analyzed mRNA levels of NF-κB target genes. p53 loss was confirmed in these cells by qPCR (Fig. A-2D). An increase in cyclooxygenase (Cox2) mRNA levels is seen upon LPS stimulation, and a +2-fold repression is seen upon Dex addition (Fig. 3-4, left panel). However, Dex can no longer regulate Cox2 mRNA levels in p53KO cells. Cells also exhibited an increase in monocyte chemotactic protein-1 (MCP-1) mRNA levels upon LPS stimulation, and a +2-fold decrease upon Dex addition (Fig. 3-4, right panel). However, Dex repression of MCP-1 mRNA levels was impaired in the absence of p53. In addition, p53 loss impaired Dex repression of both TNF and LPS-induced mRNA expression levels of IP-10, an NF-κB specific target gene (Fig. 3-5A, top and bottom panels). We conclude that p53 loss disrupted repression of NF-κB target gene transcription by GR. Interestingly, Dex could still repress
Figure 3-3. Nutlin stabilization of p53 increases GR repression of NF-κB luciferase activity. (A) NF-κB luciferase assay of THP-1 cells stably expressing a NF-κB luciferase reporter treated for 24 hours with 10uM Nutlin-3 (right), then treated for an additional 24 hours with either 10ng/ml TNF or 10ng/ml TNF + 1uM Dex. (B) Average fold change (n=3) between TNF and TNF + Dex conditions in cells with Nutlin (right) or without (left). * = P-value < 0.05.
Figure 3-4. p53 loss impairs GR repression of NF-κB target gene transcription. qPCR analysis measuring mRNA levels of NF-κB target genes, Cox2 (left) and MCP-1 (right) in BMDMs treated with 10ng/ml LPS +/- 1uM Dex for 2 hours. mRNA levels normalized to Cyclophilin A.
LPS-induced TNF mRNA expression in the absence of p53 (Fig. 3-5B) suggesting the p53 regulates Dex-mediated transcriptional repression of some, but not all NF-κB target genes.

**p53 does not alter upstream NF-κB signaling**

We next wanted to analyze the effect of p53 loss on the upstream NF-κB signaling cascade. WT and p53KO MEF cells were treated with 10ng/ml TNF +/- 1uM Dex for 10, 20, 30, or 60 minutes, and the effect of p53 loss on IκBα degradation and phosphorylation kinetics was determined by immunoblot analysis. In WT cells, IκBα total protein levels decrease starting at the 10-minute time point upon TNF addition, and the protein is re-synthesized by the 60-minute time point (Fig. 3-6A, top panel, lanes 1-5). The degradation observed at the 10-minute time point correlates to an increase in IκBα phosphorylation (Fig. 3-6A, middle panel, lane 2). Degradation and phosphorylation remain unchanged in WT cells upon Dex addition (Fig. 3-6A, top and middle panels, lanes 6-10), and these kinetics are unchanged in p53KO cells (Fig. 3-6A, top and middle panels, lanes 11-20). Protein levels were normalized to p65 loading control (Fig. 3-6A, bottom panel) and quantified using imageJ (Fig. 3-6B). We conclude that p53 loss does not alter either IκBα phosphorylation or degradation.
Figure 3-5. p53 regulates GR repression of some, but not all NF-κB target genes. (A) qPCR analysis measuring mRNA levels of IP-10, a NF-κB specific target gene, in BMDMs treated with 10ng/ml TNF +/- 1uM Dex (top) or 10ng/ml LPS +/- 1uM Dex (bottom) for 2 hours. (B) qPCR analysis measuring TNF mRNA levels in BMDMs treated with 10ng/ml LPS +/- 1uM Dex for 2 hours. mRNA levels normalized to Cycophilin A.
Figure 3-6. p53 loss does not alter IκBα phosphorylation or degradation kinetics. (A) Western blot analysis measuring total protein levels of IκBα (top panel) and phosphorylated IκBα (middle panel). p65 total protein levels (bottom panel) are shown to indicate loading control. WT MEF cells (left) and p53KO MEF cells (right) were treated for 10, 20, 30, or 60 minutes with 10ng/ml TNF +/- 1uM Dex as indicated. (B) Graphical representation of quantified total IκBα protein levels (top) and phosphorylated IκBα protein levels (bottom) normalized to the p65 loading control. Quantification performed using ImageJ.
We then evaluated the effect of p53 loss on endogenous p65 translocation. BMDMs were isolated from WT and p53KO mice, treated for 30 minutes with 10ng/ml TNF +/- 1uM Dex, and fixed with 4% paraformaldehyde. p65 nuclear accumulation is seen upon TNF treatment and remains unchanged upon Dex addition in WT cells (Fig. 3-7A, left). Similarly, we can see nuclear accumulation of endogenous p65 after treatment with TNF and after treatment with TNF + Dex in p53KO cells (Fig. 3-7A, right). We then wanted to quantify the number of cells with nuclear p65 staining in each condition. Of 100 cells counted in WT cells (n=3), 93-95% showed nuclear accumulation in the TNF and TNF + Dex conditions (Fig. 3-7B, left). p53KO cells exhibited a similar percentage of cells with p65 nuclear accumulation in each condition (Fig. 3-7B, right). We conclude that p53 loss does not alter p65 translocation to the nucleus.

p65 is phosphorylated at Serine276 by PKA and MSK1 (20, 21) and at Serine311 by ζPKC (22), two sites of phosphorylation that are important for NF-κB transcriptional activation. To analyze the effect of p53 loss on endogenous p65 phosphorylation at these two sites, we performed endogenous immunofluorescence on WT and p53KO bone marrow derived macrophages treated with 10ng/ml TNF or 10ng/ml TNF + 1uM Dex for 30 minutes. An increase in endogenous p65 phosphorylation at Ser311 is seen upon TNF treatment and remains unchanged upon Dex addition in both WT.
Figure 3-7. p53 loss does not alter p65 translocation. (A) Immunofluorescence of endogenous p65 expression in WT (left) and p53KO (right) BMDMs treated with 10ng/ml TNF +/- 1uM Dex for 30 minutes as indicated. (B) Quantification of p65 nuclear accumulation in WT (left) and p53KO (right) BMDMs. Average number of cells (n=100) in each condition with endogenous nuclear p65 accumulation is shown.
and p53KO BMDMs (Fig. 3-8A). An increase in endogenous p65 phosphorylation at Ser276 is also seen upon TNF and TNF + Dex treatment in both WT and p53KO cells (Fig. 3-8B). Together, these results suggest that p53 loss does not alter GR repression of NF-κB by affecting NF-κB phosphorylation at these two sites.

**p53 does not alter upstream GR signaling but is required for GR-mediated transcription**

To determine the effect of p53 loss on endogenous GR translocation, BMDMs were isolated from WT and p53KO mice, treated for 30 minutes with 1μM Dex, and fixed with 4% paraformaldehyde. GR nuclear accumulation is seen upon Dex treatment in WT cells (Fig. 3-9, top panels). In p53KO cells, we can see nuclear accumulation of endogenous GR upon treatment with Dex as well (Fig. 3-9, bottom panels). We conclude that p53 loss does not play a role in GR nuclear translocation upon ligand stimulation.

We then wanted to evaluate the effect of p53 loss on GR-mediated transcription. WT and p53KO BMDMs were treated for 2 hours with Dex and qPCR was performed to analyze changes in GR target gene mRNA levels. In WT cells, there is significant increase in mRNA levels of GR target genes, Mt2 and Fkbp5, upon Dex addition (Fig. 3-10A). In contrast, the increase in mRNA
Figure 3-8. p53 loss does not alter p65 phosphorylation at site Serine311 or Serine276. (A) Immunofluorescence of endogenous p65 phosphorylation at Serine311 in WT (left) and p53KO (right) BMDMs treated with 10ng/ml TNF +/- 1uM Dex for 30 minutes. (B) Immunofluorescence of endogenous p65 phosphorylation at Serine276 in WT (left) and p53KO (right) BMDMs treated with 10ng/ml TNF +/- 1uM Dex for 30 minutes.
Figure 3-9. p53 loss does not alter GR translocation. Immunofluorescence of endogenous GR localization in WT (top panels) and p53KO (bottom panels) BMDMs treated with or without 1uM Dex for 30 minutes.
Figure 3-10. p53 loss impairs transcription of GR target genes. (A) qPCR analysis measuring mRNA levels of GR target genes, Mt2 (left) and Fkbp5 (right) in BMDMs treated with or without 1µM Dex for 2 hours. (B) Time course showing GILZ mRNA levels measured by qPCR in BMDMs isolated from WT and p53KO mice and treated with or without Dex for each time point as indicated. mRNA levels normalized to Cyclophilin A.
transcription levels of Mt2 and Fkbp5 upon Dex addition, is severely impaired in p53KO cells (Fig. 3-10A).

We next wanted to analyze changes in mRNA levels of GILZ, a transcriptional target of GR known to repress NF-κB upon treatment with Dex (142-146). WT and p53KO BMDMs were treated with 1μM Dex for 30 minutes, 1hr, 2hrs, 4hrs, and 6hrs and GILZ mRNA levels were analyzed. Basal mRNA levels of GILZ were similar in WT and p53KO cells throughout the 6-hour period (Fig. 3-10B). However, Dex-induced expression of GILZ over time is impaired in p53KO cells as compared to relative mRNA levels in WT cells (Fig. 4D). From these results, we conclude that a loss in p53 impairs transcription of GR target genes.

**Biological significance confirmed in vivo**

To investigate the effect of p53 loss on GR repression of NF-κB in vivo, we developed a mouse model of LPS shock. WT and p53KO mice treated with 50mg/kg LPS or 50mg/kg LPS + 10mg/kg Dex by intraperitoneal (IP) injection were monitored for death by LPS shock every 6 hours for the first 54 hours and every 24 hours for the subsequent 4 days (7 day monitoring period total). All but one WT (n=11) and all p53KO (n=8) mice treated with LPS alone, succumbed to LPS shock within 36 hours of treatment, and the last surviving WT mouse treated with LPS died within 54 hours (Fig 3-11). Glucocorticoid
Figure 3-11. p53 loss impairs glucocorticoid rescue of LPS-induced death in a mouse model of LPS shock. Kaplan-meier survival curve analysis measuring death from LPS shock. Survival of WT and p53KO C57BL6 mice treated with 50mg/kg LPS +/- 10mg/kg Dex by IP injection is shown. Death was recorded every 6 hours for the first 54 hours, and every 24 hours up to 7 days.
treatment in 9 of 12 WT mice, rescued these animals from death (Fig. 3-11). In contrast, only 3 of 14 p53KO mice treated with LPS + Dex survived (Fig. 3-11). The Kaplan-meier survival curve shows these trends in Figure 3-11, as 75% of WT mice and only 21% of p53KO treated with Dex survived death by LPS shock.

We also wanted to analyze the effect of p53 loss on LPS shock-induced reduction in body temperature. Body temperatures from WT (n=3) and p53KO (n=3) C57BL6 mice sham treated with PBS by IP were analyzed every 6 hours over a 48-hour period using a rectal thermometer probe. The maximum recorded temperature decrease over that period of time by one individual mouse was 1.1° Celsius (Fig. 3-12A). Temperature measurements were also recorded using a rectal thermometer probe every 6 hours over a 48 hour period and for every subsequent 24 hours up to 5 days total for WT and p53KO mice treated with 50mg/kg LPS +/- 10mg/kg Dex by IP injection. 11 of 12 WT mice and all p53KO mice (n=8) treated with LPS exhibited body temperature decreases of at least 12° Celsius within 12 hours (Fig. 3-12B). One WT mouse treated with LPS did not show a 12° temperature decrease until 30 hours post IP injection (Fig. 3-12B, left). 9 of 12 WT mice treated with LPS + Dex exhibited varying degrees of temperature decrease from LPS shock, but these mice recovered to within 1° Celsius of their starting temperature within 5 days (Fig. 3-13, top). In contrast, only 3 of 14 p53KO
Figure 3-12. Time course of temperature changes in mice sham treated with PBS or treated with LPS. (A) Temperature analysis of WT (left) and p53KO mice (right) sham treated with PBS, n=3. (B) Temperature analysis of WT (left) and p53KO mice (right) treated with 50mg/kg LPS by IP injection. Temperature measurements recorded every 6 hours over a 48-hour period and every 24 hours up to 5 days post-treatment using a rectal thermometer probe.
Figure 3-13. **Time course of temperature changes in mice treated with LPS + Dex.** Temperature analysis of WT (top) and p53KO mice (bottom) treated with 50mg/kg LPS + 10mg/kg Dex by IP injection. Temperature measurements recorded every 6 hours over a 48-hour period and every 24 hours up to 5 days post-treatment using a rectal thermometer probe.
mice treated with LPS + Dex were able to recover to their starting temperature (Fig. 3-13, bottom).

*In vivo* luciferase imaging was also performed to further characterize the effect of p53 loss in a mouse model of LPS shock. WT and p53KO NF-κB luciferase reporter mice were treated with 15mg/kg LPS +/- 5mg/kg Dex by IP injection for 6 hours and imaged using the Xenogen IVIS-100. NF-κB luciferase expression was visible in the hands, feet, mouth, and tail extremities of the WT mouse treated with LPS, and a decrease in luciferase expression at these locations is visible upon Dex addition in the WT mouse to its right (Fig. 3-14, left panel). NF-κB luciferase expression was visible in the hands and mouth extremities, and high expression was seen in the liver area of the p53KO mouse treated with LPS (Fig. 3-14, right panel). In contrast, the p53KO mouse treated with Dex exhibited a decrease in NF-κB luciferase expression in the liver area, but had increased NF-κB luciferase expression in the mouse, hands, feet, and tail areas (Fig. 3-14, right panel). From these results, we conclude that p53 loss impaired GR repression of NF-κB *in vivo* in a mouse model of LPS shock.

**Discussion**

In this chapter, we have validated p53 as a novel regulator of NF-κB repression by GR, a novel finding that to our knowledge, has yet to be
Figure 3-14. NF-κB luciferase *in vivo* imaging analysis. *In vivo* luciferase imaging of WT (left) and p53KO (right) mice treated with PBS, LPS, or LPS + Dex as indicated. Images shown are a 1-minute acquisition time following 6-hour treatment. Images were taken on Xenogen IVIS-100 and are representative of n=4.
described. We originally identified a potential role for p53 in GR repression of NF-κB through high throughput screening using siRNA oligo libraries (chapter 2). Here, we provide further evidence of p53 regulation in GR repression of NF-κB by qPCR and luciferase assays in physiologically relevant cell lines and in primary cells. Our studies also show that NF-κB and GR upstream signaling cascades remain intact in the absence of the p53, but that p53 loss strongly impairs Dex-mediated transcription of GR target genes in bone marrow derived macrophages.

Importantly, we also confirm this important observation in vivo, and our studies show biological relevance in a mouse model of LPS shock. Dex rescue of LPS-induced death is strongly impaired in p53KO mice (Fig. 3-11), and we confirm this observation in a supplemental experiment using NF-κB reporter mice and by performing in vivo luciferase imaging (Fig. 3-14). We cannot explain why the p53KO mouse treated with LPS exhibits strong NF-κB expression in the liver area, an observation not seen in the WT mouse treated with LPS. Nor can we reconcile the lack of NF-κB luciferase expression observed in the tail region of this p53KO mouse, when the expression is clearly visible in the WT mouse and in the p53KO mouse treated with LPS + Dex. These differences in expression may be attributed to a mixed genetic background. Although the mice shown are littermates, C57BL6 mice were only back-crossed 3 or 4 times, and we were not able to perform our experiment
after the 7 back-crosses suggested to obtain a pure mouse strain. In the future, we would like to repeat this experiment and perform in vivo luciferase imaging once a pure C57BL6 background has been obtained.

In our studies to elucidate mechanism, we demonstrated that p53 loss has no effect on upstream NF-κB or GR signaling, but our data does suggest that p53 loss inhibits transcription of GR target genes (Fig. 3-10). We show data depicting changes in mRNA levels of three GR target genes in WT and p53KO bone marrow derived macrophages upon Dex treatment, but in fact we tested seven target genes. Compared to WT cells, p53KO cells showed a significant decrease in induction of mRNA expression levels of all seven genes tested (data not shown). We believe this strongly suggests an important role for p53 in glucocorticoid-mediated transcriptional activation. These results imply a possible mechanism of p53’s function in GR repression of NF-κB. Ultimately however, we were not able to fully elucidate mechanism to determine exactly how p53 is involved in NF-κB by glucocorticoids.

A number of experiments were performed, unsuccessfully, in an attempt to answer how p53 is aiding in GR repression of NF-κB. Three mutant p53 constructs were cloned from a lentiviral WT p53 construct by site directed mutagenesis to determine which domains of p53 were required for GR repression of NF-κB. One construct contained a deleted N-terminal transactivation domain, one p53 mutant construct lacked the DNA binding
domain located in the core region of the protein sequence, and the final p53 mutant had a C-terminal truncation, which deleted the three nuclear localization signals. Concentrated lentivirus was made for the WT p53 and the three mutant p53 constructs, however initial experiments have thus far been ineffective.

Immortalized p53KO MEF cells were transduced with a lentiviral NFκB-luciferase-mPGK-cherry reporter construct and sorted for the mcherry positive population. There was no decrease in NF-κB luciferase activity upon Dex addition in p53KO cells (Fig. 3-2B), and the repression by glucocorticoids was restored upon lentiviral transduction of the WT p53 construct (Fig. 3-2C). However, lentiviral transduction of the three mutant constructs did not give a clear indication as to which could or could not restore NF-κB repression by GR (data not shown). Because Dex repression of NF-κB was limited in MEF cells, it was difficult to determine whether the margin of change in NF-κB luciferase repression upon mutant p53 transduction was significant. Primary bone marrow derived macrophages and kidney cells were also isolated from p53KO mice in an attempt to determine which p53 regions were required for glucocorticoid repression of NF-κB, however these primary cells could not be maintained long enough in culture to perform our experiments. We were therefore unable to determine which p53 domains were required for GR anti-inflammatory effects. In the future, we would like to transduce primary p53KO
bone marrow derived macrophages with WT p53 and the three mutant constructs, treat cells with TNF +/- Dex or LPS +/- Dex and determine the effect of each mutant on NF-κB target gene mRNA expression levels by qPCR.

We also performed immunofluorescence studies to determine the effect of p53 loss on endogenous p65 and GR co-localization. Bone marrow derived macrophage (BMDM) cells were isolated from WT and p53KO mice, and treated for 30 minutes with TNF or TNF + Dex. In our initial studies, endogenous p65 and GR nuclear co-localization increased upon addition of TNF or TNF + Dex in WT cells, and p53KO cells appeared to exhibit a significant decrease in p65 and GR co-localization in these conditions (data not shown). These observations could have possibly explained how p53 may be functioning in GR repression of NF-κB, that p53 might be playing a role in GR and NF-κB protein/protein interactions and tethering, causing subsequent repression of NF-κB target gene transcription. However, incubation with our GR and p65 antibodies created a speckled phenotype in these cells, and the efficacy and specificity of each antibody were called into question. Antibody specificity was verified by knockdown of GR and p65 respectively (data not shown), and HeLa cells incubated with the GR and p65 antibodies exhibited a more diffuse pattern of protein expression, indicating that the speckled phenotype may be cell type specific. Ultimately however, this data alone could
not be used to hypothesize p53’s role in GR repression of NF-κB. To further analyze the effect of p53 loss on GR and p65 co-localization by immunofluorescence, we could isolate other primary cell types from WT and p53KO mice in which p65 and GR protein expression may appear more diffuse. In addition, we could use a siRNA targeting p53 in established cell lines to determine the effect of gene knockdown on GR and p65 co-localization. Finally, we could continue optimization experiments in BMDM cells by using a number of other commercially available antibodies in an effort to achieve a more diffuse GR and p65 protein expression pattern.

We also struggled to analyze the effect of p53 loss on endogenous p65 and GR interaction by co-immunoprecipitation experiments. BMDMs were isolated from WT and p53KO mice, and cells were treated for 30 minutes or 1 hour with TNF or TNF + Dex. We tested antibodies for pull-down of p65 and GR individual proteins, and although we do believe we detected GR and p65 interaction, we were not able to observe changes in endogenous p65 and GR interaction between the different conditions. This observation is difficult to reconcile, as we would have expected to see little interaction in the no treatment condition and greatest interaction in the TNF + Dex condition of WT cells. Perhaps, further testing of different antibodies is needed to optimize conditions for this experiment. In addition, our starting material of BMDM primary cells from WT and p53KO mice was limited (12 mice were needed for
all the conditions included in one co-IP experiment). Future studies on more readily available cells may be useful, and we may need to employ p65 and GR overexpression if we cannot observe the expected changes of p65 and GR interaction in control cells.

We also harvested tissues from WT and p53KO mice to determine whether p53 loss affected neutrophil marginalization. Mice were I.P. injected with PBS, 15mg/kg LPS, or 15mg/kg LPS + 5mg/kg Dex, and lungs, spleen, kidneys, and the liver were isolated 24 hours later. Polymorphonuclear infiltration in the lungs, spleen, and kidneys varied greatly between animals treated with LPS or LPS + Dex, and a decrease in neutrophil marginalization in animals treated with Dex was difficult to measure due to large standard deviations. In liver tissues, LPS treatment caused a marked infiltration of neutrophils within the hepatic sinusoids in both WT and p53KO mice, and this infiltration is milder in Dex treated mice (Fig. 3-15A and Fig. 3-15B). Although these results indicate that at this time point, at these doses, and in this tissue, p53 does not appear to affect neutrophil marginalization, we cannot comment fully on its role in this process. We would need to study this observation further by analyzing the effect of p53 loss when mice were treated for shorter or longer amounts of time, and it would be ideal to optimize conditions in which the standard deviations of neutrophil marginalization in lung, spleen, and kidneys were smaller.
Figure 3-15. p53 loss does not appear to alter Dex repression of LPS-induced neutrophil marginalization in liver tissue after 24 hours. (A) Representative histology images of liver sections from WT mice treated with PBS, 15mg/kg LPS, or 15mg/kg LPS + 5mg/kg Dex for 24 hours. HE staining of liver sections, arrows indicate sites of neutrophil infiltration. (B) Average quantification of neutrophil sites of marginalization in liver tissue sections from WT and p53KO mice IP injected with PBS, 15mg/kg LPS, or 15mg/kg LPS + 5mg/kg Dex.
In addition, we attempted to analyze the effect of p53 loss on ICAM-1 expression levels \textit{in vivo} by FACS analysis. WT and p53KO mice were sham treated with PBS, I.P. injected with 15mg/kg LPS for 4 hours, or treated with 15mg/kg LPS for 4 hours with a 1-hour pre-treatment of 5mg/kg Dex. Spleens were then harvested, and splenocytes from WT and p53KO mice were isolated to measure ICAM-1 expression levels in granulocyte, CD4(+), and CD4(-) cell populations. Unfortunately, standard deviations in all three cell populations treated with PBS, LPS, and LPS + Dex were too large to quantify the effect of p53 loss on Dex repression of ICAM-1 expression levels (data not shown). In the future, we would like to optimize these conditions so that the repression of ICAM-1 expression levels upon Dex addition is increased and the extent of standard deviation is each group is decreased.

\textbf{Materials and Methods}

\textbf{Luciferase experiments for validating screen results}

293T cells (American Type Culture Collection) were grown in 10\% FBS in DMEM. 293T cells were stably transfected with a 3x-NFkB-luciferase reporter and co-transfected with a RSV-lacZ construct and a sip53 oligo (Invitrogen), siGR (Invitrogen), sip65 (Invitrogen), si luciferase (Invitrogen), or a stealth RNAi negative universal control (Invitrogen). 48 hours following transfection with Lipofectamine 2000, cells were then treated with 10ng/ml
TNF or 10ng/ml TNF + 1uM Dex for 24 hours. Cells were washed with PBS and resuspended in 5X lysis buffer (Promega). Luciferase assay and normalization to LacZ transfection was then performed using Steady Glo (Promega) and Beta Glo (Promega) reagent.

THP-1 cells (American Type Culture Collection) were grown in 10% FBS in RPMI-1640 according to the manufacturer’s instructions. Cells were transduced with a concentrated lentiviral 5x-NFκB-luciferase-mPGK-mcherry reporter construct (Appendix Fig. 1A) and EF1α-GFP or EF1α-shp53-GFP. Cells were sorted for the mcherry and GFP double positive cell population (Appendix Fig. 1B). Cells were then treated with 10ng/ml TNF or 10ng/ml TNF + 1uM Dex for 6 hours, and resuspended in 5x lysis buffer (Promega) following a 5-minute centrifugation at 5000g. Luciferase assay was performed using Steady Glo reagent.

MEF cells were grown in 10% FBS in DMEM. WT and p53KO MEF cells were transduced with concentrated lentiviral 5x-NFκB-luciferase-mPGK-mcherry and sorted for the mcherry positive cell population. Cells were treated with 10ng/ml TNF or 10ng/ml TNF + 1uM Dex for 24 hours, and resuspended in 5x lysis buffer (Promega) following a 5-minute centrifugation at 5000g. Luciferase assay was performed using Steady Glo reagent.

p53KO MEF cells were transduced with concentrated lentiviral 5x-NFκB-luciferase-mPGK-mcherry and sorted for the mcherry positive cell
population. Cells were then co-transfected with RSV-LacZ and a lentiviral vector construct containing WT-p53. Cells were treated with 10ng/ml TNF or 10ng/ml TNF + 1uM Dex for 24 hours and luciferase assay was performed using Steady Glo reagent.

THP-1 cells expressing 5x-NFκB-luciferase-mPGK-mcherry were pre-treated for 24 hours with 10uM Nutlin (Sigma) and subsequently treated for another 24 hours with 10ng/ml TNF or 10ng/ml TNF + 1uM Dex. Luciferase assay was performed using Steady Glo reagent.

qPCR

Bone marrow derived macrophages were isolated from C57BL6 WT and p53KO mice and grown in 20% FBS in DMEM. After differentiation for 5 days with macrophage colony stimulating factor (M-CSF, R&D Systems), cells were treated with 10ng/ml LPS (Sigma) +/- 1uM Dex, 10ng/ml TNF +/- 1uM Dex, 1uM Dex alone, or left untreated for 2 hours. Total mRNA was extracted using TRIzol reagent (Invitrogen Life Technologies), qPCR was performed using SYBR green PCR mastermix (Applied Biosystems), and mRNA expression levels were normalized to mRNA levels of the housekeeping gene, Cyclophilin A. qPCR primer pair sequences available upon request.

Western analysis

Cells were washed and harvested in PBS, pelleted, and incubated with lysis buffer (PBS containing 1% NP40, 0.1% SDS, 0.25% Triton X-100, and 1X
Complete protease inhibitors (Roche)) for 30 min on ice. Lysates were cleared by centrifugation at 14,000g for 10 minutes at 4°C and protein concentration was measured by the Bradford assay (Biorad). Proteins were electrophoresed on poly-acrylamide gels and transferred to nitrocellulose membranes. Pre-cast 4-12% Bis-Tris gels (Invitrogen) were used to resolve phosphorylated IκBα and total IκBα protein. Novex Mini-cell gel and X-Cell 2 transfer apparatus (Invitrogen) was utilized according to manufacturer instructions. Membranes were blocked in PBS-T (PBS with 0.15% Tween-20) with 3% BSA for 1 hour at room temperature. Blots were rinsed and incubated with a mouse monoclonal Phospho-IκBα antibody (Cell Signal, 9246) or rabbit polyclonal anti-IκBα (Santa Cruz, sc-371), in PBS-T with 3% BSA overnight at 4°C. After washing with PBS-T, blots were incubated with the secondary antibody anti-rabbit (GE Healthcare) or anti-mouse (Santa Cruz) conjugated to peroxidase in PBS-T with 3% BSA for 1 hour at room temperature. After extensive washing with PBS-T, blots were treated with ECL or ECL Plus (Amersham) per the manufacturer’s instructions and exposed to film (Kodak X-omat) for varying amounts of time.

**Immunofluorescence**

Bone marrow derived macrophages isolated from WT and p53KO mice were grown in 20% FBS in DMEM, and differentiated for 5 days with M-CSF. Cells were grown on glass coverslips in 24-well dishes and treated for 30
minutes as described with either 10ng/ml TNF, 10ng/ml TNF + 1uM Dex, or 1uM Dex. The cells were then washed with phosphate-buffered saline (PBS) and fixed at room temperature with 4% paraformaldehyde. Cells were incubated with either rabbit polyclonal anti-p65 detecting phospho S311 (Abcam, ab51059), rabbit polyclonal anti-p65 detecting phospho S276 (Abcam, ab30623), mouse monoclonal anti-p65 (Santa Cruz, sc-8008), or with rabbit polyclonal anti-GR (Affinity Bioreagents, PA1-512). Secondary antibodies were Alexa488-conjugated goat anti-mouse IgG (Molecular Probes) or Alexa488-conjugated goat anti-rabbit IgG, (Molecular Probes) generating green fluorescence for both the monoclonal and polyclonal antibodies. GFP images in the GR translocation study were adjusted in photoshop to the red channel as a contrast to the green seen in the p65 translocation and phosphorylation experiments. Nuclei were stained with the fluorescent dye 4’,6-diamidino-2-phenylindole (DAPI) and cover slips were mounted using Fluoromount-G (Southern Biotechnology Associates). Fluorescence microscopy was performed on a Leica TCS SP2 AOPS confocal microscope, images were taken with a CCD camera (Cooke Sensicam), and processing was completed using Adobe photoshop.

**Kaplan-Meier survival analysis and temperature measurements**

Body temperatures from WT (n=3) and p53KO (n=3) C57BL6 mice sham treated with PBS (by intraperitoneal injection) were analyzed every 6
hours over a 48-hour period using a rectal thermometer probe to measure normal temperature changes throughout a day. WT and p53KO mice treated with 50mg/kg LPS or 50mg/kg LPS + 10mg/kg Dex by IP injection were monitored for death by LPS shock every 6 hours for the first 54 hours and every 24 hours for the subsequent 4 days (7 day monitoring period total). Temperature measurements were also recorded at these time points using a rectal thermometer probe. Mice treated with LPS or LPS + Dex exhibiting less than a 1.5° Celsius temperature decrease were excluded.

*In vivo* luciferase imaging

NF-κB luciferase reporter mice (Jackson Laboratory) were bred with p53+/- mice and back-crossed at least three times to compare WT and p53KO littermates. Mice were treated with 15mg/kg LPS +/- 5mg/kg Dex by intraperitoneal injection (IP) for 6 hours. WT and p53KO NF-κB luciferase animals then received IP injections of 4.29 mg per mouse of freshly prepared luciferin substrate (Promega) suspended in PBS. 10 minutes post IP injection of luciferin, animals were imaged using the Xenogen IVIS-100 (In vivo Imaging System) during a 1 minute acquisition time.

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Chapter 3, in part, is currently being prepared for submission for publication of the material. Murphy, Samantha H; Suzuki, Kotaro; Downes, Michael; Welch, Genevieve L; De Jesus, Paul; Miraglia, Loren; Orth, Anthony P; Chanda, Sumit K; Evans, Ron M; and Verma, Inder M. RNAi screen reveals p53 as a novel regulator of NF-κB repression by the glucocorticoid receptor. The dissertation author was the primary investigator and author of this material.
Chapter 4. Elucidation of novel regulators of glucocorticoid receptor repression of NF-κB originally identified by high throughput RNAi screening

Background

As described in chapter 2 of this dissertation, we performed a high throughput screen using 4 siRNA oligonucleotide libraries to discover novel genes that play a role in GR repression of NF-κB. After confirming screen efficacy, 290 potential genes of interest specific to the TNF + Dex condition were identified by RSA analysis and in chapter 3, we show experiments which validate and characterize the role of our number one gene of interest when genes were ranked by significance according to logP value, p53. Evaluation of 100 of the remaining 289 candidate genes identified as potential genes of interest in NF-κB repression by GR are described here.

We performed a reconfirmation screen of 100 genes, originally identified as genes of interest in our high throughput siRNA screen, and in this chapter, we report our findings describing 24 novel genes that regulate GR repression of NF-κB. We present a survey of these 24 novel genes, summarizing which were confirmed in 293T cells and which were also confirmed in a more physiologically relevant cell line. We analyze the effects of each gene on p65 nuclear translocation, and reveal which genes also alter
LPS-induced NF-κB activity as well. We then selected 8 candidate genes based on the extent of their effects on GR-mediated NF-κB repression as well as available reagents for future testing, and examined their roles in p65 and GR phosphorylation. Interestingly, our studies suggest that 4 genes may play a role in NF-κB or GR phosphorylation. Together, these results indicate that 24 novel regulators of NF-κB repression by GR have been identified and reveal a number of potential therapeutic targets that may aid in glucocorticoid suppression of inflammation.

Results

Reconfirmation screen validates 24 genes as potential genes of interest

We selected 100 genes from our list of 290 candidate genes for further testing. Genes were chosen by selecting the top genes of interest from each of the 3 RSA analyses- genes from the list of candidates created by analysis of the 2 kinase libraries, from the 2 druggable genome libraries, and from all 4 siRNA oligo libraries. siRNA oligos targeting these 100 genes were then cherry-picked from each of the 4 siRNA library collections previously used (siRNA oligos from the IDT kinase library, the Invitrogen kinase library, the Qiagen druggable genome library, and the GNF druggable library), and were pre-arrayed into 384-well plates for our reconfirmation assay.
We previously optimized high throughput screening parameters using siRNA oligo libraries (described in chapter 2) in 293T cells, and used these identical parameters to perform a reconfirmation screen in the same cell line. 293T cells stably transfected with a NF-κB luciferase reporter were plated at a density of 2,000 cells per well into wells pre-arrayed with siRNAs targeting our 100 genes, and reverse transfected for 48 hours using Lipofectamine 2000 (Fig. 4-1). Cells were then treated for 24 hours with 10ng/ml TNF or 10ng/ml TNF + 1μM Dex in duplicate and for this validation assay, we included a no treatment and Dex alone condition as well. Cells were subsequently read for luciferase activity, and raw values of every well in each 384-well plate were normalized to that plate’s average luciferase activity value.

Upon completion of the high throughput RNAi reconfirmation screen, screen efficacy was determined by luciferase analysis of siGR, sip65, si luciferase, and mock controls added to each of the 384-well plates. As expected, knockdown of GR impaired repression of NF-κB luciferase activity upon Dex addition, and knockdown of either p65 or luciferase, significantly impaired levels of NF-κB luciferase activity in both the TNF and TNF + Dex conditions (Fig. 4-2). Luciferase activity levels in non-treated and Dex treated mock cells were low, and these remained low in cells with siGR, sip65, or si luciferase transfection. From these results, we conclude that predicted changes in NF-κB luciferase activity were seen upon knockdown of GR, p65,
Figure 4-1. Schematic representation of reconfirmation screen design. siRNA oligos, pre-arrayed into 384-well plates, were reverse transfected into 293T cell stably transfected with a NF-κB luciferase reporter for 48 hours using Lipofectamine 2000. Cells were then untreated or treated with 10ng/ml TNF, 10ng/ml TNF + 1uM Dex, or 1uM Dex in duplicate for 24 hours and luciferase activity was measured upon addition of Bright Glo.
Figure 4-2. Reconfirmation screen efficacy confirmed by relative behavior of siRNA controls. 293T cells stably transfected with a NF-κB luciferase reporter were transfected with siGR, sip65, and si luciferase and treated as indicated for 24 hours in 384-well plate format.
and luciferase, validating that the screen could be used to successfully confirm potential genes of interest.

We then performed analysis using the cut-off method, a standard form of analysis that examines each well's level of luciferase activity on an individual well basis. For our assay, each well had essentially eight normalized raw values, two for each condition. The two normalized raw replicate values for each condition were averaged, and the siRNAs were ranked from high to low depending on the average normalized raw value for that well under that condition. In this way, the genes are rank ordered by activity to determine which had the highest (and lowest) normalized raw luciferase values in each condition. Then, the average normalized raw value of the TNF condition was divided by the average normalized raw value of the TNF + Dex condition for each well, to determine which genes had the highest and lowest fold change between the two conditions. The genes were rank ordered by fold change, with the gene exhibiting the smallest fold change ranked as number 1. A list of the top 24 genes ranked by fold change between the TNF and TNF + Dex condition is shown in Table 4-1.

Survey of 24 candidate genes reveals which were confirmed in 293T cells, which were confirmed in a more physiologically relevant cell line, and which affected p65 translocation
### Table 4-1. List of the top 24 genes in reconfirmation screen by cut-off method analysis.

Using cut-method analysis, genes were rank ordered according to their limited NF-κB luciferase repression upon Dex addition. The gene symbol and a brief description of each gene of interest are listed for the top 24 candidate genes. * indicates that no publications are currently found in pubmed for that particular gene of interest. (2x), (3x), or a (4x) number next to a gene symbol indicates the number of wells containing a siRNA to that gene of interest with limited repression of NF-κB luciferase activity upon Dex addition.

<table>
<thead>
<tr>
<th>Rank Order</th>
<th>Gene Symbol</th>
<th>Name and Brief Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CDK5</td>
<td>cyclin-dependent kinase 5: unique member of the serine/threonine Cdk family, primarily involved in neural development and degeneration</td>
</tr>
<tr>
<td>2</td>
<td>GALR3</td>
<td>galanin receptor 3: a G protein-coupled receptor to the neuropeptide, galanin</td>
</tr>
<tr>
<td>3</td>
<td>SETDB1</td>
<td>SET domain, bifurcated 1: histone H3, lysine 9 methyltransferase</td>
</tr>
<tr>
<td>4</td>
<td>CAD</td>
<td>carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase: trifunctional protein regulated by MAPK cascade and involved in pyrimidine biosynthesis</td>
</tr>
<tr>
<td>5</td>
<td>ALPK3</td>
<td>alpha-kinase 3: recognizes phosphorylation sites surrounded by peptides w/ an alpha-helical conformation</td>
</tr>
<tr>
<td>6</td>
<td>MAPK15</td>
<td>mitogen activated protein kinase 15: phosphorylates MBP (myelin basic protein)</td>
</tr>
<tr>
<td>7</td>
<td>NRPB1</td>
<td>nuclear receptor binding protein 1: adaptor protein involved in subcellular trafficking</td>
</tr>
<tr>
<td>8</td>
<td>MST1R</td>
<td>macrophage stimulating 1 receptor: receptor for MSP (macrophage stimulating protein), tyrosine-protein kinase activity</td>
</tr>
<tr>
<td>9</td>
<td>UCK2</td>
<td>uridine-cytidine kinase 2: catalyzes the phosphorylation of uridine monophosphate to uridine diphosphate (first step of RNA and DNA synthesis)</td>
</tr>
<tr>
<td>10</td>
<td>BRD4</td>
<td>bromodomain containing 4: contains double bromodomains and associates with mitotic chromosomes</td>
</tr>
<tr>
<td>11</td>
<td>TNK2</td>
<td>tyrosine kinase, non-receptor, 2: regulates ligand induced degradation of EGF-R</td>
</tr>
<tr>
<td>12</td>
<td>MPP2</td>
<td>membrane protein, paip protects MAGUK p55 subfamily member 2): member of membrane-associated guanylate kinase homologs family which regulate cell proliferation and signaling pathways</td>
</tr>
<tr>
<td>13</td>
<td>FGFR2</td>
<td>fibroblast growth factor receptor 2: interacts with fibroblast growth factors, creating a cascade of downstream signals and influencing mitogenesis and differentiation</td>
</tr>
<tr>
<td>14</td>
<td>MAP2K2</td>
<td>mitogen-activated protein kinase 2: phosphorylates and thus activates MAPK1/ERK2 and MAPK2/ERK3</td>
</tr>
<tr>
<td>15</td>
<td>OR2B3</td>
<td>olfactory receptor, family 2, subfamily B, member 3: member of GPCR (G-protein-coupled receptor) family and recognize odorant signals</td>
</tr>
<tr>
<td>16</td>
<td>XYLB</td>
<td>xylulokinase homolog (H. Influenzae): 22% sequence identity with Hemophilus influenzae xylulokinase, plays a role in energy metabolism</td>
</tr>
<tr>
<td>17</td>
<td>SLC7A6</td>
<td>solute carrier family 7 (cationsic amino acid transporter, y+ system), member 6: involved in the transport of L-arginine in monocytes</td>
</tr>
<tr>
<td>18</td>
<td>SGK2</td>
<td>serum/glucocorticoid regulated kinase: serine/threonine protein kinase not induced by serum or glucocorticoids</td>
</tr>
<tr>
<td>19</td>
<td>OR4A15</td>
<td>olfactory receptor, family 4, subfamily A, member 15: member of GPCR (G-protein-coupled receptor) family and recognizes odorant signals</td>
</tr>
<tr>
<td>20</td>
<td>PTPN23</td>
<td>protein tyrosine phosphatase, non-receptor type 23: may act as a negative regulator of Ras-mediated mitogenic activity</td>
</tr>
<tr>
<td>21</td>
<td>CDKN2D</td>
<td>cyclin-dependent kinase inhibitor 2D: member of the INK4 family of cyclin-dependent kinase inhibitors, complexes with CDK4 or CDK6, and prevents the activation of the Cdk kinases</td>
</tr>
<tr>
<td>22</td>
<td>PPM1E</td>
<td>protein phosphatase methylesterase 1: demethylates proteins that have been reversibly carboxymethylated</td>
</tr>
<tr>
<td>23</td>
<td>CSNK1G1</td>
<td>casein kinase 1, gamma 1: casein kinase isofrom involved in growth and morphogenesis</td>
</tr>
<tr>
<td>24</td>
<td>TLK2</td>
<td>tansied-like kinase 2: nuclear serine/threonine kinase potentially involved in the regulation of chromatin assembly</td>
</tr>
</tbody>
</table>
To further study these 24 genes, we wanted to continue to validate their role in GR repression of NF-κB. As an example for all assays performed with each of the 24 genes of interest, we will show results of one candidate gene, xylulose kinase (XYLB), which is ranked ordered as #16 in our list of 24 candidate genes from the reconfirmation screen (Table 4-1). We will then summarize the data from all genes later in this results section, and further analysis of these results will then continue in the discussion section.

One limitation of our original high throughput screen and in the reconfirmation screen, is that the assays did not include luciferase activity normalization to LacZ. We were not able to differentiate whether luciferase activity changes were due to gene knockdown, whether they were because of siRNA toxicity and subsequent cell death, or whether changes in luciferase were caused by differences in transfection efficiency or cell number. To overcome this, siRNA oligo nucleotides for each of the 24 genes were ordered from Invitrogen, and the assay was repeated in 293T cells with normalization to LacZ. 293T cells stably transfected with a NF-κB luciferase reporter were transfected with RSV-LacZ and either siGR, sip65, si luciferase, or a siRNA targeting one of our 24 genes of interest. After a 48 hour transfection, cells were then treated with 10ng/ml TNF + or 10ng/ml TNF + 1μM Dex, and luciferase activity was measured and normalized to LacZ activity in each condition. As predicted, knockdown of p65 and luciferase inhibited NF-κB
luciferase activity in both the TNF and TNF + Dex conditions, and suppression of NF-κB activity by Dex was lost upon knockdown of GR (Fig. 4-3A). Cells with a knockdown in XYLB also exhibited a loss of NF-κB luciferase repression upon Dex addition, confirming results from the original high throughput RNAi screen as well as the reconfirmation screen.

We next determined if our 24 candidate genes regulated repression of NF-κB by other synthetic glucocorticoids or if the regulation of NF-κB repression by Dex was specific. 293T cells were transfected and treated as described above, with the addition of a 10ng/ml TNF + 1μM Hydrocortisone (the synthetic equivalent to the natural glucocorticoid, cortisol) condition. Cells containing no si exhibited approximately a 2-fold decrease upon Dex addition and also upon treatment with Hydrocortisone (Fig. 4-3B). As predicted, knockdown of p65 and luciferase inhibited NF-κB luciferase activity in the TNF, TNF + Dex, and TNF + Hydrocortisone conditions, and NF-κB luciferase activity repression by both Dex and Hydrocortisone was lost upon knockdown of GR (Fig. 4-3B). Cells with a knockdown in XYLB also exhibited a loss of NF-κB luciferase repression upon Hydrocortisone addition. We conclude that XYLB regulates NF-κB repression by Hydrocortisone as well as Dex, indicating that XYLB’s role in NF-κB repression by glucocorticoids is not specific to the stereochemical structure unique to Dex.
Figure 4-3. XYLB regulates Dex and Hydrocortisone repression of NF-κB luciferase activity in 293T cells normalized to LacZ. 293T cells stably expressing a NF-κB luciferase reporter, transfected with siGR, sip65, si luciferase, or si XYLB, and treated with (A) TNF +/- Dex or (B) TNF, TNF + Dex, or TNF + Hydrocortisone as indicated. Fold change differences between the TNF and TNF + Dex treatment (A) and the TNF and TNF + Hydrocortisone treatment (B) among the transfection conditions indicated is significant by t-test. *** = P-value < 0.001.
To validate the role of the 24 candidate genes in GR repression of NF-κB in a more physiologically relevant cell line, we transduced human monocytic THP-1 cells with a NF-κB luciferase reporter (Fig. A-1A), and then transfected cells with siGR or a siRNA oligonucleotide targeting each gene of interest. Cells were then treated with 10ng/ml TNF, 10ng/ml TNF + 1μM Dex, or 1μM Dex. Untransfected cells exhibit a ~2.3-fold decrease upon Dex addition and this repression is lost upon knockdown of GR as well as upon knockdown of XYLB (Fig. 4-4A). We conclude that XYLB regulation of NF-κB repression by GR is confirmed in a more physiologically relevant setting.

To elucidate whether regulation of NF-κB repression by GR is specific to TNF-induced NF-κB activation or if regulation by each of the 24 genes also affects LPS-induced NF-κB activity, THP-1 cells stably expressing a NF-κB luciferase reporter (Fig. A-1A), were transfected with siGR or a siRNA oligo targeting each gene of interest. Cells were then treated with 10ng/ml LPS, 10ng/ml LPS + 1μM Dex, or 1μM Dex. Untransfected cells exhibit a ~2.5-fold decrease upon Dex addition and this repression is impaired upon knockdown of GR as well as upon knockdown of XYLB (Fig. 4-4B). We conclude that XYLB mediates GR repression of LPS-induced NF-κB activity, indicating that XYLB regulation of NF-κB repression is not specific to TNF signaling because it also affects Dex suppression of NF-κB transcription upon LPS addition.
Figure 4-4. XYLB regulates Dex repression of LPS and TNF-induced NF-κB luciferase activity in THP-1 cells. THP-1 cells stably expressing a NF-κB luciferase reporter, transfected with siGR or si XYLB, and treated with (A) TNF, TNF + Dex, or Dex alone as indicated or treated with (B) LPS, LPS + Dex, or Dex alone as indicated. Fold change differences between the TNF and TNF + Dex treatment (A) or the LPS and LPS + Dex treatment (B) among the transfection conditions indicated is significant by t-test. *** = P-value < 0.001.
To analyze the effect of each candidate gene on endogenous p65 nuclear translocation, HeLa cells (an immortal cell line derived from cervical cancer cells) were co-transfected with a siRNA oligo targeting one of the 24 genes of interest and a DsRed construct, to visually discriminate against the untransfected cells. Cells were then treated for 30 minutes with 10ng/ml TNF +/- 1uM Dex and fixed with 4% paraformaldehyde. p65 nuclear accumulation is seen upon TNF treatment and remains unchanged upon Dex addition in HeLa cells transfected with the DsRed construct, but no siRNA oligo (Fig. 4-5A). Cells transfected with DsRed and a siRNA targeting XYLB, also exhibit p65 nuclear translocation after a 30 minute treatment with TNF or TNF + Dex (Fig. 4-5B). This indicates that knockdown of XYLB does not alter p65 translocation.

To further investigate the role of our 24 candidate genes in endogenous p65 nuclear translocation, western blot analysis was performed on nuclear and cytoplasmic protein extracts to analyze the effect of each gene on p65 nuclear accumulation. THP-1 cells were transfected with a siRNA to each gene of interest, and cells were treated for 30 minutes with 10ng/ml TNF or 10ng/ml TNF + 1uM Dex. Nuclear and cytoplasmic protein fractions were then isolated, and western analysis was performed. Untransfected THP-1 cells exhibit an increase in p65 total protein levels in the nuclear extracts upon both TNF and TNF + Dex treatment (Fig. 4-6, left panels, lanes 1-3). THP-1 cells transfected
Figure 4-5. XYLB does not regulate endogenous p65 nuclear translocation upon TNF or TNF + Dex treatment. (A) HeLa cells transfected with DsRed and treated for 30 minutes with 10ng/ml TNF +/- 1uM Dex as indicated. (B) HeLa cells transfected with DsRed and siXYLB, and treated for 30 minutes with 10ng/ml TNF +/- 1uM Dex as indicated.
Figure 4-6. Knockdown of XYLB does not affect p65 accumulation in nuclear protein extracts upon TNF or TNF + Dex treatment. (A) HeLa cells transfected with DsRed and treated for 30 minutes with 10ng/ml TNF +/- 1uM Dex as indicated. (B) HeLa cells transfected with DsRed and siXYLB, and treated for 30 minutes with 10ng/ml TNF +/- 1uM Dex as indicated.

<table>
<thead>
<tr>
<th></th>
<th>No siRNA</th>
<th>si XYLB</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF</td>
<td>- + + +</td>
<td>- + + +</td>
</tr>
<tr>
<td>Dex</td>
<td>- - +</td>
<td>- - + +</td>
</tr>
<tr>
<td>p65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IKK2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laminin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NE | CE | NE | CE
with si XYLB also show an increase in p65 nuclear accumulation upon TNF and TNF + Dex treatment (**Fig. 4-6**, right panels, lanes 1-3). Western blots were also incubated with antibodies to Laminin, a protein exclusively nuclear, and IKK2, a protein exclusively located in cytoplasm, as controls to confirm complete isolation of the nuclear and cytoplasmic protein fractions. These results confirm our endogenous p65 immunofluorescence experiments, indicating that knockdown of XYLB does not affect p65 nuclear translocation upon treatment.

**Table 4-2** summarizes our observations from all experiments performed using siRNA oligos targeting our 24 genes of interest. When our candidate genes were tested for their effects on GR-mediated NF-κB repression by luciferase assay normalized to Lac Z activity in 293T cells, all but five genes showed at least partial rescue of NF-κB luciferase activity in the TNF + Dex condition compared to cells containing no siRNA. Cells containing a siRNA targeting OR2B3 or TNK2 exhibited a better rescue than cells containing a siRNA targeting GR. When 293T cells were treated with TNF + Hydrocortisone, another synthetic glucocorticoid, all genes which rescued NF-κB luciferase activity in the TNF + Dex condition, also rescued NF-κB luciferase activity in the TNF + Hydrocortisone condition. This indicates that knockdown of 19 candidate genes showed impaired NF-κB repression by both Dex and Hydrocortisone. Knockdown of all 24 genes led to at least partially
Table 4-2. Survey of the top 24 genes of interest identified by reconfirmation screening. Each gene abbreviation is listed on the left and a summary of the effect of each in the experiments performed is shown in each column. The number of stars indicates the extent of NF-κB luciferase activity rescue in the presence of Dex (see figure legend at bottom for explanation).

<table>
<thead>
<tr>
<th>Gene</th>
<th>293T confirmation (norm. to Lac Z)</th>
<th>Effect also on Hydrocortisone action?</th>
<th>THP-1 cell validation (NF-κB Luc Activity)</th>
<th>TNF specific (no LPS rescue)?</th>
<th>Effect on p65 translocation in HeLa cells (IF and western)?</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDK5</td>
<td>★★</td>
<td>yes</td>
<td>★★</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>GALR3</td>
<td>★</td>
<td></td>
<td>★</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>SETDB1</td>
<td>★</td>
<td>yes</td>
<td>★★</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>CAD</td>
<td>★</td>
<td></td>
<td>★</td>
<td>no</td>
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</tr>
<tr>
<td>ALPK3</td>
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<td>yes</td>
<td>★★</td>
<td>no</td>
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</tr>
<tr>
<td>MAPK15</td>
<td>★</td>
<td>yes</td>
<td>★</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
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<td>★★</td>
<td>yes</td>
<td>★★</td>
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<tr>
<td>MST1R</td>
<td>★★</td>
<td>yes</td>
<td>★★</td>
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<td>★★</td>
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<td>SLC7A6</td>
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<td>★★</td>
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<td>OR4A15</td>
<td>★</td>
<td>yes</td>
<td>★</td>
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<tr>
<td>PTPN23</td>
<td>★★</td>
<td>yes</td>
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<td>★★</td>
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<td>CSNK1G1</td>
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<tr>
<td>TLK2</td>
<td>★★★</td>
<td>yes</td>
<td></td>
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</tr>
</tbody>
</table>

No stars: no rescue of repression (fold decrease ≥ no si control)  ★ Slight rescue  ★★ Partial to almost complete rescue  ★★★ Complete rescue (fold decrease ≤ si GR control)
impaired NF-κB repression by GR in THP-1 cells, a more physiologically relevant cell line. Cells containing a siRNA oligo targeting one of seven candidate genes exhibited a greater rescue of NF-κB repression than cells containing a siRNA targeting GR. These genes include: SETDB1, ALPK3, UCK2, MAP2K2, SLC7A6, TLK, and XYL2. Interestingly, knockdown of 8 genes impaired Dex repression of TNF-induced NF-κB activity but did not affect Dex repression of LPS-induced NF-κB luciferase activity. This suggests that these genes regulate NF-κB repression by glucocorticoids only in the TNF signaling cascade and not through LPS-mediated signaling. These genes include: MST1R, UCK2, TNK2, OR2B3, OR3A15, PTPN23, CDKN2D, and TLK2. Finally, none of the 24 genes altered p65 nuclear translocation by either western or immunofluorescence (Table 4-2, last column).

The role of 8 candidate genes in p65 and GR phosphorylation events

To further investigate the role of our candidate genes in endogenous p65 and GR phosphorylation events, western blot analysis was performed on total protein extracts to analyze the effect of 8 genes on p65 phosphorylation at sites Serine276, Serine311, and Serine536 and on GR phosphorylation at site Serine211. Of the 24 candidate genes, XYL2, ALPK3, SGK2, CDKN2D, PME-1, PTPN23, SETDB1, and NRBPI were chosen for further testing to determine their effects on p65 and GR phosphorylation. A siRNA
oligonucleotide targeting each of these 8 genes was transfected into THP-1 cells for 48 hours, and cells were subsequently treated for 30 minutes with 10ng/ml TNF +/- 1uM Dex, 10ng/ml LPS +/- 1uM Dex, or 1uM Dex alone. Total protein fractions were then isolated, and western blot analysis was performed. Knockdown of each gene was confirmed at the mRNA level by qPCR analysis (data not shown), and we should note that our phosphorylation studies are thus far preliminary and should be repeated to confirm our initial observations.

Untransfected cells exhibit an increase in p65 phosphorylation at Serine536 in the TNF, TNF + Dex, LPS, and LPS + Dex conditions (Fig. 4-7A, top left panel, lanes 2-5). Unfortunately, p65 phosphorylation at Serine276 was difficult to detect in these cells, but there does appear to be increased expression in the TNF, TNF + Dex, LPS, and LPS + Dex conditions (lanes 2-5) when compared to non-treated cells in lane 1. Protein expression levels of p65 phosphorylation at Serine311 increase in the TNF and TNF + Dex conditions (lanes 2 and 3) as well as the LPS and LPS + Dex conditions (lanes 4 and 5). Finally, an up-regulation of GR phosphorylation at Serine211 is seen upon Dex addition in the TNF + Dex condition (lane 3), the LPS + Dex condition (lane 5), and the Dex alone condition (Fig. 4-7A, bottom left panel, lane 6).

There is little regulation of p65 phosphorylation by the 8 candidate genes we tested, however we did observe a few differences in protein expression levels that may indicate how PTPN23 and XYLB are functioning.
Figure 4-7. Effects of candidate gene knockdown on endogenous p65 and GR phosphorylation events. THP-1 cells transfected with a siRNA targeting one of 8 candidate genes and treated for 30 minutes with 10ng/ml TNF +/- 1uM Dex, 10ng/ml LPS +/- 1uM, or 1uM Dex as indicated. (A) Cells containing no siRNA transfection, siSETDB1, siNRBP1, or siSGK2, (B) siALPK3 or siXYLB, and (C) siPTPN23, siPME-1, or siCDKN2D.
SETDB1, NRBP1, SGK2, ALPK3, PME-1, and CDKN2D do not appear to regulate p65 phosphorylation at Serine536. A decrease in phosphorylation may be seen in the LPS + Dex condition of cells with a knockdown in XYLB (Fig. 4-7B, right top panel), and cells with a siRNA targeting PTPN23 show an increase in p65 phosphorylation at Serine536 in basal conditions (Fig. 4-7C, left top panel). It is difficult to determine the effect of gene knockdown on p65 phosphorylation at Serine276 due to the strength of our antibody. However, it does not appear that 6 of the 8 genes regulate p65 phosphorylation at this site. Cells with a siRNA targeting XYLB appear to have increased expression in the Dex alone condition (Fig. 4-7B, right), and cells with a siRNA targeting PTPN23 appear to have increased expression in the no treatment and the Dex alone condition (Fig. 4-7C, left). It does not appear that SETDB1, NRBP1, SGK2, ALPK3, PME1, or CDKN2D regulate p65 phosphorylation at site Serine311. Cells with a siRNA targeting XYLB exhibit a down-regulation in protein expression levels in the LPS + Dex condition (Fig. 4-7B, right), and cells with a siRNA targeting PTPN23 exhibit an increase in Serine311 phosphorylation in the no treatment and the Dex alone conditions (Fig. 4-7C, left).

4 of the 8 candidate genes may regulate GR phosphorylation at site Serine211. GR phosphorylation at site Serine211 appears unchanged in THP-1 cells upon knockdown of NRBP1, SGK2, ALPK3, or CDKN2D. We observed
a reduction in the up-regulation of GR phosphorylation in the Dex alone
condition of cells with SETDB1 or XYLB knockdown (Fig. 4-7A and Fig. 4-7B, right). Cells with a siRNA targeting PTPN23 have no visible increase in phosphorylation in the TNF + Dex condition, LPS + Dex condition, or the Dex alone condition (Fig. 4-7C, left). Cells with a siRNA targeting PME-1 have no visible increase in phosphorylation in the TNF + Dex condition or the Dex alone condition, but do show an increase in GR phosphorylation in the LPS + Dex condition (Fig. 4-7C, middle). These experiments should be repeated to validate our observations.

Discussion

In this chapter we have validated 24 novel genes that play a role in GR repression of NF-κB, have shown that none are regulating NF-κB upstream of p65 translocation, and have demonstrated the effects of 8 genes of interest on p65 and GR phosphorylation events. We originally identified 290 genes of interest in a high throughput luciferase assay described in chapter 2 of this dissertation, and in this chapter, validate 24 top candidates by reconfirmation screening. We selected these 24 top candidates for further testing based on their ability to rescue NF-κB luciferase activity in the presence of Dex. We then validated each gene of interest’s ability to rescue Dex repression of NF-κB activity in 293T cells and in a more physiologically relevant cell line. All genes
confirmed to regulate Dex repression of NF-κB in 293T cells, also regulated Hydrocortisone repression of NF-κB, indicating that none were specific to the stereochemical structure unique to Dexamethasone. In addition, none of the 24 candidate genes played a role in endogenous p65 nuclear translocation measured by both western and immunofluorescence. We have summarized these results in Table 4-2, and have thus provided a survey of novel candidate genes confirmed to play a role in NF-κB repression by glucocorticoids.

After performing these extensive experiments on our 24 novel regulators of NF-κB repression by GR, results of which we have summarized in our survey (Table 4-2), specific studies on each individual gene must now be performed to determine how each plays a role in GR repression of NF-κB. A number of other transcription factors are among our genes of interest as well as genes with possible kinase, phosphatase, histone methyltransferase, or dephosphatase activity, indicating that a number of novel mechanisms of GR-mediated NF-κB repression may be identified in the future.

Of the 24 genes validated by reconfirmation screening, XYLB, SETDB1, NRBP1, SGK2, ALPK3, CDKN2D, PME-1, and PTPN23 were chosen for further analysis. There were two main reasons for selecting these genes for future studies. Knockdown of each gene strongly impaired GR repression of NF-κB in both THP-1 and 293T cells (Fig. 4-3, Fig. 4-4, and summarized in Table 4-2). In addition, availability of antibody reagents as well as knockout
mouse models was considered when selecting these genes and for testing potential hypotheses. In initial studies analyzing each gene’s effect on GR or p65 phosphorylation, PTPN23, PME-1, XYL, and SETDB1 appear to regulate GR phosphorylation, and XYL and PTPN23 may regulate p65 phosphorylation under certain conditions as well. These experiments should be repeated to confirm our results, and if recapitulated, could elucidate how some of these genes are playing a role in glucocorticoid repression of NF-κB. Here, we go into more detail describing our current understanding of each gene’s known functions and hypothesize how each of these 8 genes may play a role in GR repression of NF-κB.

Xylulose kinase (XYLB) is located on chromosome 3 and shares sequence identity to gene products in *C. elegans* (45%) and *yeast* (31-35%), which are thought to belong to a family of enzymes that play an important role in energy metabolism (147, 148). Human XYL has 92% sequence identity to the mouse gene at the protein level, and GO analysis inferred from electronic annotation includes carbohydrate metabolic processing and phosphorylation, kinase activity, nucleotide binding, and transferase activity. Besides analysis of its sequence and of its genomic structure, XYL function, interaction with other proteins, and role in biological processes and signaling cascades is largely unknown. Therefore, a role in GR activity or in repression of NF-κB has yet to be described.
Set domain, bifurcated 1 (SETDB1) is located on chromosome 1, and is a histone methyltransferase that specifically trimethylates Lysine9 of histone H3 and acts as a form of transcriptional repression by recruiting HP1 proteins to methylated histones (149-151). Although there is currently no known relationship between SETDB1 and GR, it is possible that glucocorticoid binding to GR and nuclear translocation is followed by GR recruitment of SETDB1 to NF-κB target genes, causing repression of these genes by histone and DNA methylation. In addition, it has recently been shown that SETDB1 interacts with histone deacetylase complexes (HDAC) 1 and 2 (152). Another hypothesis to be tested is that SETDB1 is involved in GR recruitment of HDACs to the promoter regions of NF-κB target genes.

Nuclear receptor binding protein 1 (NRBP1) is localized to the cytoplasm and belongs to the serine/threonine protein kinase family. The features of NRBP1 predict a function as an adapter protein potentially linking signaling pathways involving nuclear receptors (153). There are no known protein/protein interactions between NRBP1 and GR, however, NRBP1 has been shown to negatively regulate AP-1, another transcription factor like NF-κB, which targets pro-inflammatory genes for transcriptional activation (154). Further studies should be performed to explore the role of NRBP1 in GR repression of NF-κB. It is possible that because NRBP1 is localized to the cytoplasm, it is involved in GR dissociation from its inhibitory complex or GR
translocation to the nucleus by acting as an adaptor to GR. In addition, because NRBP1 is thought to have protein kinase activity, it is possible that NRBP1 could directly phosphorylate GR upon ligand addition, causing a subsequent conformational change, and dissociation from the inhibitory complex.

Serum/glucocorticoid regulated kinase 2 (SGK2) is similar to serum and glucocorticoid-induced protein kinase (SGK), but has not been shown to be induced by serum or glucocorticoids (155). Like SGK, it is activated in response to phosphatidylinositol 3-kinase signals and is a serine/threonine kinase (156). It is possible that SGK2 could directly phosphorylate GR upon ligand addition, causing a subsequent conformational change, and dissociation from the inhibitory complex. In addition, SGK2 does contain GRE binding sites in its promoter region, indicating that it may be regulated by GR, despite no known transcriptional activation by GR to date. Interestingly, a recent study reported synthetic lethal interactions between p53 and SGK2 (157). In chapter 2, we identified p53 as another novel regulator of NF-κB repression by GR and went on to validate its function and to elucidate its role in chapter 3. It would be important to investigate whether SGK2 and p53 act cooperatively to aid in GR repression of NF-κB.

Alpha-kinase 3 (ALPK3) is a kinase located on chromosome 15 that is a relatively unstudied gene (no pubmed publications) thought to play a role in
cardiomyocyte differentiation based on sequence homology (158, 159). There are no known GRE binding sites in the ALPK3 promoter region, and it is thought to reside in the nucleus based on sequence similarity at the protein level. Besides sequence analysis, ALPK3 function, interaction with other proteins, and role in biological processes and signaling cascades is largely unknown. Therefore, a role in GR activity or in repression of NF-κB has yet to be described.

Protein tyrosine phosphatase, non receptor type 23 (PTPN23) is located on chromosome 3 in a region frequently mutated in many types of cancers (160). PTPN23 plays a role in endosomal cargo sorting and multivesicular body morphogenesis, but a recent report indicates that it does not harbor functional tyrosine phosphatase or lipid phosphatase activity (161, 162). In contrast, we have preliminary data that indicate p65 is phosphorylated at Ser276 and Ser311 in the absence of stimulation and in the presence of Dex when PTPN23 is knocked down in THP-1 cells (Fig. 4-7). This may indicate that PTPN23 does indeed act as a phosphatase and that PTPN23 may dephosphorylate p65 in the presence of Dex and under basal glucocorticoid conditions. It would be interesting to confirm these results and explore potential protein/protein interactions between PTPN23 and GR as well as GR regulation of NF-κB dephosphorylation by PTPN23.

Protein phosphatase methylesterase 1 (PPME1 or PME-1)
demethylates proteins that have been reversibly carboxymethylated and specifically catalyzes the demethylation of the protein phosphatase-2A catalytic subunit (PP2A), causing its inactivation (163, 164). Our preliminary data indicate that GR phosphorylation at Ser211 is impaired upon knockdown of PME-1 (Fig.4-7, bottom). It would be intriguing to test whether PP2A regulates GR dephosphorylation, and if this dephosphorylation contributes to the sequestering of GR in its cytoplasmic inhibitory complex or if dephosphorylation by PP2A contributes to GR transcriptional repression in the nucleus. If true, we might then be able to show how PME-1, which inactivates PP2A, could play a role in GR activation and subsequent repression of NF-κB by preventing GR inhibition by PP2A-mediated dephosphorylation.

Cyclin-dependent kinase inhibitor 2D (CDKN2D) forms a stable complex with CDK4 or CDK6, preventing activation of CDK kinases and functioning as a cell growth regulator that controls cell cycle progression through the G1 checkpoint (165, 166). Microarray analysis studies revealed that an increase in CDKN2D expression correlated with glucocorticoid treatment (167). It is possible that CDKN2D expression is regulated by GR or vice versa. In addition, senescence-like growth arrest is correlated with a down-regulation of CDKN2D as well as p53 (168). There is no known interaction with either GR or NF-κB, and our studies indicate that CDKN2D does not regulate either p65 or GR phosphorylation. Our finding that CDKN2D
mediates NF-κB repression by GR is novel, and further studies are needed to elucidate how it is playing a role. Considering that they play similar roles in cell cycle progression, it will be interesting to evaluate whether p53 and CDKN2D work via similar mechanisms and/or if they act together to regulate GR repression of NF-κB.

Further studies are needed to elucidate mechanism and determine how each of these 24 candidate genes regulate NF-κB repression by GR. Each gene potentially represents a novel therapeutic target that could aid in repression of inflammation by glucocorticoids. It is possible that by targeting one of these 24 genes of interest, we could ultimately develop anti-inflammatory treatment methods that employ either less or no glucocorticoids, thus reducing the negative effects that are associated with long-term steroid use.

Materials and Methods

High throughput reconfirmation screen

293T cells stably transfected with a 3x-NFκB-luciferase reporter were plated at a density of 2,000 cells per well in 384-well format. Cells were grown in 10% FBS in DMEM. 1-2 siRNAs per well were reverse transfected using Lipofectamine 2000 (Invitrogen) and cells were grown for 48 hours to allow for gene knockdown. After 48 hours, cells were left untreated or treated with 10ng/ml TNF (Calbiochem), 10ng/ml TNF + 1uM Dex (Sigma), or 1uM Dex for
24 hours and subsequently read for luciferase activity using Bright Glo (Promega) reagent. Each condition was performed in duplicate.

**High throughput reconfirmation screen analysis**

Cut-off method analysis was performed to examine the effect of each siRNA on NF-κB luciferase activity. For each siRNA, the two normalized raw replicate values for each condition were averaged, and all siRNAs were ranked from high to low according to their average normalized raw values in that condition. Then, the average normalized raw value of the TNF condition was divided by the average normalized raw value of the TNF + Dex condition for each siRNA to determine which genes had the highest and lowest fold change between the two conditions. The genes were rank ordered by fold change, with the gene exhibiting the smallest fold change ranked as number 1, generating our list of the top 24 genes ranked by fold change between the TNF and TNF + Dex condition.

**Luciferase experiments for validating screen results**

293T cells (American Type Culture Collection) were grown in 10% FBS in DMEM. 293T cells were stably transfected with a 3x-NFκB-luciferase reporter and co-transfected with a RSV-lacZ construct and siGR (Invitrogen), sip65 (Invitrogen), si luciferase (Invitrogen), or a siRNA targeting one of our 24 genes of interest (Invitrogen). 48 hours following transfection with Lipofectamine 2000, cells were then treated with 10ng/ml TNF, 10ng/ml TNF +
1uM Dex, or 10ng/ml TNF + 1uM Hydrocortisone for 24 hours. Cells were washed with PBS and resuspended in 5X lysis buffer (Promega). Luciferase assay and normalization to LacZ transfection was then performed using Steady Glo (Promega) and Beta Glo (Promega) reagent.

THP-1 cells (American Type Culture Collection) were grown in 10% FBS in RPMI-1640 according to the manufacturer’s instructions. Cells were transduced with a concentrated lentiviral 5x-NFκB-luciferase-mPGK-mcherry reporter construct (Fig. A-1A) and sorted for the mcherry positive cell population. Cells were then transfected with siGR or a siRNA targeting one of our 24 genes of interest using HiPerFect transfection reagent (Qiagen). Cells were treated with 10ng/ml TNF or 10ng/ml TNF + 1uM Dex for 6 hours, and resuspended in 5x lysis buffer (Promega) following a 5-minute centrifugation at 5000g. Luciferase assay was performed using Steady Glo reagent.

Immunofluorescence

HeLa cells, grown in 10% FBS in DMEM, were transfected with a DSRed construct or co-transfected with a DSRed construct and a siRNA oligo targeting one of the 24 genes of interest using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cells were split after 48 hours and subsequently grown on glass coverslips in 24-well dishes for 24 hours (72 hour transfection total). Cells were then treated for 30 minutes with either 10ng/ml TNF or 10ng/ml TNF + 1uM Dex, washed with phosphate-buffered
saline (PBS), and fixed at room temperature with 4% paraformaldehyde. Samples were incubated with mouse monoclonal anti-p65 (Santa Cruz, sc-8008), and the secondary antibody was Alexa488-conjugated goat anti-mouse IgG (Molecular Probes). Nuclei were stained with the fluorescent dye 4’,6-diamidino-2-phenylindole (DAPI), and coverslips were mounted using Fluoromount-G (Southern Biotechnology Associates). Fluorescence microscopy was then performed on a Leica TCS SP2 AOBS confocal microscope, images were taken with a CCD camera (Cooke Sensicam), and processing was completed using Adobe photoshop.

**Western analysis**

THP-1 cells were grown in 10% FBS in RPMI-1640, transfected for 48 hours with each siRNA oligo using HiPerFect, and treated for 30 minutes with 10ng/ml TNF or 10ng/ml TNF + 1uM Dex. Cells were washed and harvested in PBS, pelleted, and nuclear and cytoplasmic fractions were isolated using the NE-PER nuclear and cytoplasmic protein extraction kit (Thermo Scientific) according to manufacturer instructions. Proteins were electrophoresed on poly-acrylamide gels and transferred to nitrocellulose membranes. Pre-cast 4-12% Bis-Tris gels (Invitrogen) were used to resolve p65 total protein. Novex Mini-cell gel and X-Cell 2 transfer apparatus (Invitrogen) was utilized according to manufacturer instructions. Membranes were blocked in PBS-T (PBS with 0.15% Tween-20) with 3% BSA for 1 hour at room temperature.
Blots were rinsed and incubated with a mouse monoclonal p65 antibody (Santa Cruz, sc-8008) in PBS-T with 3% BSA overnight at 4°C. After washing with PBS-T, blots were incubated with the secondary antibody anti-rabbit (GE Healthcare) or anti-mouse (Santa Cruz) conjugated to peroxidase in PBS-T with 3% BSA for 1 hour at room temperature. After extensive washing with PBS-T, blots were treated with ECL (Amersham) according to manufacturer’s instructions and exposed to film (Kodak X-omat) for varying times. Blots were then stripped by boiling in PBS-T, blocked in PBS-T with 3% BSA for 1 hour at room temperature, and re-incubated with a 1:1000 dilution of rabbit polyclonal Laminin B1 antibody (Abcam, ab16048) or 1:1000 dilution of mouse monoclonal IKK2 antibody (Biosource, AH00362) in PBS-T with 3% BSA overnight at 4°C.

THP-1 were transfected for 48 hours with each siRNA oligo using HiPerFect and subsequently treated for 30 minutes with 10ng/ml TNF +/- 1uM Dex, 10ng/ml LPS +/- 1uM Dex, or 1uM Dex as indicated. Cells were washed and harvested in PBS, pelleted, and incubated with lysis buffer for 30 min on ice. Lysates were cleared by centrifugation at 14,000g for 10 minutes at 4°C and protein concentration was measured by the Bradford assay. Pre-cast 4-12% Bis-Tris gels were used to resolve the phosphorylated forms of p65 and GR. Membranes were blocked in PBS-T with 3% BSA for 1 hour at room temperature. Blots were rinsed and incubated with a rabbit monoclonal p65
Phospho-Serine536 antibody (Cell Signaling, 3033), a rabbit polyclonal p65 Phospho-Serine311 antibody (Abcam, ab51059), a rabbit polyclonal p65 Phospho-Serine276 antibody (Abcam, ab30623), or a rabbit polyclonal GR Phospho-Serine211 antibody (Cell Signal, 4161) in PBS-T with 3% BSA overnight at 4°C. After washing with PBS-T, blots were incubated with the secondary antibody anti-rabbit (GE Healthcare) conjugated to peroxidase in PBS-T with 3% BSA for 1 hour at room temperature. After extensive washing with PBS-T, blots were treated with ECL Plus (Amersham) according to manufacturer’s instructions and exposed to film (Kodak X-omat) for varying times.

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Yeddula who began collaborating with me on this project after identification of the 24 novel genes of interest by reconfirmation screening. He performed immunoblot experiments on nuclear and cytoplasmic extracts to examine the effect of each gene of interest on p65 translocation (Fig. 4-6) and is also responsible for the phosphorylation studies (Fig. 4-7).
Chapter 5. Discussion

The mechanisms explaining NF-κB repression by GR are complex and varied. Years of research have resulted in a number of hypotheses suggesting how glucocorticoids may be working to repress inflammation and the immune response, and yet, a universal mechanism of repression has not been established nor do current insights into GR repression of NF-κB entirely explain its anti-inflammatory function. Identification of novel genes involved in GR repression of NF-κB, and elucidation of their mechanisms, will ultimately be important to better understand how GR functions to repress inflammation and to potentially develop more effective forms of therapy.

The work presented in this thesis attempts to answer these outstanding questions. In chapter 2 of this dissertation, we have summarized the design and optimization of a high throughput RNAi screen, which we used to identify novel regulators of NF-κB repression by GR. We confirm the ability of our screen to successfully distinguish genes of interest and reveal a number of pathways and processes over-represented in our list of 290 candidate genes. We discovered that our number one gene of interest, when genes were ranked by significance, was p53, and our in vivo and in vitro experiments that validate this finding are described in detail in chapter 3. We also performed a reconfirmation screen, which confirmed an additional 24 novel regulators of NF-κB repression by GR. In chapter 4, we have presented a survey outlining
the effects of each these 24 genes on the upstream NF-κB signaling cascade, and our initial observations have implicated 4 genes in the regulation of p65 or GR phosphorylation events.

Notably, our studies have identified p53 as a novel regulator of NF-κB repression by the glucocorticoid receptor, and in chapter 3, we have confirmed its biological significance using both in vitro and in vivo assays. Current insight into the relationship between p53 and GR is debated. A handful of groups have suggested that p53 represses GR transcriptional activity or that both proteins inhibit each other through protein/protein interactions or binding (169-173). However, most of these studies were performed using transfection and over-expression techniques, and none include data from primary cells or in vivo mouse models as we have shown here. Other groups have reported that suppression of p53 impairs GR function in the presence of Dex and that glucocorticoid treatment enhances p53 transcriptional activity (174, 175). These findings reinforce our hypothesis that p53 is playing a supportive role in GR function and transcriptional activation. In addition, patients with rheumatoid arthritis (RA), an autoimmune disorder in which glucocorticoid treatment is often an ineffective anti-inflammatory therapy, showed reduced p53 expression levels in blood mononuclear cells (176) and were also found to exhibit p53 mutations in synoviocytes (177). The observations made in these clinical studies also give credibility to our findings, suggesting that a loss in
p53 and p53 mutations may correlate to the impaired glucocorticoid repression of inflammation seen in RA patients.

We originally identified p53 as a gene of interest through RNAi screening in HEK 293T cells (Table 2-2 and Fig. 2-7), a cell line that expresses SV40 Large T-antigen, which stabilizes p53, and also expresses viral proteins, which inactivate it. These cell line characteristics may give us an idea as to how p53 may be functioning in GR-mediated NF-κB repression. It is possible that our original observation in this cell line involves stable p53, and that p53 is playing a role once it is released from its inhibitory MDM2 complex. We must also consider the possibility that because p53 is inactive in these cells, it functions in GR repression of NF-κB through protein/protein interactions and not through p53 transcriptional activation. We later show that p53 loss does not affect GR or NF-κB nuclear translocation (Fig. 3-7 and Fig. 3-9), suggesting that p53 aids in GR repression of NF-κB when all three proteins are localized to the nucleus. It is possible that p53 plays a direct or indirect role in GR and NF-κB transrepression, that once all three are in the nucleus, p53 may target GR to NF-κB, aiding in complex formation of the two transcription factors (Fig. 5-1, top). It is also possible that p53 interacts with GR and NF-κB directly, and that all three form a complex in the nucleus, which leads to NF-κB inactivation. We also show that p53 loss impairs GR transcription of its target genes (Fig. 3-10), suggesting that p53 plays a role in
Figure 5-1. Proposed mechanisms of p53 role in GR repression of NF-κB. p53 may play a direct or indirect role in GR and p65 complex formation in the nucleus (top) and/or in GR transcriptional activity (bottom).
GR-mediated transcription. We propose that p53 aids in GR repression of NF-κB by playing a direct or indirect role in GR transcription (Fig. 5-1, bottom). p53 may directly target GR to the promoter regions of its target genes or might also help to recruit transcriptional activation machinery to the promoter regions of GR target genes. We cannot rule out the potential that p53 may be involved in removal of repressive complexes and/or recruitment of other co-activators as well. Although our original observation was made in 293T cells in which we do not believe p53 transcriptional activation played a role in GR repression of NF-κB, we must also consider this as a conceivable mechanism of action, that downstream targets of p53 may be involved in repression of NF-κB by GR as well.

Elucidating the exact mechanism, which describes the role of p53 in GR repression of NF-κB, is an important lingering question. Further studies are needed to investigate precisely how p53 functions in GR repression of NF-κB. In order to better understand the protein interactions of these three important transcription factors, mutagenesis studies, as well as endogenous immunoprecipitation and ChIP analyses, may help elucidate the domains involved and regions of interaction. We could use WT, p53KO, and p53 mutant mice, isolate bone marrow derived macrophages, treat cells with TNF +/- Dex or LPS +/- Dex and determine which p53 mutants impaired Dex repression of NF-κB target gene transcription by qPCR. This experiment might give us a
better understanding of which p53 structural components are necessary for NF-κB repression by GR. In addition, it would also be intriguing to determine if the other two p53 protein family members, p63 or p73, play a similar role in GR repression of NF-κB, and to investigate if p53 is also involved in repression of NF-κB by other nuclear hormone receptor family members.

Our discovery that p53 is a novel regulator of NF-κB repression by GR is intriguing for a number of reasons. The p53 tumor suppressor protein plays a critical role in the development of many human cancers and is often mutated or deleted, leading to tumor progression (178, 179). NF-κB is often up-regulated in tumor cells deficient in p53 and in tumor cells that have other mutations, which lead to impaired p53 signaling. In addition, there is growing evidence that NF-κB induction of inflammatory genes is an important component for tumor progression (180, 181). Our findings suggest that NF-κB could be up-regulated in tumor cells, in part, because endogenous glucocorticoids, due to a loss of p53 function, can no longer efficiently repress NF-κB. Glucocorticoid treatment, while a potent anti-inflammatory therapy, has not been shown to significantly reduce inflammation in tumor cells. Our findings suggest that a possible explanation for why glucocorticoids cannot repress inflammation and NF-κB in these cells, is because p53 is deficient. We have unveiled a new role for p53 in the repression of NF-κB by glucocorticoids in vitro and in vivo, a finding that ultimately has important implications for the
study and treatment of the pro-inflammatory microenvironments found in tumors with aberrant p53 activity.

Additional studies outlined in this dissertation describe identification of 24 novel regulators of NF-κB repression by GR (chapter 4). We have discovered novel transcription factors, histone methyltransferase, kinases, phosphatases, and dephosphatases that play a role in the regulation of NF-κB by GR, and the majority of these 24 genes have unknown mechanisms of action, functions, and their biological significance has yet to be explored. Further studies to elucidate how they mediate GR repression of NF-κB will be important to enhance our understanding of glucocorticoid’s anti-inflammatory effects. Ultimately, each of these 24 genes represents a potential therapeutic target. It is possible that development of and treatment with an agonist, which could specifically target one of our 24 candidate genes, might enable us to establish effective anti-inflammatory therapies that require lower doses of glucocorticoids, thus minimizing negative side effects associated with long-term steroid use. As a proof of concept, we have tested the effect of Triptolide, a major active component of Chinese herbal tea extracts thought to be an anti-inflammatory agent (182-184), on NF-κB luciferase activity. Our studies show that Triptolide not only reduces TNF and LPS-induced NF-κB activity in THP-1 cells, but also has an additive effect when added with Dex (Fig. A-3). This
suggests that it might be possible to develop potent anti-inflammatory therapies, which employ fewer or no glucocorticoids.

The work presented in this thesis, which identifies novel regulators of NF-κB repression by GR, has important implications for the treatment of a number of diseases in which NF-κB is misregulated. Developing agonists to the genes we have identified, may have the potential for improving therapies for patients suffering from a number of debilitating ailments including rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, arthritis, dermatitis, allergic anaphylactic shock, swelling from brain or spinal cord injuries, and graft rejection. In addition, our finding that p53 regulates GR repression of NF-κB has great significance to our understanding of why glucocorticoids are an ineffective treatment for inflammation associated with the tumor microenvironment, and our work ultimately establishes a novel explanation for why NF-κB may be up-regulated in cancer cells.

Acknowledgements

Chapter 5, in part, is currently being prepared for submission for publication of the material. Murphy, Samantha H; Suzuki, Kotaro; Downes, Michael; Welch, Genevieve L; De Jesus, Paul; Miraglia, Loren; Orth, Anthony P; Chanda, Sumit K; Evans, Ron M; and Verma, Inder M. RNAi screen reveals p53 as a novel regulator of NF-κB repression by the glucocorticoid receptor.
The dissertation author was the primary investigator and author of this material.
Figure A-1. Schematic representation of the NF-κB luciferase reporter and transduction validation in THP-1 cells. (A) Schematic representation of CMV-5x-NFκB-luciferase-mPGK-mcherry reporter construct. (B) Fluorescent microscope images of THP-1 cells sorted for mcherry and GFP positive cells after lentiviral transduction with CMV-5x-NFκB-luciferase-mPGK-mcherry reporter (left panels) and either EF1α-GFP (middle, top) or EF1α-sip53-GFP (middle, bottom). Phase contrast image of THP-1 cells indicating morphology is also shown (right).
Figure A-2. Confirmation that p53 mRNA levels decrease in cells with p53 knockdown by RNAi or in knockout cells. (A) qPCR measuring p53 mRNA levels in 293T cells (left) and in 293T cells transfected with an sip53 oligo for 72 hours (right). (B) qPCR measuring p53 mRNA levels in THP-1 cells with no virus (left) or transduced with either a GFP control (middle) or sip53-GFP(right) and sorted for GFP. (C) qPCR measuring p53 mRNA levels in WT MEF cells (left) and p53KO MEF cells (right). (D) qPCR measuring p53 mRNA levels in bone marrow derived macrophages isolated from WT mice (left) and p53KO mice (right). All qPCR experiments were normalized to Cyclophilin A levels.
Figure A-3. Triptolide reduces TNF and LPS-induced NF-κB luciferase activity and has an additive effect when combined with Dex. THP-1 cells transduced and sorted for the NFκB-luciferase-mPGK reporter were treated for 24 hours with 10ng/ml TNF +/- 1uM Dex, 5ng/ml Triptolide, or 5ng/ml Triptolide + 1uM Dex (top) or 50ng/ml LPS +/- 1uM Dex, 5ng/ml Triptolide, or 5ng/ml Triptolide + 1uM Dex (bottom).
References


34. Okret S, Poellinger L, Dong Y, Gustafsson JA (1986) Down-regulation of glucocorticoid receptor mRNA by glucocorticoid hormones and recognition by the receptor of a specific binding sequence within a receptor cDNA clone. *Proc Natl Acad Sci USA* 83:5899-5903.


184. Liu Q (2011) Triptolide and its expanding multiple pharmacological functions. *Int Immunopharmacol* [Epub ahead of print]