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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Identification of novel regulators of the Nuclear Factor Kappa B pathway in  
human macrophages

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

in

Biology

by

Suneer Verma

Committee in charge:

Professor Inder Verma, Chair  
Professor Eric Bennett  
Professor Tony Hunter  
Professor Cornelis Murre  
Professor Jing Yang

2017

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Chair

University of California, San Diego

2017

## DEDICATION

The  
dedication  
of this thesis  
is split  
seven ways:  
to Mama,  
to Papa,  
to Samman,  
to Manavi,  
to the Verma lab,  
to the Honey badgers  
and to you,  
I hope you  
find this useful

## EPIGRAPH

"The important thing is not to stop questioning. Curiosity has its own reason for existing. One cannot help but be in awe when he contemplates the mysteries of eternity, of life, of the marvelous structure of reality. It is enough if one tries merely to comprehend a little of this mystery every day."

- Albert Einstein

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Secondly, I want to thank my committee members – Dr. Eric Bennett, Dr. Tony Hunter, Dr. Kees Murre, Dr. Jing Yang for always giving me excellent advice and keeping my project on track. I was lucky to have been mentored by them over the course of my PhD.

Thirdly, I am grateful to my fellow lab members for being an endless source of scientific advice and encouragement. For 4 years, I was the only graduate student in a lab full of senior post-docs and rather than being intimidated by this, I felt that I had numerous channels to talk about why my loading control wasn't working or why does Glioblastoma demonstrate explosive growth. I have no doubt that they are the smartest and most diverse group of people I have ever met and I would like to thank a few in particular – Mark, our lab manager, for being ever so resourceful and kind; Beth – for her wonderful support throughout my tenure in lab and for always being so cheerful; Nari, for

his mentorship during my initial years in the lab; and Eugene, for in-depth and fascinating discussions on the NF- $\kappa$ B pathway – I hope we can find out one day what exactly this heterodimer does in the nucleus.

Fourthly, I am thankful to our collaborators Dr. Sumit Chanda and Paul De Jesus at the Sanford Burnham Prebys Medical Discovery Institute. They were very generous with their time and facilities and had invaluable contributions in running and analyzing our RNAi screens.

And finally, I am thankful for being able to work at the Salk Institute. Some students swear by their college football teams or their fraternities, but I am proud to be a future alumnus of this magnificent research institute.

Chapters 2, 3, and 4, in part, are currently being prepared for submission for publication of the material. Verma, Suneer; De Jesus, Paul; Chanda, Sumit K; and Verma, Inder M. RNAi screens reveal SNW1 as a novel regulator of NF- $\kappa$ B in macrophages.

## VITA

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## ABSTRACT OF THE DISSERTATION

Identification of novel regulators of the Nuclear Factor Kappa B pathway in  
human macrophages

by

Suneer Verma

Doctor of Philosophy in Biology

University of California, San Diego, 2017

Professor Inder Verma, Chair

The nuclear factor-kappa B (NF- $\kappa$ B) family of transcription factors has a central role in coordinating the expression of genes that control inflammation, immune responses, cell-proliferation, and a variety of other processes. Ever

since its discovery in 1986 in David Baltimore's lab, the NF- $\kappa$ B pathway has been the prime model of inducible transcription in various cell types, and in response to multiple stimuli. Despite being one of the most well-studied pathway in biology, it still has a lot of unanswered questions associated with it including the events that lead up to its activation in the cytoplasm as well as the sequence of events that lead to the transcription of hundreds of its target genes in the nucleus. Given the pathway's implication in development and diseases, it has become increasingly important to answer these questions. Here, we present two whole-genome RNAi screens conducted to find novel regulators of this pathway in the physiologically relevant human macrophages in response to Lipopolysaccharides (LPS) and Tumor Necrosis Factor Alpha (TNF). After three levels of screening we have found over 25 potential novel regulators of this pathway, summarized in Chapters 2 and 4. The top hit is the splicing factor and transcriptional co-activator SNW1. We have further validated it as a regulator of the NF- $\kappa$ B pathway in response to multiple stimuli and in five different cell lines (THP-1, U87, 293T, A549, and U2-OS). SNW1 does not seem to affect general constitutive transcription in THP-1 cells but does seem to repress some transcription programs e.g. CREB and NFE2. SNW1 does not regulate the cytoplasmic part of the NF- $\kappa$ B pathway but does complex with the NF- $\kappa$ B hetero dimer in the nucleus on pathway activation. We have shown that it binds to NF- $\kappa$ B's transcriptional elongation partner p-TEFb and helps recruit it to the NF- $\kappa$ B nuclear complex that contains RNA Polymerase II. We have also shown that SNW1 loses binding from its splicing complex

(SNRNP200, SNRNP220) on NF- $\kappa$ B activation. SNW1 is a unique protein shown to be involved in both splicing and transcription and in the case of NF- $\kappa$ B, its role seems to involve recruitment of p-TEFb for effective transcriptional elongation of NF- $\kappa$ B target genes.



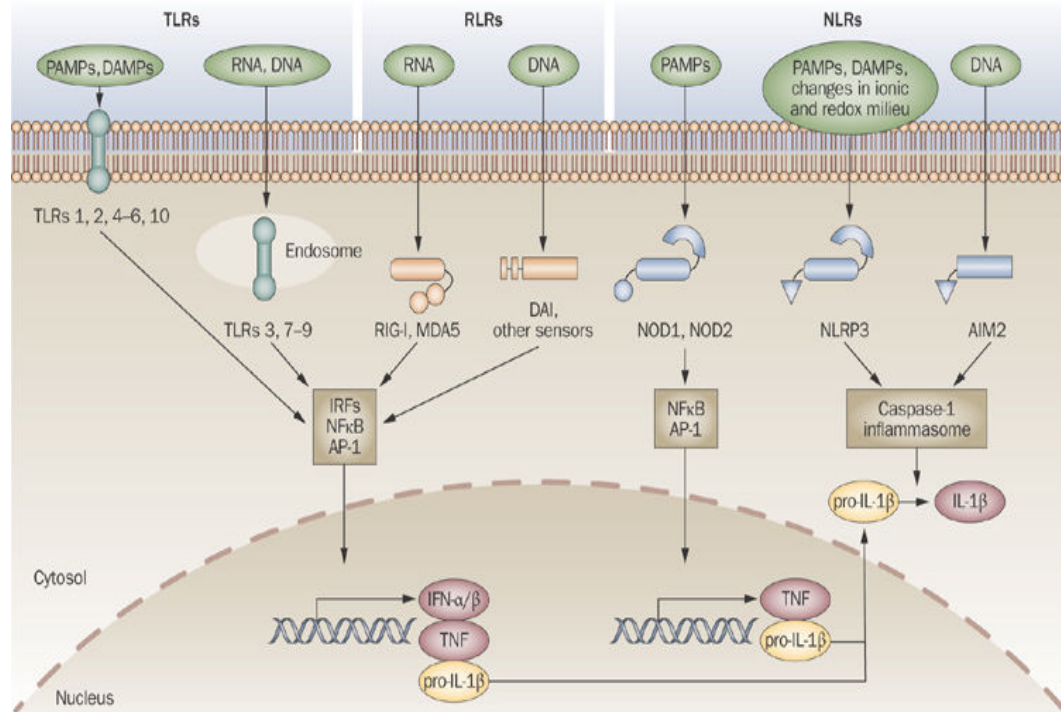
## **Chapter 1: NF- $\kappa$ B pathway and the quest to find its regulators**

## **An introduction to NF- $\kappa$ B**

### Innate immunity and the discovery of NF- $\kappa$ B

Once our body detects a pathogenic attack, we rely on our innate immune system to mount a response to fight off potential infection and disease. This response is initiated via diverse families of pattern recognition receptors (PRRs) that recognize microbial components known as pathogen associated molecular patterns (PAMPs). Leukocytes like neutrophils and macrophages express these PRRs such as Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), and Nod-like receptors (NLRs).

Once the infectious pathogen is recognized by the leukocytes, the cells undergo a quick and coordinated activation of various signaling pathways typically leading to the activation of one or more transcription factors (Figure 1.1). The factors express a variety of target genes by interacting with their *cis*-regulatory elements (promoters, enhancers, etc.) and recruiting the general transcription machinery. The target genes include anti-microbial peptides, cytokines, chemokines, stress-response proteins, and anti-apoptotic proteins which collectively help the infected tissue ward off the pathogenic insult and heal efficiently. Hence, these signaling pathways are our first line of defense against a variety of infective agents. The central and most well-studied (over 46,000 papers indexed on PubMed) member of these signaling pathways is Nuclear factor- $\kappa$ B or NF- $\kappa$ B.



**Figure 1.1.**  
**Activation of pattern recognition receptors leads to various transcriptional programs**

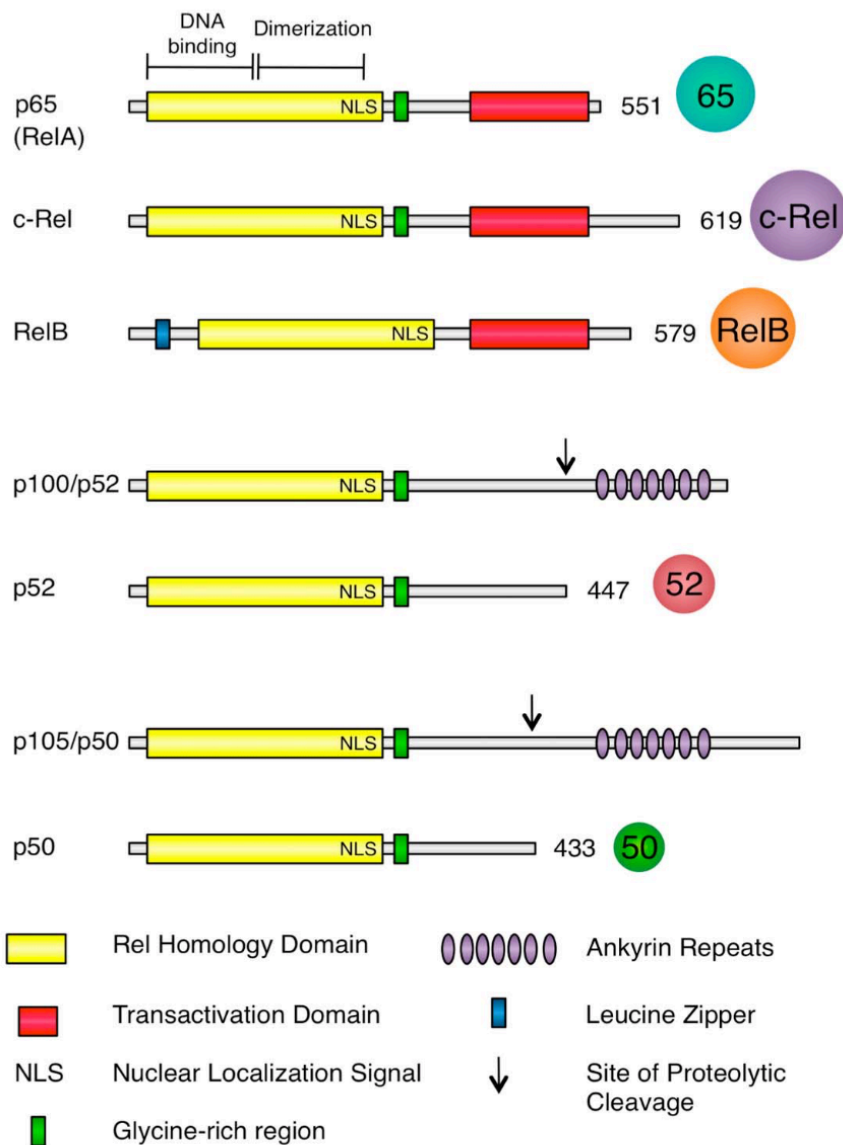
Pattern recognition receptors (like TLRs, RLRs (for example, RIG-I) and NLRs recognize danger signals derived from pathogens (PAMPs), damaged cells (DAMPs) or associated nucleic acids at the cell surface, in endo-lysosomes or in the cytoplasm. Their activation leads to downstream activation of transcription factors like IRFs, NF-κB and AP-1, that drive expression of cytokines (IFN-α/β, TNF and pro-IL-1β), or the assembly of the caspase-1 inflammasome and subsequent maturation of IL-1β from pro-IL-1β. Reproduced from Theofilopoulos et al. 2010.

Around three decades ago, Ranjan Sen and David Baltimore identified a protein binding to a specific, conserved DNA sequence in the nuclei of activated B lymphocytes (Sen and Baltimore, 1986). They named it after the cell type in which they had identified it and the gene it affected calling it 'nuclear factor binding near the  $\kappa$  light-chain gene in B cells', or NF- $\kappa$ B.

### The biology of the NF- $\kappa$ B pathway

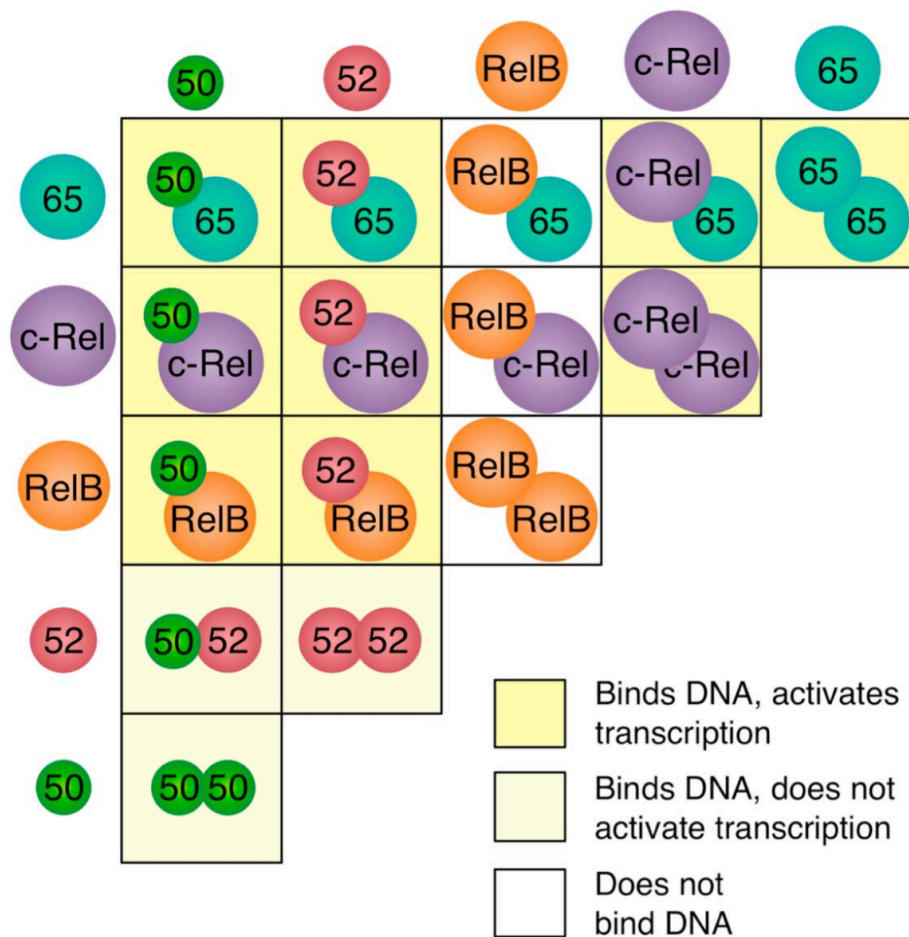
NF- $\kappa$ B family members in mammals contain five Rel proteins, p50 (NF- $\kappa$ B1), p52 (NF- $\kappa$ B2), p65 (RelA), RelB, and c-Rel, and these subunits form homo- and heterodimerized complexes (Lawrence, 2009). Their name comes from a common Rel homology domain (originally identified in chicken Reticuloendothelial virus, REV) in their N-terminus which is a 300 amino acids motif that mediates dimerization, DNA binding, nuclear localization, and interaction with I $\kappa$ B $\alpha$ , an inhibitory molecule of NF- $\kappa$ B. Also, p65, c-Rel, and RelB, but not p50 or p52, contain C-terminal transactivation domains. Schematic diagrams depicting the modular domains of each NF- $\kappa$ B family member and summarizing which dimerization pairs among family members are transcriptionally active or inactive are shown in Figures 1.2 and 1.3.

In the classical NF- $\kappa$ B signaling pathway (Figure 1.4), p65 and p50 subunits heterodimerize and are sequestered in an inactive complex in the cytoplasm bound to I $\kappa$ B $\alpha$  (Verma *et al.*, 1995). Upon activation by pro-inflammatory stimuli such as tumor necrosis factor-alpha (TNF) or



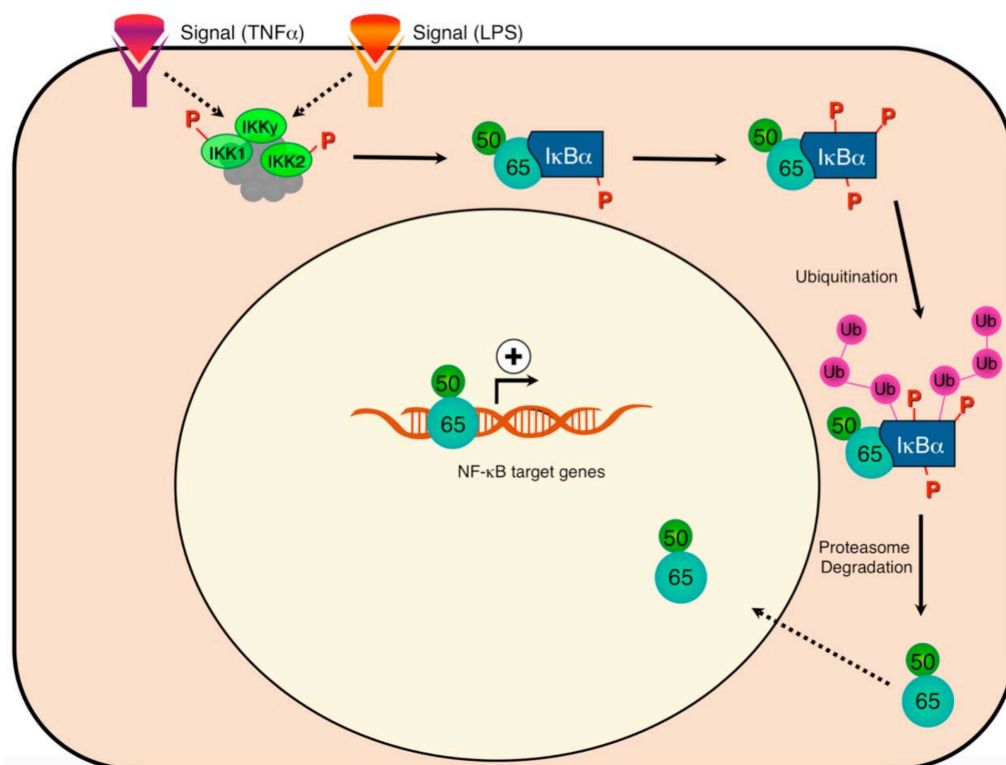
**Figure 1.2.**  
**NF- $\kappa$ B family members.**

These representations denote the protein domains of the five NF- $\kappa$ B family members (p65, c-Rel, RelB, p52, p50). The latter two (p52 and p50) need to be processed from their pre-cursors (p100 and p105) via the Ankyrin repeats. Reproduced from Murphy et al. 2011.



**Figure 1.3.**  
**NF-κB homo- and hetero-dimers.**

These representations denote the dimers which translocate to the nucleus to activate NF-κB target genes. The figure also shows the DNA binding and transcription activation potential of all the possible dimers. Reproduced from Murphy et al., 2011.



**Figure 1.4.**

**The canonical NF- $\kappa$ B pathway.**

NF- $\kappa$ B protein family members, p65 and p50, are located in the cytoplasm in an inhibitory complex associated with I $\kappa$ B $\alpha$ . Upon pro-inflammatory signals such as TNF or LPS, the IKK complex phosphorylates I $\kappa$ B $\alpha$ , 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- $\kappa$ B target genes to activate transcription. Reproduced from Murphy et al., 2011.

lipopolysaccharide (LPS), the I $\kappa$ B kinase (IKK) complex, which is composed of two functionally non-redundant kinases, IKK1 and IKK2 (Liu *et al.*, 2012), the stoichiometric regulatory subunit NEMO, and ELKS (Rothwarf *et al.* 1998; Sigala *et al.* 2004), phosphorylates I $\kappa$ B $\alpha$  at Serine 32 and 36 (Mercurio *et al.*, 1997), targeting it for ubiquitination and proteosomal degradation (Spencer, Jiang and Chen, 1999). This allows the p65/50 complex to translocate to the nucleus (Figure 1.4) where p65 can then bind to the promoter regions of its target genes, recruit the general transcription machinery, and induce the corresponding mRNA expression. One of NF- $\kappa$ B's target genes is I $\kappa$ B $\alpha$  itself, which acts as an auto-regulatory feedback loop to represses NF- $\kappa$ B activity (Baeuerle and Baltimore, 1996). Now that we have a basic picture of the NF- $\kappa$ B pathway in mind, we will describe its key roles in physiological and developmental processes. Additionally, we recommend Hayden and Ghosh 2012 as an excellent review on the biology of activation of the NF- $\kappa$ B pathway.

## **NF- $\kappa$ B in human physiology**

### NF- $\kappa$ B in innate immunity

Inflammation in response to pathogenic infection typically begins at the level of the Toll-like receptors (TLRs). These function as sentinels of the innate immune system (Akira and Takeda, 2004). Each TLR is able to recognize distinct PAMPs found in bacteria, viruses, fungi, and protozoa. TLR1, 2, 4, and 6 recognize bacterial lipids, with TLR4 specifically detecting lipopolysaccharide



(LPS) found in gram-negative bacteria. TLR5 binds to micro-bacterial protein components. Some TLRs are also located inside the cell which recognize viral and bacterial nucleic acids that are internalized during an infection via endocytosis. These include TLR3 which responds to double-stranded RNA, along with TLR7 and 8 which recognize single-stranded RNA. Finally, TLR9 binds to double-stranded CpG motifs found in pathogen DNA. The activation of these TLRs in innate immune cells leads to downstream activation of NF- $\kappa$ B and expression of its target genes.

Examples of immediately expressed target genes include defensins - cationic peptides that exert direct bactericidal activity by inducing membrane permeabilization. NF- $\kappa$ B also induces production of antimicrobial nitrogen and oxygen species (via activation of inducible Nitric Oxide Synthase) that are acutely toxic to microbes and complement the activity of defensins. Along with production of anti-microbial compounds, NF- $\kappa$ B induces production of vascular endothelium modifying proteins. These genes, such as VCAM1 and ICAM1, assist in recruiting circulating leukocytes and provide them with a means of exiting the vasculature into the infected tissue. NF- $\kappa$ B also induces adhesion molecules, both on leukocytes and endothelial cells, which allow the extravasation of leukocytes from the circulation to the site of infection. Further, NF- $\kappa$ B transcribes cytokines and chemokines whose gradient helps leukocytes migrate towards the site of infection. These molecules also act as messengers and activate the pathway in these recruited leukocytes using positive feedback (Hayden, West

and Ghosh, 2006). Hence NF- $\kappa$ B regulates many key aspects of the immediate immune response. It is not a surprise that mouse models with defective NF- $\kappa$ B components (e.g. p65, NF- $\kappa$ B1, NF- $\kappa$ B2, c-Rel knockouts) have impaired macrophage activation and increased susceptibility to damage from infections (Li and Verma, 2002) . For further reference, we recommend a comprehensive book chapter written by Dev et al. 2011 for NF- $\kappa$ B's role in innate immunity.

#### NF- $\kappa$ B and adaptive immunity

NF- $\kappa$ B was discovered during experiments on B-cell maturation (Sen and Baltimore, 1986) providing early hints of its role in the development of adaptive immunity involving B-cell, T-cell, and Dendritic cells. We now know that mice that lack individual NF- $\kappa$ B proteins have defects in B- and T-cell proliferation, activation, cytokine production, but no important defects in B- and T-cell development, probably owing to the functional redundancy between the NF- $\kappa$ B family members (Gerondakis *et al.*, 2006). T cells from transgenic mice that express I $\kappa$ B $\alpha$ M (an non-degradable mutant of I $\kappa$ B $\alpha$ ) under the control of a T-cell specific promoter have markedly impaired proliferative responses (Boothby *et al.*, 1997). Inhibitors of NF- $\kappa$ B activation have been shown to block the maturation of dendritic cells (Caamano and Hunter, 2002). B-cell defects that involve NF- $\kappa$ B proteins — include a lack of immunoglobulin class switching, lack of germinal centers and disruption of splenic microarchitecture leading to B-cell abnormalities and defective maturation (Franzoso *et al.*, 1997; Li and Verma, 2002). NF- $\kappa$ B1

haplo-insufficiency and NF- $\kappa$ B2 mutations have been known to be associated with common variable immuno-deficiency (Chen *et al.*, 2013; Boztug *et al.*, 2016). The deletion of NIK or IKK1 results in defects in B-cell maturation, but not in early B-cell development (Brightbill *et al.*, 2015). The review paper by Steve Gerondakis and Siebenlist 2010 covers the role of NF- $\kappa$ B in lymphocyte function in great detail but the overall consensus is that NF- $\kappa$ B family members regulate lymphocyte development through regulation of proliferation and protection from TNF-induced apoptosis (Li and Verma, 2002).

#### NF- $\kappa$ B in development of other tissues

Although NF- $\kappa$ B is most researched in the context of immunity and the inflammatory response, there is a plethora of evidence that it plays a role in the development and maintenance of a variety of tissue types including Brain, Gut, Liver, Skin, etc. These have been summarized in **Table 1.1**.

#### **The regulation of NF- $\kappa$ B**

Since the NF- $\kappa$ B pathway is so important for both immunity and development, it needs to be very tightly regulated. We know that the pathway has hundreds of diverse target genes (thanks to the excellent database maintained by Boston University), depending on the stimulus and tissue type,

**Table 1.1****The role of NF- $\kappa$ B in the development and maintenance of tissues.**

The NF- $\kappa$ B pathway plays key roles in a variety of tissue and organ types. The pathway has thousands of target genes and differential expression of these genes in different tissues contributes to these phenomenon. Most of the mentioned roles were discovered via the tissue specific knockouts of NF- $\kappa$ B components.

Organ	Role	Evidence	Reference
CNS	NF- $\kappa$ B is important for translating short-term synaptic events into changes in gene expression	<ul style="list-style-type: none"> <li>• Constitutive NF-<math>\kappa</math>B activity in glutamatergic neurons that is suppressed by glutamate antagonists and L-type Ca<sup>2+</sup> channel blockers</li> <li>• p65-GFP fusion protein is transported to the nucleus in sites of active synapses</li> </ul>	(Kaltschmidt and Kaltschmidt, 2009)
Brain	NF- $\kappa$ B has a role in maintaining learning and memory	<ul style="list-style-type: none"> <li>• Deletion of DNA-binding NF-<math>\kappa</math>B units in neurons or glia resulted in lower performances in multiple behavior tests in mice</li> <li>• Above-mentioned observation is also true for glutamatergic neurons with ablated NF-<math>\kappa</math>B</li> </ul>	(Kaltschmidt and Kaltschmidt, 2009)
Gut	NF- $\kappa$ B (through NEMO) protects intestinal epithelial cells (IECs) from gut commensal bacterial infection	<ul style="list-style-type: none"> <li>• Ablation of NEMO in IECs results in severe colitis from gut microbial infection</li> <li>• Ablation of NEMO in IECs decreases production of gut-protective defensins</li> </ul>	(Pasparakis, 2009)
Hair	NF- $\kappa$ B has a role in hair follicle organogenesis	<ul style="list-style-type: none"> <li>• Mouse model expressing NF-<math>\kappa</math>B super repressor in a Beta-catenin locus shows impaired hair follicle development</li> <li>• Repressed NF-<math>\kappa</math>B leads to decreased protection from apoptosis in follicular cells</li> </ul>	(Schmidt-Ullrich and Paus, 2005)

**Table 1.1.**  
**The role of NF- $\kappa$ B in the development and maintenance of tissues -**  
**continued**

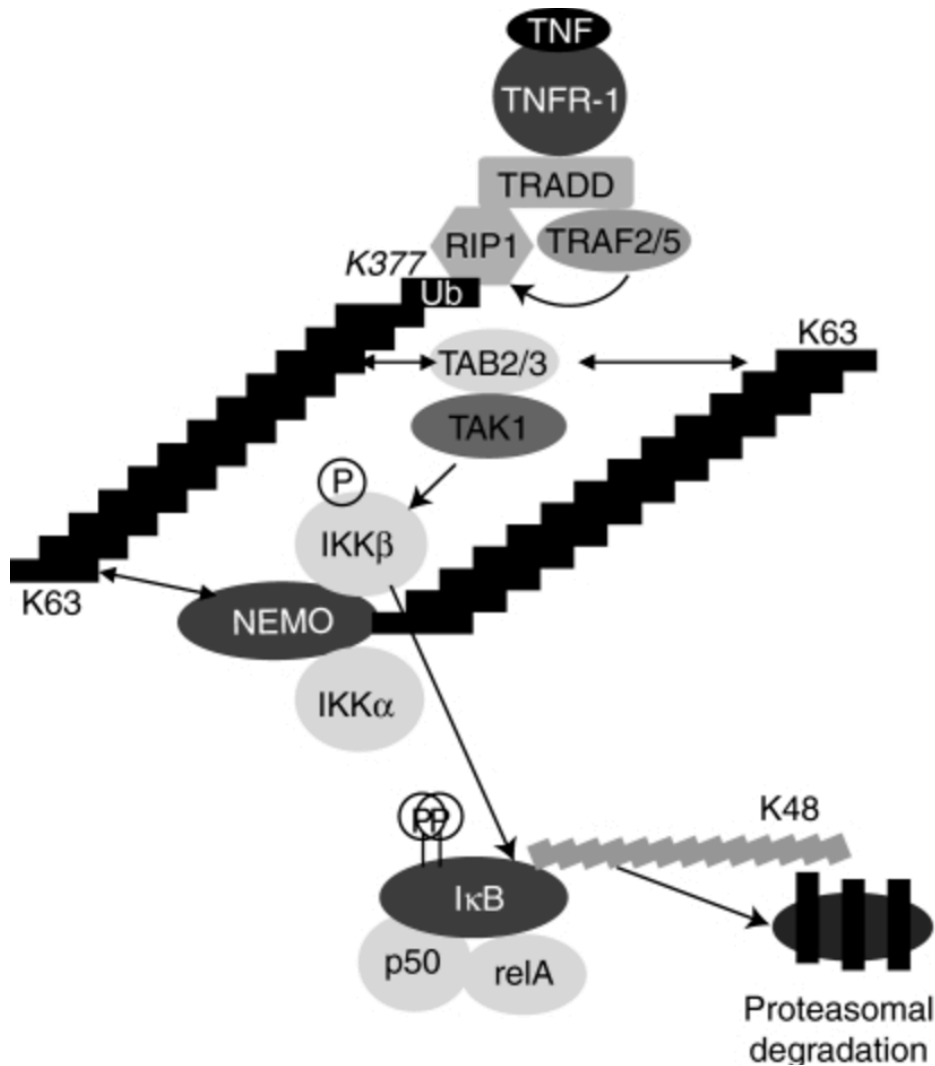
Organ	Role	Evidence	Reference
Liver	NF- $\kappa$ B helps maintain homeostasis in adult liver parenchymal cells (LPCs)	<ul style="list-style-type: none"> <li>• LPC specific ablation of NF-<math>\kappa</math>B (via NEMO) results in severe hepatitis &amp; hepato-cellular carcinoma</li> <li>• Active NF-<math>\kappa</math>B helps protect LPCs from TNF mediated apoptosis</li> </ul>	(Pasparakis, 2009)
Skin	NF- $\kappa$ B helps maintain immune homeostasis in Epidermal Keratinocytes (EKs)	<ul style="list-style-type: none"> <li>• EK specific IKK2 knockout mice show severe inflammation 3-4 days post birth</li> <li>• This defect is rescued in TNF knock-out mice which highlights the importance of the TNF dependent NF-<math>\kappa</math>B pathway</li> </ul>	(Pasparakis, 2009)
Limbs	NF- $\kappa$ B is important for vertebrate limb outgrowth during development	<ul style="list-style-type: none"> <li>• Rel/NF-<math>\kappa</math>B genes are expressed in the progress zone of the developing chick limb bud</li> <li>• Limb outgrowth is arrested when NF-<math>\kappa</math>B is blocked via dominant negative I<math>\kappa</math>B<math>\alpha</math></li> </ul>	(Kanegae <i>et al.</i> , 1998)

and their expression is often regulated at four different points in the pathway. These include at the cytoplasmic level (via IKKs and I $\kappa$ B $\alpha$ ), post-translational modifications of NF- $\kappa$ B itself (especially p65), at the level of transcriptional co-activators and the general transcription machinery, and via cross-talk with other pathways. Some important instances of these regulations are described below:

#### Regulation via core pathway proteins IKK and I $\kappa$ B $\alpha$

It has been clearly and unambiguously shown that most NF- $\kappa$ B signaling pathways proceed through the IKK complex (consisting of IKK1, IKK2, NEMO, ELKS and possibly other proteins (Figure 1.4). The three key and co-dependent aspects of IKK regulation that we want to draw your attention to are its ubiquitination (K63), oligomerization and phosphorylation. A 'simplified' elaboration of these steps is shown in Figure 1.5.

Once the IKK complex phosphorylates I $\kappa$ B $\alpha$ , the latter is subsequently ubiquitinated at Lys21 and Lys22 by  $\beta$ -TRCP ( $\beta$ -transducin repeat-containing



**Figure 1.5.**

**The IKK regulation of the NF-κB pathway.**

On NF-κB activation (via TNF in this case), the TNF receptor recruits TRADD (an E3 ubiquitin-ligase), TRAF2/5 and the kinase RIP1. K63 ubiquitination of RIP1 leads to the recruitment of TAB2/3 and eventual activation of the TAK1 kinase. At this point the IKK complex (which is scaffolded and organized via K63 ubiquitination of NEMO and oligomerization) is phosphorylated by TAK1. This activated IKK complex phosphorylates IκBα which in turn is ubiquitinated and degraded through K48 chains and the SCF-TrCP E3 Ligase, freeing up NF-κB to translocate to the nucleus and transcribe its target genes. Reproduced from Israël 2010

protein) which targets it for degradation by the 26S proteasome, thereby releasing NF- $\kappa$ B dimers from the cytoplasm and allowing them to translocate to the nucleus (Karin and Ben-Neriah, 2000) . Proteolysis-associated ubiquitination is required not only for I $\kappa$ B $\alpha$  degradation, but also for the processing of p100 and p105 NF- $\kappa$ B precursors. The review by Liu et al. 2012 is a great compendium on the biology of activation of the IKK complex.

#### Regulation via post translational modifications (PTMs) of p65

Post translational modifications targeting p65 can be mediated by components of both the NF- $\kappa$ B or heterologous signaling pathways. They provide an additional layer of regulation to NF- $\kappa$ B's transcriptional responses. The consensus is that these PTMs prevent inadvertent induction of target gene transcription and also provide an additional means of generating specificity in transcriptional programs (Huang *et al.*, 2010). They include phosphorylation, acetylation, and methylation of p65. We have summarized the key PTMs and their possible role in NF- $\kappa$ B regulation in Table 1.2. Although most of the established PTMs are on p65, other NF- $\kappa$ B factors, including p50, are known to be modified as well. In fact, phosphorylation of the NF- $\kappa$ B p50 subunit in response to IL-1-stimulated phosphatidylinositol 3-kinase (PI3K)/AKT increases the DNA-binding activity of the NF- $\kappa$ B complex (Koul *et al.*, 2001).



**Table 1.2****The key post-translational modifications (PTMs) of p65.**

PTMs add additional layers of regulation on the NF- $\kappa$ B pathway in the following ways. For references – <sup>1</sup>(Christian, Smith and Carmody, 2016), <sup>2</sup>(Chen, Mu and Greene, 2002), <sup>3</sup>(Ea and Baltimore, 2009), <sup>4</sup>(Collins, Mitxitorena and Carmody, 2016), <sup>5</sup>(Kelleher *et al.*, 2007).

Type of PTM	Site on p65	Causal protein	Role
Phosphorylation	S276 <sup>1</sup>	Protein Kinase A	Essential for p65-CBP (histone acetylase) binding for efficient transcription
Phosphorylation	S529 <sup>1</sup>	Casein Kinase II	Increases transcriptional activity (on endogenously supplied reporter)
Phosphorylation	S536 <sup>1</sup>	IKK2	Required for transactivation function
Acetylation	K310 <sup>2</sup>	CBP/p300	Required for transcriptional activity (but no role in DNA binding)
Monomethylation	K37 <sup>3</sup>	Set9	Required for NF- $\kappa$ B's promoter-binding and transcription of a subset of target genes
Ubiquitination	Unknown <sup>4</sup>	PDLIM2, COMMD1	Required for termination of the NF- $\kappa$ B transcription program
Nitrosylation	C38 <sup>5</sup>	NOS2	Inhibits NF- $\kappa$ B dependent transcription

### Regulation via co-activators and transcription machinery

NF- $\kappa$ B family members, like other transcription factors, lack enzymatic activity and can be considered specialized adapter proteins linking DNA sequences to enzymatic transcriptional co-regulatory proteins. The most well-studied is the CBP/p300 complex. This complex binds to NF- $\kappa$ B, and acetylates histones thereby influencing the folding and functional state of the chromatin fiber and increasing the accessibility of DNA to the transcriptional machinery (TATA binding protein, TFIID, RNA Polymerase II etc.) for transcription initiation (Gerritsen *et al.*, 1997; Mukherjee *et al.*, 2013). NF- $\kappa$ B also plays a role in transcription elongation by recruiting p-TEFb (there is proof of direct binding of p65 and p-TEFb in Barboric *et al.* 2001, while other studies including Hargreaves, Horng, and Medzhitov 2009 mention GCN5 and Brd4 as intermediaries). p-TEFb's kinase activity is necessary to de-repress the stalled polymerase complex leading to active transcription, especially of the immediately transcribed target genes. While p-TEFb and CBP/p300 positively regulate NF- $\kappa$ B dependent transcription, there are a few negative regulators as well. Chief amongst them are the HDAC (histone deacetylase) proteins HDAC1, HDAC2, and HDAC3 whose histone-deacetylation activities help in transcription deactivation (Ashburner, Westerheide and Baldwin, 2001). HDAC3 also deacetylates p65, hence enhancing its binding to I $\kappa$ B $\alpha$ , which results in its export back to the cytoplasm and eventual reset of the NF- $\kappa$ B transcriptional program (Kiernan *et al.*, 2003). Recently, another protein, ATF3 has been found to recruit

HDAC1 to p65 to help in deacetylation of its lysine 310, and reducing its transcriptional activity (Kwon *et al.*, 2015). The incredibly detailed reviews by Bhatt and Ghosh 2014 is an excellent resource for more information on the nuclear regulation of NF- $\kappa$ B.

#### Regulation via cross-talk with other pathways

NF- $\kappa$ B regulation via its own pathway proteins, post-translational modifications and co-activators (and repressors) ensure multiple levels of control over the induction of hundreds of genes. Since this pathway is implicated in multiple cellular processes including proliferation, inflammation, apoptosis etc. (Hayden and Ghosh, 2012) there is plenty of expected cross-talk and mutual regulation with other signaling pathways. For our purposes, we have focused on pathways that feed into NF- $\kappa$ B rather than the other way around. We have summarized the key ones in Table 1.3. Hoesel and Schmid 2013 have summarized the cross-talk of NF- $\kappa$ B with other pathways in an excellent review.

**Table 1.3****Biological pathways that lead to downstream activation or repression of NF- $\kappa$ B.**

For references – <sup>1</sup>(Wen, Sakamoto and Miller, 2010), <sup>2</sup>(Kwon *et al.*, 2015), <sup>3</sup>(Kawauchi *et al.*, 2008), <sup>4</sup>(Webster and Perkins, 1999), <sup>5</sup>(Xia, Shen and Verma, 2014), <sup>6</sup>(Pak and Miyamoto, 2013), <sup>7</sup>(Du and Geller, 2010), <sup>8</sup>(Criollo *et al.*, 2012), <sup>9</sup>(Salah *et al.*, 2016).

Pathway	Interaction with NF- $\kappa$ B	Hypothesis
CREB	Activation of CREB inhibits NF- $\kappa$ B	<ul style="list-style-type: none"> <li>• CREB and NF-<math>\kappa</math>B compete for the same pool of CBP/p300 for activation<sup>1</sup></li> <li>• Activated CREB expresses ATF3 that inhibits NF-<math>\kappa</math>B<sup>2</sup></li> </ul>
p53	Activation of p53 inhibits NF- $\kappa$ B	<ul style="list-style-type: none"> <li>• p53 suppresses GLUT3 (glycolysis), which impairs the GlcNAc modification of IKK2 thereby diminishing its kinase activity<sup>3</sup></li> <li>• p53 and NF-<math>\kappa</math>B also compete for the same pool of CBP/p300<sup>4</sup></li> </ul>
Kras	Activated Kras leads to activation of NF- $\kappa$ B	<ul style="list-style-type: none"> <li>• Activation of Kras induces the Erk and Akt pathways which further induce NF-<math>\kappa</math>B<sup>5</sup></li> <li>• Activation of Kras also up-regulates GSK-3alpha which induces NF-<math>\kappa</math>B<sup>6</sup></li> </ul>
Wnt	Wnt-Beta-Catenin pathway represses NF- $\kappa$ B in some cancer cells	<ul style="list-style-type: none"> <li>• <math>\beta</math>-catenin complexes with NF-<math>\kappa</math>B and reduces the latter's DNA binding<sup>7</sup></li> </ul>
Autophagy	Autophagy induces NF- $\kappa$ B target gene expression	<ul style="list-style-type: none"> <li>• Autophagy gene ATG5 induces NF-<math>\kappa</math>B in 293T and A549 cells<sup>8</sup></li> <li>• ATG7 and GABARAP (autophagy gene) knockout mice have repressed NF-<math>\kappa</math>B<sup>9</sup></li> </ul>

## **The mis-regulation of NF- $\kappa$ B**

In the previous sections, we have summarized the involvement of the NF- $\kappa$ B pathway in various aspects of immunity and development. Hence it is not a surprise that the mis-regulation of this pathway (via mutations or aberrant activation) plays an important and sometimes driving role in a number of diseases.

### NF- $\kappa$ B in genetic diseases

The role of NF- $\kappa$ B in the immune system becomes even more apparent when analyzed from the perspective of monogenic diseases stemming from the members of the pathway (Zhang, Lenardo and Baltimore, 2017). These diseases have only been discovered recently (early 2000s onwards) because of the progress made in next-generation sequencing, micro-arrays and linkage studies. Diseases affecting key members of the NF- $\kappa$ B pathway are summarized in Table 1.4. This recently acquired data shows the striking phenotypes of mutations in NF- $\kappa$ B proteins in humans and will be very useful in developing therapy as well as studying the consequences of signaling mis-regulation in the most relevant physiological context. The review by Zhang, Lenardo, and Baltimore 2017 goes into incredible detail about these diseases.

**Table 1.4****Monogenic diseases stemming from core NF- $\kappa$ B pathway components.**

This table is partially adopted from Zhang, Lenardo and Baltimore, 2017.

<b>Modified NF-<math>\kappa</math>B pathway gene</b>	<b>Resulting disease</b>
NEMO	Incontinentia Pigmenti
NEMO	Anhidrotic ectodermal dysplasia with immunodeficiency (EDA-ID)
I $\kappa$ B $\alpha$	Anhidrotic ectodermal dysplasia with immunodeficiency (EDA-ID)
IKK1	Severe fetal encasement malformation
IKK2	Severe combined immuno-deficiency (SCID)
p52, p50, RelB	Common variable immune deficiency (CVID)
NIK	Combined immuno-deficiency (CID)
CYLD (De-ubiquitinating enzyme)	Cylindromatosis

### NF- $\kappa$ B in inflammatory diseases

NF- $\kappa$ B is the main pathway that mediates inflammation; hence its up-regulation is an important factor in multiple diseases with an inflammatory phenotype. Such diseases include Rheumatoid Arthritis, Atherosclerosis, Asthma and Inflammatory bowel disease. In the synovial cells of patients with Rheumatoid Arthritis, activation of the NF- $\kappa$ B pathway has been shown to lead to activation of multiple genes that contribute to the inflammatory phenotype, including TNF $\alpha$ , chemokines and matrix metalloproteinases (Simmonds and Foxwell, 2008). The activation of the NF- $\kappa$ B pathway has been shown during different stages of Atherosclerosis including plaque formation, destabilization, and rupture. NF- $\kappa$ B target genes contribute to these angiogenic, apoptotic, and neoplastic processes (Pamukcu, Lip and Shantsila, 2011). The airway tissue of Asthma patients has shown increased NF- $\kappa$ B nuclear translocation and activation (Schuliga, 2015). Mice with attenuated NF- $\kappa$ B signaling (deficient in p65, TLR4, or TLR2) show decreased allergen-induced airway inflammation, providing evidence for the role of the innate immunity in this inflammatory phenotype (Schuliga, 2015). Finally, in Inflammatory Bowel Disease (both Crohn's Disease and Ulcerative Colitis), effector immune cells produce high levels of NF- $\kappa$ B activating cytokines (TNF, IL-6) that results in colonic tissue damage (Atreya, Atreya and Neurath, 2008).

## NF- $\kappa$ B in cancers

The study of NF- $\kappa$ B in cancers started when pathway members were found to be mutated in certain cancers, mostly of hematopoietic origins. Some non-Hodgkin's B-cell lymphomas have amplification and rearrangement of c-Rel. Also, NF- $\kappa$ B2/p100 is frequently activated through chromosomal translocations in lymphomas and leukemias (Xia, Shen and Verma, 2014). Direct NF- $\kappa$ B-activating mutations are extremely rare in solid tumors. That said, the NF- $\kappa$ B pathway is known to be active in multiple tumors including glioblastoma (Friedmann-Morvinski *et al.*, 2016), lung cancer (Xia *et al.*, 2012), etc. This leads to a chronic inflammatory phenotype which contributes to genomic instability that drives tumor development (Xia, Shen and Verma, 2014), so much so, that 'tumor promoting inflammation' is now a recognized 'hallmark' of cancer (Hanahan and Weinberg, 2011). Besides inflammation, NF- $\kappa$ B can also contribute to tumor development by inhibiting apoptosis (expressing anti-apoptotic genes like FLIP, c-IAP1/2, Bcl2), regulating angiogenesis (expressing matrix metalloproteinases, fibroblast growth factors, and IL8), and promoting metastasis (Twist1, a transcription factor regulating EMT is an NF- $\kappa$ B target in breast cancer). The reviews by Hoesel and Schmid (2013); Xia, Shen, and Verma (2014) offer great insights into NF- $\kappa$ B's proven and potential involvement in tumor progression. The comprehensive database maintained by Thomas Gilmore and Boston University cites evidence of the constitutive activation of NF- $\kappa$ B in over 30 different human cancers and cancer cell-lines.



### NF- $\kappa$ B and therapy

In theory, NF- $\kappa$ B seems to be a valuable pharmaceutical target, given its role in cancers and inflammatory diseases. In reality, NF- $\kappa$ B's involvement in multiple important processes including immunity leads to a lot of pleiotropic side effects once the pathway is therapeutically targeted. The following paragraph quoted from (Zhang, Lenardo and Baltimore, 2017) illustrates the challenges with using NF- $\kappa$ B as a target:

“Since NF- $\kappa$ B inhibits apoptosis and this is obligatory for cell survival, for example, in ABC-DLBCL lymphoma, then blocking NF- $\kappa$ B should be an effective treatment. Furthermore, upregulated NF- $\kappa$ B promotes cell proliferation, metastasis, metabolic changes, and other abnormalities that favor the expansion and spread of malignancy. Thus, strategies to suppress NF- $\kappa$ B have been clinically tested, most prominently proteasome blockers and IKK inhibitors. Bortezomib (Velcade) is approved for multiple myeloma, a plasma cell malignancy, whose aggressiveness depends in part on NF- $\kappa$ B. Also, thalidomide and other putative IKK inhibitors have been successful in myeloma. These agents, however, have been thwarted by side effects potentially due to NF- $\kappa$ B suppression including nephrotoxicity, neuropathy, and also the relapse of more aggressive forms of malignancy. Finally, as the pivotal role of the immune system in fighting malignancy has come into focus with new discoveries in immunotherapy, NF- $\kappa$ B inhibition is cast into a new light. Potent NF- $\kappa$ B inhibitors may emasculate T cells that antigenically recognize and kill tumor cells, thereby

worsening disease. Thus, the broad role of NF- $\kappa$ B in cellular regulation makes its druggability complicated.”

### **NF- $\kappa$ B – the questions unanswered**

Until now we have focused on the facts about NF- $\kappa$ B that “we know that we know” (as Donald Rumsfeld put it). We now want to draw your attention to the known unknowns – what we know that we don’t know-

1) IKK activation – The key question is how does a signaling cascade from receptors (TNFR, TLR4 etc.) that lack inherent kinase activity lead to the phosphorylation and activation of a kinase complex (IKK in this case). Despite the discovery of an increasing number of proteins between the receptor and IKK (including ubiquitin ligases), the model of auto-phosphorylation of IKK still holds water (Hayden and Ghosh, 2012). The other model (described in Figure 1.5) that describes IKK and TAK1 scaffolding via K63 ubiquitin chains originating from RIP1 is also supported in literature. Neither of these models have been conclusively proven *in vivo*, and there is a quest to seek more regulators of the NF- $\kappa$ B pathway upstream of IKK that will help answer these questions.

2) NF- $\kappa$ B regulation of transcription – The canonical NF- $\kappa$ B transcription factor (p65-p50) responds to a variety of stimuli and is activated by very similar steps (phosphorylation and degradation of I $\kappa$ B $\alpha$ , translocation of the hetero-dimer into the nucleus, binding to CBP/p300 and recruitment of the general transcription machinery). Yet, the genes transcribed by the pathway differ significantly based

on the stimulus and cell background. Also some NF- $\kappa$ B target genes are transcribed at different times in response to the same stimuli. Observations like these provide a hint that there are many layers (and hence permutations and combinations) in the nuclear regulation of NF- $\kappa$ B target gene transcription. Some relevant open questions in the field include – What are the co-factors that bind to NF- $\kappa$ B in the nucleus? What is the order of events of NF- $\kappa$ B dependent transcription for different genes? How are stimulus specific signals relayed to the nucleus to cause differences in the gene transcribed? We also know relatively little about the cause and status of chromatin remodeling, or the recruitment of the splicing machinery, at NF- $\kappa$ B transcribed genes. Integrative studies, such as the brilliant work by Aviv Regev's lab (Garber *et al.*, 2012) have given us snapshots of the different hierarchies of transcription factor binding and chromatin modification. They only show that multiple chromatin modifiers, enhancers, transcription factors, the pre-initiation complex, the elongation complex must come together to induce gene transcription and the composition and order of events in this milieu is stimulus, pathway, and even gene specific. Hence there is much to be learned still and we have explored these aspects of NF- $\kappa$ B regulation in Chapter 4.

3) Post-translational modifications of p65 (hows and whys) – PTMs contribute to an added layer of regulation to the NF- $\kappa$ B pathway. p65 is known to undergo at least 20 PTMs (Huang *et al.*, 2010). Only a few of them (including S276, S536 phosphorylations and K310 acetylation) are well characterized in terms of the

upstream proteins that cause them (e.g. PKA for S276 phosphorylation) and their roles in NF- $\kappa$ B dependent transcription. Their role *in vivo* is even less understood (Bhatt and Ghosh, 2014). It is possible that the PTMs are stimulus and target gene specific and discovering the proteins that cause them and the effects of removing these PTMs on NF- $\kappa$ B dependent transcription would help uncover this key layer of regulation.

4) Therapeutically targeting NF- $\kappa$ B – For a pathway that is known to be activated in many diseases and cancers, the lack of NF- $\kappa$ B targeting therapy, due to pleiotropic effects, further underscores its importance in general human physiology (including immunity and development). Research groups (including ours) have focused on targeting the disease promoting NF- $\kappa$ B transcribed genes rather than the pathway itself. This approach has its merits but the typical NF- $\kappa$ B dependent inflammatory phenotype in diseases is generally a result of a plethora of NF- $\kappa$ B target genes. One attempt to solve this conundrum would be to discover stimulus specific regulators of this pathway which can then be targeted, thereby reducing or potentially eliminating the harmful side effects of targeting the pathway as a whole.

Besides the unanswered questions mentioned above, we believe that the 31 year old collective effort put into learning more about NF- $\kappa$ B makes it an even more attractive pathway to study (“the most well studied transcription factor” according to David Baltimore). We say this because any future discoveries we make will be easier to put into context of the rest of the pathway given the

amount of work that has been done on it. This will give us more hints not just on the workings of the NF- $\kappa$ B pathway, but also of transcriptional regulation. With all this in mind, research groups including ours, have used high throughput methods to discover novel regulators of NF- $\kappa$ B. In the next sections we will summarize previously published efforts and introduce our own method to learn more about this pathway.

### **High throughput assays to discover regulators of the NF- $\kappa$ B pathway**

Given the importance of the NF- $\kappa$ B pathway in human biology, and the advent of high-throughput screening technology (RNAi, CRISPR, Mass-Spec analysis), it isn't a surprise that around 30 screens (Figure 1.6) have been published over the last decade, that have tried to uncover genes involved in this pathway. Their key aspects are summarized below:

**1) RNAi dominates the type of screening method used but proteomics is catching up** – RNAi is the high throughput assay most often used for screening for NF- $\kappa$ B regulators. In combination with an NF- $\kappa$ B promoter driven luciferase system, it is an easily tractable and scalable system (Sharma and Rao, 2009).

27 Screens, published from 2004 – 2015			
Type	Background	Scale	Stimulus
RNAi (48%)	293T cells (37%)	Whole Genome (48%)	TNF $\alpha$ (56%)
	Human Non-Immune cells (22%) (HeLa, A549, U2OS)		
Proteomics (26%)	Mouse Macrophages (15%)	Protein Specific (26%)	Bacterial stimulants (15%)
	Drosophila (15%)		
cDNA (7%)		Genome subset (26%) (Kinome, proteases, deubiquitinases etc.)	No stimulant (15%)
Others (19%)	Others (11%)		
<b>Regulators – CYLD, WIP1, USP2, AKIRIN1</b> are some of the novel NF- $\kappa$ B regulators that were comprehensively validated as follow-ups to the screens			

**Figure 1.6.**

**Summarizing previously published screens to discover regulators of the NF- $\kappa$ B pathway.**

We have used the parameters of Type (RNAi, Mass-spectrophotometry proteomics, over-expression cDNA, others including CRISPR), Background (different types of cells used to conduct these screens), Scale (the gene-set from which the regulators were discovered), and the Stimulus used to activate the pathway.

Potential off-target effects have continuously been minimized by verified and better-designed siRNA screening sets. The main limitation of RNAi is the transfection efficiency achieved in cells. It is known that some signaling proteins (especially kinases) can exert their impact on pathways even when they are knocked down by up to 80%.

Proteomics using mass-spectrophotometry has been an emerging alternative (or even complement) to RNAi screening. An elegant example is the study done by W. H. Wang et al. (2010) where they detect phospho-proteins that interact with p65 in response to TNF stimulation. Studies like these can be used to ask more pointed questions about various protein-protein interactions in the NF- $\kappa$ B pathway and how they change in response to stimulus. The advent of CRISPR technology provides an exciting avenue, especially in *in vivo* screening, as shown by the comprehensive screen by Parnas et al. 2015.

## **2) NF- $\kappa$ B screens have been predominantly done in less relevant backgrounds –**

The majority of human siRNA screens have been done in easily transfectable and tractable 293T cells (along with HeLa and A549 cells). The NF- $\kappa$ B pathway is most relevant in innate immune cells (monocytes, macrophages, neutrophils, etc.) and only one proteomics screen has been done in a human macrophage cell line (THP-1; S. Li et al. 2011). Non-immune cells such as 293T don't have all the components of the NF- $\kappa$ B pathway (for instance, these cells do not respond to LPS, which is a key stimulus of the pathway). With the improvement in efficiency of siRNA transfection reagents, we expect an increased trend

(including our own screens) in using relevant cell-types as the background for such experiments.

3) Over 90% of the screens have used a single stimulus, with TNF leading the way – Although TNF is a key activator of the NF- $\kappa$ B pathway, other important stimuli (especially TLR including bacterial products) represent less than 30% of the screens published on this pathway. We also found only one screen published by Chiang et al. 2012 that simultaneously tested more than one stimuli in a quest to find regulators that overlap between, or are unique to different stimulus driven NF- $\kappa$ B pathways.

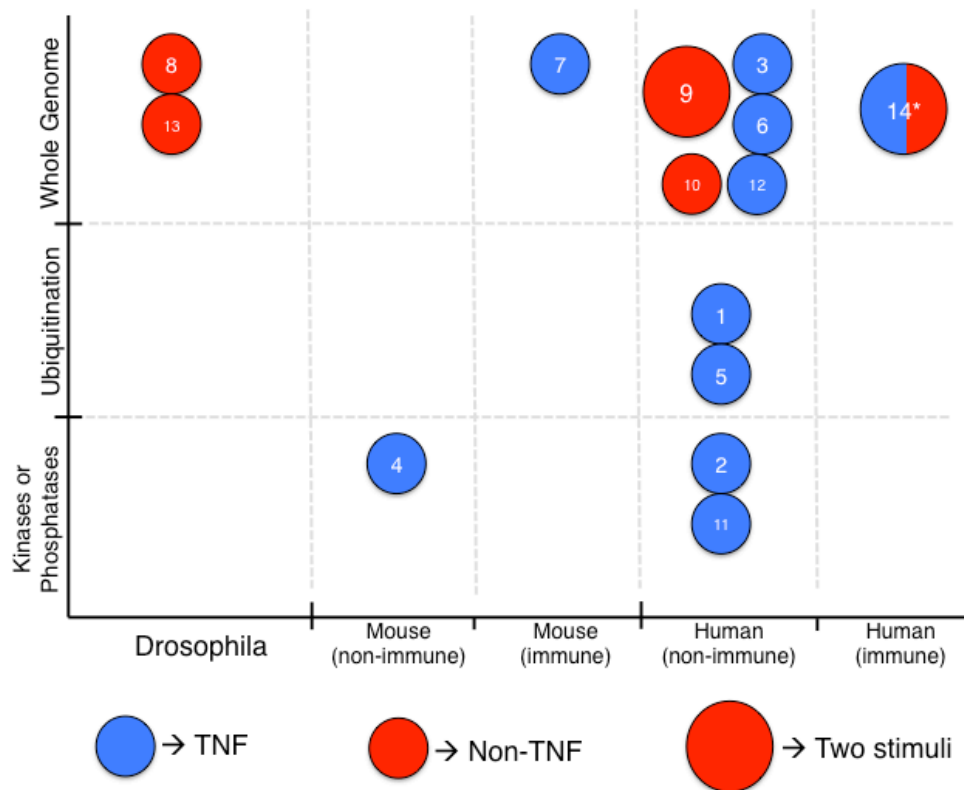
4) Screens have not provided in-depth mechanistic insight into the NF- $\kappa$ B pathway – The published screens on the NF- $\kappa$ B pathway have provided extensive validated datasets on potential regulators of the pathway but are overall lacking in new mechanistic insight about the pathway. Out of the 13 published RNAi screens that we looked at, only Brummelkamp et al. (2003) have proposed and verified a model for a novel gene's (CYLD) regulation of the NF- $\kappa$ B pathway. Other screens like Chew et al. (2009) and Metzger et al. (2011) have verified WIP1 and USP2 respectively to be regulators of NF- $\kappa$ B at but have stopped short of proposing and testing an exact mechanism. Also, the CRISPR screen from Parnas et al. (2015) has found the Oligosaccharyltransferase Complex to be a regulator of NF- $\kappa$ B, which is potentially very interesting. Directed proteomic screens (using mass-spec) as done by Kliza et al. (2017) have offered interesting insights into the binding partners of various proteins in the TNF



dependent NF- $\kappa$ B pathway. They have used a creative method using internally tagged ubiquitin to identify the linear poly-ubiquitin targets in the NF- $\kappa$ B pathway in response to TNF.

### **Our proposal to screen for novel NF- $\kappa$ B regulators**

With the unanswered questions on NF- $\kappa$ B, and the knowledge of previous screens, in mind, we have run two whole-genome RNAi screens in human macrophages in response to TNF and LPS. We believe that the relevant physiological background, multiple stimuli, stringency of analysis, and focused pursuit of a novel NF- $\kappa$ B regulator (SNW1/SKIP) are the key differentiators of our screens (Figure 1.7). We have described the screens and their analysis in Chapter 2, and the data supporting SNW1's exact role in the NF- $\kappa$ B pathway in Chapter 3. We have also described evidence for other novel genes' involvement in the NF- $\kappa$ B pathway in Chapter 4 along with defining a place for our body of work (the screens and experiments on SNW1) in the immensely populated field of NF- $\kappa$ B research.



**Figure 1.7.**

**Differentiating our screens (14) from 13 previously published RNAi screen on novel regulators of NF- $\kappa$ B**

Our key differentiators include a relevant physiological background (human macrophages), along with the fact that we are testing two stimuli at the same time (LPS and TNF). Most previous screens (1-13) have used a single stimulus and/or were conducted in non-immune human cells (like 293T, HeLa, A549) which are relatively less useful in studying the NF- $\kappa$ B pathway.

## **Chapter 2: Screening for regulators of NF- $\kappa$ B in human macrophages**

## BACKGROUND

Nuclear factor-kappa B (NF- $\kappa$ B), as previously described, is the major inflammatory signaling pathway activated when cells are exposed to a variety of stimuli, including cytokines (such as tumor necrosis factor alpha (TNF) and lipopolysaccharides (LPS)), ultraviolet (UV) radiation, stress, and pathogenic assaults. Here we describe three levels of RNAi screens to identify genes regulating the pathway in human macrophage cells in response to TNF and LPS, using an exogenous NF- $\kappa$ B-Luciferase reporter. LPS activates the NF- $\kappa$ B pathway via Toll-like receptor 4 (TLR4) that is the main sensor for microbial stimuli in innate immune cells (Lu et al., 2008), while TNF activates the pathway through the TNF-receptor (TNF-R) family which is involved in the regulation of a variety of processes including inflammation, proliferation, differentiation, apoptosis and lipid metabolism (Kallioli et al., 2016). These data provide a resource for analyzing and comparing mediators of the NF- $\kappa$ B pathway across two different stimuli in the physiologically relevant human macrophage background. Our list of novel regulators of NF- $\kappa$ B is led by SNW1, a transcription co-activator and splicing factor. We have also performed a number of bioinformatics analyses to help characterize new genes and pathways of interest that have shown positive results in our screening process.

## RESULTS

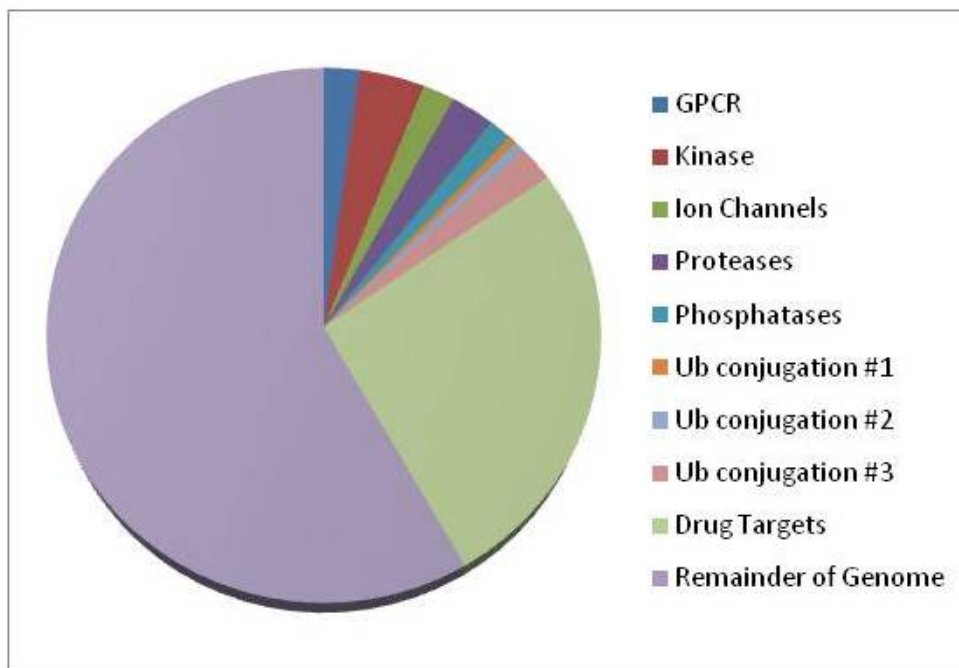
### Primary Screens

#### Methodology

We developed and optimized a high-throughput luciferase assay using a whole genome siRNA oligo library from GE Dharmacon to search for potential genes that play a role in NF- $\kappa$ B activation by LPS and TNF. The background we used was the human acute monocytic leukemia line THP-1 differentiated into macrophages by adding phorbol 12-myristate 13-acetate (PMA; optimized by Park et al. 2007). We had pre-optimized siRNA transfection conditions for signal versus background with p65-specific siRNAs versus control siRNAs.

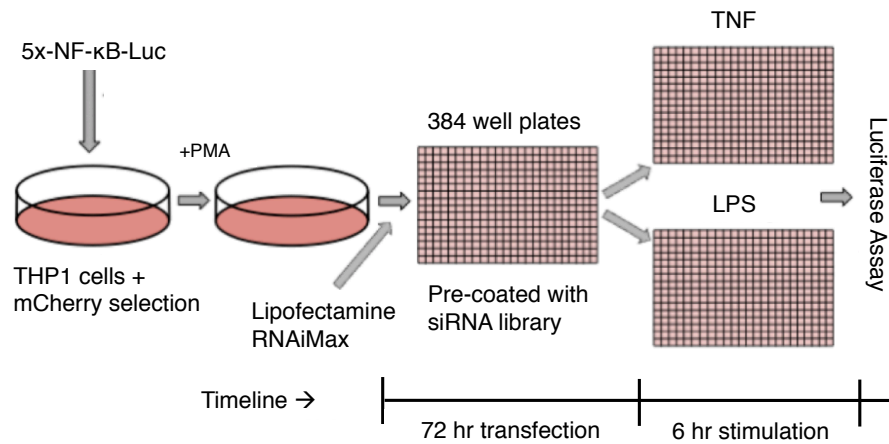
Differentiated THP-1s were further validated for NF- $\kappa$ B signaling by finding that both LPS and TNF $\alpha$  induced I $\kappa$ B $\alpha$  phosphorylation and degradation, and p65 phosphorylation and nuclear translocation.

The number of genes represented and characteristics of the siRNA libraries used for the primary screens are summarized in Figure 2.1. siRNA oligos from each of the libraries were pre-spotted into 384-well plates and reverse transfected for 72 h into differentiated THP-1 macrophages stably transduced with a 5X-NF- $\kappa$ B luciferase reporter. These cells were then treated for 6 hours with 10 ng/mL TNF or LPS (each in duplicate) and assayed for



**Figure 2.1.**  
**Characteristics of the Dharmacon On Target Plus Whole Genome library (GE) used for the primary screen**

The library covered siRNAs for 18090 genes with potential 'drug targets' representing the largest group of genes covered. Each gene was targeted simultaneously by 4 different siRNAs (pooled) in the primary screen to ensure better knockdown.



**Figure 2.2.**

**Schematic representation of the primary screen**

siRNA oligos (4 for every gene, and one gene per well) were pre-arrayed into 384-well plates. THP-1 macrophages with the NF-κB reporter were reverse transfected for 72 h before being treated with TNF or LPS for 6 h and assayed for Luciferase activity. Each whole genome library was assayed in duplicate for each of the two stimuli.

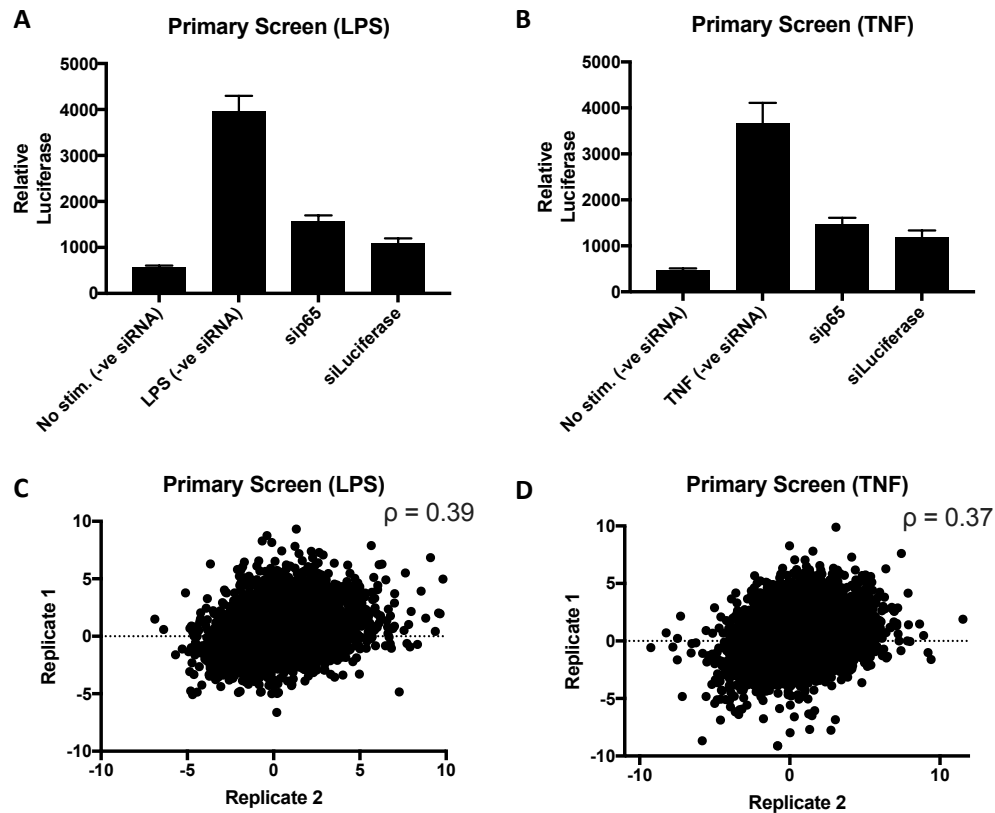
luciferase activity (methodology described in Figure 2.2). Each plate had pre-spotted negative controls (Scrambled siRNAs) and positive controls (Luciferase and p65 siRNAs).

#### Assessing screen efficiency

Upon completion of the screen, we wanted to assess its ability to successfully identify genes of interest. Screen efficacy was determined by the induction of luciferase on treatment with LPS and TNF along with the knockdown in presence of siLuciferase (siLuc), sip65 and other positive controls that had been pre-added to each of the 384-well plates (Figure 2.3A-B). The results showed that each of these screens could successfully identify potential genes of interest. Screen efficacy was also determined by replicate well analysis. 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. The correlation coefficients for both the stimulants were positive and statistically significant (Figure 2.3C-D) indicating the reproducibility of the screen.

A final analysis was done to assess intra-plate variability caused from liquid dispensing and plate-reading errors. This included siphoning off outliers





**Figure 2.3.**

**Assessing primary screen efficiency**

(A, B) Average normalized NF- $\kappa$ B luciferase values for the negative controls (-ve siRNA) and positive controls (sip65, siLuciferase) across both screens. (C, D) High co-relation coefficients for the replicates of both the screens indicates their reproducibility

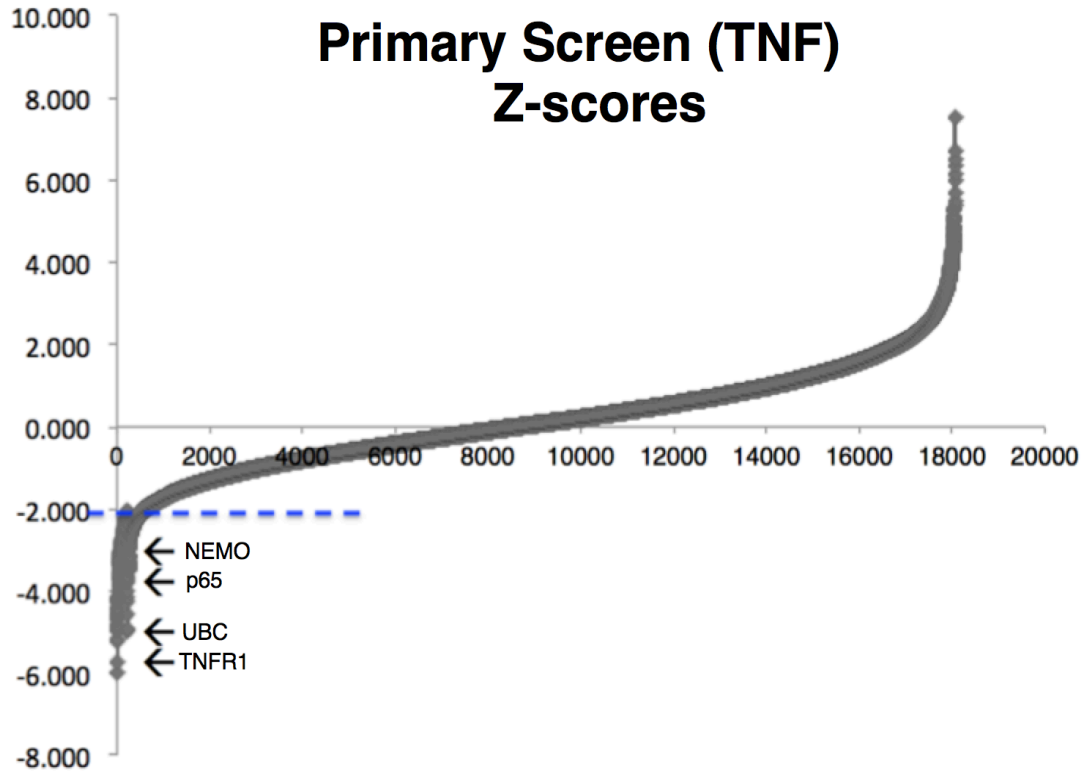
that weren't replicated and detecting and normalizing for geometric patterns (for e.g. increased Luciferase values from Left to Right of the plate indicating an error in the geometric calibration of the plate-reader).

### Analysis

Our method of "hit" analysis used the z-score based on every plate's median and median absolute deviation (MAD). This method, known for its robustness (Chung *et al.*, 2008), safely assumed that most of the genes in every plate are non-regulators of the NF- $\kappa$ B pathway. A plate median and MAD is calculated for every plate, and every well (1 gene = 1 well) is assigned a z-score based on the formula shown in Table 2.1. Hence every gene has two z-scores per stimulus (LPS or TNF). A z-score cut-off of less than -2.0 was used for each replicate for both the LPS and TNF screens. This cut-off is more stringent than other published whole-genome RNAi screens on the NF- $\kappa$ B pathway (Gewurz *et al.*, 2012). A fraction of the known regulators of both the LPS and TNF NF $\kappa$ B pathways were identified from this primary screen proving its validity as shown in Figures 2.4 and 2.5. The TNF and LPS screens gave 232 and 104 hits respectively with 43 of them being common, including known regulators like RELA, NEMO, and UBC (Chen ZJ, 2005). Figures 2.4 and 2.5 also shows hits that are stimulus specific mediators (e.g. TNFR for TNF, TLR4 for LPS), providing further validation for both the screens.

**Table 2.1.**  
**Calculating the z-score of individual genes in the primary screen**

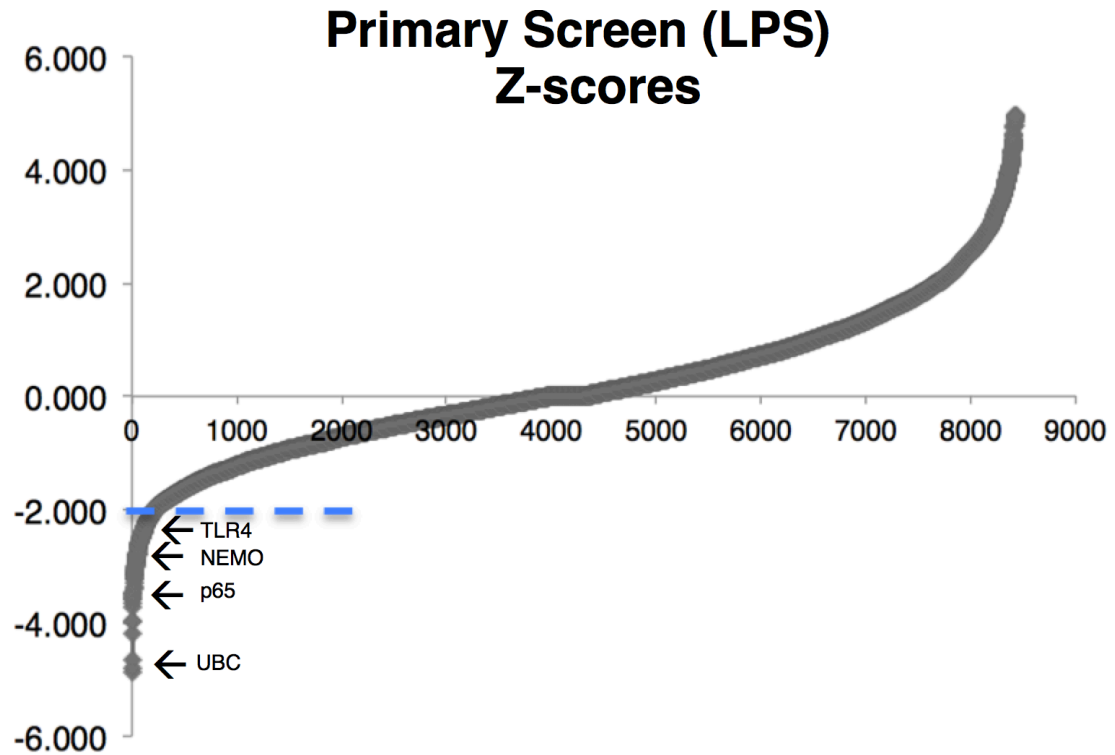
Steps to calculate z-score for a gene	Formulas
1) Calculate the plate median luciferase readout of the plate in which the gene's siRNA pool was transfected	$MAD = \frac{\sum_{i=1}^n  x_i - \bar{x} }{n}$ $z_i = (x_i - \bar{x})/MAD$ <p><math>\bar{x}</math> = plate median, <math>x_i</math> = reading of the <math>i</math>th well, <math>n</math> = total wells in a plate, <math>z_i</math> = z-score of the gene who's siRNAs are in the <math>i</math>th well</p>
2) Calculate the same plate's median absolute deviation (MAD)	
3) Calculate z-score which represents the number of MADs that a gene's luciferase readout is away from its plate median	
4) If z-score in both the duplicates (for one stimulus LPS or TNF) is $\leq -2.0$ then mark it as a 'hit'	



**Figure 2.4.**

**The TNF Primary Screen**

Most genes have a z-score between -2 and +2, as evident from the smooth line in that interval. Only 232 genes have a z-score lower than the cut-off of -2 (marked by the dashed line). They include known NF- $\kappa$ B regulators such as NEMO and p65 and the TNF specific regulator TNF Receptor (TNFR1).



**Figure 2.5.**

**The LPS Primary Screen**

Most genes have a z-score between -2 and +2, as evident from the smooth line in that interval. Only 104 genes have a z-score lower than the cut-off of -2 (marked by the dashed line). They include known NF- $\kappa$ B regulators such as NEMO and p65 and the LPS specific regulator Toll-like receptor 4 (TLR4).

## Next Steps

Although the primary screen hits include known NF- $\kappa$ B mediators, it also includes genes that are essential for transcription and translation including ribosomal proteins and elongation factors (RPL18, RPL23, RPL8, RPL4, EIF4A1 etc). These genes passed the z-score cut-off since knocking them down reduces cell-viability which shows as a reduction in Luciferase readout. Along with that, every well had a pool of four siRNAs that targeted the same gene and it was necessary to remove false positives that arose from potential off-target effects of one of those four siRNAs, and to make sure that multiple individual siRNAs targeting the same gene gave the same positive result (reduction in NF- $\kappa$ B dependent Luciferase activity). These two key factors necessitated stringent secondary screens that are elaborated upon in the following section.

## **Secondary Screens**

### Methodology

We addressed the caveats of the primary screen (off-target effects of siRNAs and counting 'essential' genes as false-positives) by setting up a second round of screening using the 232 and 104 hits from the TNF and LPS screens respectively. The process (illustrated in Table 2.2) included testing all four siRNAs (in four different wells) per gene in triplicates for knockdown of NF- $\kappa$ B Luciferase induction in response to TNF or LPS. We also normalized

**Table 2.2****Key differences between the primary and secondary screens**

The secondary screen is an attempt to deconvolute the effects of single siRNAs from the siRNA pool, along with filtering out the false positives from the primary screen that have been counted due to the loss of cell viability. Also, the secondary screen has more replicates and a stringent RSA analysis for 'hits' for greater stringency

<b>Parameter</b>	<b>Primary Screen</b>	<b>Secondary Screen</b>
siRNAs	4 siRNAs per gene were transfected together in a single well	4 siRNAs per gene were transfected in individual wells
Replicates	2 replicates per stimuli	3 replicates per stimuli
Readout	NF- $\kappa$ B driven luciferase	NF- $\kappa$ B driven luciferase and cell viability
Analysis	Z-score cut-off	Redundant siRNA analysis

these results for cell viability by including a toxicity assay performed in duplicate for both stimuli.

### Analysis and Results

Redundant siRNA activity (RSA) analysis is a convincing statistical method to interpret data from RNAi screens, where individual siRNAs are tested, while minimizing off-target effects (König *et al.*, 2007). We could not use it for the primary screen since different siRNAs for the same gene were not tested individually. Genes in the secondary screen however were ranked based on the RSA method. We also included two other pre-requisites for genes to be classified as positives – first that all 4 siRNAs of the gene showed less than 20% loss in cell viability (to eliminate ‘essential’ genes like elongation factors, ribosomal proteins that showed up as false-positives in the primary screen), and second was that at least 2 out of 4 siRNAs for each gene shows more than 50% reduction in NF- $\kappa$ B dependent luciferase activity (to eliminate false-positives due to off-target effects from single siRNAs).

The aforementioned analysis resulted in 41 and 35 genes that mediated TNF and LPS dependent NF- $\kappa$ B Luciferase activity respectively. The top 20 genes for each stimulus are shown in Tables 2.3 and 2.4, in order of their RSA ranks. The list contains known mediators like NEMO, p65, Importin and stimulus specific mediators like TLR4 (LPS) and TNFR1 (TNF). Along with those genes,



**Table 2.3****Top 20 hits from the LPS Secondary Screen**

Known NF- $\kappa$ B regulators are underlined while genes common between the TNF and LPS screens are bolded

Rank	Gene Name	Known Gene Function(s)
1	<b>SNW1</b>	Transcription Co-activator, Splicing Factor
2	<b>UCHL3</b>	De-ubiquitinating Enzyme
3	<b>CFLAR</b>	Apoptosis Regulator
4	<b>PRKCG</b>	Protein Kinase C Gamma
5	<b>TRPC6</b>	Ion Channel
6	DUSP15	Dual Specificity Phosphatase
7	<u>KPNA1</u>	Importin
8	<b>BHMT2</b>	Methyltransferase
9	GRIN2B	NMDA Receptor
10	ITCH	E3 Ligase
11	APC2	Wnt Regulator
12	CINP	CdK Interacting Protein
13	<u><b>RELA</b></u>	NF- $\kappa$ B
14	<u>RIP1</u>	NF- $\kappa$ B Receptor Activator
15	FOSB	Transcription Factor (with Jun)
16	<u>TLR4</u>	LPS Receptor
17	KEL	Metallo-peptidase
18	<b>KDM4A</b>	Histone Demethylase
19	BFAR	Apoptosis Regulator
20	<b>ATG7</b>	Autophagy Regulator

**Table 2.4****Top 20 hits from the TNF Secondary Screen**

Known NF- $\kappa$ B regulators are underlined while genes common between the TNF and LPS screens are bolded

Rank	Gene Name	Known Gene Function(s)
1	<b>SNW1</b>	Transcription Co-activator, Splicing Factor
2	TNFR1	TNF Receptor
3	<b>UCHL3</b>	De-ubiquitinating Enzyme
4	<u>GHRH</u>	Growth Hormone Releasing Hormone
5	BMP10	TGF Beta Activator
6	<u>NDP52</u>	Autophagy Regulator
7	CRHR2	Corticotropin Releasing Hormone
8	<b>ATG7</b>	Autophagy Regulator
9	NEF3	Neurofilament Protein
10	<b>CFLAR</b>	Apoptosis Regulator
11	CLCN2	Ion Channel
12	<b>KDM4A</b>	Histone Demethylase
13	HAPLN2	Extra-cellular Matrix Protein
14	<b>BHMT2</b>	Methyltransferase
15	<b>PARVA</b>	Matrix Remodeller
16	<u>RELA</u>	NF- $\kappa$ B
17	C5ORF11	Unknown Function
18	<b>TRPC6</b>	Ion Channel
19	LCE1B	Keratinization
20	CX3CL1	Chemokine

there are genes like ATG7, GABARAP, CFLAR, NDP52 that have a previously published role in the NF- $\kappa$ B pathway (Salah *et al.*, 2016), (Kataoka and Tschopp, 2004; Till *et al.*, 2013) but the mechanism by which they affect the pathway had not been completely elucidated. Most interestingly, we discovered some novel regulators like SNW1, UCHL3, KDM4A etc. and we have hypothesized their role in the NF- $\kappa$ B pathway in Table 2.5.

We then performed a number of analysis to identify pathways, and biologic processes over-represented in our list of novel candidate genes. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis showed enrichment for 10 pathways with p-values < 0.05. As expected, the TNF and LPS signaling pathway were the top 2 hits. Interestingly, the apoptosis pathways (due to hits like CFLAR, BFAR) and the autophagy pathways (due to hits like ATG7, NDP52) was also over-represented.

### Next steps

The potential novel mediators of the NF- $\kappa$ B pathway discovered through two rounds of screening seemed interesting, especially the ones that were hits in both the LPS and TNF secondary screens. Hence, we wanted to validate them even further using an endogenous readout of NF- $\kappa$ B (as opposed to the exogenous NF- $\kappa$ B driven Luciferase reporter), and in a different cell-line.

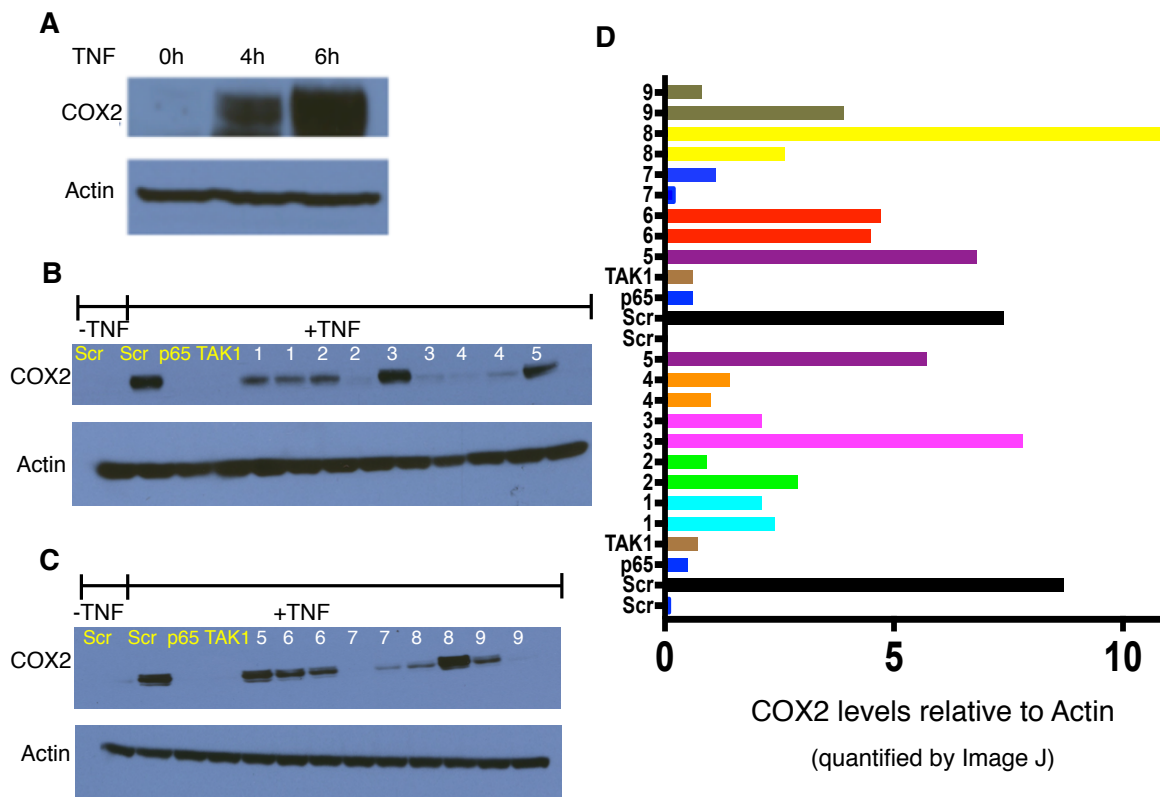
## Final validation of hits

### Methodology

To further validate our top candidates (common for the LPS and TNF pathways), we used the U87 Glioblastoma-like cell line as it had been previously shown by our lab to have an inducible NF- $\kappa$ B pathway (Friedmann-Morvinski *et al.*, 2016) and could be transfected with siRNAs at a very high efficiency (data not shown). We reproduced the activation of the endogenous NF- $\kappa$ B pathway in this cell line by treating it with TNF and immuno-blotting for COX2 protein (one of the most well established target genes of NF- $\kappa$ B) after 6 hours of stimulation as shown in Figure 2.6A. Once that was established, we took the two best siRNAs for every gene (out of four tested in the secondary screen) from the secondary screen and tested the induction COX2 on TNF treatment as shown in Figure 2.6B and Figure 2.6C. siRNAs against p65, TAK1, NEMO were used as positive controls. The protein readouts were normalized to Actin (loading control).

### Analysis and Results

We used Image J to quantify induction and subsequent knockdown of COX-2 protein expression as shown in Figure 2.6D. As shown, a good majority of the siRNAs tested showed attenuation of endogenous NF- $\kappa$ B dependent COX2 expression. Table 2.5 shows the top genes (in order of COX2 attenuation) that passed this level of screening. The top gene from both



**Figure 2.6.**

### Endogenous validation using U87 cells

**(A)** shows activation of COX2 on TNF treatment in U87 cells to verify its utility as a cell-line to test regulators of NF- $\kappa$ B. **(B)**, **(C)** show the validation methodology – U87 cells treated with Scrambled siRNAs (Scr) are induced with TNF and COX2 expression is checked. U87 cells transfected with positive controls siRNAs against p65 and TAK1 along with pairs of siRNAs of test genes (1,1), (2,2), etc. are also treated with TNF to measure COX2 induction via the NF- $\kappa$ B pathway. COX2 induction (with respect to Actin) is quantified in **(D)** – as expected siRNAs against p65 and TAK1 down-regulate COX2; positive hits include pairs such as (1,1), (2,2), (4,4), (7,7), etc. The genes tested (with their numbers) were BHMT2(1), UCHL3(2), NEF3(3), GHRH(4), HAPLN2 (5), GABARAP (6), SNW1(7), ATG7(8), TRPC6 (9)

**Table 2.5****The top hits from endogenous validation in U87 cells.**

The genes that have been previously implicated in the NF- $\kappa$ B pathway, or innate immunity, are bolded.

<b>Rank</b>	<b>Gene Name</b>	<b>Possible mechanism for regulating NF-<math>\kappa</math>B</b>
1	SNW1	Co-activator, NF- $\kappa$ B target gene splicing
2	GHRH	Via PAK-1 or STAT3 (Gan <i>et al.</i> , 2016)
3	UCHL3	De-ubiquitination of complexes upstream of IKK
4	BHMT2	Methyl-transferase (PTM of p65)
5	KDM4A	Histone Demethylase – Regulating chromatin
6	<b>CFLAR</b>	via reported RIP1 association
7	TRPC6	Ion channel – at the receptor level
8	BFAR	Cross talk with Apoptosis
9	DGKK	Kinase – phosphorylation of a key pathway protein
10	<b>CUL1</b>	Via ubiquitination of complexes upstream of IKK
11	PRKCG	Kinase – phosphorylation of a key pathway protein
12	PARVA	Via matrix remodeling
13	<b>GABARAP</b>	Cross talk with autophagy (ATG5, ATG7)
14	NEF3	Via matrix remodeling

the LPS and TNF tertiary screens was SNW1 (SKIP), a transcription co-activator and splicing factor. Hence, we chose to validate this gene further and delineate its role in the NF- $\kappa$ B pathway as described extensively in Chapter 3.

## **DISCUSSION**

### **Screens**

In this chapter, we have described a comprehensive method of finding novel regulators of the NF- $\kappa$ B pathway using RNAi screens in a human macrophage cell line. Our primary screen identified 232 and 104 potential regulators in response to TNF and LPS respectively. Normalizing for siRNA off-target effects and cell-viability, the secondary screen narrowed the list to 41 and 35 regulators for the TNF and LPS induced pathways respectively. We went a step further and conducted a tertiary round of screening in a glioblastoma cell line and the top 14 regulators of the TNF and LPS NF- $\kappa$ B pathways that passed that screen are listed in Table 2.5. These regulators have hence demonstrated a potential role in the NF- $\kappa$ B pathway in two different cell-lines and using both exogenous and endogenous NF- $\kappa$ B dependent reporters.

These were the first NF- $\kappa$ B RNAi screens conducted in the physiologically relevant background of differentiated THP-1 macrophages, due to the previous lack of efficient transfection protocols in these cells. These were also one of the first RNAi screens that directly compared NF- $\kappa$ B modulators from two distinct stimuli, LPS and TNF. A commentary on the comparison of regulators between

those two stimuli is in Chapter 4. Finally, these screens provide a comprehensive data set for comparison with other NF- $\kappa$ B regulators discovered using screens in different cell lines, described in Chapter 1.

### **Novel mediators of the NF- $\kappa$ B pathway**

Our three levels of screening found known and novel regulators of the NF- $\kappa$ B pathway. Hits such as the core pathway genes, including NEMO, p65, Importin, TNFR1 (TNF screens) and TLR4 (LPS screens), validated our methods. Other previously-reported modulators in the top hits include ATG7, GABARAP, CFLAR, CUL1 and NDP52 (Kataoka and Tschopp, 2004; Singleton and Wischmeyer, 2008; Till *et al.*, 2013; Salah *et al.*, 2016). They are not known to be the core members of the NF- $\kappa$ B pathway in multiple cell-lines, but there is evidence for their potential roles in the pathway. Most interestingly, the tertiary screens provided a list of novel genes that potentially affect the NF- $\kappa$ B pathway. We have summarized their potential roles in this pathway in Table 2.5.

The top novel gene discovered from this process is SNW1 (a known splicing factor and transcription regulator) and we will focus Chapter 3 on uncovering its potential role in the NF- $\kappa$ B pathway.

Other genes of interest include the kinases PRKCG (Protein Kinase C Gamma) and DGKK (Diacylglycerol Kinase Kappa). Since the NF- $\kappa$ B is a fast-acting pathway (the first transcription of target genes after induction happens within 10 minutes (Hao and Baltimore, 2013), it relies a lot on kinases on signal



transduction (eg TAK1, IKK complex etc.), and it will be interesting to follow-up on these two kinases. Our hypothesis is that since they are membrane proteins, they affect the NF- $\kappa$ B pathway at the level of the receptor, IKK or p65-p50 phosphorylation in the cytoplasm.

GHRH (Growth hormone releasing hormone; discovered in our screen) was recently reported to influence the inflammatory phenotype in patients with gastric cancer (Gan *et al.*, 2016). The authors hypothesize that the influence is via PAK1's activation of the NF- $\kappa$ B pathway (which is regulated through NIK). We believe that there might be a more direct mechanism at play since, in our screens, knocking down GHRH affects the TNF and LPS arc of the NF- $\kappa$ B pathway (not related to NIK). This is especially interesting since GHRH agonists are available and blocking the TNF arc of NF- $\kappa$ B will be useful in attenuating the inflammatory component of multiple diseases and cancers.

One more gene that is very interesting is TRPC6 – a transient receptor potential (TRP) channel. NF- $\kappa$ B is known to activate this gene in pulmonary artery smooth muscle cells in the context of pulmonary arterial hypertension (PAH), (Yu *et al.*, 2009). Once activated this channel further activates the NF- $\kappa$ B pathway in a feed-forward manner via an unknown mechanism. While in Kidney cells, NF- $\kappa$ B is known to mediate the suppression of TRPC6 via Protein Kinase C (Wang *et al.*, 2013). Hence the interaction between TRPC6 and the NF- $\kappa$ B pathway might be very context specific, and hence it'll be fascinating to see its

role in mediating NF- $\kappa$ B in immune cells, as the general role of TRP channels in the immune system is not well established.

## **Limitations**

Although our data suggests that a number of genes may play a role in NF- $\kappa$ B induction, we must also take note of our assay limitations. Firstly, the siRNA library (4 siRNAs per gene) is unfortunately, not all validated. The library was designed via algorithms for each siRNA to specifically target a particular gene so their efficiency in knocking down the target gene could be over-stated. In this way, we might be underestimating our list of genes of interest, especially because siRNA transfection efficiency in THP-1 cells itself was around 75-80% (data not shown). Hence, when we followed up on SNW1 as a mediator of the NF- $\kappa$ B pathway, one of the first experiments we did was to validate SNW1 protein knockdown using the siRNAs that we used for the screen (Chapter 3). Secondly, our screens did not normalize NF- $\kappa$ B luciferase activity for transfection efficiency (using Renilla luciferase). We partially compensated for this by normalizing for total number of alive cells in the secondary screen but our method assumes similar siRNA transfection efficiency for THP-1 cells in every round of screening and for all siRNAs transfected. We are confident about this assumption since we had consistently achieved 75-80% siRNA transfection in THP-1 cells using the screening protocol (measured by a fluorescent siRNA; data not shown). Also, all the screens were done using low passage THP-1 cells (Passage 5-10

after receiving from ATCC) from the same lot. Hence we believe that siRNA transfection efficiency variability was minimal. Thirdly, our screens' results are limited to THP-1 cells (and U87 cells for the validation tertiary screen) and even though it is a relevant cell-line, the NF- $\kappa$ B pathway is active in most cells and with a growing interest in the activation of the pathway in various cancerous tissues, it will be interesting to apply this screening method to those different types of cells. And fourthly, even though our screens uncovered some potential negative regulators of NF- $\kappa$ B (siRNAs against those genes up-regulated NF- $\kappa$ B dependent luciferase), we believe that the TNF and LPS concentration used was potentially saturating and hence we would be skeptical about these negative regulators and would want to validate them further in un-stimulated basal conditions before proceeding further.

### **Next steps**

Our overall goal with these screens was not only to provide a potentially useful data set of NF- $\kappa$ B mediators but to define a specific role for one new mediator. With that in mind, we followed up on our top hit from the tertiary screens – SNW1. Chapter 3 is composed of our results and analysis on that topic. Along with that, we followed up on a few other positive and negative regulators of NF- $\kappa$ B and Chapter 4 summarizes our results and hypothesis for them.

## **MATERIALS AND METHODS**

### **Screening Reagents**

#### 1) siRNAs

- Library: GE Dharmacon Human On-TARGETplus siRNA SMARTPOOL library (Catalog Number G-105005-025)
- Negative Control: Qiagen AllStars Negative Control siRNA (Catalog Number SI03650318)
- Positive Controls: Qiagen Luciferase GL2 siRNA (Catalog Number SI03650353), Qiagen FlexiTube p65 siRNA (Catalog Number SI00301672)

#### 2) Transfection

- Plates: Falcon 384 well plates (Catalog Number 353988)
- Transfection Reagent: ThermoFisher Lipofectamine RNAiMAX (Catalog Number 13778150)
- Transfection Media: ThermoFisher Opti-MEM (Catalog Number 31985070)

#### 3) Cell differentiation and stimulation

- Macrophage differentiation: Sigma-Aldrich PMA (Catalog Number P1585)
- Stimulants: Lipopolysachharide (Invivogen, Catalog Number tlrl-eb1ps), Tumor Necrosis Factor Alpha (Cell Signaling, Catalog Number 8902SC)

#### 4) Readout

- Luciferase reagent: Promega Bright-Glo Assay System (Catalog Number E2620)
- Cell viability reagent: Promega CellTiter-Glo Assay System (Catalog Number G7571)

#### 5) Antibodies

- COX2 (Cayman Chemicals, Catalog Number 160107)
- VCAM1 (Cell Signaling, Catalog Number 13662S)
- Actin (Sigma Aldrich, Catalog Number A3854)

### **Cell Culture Reagents**

#### 1) Cells

- ATCC THP-1 (Catalog Number TIB-202)

#### 2) Media

- ThermoFisher RPMI 1640 (Catalog Number 11875-085)
- VWR Fetal Bovine Serum (Catalog Number 97068-085)
- ThermoFisher HEPES 1M (Catalog Number 15630080)
- ThermoFisher Sodium Pyruvate 100mM (Catalog Number 11360-070)
- ThermoFisher Anti-Anti 100X (Catalog Number 15240062)

## Primary and Secondary Screen

- Day 0 – THP-1 monocytes were treated with PMA (final concentration of 10ng/ml). The cells were at a confluency of ~250,000 cells per ml at time of treatment
- Day 1
  - All siRNAs were pre-arrayed in 384-well plates with 2µl of a 1.25 µM stock (Primary Screen had 4 siRNAs per gene per well while the Secondary Screen had 1 siRNA per gene per well, hence 4 wells in total per gene per replicate)
  - A master stock containing 10µl of pre-warmed Opti-MEM and 0.1µl Lipofectamine RNAiMAX for each well was prepared, incubated for 5 min at Room Temperature, then added to each well.
  - The plates were shaken for 1 min to generate a homogenous siRNA-Lipid solution and incubated for 20 min at room temperature
  - A 50µl suspension of 7500 THP-1 differentiated cells in growth media was added and cells were incubated at 37 C and 5% CO<sub>2</sub> for 72 h
- Day 4
  - Cells were stimulated with LPS or TNF diluted in growth media, to a final concentration of 10 ng/ml for 6 h each

- Cells were then read using a plate reader and the Bright-Glo and CellTiter-Glo (secondary screen only) assay systems according to the manufacturer's protocols

### **Tertiary Screen**

- Day 0
  - U87 cells were seeded in 6-well plates at a ~50% confluency
- Day 1
  - The seeded cells were transfected with individual siRNAs (two siRNAs per gene, and hence 2 wells per gene) using Lipofectamine RNAiMAX using the manufacturer's default protocol
- Day 4
  - 72 h post transfection, the cells were stimulated with TNF for a final concentration of 10 ng/ml for 6 h
  - After 6 h of stimulation, cells were washed once with ice-cold PBS and collected in RIPA buffer (Sigma Aldrich) mixed with Protease and Phosphatase Inhibitors (Cell Signaling)
  - The cells were lysed, protein quantified and run on an SDS-PAGE system as per the standard western blotting protocol (<http://www.abcam.com/protocols/general-western-blot-protocol>) using NuPage (ThermoFisher) reagents and apparatus

- The gels used were 4-12% Bis-Tris NuPage gels and the proteins were transferred to a PVDF membrane. Primary antibodies were incubated overnight in 5% milk at a final concentration of 1:2000, followed by secondary antibody incubation at 1:10000 for 2 hours at room temperature.
- The blots were developed using the Amersham ECL Western Blotting Detection Reagent and X-Ray films as mentioned in the above-linked protocol

## **ACKNOWLEDGEMENTS**

We are grateful to our collaborators at the Sanford Burnham Prebys Medical Discovery Institute, Dr. Sumit Chanda and Paul De Jesus. They trained us to perform our primary and secondary screens using their automated liquid handlers and plate readers. In addition, Paul performed the RSA analysis and rank ordered the genes from the secondary screen in order of significance.

Chapter 2, in part, is currently being prepared for submission for publication of the material. Verma, Suneer; De Jesus, Paul; Chanda, Sumit K; and Verma, Inder M. RNAi screens reveal SNW1 as a novel regulator of the NF- $\kappa$ B pathway. The dissertation author is the primary investigator and author of this material.



**Chapter 3: Validation and characterization of SNW1's role in the NF- $\kappa$ B pathway**

## BACKGROUND

In Chapter 2 of this dissertation, we developed high throughput screens using a whole genome siRNA oligo library to discover novel genes that regulate the NF- $\kappa$ B pathway in response to LPS and TNF in human macrophage cells. Out of the 18095 genes tested, SNW1 was one of the top 50 hits in the primary screen, and the number 1 ranked hit in both the secondary and tertiary screens. Hence we decided to try and delineate its role in the NF- $\kappa$ B pathway.

### Introduction to SNW1

SNW1 (also termed NCoA62, SKIP in vertebrates; Prp45 in *S. cerevisiae*; BX42 in *D. melanogaster*) is a highly conserved protein associated with splicing and transcription. It was discovered as a binding partner of the Ski oncoprotein using a two-hybrid system (called Skip – Ski interacting protein by Dahl, Wani, and Hayman 1998). It was found to be highly homologous to the Bx42 protein in *Drosophila* that is found to be associated with chromatin in transcriptionally active puffs of the salivary gland. In the same year Baudino et al. 1998 discovered it independently – as a co-activator of Vitamin D mediated transcription, naming it NCoA62 (Nuclear Receptor Co-activator 62 KDa). The acronym SNW stems from the SNWKN conserved motif (Folk, Půta and Skružný, 2004). SNW1 is known to be primarily a nuclear protein, which is in line with its role in gene regulation at the level of transcription and splicing. SNW1 mRNA and protein forms are constitutively expressed in a variety of human tissues (source: The

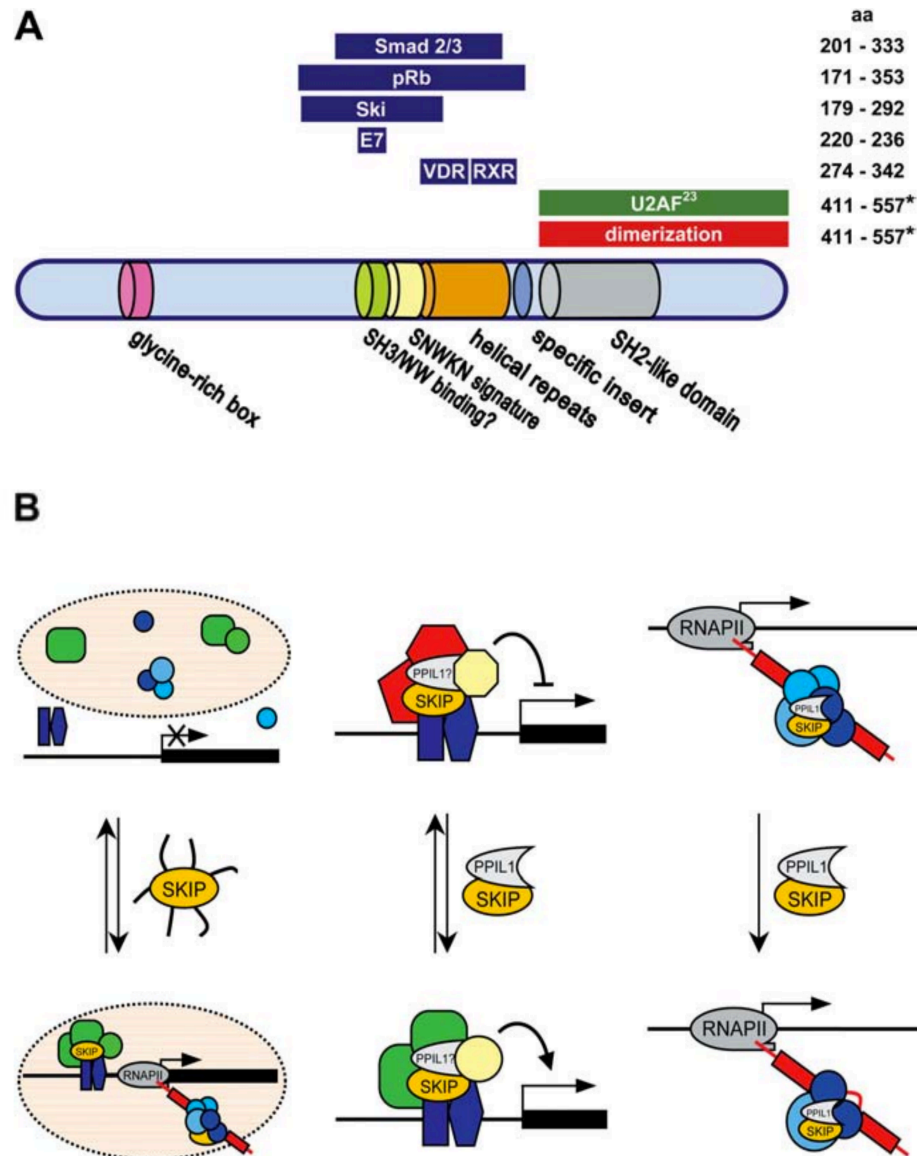
Protein Atlas) and cancer cell lines (source: The Cancer Cell Line Encyclopedia by the Broad Institute).

### SNW1 Function (Splicing and Transcription)

**Splicing:** SNW1 was discovered as part of the human spliceosome and was shown to be involved in various aspects of the splicing cycle (Folk et al. 2004). The analyses also found the spliceosomal proteome to be very large and containing a significant number of factors that couple splicing with other steps in gene expression including transcription.

**Transcription:** The well-established roles of SNW1 in transcriptional regulation are its co-regulatory effect on nuclear hormone receptors, including the Vitamin D receptor (VDR; C. Zhang et al. 2001), Androgen Receptor (AR; Abankwa et al. 2013) and Retinoid X Receptor, which it antagonistically regulates in association with SIRT1 (Kang *et al.*, 2009). SNW1 also interacts with a large range of DNA binding proteins, including Smad2 and 3 proteins of the TGF- $\beta$  pathway (Leong *et al.*, 2001), and proteins involved in MyoD and Notch signalling (Zhou *et al.*, 2000; Kim *et al.*, 2001). SNW1's gene regulation functions have been summarized into three non-exclusive hypothesis as summarized in Figure 3.1.

Since splicing and transcription are closely regulated, SNW1's exact role in mediating them individually is unclear and Figure 3.1 (borrowed from the excellent review by Folk et al., 2004) describes the SNW1 structure, known



**Figure 3.1.**

**SNW1's interactions and proposed role in gene regulation**

(A) shows the motifs in SNW1 and their potential role in binding with transcription factors (such as VDR, RXR, Smad proteins, via the SNWKN signature domain) or for splicing (via U2AF). (B) shows three hypothesis of SNW1's mechanisms for gene regulation. First, it can act as a scaffolding protein to help compartmentalize the complexes for splicing and transcription. Second, it can help bring about an activating conformational change in these complexes by sequestering effector molecules like prolyl isomerase PPIL1. And third, it can be a direct part of the spliceosome and increase splicing efficiency. Reproduced from Folk., et al 2004.

binding partners and potential hypothesis for SNW1's influence on gene regulation.

### SNW1 and NF- $\kappa$ B

To develop our hypothesis, we searched for SNW1's potential interactions with proteins involved in NF- $\kappa$ B regulation, especially in the nucleus. We found three relevant pieces of evidence. Firstly, SNW1 is known to promote HIV-1 Tat Transactivation by interacting with the elongation factor p-TEFb, composed of CDK9 and Cyclin T1 (Brés et al. 2005, 2009). p-TEFb has been previously shown to be recruited to the promoters of NF- $\kappa$ B target genes (IL-8 etc.) on TNF treatment (Barboric *et al.*, 2001). In the same study, Barboric et al., show that p-TEFb binds to p65 (on TNF treatment) to start transcriptional elongation and help activate the pre-initiation complex (PIC) which includes RNA Polymerase 2 (RNA POL II). Hargreaves, Horng, and Medzhitov in 2009 have also shown that p-TEFb is bound to the promoters of fast-transcribed NF- $\kappa$ B target genes on pathway activation. Inhibition of p-TEFb has also been shown to attenuate NF- $\kappa$ B dependent transcription. This hypothesis is covered in more detail in Chapter 4.

Secondly, SNW1 is known to interact with the transcription co-activator SRC-1 in the context of activating transcription through the Vitamin D Receptor (Baudino *et al.*, 1998). SRC-1 itself is recruited to the promoter of NF- $\kappa$ B target gene I $\kappa$ B $\alpha$  upon TNF treatment (Gao *et al.*, 2005).

Thirdly, SNW1 is a known co-binder of Notch-IC (Intra-cellular domain of Notch; Zhou et al. 2000) and helps with Notch-IC transactivation function. On the other hand, constitutively active Notch transcribes the gene Hes1 which, in turn, activates the NF- $\kappa$ B pathway by repressing CYLD, in the context of T-cell leukemias (Espinosa *et al.*, 2010).

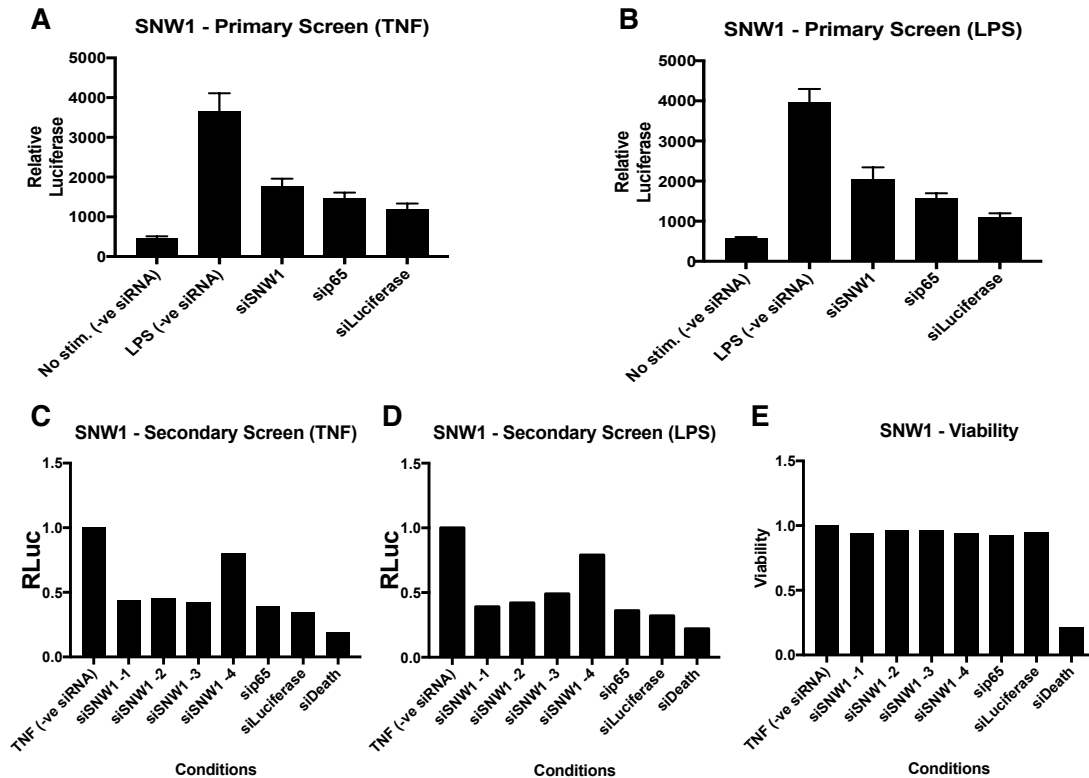
In this chapter, we show that SNW1 indeed regulates a variety of NF- $\kappa$ B target genes, by serving as an adapter that facilitates the binding of p-TEFb to p65 on pathway activation. Importantly, SNW1 knockdown attenuates p-TEFb binding to p65 hereby suggesting the former's role as an adapter protein necessary for NF- $\kappa$ B dependent transcriptional elongation. This role seems to be independent of SNW1's binding to its splicing co-factors SNRNP200 and SNRNP220.

## **RESULTS**

### **SNW1 validated as a novel mediator of the NF- $\kappa$ B pathway**

#### Validation of Primary and Secondary Screens

To confirm initial screening results, we replicated the primary screening process for SNW1 by transfecting differentiated THP-1 cells (NF- $\kappa$ B Luciferase reporter) with sip65, siLuciferase, siSNW1 (pool of four) and a non-targeting siRNA. After stimulation with LPS or TNF (Figure 3.2A-B), we saw a significant knockdown of relative luciferase activity in siSNW1 along with the positive controls sip65 and siLuciferase. SNW1 was ranked 68<sup>th</sup> in our primary screen



**Figure 3.2.**

**SNW1 depletion attenuates NF- $\kappa$ B dependent Luciferase expression with minimal loss in cell-viability**

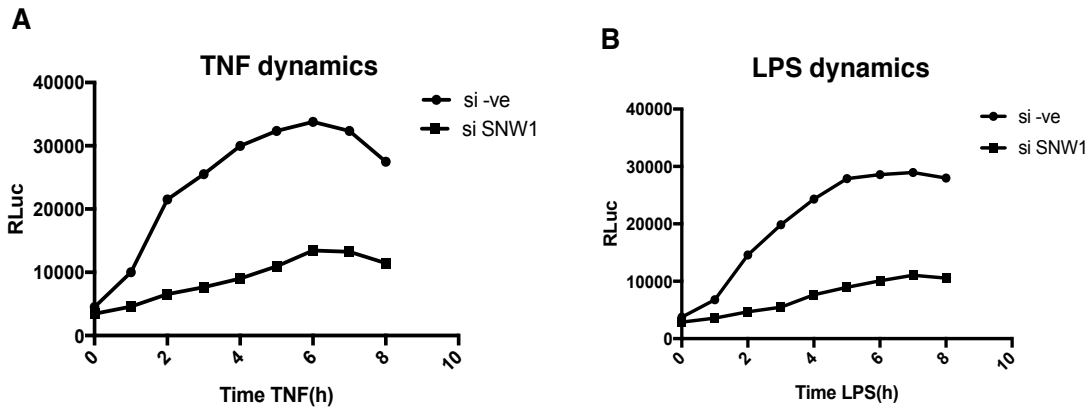
(A) and (B) show reduction of activated NF- $\kappa$ B dependent luciferase (via TNF or LPS) under SNW1 RNAi (pool of 4 siRNAs) conditions in THP-1 cells. The reduction is comparable to positive controls (RNAi against p65 or Luciferase). (C) and (D) show that 3 out of 4 individual siRNAs against SNW1 used in our secondary screen attenuated NF- $\kappa$ B dependent luciferase response on stimulation with TNF or LPS. Finally, (E) shows that the reduction in NF- $\kappa$ B dependent luciferase by siRNAs against SNW1 is not due to loss in cell viability. siRNA against an essential ribosomal protein (siDeath) is used as a positive control.

(Top 0.4 percentile out of 18,095 genes) and the results confirmed it to be a strong hit). We next replicated the secondary screening conditions for SNW1 by transfecting differentiated THP-1 cells (NF- $\kappa$ B reporter) with sip65, siLuciferase, siDeath (pool of siRNAs against essential protein translation genes), and the four siRNAs against SNW1 as shown in Figure 3.2C-D. Upon stimulation with either LPS or TNF, we saw that three out of four siRNAs against SNW1 significantly knocked down NF- $\kappa$ B dependent luciferase, comparable to the levels of the positive controls (sip65, siLuciferase). Also, none of the siRNAs against SNW1, p65, Luciferase showed significant cell toxicity, but siDeath (positive control for toxicity) killed almost 90% of the transfected cells. SNW1 was ranked as the number 1 hit out of both our secondary screens and the results supported that. The lack of cell toxicity of SNW1 siRNAs provide evidence against SNW1 being involved in general cell transcription and later in this chapter, we provide evidence for SNW1's specificity as an NF- $\kappa$ B regulator. We also tested the dynamics of NF- $\kappa$ B activation on SNW1 depletion. Figure 3.3 shows that knocking down SNW1 attenuates NF- $\kappa$ B dependent Luciferase activity from the very onset.

#### SNW1 regulates endogenous targets of NF- $\kappa$ B in U87 and THP-1 cells

Next, to replicate the tertiary screen for SNW1, we transfected U87 cells with siSNW1 or a non-targeting siRNA and activated the NF- $\kappa$ B pathway by treating them with TNF. As shown in Figure 3.4A-B, NF- $\kappa$ B activation induced the

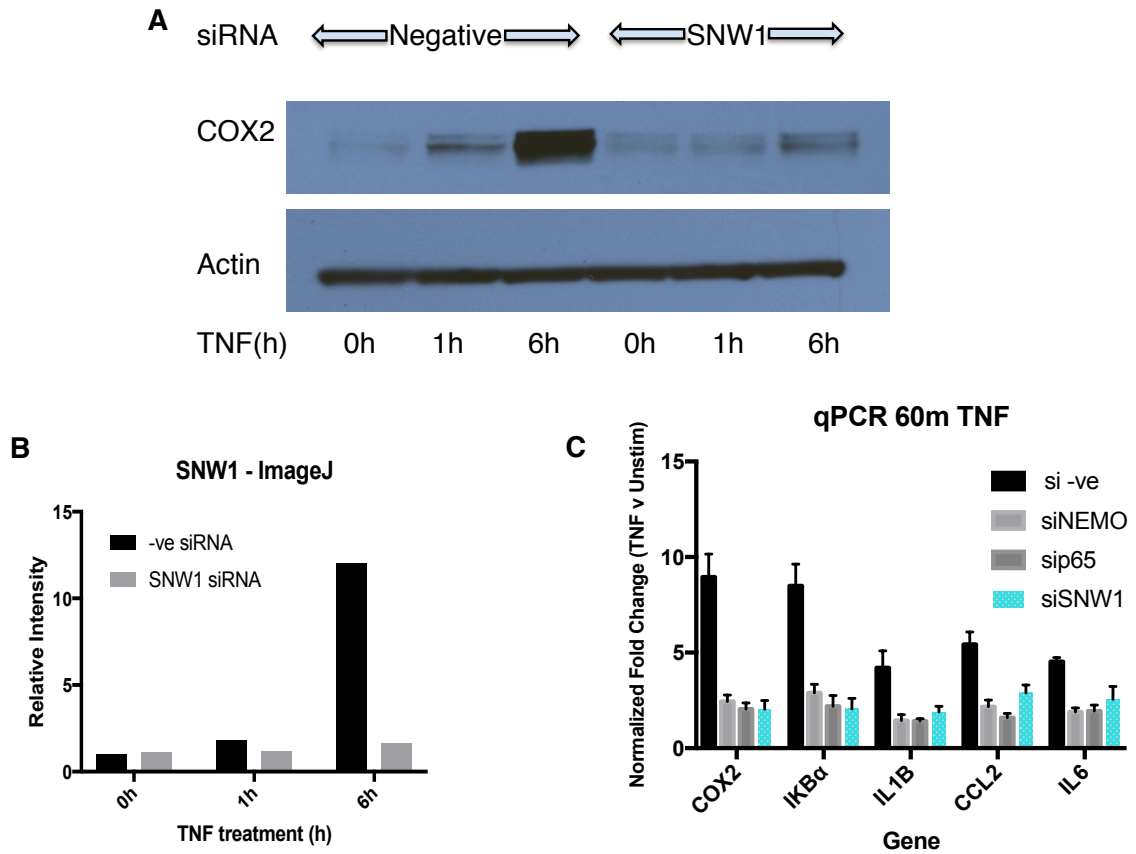




**Figure 3.3.**

**SNW1 depletion attenuates NF- $\kappa$ B transcription dynamics**

(A) and (B) use THP-1 cells and a NF- $\kappa$ B luciferase reporter to show that SNW1 knockdown cells mimic NF- $\kappa$ B induction dynamics of wild-type cells albeit at much lower levels. This shows that SNW1's regulation on the NF- $\kappa$ B pathway is probably not time-specific.



**Figure 3.4.**

**SNW1 depletion represses the endogenous NF-κB pathway**

(A) shows that TNF stimulated NF-κB dependent expression of COX2 is attenuated in U87 cells transfected with siRNA against SNW1. (B) is a quantification of COX2 expression from A. (C) shows that knocking down SNW1 down-regulates the expression of NF-κB target genes on TNF treatment in THP-1 cells. The down-regulation is similar to sip65 and siNEMO conditions (positive controls). Note – the Y axis in (C) is showing fold-change of NF-κB target gene expression with respect to non-TNF stimulated control.

production of COX2 up to 12-fold, while knocking down SNW1 almost completely attenuated that process. This confirmed SNW1's involvement with NF- $\kappa$ B activation in another cell-line, using an endogenous target gene as a reporter. Notably, SNW1 was ranked number 1 amongst the hits from the tertiary screen.

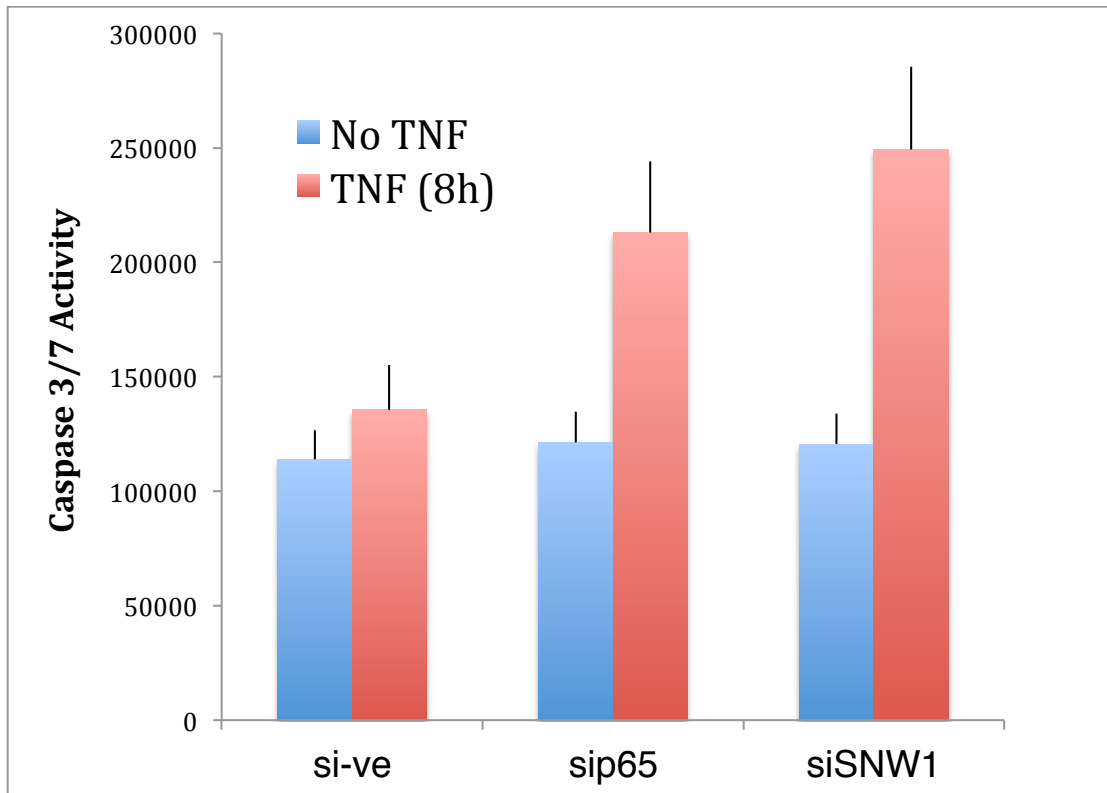
We also tested SNW1's involvement in the transcription of other NF- $\kappa$ B target genes on induction by using qPCR. Figure 3.4C shows that knocking down SNW1 in differentiated THP-1 cells reduces the TNF induced expression of I $\kappa$ B $\alpha$ , IL1, CCL2 and IL6 – all canonical NF- $\kappa$ B target genes after 60 min of TNF stimulation. The qPCR knockdown is also consistent after 150 min of TNF induction (data not shown). It is interesting to note that the knockdown of SNW1 causes a near complete repression of NF- $\kappa$ B induced COX-2 (Figure 3.4A,C) but a partial (~65%) repression of NF- $\kappa$ B dependent luciferase (Figure 3.2A,B). This might be because the NF- $\kappa$ B dependent Luciferase has 5 NF- $\kappa$ B binding sites on the promoter element and hence is super-sensitive to NF- $\kappa$ B activity. Hence presence or absence of SNW1 might not be the rate limiting factor in that case.

#### SNW1 regulates NF- $\kappa$ B's anti-apoptotic transcription program

Next, we tested SNW1's ability to mediate suppression of TNF-induced apoptosis through the NF- $\kappa$ B pathway. TNF stimulation of cells is known to activate two programs – the NF- $\kappa$ B pathway and programmed cell death. Our lab had previously established (Van Antwerp *et al.*, 1996) that the TNF induced pro-

survival NF- $\kappa$ B pathway suppresses the induced programmed cell death in these cells, but, when the NF- $\kappa$ B pathway is attenuated, cells undergo apoptosis at a much higher rate due to the dominance of the programmed cell death pathway. Hence our hypothesis was that SNW1 depletion that led to NF- $\kappa$ B repression would increase apoptosis in TNF treated cells supporting the evidence that SNW1 is directly and specifically affecting the NF- $\kappa$ B pathway. To test this, we transfected THP-1 cells with siRNAs against p65, SNW1 and a non-targeting control, treated them with TNF and checked Caspase 3/7 activity as a readout of apoptosis. As expected, apoptotic activity increased in sip65 cells post TNF treatment but not in the si-ve (scrambled siRNA) cells. SNW1 depletion also increased apoptosis activity on TNF treatment (Figure 3.5) supporting our hypothesis.

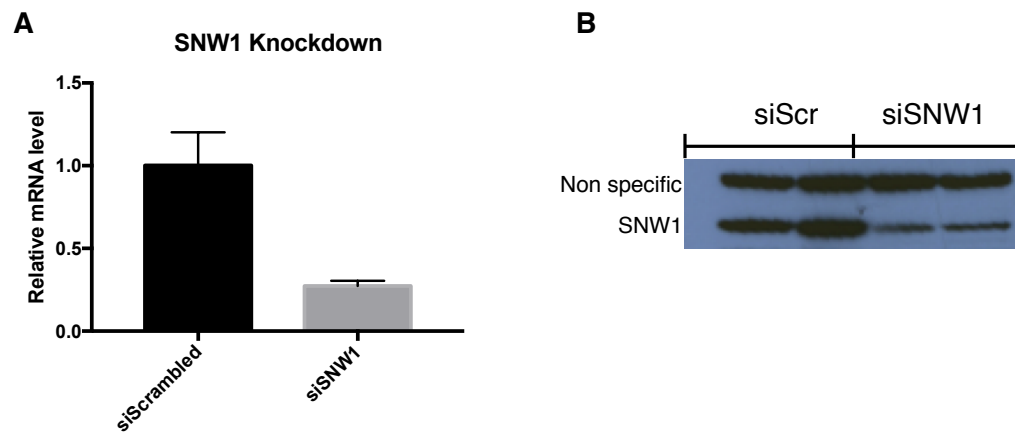
Finally, as a verification, we checked SNW1 RNA and protein levels after transfecting THP-1 cells with siSNW1s to confirm that it was knocked down (Figure 3.6). Taken together, these results support the hypothesis that the SNW1 is a regulator of the NF- $\kappa$ B pathway. Our next set of experiments test the specificity of SNW1 in regulating NF- $\kappa$ B and if it affects transcription from other transcription factors as well.



**Figure 3.5.**

**SNW1 depletion shows increased TNF induced apoptosis**

Knocking down the NF- $\kappa$ B pathway (via sip65) increases TNF mediated apoptosis in THP-1 cells. A similar result is seen when SNW1 is knocked down providing evidence for SNW1's role in the NF- $\kappa$ B pathway on TNF treatment.



**Figure 3.6.**

**Verification of SNW1 knockdown using siRNAs**

THP-1 cells show SNW1 knockdown by around 80% in both mRNA (normalized to GAPDH mRNA) (**A**) and protein (**B**) levels.

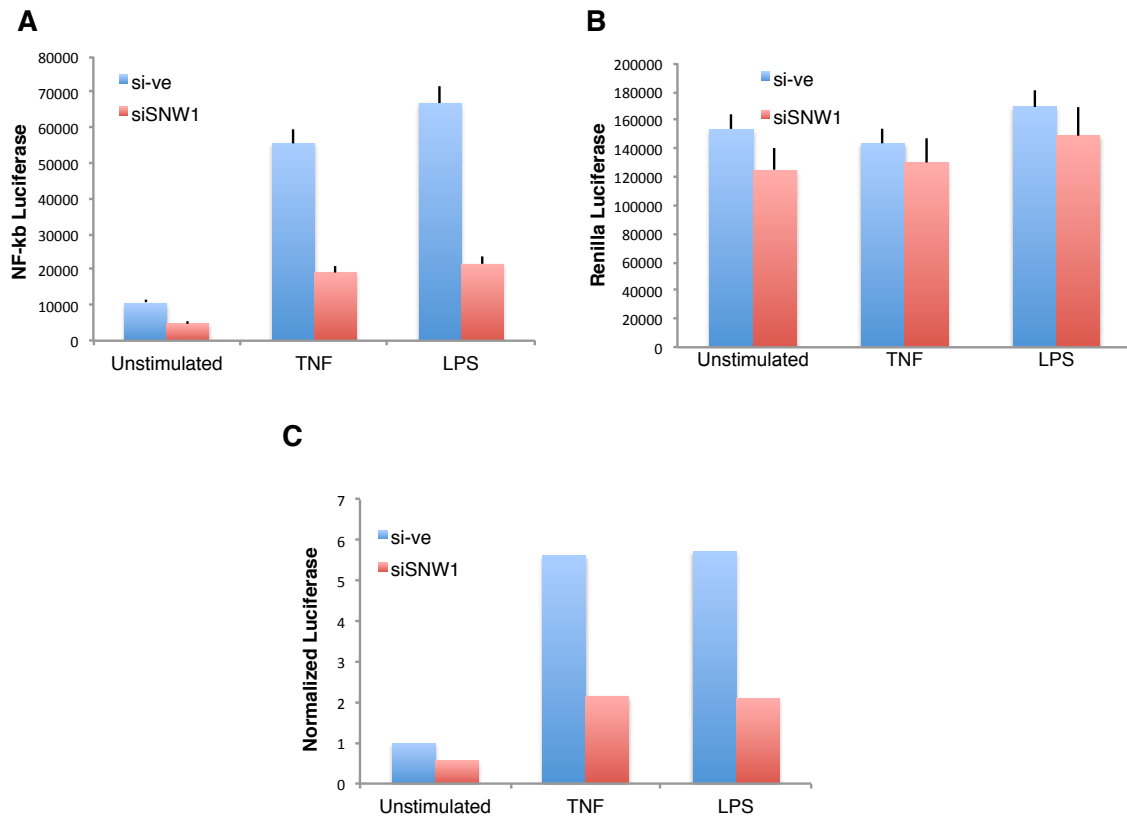
## **SNW1 is not a general transcription regulator**

### SNW1 does not affect general constitutive transcription

Once we had established SNW1 to be a regulator of NF- $\kappa$ B and its target genes on induction with LPS and TNF, we wanted to check its involvement in general constitutive transcription. To test that, we transfected THP-1 cells with both an NF- $\kappa$ B driven Luciferase and a pTK (weak constitutive promoter) driven Renilla Luciferase. We knocked down SNW1 in these cells using siRNA (along with a negative control) and read luminescence values for both the luciferases. As previously reported, SNW1 knockdown decreased NF- $\kappa$ B dependent luciferase expression but had no significant affect on Renilla luciferase levels. The results are shown both individually and normalized together in Figure 3.7. We repeated this experiment in U87 cells and got similar results (data not shown). These observations support the hypothesis that knocking down SNW1 does not affect general transcription in multiple cell lines.

### SNW1 activates and represses transcription from some other transcription factors

Now that we had evidence that knocking down SNW1 does not affect constitutive transcription, we wanted to find other transcription factors whose activity might be regulated by SNW1, because of its previously established role as co-activator or repressor for multiple transcription factors. For this process, we transfected multiple luciferase plasmids driven by different transcription factors in

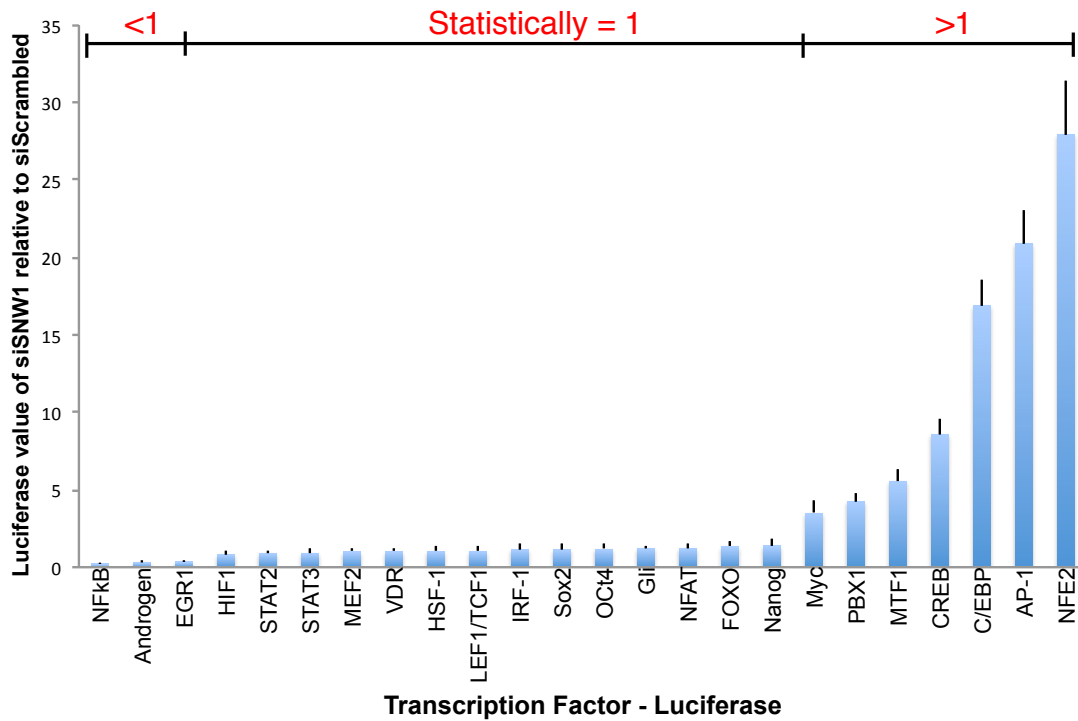


**Figure 3.7.**

**SNW1 knockdown does not affect general constitutive transcription**

THP-1 cells (with negative control siRNA or SNW1 siRNA) were transfected with either **(A)** NF- $\kappa$ B dependent luciferase or **(B)** Constitutively active pTK Renilla Luciferase, and in both cases treated with LPS or TNF. **(A)** shows knockdown of NF- $\kappa$ B luciferase in siSNW1 conditions. **(B)** shows no significant change in constitutively active Renilla Luciferase in siSNW1 conditions. **(C)** shows the results of **(A)** normalized by **(B)**





**Figure 3.8.**

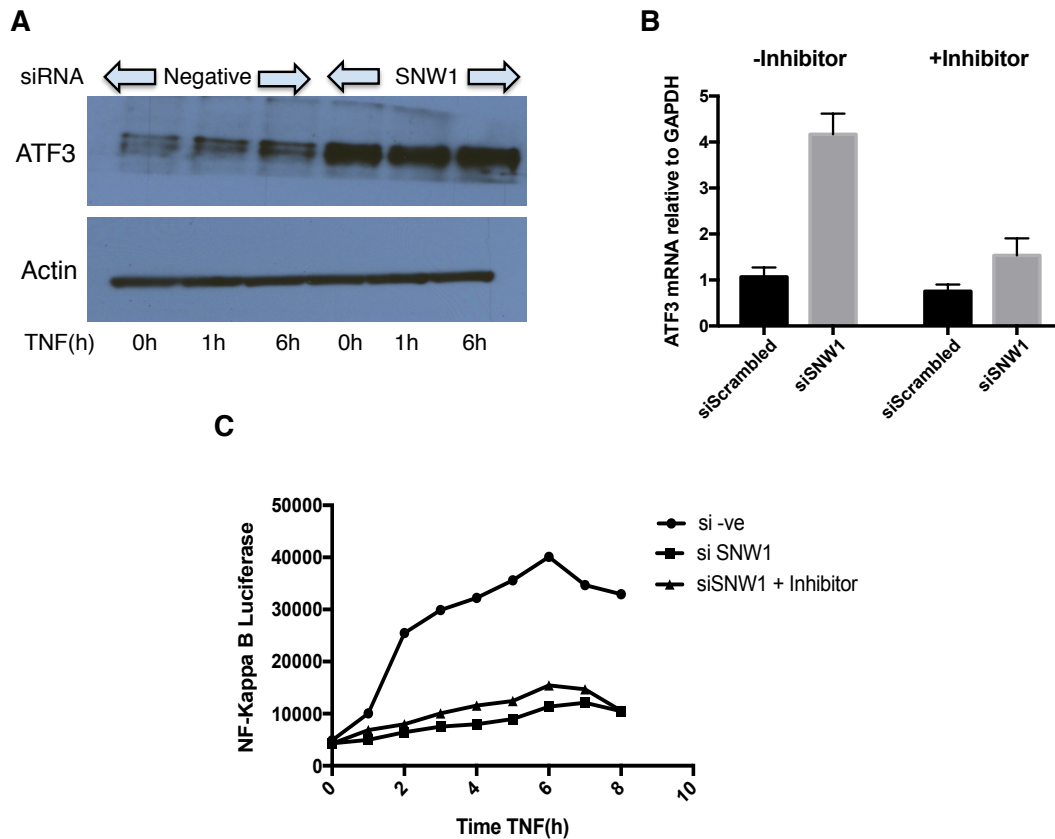
**Effects of SNW1 on various transcription factor dependent luciferase reporters.**

293T cells were transfected with Transcription Factor driven Luciferase reporters along with either siScrambled or siSNW1. Knocking down SNW1 decreases the activity of NF-κB, Androgen Receptor and EGR1 driven Luciferases, while increases the activity of CREB, C/EBP, AP-1 and NFE2 dependent Luciferase

293T cells (chosen for high transfection efficiency). Following that, we knocked down SNW1 (or a negative control) in these cells using siRNAs and checked for luciferase expression. The final values in SNW1 knocked down cells were normalized to values in the negative control cells. Figure 3.8 shows that knocking down SNW1 does not significantly affect basal transcription activity from 15 out of the 24 transcription factors tested. SNW1 depletion reduces basal transcriptional output from 3 transcription factors including NF- $\kappa$ B, EGR1 and Androgen Receptor. The effect on the latter has been previously published (Abankwa *et al.*, 2013). Surprisingly, depletion of SNW1 led to up-regulation of luciferase expression from 6 transcription factors including CREB, C/EBP and NFE2.

#### SNW1 knockdown up-regulates CREB activity but that does not drive NF- $\kappa$ B target gene attenuation

The up-regulation of CREB transcription on SNW1 depletion was interesting since CREB up-regulation has been causally associated with NF- $\kappa$ B suppression in multiple contexts (Kwon *et al.*, 2015). We wanted to investigate this further so we checked the expression of CREB target gene ATF3 upon SNW1 depletion in THP-1 cells and found that it was indeed upregulated at both the RNA and protein level as shown in Figure 3.9A-B. To investigate if this activation of CREB on SNW1 depletion was causing NF- $\kappa$ B repression, we treated SNW1 depleted cells with a CREB inhibitor (CAS 92-78-4; Calbiochem)



**Figure 3.9.**

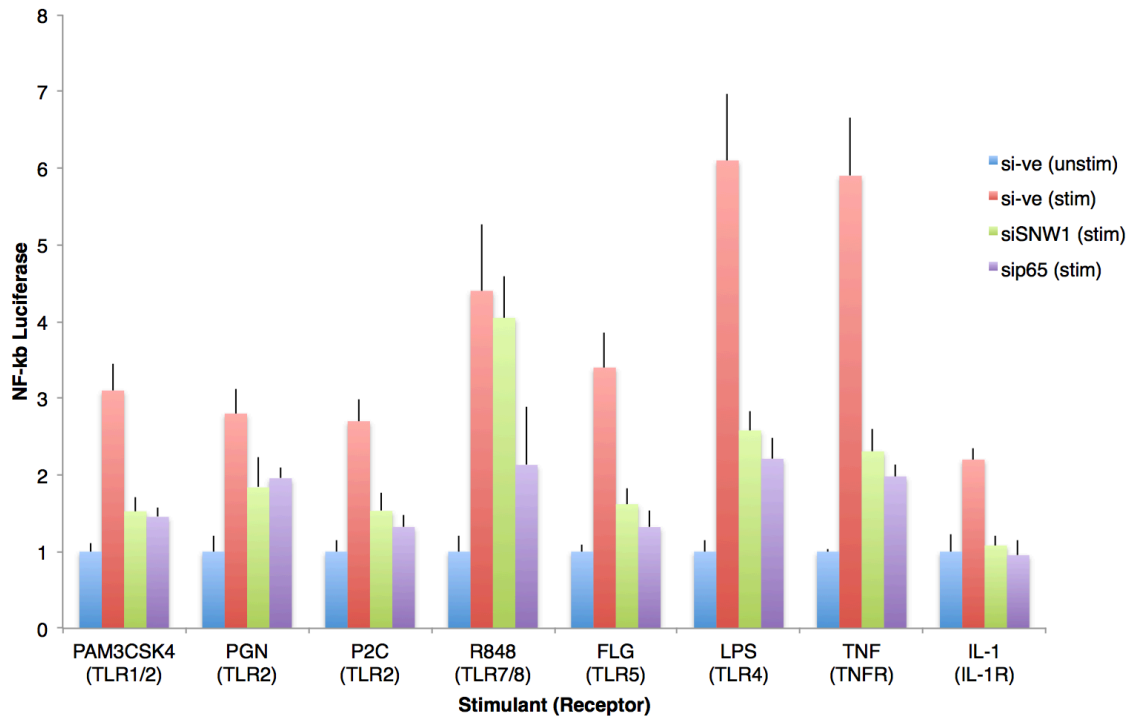
**SNW1 knockdown increases CREB activity but that does not drive the repression of NF- $\kappa$ B**

(A) shows that knocking down SNW1 in THP-1 cells increases the protein expression of CREB target gene ATF3 (consistent with the finding in Figure 3.8). (B) shows that ATF3 mRNA up-regulation is attenuated by a CREB inhibitor but this inhibitor fails to rescue the repression of NF- $\kappa$ B dependent luciferase under siSNW1 conditions, as shown in (C).

and checked for NF- $\kappa$ B induction levels versus control cells. As shown in Figure 3.9C, inhibition of CREB had no bearing on NF- $\kappa$ B repression in SNW1 depleted cells giving evidence for the hypothesis that SNW1's effect on the NF- $\kappa$ B pathway is not due to its effect on the CREB pathway. The CREB inhibitor's efficacy on ATF3 induction is shown in Figure 3.9B.

#### SNW1 knockdown represses NF- $\kappa$ B activity in response to a variety of stimuli

Finally, we wanted to check SNW1's specificity in regulating NF- $\kappa$ B transcription itself. To try and answer that we knocked SNW1 down in THP-1 cells (transduced with the NF- $\kappa$ B Luciferase reporter) and treated them with NF- $\kappa$ B inducing stimuli – PAM3CSK4 (Lipoprotein mimic; TLR1 and TLR2 agonist), PGN (Peptidoglycan; TLR2 agonist), P2C (Lipoprotein; TLR2 agonist), R848 (Resiquimod; TLR8 agonist), FLG (Flagellin; TLR5 agonist) and IL-1 (cytokine; IL1-R agonist). We found the knocking down SNW1 reduced NF- $\kappa$ B activation from all stimuli except R848 (TLR7/8 agonist). Since knocking down SNW1 attenuated the NF- $\kappa$ B pathway induced by multiple stimuli, we hypothesized that it is not involved upstream of the IKK complex (at the ligand-receptor level, which is different for individual receptors). We also confirmed the reduction in NF- $\kappa$ B luciferase levels on knocking down SNW1 in U-2OS (human bone osteosarcoma) and A549 (human adenocarcinoma) cells supporting its role in the pathway across multiple tissue lineage (data not shown).



**Figure 3.10.**

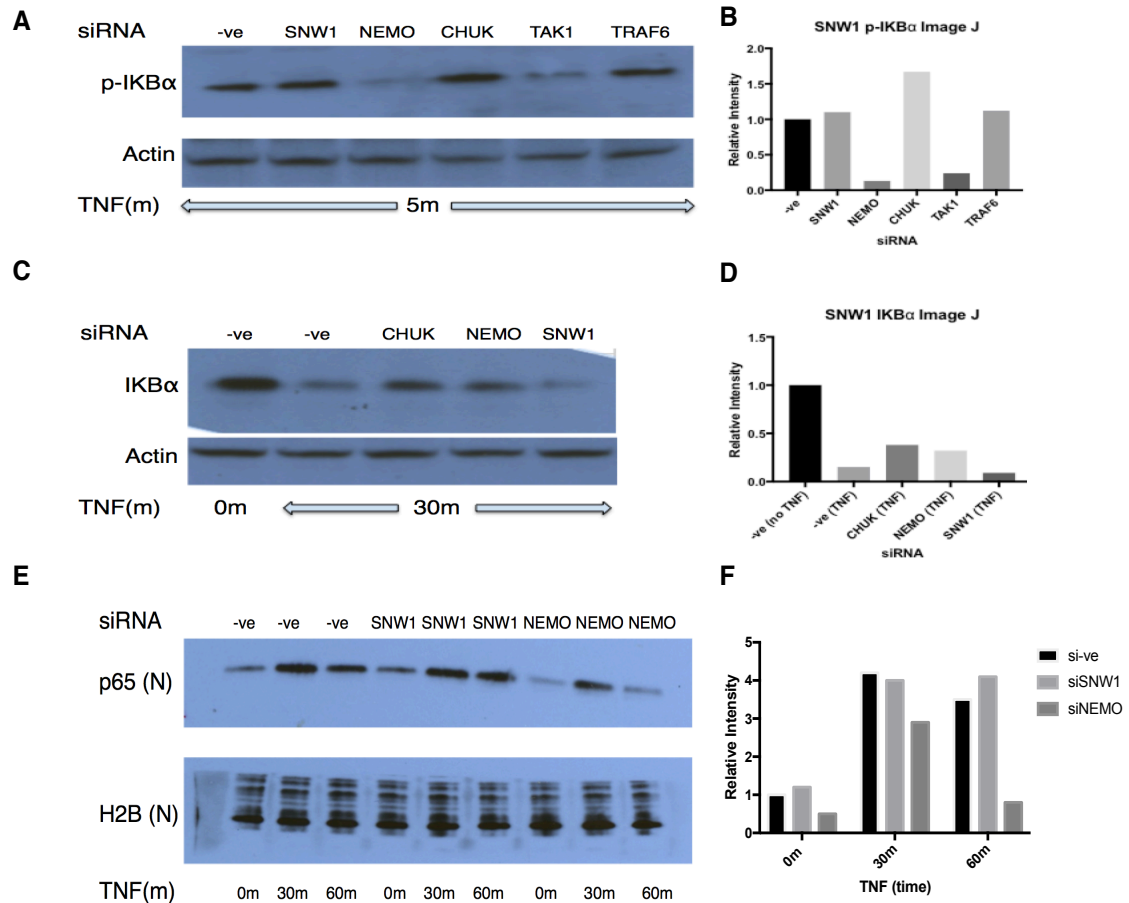
**SNW1 knockdown represses NF- $\kappa$ B dependent transcription in response to multiple stimuli**

THP-1 cells (transduced with NF- $\kappa$ B luciferase) were transfected with a negative control siRNA (si-ve), siSNW1, or sip65 and stimulated for 6 hours with the above-mentioned stimuli. siRNA against SNW1 attenuates NF- $\kappa$ B driven luciferase in all but one stimulus (R848) while siRNA against p65 shows a similar effect in all stimuli.

In total, we have provided evidence that SNW1 depletion does not interfere with general constitutive transcription, but affects induced NF- $\kappa$ B transcription on stimulation with multiple stimuli. Some other relevant transcription pathways that are seemingly affected by SNW1 include CREB, NFE2 and AP-1. In the next section, we have tried to narrow down SNW1's role in the NF- $\kappa$ B pathway.

### **SNW1 does not affect the cytoplasmic part of NF- $\kappa$ B signaling**

We next wanted to analyze the effect of SNW1 depletion on the upstream cytoplasmic NF- $\kappa$ B signaling cascade. We started off by testing SNW1's involvement in I $\kappa$ B $\alpha$  phosphorylation and degradation (required to activate the canonical NF- $\kappa$ B pathway). Figure 3.11A-D shows that knocking down SNW1 in THP-1 cells has little to no effect on I $\kappa$ B $\alpha$  dynamics on treatment with TNF. Next, we checked the role of SNW1 depletion on NF- $\kappa$ B's (p65) translocation to the nucleus. Figure 3.11E-F shows that knocking down SNW1 had no effect on the amount of nuclear p65 (on TNF treatment), but knocking down NEMO reduced the amount of p65 in the nucleus as expected. We also observed that I $\kappa$ B $\alpha$  was still partially degraded on knocking down CHUK (IKK1) and NEMO in Figure 3.11C. We believe this is due to lower levels of siRNA transfection in THP-1 cells (compared to 293T, HeLa cells) and that residual CHUK, NEMO were enough to degrade the majority of I $\kappa$ B $\alpha$  in response to TNF. These results were expected since SNW1 is primarily a nuclear protein so it would have been surprising (and interesting!) if it had any effect on I $\kappa$ B $\alpha$  dynamics or p65 nuclear translocation. We also checked the effect of knocking down SNW1 on two post translational



**Figure 3.11.**

**SNW1 knockdown doesn't affect cytoplasmic signaling of NF- $\kappa$ B**

THP-1 cells were transfected with a negative control siRNA (si-ve), siSNW1, or siRNAs against NEMO, CHUK (IKK1), TAK1, TRAF6 and stimulated with TNF.

(A), (B) show that knocking down NEMO or TAK1, but not SNW1 reduces phosphorylation of IKB $\alpha$  on TNF treatment. (C), (D) show that knocking down NEMO or CHUK, but not SNW1 prevents the complete degradation of IKB $\alpha$  on TNF treatment. (E), (F) show that knocking down NEMO, but not SNW1, decreases the amount of nuclear NF- $\kappa$ B (p65(N)) on 30 and 60 min of TNF treatment.

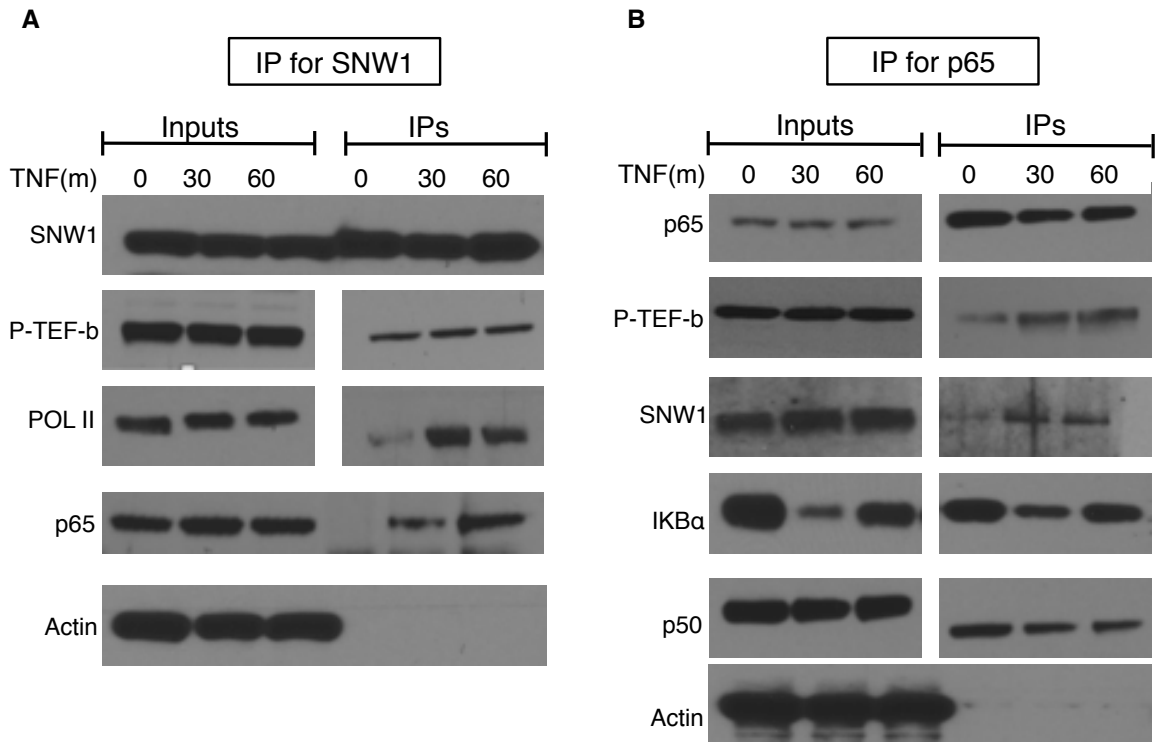
modifications (PTMs) of p65 – phosphorylation (S276) and acetylation (K310). Neither of the two PTMs were affected by SNW1 depletion in the presence of TNF (data not shown). In total, these results suggest that SNW1 does not affect the cytoplasmic part of the NF- $\kappa$ B pathway.

### **SNW1's role in NF- $\kappa$ B dependent transcription inside the nucleus**

#### SNW1 and p65 both complex with p-TEFb on TNF treatment

We wanted to test SNW1's involvement in the nuclear part of the NF- $\kappa$ B pathway. As previously mentioned, SNW1 was known to assist in HIV-1 Tat mediated transcription via its association with p-TEFb. Hence, we tested the binding of SNW1 with p-TEFb on TNF treatment in THP-1 cells. Figure 3.12A shows that SNW1 is in the same complex as p-TEFb in untreated cells and it maintains this interaction while the cells are treated with TNF. p-TEFb is known to be an elongation factor for NF- $\kappa$ B target genes (Barboric *et al.*, 2001) and it complexes with the pre-initiation complex (consisting of RNA POL II and core transcription factors) on TNF treatment (Hargreaves, Horng and Medzhitov, 2009). Hence we checked SNW1's interaction with POL II and sure enough, we saw a considerable increase in SNW1-Pol II interaction on TNF treatment (Figure 3.12A) indicating that SNW1 is part of the elongation complex that interacts with the pre-initiation complex on TNF treatment. As a positive control for SNW1 immuno-precipitations, we probed for SNW1 itself, and for a negative control we probed for  $\beta$ -actin. Next, we wanted to check p65's interaction with this





**Figure 3.12.**

**SNW1 and p65 both complex with p-TEFb on TNF treatment**

THP-1 cells were treated with TNF for 30 or 60 minutes and immuno-precipitated for either **(A)** SNW1 or **(B)** p65. **(A)** shows constitutive binding of p-TEFb (CDK9) to SNW1 inside THP-1 cells while a significantly increased binding to RNA Pol II, along with p65, on TNF treatment. As expected, **(B)** shows an increased binding of p65 to p-TEFb and SNW1 on treatment with TNF. The binding of IκBα and p50 to p65 are used as positive controls while the lack of binding of Actin (a ubiquitous highly-expressed protein) to either SNW1 or p65 is used as a negative control.

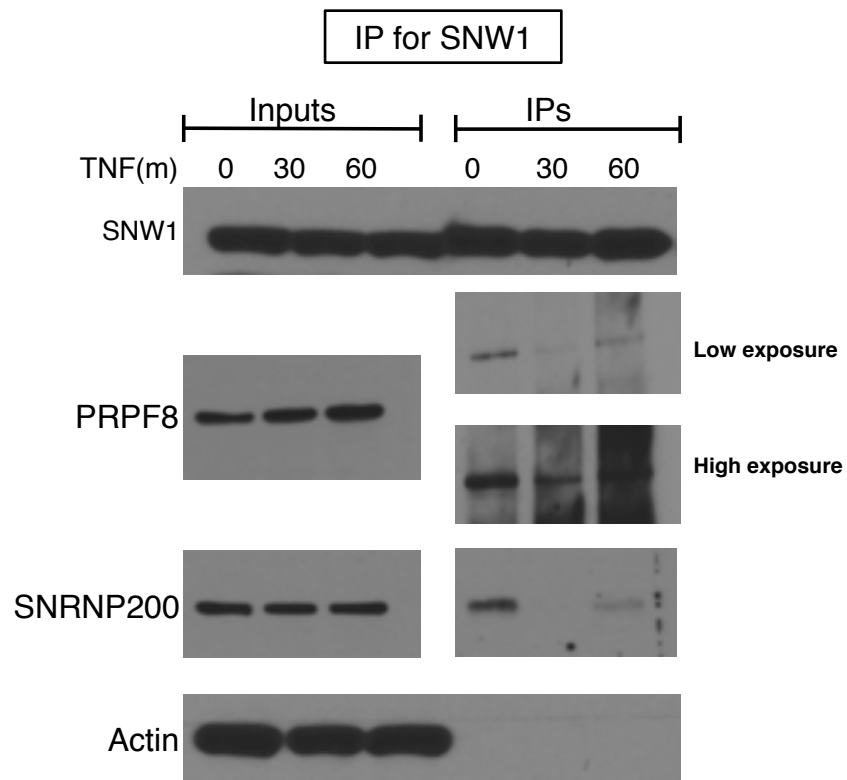
complex so we treated THP-1 cells with TNF, immuno-precipitated p65 and probed for proteins bound to it. Sure enough, p65 showed increased interaction with p-TEFb on TNF treatment (Figure 3.12B) hinting that SNW1 and p-TEFb are recruited to p65 on TNF treatment. And as expected (based on these results), we saw that SNW1 and p65 were part of the same complex on TNF treatment as shown in Figures 3.12A-B. We used I $\kappa$ B $\alpha$  and p50 as positive controls for p65 binding while Actin served as a negative control. Taken together, these results suggest SNW1's involvement as an adapter in the p65-pTEFb complex, that co-binds them to RNA Polymerase II on treatment with TNF. Brés et al. 2009 had suggested that SNW1's association with p-TEFb in context of HIV-1 Tat transcription was dependent on its binding to c-Myc and Menin and our attempts to detect both these proteins potentially bound to p65 or SNW1 on TNF treatment yielded negative results (data not shown). Another hint was that neither c-Myc nor Menin were hits in our NF- $\kappa$ B screens suggesting that they weren't essential for NF- $\kappa$ B transcriptional activity in THP-1 cells.

#### SNW1's role in NF- $\kappa$ B transcription is independent of splicing

We have provided evidence for SNW1's role as an adapter protein between p-TEFb, p65, and RNA Polymerase II on TNF treatment. SNW1 is also a known splicing protein that binds with other splicing factors (SNRNP200, EFTUD2 and PRPF8) to facilitate splicing of certain genes (Sato *et al.*, 2015). Interestingly, it has been known to act as a transcriptional co-activator for a target

gene of the transcription factor p53, while acting as a splicing factor for another p53 target (Chen, Zhang and Jones, 2011). Hence, we wanted to check its role as a splicing factor in the context of NF- $\kappa$ B and TNF treatment.

We took THP-1 cells and immuno-precipitated SNW1 with and without TNF treatment and probed for known splicing binding partners of SNW1. As shown in Figure 3.13, SNW1 did bind to PRPF8 and SNRNP200 under basal conditions but lost that binding on treatment with TNF. Interestingly, the SNW1-PRPF8 and SNW1-SNRNP200 binding is partially recovered after 60 minutes of TNF treatment. This shows that SNW1's involvement in the NF- $\kappa$ B pathway is potentially independent of its splicing role. The fact that the NF- $\kappa$ B driven Luciferase construct used to conduct the primary and secondary screen did not have an intron in it, supports this claim. In totality, we have provided evidence towards SNW1 being an adapter protein, required for the recruitment of p65 by p-TEFb. On the activation of the NF- $\kappa$ B pathway by TNF, SNW1 appears to unbind from its splicing co-factors, PRPF8 and SNRNP200, and form a complex with p65 and RNA Polymerase II. These data not only point to SNW1's role as a novel adapter in the NF- $\kappa$ B pathway but also towards its potential role as a key player in co-transcriptional splicing (in TNF untreated conditions) given its unique ability to bind to p-TEFb (the most well established link between transcription and splicing; Merkhofer, Hu, and Johnson 2014) and other splicing factors.



	Inputs			IPs				
	TNF(m)	0	30	60	0	30		60
SNW1		[Input bands]			[IP bands]			
PRPF8		[Input bands]			[IP bands]			Low exposure
		[Input bands]			[IP bands]			High exposure
SNRNP200		[Input bands]			[IP bands]			
Actin		[Input bands]			[IP bands]			

**Figure 3.13.**

**SNW1 binds to splicing factors in basal (but not TNF treated) state.**

THP-1 cells were treated with TNF for 30 or 60 min, immuno-precipitated for SNW1 and probed for its binding partners. Splicing factors PRPF8 and SNRNP200 appear to bind to SNW1 in basal conditions (absence of TNF) but lose that binding 30 min after TNF treatment, only to partially regain it 60 min after TNF treatment.

## DISCUSSION

In this section, we will summarize the questions we asked, our findings and their limitations.

### Validation of SNW1 as an NF- $\kappa$ B modulator

In this chapter, we have validated SNW1 as a novel regulator of the NF- $\kappa$ B pathway in human macrophages. We originally identified a potential role for SNW1 in the TNF and LPS stimulated NF- $\kappa$ B pathway through high throughput screenings using siRNA libraries (Chapter 2). SNW1 depletion led to a reduced expression of NF- $\kappa$ B target genes, like IL1, CCL2, COX2 (both RNA and protein), on pathway activation (Figure 3.4). SNW1 seemed to be regulating the NF- $\kappa$ B pathway in multiple cell lines (we have tested the down-regulation of NF- $\kappa$ B luciferase in THP-1, U87, A549, 293T and U2-OS cell lines) and in response to various stimulants (Figure 3.10). The common theme amongst the stimulants was that they activated the canonical NF- $\kappa$ B pathway (p65-p50 heterodimer as the transcription factor).

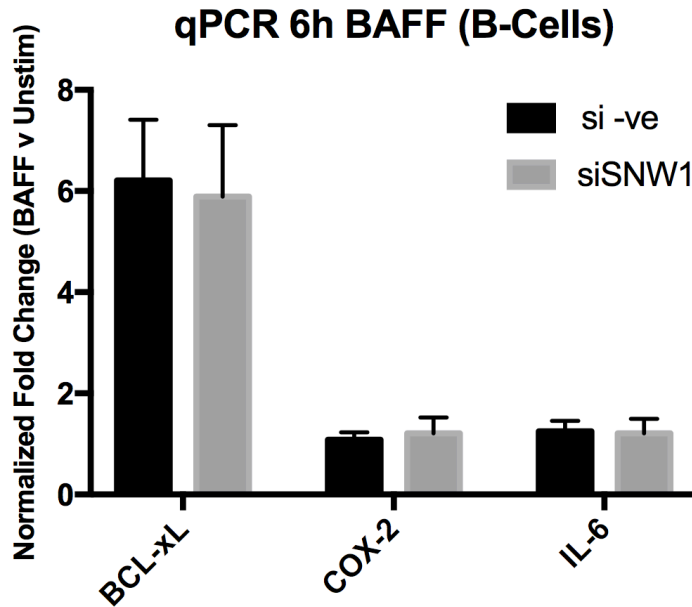
### SNW1 and non-canonical NF- $\kappa$ B

We did conduct an experiment to test SNW1 knockdown would affect the non-canonical NF- $\kappa$ B pathway (RelB-p52 heterodimer). We tested this by looking at the mRNA levels of Bcl-xl in response to B-cell activating factor (BAFF) in B-cells (one of the few established models of the non-canonical pathway; Claudio

et al. 2002). We saw an over-expression of Bcl-xl in B-cells on treatment with BAFF and this was not repressed by knocking down SNW1 (Figure 3.14). We also did not see any overexpression of COX-2 or IL-6 on BAFF treatment hinting that only the non-canonical pathway was activated. SNW1's inability to regulate the non-canonical NF- $\kappa$ B pathway in this context was not surprising, since we eventually found out that SNW1 is linked to p-TEFb, and there is no prior link between p-TEFb and non-canonical NF- $\kappa$ B. That said, this experiment was the only one in which we tested the non-canonical pathway and we would need more data points in other non-canonical pathway model systems to dismiss SNW1's role in them.

#### SNW1 and TNF-mediated apoptosis

SNW1 depletion also led to increased apoptosis of THP-1 cells on TNF treatment (Figure 3.5). This was a particularly satisfying result for two reasons, firstly – it connected nicely with a result that our lab had published 21 years ago (Van Antwerp *et al.*, 1996) that a compromised NF- $\kappa$ B pathway (in this case due to SNW1 depletion) would lead to elevated apoptosis in TNF treated cells, and secondly – it highlights the specificity of SNW1 as an NF- $\kappa$ B regulator in the context of TNF activation, since knocking down SNW1 behaves like knocking down p65 in TNF treated cells (only affecting the NF- $\kappa$ B pathway, and not the TNF-mediated activation of Caspase 8 which leads to apoptosis (Wang, Du and Wang, 2008). In fact, p-TEFb, another key regulator of NF- $\kappa$ B that binds to



**Figure 3.14.**

**SNW1 depletion does not affect Bcl-xl induction in B-cells.**

B-cells (GA-10) were transfected with a negative control siRNA (si-ve) or an SNW1 siRNA, and treated with BAFF for 6 hours. Bcl-xl was induced around 6 fold while COX-2 and IL-6 were maintained at basal levels. Knocking down SNW1 had little to no effect on the induction of Bcl-xl hinting that SNW1 is not involved in the non-canonical NF- $\kappa$ B pathway.

SNW1 was also validated using a similar experiment (p-TEFb inhibition led to increased TNF-mediated apoptosis in A549 cells; Barboric et al. 2001).

#### SNW1's role in NF- $\kappa$ B mediated transcription

The next and most important question we tried to answer was – What is SNW1's role in the NF- $\kappa$ B pathway? Published literature has shown SNW1 as a splicing co-factor, transcription co-activator, and transcription repressor. In our cell-lines SNW1 (tagged with GFP) is always localized to the nucleus (data not shown) and hence it was not a surprise when we found that it does not affect the cytoplasmic part of the NF- $\kappa$ B pathway (Figure 3.11), including NF- $\kappa$ B's translocation into the nucleus.

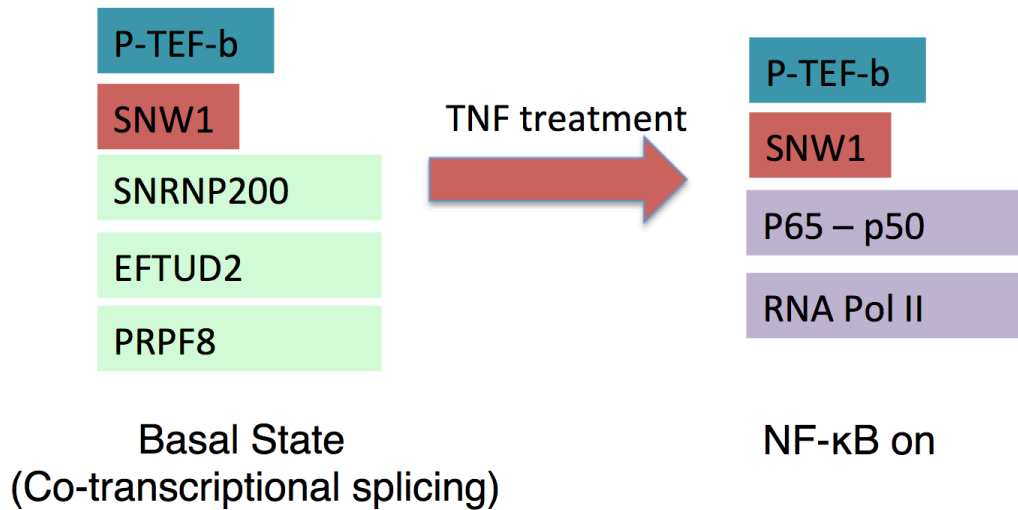
NF- $\kappa$ B's transcriptional regulation in the nucleus, as mentioned in Chapter 1, has always been a black box for the field. The nature and role of NF- $\kappa$ B's binding partners and transcriptional co-activators is still not completely characterized (with the exception of CBP/p300). It is also unknown how NF- $\kappa$ B binding partners help in the transcription of a different genes, both in terms of – in response to different stimuli (TNF, LPS, IL-1 etc.), or the order in which genes are transcribed (early versus late) in response to a single stimulus. NF- $\kappa$ B's role in transcription itself is debated between it being a pioneer transcription factor (able to remodel chromatin, bind to DNA and directly activate transcription) or being the final regulatory switch (that enables productive elongation of nascent



transcription). We have discussed these phenomena in greater detail in Chapter 4, but given the complexity of NF- $\kappa$ B transcriptional activity inside the nucleus, we were happy to get some hints from literature about SNW1's potential role in this jigsaw.

A common protein complex that had been independently shown to interact with SNW1 and p65 in the context of transcription, is p-TEFb. SNW1 had been shown to be p-TEFb's binding partner in the context of transcriptional activation via HIV-1 Tat, while p65 had been shown to use p-TEFb as a transcription elongator. Using this information as our basis, we tested for SNW1's interaction with p-TEFb in the context of NF- $\kappa$ B. We have reported that SNW1 consistently binds to p-TEFb inside the nucleus with or without NF- $\kappa$ B activation (Figure 3.12). We also reported that once NF- $\kappa$ B translocates to the nucleus on TNF treatment, it interacts with the SNW1-p-TEFb complex (Figure 3.12). Hence, we believe that SNW1 is a key adapter protein that facilitates the recruitment of p-TEFb to NF- $\kappa$ B and assists with transcriptional elongation of NF- $\kappa$ B target genes.

The next part that we wanted to understand was how SNW1's role changes before and after the NF- $\kappa$ B pathway is activated. Along with transcription co-activation, SNW1 was known to be involved in splicing. Hence we checked that potential role with and without TNF treatment. We found that SNW1 binds to its splicing co-factors SNRNP200 and SNRNP220 in basal state (no TNF treatment) but dissociates from them when the NF- $\kappa$ B pathway is activated (Figure 3.13). Given that SNW1 was always bound to p-TEFb (Figure



**Figure 3.15.**

**SNW1's potential role in NF-κB mediated transcription.**

In basal state, SNW1 seems to be playing a role in co-transcriptional splicing since it's bound to both p-TEFb and splicing proteins, but on TNF treatment it dissociates from its splicing partners and complexes with the NF-κB transcription machinery (p65, RNA Pol II)

3.12), we believe that in basal state, SNW1 is involved in co-transcriptional splicing connecting nascent RNAs that are being transcribed with the help of p-TEFb to various splicing factors. Similar observations regarding SNW1 have been reported in multiple papers (Brés et al. 2005; C. Zhang et al. 2003) in the context of Vitamin D and HIV-1 Tat transcription. Interestingly, on the activation of the NF- $\kappa$ B pathway, it seemingly loses its binding to splicing partners and facilitates the formation of the NF- $\kappa$ B, p-TEFb complex for efficient transcription of NF- $\kappa$ B target genes. This hints towards a more transcriptional and less splicing mediated role of SNW1 in NF- $\kappa$ B transcription. This hypothesis garners more support when coupled with the fact that the NF- $\kappa$ B luciferase construct used for in the screening did not have an intron in it and yet SNW1 affected its transcription.

We have summarized our observations in Figure 3.15 but these do not come without caveats and limitations. Firstly, although SNW1's involvement in the NF- $\kappa$ B pathway does seem specific in the context of TNF activation (Figure 3.5), it also affects (activates and represses) other transcription factors (Figure 3.8). Out of these factors, AP-1, CREB are known to cross-talk with NF- $\kappa$ B (Wen, Sakamoto and Miller, 2010). We have tried to prove that SNW1's regulation of CREB does not directly impact its regulation of NF- $\kappa$ B but it will be interesting to see how SNW1 co-regulates multiple transcriptional programs. Secondly, we do not know if the SNW1-p65 interaction on TNF treatment (Figure 3.12) is direct or indirect. Since SNW1 has not been found in mass-spectrophotometry

experiments run on p65 on TNF treatment (Wang *et al.*, 2010), we are inclined to conclude this interaction to be indirect. Thirdly, despite the fact that SNW1 modulates the NF- $\kappa$ B pathway, it will be a challenge to target or knock it down as part of a potential anti-inflammatory therapy due to its role in transcription and splicing. To our knowledge, there are no SNW1 knockout mice and our attempts to knock it out from 293T and THP-1 cells using CRIPSR-Cas9 failed due to lethal cell-cycle defects in the knockout lines (data not shown). Fourthly, we have limited knowledge about SNW1's interactions with other binding partners of NF- $\kappa$ B. For example, we were unable to see any co-binding of SNW1 with either CBP/p300, SRC-1 or HDAC1 (three known binding partners of p65) on TNF treatment. We can not discount the fact that SNW1's role in NF- $\kappa$ B transcription might be independent of these two proteins, but more decisive experiments are needed to find out the exact context in which SNW1 is affecting NF- $\kappa$ B transcription. We have built testable hypothesis on SNW1's role in the NF- $\kappa$ B pathway in Chapter 4.

## **MATERIALS AND METHODS**

### **Validating SNW1 as an NF- $\kappa$ B mediator**

- siRNAs: siSNW1 (Dharmacon, Catalog Number J-012446-05), siDeath (Qiagen, Catalog Number SI04381048), sip65, siLuciferase
- Cell culture: THP-1, U87 (from ATCC)
- Antibodies: COX-2 (Cayman Chemical, Catalog Number 160112), SNW1 (Atlas Antibodies, Catalog Number HPA002457)
- Primers: IKBa, IL1, CCL2, IL6 (PrimerBank – MGH-PGA)
- Cytokines: TNF, LPS
- Transfection reagents: Lipofectamine RNAiMax (for siRNAs, using the manufacturer's protocol) and Lipofectamine 3000 (for plasmid DNA, using the manufacturer's protocol)
- Apoptosis readout: Caspase Glo (Promega, Catalog Number G8090 – using the manufacturer's protocol)

### **Testing SNW1's specificity in regulating transcription**

- Plasmids: NF- $\kappa$ B driven luciferase, pTK Renilla luciferase (Addgene, Catalog Number 12179)
- Cell culture: 293T cells, B-cells, U-2OS, A549 (from ATCC)
- Transcription Factor Luciferase Assay ('PathwayScan' Multi-Pathway Reporter Kit, GM Biosciences, Catalog Number GM8001 – using the manufacturer's protocol)

- Antibodies: ATF3 (Cell Signaling, Catalog Number 33593S)
- CREB inhibitor (Millipore Sigma, Catalog Number 217505-250MG)
- Stimuli: PAM3CSK4 (Invivogen, Catalog Number tlr1-pms), PGN (Invivogen, Catalog Number tlr1-pgnb3), P2C (Invivogen, Catalog Number tlr1-pm2s-1), R848 (Invivogen, Catalog Number tlr1-r848), FLG (Invivogen, Catalog Number tlr1-stfla), IL-1 (R&D Systems, Catalog Number 201-LB-005), IDCGAP (Invivogen, Catalog Number tlr1-c12dap), CD40L (Peprotech, Catalog Number AF-310-02), BAFF (R&D Systems, Catalog Number 2149-BF-010).

#### **Testing SNW1's role in the cytoplasmic part of the NF- $\kappa$ B pathway**

- Antibodies: IKK2-p (Cell Signaling, Catalog Number 2697S), IKK2 total (Cell Signaling, Catalog Number 2684S), IKBa-p (Cell Signaling, Catalog Number 2859S), IKBa total (Santa Cruz, Catalog Number sc-7218), Beta-actin, p65 (Santa Cruz, Catalog Number sc-8008), phospho-p65 (Serine 536, Cell Signaling, Catalog Number 3031S), phospho-p65 (Serine 276, Cell Signaling, Catalog Number 3037), acetyl-p65 (K310, Cell Signaling, Catalog Number 3045S), H2A (Cell Signaling, 2578S)
- Nuclear Translocation Assay (ThermoFisher Scientific, Catalog Number 78833 – used as per the manufacturer's protocol)

**Testing SNW1's role in the nuclear part of the NF- $\kappa$ B pathway**

- Antibodies: p-TEFb (Cell Signaling, Catalog Number 2316), RNA Pol II (Abcam, Catalog Number ab5095), c-Myc (Cell Signaling, Catalog Number 9402), Menin (Abcam, Catalog Number ab2605)

**Testing SNW1's role in splicing in the context of NF- $\kappa$ B**

- Antibodies: SNRNP200 (Bethyl Laboratories, Catalog Number A303-453A), PRPF8 (Abcam, Catalog Number ab79237)

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Chapter 3, in part, is currently being prepared for submission for publication of the material. Verma, Suneer; De Jesus, Paul; Chanda, Sumit K; and Verma, Inder M. RNAi screens reveal SNW1 as a novel regulator of the NF- $\kappa$ B pathway. The dissertation author is the primary investigator and author of this material.

## Chapter 4: Perspectives



## INTRODUCTION

The mechanisms explaining NF- $\kappa$ B's inducible transcriptional programs in response to activating stimuli are complex and varied. Over three decades of research and 45000 papers later we do have a lot of answers, especially at the macro level. We have a great understanding of what activates this pathway, in terms of extra- (or even intra-) cellular stimuli; the key proteins that are involved in relaying that signal and converting it into a transcriptional program; and the consequences of mis-regulation of this pathway in the context of human diseases. That said, the more we zoom into this pathway, the fuzzier it gets.

If we divide the NF- $\kappa$ B pathway into its cytoplasmic and nuclear components, we would argue that we have a great handle on the former. We have detailed understanding of the receptor complexes for different activating stimuli (TNF receptor superfamily, Toll-like receptors, IL-1 receptor, etc. along with their adapter proteins) and the major protein-protein interactions (IKK complex, I $\kappa$ B $\alpha$ ) that lead to the NF- $\kappa$ B dimer entering the nucleus. Although one can argue about our gaps in knowledge on the activation of the IKK complex and cytoplasmic post-translational modifications of NF- $\kappa$ B, but it is after NF- $\kappa$ B enters the nucleus, that we have much more to learn. What are the proteins that NF- $\kappa$ B binds to inside the nucleus that assist in transcription? What is the connection between NF- $\kappa$ B and the core-transcription and RNA processing machinery? What is the order of protein-protein interactions that lead to the assembly of the transcription machinery on the promoter and enhancer elements of NF- $\kappa$ B target

genes? The heart of the problem is that the canonical NF- $\kappa$ B pathway, in response to different stimuli, leads to the same hetero-dimer (p65-p50) entering the nucleus and yet it results in a different transcriptional output depending on the stimulus, the cell-type, and the amount of time passed after pathway activation. The answers to this conundrum will not only assist in a greater understanding of this ubiquitous pathway, but also that of general transcription and gene regulation.

The work presented in this thesis has attempted to find novel regulators of the NF- $\kappa$ B pathway in relevant background of human macrophage cells (Chapter 2). We have identified and characterized SNW1 as a nuclear modulator of NF- $\kappa$ B that assists in the recruitment of p-TEFb for efficient transcriptional elongation of NF- $\kappa$ B target genes (Chapter 3). In this chapter, we have given our high-level perspective on the quest to find new modulators of the NF- $\kappa$ B pathway, the relevance of our RNAi screens, and where we think the next discoveries will come from. We have also commented on the NF- $\kappa$ B mediated regulation of POL II elongation and the potential role of SNW1 in it.

### **Novel modulators of NF- $\kappa$ B**

In Chapter 1, we have summarized previously published high throughput screens on the NF- $\kappa$ B pathway in Figures 1.6 and 1.7. The key differentiator of our screens was that they were conducted in a more physiologically relevant background (THP-1 macrophages) compared to previous screens. Macrophages

are the sentinels of the innate immune system and hence have an NF- $\kappa$ B pathway that responds to various stimuli (as opposed to 293T cells, for example, where the NF- $\kappa$ B pathway does not respond to LPS). This brings us to our next differentiator – our screens have the ability to identify and compare LPS and TNF specific (and common) modulators.

#### Stimulus specific modulators of NF- $\kappa$ B

High throughput approaches have rarely been used to find and compare stimulus specific modulators of NF- $\kappa$ B. As we pointed out, only one previous RNAi screen compared NF- $\kappa$ B regulators across two stimuli (TLR7 and TLR9 agonists; Chiang et al. 2012). Our secondary screens identified 41 and 35 regulators of the TNF and LPS dependent NF- $\kappa$ B pathway respectively. Out of them, 26 were common to both pathways, including our top hit SNW1, and the signal specific regulators are summarized in Tables 4.1 and 4.2. We have also included the results of further screening experiments we performed on some of them – including their ability to regulate I $\kappa$ B $\alpha$  phosphorylation (via IKK activity) and/or their ability to regulate NF- $\kappa$ B translocation to the nucleus. These experiments were performed in U87 glioblastoma-like cells, since their siRNA transfection efficiency is higher versus THP-1 cells which led to cleaner results.

For the LPS pathway, we are especially interested in genes FOSB and B2M (Table 4.1). FOSB, as part of the AP-1 transcription factor, is known to have a positive synergistic effect with NF- $\kappa$ B on the transcription of cytokines in T-cells

**Table 4.1****Secondary screen hits specific to the LPS activated NF- $\kappa$ B pathway.**

The table shows 11 hits (other than TLR4) that passed the LPS (but not TNF) activated secondary screening process. The results of their effects on phosphorylation of I $\kappa$ B and translocation of NF- $\kappa$ B in U87 cells are also mentioned.

<b>Gene Name</b>	<b>Function</b>	<b>Effect on p-I<math>\kappa</math>B<math>\alpha</math>?</b>	<b>Effect on NF-<math>\kappa</math>B translocation?</b>
DUSP15	Phosphatase	Yes	Yes
KPNA1	Importin	No	Yes
GRIN2B	Receptor	No	No
ITCH	E3 Ligase	Yes	Yes
APC2	Wnt regulator	No	Yes
CINP	CDK interacting protein	No	No
RIP1	Receptor Activator	Yes	Yes
FOSB	Transcription Factor	No	No
KEL	Metallo-peptidase	No	No
BFAR	Apoptosis regulator	No	No
B2M	MHC complex	Yes	Yes

**Table 4.2****Secondary screen hits specific to the TNF activated NF- $\kappa$ B pathway.**

The table shows 15 hits (other than TNFR1) that passed the LPS (but not TNF) activated secondary screening process. The results of their effects on phosphorylation of I $\kappa$ B and translocation of NF- $\kappa$ B in U87 cells are also mentioned.

<b>Gene Name</b>	<b>Function</b>	<b>Effect on p-I<math>\kappa</math>B<math>\alpha</math>?</b>	<b>Effect on NF-<math>\kappa</math>B translocation?</b>
GHRH	Growth Hormone Releasing Hormone	Yes	Yes
BMP10	TGF Beta Agonist	No	No
NDP52	Autophagy Regulator	No	Yes
CRHR2	Corticotropin Releasing Hormone	Yes	Yes
NEF3	Neurofilament	No	No
CLCN2	Ion Channel	Yes	No
HAPLN2	ECM protein	Yes	Yes
C5ORF11	Unknown	No	No
LCE1B	Keratinization	No	No
CX3CL1	Chemokine	Yes	Yes
GRIPAP1	Glutamate Receptor Interacting Protein	No	No
PACSIN2	Protein Kinase C substrate	Yes	Yes
ADD2	Membrane Skeletal protein	Yes	Yes
BAZ1A	Chromatin Remodeler	No	No
TRPM8	Temperature Regulation	No	No

(Khalaf, Jass and Olsson, 2010). Since FOSB is part of a nuclear transcription factor, it wasn't a surprise that it did not affect the phosphorylation of I $\kappa$ B $\alpha$  and the nuclear translocation of NF- $\kappa$ B. B2M on the other hand is involved in the presentation of peptide antigens to the immune system. It is known to be a part of the immune response to a bacterial infection by *M.tuberculosis* (Chiou et al. 2016) and hence it isn't a surprise that it was part of our LPS-specific NF- $\kappa$ B mediator list. For the TNF pathway, we would love to find out more about the roles of GHRH and CRHR2 in NF- $\kappa$ B activation. The activities of both Growth Hormone Releasing Hormone and Corticotropin Releasing Hormone has been implicated in the NF- $\kappa$ B pathway in very limited contexts (gastric cancer cells and human keratinocytes respectively (Zbytek, Pfeffer and Slominski, 2004; Gan *et al.*, 2016). GHRH is hypothesized to regulate the NF- $\kappa$ B through the STAT3 pathway while CRHR has been shown to increase DNA binding by NF- $\kappa$ B via increased nuclear translocation of NF- $\kappa$ B. It will be interesting to see their interaction with the TNF dependent NF- $\kappa$ B pathway especially because they both are evidently involved before the phosphorylation of I $\kappa$ B $\alpha$  (and eventual nuclear translocation of NF- $\kappa$ B; Figure 4.2).

One limitation of finding a stimulus specific factor through our screen was the presence of false negatives. For example, the genes ITCH and RIP1 appeared to be LPS specific but there is published evidence of them being involved in the TNF-dependent NF- $\kappa$ B pathway (Festjens *et al.*, 2007; Shembade *et al.*, 2008). They had barely missed the cut in our TNF secondary screen

because of stringency. Hence, we will do better to examine each stimulus specific regulator on its own merits and develop relevant hypothesis before proceeding.

#### Future of NF- $\kappa$ B screening

With the advent of high-throughput assay development including RNAi and CRISPR screening, Mass Spectrophotometry, High content imaging, we believe the rate limiting factor in research today is not what system you screen in but the question you ask. In our opinion, majority of the previous screens have tried to answer questions on the biology of the NF- $\kappa$ B pathway and the next wave of screens would be done with a therapeutic motivation in mind. Some ways in which we can envision this include:

- Small compound screens done by pharmaceutical companies to find inhibitors of stimulus specific NF- $\kappa$ B pathway and hence to mitigate the pleiotropic side effects of inhibitors available today (Chapter 1)
- Screening on modulators of the NF- $\kappa$ B pathway in cancer vs. normal cells to find modulators that contribute to the tumorigenic inflammatory phenotype specifically in cancer
- Screening kinases or other druggable genes for stimulus specific modulators of NF- $\kappa$ B. One caveat is that kinases can show potent residual activity despite being highly knocked down. Hence, a CRISPR based knockout screen would be ideal.

## **NF- $\kappa$ B mediated transcription**

### Intricacies of induced transcription

Inducible transcription is a function of – the inducible transcription factor(s) which can act synergistically, chromatin environment at the gene's regulatory elements, lineage specific transcription factors, and the core transcription machinery. This example from Bhatt and Ghosh 2014 on the transcription of NF- $\kappa$ B target gene Interferon Beta (IFNB) gives a good perspective –

“Stimulus-dependent expression of this gene requires the cooperative binding of three transcription factors: NF- $\kappa$ B, IRF3/IRF7, and ATF-2/c-JUN. NF- $\kappa$ B initially binds to the conserved PRDII element in the promoter. This in turn facilitates the recruitment of IRF and ATF-2/c-Jun. Once properly assembled at the promoter, these transcription factors serve as a platform for the sequential recruitment of the PCAF chromatin modifying complex, the p300/CBP acetyltransferase, and subsequently the SWI/SNF chromatin remodeling complexes. SWI/SNF remodels the downstream nucleosome that encompasses the TATA box, thus allowing TBP binding and subsequent pre-initiation complex assembly.”

### NF- $\kappa$ B in the nucleus

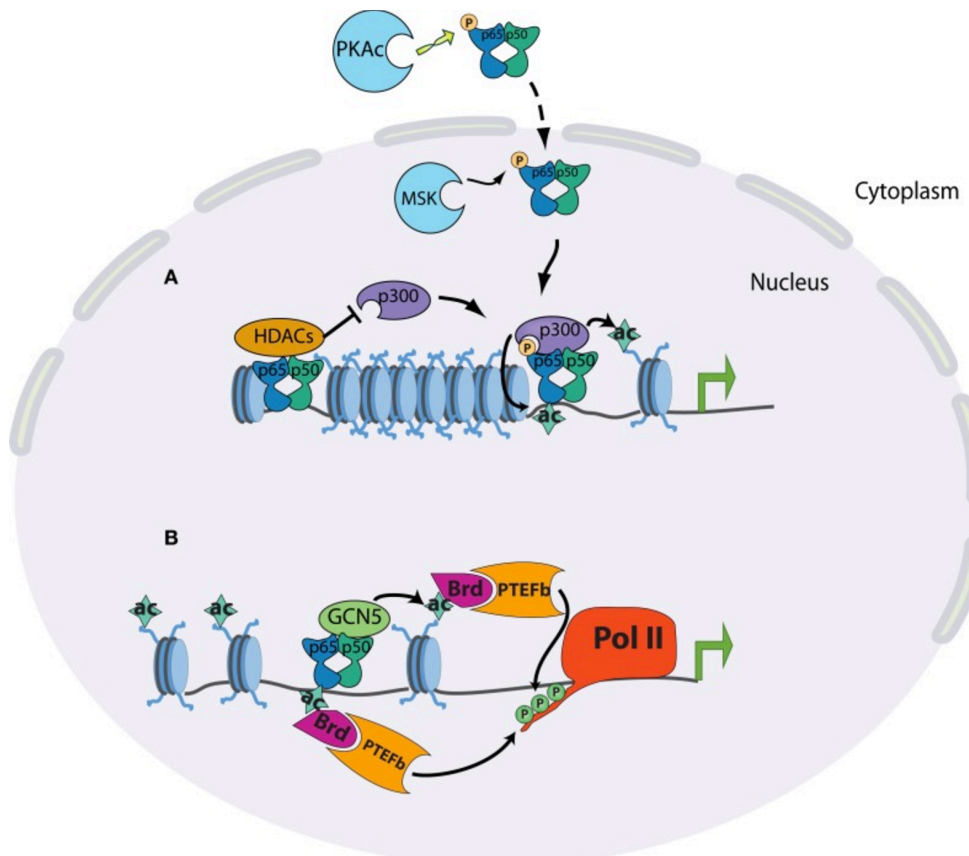
NF- $\kappa$ B has two main functions that help transcribe target genes. Firstly, it can recruit co-activators that help in increasing chromatin accessibility at the



gene promoter. This leads to the recruitment of the pre-initiation complex and core-transcription machinery (POL II). Secondly, it can recruit and induce the activity of p-TEFb which helps with transcriptional elongation from stalled polymerases. A very simplified consensus is that once NF- $\kappa$ B enters the nucleus it binds to the promoters of easily accessible genes (higher level of chromatin acetylation) or waits to recruit histone acetylases (CBP/p300) along with the SWI/SNF histone remodeling complexes to the promoters of less accessible genes. Once the gene promoters are made accessible to the pre-initiation complex, consisting of RNA POL II and other core transcription factors, NF- $\kappa$ B (acetylated) recruits Brd4 and p-TEFb, which can phosphorylate the POL II large subunit C-terminus leading to transcriptional elongation. This process is shown in Figure 4.1. Our RNAi screens help discover SNW1, which assists in the second part of the process as detailed below.

#### NF- $\kappa$ B and p-TEFb (and SNW1)

Hargreaves, Horng, and Medzhitov 2009 published a seminal paper (in 2009) that suggests that the switch from transcription initiation to transcription elongation is the rate-limiting step in the expression of primary NF- $\kappa$ B target genes (primary genes do not require de novo protein synthesis for transcription). The working model from that paper is as follows – Primary NF- $\kappa$ B target genes have S5-phosphorylated POL II present at their promoters. This signifies an



**Figure 4.1.**

**Steps leading up to NF- $\kappa$ B target-gene transcription**

(A) shows that phosphorylation of p65 at S276A by PKA or MSK (kinases) leads to preferential recruitment of histone acetylase CBP/p300 which leads to acetylation of relevant histones and hence increasing accessibility around the NF- $\kappa$ B target gene promoter. (B) shows that once RNA POL II is bound to the promoter of the target gene, p65 (and p50) recruit co-factors GCN5 and Brd4 to complex with p-TEFb. The latter phosphorylates the stalled RNA POL II causing efficient elongation. Reproduced from Bhatt and Ghosh 2014.

activated, but stalled POL II and hence suggests that their transcription is controlled at the level of elongation. Sure enough, once these cells were treated with LPS, the promoters accumulated S2-phosphorylated POL II (signifying elongation; POL II is S2-phosphorylated by CDK9), along with CDK9 and CyclinT1 (the components of p-TEFb). Blocking of NF- $\kappa$ B using an inhibitor reversed the LPS induced recruitment of p-TEFb and S2-phosphorylation of POL II. These findings have nicely built upon the paper by Barboric et al in 2001 that originally showed the requirement of p-TEFb for NF- $\kappa$ B dependent transcription. The key question, relevant to NF- $\kappa$ B, that Hargreaves, Horng, and Medzhitov ponder over in their discussion section is if NF- $\kappa$ B directly recruits p-TEFb (if so, then how does it do it?). Huang et al. (2009) have suggested Brd4 to be a mediator between NF- $\kappa$ B and p-TEFb but our RNAi screens did not pick up Brd4 as a potential regulator of NF- $\kappa$ B dependent transcription. Hargreaves, Horng, and Medzhitov have suggested that Brd4 may function to recruit and maintain P-TEFb throughout the general transcribed region, in proximity to elongating Pol II (as opposed to being specifically recruited to NF- $\kappa$ B) Our RNAi screens have discovered SNW1 (splicing factor and transcription co-activator) to be a mediator of the NF- $\kappa$ B pathway in response to various stimuli (Figure 3.10). Importantly, as Figure 3.15 suggests, on TNF treatment, SNW1 disassociates from its splicing complex to complex with p-TEFb, p65, and RNA POL II.

### SNW1 and NF- $\kappa$ B – looking ahead

We have looked into previously published papers about SNW1's role in transcription as a guide for future hypothesis and believe there are three important questions that are still to be answered about SNW1's role in the NF- $\kappa$ B pathway.

Firstly, it is important to check if SNW1's regulation of the NF- $\kappa$ B pathway is specific to certain target genes – to answer this, we are performing a p65 and p-TEFb ChIP-Seq experiments in THP-1 cells, on TNF treatment, with the conditions of wild-type and knocked-down SNW1. In parallel, we will perform phosphorylated RNA POL II and H3K4Me3 ChIP-Seq to monitor general transcription activity. We hypothesize that SNW1 depletion will lead to a significant decrease in p65 binding to the promoters of the p-TEFb dependent NF- $\kappa$ B target genes (primary target genes that are not dependent on *de novo* protein synthesis for transcription; Hargreaves, Horng, and Medzhitov 2009).

Secondly, we would love to understand the process by which SNW1 loses its binding with splicing factors and forms the complex with NF- $\kappa$ B and p-TEFb on TNF treatment. Our hypothesis is that SNW1 undergoes some sort of post-translational modification (eg. phosphorylation or acetylation) on treatment with TNF that helps it undergo the transition shown in Figure 3.15. There is a recent paper which suggests that SNW1 undergoes a c-Abl-mediated tyrosine phosphorylation to act as a transcription co-activator for the TGF- $\beta$  pathway (Kuki et al 2017). Hence, we are investigating this by trying to detect a change in the

electrophoretic mobility of SNW1 protein on treatment with TNF using high resolution gel electrophoresis and by immuno-precipitating SNW1 with and without TNF treatment and probing for PTMs (tyrosine and serine phosphorylations etc).

Thirdly, it would be interesting to know the status of splicing in cells treated with TNF – we have observed that SNW1 dissociates from its splicing partners on TNF treatment and a global splicing analysis would reveal if this dissociation affects the splicing of certain pre-mRNAs in the transcriptome. Although we don't know how 'complete' this dissociation is but Brés et al., have suggested in their 2005 paper that SNW1's transcriptional co-activation in the context of HIV-1 Tat induced genes is independent of its binding with its splicing factors. This has put SNW1 in a unique place as a regulator of splicing, transcription activation and transcription repression. We know that these processes are co-dependent and SNW1 seems like a key protein at the heart of all three.

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Chapter 4, in part, is currently being prepared for submission for publication of the material. Verma, Suneer; De Jesus, Paul; Chanda, Sumit K; and Verma, Inder M. RNAi screens reveal SNW1 as a novel regulator of the NF- $\kappa$ B pathway. The dissertation author is the primary investigator and author of this material.

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