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A novel protein NFKBIL-1 regulates the activity of key transcription factors

A thesis submitted to the University of Dublin for the degree of Doctor of Philosophy

By

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2008

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Declaration

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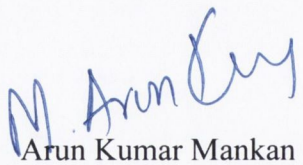

Arun Kumar Mankan

Summary:

Inflammation is a physiological process initiated in response to an injury or infection. Proper execution of the inflammatory response requires activation of several pro-inflammatory transcription factors. Nuclear Factor kappa B (NFκB), Activator protein-1 (AP-1) and Signal Transducers and Activators of Transcription (STAT) proteins are some of the well-characterised pro-inflammatory transcription factors. These transcription factors are inducible and regulate the expression of a range of genes involved in important biological processes such as the innate and adaptive immunity, inflammation, cellular stress responses, cell adhesion, apoptosis and proliferation. Regulation of these transcription factors is crucial since dysregulated inflammatory response can lead to acute or chronic inflammatory diseases. Investigations in our laboratory revealed that SNP in a potential immune/inflammation regulatory gene NFKBIL-1 was associated with increased susceptibility to Coeliac Disease in Irish population. In this regard, we characterized the function of a novel protein, thought to be a potential inhibitor of NFκB, Nuclear Factor Kappa B Inhibitor Like-1 protein (NFKBIL-1). We demonstrate that NFKBIL-1 inhibits IL-1β, TNF-α, TLR2 and TLR4 stimulated NFκB activity. Furthermore, we also report that AP-1 and STAT1 are also regulated by NFKBIL-1. We did not observe any translocation of NFKBIL-1 out of the nucleus in cells stimulated with various pro- and anti- inflammatory agents, suggesting that it regulates these transcription factors from within the nucleus. Finally, we demonstrate a colocalisation of NFKBIL-1 and HDAC3, providing a potential mechanism by which NFKBIL-1 may exert its broad based anti-inflammatory effects.

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Last but in many ways most important my family has been main source of support for me during these years. We all made it against odds. Lets keep the flag flying high.

Dedicated to my mother

Passive support is always under appreciated, but sometimes it has a bigger influence than expected

Contents

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Abbreviations

Amp	Ampicillin
AP-1	Activated Protein-1
APS	Ammoniumpersulphate
ARD	Ankyrin repeat domain
ATF	Activated Transcription Factor
ATM	Ataxia telangiectasia mutated
ATP	Adenosine 5'-triphosphate
Bcl3	B cell lymphoma 3 protein
BLAST	Basic local allignment tool
cAMP	Cyclic Adenosine monophosphate
CCAAT	Cytidine-cytidine-adenosine adenosine-thymidine transcription factor
CARD	Caspase recruitment domain
CBP	CREB binding protein
CD	Crohn's Disease
CIP	Calf Intestinal Phosphatase
CREB	cAMP responsive element binding
CRM	Chromosome maintenance region
(d)dNTP	(Di)Desoxynucleotide triphosphate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
E. coli	*Escherichia coli*
ECL	Enhanced Chemoluminiscence
EDTA	Ethylendiamintetra acetic acid
EGTA	Ethylene glycol tetraacetic acid
EMSA	Electrophoretic Mobility Shift Assay
FBS	Fœtal bovine serum
FCS	Foetal calf serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GSK	Glycogen synthase kinase
HAT	Histone Acetylase Transferase
HBSS	Hank's balanced salt solution
HDAC	Histone Deacetylase
His	Histidine
HLA	Human Leukocyte Antigen
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HEK	Human Embryonic Kidney Cells
HTLV	Human T Lymphotropic virus
IBD	Inflammatory Bowel Disease
IFN	Interferon
IκB	Inhibitor of κB
IKK	IκB kinase
IL	Interleukin
INAP	IL-1β induced nuclear ankyrin repeat protein
IP	Immunoprecipitation
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRAK	Interleukin-1 receptor-associated kinase 1
ISRE	Interferon-stimulated response elements
ITA	Inhibitor of T-cell apoptosis
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
JNKK	JNK kinase
bp	base pairs
kD	Kilo Dalton
Leu	Leucine
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MAP2K	MAPK kinase
MAP3K	MAP2K kinase
MCP	Monocyte chemoattractant protein
MEK	MAPK/ERK activating kinase
MEKK	MAPK/ERK activating kinase kinase
MKK	MAP kinase kinase
MMP	Matrix metalloproteinases
N-Cor	Nucler receptor Co repressor
NFκB	Nuclear factor kappa B
NEMO	NF-Kappa-B Essential Modulator
NFAT	Nuclear factor of activated T cells
NFKBIL-1	Nuclear Factor kappa B inhibitor Like-1
NLS	Nuclear localisation signal
NOD2	Nucleotide-binding oligomerization domain containing 2
NP40	Nonidet 40
OD	Optical density
ON	Overnight
ORF	Open reading frame
PAGE	Polyacrylamide-Gel

	electrophoresis
PBS	Phosphate buffered saline
PMA	Phorbol 13-myristate 12-acetate
PML	Promyelocytic Leukemia protein
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PKC	Protein kinase C
RHD	Rel homology domain
RIPA	Radio Immuno precipitation assay buffer
PMSF	Phenylmethanesulphonylfluoride
RNA	Ribonucleic Acid
RNAP	RNA polymerase
rpm	revolutions per minute
RT	Room temperature
RXR	Retinoid X receptor
SAPK	Stress-activated protein kinase
Ser	Serine
SH	Src Homology
SMRT	Silencing mediator for retinoid and thyroid-hormone receptor
SNP	Single Nucleotide polymorphism
SRC	Steroid receptor coactivator
STAT	Signal transducers and activators of transcription
TAK-1	TGF-β-activating kinase
TEMED	Tetramethylethylenediamine
TF	Transcription Factor
TGF	Transforming growth factor
Thr	Threonine
TIRAP	Toll-interleukin 1 receptor (TIR) domain containing adaptor protein
TLR	Toll like receptors
TNF	Tumour necrosis factor
TNFR	TNF receptor
TORC	Transducer of regulated CREB activity
TRADD	TNF-R1 associated death domain protein
TRAF	TNFR associated factor
Trp	Tryptophan
Tyr	Tyrosine
TSA	Trichostatin A
Ub	Ubiquitin
UC	Ulcerative colitis
USF	Upstream transcription factor
UXT	Ubiquitously-expressed transcript
v	Volume
w	Weight

Chapter 1
Introduction

1.0 Introduction

1.1 Inflammation

Inflammation is part of the inherent survival mechanism that enables an organism to defend itself against external biological and non-biological agents. It is a protective response intended to eliminate the initial cause of cell injury as well as the necrotic cells and tissues resulting from the original insult (Vinay Kumar 2007). This response is achieved by either diluting, destroying or neutralizing the injurious agent.

Although inflammation is an essential requirement for maintaining homeostasis, the inflammatory reaction and the subsequent repair process can cause considerable harm. Indeed inflammation is a very common response initiated by the body in various diseases and is caused by inappropriate reaction. This inflammatory response can be either acute or chronic. Acute inflammation is rapid in onset and of short duration, lasting from a few minutes to as long as a few days, and is characterized by fluid exudation and a predominantly neutrophilic leukocyte accumulation. Chronic inflammation is usually of longer duration and is typified by influx of lymphocytes and macrophages with associated vascular proliferation and fibrosis. Prolonged inflammation is indeed a hallmark of several chronic and autoimmune diseases (Henson, 2005; Vinay Kumar 2007). Examples of diseases where inflammation is dysregulated include rheumatoid arthritis, asthma, sepsis, atherosclerosis and inflammatory bowel disease.

Sepsis is an example of an acute inflammatory condition, encompassing multiple pathological processes including systemic inflammation, coagulopathy, hemodynamic

abnormalities and multiple organ dysfunctions. The pathophysiology of sepsis involves complex cytokine and inflammatory mediator networks while septic shock results from the host innate immune response to infectious organisms that may be blood borne or localized to a particular site (Cinel and Dellinger, 2007).

Inflammatory Bowel Disease (IBD) is on the other hand, characterised by a chronic, uncontrolled inflammation of the intestinal mucosa that can affect any part of the gastrointestinal tract. It comprises primarily two disorders: Crohn's disease (CD) and Ulcerative colitis (UC). Crohn's disease can affect any portion of the gastrointestinal tract but it is mainly seen in the ileum. Ulcerative colitis is a disease limited to the colon. A number of different genetic, environmental and immunoregulatory factors have been implicated in the pathogenesis of IBD (Bouma and Strober, 2003). It seems likely that these conditions result from an abnormal local immune response against the normal flora of the gut and probably against some self-antigens, in genetically susceptible individuals. Ulcerative colitis has been associated with *HLA-DRB1* whereas *HLA-DR7* and *DQ4* alleles are associated with approximately 30% of Crohn's disease cases (Sartor, 2006; Xavier and Podolsky, 2007). More recently mutations in *NOD2* gene were found to be associated in as many as 25% of Crohn's patients (Cario, 2005). Inflammation is the final common pathway in the pathogenesis of IBD. The morphological changes seen in IBD are the result of activation of inflammatory cells, neutrophils initially and mononuclear cells later in the course, terminating with destruction of the mucosal membrane (Bouma and Strober, 2003) .

Rheumatoid arthritis is another common, chronic inflammatory disease characterized by progressive destruction of cartilage and bone (Lee and Weinblatt, 2001). It is caused by an autoimmune response against an unknown self-antigen that initiates an immune response in the joint with the production of cytokines, including TNF-α that activates phagocytes. These phagocytes further initiate an inflammatory response leading to the damage of the joint tissue. The increased incidence of the disease in first degree relatives, a high concordance rate in monozygotic twins and association of *HLA-DR4* suggests the involvement of an genetic component in the pathogenesis of this disease (Weissmann, 2006). Although these three diseases represent the spectrum of inflammatory diseases, there are very few clinical conditions that do not have some element of inflammation associated with them.

The classical signs of inflammation, especially acute inflammation, were recognized very early on and include redness of the inflamed area, swelling, pain, heat and loss of function. Broadly the inflammatory response can be divided into several stages: Stage 1: Recognition of the injurious agent, Stage 2: migration of leukocytes to the site of injury; Stage 3: clearance of these cells once inflammation has been controlled; Stage 4: regulation of the response and finally Stage 5: restoration of the normal cellular architecture and the return of homeostasis (Vinay Kumar 2007). At the histological level, resolution can be defined as the interval from maximum neutrophilic infiltration to the point when they are lost from the tissue. In fact inflammation is an active process in all of its facets and which also requires endogenous processes to initiate and sustain the expression of pro-resolution proteins (Serhan et al., 2006; Serhan et al., 2007).

At a cellular and molecular level, inflammation initiates a response from the injured cell and the neighbouring normal tissue in the form of release of various chemokines, cytokines, eicosinoids and other signalling agents leading to the migration of specialised leukocytes including neutrophils and eosinophils to the site of the damage/injury. Subsequently peripheral blood monocytes accumulate at the site of inflammation and differentiate locally into larger, more granular, phagocytosing macrophages. Once the inflammatory agent has been neutralized, these macrophages clear away the dead neutrophils and eosinophils by phagocytosis. These cells in turn release cytokines and other mediators that not only limit any further damage but also enable the cell to recover from the injury (Figure 1.1) (Lawrence and Gilroy, 2007; Serhan et al., 2007). Although the initial response involves components of the innate immune system, other players such as dendritic cells act as a link between innate and adaptive immune systems. Dendritic cells are part of the antigen presenting system and upon activation present antigen to specific T cells. T cells and antibodies react directly or indirectly to neutralize or destroy the harmful agent. The adaptive immune response acts specifically against the harmful agent and remains active over a long period of time.

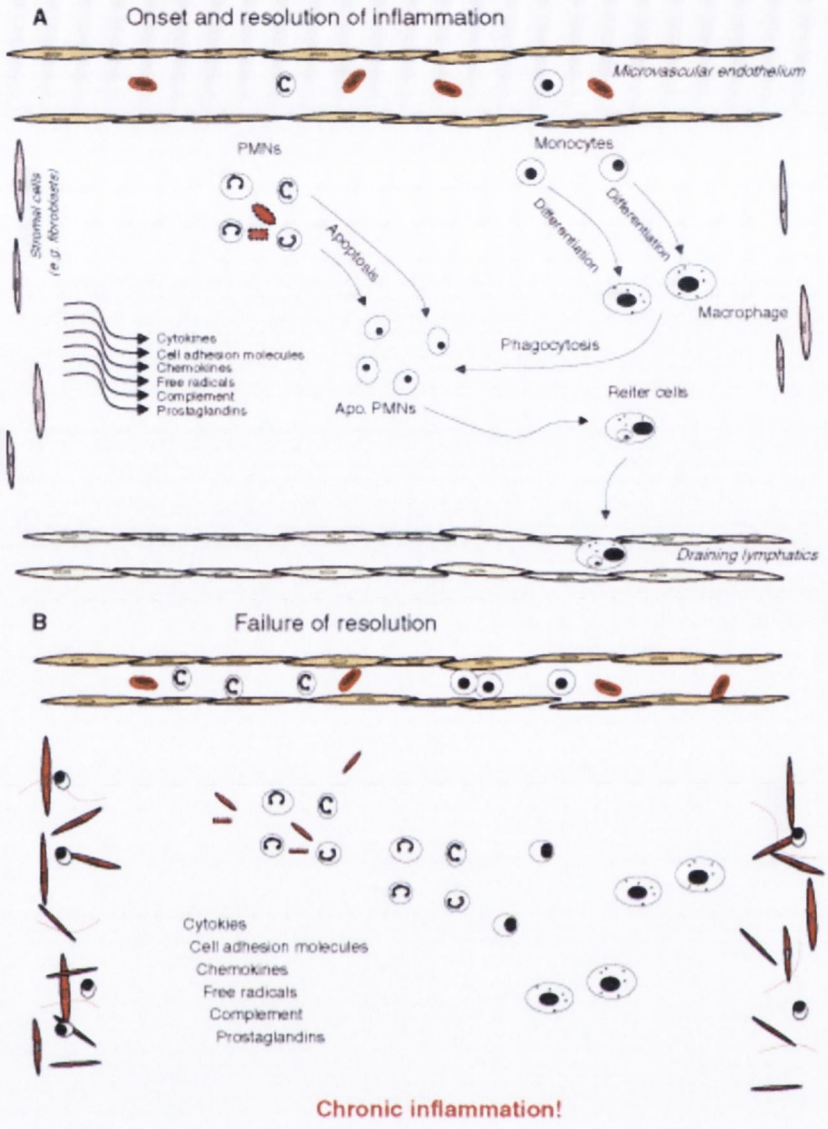


Figure 1.1: Illustration of the sequence of events initiated by an inflammatory response. This response requires interactions between various transcription factors and can terminate as either fully resolved response (A) or progress to chronic inflammation (B). In the presence of an appropriate immune response various chemokines and cytokines activate different effector cells. These cells include Neutrophils and macrophages that phagocytose the harmful agent and dead cells. In addition the adaptive immune system is also activated. However, when the harmful agent cannot be removed or the inflammatory reaction is unbalanced, an active pro-inflammatory states ensues leading to chronic inflammation (adapted from Lawrence and Gilroy, 2007).

Inflammation caused by biological (infectious), physical or chemical agents is characterized by coordinated activation of various signalling pathways that regulate expression of both pro- and anti-inflammatory mediators. IL-1β and TNF-α represent the archetypal pro-inflammatory cytokines that are rapidly released upon tissue injury or infection. On the other hand, the initiation of the inflammatory response during infection or in response to sterile tissue injury involves two families of receptors, the toll-like receptors (TLRs) and the nod-like receptors (NLRs). These cytokines and ligands bind to selective receptors and cause activation of several signalling complexes, terminating with the induction of transcription factors. A number of transcription factor families, including those for AP-1, NFκB, NFAT and the STATs have been implicated as critical regulators of gene expression in the setting of inflammation.

1.2 Transcription factors

Transcription factors represent a critical cog in the response of eukaryotic cells to the numerous physiological and pathological stimuli to which they are exposed. This process requires robust machinery within the cells that responds to the stimuli and regulates the expression of genes (Latchman, 1990; Latchman, 1992) including receptors, signalling pathways and effector proteins such as transcription factors.

Transcription factors are key components of a complex process by which genes are regulated. Other components of this machinery include a host of proteins which may be assembled into the RNA polymerase (RNAP) or comprise the general transcription factors (GTFs), co-activators, co-repressors, chromatin remodelers, histone acetylases,

deacetylases, kinases and methylases, to list some of the main participants that are involved in the initiation of transcription. The RNAP II transcription pre-initiation complex (PIC) is composed of RNAP II and a set of GTFs that includes the TATA-binding protein (TBP), TFIIB, TF IIE, TFIIF, and TFIIH. These crucial proteins are present in all eukaryotic cells and contribute to the initiation of every RNA polymerase II primary transcript that eventually becomes messenger RNA (Maldonado et al., 1999).

While these members of the transcriptional machinery are critical, the cells still require specific proteins called transcription factors. The number of proteins characterised as transcription factors is extensive, with estimates ranging from 2000~3000. These proteins share two characteristic domains: a DNA binding domain that binds with gene-specific regulatory sites directly and a second domain that exhibits transcriptional activation potential (Brivanlou and Darnell, 2002). Not all transcription factors possess these two domains and sometimes it is essential for two different proteins to form a complex with each of them contributing a domain. These site-specific transcription factors recruit coactivators and the transcriptional machinery to initiate gene-specific transcription. The large number of proteins having this property ensures not only redundancy in function but also allows a possibility of different combinatorial subsets that ensures that the complete set of regulators for each gene is unique leading to the right amount of the right protein at the right time. In order to understand the function of these various transcription factors a novel classification was recently put forward (Figure 1.2) (Brivanlou and Darnell, 2002). The authors proposed a broad classification of transcription factors into either being constitutively active or conditionally induced. A small set of transcription factors such

as Sp1 and CCAAT are present in the cell nucleus at all times. These transcription factors, although unable to induce gene expression on their own, facilitate maintenance of gene expression of proteins that are constitutively present. The second group of transcription factors are induced either in a cell-specific manner or in response to a specific signal. These signal dependent transcription factors can be further divided into three groups: 1) activated by steroid receptor superfamily (for example Glucocorticoid receptor and Estrogen receptor); 2) activated by internal signals (for example p53); 3) activated by cell surface receptor-ligand (for example AP-1 and STAT). Finally the last group can be additionally subdivided based on the sub-cellular location of the transcription factors into two classes: 1) resident nuclear factors and 2) latent cytoplasmic factors. Proteins, such as AP-1, CREB and ATMs, belong to the resident nuclear factors class and are localised within the nucleus but need to be activated by different signal pathways originating at the cell surface. Members of the second group of cell surface receptor-ligand family are present as inactive transcription factors in the cytoplasm. When these factors are activated by a series of signals they translocate to the nucleus, bind to the specific DNA sequence and regulate target gene expression. A classic example of this class of transcription factor is NFκB. This transcription factor is a complex of two different proteins and is sequestered in the cytoplasm by a class of inhibitors called IκB. When phosphorylated by specific stimuli, IκB's undergo destruction by proteolysis thereby releasing the NFκB, which then translocates to the nucleus and targets several different proteins including pro- and anti-inflammatory genes and genes involved in immune regulation.

The control of eukaryotic transcription requires the sequential interaction and precise coordination of a variety of large enzymatic complexes that are recruited by sequence-specific promoter/enhancer-binding proteins. Regulation is exerted at all levels of the process, which include chromatin recognition and reconfiguration, covalent modification of histones, recruitment of coactivators and basal transcription components (Jones and Kadonaga, 2000).

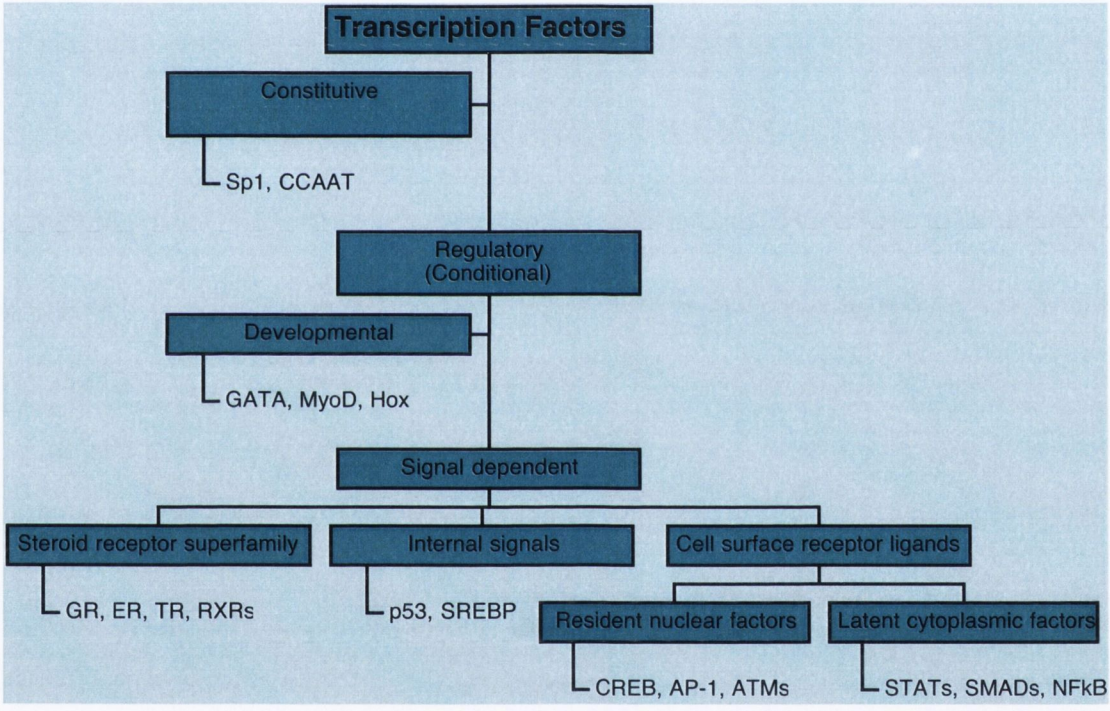


Figure 1.2: A general classification of transcription factors. Transcription factors can be grouped based on the temporal and spatial activity. While some transcription factors such as Sp1 are constitutively active others such as AP-1 and STATs are signal induced (from Brivanlou and Darnell, 2002).

Apart from above mentioned transcription factors activated by intracellular signals, a whole gamut of transcription factors exist that are activated by extracellular signals including osmolarity, hypoxia, heat shock and pH. These include Hypoxia inducing

factor, Nrf2 and Heat shock factor (Coleman and Ratcliffe, 2007; Thimmulappa et al., 2002) .

Regulatory factors can alternatively act as activators or as repressors, depending on their context or interacting partners, and regulatory complexes of apparently distinct function in transcription may be linked through sharing common subunits. Moreover reversible chemical modifications of chromatin such as methylation or acetylation can variably affect gene expression (Jones and Kadonaga, 2000; Korzus et al., 1998).

Transcription repressor proteins associate with their target genes either directly through a DNA-binding domain or indirectly by interacting with other DNA-bound proteins. To inhibit transcription in a selective manner, repressor proteins can 1) Mask transcription activation domains; 2) Block interaction of an activator with other components of the transcription machinery or 3) displace an activator from the DNA (Maldonado et al., 1999).

Examples of coactivators include CBP and p300 which have been shown to interact and serve co-activator roles for numerous transcription factors including CREB, AP-1, and STAT as well as nuclear receptors (Na et al., 1998b; Smith and O'Malley, 2004). The action of CBP and p300 appears to require both their intrinsic enzymatic function that adds an acetyl group to a recipient protein and their role as a scaffolding platform facilitating the interaction of a large number of potentially important associated proteins. In contrast a family of proteins belonging to the class of Histone Deacetylases (HDACs) remove the acetyl group from the chromatin and other acetylated proteins and consequently repress gene expression (Torchia et al., 1998).

A number of different proteins have been identified with similar HAT or HDAC activities. One explanation for this multiplicity of proteins with similar functions is that individual HAT activities could be restricted to specific substrates that may include non-histone proteins. Another possibility is that different classes of transcription factors require specific acetyltransferases which enables them to target specific genes (Torchia et al., 1998).

Furthermore multifunctionality of proteins within the repressor complex may allow co repressors to shut off gene expression in ways that are tailored to the goal of the repression. For example if the goal is to transiently repress transcription in response to a temporary change in the environment, then repression via transcriptional machinery interactions, which should be rapidly reversible, might be the best option. In contrast, if the goal is to generate a repressed epigenetic state, then repression via covalent changes in histone structure might be the preferred option (Courey and Jia, 2001).

Although the primacy of transcription as the necessary first step in gene expression is undeniable, this does not imply that transcriptional regulation has the largest effect on the final concentration of the active gene product, which is the most relevant quantity to the phenotype (He and Hannon, 2004) .

miRNAs are generally 21–25nucleotide, non-coding RNAs that are derived from larger precursors that form imperfect stem-loop structures (Bartel, 2004; Lai, 2003). The mature miRNA is most often derived from one arm of the precursor hairpin, and is released from the primary transcript through stepwise processing by two ribonuclease-III (RNase III) (Lee et al., 2003). One of the best studied example of

miRNA is lin-4 and although the precise molecular mechanisms that underlie post-transcriptional repression by miRNAs still remain largely unknown, most miRNAs bind to the target-3' UTR with imperfect complementarity and function as translational repressors (Bartel, 2004; He and Hannon, 2004).

In addition to transcription factors and miRNAs, there are many other layers of gene regulation, including: cell signalling; mRNA splicing, mRNA stabilization, polyadenylation and localization; chromatin modifications; and mechanisms of protein localization, modification and degradation.

1.3 Transcription factors activated during inflammation

1.3.1 Transcription factors activated during inflammation: NF Kappa B (NFκB)

NFκB is an important transcription factor activated by pro-inflammatory cytokines and numerous other stimuli involved in the regulation of a number of key inducible genes. It was initially identified as a protein that binds to a specific DNA sequence within the intronic enhancer of the immunoglobulin kappa light chain in mature B and plasma cells (Sen and Baltimore, 1986). Subsequent advances have revealed that it is a widely expressed transcription factor that plays a critical role in the regulation of inflammatory, apoptotic and immune processes and regulates the expression of proteins such as cytokines, chemokines, cellular adhesion molecules and those involved in apoptosis (Table 1) (Ghosh et al., 1998; May and Ghosh, 1998).

Class of gene	NFκB-dependent gene
Cytokines/growth factors	IL-1α and β IL-2, -3, -6, -8, -12 TNF-α IFNβ G-CSF M-CSF GM-CSF
Cytokine receptors	IL-2Rα
Stress proteins	SAA Complement factors B, C3 and C4
Leukocyte adhesion molecules	ICAM-1, VCAM-1, E-selectin
Immunoregulatory molecules	MHC class I and class II TCRα and β

Table 1.1: Examples of genes regulated by NFκB. NFκB regulates a number of genes including interleukins, growth factors and complement factors.

NFκB consists of homo- or hetero- dimers of a family of structurally related proteins that share a common Rel homology domain (RHD) located at the N-terminal. The RHD consists of DNA-binding and dimerization domains and a nuclear localization signal (NLS). This family is made up of five proteins: p65, c-Rel, Rel B, p50/p105 and p52/p100. Both p105 and p100 are precursor proteins that are subsequently processed to the smaller active forms i.e. p50 and p52 respectively. The p65/p50 heterodimer is the most conspicuously active form of the NFκB protein followed by the p50/p50 and p65/p65 homodimer complexes (Ghosh et al., 1998; Grossmann et al., 1999; Li and Verma, 2002; Moynagh, 2005).

As depicted in Figure 3, various different stimuli including oxidative stress, inflammatory cytokines, radiation, viruses and bacteria activate NFκB. It has been shown that signal pathways from these various stimuli generally converge upon the IKK complex. This complex consists of three proteins two of which IKK α and IKK β are catalytic in activity while the third, IKK gamma/NEMO has a regulatory role serving as an adapter protein, connecting both the catalytic subunits with the upstream activators. The IKK's are activated by phosphorylation of serine residues *via* kinases such as NFκB inducing kinase (NIK). The now activated IKK can phosphorylate two critical serine residues in IκB α (Ser 32 and Ser 36) allowing targeting of the IκB α for ubiquitination and degradation (Karin, 1999; Senftleben and Karin, 2002). The degradation of IκB α unmasks the nuclear localisation signal of p65, allowing NFκB to translocate to the nucleus where it regulates target gene expression. However recent studies have shown the existence of IKK-complex independent activation of NFκB.

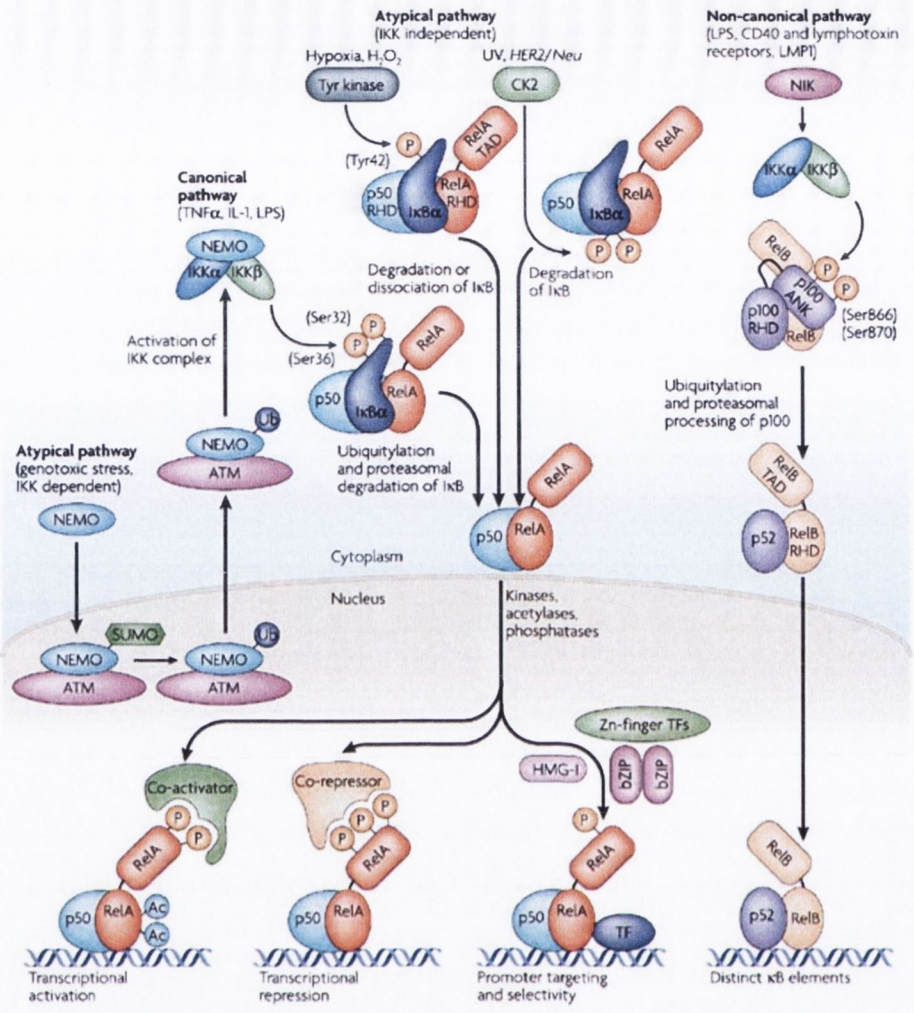


Figure 1.3: Activation of NF kappa B. Several different stimuli activate NFκB. The most studied canonical pathway is induced by TNFα, IL-1β and other stimuli, and is dependent on activation of IKKβ. This activation results in the phosphorylation (P) of IκBα leading to its ubiquitylation (Ub) and subsequent degradation by the 26S proteasome. Release of the NFκB complex allows it to relocate to the nucleus. IKK-dependent activation of NFκB can occur following genotoxic stress. Here, NFκB essential modifier (NEMO) localizes to the nucleus. NEMO relocates back to the cytoplasm together with ATM, where activation of IKKβ occurs. IKK-independent atypical pathways of NFκB activation have also been described. The non-canonical pathway results in the activation of IKKα by the NFκB-inducing kinase (NIK), followed by phosphorylation of the p100 NFκB subunit by IKKα. This results in proteasome-dependent processing of p100 to p52, which can lead to the activation of p52–RelB heterodimers that target distinct κB elements (adapted from Perkins, 2007).

This alternative pathway involves selective activation of the [p100/p52]/Rel B heterodimer complex, triggered by members of the TNF-α family and certain chemokines, and involves an IKK α dimer formation. The importance of both of these pathways have been demonstrated by their ability to regulate gene expression and functional outcome *in vivo* (Luo et al., 2005; Shen et al., 2005) (Ghosh et al., 1998). In addition, Saccani *et al.* demonstrated that recruitment of NFκB to the promoters of responsive genes occurs in two phases (Saccani et al., 2001). During the first phase, NFκB binds to and regulates the expression of constitutively and immediately accessible promoters and during the second phase it binds to the promoters of genes that first require modification in the chromatin structure to allow NFκB to access them. Thus as described in the above section, the activation of NFκB is indeed a very complex event, and proper execution of this event requires an equally complex sequence of regulatory activities to take place in the cell to terminate the activity of this critical transcription factor.

1.3.1.1 Regulators of NFκB activation

NFκB is regulated at multiple levels. While some of the mechanisms target the Rel proteins themselves by regulating synthesis of individual subunits of NFκB with subsequent post-translational modifications (e.g. phosphorylation and acetylation), others involve the group of regulators of NFκB, termed as IκB proteins. IκB proteins belong to a structurally and functionally distinct family who's members include IκB α, IκB β, IκB ε, IκB ζ, Bcl3, p100 and p105; all of which are important regulators of NFκB (Gerondakis et al., 1999). Members of this family share a common structure called the ankyrin repeat domains. Most members of this family have 6-7 ankyrin

repeats (each being 33 amino acids in length). Ankyrin repeat domains have been found in proteins as diverse as Cdk inhibitors, signal transduction and transcriptional regulators, cytoskeletal organizers and developmental reSgulators (Mosavi et al., 2004). No enzymatic function has been attributed to these motifs that are believed to function primarily in the facilitation of protein-protein interactions (Mosavi et al., 2004). Another common protein motif found in these proteins is the PEST (region rich in the amino acids proline (P); glutamic acid (E); serine (S); or threonine (T)) sequence present in most members of the IκB family, which targets them for degradation *via* the 26 S proteasome pathways (Rogers et al., 1986; Shumway et al., 1999)

IκB α plays a pivotal role in the regulation of NFκB activity. Indeed, mice deficient in IκB α have constitutive NFκB activation resulting in pathology of immunodeficiency with extensive inflammatory dermatitis and secondary granulocytosis with mortality occurring 10 days after birth (Beg et al., 1995a; Beg et al., 1995b). As mentioned before, degradation of IκB α after phosphorylation releases NFκB permitting its translocation to the nucleus. In the nucleus NFκB binds to the promoter of IκB α, among other genes, and in turn increases IκB α protein expression. Newly synthesized IκB α enters the nucleus and facilitates the removal of NFκB from the DNA causing it to be exported back to the cytoplasm. This feedback cycle is tightly regulated and essential for terminating the action of NFκB which otherwise could lead to sustained inflammation of the tissue (Moynagh, 2005).

Similar to IκB α, the other major well-characterised inhibitor, IκB β, predominantly associates with the p65/ p50 and p50/ cRel heterodimers. Unlike IκB α however, IκB

β degradation is only affected by a more limited range of stimuli such as LPS, IL-1 or HTLV-1 Tax protein. It has been shown that IκB β is involved in persistent activation of NFκB (Bourke et al., 2000). Interestingly, even though both these inhibitors are degraded following phosphorylation of specific serine residues, phosphorylation of IκB β occurs at a slower rate and with less efficiency. Thus stimuli that only weakly activate IKKα may not cause phosphorylation of IκB β at all. Moreover, it has been established that binding of NFκB to the free form of IκB β prevents it from binding to IκB α thus preventing the nuclear export of p65 leading to persistent activation of NFκB (Suyang et al., 1996).

The other known cytoplasmic inhibitor of NFκB, IκB ε was first identified by yeast two-hybrid screen (Whiteside et al., 1997). IκB ε is mainly expressed in T cells in the thymus, spleen, and lymph nodes. Mice lacking IκB ε are viable, fertile, and indistinguishable from wild-type animals in appearance and histology. However these mice exhibit a subtle immunologic phenotype with specific defects in T cell development, Ig isotype switching, and cytokine gene expression. Furthermore, enhanced up-regulation of IκB α and IκB β has been demonstrated in these mice, suggesting that IκB members may functionally compensate for each other (Memet et al., 1999). Athough there has been remarkable advances in the understanding of NFκB and its regulation, the exact mechanism of how this important transcription factor regulates the expression of so many genes and the actual sequence of events leading to the termination of its activity remains unclear.

While IκB α, IκB β and IκB ε are present in the cytoplasm, recent studies reveal a more complex pattern of regulation of NFκB in the nucleus. Although IκB β protein

has been observed in the nucleus, its role in the nucleus is still unknown. On the other hand, it has been established that IκB α binds with p65 and facilitates the export of p65 back to cytoplasm, terminating NFκB activation and thus replenishing a stockpile of cytoplasmic p65 available for the next wave of NFκB activation when required (Chen and Greene, 2003). More recently, a number of nuclear regulators of NFκB have been reported. How these novel regulators of NFκB function is the discussed in the subsequent sections.

1.3.1.2 Nuclear regulation of NFκB

Bcl3 and IκB ζ are two novel inhibitors of NFκB. These two inhibitors are exclusively localised within the nucleus and regulate NFκB activity. Identification of these and other inhibitors of NFκB localised within the nucleus provides another layer in the understanding of NFκB regulation.

1.3.1.2.1 Bcl3

Unlike the cytoplasmic inhibitors IκB α, IκB β and IκB ε, Bcl3 is a nuclear protein that preferentially promotes κB-dependent gene transcription (Zhang et al., 1994). Bcl3 can cause derepression of transcription by removing p50 and p52 dimers, which are transcriptionally inactive, from the κB sites, thus facilitating the binding of the active complex of p65, Rel-B or c-Rel to the consensus site (Bundy and McKeithan, 1997). While the ankyrin repeat domain of Bcl3 protein is required for its activity as an IκB, its N- and C- terminal regions are not homologous to those of other IκB proteins being, very proline rich, which indicates a potential role as a transactivator.

Interestingly, unlike IκB α, Bcl3 does not undergo proteasomal degradation. Zhang *et al.* demonstrated Bcl3 colocalises with p50 subunit in a variety of punctate or speckled patterns that strongly implies an interaction between these two proteins. This pattern of distribution along with the proline rich sequence supports the possible role of this protein as a transactivator. Indeed transactivation by Bcl3 has been shown to occur with the formation of a ternary complex with DNA and p50 or p52 homodimers.

1.3.1.2.2 IκB ζ

Haruta *et al.* isolated a novel gene termed IL-1β induced Nuclear Ankyrin repeat Protein (INAP; also called IκB ζ) (Haruta et al., 2001). They showed that INAP/ IκB ζ was induced specifically by IL-1β and subsequently translocated to the nucleus. They reported a lack of interaction of INAP with either p65, p50, CBPβ or RXR. Yamazaki and co-workers reported that IκB ζ inhibition is essential for the induction of inflammatory genes such as IL-6, the IL-12 p40 subunit and granulocyte-macrophage colony-stimulating factor (Yamazaki et al., 2001). Further studies revealed that the expression of IκB ζ was induced by IL-1β, LPS, peptidoglycan, bacterial lipoprotein, flagellin, and CpG DNA but not with TNF-α (Yamamoto et al., 2004). Totzke *et al.* identified IκB ζ by differential screening approach of apoptosis-sensitive and resistant tumour cells (Totzke et al., 2006). They demonstrated that it associates with both p65 and p50 and is localized in the nucleus where it aggregates in matrix-associated deacetylase bodies indicating that it regulates nuclear NFκB activity rather than its nuclear translocation from the cytoplasm. They also found that it did not colocalize with known proteins distributed within speckles such as either

PML protein or with SC-35 but with the nuclear transcriptional co-repressor SMRT that interacts with HDAC. These characteristics indicate that IκB ζ is a nuclear IκB protein that might function through modulating HDAC activity. Functionally the inhibitory function of IκB ζ was found to be essential for IL-6 production (Totzke et al., 2006; Yamamoto et al., 2004). When stimulated it associates with the p50 subunit and is recruited to the NFκB binding site of the IL-6 promoter (Totzke et al., 2006).

1.3.1.3 Post-translational modification of NFκB complex in the nucleus

Proteins within the NFκB complex are also subjected to signal-induced post-translational modifications that may influence their physiological functions. These post-translational modifications include phosphorylation, acetylation and ubiquitinylation (Neumann and Naumann, 2007; Perkins, 2007). The effect of these post-translational modifications can range from transcriptional activation to repression. p65 is phosphorylated at nine different sites including six serine and three threonine residues. These sites are Ser-536, Ser-535, Ser-529, Ser-468, Ser-311, Ser-276, Thr-435, Thr-505 and Thr-254 (Neumann and Naumann, 2007; Viatour et al., 2005). Ser-468 is phosphorylated by IKKβ, IKKε and GSK-3β. GSK-3β catalyzed-phosphorylation inhibits the basal activity of NFκB in the cell (Neumann and Naumann, 2007). p65 phosphorylated at Ser-468 is localised within the nucleus. Ser-276 is also a target of mitogen- and stress- activated protein kinase 1 (MSK-1). This phosphorylation occurs exclusively within the nucleus (Duran et al., 2003; Vermeulen et al., 2003). One common consequence of p65 phosphorylation is the increased recruitment of CBP/p300 leading to the export of repressor proteins such as HDACs (Zhong et al., 2002; Zhong et al., 1998). In addition to phosphorylation, p65 also

undergoes ubiquitinylation by the E3-ubiquitin ligase, suppressor of cytokine signalling-1 (SOCS-1), leading to its proteolysis and degradation (Maine et al., 2007) and thus termination of NFκB activity. Both RelB and C-Rel also undergo phosphorylation – but while phosphorylation promotes RelB degradation, it enhances C-Rel transactivation (Maier et al., 2003; Marienfeld et al., 2001).

Of the known inhibitors of NFκB in the nucleus, only Bcl3 undergoes GSK-3 mediated phosphorylation. This targets the protein for degradation through the proteasome pathway modulating its association with HDAC 1-, 3- and 6 (Viatour et al., 2004).

Understanding the mechanism of NFκB activation and its regulation is important since it is one of the main transcription factors activated during inflammation. At the same time a number of other transcription factors such as AP-1 and STATs, are also essential for a initiating and executing the appropriate inflammatory response.

1.3.2 Transcription factors activated during inflammation: Activator Protein-1 (AP-1)

The transcription factor AP-1 collectively describes a group of structurally and functionally related members of the Jun protein family (c-Jun, JunB and JunD) and Fos protein family (c-Fos, FosB, Fra-1 and Fra-2). Other members of this group include proteins such as ATF and JDP. It is activated by various physiological and pathological stimuli such as cytokines, growth factors, stress signals, infections and oncogenic signals. AP-1 regulates the basal and inducible expression of a number of different genes which have as a regulatory factor the palindromic consensus sequence

5'-TGAG/CTCA-3' also known as TPA responsive elements (TREs) (De Bosscher et al., 2003; Herdegen and Leah, 1998; Hess et al., 2004; Karin et al., 1997; Shaulian and Karin, 2002).

A common structural feature of all the members of this family is the conserved bZIP motif that is responsible for the dimerization of the protein subunits and their binding to TREs. Of all the members of the group, Jun proteins can form both hetero- and homo- dimers while Fos proteins can only form Fos/Fos homodimers (Bakiri et al., 2002). The various different dimers of an AP-1 complex differ in their affinity and specificity of DNA binding and also in their distribution in different cell types. For example, the predominant form of AP-1 in most cells is the Fos/Jun heterodimer that binds to DNA with very high affinity whereas the Jun/Jun homodimer binds to DNA with less affinity (Shaulian and Karin, 2002). Components of AP-1 are activated by three distinct but parallel MAP kinase pathways i.e. ERKs, JNKs and p38 kinase. Each pathway consists of a module of three kinases: MAPK, a MAPK kinase (MAPKK) that phosphorylates MAPK and a MAPKK kinase (MAPKKK/MEK) that phosphorylates MAPKK (Cano and Mahadevan, 1995; Chang and Karin, 2001; Ding et al., 1999; Feng et al., 2005). Once activated by the upstream kinases, the proteins such as JNK translocate to the nucleus and phosphorylate the components of AP-1 leading to dimerization and binding of the AP-1 complex to the TREs (Chen et al., 1996; Davis, 2000; Glover and Harrison, 1995). In the nucleus, the AP-1 dimers are part of a bigger transcriptional complex that includes co-factors such as CBP/p300, RNA pol II and HAT. A wide range of genes including for example a variety of transcription factors, matrix–degrading enzyme, cell adhesion molecules, cyclins and cytokines are regulated by AP-1 suggesting a profound ability of AP-1 to regulate

cellular homeostasis (Ameyar et al., 2003; Bakiri et al., 2002; Eferl and Wagner, 2003; Florin et al., 2004).

Regulation of AP-1 itself is very complex and occurs at multiple levels. It can be achieved through changes in transcription of genes encoding AP-1 subunits, control of the stability of their mRNAs, post translational processing and turnover of pre-existing or newly synthesized AP-1 subunits and the specific interactions between AP-1 proteins and other transcription factors and co-factors. For example, the protein Elk-1 regulates c-Fos transcription and consequently the composition of AP-1 complex (Marais et al., 1993).

1.3.3 Transcription factors activated during inflammation: Signal transducers and activators of transcription (STAT)

STAT proteins are a group of transcription factors activated in response to various ligands such as cytokines, growth factors and hormones and transduce extracellular and intracellular signals by translocating to the nucleus and regulating the expression of a number of genes (Darnell, 1997; Darnell et al., 1994; Ihle, 1996). The seven known members of this family are characterized by the presence of the Src homology 2 (SH2) domain and a carboxyl terminal tyrosine phosphorylation site. In an unstimulated cell the STAT proteins are present in an inactive form in the cytoplasm. Signal transduction pathways are activated when specific ligands bind to the receptor. Jak proteins, located at the intracellular part of the STAT receptor bound to the membrane, are subsequently phosphorylated and undergo dimerization becoming catalytically active. Jak in turn phosphorylate STAT proteins. Phosphorylated STATs then detach from the receptor, heterodimerize or homodimerize and translocate to the

nucleus where they bind to specific DNA sequences. The Jak protein family consists of 4 members and includes Jak1, Jak2, Jak3 and Tyk2. Jak proteins are characterized by their ability to undergo autophosphorylation and also to phosphorylate and activate STAT proteins.

Interferon is the most intensively studied inducer of STAT proteins. Type I Interferon activates both STAT1 and STAT2. The STAT1-STAT2 heterodimer associates with p48 also known as ISGF3γ to form a heterotrimeric complex in the nucleus (Fu et al., 1992; Schindler et al., 1992). This complex called the ISGF3 binds to interferon-stimulated response elements (ISREs) in promoters of IFN-stimulated genes (ISGs) (Levy et al., 1988). Type II interferon activates predominantly STAT1, which homodimerizes, translocates to the nucleus and binds to IFN-γ activated sequences (GAS) (Shuai et al., 1992; Shuai et al., 1993).

Like most other signal transducers, the activity of STAT proteins is transient. STAT proteins shuttle dynamically between the nucleus and the cytoplasm. Regulation of STAT activity involves several different mechanisms. STAT proteins have been shown to be degraded following ubiquitination via the Ubiquitin-proteasome pathway. STATs may also be inactivated by dephosphorylation by phosphatases in the nucleus (Kim and Maniatis, 1996). Novel inhibitors of STAT proteins identified in recent times include suppressor–of-cytokine-signalling (SOCS) that bind with Jak proteins and prevent activation of STATs, and a family of protein inhibitors of activated STAT (PIAS) that inhibit binding of STAT proteins to the DNA (Chung et al., 1997; Endo et al., 1997; Liu et al., 1998; Naka et al., 1997; Shuai, 2000; Starr et al., 1997).

Although numerous different stimuli activate the transcription factors NFκB, AP-1 and STAT more recently a novel group of receptors called the toll like receptors were identified that play a crucial role in the host's immune response to invading microorganisms. The role of these receptors in immunity and inflammation is dealt with in the next section.

1.4 Toll like receptors

The immune system recognises challenges and destroys invading microorganisms and other foreign antigens. It is broadly divided into the innate immune system and the adaptive immune system. While the innate immune system acts as the first line of defence and protects the body from invading pathogens, the adaptive immune system specifically identifies antigens and targets them for destruction (Charles Janeway, 2004). It was assumed until very recently that the innate immune system was non-specific and destroyed invading pathogens by phagocytosis. Initial studies on Drosophila identified that a loss of function mutation in the toll like receptor resulted in severe fungal infection suggesting that this receptor was responsible for detection of fungal infection (Hoffmann, 2003; Lemaitre et al., 1996). Subsequently Medzitov *et al.* reported the human homolog of Toll and demonstrated that it induced the production of inflammatory cytokines (Medzhitov et al., 1997).

TLRs are a family of proteins that are distributed on many immune related cells such as macrophages, dendritic cells, neutrophils, B cells and mucosal epithelial cells. While TLR 1, 2, 4, 5 and 6 are associated with the plasma membrane, TLR 3, 7, 8 and 9 are located intracellularly. Most of the TLRs are activated by specific ligands, for example TLR4 is activated by bacterial LPS, while TLR2, in concert with TLR1 or

TLR6, recognizes various bacterial components, including peptidoglycan, lipopeptide and lipoprotein of Gram-positive bacteria and mycoplasma lipopeptide. Double-stranded DNA binds to TLR3 receptor and TLR5 recognizes bacterial flagellin. The ligands for TLR7 are imidazoquinoline-like molecules, ssRNA derived from influenza virus and HIV-1. Human TLR8 mediates the recognition of imidazoquinolines and ssRNA while bacterial and viral CpG DNA activates TLR9 (Akira, 2000; Bauer et al., 2001; Chow et al., 1999; Diebold et al., 2004; Heil et al., 2004; Hemmi et al., 2002; Hoshino et al., 1999; Marshall-Clarke et al., 2006; Ozinsky et al., 2000; Schnare et al., 2000; Schwandner et al., 1999; Shimazu et al., 1999; Stanley, 2002; Takeuchi et al., 1999; Underhill and Ozinsky, 2002; Verstak et al., 2007; Yoshimura et al., 1999).

The signalling pathways activated by the TLRs are very complex and include the involvement of a number of different proteins. The two major pathways include the MyD88 dependent pathway common to most TLRs and the MyD88 independent pathways activated by TLR2 and TLR4. While these two receptors require an additional molecule TIRAP for signal transduction to occur the rest of the TLRs initiate signal transduction via the activation of MyD88 (Burns et al., 1998; Horng et al., 2001; Kawai et al., 1999; Medzhitov et al., 1998; Wesche et al., 1997). MyD88 triggers sequential activation of IRAK-4, IRAK-1 and TRAF 6 (Li et al., 2002; Suzuki et al., 2002). Together with an ubiquitination enzyme complex, TRAF 6 catalyses auto-polyubiquitination of IKK-γ/NFκB essential modulator (NEMO) (Ye et al., 2002). TRAF 6 also activates TGF-β-activated kinase 1 (TAK1), which phosphorylates IKKβ and MAP kinase kinase 6. These activated proteins in turn modulate the activation of NFκB and MAP kinases respectively, which directly or indirectly induce expression of several different classes of genes (Akira, 2006; Akira and Takeda, 2004; O'Neill, 2006; Takeuchi and Akira, 2007; Verstak et al., 2007).

Additionally, the other pathway stimulated by TLRs includes the IRF that induces STAT activation with the subsequent induction of IFN responsive genes (Figure 1.4) (O'Neill, 2006; Verstak et al., 2007).

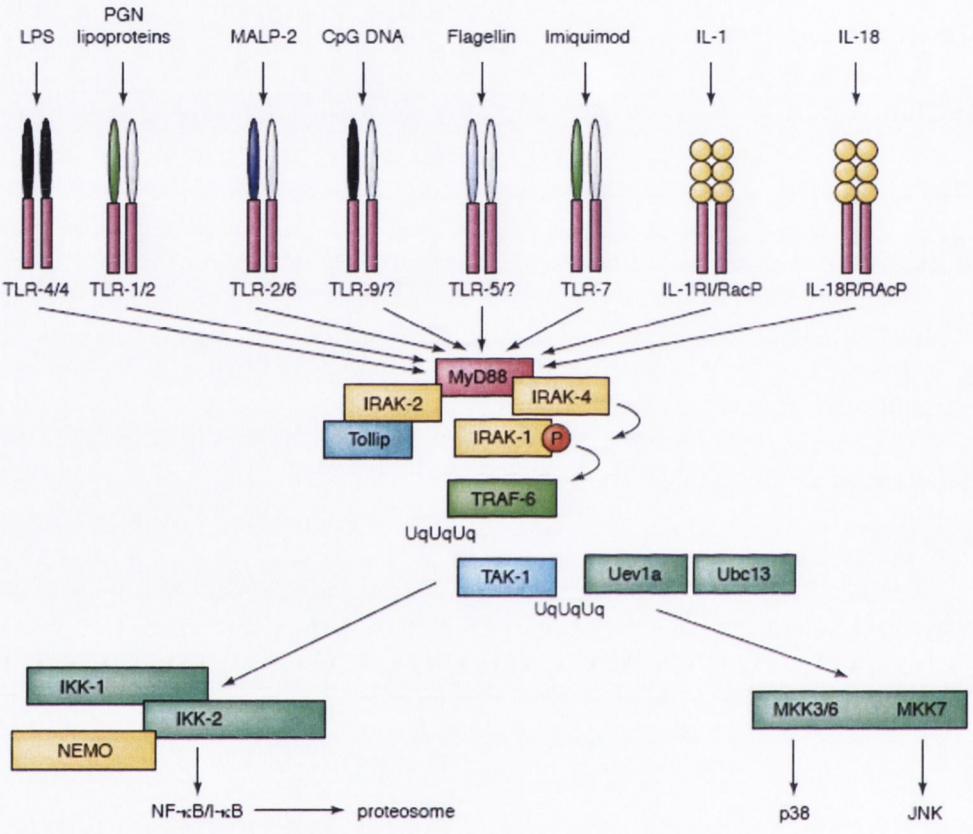


Figure 1.4: Signal transduction pathways activated by Toll like receptor pathways. Different ligands bind to specific TLRs and activate the downstream signal pathways. Myd88 is central to this pathway that terminates with the activation of NFκB and AP-1 and other transcription factors. IKK-1 and IKK-2 refer to IKK-α and IKK-γ respectively. This figure provides details only regarding some of the signal transduction pathways activated (Adapted from O'Neill, 2003).

The different stimuli that activate TLR induced signal pathways ultimately cause up regulation of important inflammatory mediators such as TNF-α, IL-1β, IL-12, chemokines, cyclooxygenase, adhesion molecules, and also increased production of reactive oxygen species that promote death of the invading microbes (Brightbill et al., 1999; Das, 2000; Jimenez et al., 2005; Rhee and Hwang, 2000; Takeuchi and Akira, 2007; Whiteside and Israel, 1997).

TLR	Diseases
TLR1/2	Bacterial/fungal diseases Gram-positive sepsis
TLR3	Viral diseases
TLR4	Bacterial diseases Gram-negative sepsis Chronic inflammation Autoimmune diseases Cancer
TLR5	Bacterial diseases
TLR2/6	Mycobacterial diseases
TLR7	Viral diseases
TLR8	Viral diseases
TLR9	Bacterial and viral diseases Autoimmune diseases Cancer

Table 1.2: Toll like receptors are implicated in the pathogenesis of various diseases. Identification of the contribution of the toll like receptors in diseases provides us with an opportunity to target them as potential therapy (O'Neill, 2003).

Persistent stimulation by TLRs such as TLR2 or TLR4 can lead to sustained inflammation and consequent tissue damage. Additionally, when challenged by an agent, the immune system needs to regulate the amount of inflammation and at the

same time mount adequate resistance to the antigen. Using a series of elegant experiments, Foster *et al.* demonstrated the existence of two classes of TLR 4 induced genes and showed the presence of a biphasic, co-ordinated response, wherein, after the initial antigenic challenge, the inflammatory genes were down regulated while the antimicrobial genes were primed for further action against the antigen (Foster et al., 2007). Apart from this biphasic response, a number of other proteins have also been identified that negatively regulate TLR responses. For example, a splice variant form of MyD88, termed MyD88s, inhibits LPS/IL-1β induced NFκB activity by preventing the association of IRAK-1 with IRAK-4 that normally leads to activation of IRAK-1 (Burns et al., 2003; Janssens et al., 2003; Liew et al., 2005). IRAK-M, a kinase-inactive IRAK family member, prevents the dissociation of IRAK-1 from MyD88 and the subsequent association of IRAK-1 with the downstream adaptor TRAF6 (Adib-Conquy et al., 2006; Kobayashi et al., 2002; Li and Qin, 2005; Liew et al., 2005). Two novel proteins termed SIGIRR and T1/ST2 have been shown to be involved in the negative regulation of TLR signalling. Thus the regulation of TLR stimulated pathways is critical for execution of an appropriate response to various stimuli.

The amplitude and the qualitative response to any stimulus require a co-ordinated cross regulation between various transcription factors. The balance of pro-inflammatory and anti-inflammatory cytokines is regulated in a dynamic manner. NFκB, AP-1 and STAT proteins regulate a number of similar or different downstream signals depending on the stimuli; and the cross talk between these transcription factors determines the outcome in terms of gene expression profile both in homeostatic and pathological conditions.

1.5 Cross talk between NFκB, AP-1 and STAT transcription factors

As discussed above, maintenance of homeostasis and response to any stimuli in normal or in pathological states necessitates constant, yet dynamic, cross talk between various regulating proteins.

Transcription factors can be activated individually by specific stimuli or a common ligand can activate several transcription factors together. For example TLR signalling results in the downstream activation of major families of proteins important in initiating inflammatory gene expression including NFκB, IRFs and MAPKs (Kawai and Akira, 2006). Since these transcription factors activate several genes of different classes, there is a requirement for cross talk between them.

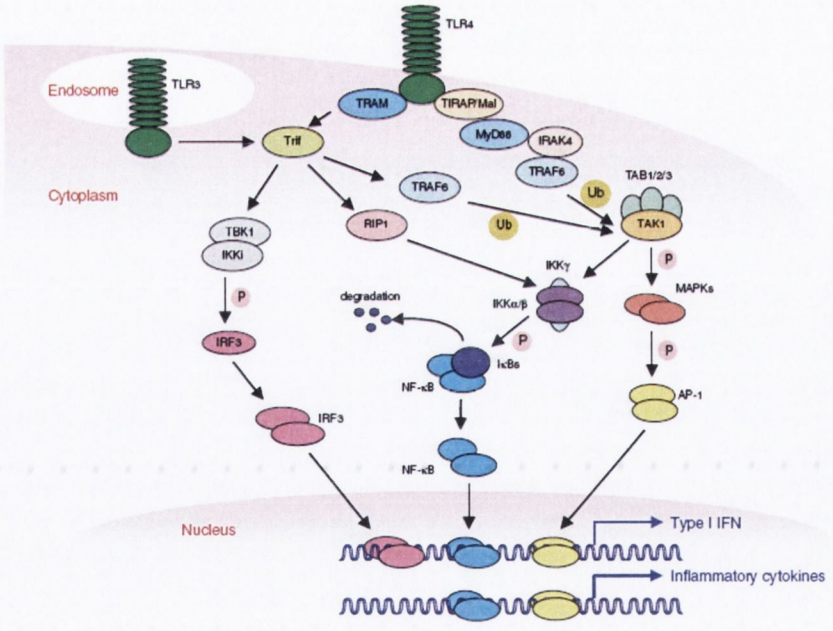


Figure 1.5: Cross talk between NFκB, AP-1 and the STAT transcription factors. TLR4 activates several transcription factors, including AP-1, NFκB and STATs, that dynamically interact with each other (Kawai and Akira, 2006).

One of the many modes by which termination of these activated pathways is effected (an essential step for limiting the extent of a pro-inflammatory response) results from cross talk deriving from both positive and negative stimuli taking place between transcription factors. It has been demonstrated that LPS stimulated signalling pathways activate IKK-NFκB, Jnk-AP1 and STAT transcription factors (figure 1.5). More recent evidence suggests that there might be a connection between the NFκB signalling pathway and the AP-1 pathway. Indeed, the activation of JNK by inflammatory cytokines or by stress is often accompanied by the nuclear translocation of NFκB and many genes require the concomitant activation of AP-1 and NFκB suggesting that these transcription factors work cooperatively (Verma et al., 1995). Stein *et al.* also showed that the bZIP regions of c-Jun and c-Fos are capable of physically interacting with NFκB/p65 through the Rel homology domain, which enhances DNA binding and biological function via both the κB and AP-1 response element (Stein et al., 1993). It has also been reported that the activation of the MAPK pathway leads to the activation of both the JNK and IκB kinase complexes (Yang et al., 2001). Furthermore, the response to AP-1 is very much enhanced when NFκB subunits are present and vice versa. This was demonstrated by Fujioka *et al.* in their elegant study whereby they showed that inhibition of NFκB reduced the level of Elk-1 protein activated by MAPK, thus impeding AP-1 activity and AP-1 dependent gene expression (Fujioka et al., 2004). IFN-γ is a potent activator of a Jak/STAT pathway, while being a weak activator of NFκB and MAPKs. In fact, it has been shown that IFN-γ can negatively regulate c-Fos and c-Jun expression by promoting their degradation via the Ubiquitin-proteasomal pathway. Many other examples of cross talk are known and thus it appears that extensive cross talk and negative feedback mechanisms are common and probably essential to limit uninhibited action of a single

transcription factor and leading to coordinated outcomes such as curtailing the inflammatory response.

1.6 Inhibitor of NFκB like protein (NFKBIL-1, IκBL)

A novel potential regulator of NFκB, encoded by the gene *NFKBIL-1,* was first reported by Albertella *et al.* in 1994 (Albertella and Campbell, 1994). They identified a full-length cDNA clone which was isolated from a premonocytic leukaemic cell line. This cDNA was identified as a novel gene located in the MHC class III region of chromosome 6p21.3 between *TNFB* and *BAT1* (Albertella and Campbell, 1994). Further characterization of this gene illustrated that it spans 13.5 kb of DNA, with the 3' end of the gene lying 12 kb from the 5' end of the *TNFB* gene (Albertella and Campbell, 1994; Shiina et al., 1998). It was reported that NFKBIL-1 was expressed in monocytes, T cells, B cells and hepatocytes and structurally consisted of 381 amino acids with two and half ankyrin repeat domains linked to an acidic region in the structure. Thus it showed homology with the IκB family of proteins in that it contains ankyrin repeat regions and the PEST motif. Ankyrin repeat domains have been found in proteins as diverse as Cdk inhibitors, signal transduction and transcriptional regulators, cytoskeletal organizers and developmental regulators. However, while no enzymatic function has been attributed to these motifs it is well known that they all commonly function to facilitate protein-protein interactions. In spite of being the most common protein motif, ankyrin repeats allow specific interactions due to variation in the surface area of the protein (Mosavi et al., 2004). The PEST (region rich in the amino acids proline (P); glutamic acid (E); serine (S); or threonine (T)) sequence present in most members of the IκB family targets them for degradation *via* the 26 S

proteasome pathways (Rogers et al., 1986; Shumway et al., 1999). Sequence analysis of NFKBIL-1 coding sequence using the Prosite tool (http://www.expasy.ch/prosite/) predicted that apart from the 2-3 ankyrin repeat regions, translated NFKBIL-1 protein had one PKC phosphorylation site, one Casein Kinase 2 phosphorylation site and an arginine rich region. Unlike other IκBs however, in NFKBIL-1 there is a motif of three leucine residues and one valine residue recurring with a periodicity of every seven amino acids at the C-terminal end of the protein and this may constitute a leucine zipper domain. Leucine zipper domains are a common dimerization domain seen in proteins involved in gene regulation (Landschulz et al., 1988). NFKBIL-1 also has a PEST sequence near its 5' terminal (Semple et al. 2002).

Three potentially important promoter polymorphisms have been reported in *NFKBIL-1*: the two common ones –63 and -421 and a rarer deletion at position –473 (Allcock et al., 2001; Allcock et al., 1999). A number of different association studies have shown polymorphisms for *NFKBIL-1* promoter region to be associated with Ulcerative colitis (UC), Diabetes Mellitus (DM) and Rheumatoid Arthritis (RA) (Table1.3) (de la Concha et al., 2000; Fernandez et al., 2005; Martinez et al., 2003; Miterski et al., 2004; Okamoto et al., 2003; Price et al., 2001; Windsor et al., 2005; Yamashita et al., 2004). In a genome wide association study performed using samples from 1133 affected individuals and 1006 controls, Ozaki *et al.* (2002) reported that *NFKBIL-1* –63T/A was associated with increased susceptibility to myocardial infarction. Functional studies were performed to progress these findings in the context of potential effects of this –63T/A SNP on the protein expression. These studies demonstrated a decrease in expression of NFKBIL-1 as a result of the –63T/A SNP (Ozaki et al., 2002). It should be noted that *NFKBIL-1* is located on a well defined haplotype block in the class III region of the MHC, which carries proven or

potentially functional polymorphisms of several important genes with immune or inflammatory actions, such as TNF-α and LTA and individual SNP associations need to be interpreted with this in mind ((Allcock et al., 2004; Miretti et al., 2005).

Authors	Disease	*NFKBIL-1* Locus	Control	Cases	OR
De la Concha EG *et al.*	Ulcerative Colitis	+738	298	155	9.25
Okamoto K *et al.*	Rheumatoid Arthritis	-63T/A	100	116	2.08
Yamashita T *et al.*	Type 1 Diabetes	-263A	660	237	4.55
Fernandez L *et al.*	Ulcerative Colitis	+738	315	253	5.90
Lin CH *et al.*	Rheumatoid Arthritis	-421, -62	110	129	14.6 and 1.7
Ozaki *et al.*	Myocardial Infarction	-63T/A	2 sets of controls: 1006; 872	1133	Vs 1st control 1:1.6. Vs 2nd control: 1.57

Table 1.3: Genetic studies showing positive association between *NFKBIL-1* SNPs and various diseases (see text for references).

In spite of the evidence of a possible role in diseases raised by these association studies, the exact function of NFKBIL-1 has not been reported before. The association of SNPs in *NFKBIL-1* with several autoimmune/inflammatory diseases both individually and by virtue of its location in the class III region of the MHC, and its sequence homology with members of IκB family of proteins, suggests a possible immunomodulatory role for NFKBIL-1 (Figure 1.6).

Sequence analysis of NFKBIL-1 has revealed the existence of two start codons. The upstream start codon at nucleotide 69 had a weak Kozak consensus sequence. However another start codon at nucleotide 139 had an adequate Kozak sequence suggesting that the second start codon might support stronger translational initiation. Indeed in 2000, Semple *et al.* first showed NFKBIL-1 is expressed as two isoforms denoted by full-length (α) and alternative splice variant (β), with the splice variant isoform being 15 amino acids shorter than the α isoform (Semple et al., 2002). The shorter isoform of NFKBIL-1 lacks 15 amino acids including the PEST sequence, suggesting that it might be more stable, whereas the full length variant possessing the PEST sequence might be degraded more rapidly as in the case of IκB α (Lin et al., 1996). They also showed that NFKBIL-1 was expressed constitutively in the nucleus in a number of different cell types (and all cells tested) within specific structures called nuclear speckles. Interestingly, it was observed that the ankyrin repeats were essential to direct the NFKBIL-1 expression to the nuclear speckles. Furthermore they reported that NFKBIL-1 co-localised with Sm protein, the classical marker of nuclear speckles (Semple et al., 2002). More recently, Boodhoo *et al.* identified that the E-box transcription factor, E47 and the ubiquitously expressed transcription factor USF-1 bind to the E-box element of the 7.1 ancestral haplotype (Boodhoo et al., 2004). They found that this binding was specifically seen in the Jurkat cells and not in other cell lines tested suggesting, that NFKBIL-1 affects autoimmunity through an effect on T-cell selection. Allcock *et al.* observed expression of NFKBIL-1 in several different murine tissues with decreased levels of NFKBIL-1 detected in brain and lung (Allcock et al., 2006). Thus, while all these studies commented on some aspects of NFKBIL-1 protein function, none of them reported on the central issue of whether NFKBIL-1 was an inhibitor of NFκB and/or other transcription factors.

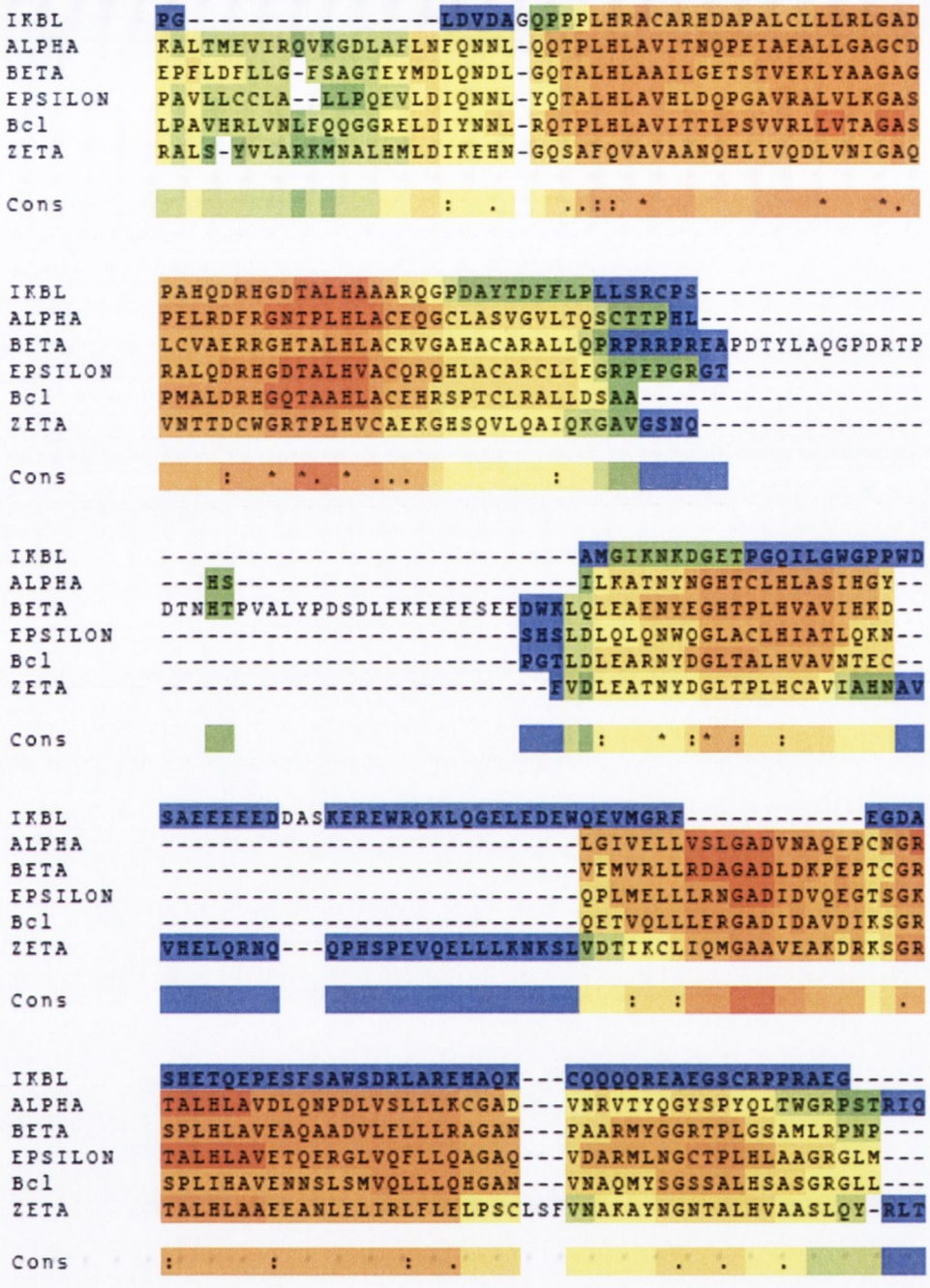


Figure 1.6: Multiple amino acid sequence alignment of the known inhibitors of NF kappa B with NFKBIL-1. The shaded area in red shows regions of high homology that corresponds to the ankyrin repeats, while blue indicates little or no homology Only partial data is shown here. Inhibitors are members of the IκB family and include IκB α, IκB β, IκB ε, IκB ζ and Bcl3. Cons denotes the degree of conservation seen, more number of asterixs implies greater degree of conservation.

Biological activities specifically related to DNA and RNA processing take place in the nucleus. These include the replication of the DNA, transcription and regulation of gene expression, RNA processing to generate mature RNA species. In a cell under going active transcription, the distribution of essential proteins such as splicing factors needs to be organised in special compartments. Nuclear speckles, Cajal bodies, PML bodies and para-speckles are some of the sub nuclear compartments seen within the nucleus (Lamond and Sleeman, 2003). Speckles are irregularly shaped structures that contain high concentrations of splicing factors and other proteins and appear as clusters of interchromatin granules (Hall et al., 2006; Lamond and Sleeman, 2003; Sleeman and Lamond, 1999; Zimber et al., 2004). Apart from NFKBIL-1, the proteins reported to be distributed in speckles include among others, Nuclear eukaryotic initiation factor 4E (eIF4E), CREB-binding protein (CBP)/p300, RNA polymerase II, Cdk9 and cyclin T subunits of TAK/P-TEFb, Phosphatidylinositol 3-kinase c 2 alpha, Profilin I, Diacylglycerol kinase-theta, small heat shock protein alpha B-crystallin, histone deacetylase 3, Human transcription elongation factor CA150, XE7 and the DNA repair protein, hOGG1 (Table 1.4) (Bryantsev et al., 2007; Campalans et al., 2007; Didichenko and Thelen, 2001; Djabali and Christiano, 2004; Dostie et al., 2000; Hall et al., 2006; Herrmann and Mancini, 2001; Mangs et al., 2006; Neugebauer and Roth, 1997; Sanchez-Alvarez et al., 2006; Skare et al., 2003; Tabellini et al., 2003; van Rijk et al., 2003; von Mikecz et al., 2000).

Splicing factors	**Other mRNA processing**	**Transcription**
snRNPs (U1, U2, U4/U6, U5), SR proteins (SC35, ASF/SF2, 9G8, SRp20, SRp30c, SRp40, SRp55, Srp75, p54),SRm, 160, SRm 300, U2AF, Pinin, RNPS1, PAP-1	Poly (A) polymerase, Poly (A) binding protein II, Eukaryotic initiation factor 4E (eIF4E)	Hyperphosphorylated RNA pol II, WT1, FBI-1, HMG-17 Fibroblast growth factor receptor 1, RNA helicase, p68, PRCC
Structural proteins	**Post translation modifications**	**RNA transport**
Lamin A, B-crystallin and HSP27, Profilin	Protein phosphatase 1, PSKH1, PRP4, CLK/STY, HDAC3	Aly/REF MLN51 Magoh Y14

Table 1.4: Proteins localized to splicing factor sub-nuclear organelle. Nuclear speckles are sub-nuclear bodies with a characteristic distribution pattern in which a number of proteins accumulate. Some of the proteins belonging to different functional classes distributed in the speckles are described in this table.

Speckles are dynamic structures and the size of the granules changes based on the transcription status of the cell. In resting cells, the size of the speckles is greatly enlarged due to concentration of various proteins within the speckles. Apart from their role in transcription, proteins found in the speckles have been reported to contain factors that enable the transport of mRNA on its journey outside the nucleus (Politz et al., 2006). The distribution of NFKBIL-1 in nuclear speckles and its co-localisation with the Sm protein allows us to speculate a possible role for this protein as a regulator of transcription process.

Investigations in our laboratory revealed that SNP in a potential immune/inflammation regulatory gene NFKBIL-1 was associated with increased susceptibility to Coeliac Disease in Irish population. Based on the available evidence it appeared that NFKBIL-1 might be involved in the regulation of NFκB. We decided to investigate the potential nature of this regulation and to identify the various other biological functions requiring NFKBIL-1 participation. Thus the aim of this project was to characterize the function of NFKBIL-1 and the identification of its involvement in the regulation of transcription factors.

Chapter 2
Material and Methods

2. Materials and methods

2.1 Reagents

General reagents: PMA, EDTA, EGTA, sodium chloride, sodium hydroxide, magnesium chloride, calcium chloride, potassium chloride, Tris(hydroxylmethyl)aminomethane (Tris), glycine, dimethyl sulfoxide, sodium dodecyl sulphate, iminodiacetic acid-Sepharose 6B, IPTG were obtained from Sigma Aldrich (Poole, Dorset, U.K. and St. Louis, M.O., U.S.A). Ethanol, methanol, glacial acetic acid, hydrochloric acid, acetone and glycerol were obtained from BDH (VWR International Ltd., Poole, Dorset, U.K.).

Reagents for Cell culture: HCT-116 cell line was purchased from LGC (LGC, Middlesex, UK), HEK 293 cell line was kind gift from Dr. Matthew Lawless, Trinity College Dublin, Professor Luke O'Neill, Department of Biochemistry, Trinity College Dublin gifted HEK 293 with stable overexpression of TLR4 and HEK 293 with stable expression of TLR2, PAM_2CSK_4 and Blasticidin was purchased from Invivogen (San Diego, California 92121, USA). Cell culture media, Foetal Bovine Serum, Trypsin, EDTA, HBSS and HEPES buffer was purchased from Invitrogen (Invitrogen Corp, Carlsbad, CA, USA). Culture flasks, culture plates and Lab Tek II chamber slides were purchased from Nunc (Nalge Nunc International, Rochester, NY, USA). Gene Juice transfection reagent was purchased from Merck Biosciences (Merck Chemicals Limited, Nottingham, UK), Trypan blue and Geneticin (G418) was purchased from Sigma-Aldrich. Recombinant human IL-1ß and TNF-α proteins were purchased from R&D systems and recombinant human IL-6 and IFN-γ proteins were kind gifts from Professor Kingston Mills, Trinity College Dublin. NF kappa B reporter plasmid and

IL-8 reporter plasmids were kind gifts from Dr. Matthew Lawless, Trinity College Dublin. Activator Protein-1 luciferase vector was bought from Stratagene (Stratagene, La Jolla, CA, USA); Lipopolysaccharide was purchased from Alexis Corporation, Switzerland.

Reagents for RNA manipulations: Trireagent was purchased from Sigma-Aldrich, DTT, Random Primers, Moloney Murine Leukemia Virus Reverse Transcriptase, First Strand Buffer from Invitrogen (Invitrogen Corp, Carlsbad, CA, USA), the Deoxynucleotide Triphosphate mix and RNasin from Promega (Promega Corp, Madison MI, USA).

Reagents for DNA manipulations: Electrophoresis grade agarose, TAE buffer was purchased from Sigma-Aldrich, DNA ladders, loading dye and restriction enzymes, DNA ligase and the required restriction digestion reaction buffers were purchased from New England Biolabs, UK; primers for PCR was ordered from Invitrogen and reagents for Real time PCR were purchased from Applied Biosystems, UK. Hot start Taq polymerase was purchased from Qiagen (Qiagen, Hilden, Germany).

Reagents for Protein manipulations: General Reagents: Paraformaldehyde, Triton-X, Bovine serum albumin (BSA), acrylamide:bisacrylamide (29:1), ammonium persulphate, Nonidet- P40, leupeptin, PMSF, ß-mercaptoethanol, Sepharose A beads, RIPA buffer, TEMED and Tween-20 were purchased from Sigma-Aldrich; Bio-Rad Protein Assay Concentrate was purchased from Bio-Rad (Alpha Technologies, Ireland)

Reagents for *E.coli* manipulations: Luria-Bertani Medium, SOC media, Agar Select, ampicillin, kanamycin, and chloramphenicol were purchased from Sigma-Aldrich.

2.2 Cell culture

HEK 293 and HEK-TLR4 cells were grown in MEM alpha medium supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamate and 10% (v/v) fetal calf serum. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. HEK-TLR2 cells were grown in DMEM medium supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamate and 10% (v/v) fetal calf serum. HEK 293-TLR4 and TLR2 cells were maintained by adding G418 (500µg/ml) and Blasticidin (10µg/ml) respectively in a T-75 flasks. HCT-116 cells were cultured in McCoy's modified media supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamate and 10% (v/v) fetal bovine serum. (Table 2.1)

CELL LINE	MEDIUM
HEK 293	Minimum Essential Medium with L-Glutamine and Non Essential amino acids.
HEK 293 + TLR4 stable cell line	Minimum Essential Medium with L-Glutamine and Non Essential amino acids. To maintain stable transformants medium was supplemented with G418 (500µg/ml).
HEK 293 + TLR2 stable cell line	Minimum Essential Medium with L-Glutamine and Non Essential amino acids. To maintain stable transformants medium was supplemented with Blasticidin (10µg/ml).
HCT-116	McCoy's 5A Medium with L-Glutamine.

Table 2.1: Cell lines and the medium used for propagation.

2.2.1 Passaging of cells

The cells were passaged when they reached confluency of ~90% in the T 25 flasks. Briefly media was removed and the cells were washed with appropriate amount of HBSS supplemented with 1M HEPES solution for 5 minutes. The buffer was removed and 1ml of 1X trypsin EDTA solution was added to the flask. The cells were incubated at 37°C for 1-3 minutes until all the cells were detached from the base of the flask. 9ml of the complete media was added and the cells collected by centrifugation at 1200rpm for 5 minutes at 4°C. 50µl of the resuspended cells were added to 50µl of Trypan Blue solution and the cells were counted using Neubarrs chamber slide. The number of cells present was calculated using the formula below and an appropriate number of cells were then seeded into new flasks.

Average number of cells counted X dilution factor X 10000 =

Total number of cells / ml

2.2.2 Frozen stock of cells

When cells reached a confluency of ~90%, they were collected by trypsinisation as described above and counting was performed. Frozen stocks of approximately 1 million cells/ vial were made by using a 10% DMSO solution in FBS media. The cells were immediately stored in liquid nitrogen or at –80°C.

2.2.3 Thawing

Frozen vials of the cell line were thawed quickly in a 37°C water bath and the cells washed in 10 mls of complete medium. Cells were centrifuged by spinning at 1200 rpm (Eppendorf Centrifuge 5810 R) for 5 minutes at 4°C. 5ml of media with 20% (v/v) FBS was added to the pellet and the cells were resuspended gently and plated in T 25 tissue culture flasks.

2.2.4 Transfection of cells

The required numbers of cells were plated in 6 well plates. 24 hours post seeding the cells were transfected with plasmids. Briefly 2µg of plasmid DNA was added to a premix of serum free medium and 2.5µl of Gene Juice transfection reagent. The reagents were incubated at room temperature for 20 minutes and then added to a single well of the 6 well tissue culture plate in 2mls of serum free media. Cells were incubated for 4 hours, in a 37°C incubator in a humidified atmosphere of 5% CO_2. After 4 hours, complete media with 10% serum was added and the cells were placed in a 37°C incubator and allowed to recover for 24 hours. Transfection of plasmids was confirmed by observing the cells for the amount of green fluorescence emitted by the GFP under a microscope.

2.3 RNA manipulations

2.3.1 Total RNA extraction and Reverse Transcription

Cells were transfected with appropriate plasmids as described above. The cells were treated for various time points, washed in 1 X PBS buffer, collected in 1ml of Trireagent and stored at –80°C. RNA extraction was performed using a column-based method in accordance with the manufacture's instructions (Macherey Nagel GmbH, KG, Germany). In order to avoid amplification of contaminating genomic DNA, all samples were treated with RNase-free DNase for 15 minutes. The quantity and purity of extracted RNA was measured with a spectrophotometer (Eppendorf BioPhotometer, Eppendorf AG, Hamburg, Germany). The quality of the extracted mRNA was verified on an Agilent 2100 Bioanalyzer using the RNA Nano LabChip kit (Agilent trechnologies, Palto Alto,CA, USA).

Total RNA was then reverse transcribed as follows: 11.15µl of water containing 500ng of total RNA was first incubated at 65°C for 10 minutes. 18.85µl of the reverse transcription mix containing the following components were added: (1) 3µl 0.1M DTT; (2) 4.5µl Dimethyl Sulfoxide: (3) 2µl 100µM Random Primers; (4) 1.25µl Moloney Murine Leukemia Virus Reverse Transcriptase; (5) 6µl 5X First Strand Buffer; (6) 1.5µl 4mM deoxynucleotide triphosphate mix; (7) 0.6µl RNasin 10U/µl. The samples (final volume 30µl) was then incubated at 37°C for 1 hour.

2.3.2 Primers and probes

All primer and probes used in this study were synthesized at Applied Biosystems (Foster City, CA, USA). Primers and probes were obtained as a pre-customised mix (Assay ID for NFKBIL-1 is Hs 00428211_ml, TNF-α is Hs 00174128_m1, for IL-10 Hs 00174086_ml and for GAPDH Hs 99999905_ml). β-Actin primers and probes were kind gifts from Dr. Michael O 'Dwyer, Trinity College Dublin.

2.3.3 Preparation of NFKBIL-1 standards

Preparation of the standards was the first step for absolute quantification of mRNA. To do this first the mass of a single plasmid molecule was calculated using the following formula:

$$m = (n)(1.096 \times 10^{-21}\ g/bp);$$

where m=mass and n= plasmid size (bp), where the size of the entire plasmid (vector + insert) is used in this calculation.

For NFKBIL-1-GFP the size of the vector is 4700 bp and the insert size is 1155 bp.

Hence the total size of the recombinant vector is 5855 bp

$$m = 5855\ bp(1.096 \times 10^{-21})\ g/bp$$

$$= 6.41 \times 10^{-18}\ g = \text{mass of 1 plasmid molecule}$$

The mass of plasmid containing the copy number (CN) of interest is then calculated, in this example 10^{-11} copies:

$$10^{-11}\ \text{copies} \times 6.41 \times 10^{-18}\ g/\ \text{copy}$$

$$6.41 \times 10^{-7}\ g$$

Then concentrations of plasmid DNA needed to achieve the CNs of interest are calculated. The mass of DNA needed is next divided by the volume to be pipetted into each reaction. For this example 5µl of plasmid DNA solution is pipetted into each PCR reaction.

For 10^{-11} copies: 6.41×10^{-7} g / 5µl = 1.283×10^{-7} g/µl.

A serial dilution of the plasmid DNA is then prepared. We used the following formula to calculate the volume needed to prepare the 10^{-11} copy standard dilution. For this example the stock solution of plasmid DNA was taken as having a concentration 1.5µg/µl as determined by spectrophotometric analysis.

$C_1V_1 = C_2V_2$

$(1.5\mu g/\mu l)(V_1) = (1.283 \times 10^{-7}\ g/\mu l)(100\mu l)$; $V_1 = 8.35\mu l$

For 10^{11} CN NFKBIL-1-GFP / µl we added 7.73 µl to 91.65µl of dilutent and from this point prepare a serial dilution of the plasmid DNA.

2.3.4 Real Time PCR

2.3.4.1 Absolute QRTPCR

β-Actin plasmid and NFKBIL-1-GFP plasmid standards were created by serial dilutions. The plasmid number in these standards varied from 10^7 copies to 10^2 copies of plasmid/µl. The PCR reactions were carried out in an ABI Prism 7000 (Applied Biosystems, Foster City, CA, U.S.A.). All reactions were performed either in triplicate or in duplicate. Thermo cycling was carried out in a 20µl final volume containing: (1) water up to 20µl; (2) 10µl Master mix (Applied Biosystems); (3) 2 µl of 6 pmol/µl forward and reverse primers (final concentration 600nM); (4) 1µl of 4

pmol/μl Taqman Probe (final concentration 200nM) or 1μl of pre-customised primer/probe mix with default primer and probe concentrations; (5) 0.8μl standard dilution or 2.4μl cDNA. After an initial denaturation step at 95°C for 10 minutes, temperature cycling was initiated. Each cycle consisted of 95°C for 15 seconds and 60°C for 60 seconds, the fluorescence being read at the end of this second step. In total, 40 cycles were performed. The standard curve was generated using the number of plasmid copy numbers and the corresponding amount of the amplicon. Linear regression analysis was performed to quantify the amount of mRNA copy numbers in the target sample.

2.3.4.2 Relative QRTPCR

The PCR reactions were carried out in an ABI Prism 7000 (Applied Biosystems). All reactions were performed either in triplicate or in duplicate. Thermo cycling was carried out in a 20μl final volume containing: (1) water up to 20μl; (2) 10μl Master mix (Applied Biosystems); (3) 1μl of pre-customised primer/probe mix with default primer and probe concentrations; (4) 2.4μl cDNA. After an initial denaturation step at 95°C for 10 minutes, temperature cycling was initiated. Each cycle consisted of 95°C for 15 seconds and 60°C for 60 seconds, the fluorescence being read at the end of this second step. In total, 40 cycles were performed. The final fold change in the expression of the target mRNA was measured by normalising with the amount of house keeping gene GAPDH.

2.4 DNA manipulations

2.4.1 PCR amplification

Polymerase Chain Reactions were performed in a Perkin-Elmer 2400 thermal cycler (Perkin-Elmer, U.K.) using Hot Start Taq DNA Polymerase. Custom made primers were bought from Invitrogen. For standard amplifications, typically, in each reaction approximately 100 ng of template DNA was mixed with 5 pmol of each specific primer in a reaction volume of 25 µl. Initial denaturation was carried out by incubation the samples at 94˚C for 15 minutes. Subsequent reaction was performed by denaturing DNA at 94˚C for 1 minute, annealing at 58˚C for 1 minute and allowing the extension step at 72˚C for further 1 minute. A total of 30 cycles were performed. The reaction was allowed to complete by incubating samples at 72˚C for 10 minutes. The samples were then analyzed using an agarose gel system.

2.4.2 Agarose gels

To separate different DNA fragments based on their weight agarose gel electrophoresis was performed. 1% agarose (w/v) was dissolved in 1x TAE buffer and melted. The solution was mixed and allowed to cool down. 0.5µg/ml Ethidium Bromide was added to the solution and poured on a gel rig (Thermo EC) with combs to create the wells. Loading dye was added to the DNA samples and the samples were loaded into the wells. Electrical circuit was established and the gel was run at 10 volts/cm. The gel was photographed using a Kodak Image Station.

2.4.3 Plasmid preparation

Plasmids were purified using the QIAprep miniprep kit (Qiagen, Germany) or for larger volumes, using the Sigma Endotoxin free plasmid purification kit following the directions specified by the manufacturer.

2.4.4 Restriction Digestion

Digests were routinely carried out at 37°C overnight, after which the enzyme was inactivated by heat inactivation where possible or otherwise the enzyme was removed during DNA extraction. Where required, the reactions were supplemented with 100μg/ml BSA.

2.4.5 Ethanol precipitation of DNA

Ethanol precipitation of digested DNA facilitated purification and recovery of the DNA in a concentrated form. A 1/5 volume of 3 M Sodium Acetate was added to the reaction mixture followed by the addition of 2 volumes of cold 96% ethanol. The mixture was then normally kept at -20 °C for approximately 1 hour and afterwards centrifuged at 12,000 rpm at 4°C for 15 minutes. The supernatant was drained off and the resultant pellet washed with 2 volumes of cold 80% ethanol. It was allowed to stand for 5-10 minutes and then centrifuged as before. The supernatant was drained off and the pellet was allowed to dry and then resuspended in ddH_2O or Tris buffer.

2.4.6 Gel extraction of DNA fragments

DNA fragments were extracted from agarose gels using QIA quick Gel Extraction Kit following manufacturers' instructions (Qiagen, Hilden, Germany).

2.4.7 Ligation

Gel purified vector and PCR amplified insert were mixed together in 1:3 or 3:1 ratio (vector: insert). Appropriate amount of DNA ligase and DNA ligase buffer was added to the reaction mix and the samples were incubated for 12 hours at 16°C. Single and double digested vector were used as positive and negative controls respectively for the ligation reactions.

2.5 Protein manipulations

2.5.1 Extraction of proteins

The cells were plated for 24 hours and transfected with the required vectors. After 24 hours, the transfection efficiency was calculated and the culture plate was placed on ice. The supernatant was removed and the cells were washed three times with ice-cold 1X PBS solution. 150µl of RIPA buffer was added and the culture plate was swirled to ensure that all the cells were covered with lysis buffer. The cells were scrapped and collected in a sterile microcentrifuge tube. The samples were allowed to stand for 15 minutes on ice and then pelleted by centrifuging at 12,000 rcf for 10 minutes. The supernatant was collected in a fresh microcentrifuge tube and stored at –20°C.

2.5.2 Sub-cellular fractionation of total cellular proteins and isolation of cytoplasmic and nuclear fractions

Confluent cells were washed with ice-cold 1 X PBS solution and resuspended in 1 ml of hypotonic buffer (10mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl,), 5 mM PMSF, and 0.5 mM DTT) (Sigma). The cells were then pelleted by centrifugation at 13,000 rcf for 10 minutes at 4°C and then lysed for 10 minutes on ice in 20µl hypotonic buffer containing 0.1% NP-40 (Sigma). Lysates were centrifuged as before, the supernatants taken at this point contained the cytoplasm extracts. The nuclear pellet was then lysed in 15µl of lysis buffer (20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 25% (v/v) glycerol, 0.5 mM PMSF) (Sigma) for 15 minutes on ice. After centrifugation at 13,000 rcf for 15 minutes at 4°C, nuclear extracts were removed into 50µl storage buffer (10 mM HEPES (pH 7.9), 50mM KCl, 0.2 mM EDTA, 20 % (v/v) glycerol, 0.5 mM PMSF, and 0.5 mM DTT). Protein concentrations were determined by Bradford method and the extracts were stored at –80°C.

2.5.3 Protein Quantification

The protein concentration of various cellular extracts was determined primarily by the method of Bradford (Bradford, 1976) using Bio-Rad Protein Assay Concentrate. Assay concentrate was freshly diluted 1:5 with distilled water and mixed well prior to each assay. A BSA standard was prepared in the appropriate buffer to a final concentration of 20 mg/ml BSA; this was serially diluted to yield standards at 20, 10, 5, 2.5, 1.25, 0.312, 0.0625 and 0 µg/ml protein. The absorbance of protein solutions was read at 595 nm in duplicate in a Tecan Spectra Fluor Plus spectrophotometer. In

some instances; a protein standard of known concentration was included with unknown samples. A commercial BSA solution at a concentration of 2 mg/ml (BSA Protein Standard, Sigma, Poole, U.K.) was used to monitor accuracy and reproducibility of the Bradford method. Linear regression was applied to quantify the amount of the target protein.

2.5.4 Luciferase Assays

Luciferase reporter gene assays were used to measure the activity of genetic regulatory sequences. The Luciferase Assay System from Promega was used to quantify the luciferase expression. Cells were plated in 6 well plates and 24 hours later co-transfected with target plasmids and the luciferase reporter vector. Plasmid coding for ß-galactosidase was included in each experiment. 24 hours later the cells were placed on ice and washed with sterile 1X PBS solution and 250µl of Promega Cell Lysis Buffer was added. The plate was swirled to ensure that the cells were suspended in the solution and allowed to stay on ice for 5 minutes. The cells were then scraped and collected in microcentrifuge tubes, spun at 15000 rpm for 10 minutes and the supernatant was transferred to fresh microcentrifuge tubes. Following the manufactures instructions, the Luciferase activity was quantified by luminometry using a Wallac plate reader. In order to normalise for the variations in transfections ß-galactosidase assay was performed and the total protein in each sample was also quantified by Bradford method and the final value expressed as relative luciferase activity.

2.5.5 ß- Galactosidase Assay

All transfections included a plasmid encoding for ß-galactosidase, as an internal control to normalise for the variation is the efficiency of transfection. The ß-galactosidase plasmid was kind gift from Dr. Sinead Smith, Trinity College Dublin. ONPG (o-nitrophenyl-ß-D-galactopyranoside) was used as the substrate for this enzyme assay and when it is hydrolyzed to its free form the colour of the solution changes to yellow. For each 30µl of the lysate to be assayed, the following were added to the reaction mix:

100x Mg^{2+} solution	3µl
1x ONPG	66µl
Cell extract	30µl
0.1 M Sodium Phosphate (pH 7.5)	201µl

The sample was then incubated 37°C until the solution changed from colourless to yellow colour. Finally 500µl of Sodium Carbonate was added to stop the reaction and the sample was assayed by reading the optical density at 420 nm in a spectrophotometer.

2.5.6 Enzyme linked immunosorbent assay (ELISA)

ELISA is a powerful technique to quantify the amount of protein present in biological samples such as serum, plasma, cell medium or cellular lysates. An IL-8 ELISA kit (Catalogue Number: DY208; R&D systems, Minneapolis, USA) was used according

to the manufacturers instructions. Recombinant human IL-8 included in the kit was used to generate standard curve and by applying linear regression, the amount of protein in the target samples was estimated.

2.5.7 Immunoprecipitation assay

Immunoprecipitation allows identification of interacting proteins. Total cellular lysates from cells was collected in RIPA buffer and the amount of protein quantified as described in section 2.8.3. Equal amounts of protein were transferred to a new Eppendorf and 20µl of Protein A agarose was added to pre-clear the lysates. After 30 minutes, the samples were centrifuged at 2000 rpm for 2 minutes and the supernatant was transferred to a new Eppendorf. Anti-GFP antibody (1:100) was added and the solution was allowed to equilibrate for 1 hour on ice. 50µl of Protein A agarose beads was then added and the samples were incubated overnight in a cold room (4°C) on a slowly rotating rocker. The samples were then washed by adding 500µl of the RIPA buffer and centrifuged at 7000 rpm for 2 minutes. The wash procedure was repeated three times. After the final centrifugation, the supernatant was discarded and 5X sample buffer was added to the beads. The solution was boiled for 10 minutes and during this period it was intermittently vortexed and centrifuged to dissociate all the complexes. The sample was then loaded on to a Western blot gel and probed with appropriate antibodies.

2.5.8 Protein Electrophoresis Analysis and Immunoblotting

2.5.8.1 Sample and Molecular Weight Standard Preparation

Following protein estimation, samples were generally resuspended in 5 to 20µl 1X reducing or non-reducing sample buffer (diluted from 5X stock) as required. Protein samples and appropriate molecular weight standards were boiled at 100°C for 5 minutes and centrifuged briefly (30 seconds) to remove any insoluble solids.

2.5.8.2 SDS-PAGE

An ATTO system was used for all SDS/PAGE gels (ATTO Corporation, Japan) and protein samples were electrophoresed using a Consort electrophoresis power supply unit. Proteins were separated on reducing gels prepared using a discontinuous buffer system as described by Laemmli (Laemmli, 1970) and adapted by Sambrook *et al.* (Joseph Sambrook, 2001). Resolving and stacking acrylamide gels were prepared to the required percentage acrylamide in the order indicated in Tables 2.2 and 2.3. APS and TEMED were added last with gentle swirling of the mixture. Electrophoresis was carried out at 25 mAmps per gel for approximately 1.5 hour until the dye front had reached just above the gel base, at which stage electrophoresis was discontinued. When required the gel was stained with Coomassie Blue R-250 followed by destaining in 50% methanol.

Component	**10%**	**12.5%**	**15%**	**20%**
Acryl/Bis	6.66 ml	8.32 ml	10 ml	13.32 ml
Tris 1.5M	5.0 ml	5.0 ml	5.0 ml	5.0 ml
H_2O	8.32 ml	6.56 ml	4.89 ml	2.78 ml
APS 10%	100 µl	100 µl	100 µl	100 µl
TEMED	10 µl	10 µl	10 µl	10 µl

Table2.2: Composition of resolving gels for SDS/PAGE

Component	*Amount*
Acryl/Bis 30%	1.33 ml
Tris 1.0M	3.05 ml
H_2O	5.55 ml
APS 10%	50 µl
TEMED	10 µl

Table 2.3: Composition of stacking gel for SDS/PAGE

2.5.8.3 Western Immunoblotting

Western immunoblotting was carried out using the semi-dry method for the transfer of electrophoresed proteins to immobilising membranes as described by Towbin (Towbin et al., 1979) and was performed using an ATTO semi-dry transfer system (ATTO Medical Supplies, Japan). During SDS-PAGE, polyvinylidene fluoride (PVDF) transfer membrane (0.45µm, Pall Life Sciences) of dimensions 6.5 x 9 cm was briefly saturated with methanol for 10 –15 seconds to activate the membrane and then equilibrated in transfer buffer for approximately 20 –30 minutes prior to semi-dry transfer. Whatmann 3mm filter paper cut to 6.5 x 9 cm was also saturated in transfer buffer prior to semi-dry blot sandwich construction, which was assembled in the order of cathode, filter paper, PVDF, acrylamide gel, filter paper and finally anode. Electrophoretic transfer was performed at 100 mA per gel for 60 minutes. Following semi-dry transfer; PVDF membrane was removed and processed for immunoblotting. The lane containing the molecular weight marker was stained with Coomassie Blue R-250 followed by destaining in 50% methanol.

2.5.8.4 Immunoblot Detection and Development

Non-specific sites on the membrane following semi-dry transfer were blocked by incubation with freshly prepared PBS buffer containing 5% non-fat skimmed milk (Blocking solution) for 1 hour with gentle agitation on an orbital shaker (Stuart Scientific, U.K.) at room temperature. Blots were then washed twice with PBS and incubated with appropriate primary antibodies diluted (range 1:200 to 1:2000) in blocking solution for 1 hour with shaking in a sealed plastic bag. Following

incubation with primary antibody, blots were washed several times over 30 minutes with 0.1% PBS-Tween Washing Solution. Blots were then incubated with the relevant horseradish peroxidase-conjugated secondary antibody in Primary and Secondary Antibody Diluent Solution for 1 hour with shaking in a sealed plastic bag. Following incubation with secondary antibody, blots were finally washed 4 to 5 times with 0.1% PBS-Tween Washing Solution over 30 minute. Prior to development, blots were placed in PBS. Blots were stored in PBS at 4°C if development could not be undertaken immediately.

Detection of immunblots was performed using the enhanced chemiluminescence (ECL) method (GE Healthcare UK Limited Amersham Place, Little Chalfont, Buckinghamshire, UK). The membrane was removed after 5 minutes and placed between acetate sheets that were then exposed to Kodak X-OMAT S film for the appropriate time period (range 30 seconds to 30 minutes). Exposed films were developed using an automatic developer (CURIX 60, AGFA, Type 9462/100/140, Agfa-Gevaert AG, Munich, Germany).

2.5.8.5 Antibodies

Target	Species	Dilution	Company
Anti- p65	Rabbit	1:1000 for WB, and 1:100 for IHC.	Santa Cruz Biotechnologies Inc. Germany.
Anti- p50	Rabbit	1:1000 for WB, and 1:100 for IHC.	Santa Cruz Biotechnologies Inc. Germany.
Anti-HDAC 3	Rabbit	1:1000 for WB, and 1:50 for IHC.	Santa Cruz Biotechnologies Inc. Germany.
Anti-GFP	Mouse	1:1000 for WB	Roche
Anti-GR	Mouse	1:1000 for WB, and 1:100 for IHC.	Santa Cruz Biotechnologies Inc. Germany.
Penta-His Antibody	Mouse	1:1000 for WB	Qiagen

Table 2.4: Antibodies used for Western blotting (WB) or Immunocytochemistry (IHC).

2.5.9 Immunostaining

The cells were plated on the 8 well chamber slide (Lab Tek II, Nunc). Transfection of the plasmids was carried out using the protocol described above. If required by the experimental conditions, the cells were stimulated for various time points. The media was removed and the cells washed with 1X PBS three times. Cells were fixed in 4% paraformaldehyde (Sigma) and permeabilised using 0.3% Triton-X (Sigma) or fixed in pre-chilled Methanol (-20˚C) for 10 minutes followed by pre-chilled Acetone (-20˚C) for 2 minutes. The primary antibody incubation was done overnight at 4˚C. The cells were incubated with fluorescently labelled secondary antibody for 1 hour at room temperature. After washing in 1X PBS, Hoecsht staining was performed for 1 minute. The slides were observed under a fluorescent microscope. Adobe Photoshop software was used for capturing the images.

2.6 Cytokine profile of cells using the Randox Platform

Cytokine measurements were performed using a biochip array analyzer, The Evidence Investigator system (Randox Laboratories Ltd, Crumlin, UK). Evidence assay reagents, controls, calibrators, and Evidence analyzer, were from Randox Laboratories. (Randox Laboratories Ltd, Crumlin, UK). Samples were run in duplicate according to the manufacturer's instructions. The cytokine biochips were precoated with a monoclonal antibody to 12 human cytokines that include IL-1A, IL-1B, VEGF, TNFA, MCP-1 EGF, IL-8 and IL-6. After addition of the plasma sample or standard controls, a polyclonal biotin conjugated antibody was added and an ELISA procedure followed according to manufactures instructions. Light emission

from chemiluminescent reactions generated were detected and quantified at each spot on the array, captured by a charge-coupled device camera as part of an imaging station and converted by image-processing software to provide results compared with calibration curves for each location on the biochip.

2.7 *Escherichia coli* (*E. coli*) manipulations

2.7.1 Preparation of competent *E. coli* cells for transformation

An overnight culture of the appropriate *E. coli* strain (DH5α/ BL21) was grown in LB liquid media at 37°C. The overnight culture (1 ml) was used to inoculate 100 ml of fresh LB. The culture was shaken at 200 rpm at 37°C until cells reached a mid-log phase. The samples were checked for growth by using a spectrophotometer (OD read at 600 nm should be approximately 0.4-0.5). The cells were placed on ice for 1 hour, decanted into 2 x 50 ml falcon tubes and centrifuged at 2000 rpm for 15 minutes at 4°C. Each pellet was resuspended in 7.5 ml of 100 mM $MgCl_2$ (sterile & ice cold) and centrifuged at 2000 rpm for 5 minutes at 4°C. Each pellet was then resuspended in 25 ml of 100 mM $CaCl_2$ (sterile & ice cold) and centrifuged at 2000 rpm for 5 minutes at 4°C. Finally the pellet was resuspended in 5.25 ml of 100 mM $CaCl_2$ and gradually 750 µl of sterile ice-cold 80% glycerol was added in a swirling motion (to give a final concentration of 10%). The cells were aliquoted (0.5 ml), snap frozen using liquid nitrogen and stored at -80°C.

2.7.2 Transformation of competent *E. coli* cells

Competent cells were thawed on ice and 10-20 ng of plasmid DNA was added. The cells were then incubated on ice for approximately 30 minutes, heat shocked at 42°C for 45 seconds and cooled on ice for 1 minute. SOC medium (1 ml) was then added to the cells and they were incubated at 37°C for 1 hour (120-150 rpm). The culture was then removed and about 50µl of it added to the LB agar plate.

2.7.3 LB Agar plates and LB broth preparation

LB broth and LB agar plates were made freshly as required a day before the experiment was set up. 50-100µl of the SOC media with transformed *E.coli* was added to the LB agar plates supplemented with the appropriate antibiotic and the media was evenly distributed over the plate surface using a sterile spreader. The plates were then incubated for 12-16 hours (usually overnight) at 37°C.

2.7.4 Screening of the colonies

Selected colonies from the target LB agar plates were inoculated into 5ml of LB broth supplemented with appropriate antibiotics and incubated overnight by shaking in a 37°C incubator at 200rpm. Cells were collected by centrifugation at ~4000rpm for 10 minutes. The Mini prep Kit from Qiagen was used to isolate and purify the plasmid DNA which was subsequently subjected to restriction digestion using appropriate enzymes. Samples were run on a 1% agarose gel in TAE buffer and observed under UV light and the image captured using the Kodak Image system.

2.7.5 Sequencing

The DNA sequencing described in this project was carried out at the Cambridge Sequencing facility, Department of Biochemistry, University of Cambridge. Sequence data was analyzed using the software 'Editview' and compared with the original sequence using the NCBI Blast program.

2.7.6 Storage of *E.coli* Bacterial Strains

Transformed *E.coli* cells grown in LB liquid broth media for 8-12 hours at 37°C augmented with the appropriate antibiotic for positive selection. 600 µl of the liquid culture was added to 400µl of sterile glycerol solution in a cryovial. The samples were vortexed very briefly and then stored at –80°C.

2.8 Cloning and expression of recombinant protein

2.8.1 Bacterial strains and plasmids

E.coli DH5α (Invitrogen) was used for routine cloning procedures. This has the genotype: F-, endA1, gyrA96, thi-1, hsdR17(rK-,mK+), supE44, relA1, 80D lacZD M15, D (lacZYA-argF),U169. *E coli* BL21 (DE3) pLysS (Genotype: E. coli B, F^-, dcm, ompT, hsdS (rB-mB-), galλ(DE3), [pLysE Camr])was used for expression of the target protein (Novagen, Darmstadt, Germany). This strain is designed for protein expression and to this end, contains the T7 RNA polymerase gene under control of the lacUV5 promoter. The polymerase gene is integrated into the bacterial chromosome from (DE3). These cells have the pLysS plasmid added to them containing T7

lysozyme, a T7 RNA polymerase inhibitor to prevent leaky expression in uninduced cells. Protein expression is induced with IPTG.

PCR was performed with primers incorporating novel *Nde*1 and *Xho*1 restriction sites allowing for subsequent cloning steps involving these enzymes. The amplified product was cloned into the pET 16b vector (Novagen) and over expressed in *E.coli* BL21 (DE3) plysS. *E.coli* was grown at 37°C in LB medium supplemented with ampicillin (100 μg/ml) and chloroamphenicol (34 μg/ml) to select for the desired constructs.

2.8.2 Purification of recombinant proteins

Over production of cloned recombinant protein was achieved in *E. coli* BL21 (DE3) plysS. Cells harbouring pNFKBIL-1, pNFKBIL-1-NT (encoding for partial N-terminal protein without the ankyrin repeats) and pNFKBIL-1-CT (encoding for partial C-terminal protein without the ankyrin repeats) were grown to an OD600 of 0.6, in LB media containing ampicillin (100 μg/ml) and chloroamphenicol (34 μg/ml). Production of recombinant protein was initiated by addition of 1 mM isopropyl ß-D-thiogalactoside (IPTG), followed by incubation at room temperature, to minimise inclusion body i.e. intracellular deposits of insoluble protein formation. After 14 hours the cells were harvested by centrifugation at 5,000 rcf for 30 minutes at 4°C. For protein purification, the cells from a 600 ml culture were resuspended in 30 ml of binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) and sonicated on ice for 3-5 min, (Soniprep 150, Sanyo). The resulting cell lysate was centrifuged at 5,000 rcf for 1 hour at 4°C, and the supernatant filtered (0.45 μm) prior to loading onto a nickel charged iminodiacetic acid column. Any unbound material

was eluted using 10 column volumes of binding buffer and 6 column volumes of wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). The recombinant protein was then eluted using 7 column volumes of elution buffer (500 mM imidazole, 0.5 M NaCl, 20mM Tris-HCl, pH 7.9). The purified protein was dialysed against 75 mM sodium phosphate buffer (pH 7.5) containing 5 mM dithiothreitol (DTT). Protein concentrations were determined by the Bradford method (Bradford, 1976).

2.9 Statistical Analyses

Data were analyzed with the PRISM 4.0 software package (GraphPad, San Diego, CA). Data are presented mean ± SD, unless otherwise stated. Statistical comparisons were made using paired Student's t test. Significance was set at $P < 0.05$.

Chapter 3

Results

3.0 Results

The results chapter describes the findings from various experiments conducted to identify the function of NFKBIL-1. This chapter is divided into three sections: in the first section we investigate the role of NFKBIL-1 in the regulation of transcription factors; in the second section we report on the functional characterization of NFKBIL-1 gene products and in the final section we provide details on the potential interacting partners of NFKBIL-1.

Results section 1:
3.1 Regulation of transcription factors by novel protein NFKBIL-1

3.1.1 Introduction

The function of the novel protein NFKBIL-1 has not as yet been reported. However, as described in the Introduction, it shares certain similarities with known IκB inhibitors. Allied to this observation, its location in the MHC region between *TNF*-α and *BAT 1a* and the previous association of a number of SNPs in the gene with autoimmune diseases provides a clue that NFKBIL-1 might function as an immune regulator. Semple *et al.* (2002) showed that there are potentially two different transcripts of this gene and that it co-locates with splicing factors in the nucleus showing a speckled pattern. Although suggestive, this information does not provide any concrete evidence regarding the function of NFKBIL-1 and does not answer the specific question of whether it is a putative regulator of NFκB. It should be noted that speckles are probably large aggregations of proteins with an undetermined number of protein constituents. Another study reported that the E-box transcription factor, E47 and the ubiquitously expressed transcription factor USF-1 bind to the promoter of NFKBIL-1 in T cells (Boodhoo et al., 2004). Initial experiments in our laboratory

identified NFKBIL-1 as an inhibitor of NFκB. Further studies were undertaken to confirm this finding and to identify if other transcription factors were also regulated by NFKBIL-1. In addition, we undertook experiments to identify whether other transcription factors were regulated by NFKBIL-1 and also the regions of the protein essential for this activity. Furthermore, we extend our studies to characterize the function NFKBIL-1 protein and report on the potential interactions of NFKBIL-1 with proteins.

3.1.2 Results:

3.1.2.1 NFKBIL-1 inhibits NFκB activity

Preliminary studies into the function of NFKBIL-1, using Electrophoretic Mobility Shift Assay (EMSA), demonstrated that overexpression of NFKBIL-1 was capable of inhibiting the binding of DNA by NFκB (Daly, J and McManus, R; unpublished data). For these experiments the NFKBIL-1 coding sequence was cloned into a pGFPN-1 plasmid (Clontech-Takara Bio Europe, France). This vector was subsequently used for further characterization of NFKBIL-1. We confirmed our initial finding that NFKBIL-1 inhibited NFκB activity by performing Luciferase Assays using a luciferase vector driven by an NFκB responsive promoter comprising 5 κB consensus sites in tandem directing expression of the luciferase protein encoding sequence. Therefore any increase in NFκB activity in the cell leads to increased binding of NFκB to the promoter and consequently increases the expression of luciferase protein. The increase in luciferase expression can be quantified by using a Luminometer. HCT-116 cells were co-transfected with either an NFKBIL-1 expressing vector or the equivalent empty vector, and NFκB-luciferase reporter

construct. Cells were either stimulated with IL-1ß (10ng/ml) (Figure 3.1A) or TNF-α (20ng/ml) (Figure 3.1B) for 2 hours and the luciferase activity was then measured. Our results demonstrate that NFKBIL-1 inhibits NFκB activity when compared with empty vector (Figure 3.1.1A and 3.1.1B).

3.1.1A

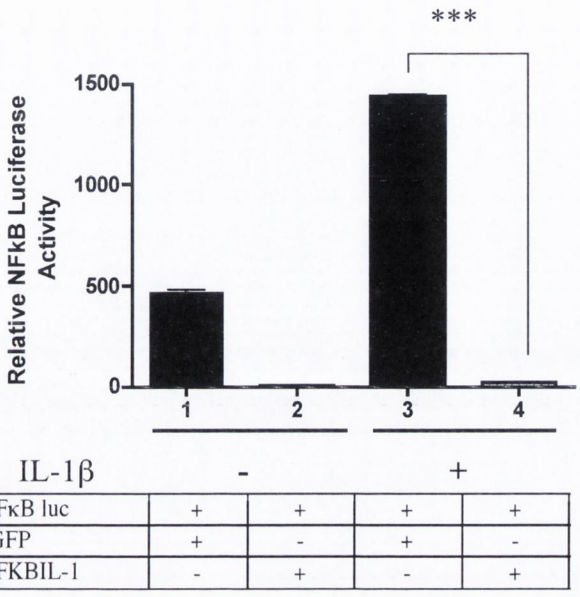
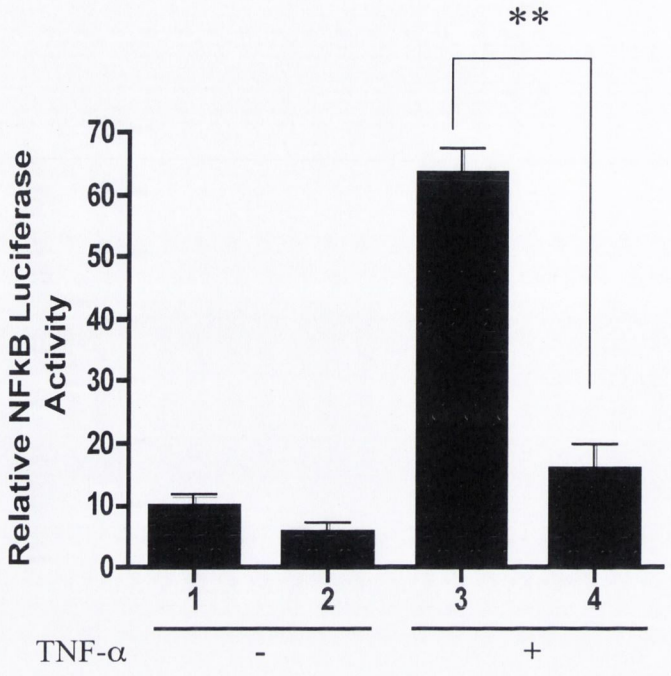


FIGURE 3.1.1A: NFKBIL-1 inhibits IL-1ß stimulated NFκB reporter expression. HCT-116 cells were co-transfected with a luciferase linked NFκB reporter vector and NFKBIL-1/empty GFP expression vector. All transfections contained 2μg of plasmid DNA. Cells were unstimulated (lanes 1, 2) or stimulated with IL-1ß (10ng/ml) for 2 hours (lanes 3, 4) and luciferase production in cell lysates was measured by luminometry. Levels are expressed as relative luciferase activity after normalizing for ß-galactosidase expression. Each experiment was done in duplicate and repeated three times. NFKBIL-1 decreases IL-1ß induced NFκB luciferase expression ***, $p<0.0001$, compared to eGFP.

3.1.1B



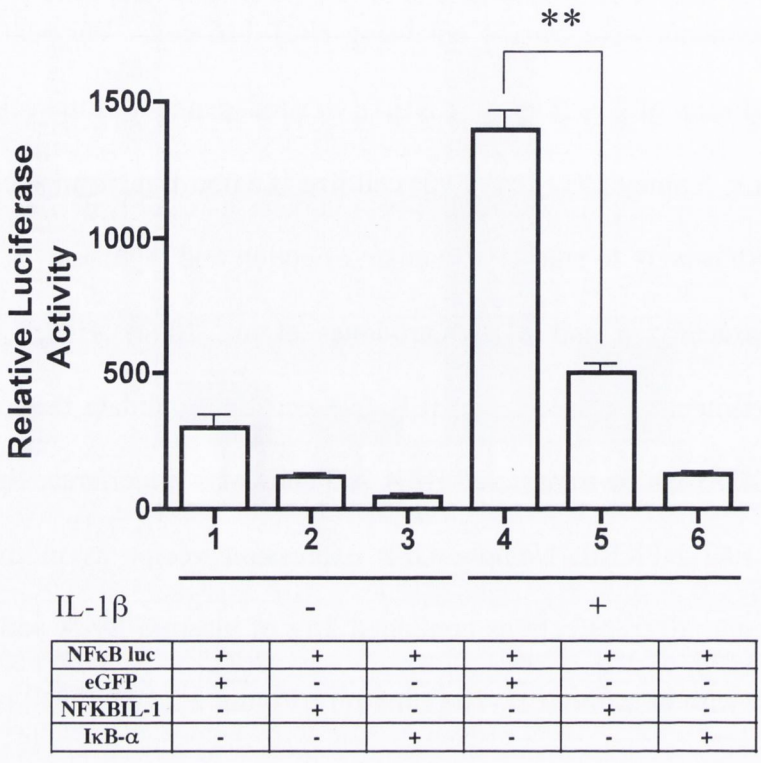
NFkB luc	+	+	+	+
eGFP	+	-	+	-
NFKBIL-1	-	+	-	+

FIGURE 3.1.1B: NFKBIL-1 inhibits TNF-α stimulated NFκB reporter expression. HCT-116 cells were co-transfected with a luciferase linked NFκB reporter vector and NFKBIL-1/empty GFP expression vector. All transfections contained 2μg of plasmid DNA. Cells were stimulated with TNF-α (20ng/ml) for 2 hours and luciferase production in cell lysates was measured by luminometry. Levels are expressed as relative luciferase activity after normalizing for ß-galactosidase expression. Each experiment was done in duplicate and repeated three times. NFKBIL-1 decreases TNF-α induced NFκB luciferase expression **, p= 0.0036, compared to eGFP.

3.1.2.2 NFKBIL-1 inhibits NFκB reporter activation in HEK 293 cells

Our initial experiments were carried out using HCT-116 cells. We next wanted to confirm that inhibition of NFκB by NFKBIL-1 was independent of the cell line used. Human Embryonic Kidney 293 (HEK 293) cell line is a non-transformed cell line that has been used extensively to study mechanism of action and regulation of NFκB and it is a well characterized cell line (Kurt-Jones et al., 2004). Hence for all the subsequent experiments we used only HEK 293 cells. To validate the inhibition of NFκB by NFKBIL-1 we co-transfected HEK 293 cells with a luciferase linked NFκB reporter vector and NFKBIL-1/empty GFP expression vector as in the previous experiments. Again, all transfections contained 2μg of plasmid DNA and cells were either stimulated with or without IL-1ß (10ng/ml) (Figure 3.2A) or TNF-α (20ng/ml) (Figure 3.2B) for 2 hours before luciferase activity was measured. Furthermore, IκB α, a known inhibitor of NFκB, was also co-transfected with the luciferase vector as a positive control. When compared with the empty GFP vector, NFKBIL-1 inhibited NFκB activity in both IL-1ß stimulated cells (Figure 3.1.2A; **, p= 0.0065) and in TNF-α induced cells. (Figure 3.1.2B; **, p= 0.0012), confirming the findings in the HCT-116 cell line described above.

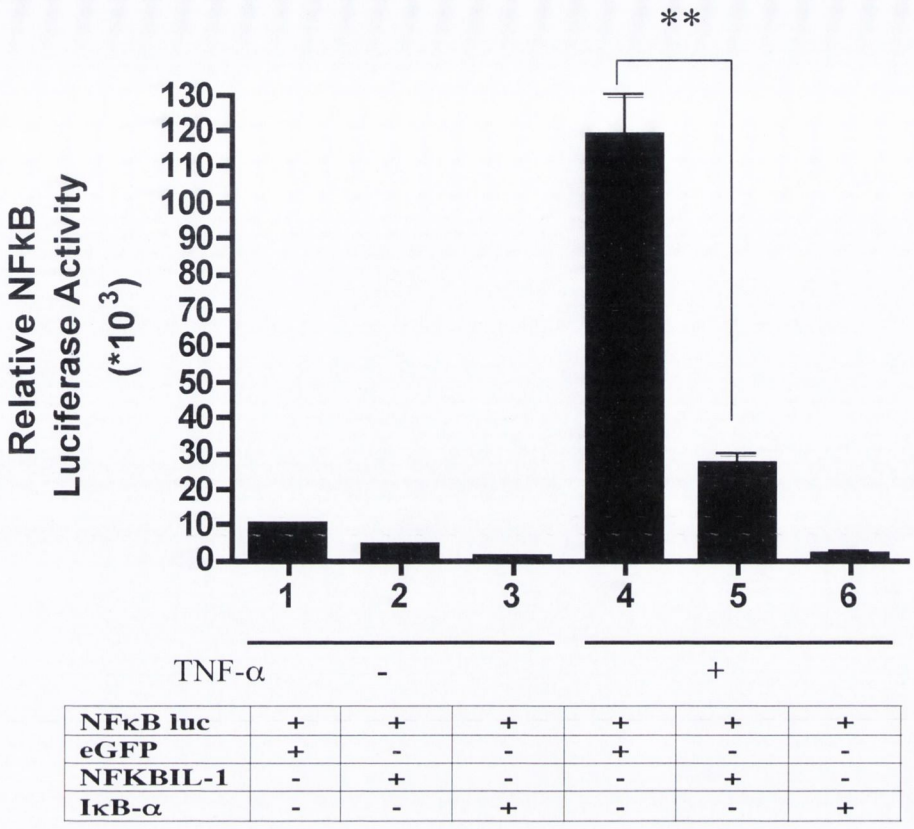
3.1.2A



NFκB luc	+	+	+	+	+	+
eGFP	+	-	-	+	-	-
NFKBIL-1	-	+	-	-	+	-
IκB-α	-	-	+	-	-	+

FIGURE 3.1.2A: NFKBIL-1 inhibits IL-1ß activated NFκB reporter expression. HEK 293 cells were co-transfected with a luciferase linked NFκB reporter vector and NFKBIL-1/empty GFP expression vector. All transfections contained 2μg of plasmid DNA. Cells were stimulated with IL-1ß (10ng/ml) for 2 hours and luciferase production in cell lysates was measured by luminometry. Levels are expressed as relative luciferase activity after normalizing for ß-galactosidase expression. As a positive control, IκB α linked to GFP was co-transfected along with NFκB reporter vector. Each experiment was done in duplicate and repeated three times. NFKBIL-1 decreases NFκB luciferase expression **, p= 0.0065, compared to eGFP.

3.1.2B



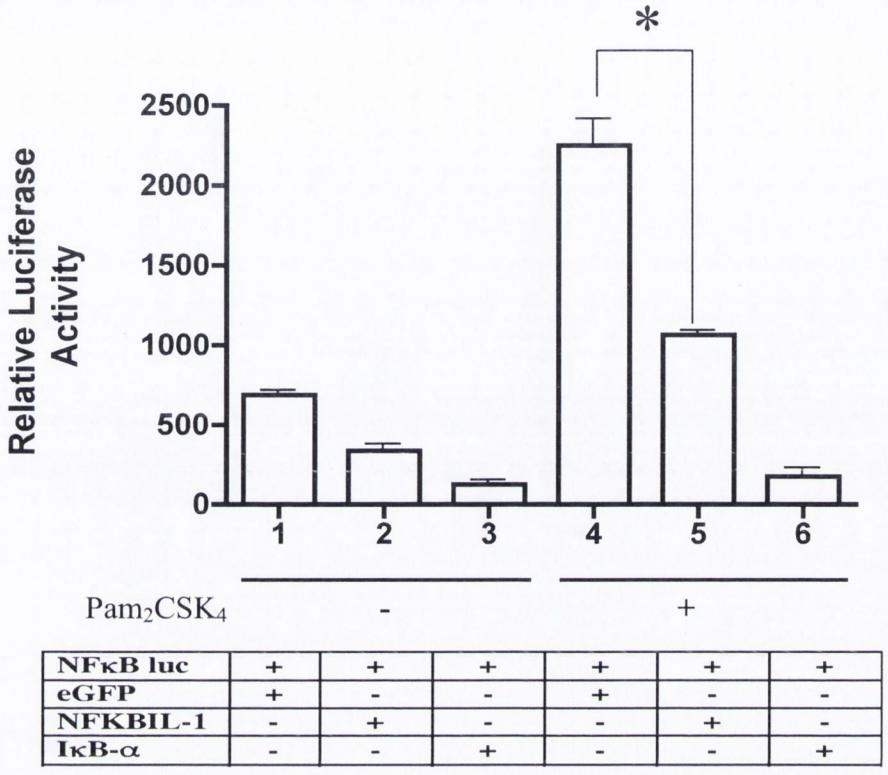
NFκB luc	+	+	+	+	+	+
eGFP	+	-	-	+	-	-
NFKBIL-1	-	+	-	-	+	-
IκB-α	-	-	+	-	-	+

FIGURE 3.1.2B: NFKBIL-1 inhibits TNF-α NFκB reporter activation. HEK 293 cells were co-transfected with a luciferase linked NFκB reporter vector and NFKBIL-1/empty GFP expression vector. All transfections contained 2μg of plasmid DNA. Cells were stimulated with TNF-α (20ng/ml) for 2 hours and luciferase production in cell lysates was measured by luminometry. Levels are expressed as relative luciferase activity after normalizing for ß-galactosidase expression. As a positive control IκB α linked to GFP was co-transfected along with NFκB reporter vector. Each experiment was done in duplicate and repeated three times. NFKBIL-1 decreases NFκB luciferase expression **, p= 0.0012, compared to eGFP.

3.1.2.3 NFKBIL-1 inhibits NFκB activated *via* the Toll Like Receptors (TLR) pathway

In the previous experiments we have demonstrated that NFKBIL-1 inhibits the activation of NFκB in response to the powerful pro-inflammatory immune mediators IL-1β and TNF-α, influencing both innate and adaptive immune responses. However regulation of NFκB via the TLR pathways is an important component specifically of innate immunity. Although a number of different TLRs have been reported, as described in section 1.4, TLR4 and TLR2 are important in response to bacterial infection and both are well-characterized members of the system and are known to activate NFκB (Akira, 2006). We wished therefore to investigate if activation of NFκB by these receptors can also be regulated by NFKBIL-1. HEK 293 cells (which do not normally express all the TLRs) stably transfected with TLR2 or TLR4 were co-transfected with either NFKBIL-1 vector or empty vector and NFκB-luciferase vector as before. Cells were either stimulated with PAM_2CSK_4, a TLR2 specific stimulant, or LPS, a TLR4 specific stimulant for 6 hours and the luciferase activity was measured compared to a baseline provided by unstimulated cells. As before, IκB α, a known inhibitor of NFκB, was also co-transfected with the luciferase vector as a positive control. We observed a significant reduction in NFκB activity in both TLR2 (Figure 3.1.3A) and TLR4 (Figure 3.1.4A) expressing HEK 293 cells in the presence of overexpressed NFKBIL-1. HEK 293 cells lacking TLR2 and TLR4 receptors were used as negative control (Figure 3.1.3B and 3.1.4B).

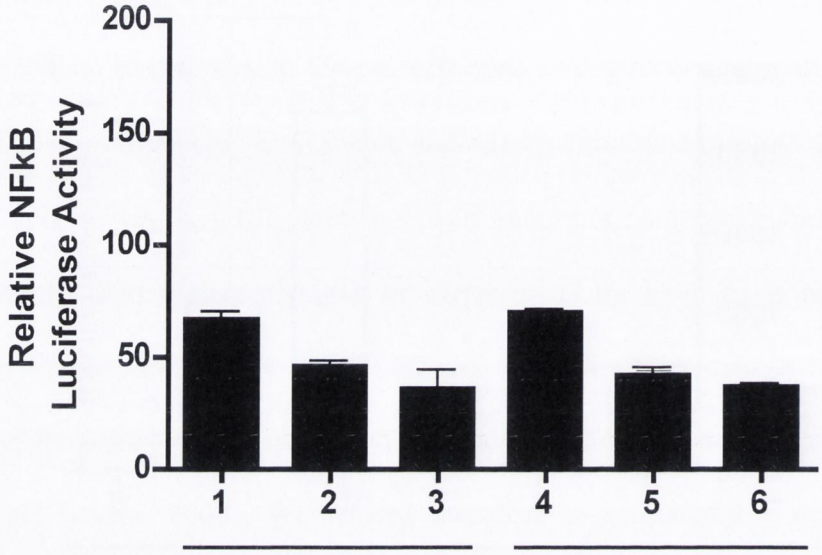
3.1.3A



NFκB luc	+	+	+	+	+	+
eGFP	+	-	-	+	-	-
NFKBIL-1	-	+	-	-	+	-
IκB-α	-	-	+	-	-	+

FIGURE 3.1.3A: NFKBIL-1 inhibits NFκB reporter activation in HEK 293 TLR2$^+$ cells. Stably transfected HEK 293 TLR2$^+$ were co-transfected with a luciferase linked NFκB reporter vector and NFKBIL-1/empty GFP expression vector. All transfections contained 2μg of plasmid DNA. Cells were stimulated with PAM_2CSK_4 (100ng/ml) for 6 hours and luciferase production in cell lysates was measured by luminometry. Levels are expressed as relative luciferase activity after normalising for vector encoding for ß-galactosidase expression. As a positive control, IκB α linked to GFP was co-transfected along with NFκB reporter vector. Each experiment was done in duplicate and repeated three times. NFKBIL-1 decreases NFκB luciferase expression **, p= 0.0186, compared to eGFP

3.1.3B



NFκB luc	+	+	+	+	+	+
eGFP	+	-	-	+	-	-
NFKBIL-1	-	+	-	-	+	-
IκB-α	-	-	+	-	-	+

FIGURE 3.1.3B: NFKBIL-1 has no effect on TLR2 activated NFκB reporter activation in HEK 293 cells. As a control for the previous experiment. HEK 293 cells (i.e. without the TLR2 expression module) were co-transfected with a luciferase linked NFκB reporter vector and NFKBIL-1/empty GFP expression vector. All transfections contained 2μg of plasmid DNA. Cells were stimulated with PAM_2CSK_4 (100ng/ml) for 6 hours and luciferase production in cell lysates was measured by luminometry. Levels are expressed as relative luciferase activity after normalizing for ß-galactosidase expression. As a positive control IκB α linked to GFP was co-transfected along with NFκB reporter vector. Each experiment was done in duplicate and repeated three times. As HEK 293 cells lack TLR2 receptors, only basal NFκB activity was observed.

3.1.4A

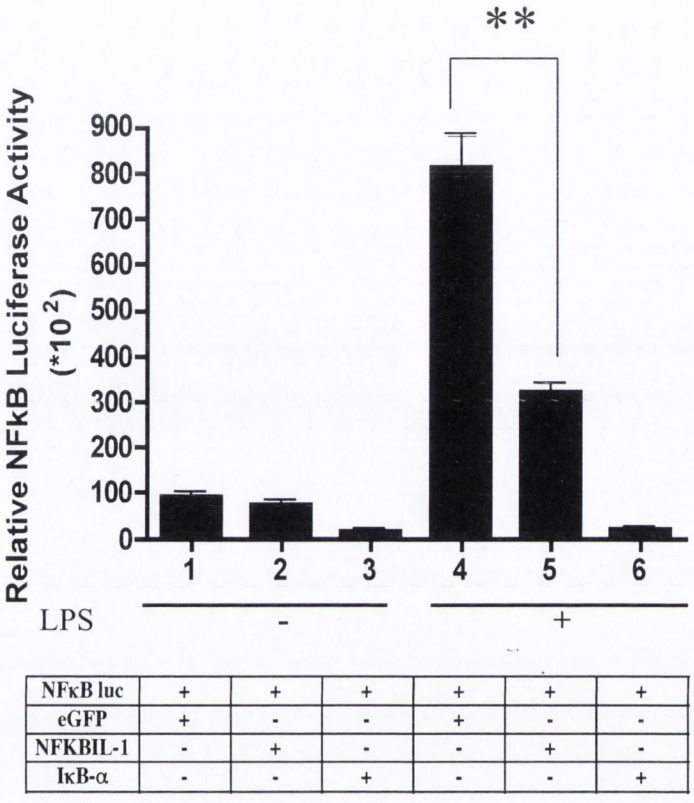
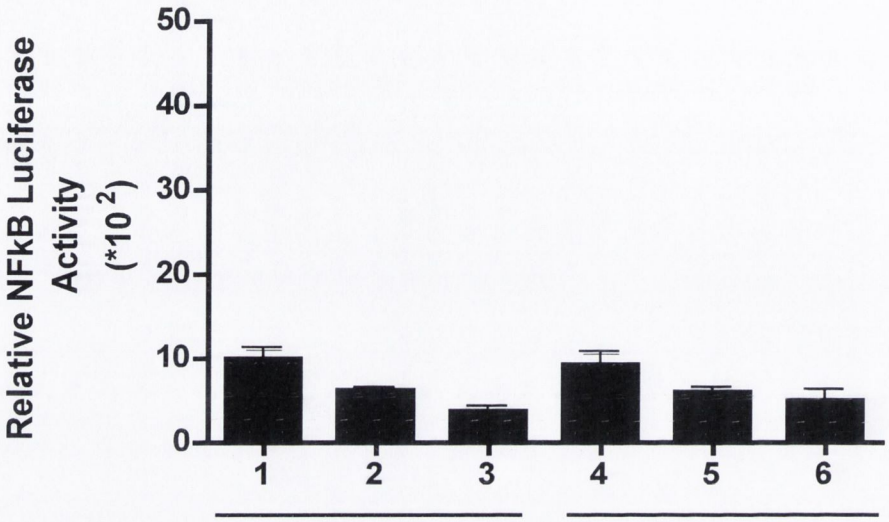


FIGURE 3.1.4A: NFKBIL-1 inhibits NFκB reporter activation in HEK 293+TLR4. Stably transfected HEK 293+TLR4 were co-transfected with a luciferase linked NFκB reporter vector and NFKBIL-1/empty GFP expression vector. All transfections contained 2μg of plasmid DNA. Cells were stimulated with LPS (100ng/ml) for 6 hours and luciferase production in cell lysates was measured by luminometry. Levels are expressed as relative luciferase activity after normalising for ß-galactosidase expression. As a positive control, IκB α linked to GFP was co-transfected along with NFκB reporter vector. Each experiment was done in duplicate and repeated three times. NFKBIL-1 decreases NFκB luciferase expression **, p= 0.0029, compared to eGFP.

3.1.4B



NFκB luc	+	+	+	+	+	+
eGFP	+	-	-	+	-	-
NFKBIL-1	-	+	-	-	+	-
IκB-α	-	-	+	-	-	+

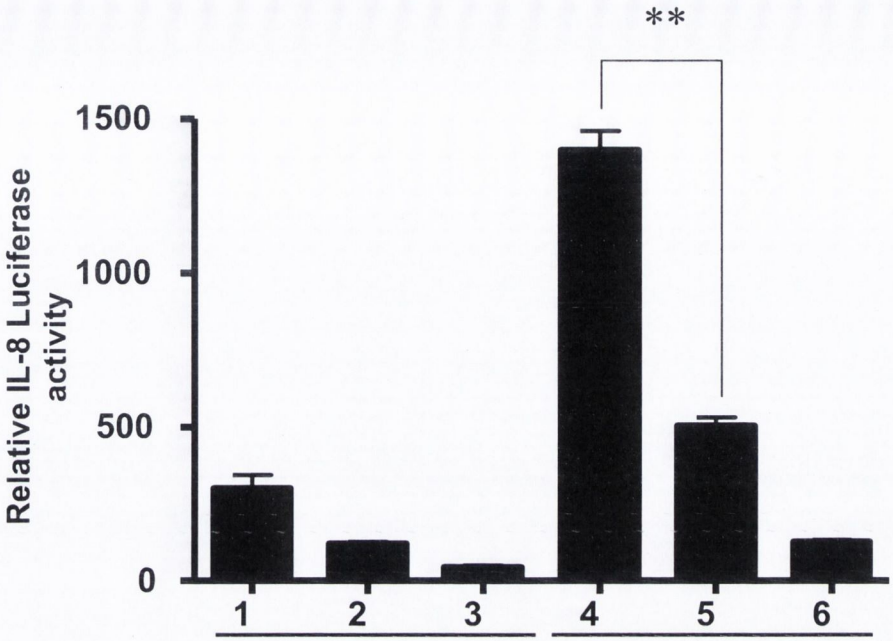
FIGURE 3.1.4B: NFKBIL-1 has no effect on TLR4 mediated NFκB reporter activation in HEK 293. HEK 293 were co-transfected with a luciferase linked NFκB reporter vector and NFKBIL-1/empty GFP expression vector. All transfections contained 2μg of plasmid DNA. Cells were stimulated with LPS (100ng/ml) for 6 hours and luciferase production in cell lysates was measured by luminometry. Levels are expressed as relative luciferase activity after normalising for ß-galactosidase expression. As a positive control IκB α linked to GFP was co-transfected along with NFκB reporter vector. As HEK 293 cells lack TLR4 receptors, only basal NFκB activity was observed.

3.1.2.4 Identification of downstream pathways of NFκB signalling regulated by NFKBIL-1

NFκB modulates the expression of a number of pro and anti-inflammatory cytokines. Consequent to the inhibition of NFκB by NFKBIL-1 we expected to observe changes in the expression of these cytokines. Various types of cytokines are produced during inflammation and a single cell is exposed to a mixture of cytokines. For this we first performed a biochip array to identify the cytokines expressed by the HEK 293 cells. HEK 293 cells were plated in 6 well plates and stimulated with/without IL-1β (10ng/ml) for 2 hours. The supernatant was removed and used for measuring the cytokine expression levels. We observed that the major cytokines expressed were IL-8, VEGF and MCP-1. A number of other cytokines including TNF-α and IL-10 were expressed at very low levels (Figure not shown).

3.1.2.5 NFKBIL-1 decreases IL-8 expression.

From our previous experiments we were able to identify that IL-8 was one of the main chemokines secreted by HEK 293 cells. IL-8 is a pro-inflammatory chemokine known to attract neutrophils to the site of inflammation (Kumar V, 2007). The promoter of IL-8 has consensus sites for various transcription factors such as NFκB and AP-1 (Blackwell and Christman, 1997; Wolf et al., 2001). Based on these observations, we selected IL-8 as a candidate to study the functional consequence of inhibition of NFκB. Therefore, using an IL-8 promoter driven luciferase vector, we investigated if NFKBIL-1 could regulate the expression of IL-8 in HEK-293 cells. To do this, HEK 293 cells were co-transfected with an IL-8 promoter luciferase reporter vector and NFKBIL-1 or empty vector. Cells were either stimulated with or without IL-1ß for 2 hours and the luciferase activity was measured. IκB α, a known inhibitor of NFκB and demonstrated to decrease IL-8 expression was also co-transfected with the luciferase vector as a positive control (Dai et al., 2004). Our results show clearly that activity of the IL-8 promoter is strongly induced by IL-1ß as expected and that this is significantly inhibited in the presence of both NFKBIL-1 and IκB α expression vectors. Hence we conclude that overexpression of NFKBIL-1 inhibits activation of the IL-8 promoter as adjudged by luciferase protein expression, when compared to empty vector (Figure 3.1.5, **, p= 0.0077).



NFκB luc	+	+	+	+	+	+
eGFP	+	-	-	+	-	-
NFKBIL-1	-	+	-	-	+	-
IκB-α	-	-	+	-	-	+

FIGURE 3.1.5: NFKBIL-1 inhibits IL-8 reporter expression. HEK 293 cells were co-transfected with a luciferase linked IL-8 promoter reporter vector and NFKBIL-1/empty GFP expression vector. All transfections contained 2μg of plasmid DNA. Cells were stimulated with IL-1ß (10ng/ml) for 2 hours and luciferase production in cell lysates was measured by luminometry. Levels are expressed as relative luciferase activity after normalising for ß-galactosidase expression. As a positive control, IκB α linked to GFP was co-transfected along with IL-8 reporter vector. Each experiment was carried out in duplicate and repeated three times. NFKBIL-1 decreases IL-8 luciferase expression **, p= 0.0077, compared to eGFP.

3.1.2.6 NFKBIL-1 IL-8 protein expression

Luciferase assays provide us with information about the promoter activity of the gene and its level of transcription. We wanted to identify if the inhibition of *IL-8* expression by NFKBIL-1 as seen in experiments using luciferase reporter constructs leads to an actual decrease in IL-8 protein production in a cell culture system. For this we carried out ELISA for IL-8 using the standard protocol. To this end, HEK 293 cells were plated in 6 well plates and incubated for 24 hours. Subsequently, fresh media was added to the cells and they were stimulated with IL-1ß (10ng/ml). This was performed for six different time points ranging from 0-8 hours. As expected, we observed a significant increase in IL-8 protein production in response to IL-1ß stimulation, which is significantly inhibited in cells transfected with NFKBIL-1 ((Figure 3.1.6, **, p= 0.0076 compared with empty vector).

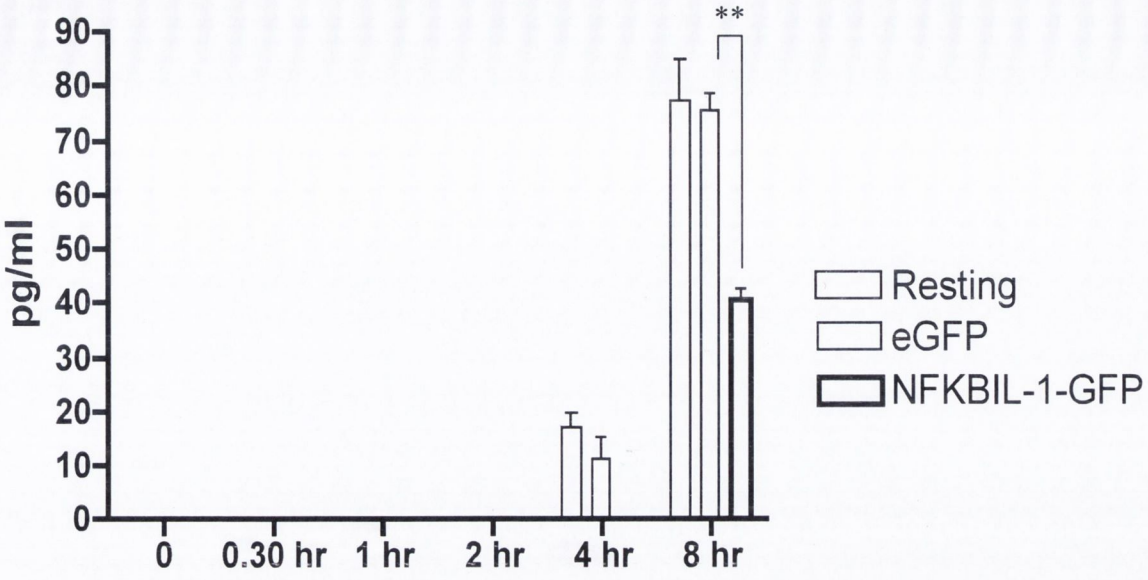


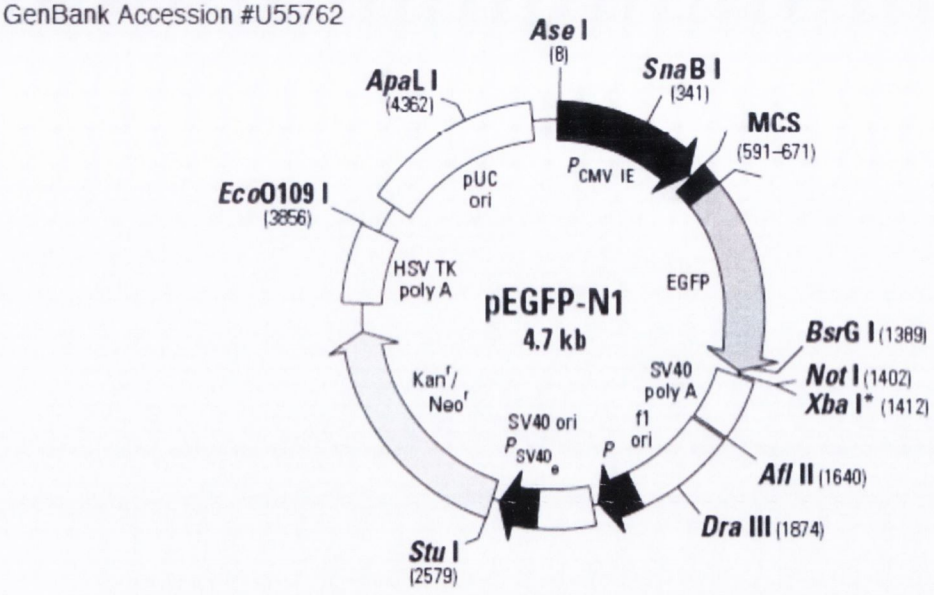
FIGURE 3.1.6: NFKBIL-1 decreases IL-8 production. ELISA was performed to measure the protein production in cell supernatants from resting HEK 293 cells and cells transfected with NFKBIL-1 and empty GFP vector and stimulated for various time points up to 8 hours with IL-1ß (10ng/ml). NFKBIL-1 decreases IL-8 production **, p= 0.0076 compared with eGFP.

3.1.2.7 Identification of domains of NFKBIL-1 protein involved in the inhibition of NFκB

The NFKBIL-1 gene codes for various different domains including an ankyrin repeat domain reported to be necessary for protein-protein interactions. Our initial study looking at the inhibition of NFκB by NFKBIL-1, focused on the full-length protein. Apart from the ankyrin repeat domain, the other interesting sequences encoded include a potential alternative start site, a PEST sequence, and couple of phosphorylation sites as described in section 1.3.1.2. We wanted to identify the regions of the protein involved in the inhibition of NFκB. To do this we cloned coding sequences for various domains into an eGFP vector, pEGFP-N1 which generates a fusion protein with eGFP attached to the C-terminal of the protein of interest (Figure 3.1.7). These included coding sequences for an alternative transcript (NFKBIL-1-ALT) and the N-terminal and C-terminal sequences excluding the ankyrin repeat domains (NT-NFKBIL-1 and CT-NFKBIL-1 respectively) (Figure 3.1.8). In order to generate these gene fragments for cloning, PCR was performed using primers described in Table 3.1.1. The PCR products were subjected to restriction digestion, extracted from 1% agarose gel and the ligation and cloning was performed using standard protocols. Restriction digestion was performed and the plasmid DNA was sequenced to confirm successful cloning (Figure 3.1.9).

pEGFP-N1 Vector Information

GenBank Accession #U55762



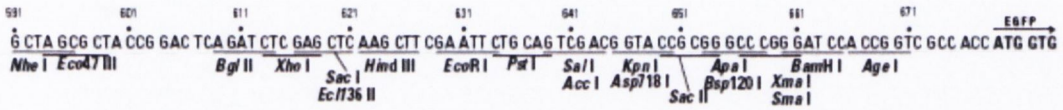


Figure 3.1.7: Vector map and sequence of multiple cloning site of pEGFP plasmid. Amplified PCR fragments with primers coding for *BglII* and *Asp718I* were inserted between the two sites by ligation.

GCTAGCGCTACCGGACTCAGATCTC*ATG*AGTAACCCCTCCCCCCAGGTTCCAGAGGA
AGAAGCCTCCACATCTGTCTGAGATCTCAAGAGTTCC*ATG*GCCTCCACTTCCCGCCGC
CAACGCCGAGAACGTCGCTTTCGTCGTTACTTGTCTGCAGGACGGCTGGTCCGGGCCC
AGGCCCTCCTCCAGCGACACCCGGTACCCGATGTAGATGCTGGGCAGCCCCCACCAC
TGCACCGGGCCTGTGCCCGCCACGATGCCCCTGCCCTGTGCCTGCTGCTTCGGCTCGG
GGCTGACCCTGCCCACCAGGACCGCCATGGGGACACGGCACTGCATGCTGCTGCCCG
CCAGGGCCCAGATGCCTACACCGATTTCTTCCTCCCGCTGCTAAGCCGCTGTCCCTCC
GCCATGGGAATAAAGAATAAGGATGGGGAGACCCCTGGCCAAATTTTGGGCTGGGG
ACCCCCCTGGGATTCTGCTGAAGATCTCATGGAAGAAGATGATGCCTCCAAGGAGCG
GGAATGGAGACAGAAGCTCCAGGGTGAGCTGGAGGACGAGTGGCAGGAAGTCATGG
GGAGGTTTGAAGGTGATGCCTCCCATGAAACCCAGGAACCTGAGTCCTTCTCAGCCT
GGTCAGATCGCCTGGCCCGGGAACATGCCCAGAAGTGCCAGCAGCAGCAGCGAGAA
GCAGAGGGATCCTGTCGACCCCCACGTGCTGAGGGCTCCAGCCAGAGCTGGCGACAG
CAGGAGGAGGAGCAGCGGCTCTTCAGGGAGCGAGCCCGGGCCAAGGAGGAAGAGCT
GCGTGAGAGCCGAGCCAGGAGGGCGCAGGAGGCTCTAGGGGACCGAGAACCCAAGC
CAACCAGGGCCGGGCCCAGGGAAGAGCACCCCAGAGGAGCGGGGAGGGGCAGCCTC
TGGCGATTTGGTGATGTGCCCTGGCCCTGCCCTGGGGGAGGGGACCCAGAGGCCATG
GCTGCAGCCCTGGTGGCCAGGGGCCCCCCTTTGGAGGAACAGGGGGCTCTGAGGAGG
TACTTGAGGGTCCAGCAGGTCCGCTGGCACCCTGACCGCTTCCTGCAGCGATTCCGAA
GCCAGATTGAGACCTGGGAGCTGGGCCGTGTGATGGGAGCAGTGACAGCCCTTTCTC
AGGCCCTGAATCGCCATGCAGAGGCCCTCAAGGTACCGCGGGCCCGGGATCCACCG
GTCGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTG
GTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGA
GGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCT
GCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGC
CGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGC
TACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCC
GAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGAC
TTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCAC
AACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATC
CGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACC
CCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCC
GCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTG
ACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAA

Figure 3.1.8: Sequence of NFKBIL-1 coding DNA. The start codon is italicized and highlighted (yellow) and the alternative start codon is underlined and highlighted (yellow). The *NFKBIL-1* coding sequence is in blue colour. Green colored sequences denote the vector sequences encoding for the green fluorescent protein (GFP). Bold sequence (highlighted in Green) denotes the restriction enzyme sites inserted into the PCR amplicon.

Name of the Coding DNA	*Description ATG (bold, italics)=position no.1 in figure 3.1.8*	*PCR primers (5' to 3')*
NFKBIL-1	Size 1165bp. From –11 to 1154	Fwd: ACTCAGATCTCATGAGTAA Rev: CGCGGTACCTTGAGGGCCTCT
NFKBIL-1-ALT	Size 1085bp. From 70 to 1154	Fwd: TGTCTGAGATCTCAAGAGT Rev: CGCGGTACCTTGAGGGCCTCT
NFKBIL-NT	Size 197bp. From -11 to 186	Fwd: ACTCAGATCTCATGAGTAA Rev: ATCTACATCGGGTACCGGGTGT
NFKBIL-CT	Size 702bp. From 452 to 1154	Fwd: TGAAGATCTCATGGAAGAAGAT Rev: CGCGGTACCTTGAGGGCCTCT

Table 3.1.1: **Description of the coding sequences cloned into the eGFP vector.** Forward and reverse primers used in the PCR reaction are also described. Numbers denote the nucleotide position in the gene sequence. See previous figure for details regarding the sequence position.

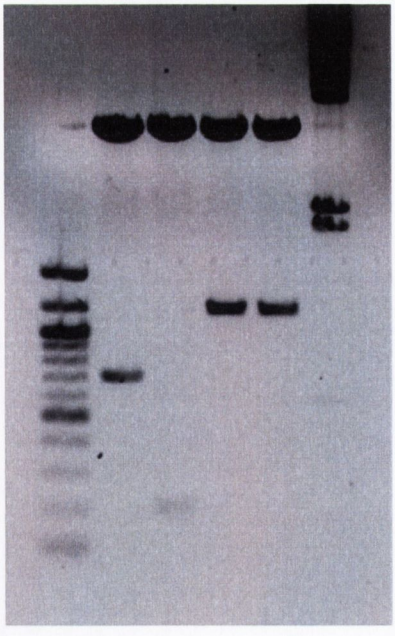


Figure 3.1.9: Restriction fragments identifying successfully cloned vectors. Plasmid DNA from selected clones was extracted from LB liquid cultures and subjected to restriction digestion using enzymes *BglII* and *Asp718I* in the same reaction. The digests were run on a 1% agarose gel and observed under UV source. Left to right: 100 bp ladder, CT-NFKBIL-1 (702bp), NT-NFKBIL-1 (197 bp), Full length NFKBIL-1 (1165 bp) and ALT-NFKBIL-1 (1085 bp) and *HindIII* digested Lambda DNA.

3.1.2.8 Partial NFKBIL-1 constructs do not inhibit NFκB

After identifying vectors successfully cloned with the required insert we next proceeded to perform luciferase assays to detect the region of NFKBIL-1 protein essential for inhibition of NFκB. For this assay all the different vectors i.e. Full length NFKBIL-1, Alt-NFKBIL-1, NT-NFKBIL-1 and CT-NFKBIL-1 were co-transfected with κB or IL-8 promoter luciferase vector in HEK 293 cells and after 24 hours, stimulated with IL-1ß for 2 hours. Cells were also transfected with eGFP vector as a negative control. Both the full length NFKBIL-1 and the ALT-NFKBIL-1 inhibited NFκB activity while partial sequences did not (NFκB: Figure 3.1.10, Full length, ***, $p=0.0002$, Alt, **, $p<0.0195$) and (IL-8: Figure 3.1.11, Full length, ***, $p=<0.0001$, Alt, $p<0.0001$).

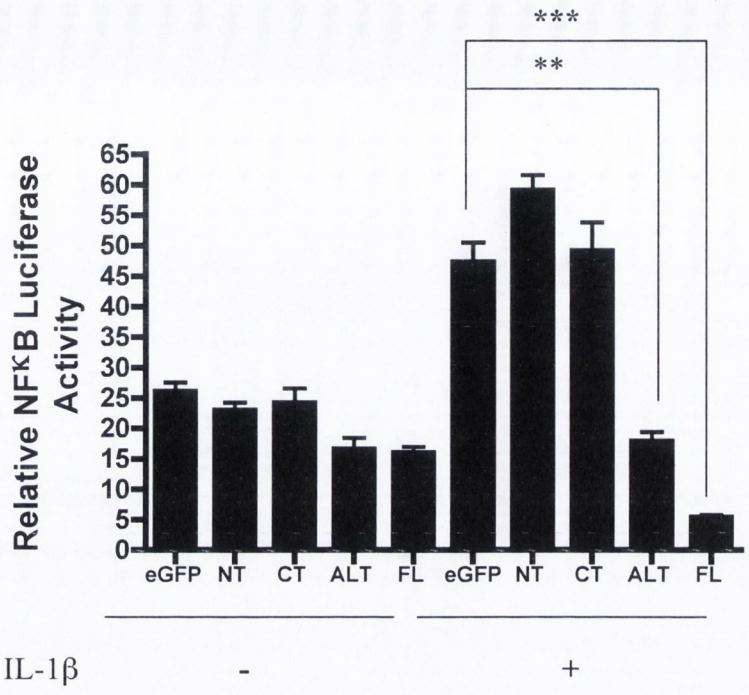


Figure 3.1.10: Identification of regions of NFKBIL-1 protein that are essential for inhibition of NFκB. Different domains of NFKBIL-1 were cloned into eGFP vector and co-transfected with the κB-driven luciferase reporter vector. Luciferase Assay was performed as described before. eGFP=empty vector, NT=N-terminal, CT=C-terminal, ALT=NFKBIL-1 alternative isoform, FL=full length NFKBIL-1 protein. Both the full length and the Alt variant significantly inhibited NFκB activity. (Figure 3.1.10, Full length, ***, p=0.0002, Alt, **, p<0.0195).

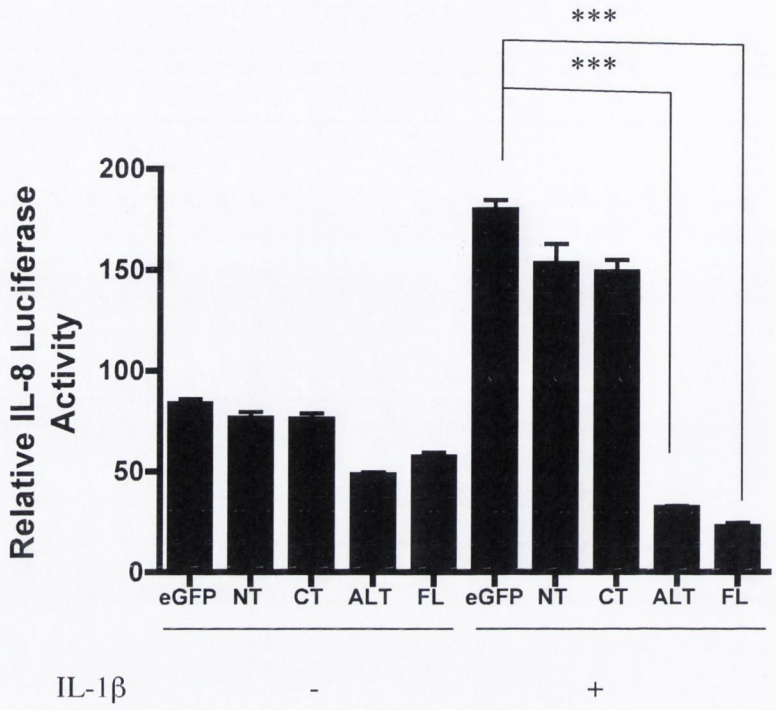
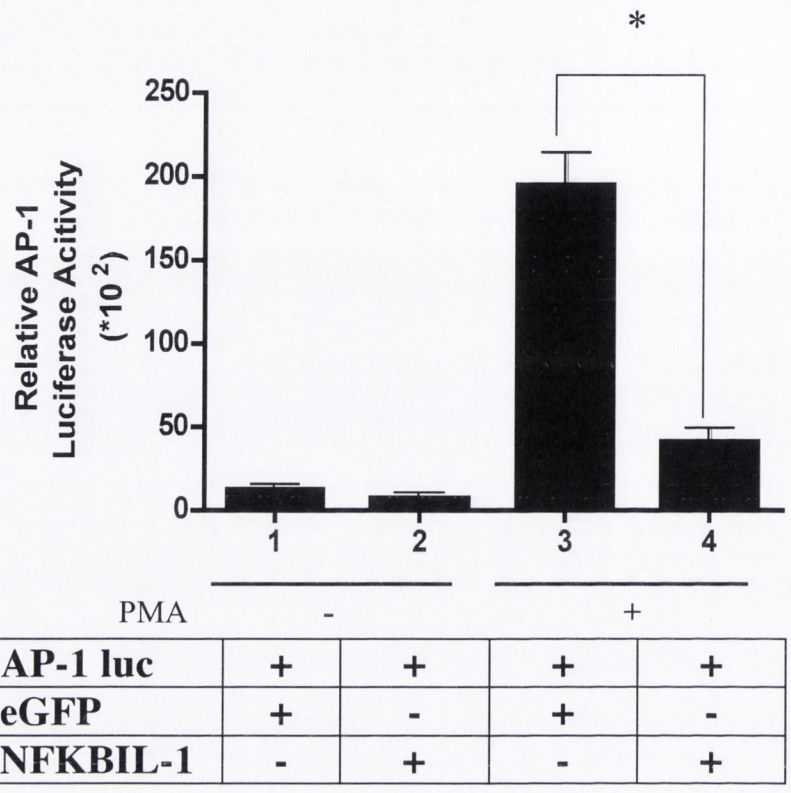


Figure 3.1.11: Identification of regions of NFKBIL-1 protein essential for inhibition of IL-8. Different domains of NFKBIL-1 were cloned into eGFP vector and co-transfected with IL-8 promoter luciferase vector. Luciferase Assay was performed as described before. eGFP= empty vector, NT=N-terminal, CT=C terminal, ALT= NFKBIL-1 alternative isoform, FL= full length NFKBIL-1 protein. (Full length, ***, p=<0.0001, Alt, p<0.0001)

3.1.2.9 NFKBIL-1 inhibits Activated Protein-1 (AP-1) activity

The activator protein-1 (AP-1) transcription factor is a dimeric complex that comprises members of the Jun, Fos, ATF and MAF protein families with Fos and Jun being the most common proteins in mammalian cells. AP-1 activity is induced by growth factors, cytokines and oncoproteins. Upon activation, AP-1 binds to the TPA Responsive element (TRE) and induces transcription of a variety of genes involved in multiple cellular functions such as proliferation, survival, differentiation and transformation (Shaulian and Karin, 2002). AP-1 transcription factor is also induced by the Toll like receptor pathway. We have demonstrated above that NFKBIL-1 inhibited TLR stimulated NFκB activity. As TLR also activate AP-1 transcription factor, we next wanted to identify if NFKBIL-1 could regulate AP-1 activity. For this we used an AP-1 responsive luciferase vector designed for monitoring induction of the AP-1 and the stress-activated protein kinase/Jun N-terminal kinase (SAPK/JNK) signal transduction pathway. HEK 293 cells were co-transfected with either NFKBIL-1 vector or empty vector and AP-1-luciferase vector. Cells were stimulated with PMA (10ng/ml) for 24 hours and the luciferase activity was measured. NFKBIL-1 inhibits AP-1 activity when compared with empty vector (Figure 3.1.12, *, p= 0.0167).

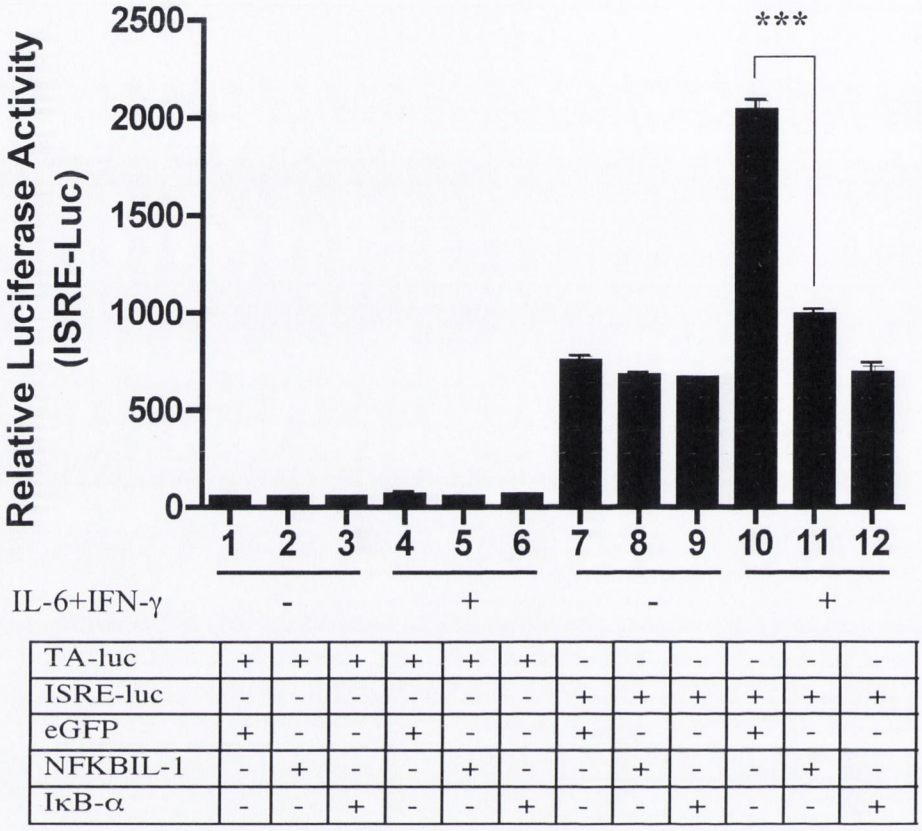


AP-1 luc	+	+	+	+
eGFP	+	-	+	-
NFKBIL-1	-	+	-	+

FIGURE 3.1.12: NFKBIL-1 inhibits AP-1 driven luciferase expression. HEK 293 cells were co-transfected with a luciferase linked AP-1 reporter vector and NFKBIL-1/empty GFP expression vector. All transfections contained 2μg of plasmid DNA. Cells were stimulated with PMA (10ng/ml) for 24 hours and luciferase production in cell lysates was measured by luminometry. Levels are expressed as relative luciferase activity after normalizing for ß-galactosidase expression. Each experiment was carried out in duplicate and repeated three times. NFKBIL-1 decreases AP-1 luciferase expression *, p= 0.0167, compared to eGFP.

3.1.2.10 NFKBIL-1 inhibits the activation of STAT responsive ISRE reporter

As described in section 1.3.3 STAT proteins are a group of transcription factor important for cell growth, survival and proliferation. Upon activation, STAT proteins are phosphorylated and translocate to the nucleus, where the STAT1 and STAT2 heterodimers regulate transcription by binding to the IFN-γ stimulated response element (ISRE). pISRE-luc is designed for monitoring the induction of the STAT1 and STAT2 components of Jak/STAT-mediated signal transduction pathways. pISRE was a kind gift from Dr. Carmen Gomez-Guerrero, Fundacion Jimenez Diaz (Autonoma University), Madrid and the experiment was designed as described in Hernández-Vargas *et al.*, 2005. HEK 293 cells were co-transfected with either NFKBIL-1 vector or empty vector and ISRE-luciferase vector. As an additional control in this set of experiments, the Luciferase vector without the ISRE regulatory sequences was included (TA-Luc). Cells were stimulated with IL-6 and IFN-γ (100U/ml each) for 24 hours and the luciferase activity was measured. NFKBIL-1 inhibits ISRE expression when compared with empty vector (Figure 3.1.14, ***, $p<0.0001$).



TA-luc	+	+	+	+	+	+	-	-	-	-	-	-
ISRE-luc	-	-	-	-	-	-	+	+	+	+	+	+
eGFP	+	-	-	+	-	-	+	-	-	+	-	-
NFKBIL-1	-	+	-	-	+	-	-	+	-	-	+	-
IκB-α	-	-	+	-	-	+	-	-	+	-	-	+

FIGURE 3.1.14: NFKBIL-1 inhibits STAT1/2 driven luciferase expression. HEK 293 cells were co-transfected with a luciferase linked ISRE reporter vector and NFKBIL-1/empty GFP expression vector. All transfections contained 2μg of plasmid DNA. Cells were stimulated with IL-6 and IFN-γ (100U/ml each) for 24 hours and luciferase production in cell lysates was measured by luminometry. IκB α plasmid was used as a positive control. TA-luc is empty vector without any insert and is used as a negative control. Levels are expressed as relative luciferase activity after normalizing for ß-galactosidase expression. Each experiment was done in duplicate and repeated three times. NFKBIL-1 decreases ISRE luciferase expression ***, $p<0.0001$, compared to eGFP.

Results Section 2
3.2 Characterisation of products of NFKBIL-1 gene

Our findings demonstrate that NFKBIL-1 inhibits the activity of various stress responsive transcription factors such as NFκB, AP-1 and STAT1/2. These transcription factors play an important role in the regulation of various cellular processes. In order to identify the mechanism of inhibition of these transcription factors by NFKBIL-1, we first wished to characterise the products of *NFKBIL-1* gene. Previous studies have reported that NFKBIL-1 is localised within the nucleus where it is distributed in a speckled pattern. We wanted to investigate whether different stimuli affect the expression of the *NFKBIL-1* gene or alter mRNA and protein levels or distribution. Here we report the effect of pro-inflammatory stimuli such as IL-1ß, TNF-α and PMA, and anti-inflammatory stimuli such as the glucocorticoid, dexamethasone on the expression and distribution of NFKBIL-1. We further extend our study to identify the proteins interacting with NFKBIL-1.

3.2.1 Effect of pro- and anti- inflammatory stimuli on NFKBIL-1 mRNA expression

We first studied the effect of both pro-inflammatory and anti-inflammatory stimuli on the expression of NFKBIL-1 mRNA. To do this, 10^6 HEK 293 cells were plated on 6-well plates. The cells were stimulated with IL-1ß (10ng/ml) or Dexamethasone (10μM) for a range of time points from 0-48 hours. mRNA was collected and quantitative RT-PCR was performed as described in section 2.3. Each of these experiments were performed in duplicate and repeated twice. Data was analysed and the results were expressed as number of mRNA copies of NFKBIL-1 compared to the number of mRNA copies of the house keeping gene ß-actin. Our data showed that

NFKBIL-1 was expressed constitutively in large amounts. We observed an increase in the expression of NFKBIL-1 in cells stimulated with Dexamethasone. This increase was immediately seen after 5 minutes and remained increased until 24 hours. There was no significant change in the NFKBIL-1 expression pattern in IL-1ß stimulated cells (Figure 3.2.1).

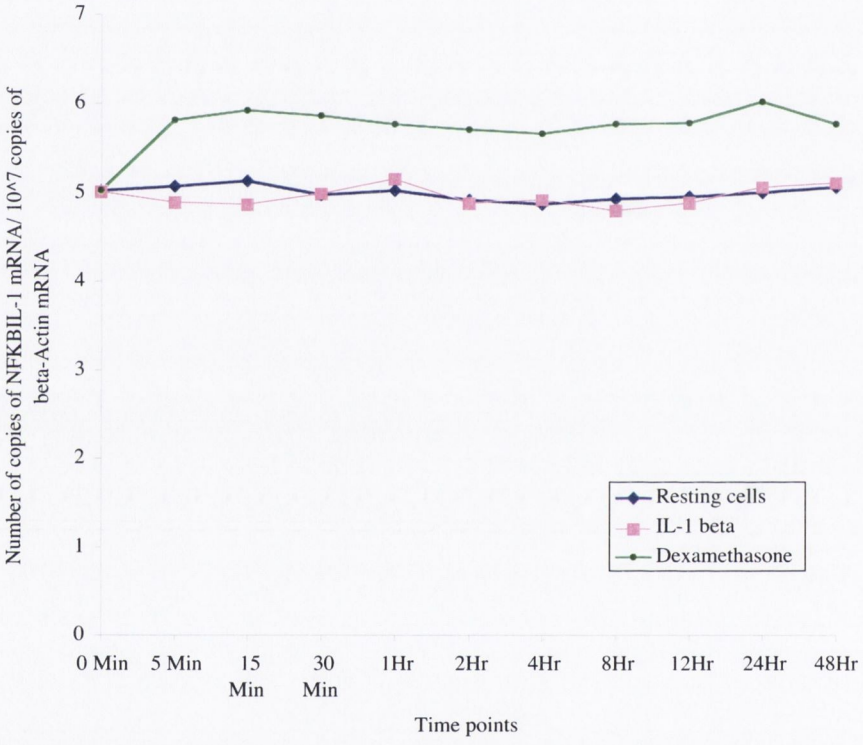


Figure 3.2.1: Dexamethasone up regulates NFKBIL-1 expression. $1x10^6$ HEK 293 cells were plated in 6 well plates and 24 hours later stimulated with IL-1ß (10ng/ml) and Dexamethasone (10µM) for various time points. mRNA was extracted and the expression of NFKBIL-1 mRNA was quantified by absolute RT-PCR. Results shown here are from N=2 experiments done in duplicate. NFKBIL-1 was expressed constitutively and while no change was observed in cells stimulated with IL-1ß, its expression was upregulated by Dexamethasone.

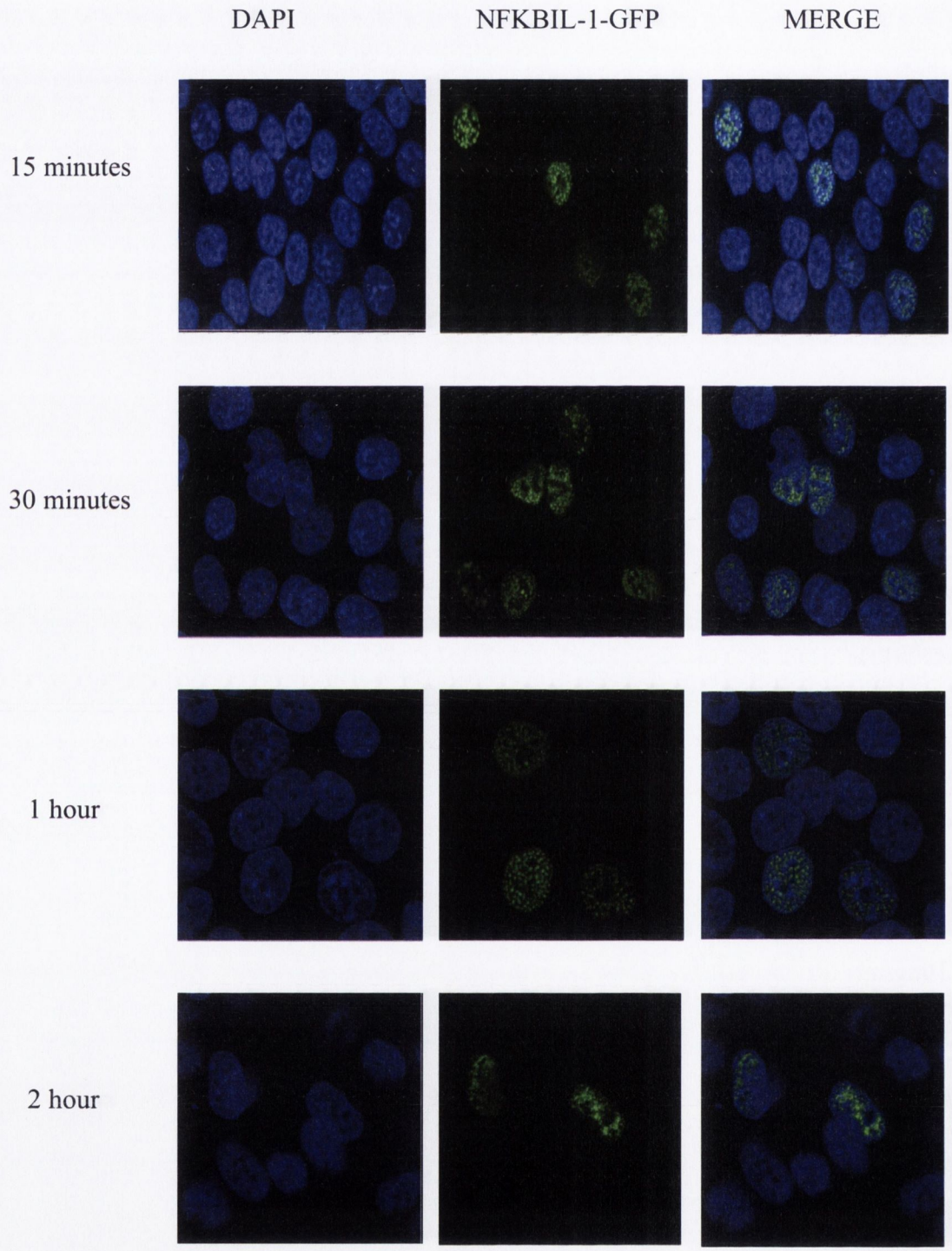
3.2.2 Intracellular localisation of NFKBIL-1 protein and effect of various stimuli on its distribution

Semple *et al.* had reported that NFKBIL-1 is distributed in the nucleus as speckles (Semple et al., 2002). As demonstrated before NFKBIL-1 regulated the activity of NFκB, AP-1 and STAT-1. Interestingly, all these three transcription factors are implicated in inflammatory response and translocate to the nucleus and regulate the gene expression. Thus we proceeded to examine the effect of various pro- and anti-inflammatory stimuli on the localisation and distribution of NFKBIL-1 protein. HEK 293 cells were plated in 8 well chamber slides. The cells were incubated for 24 hours. Subsequently, they were transfected with NFKBIL-1-GFP vector. The cells were allowed to recover over night and stimulated with IL-1ß, TNF-α, Dexamethasone and PMA. The cells were fixed, permeabilised and stained with the nuclear stain DAPI and observed under Ziess LSM 510 Con-focal microscope. Image acquisition was performed using the software provided by the manufacturer.

3.2.2.1 NFKBIL-1 does not translocate in cells stimulated with pro-inflammatory cytokine IL-1ß

HEK 293 cells transfected with NFKBIL-1-GFP vector were stimulated with IL-1ß (10ng/ml) for various time points starting at 15 minutes up to 8 hours. The cells were fixed and stained with the nuclear stain DAPI and observed under the microscope. We did not observe translocation of NFKBIL-1 out of the nucleus at any time-point. The translocation of p65 confirmed that the cells were adequately stimulated (Figure 3.2.2).

Figure 3.2.2: NFKBIL-1 does not translocate from the nucleus in cells stimulated with IL-1ß (more information on page 115).



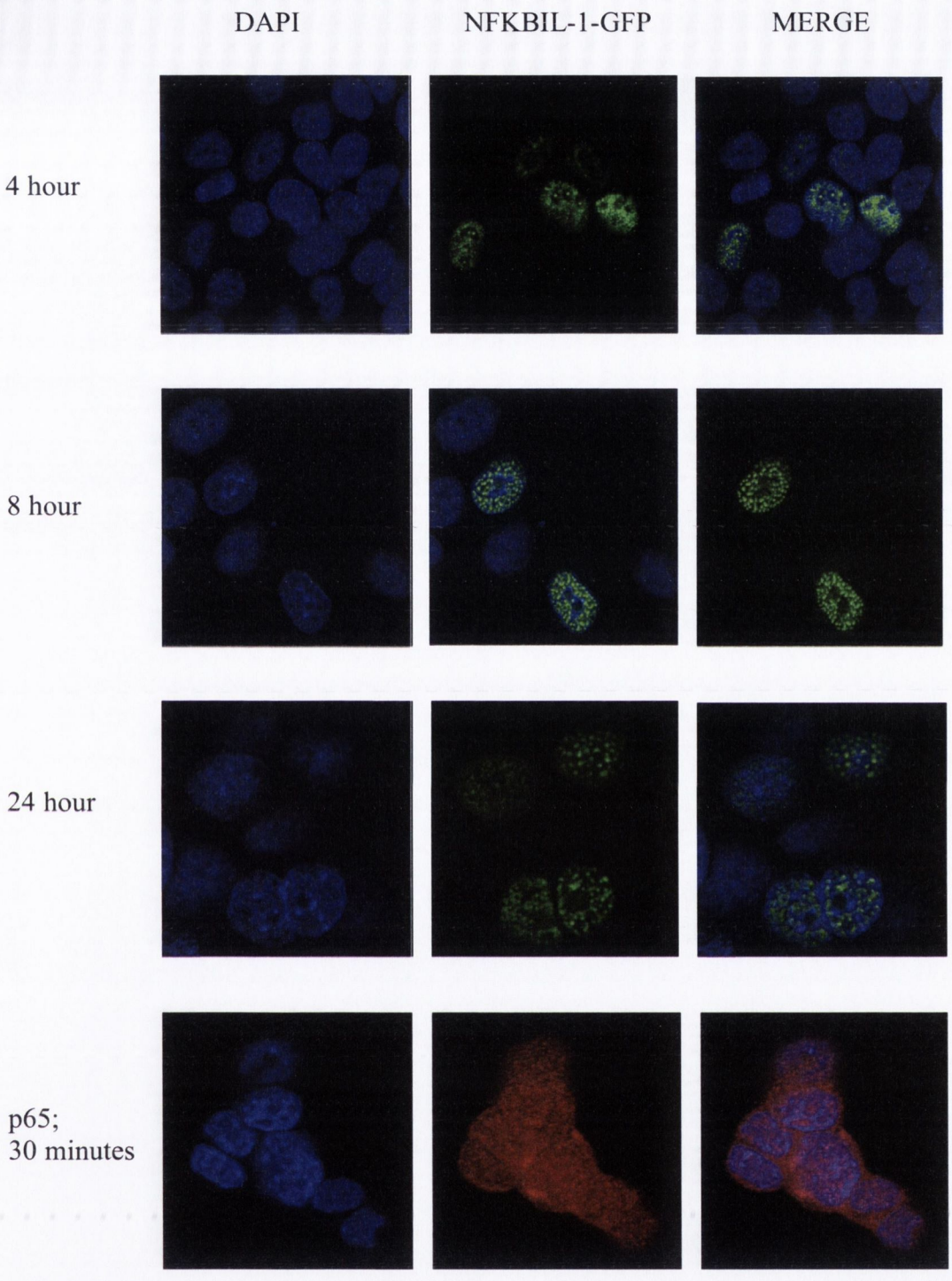


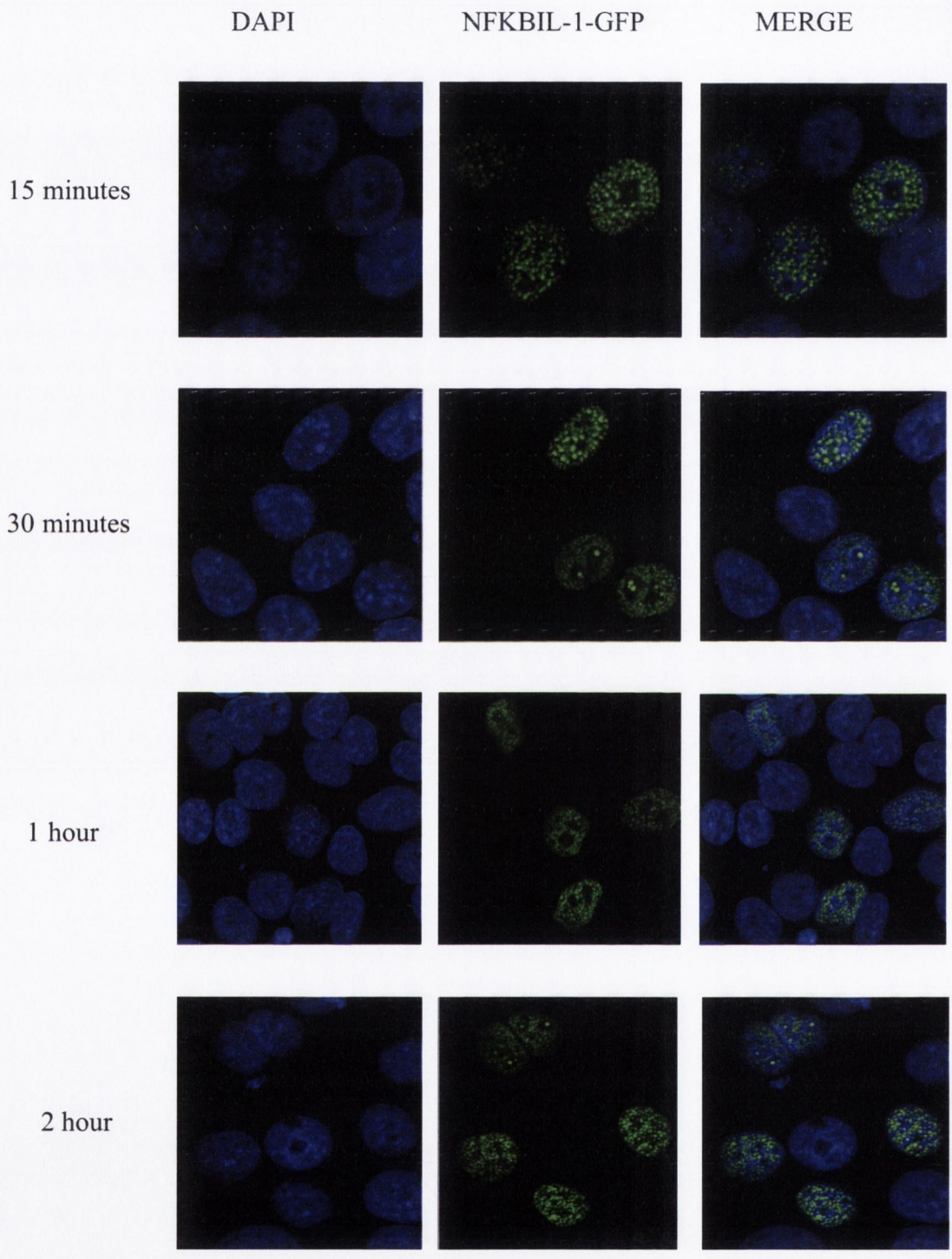
Figure 3.2.2 ***(Continued)*****: NFKBIL-1 does not translocate from the nucleus in cells stimulated with IL-1ß.** As a positive control cells were stimulated for 30 minutes and probed for the p65 subunit of NFκB (bottom).

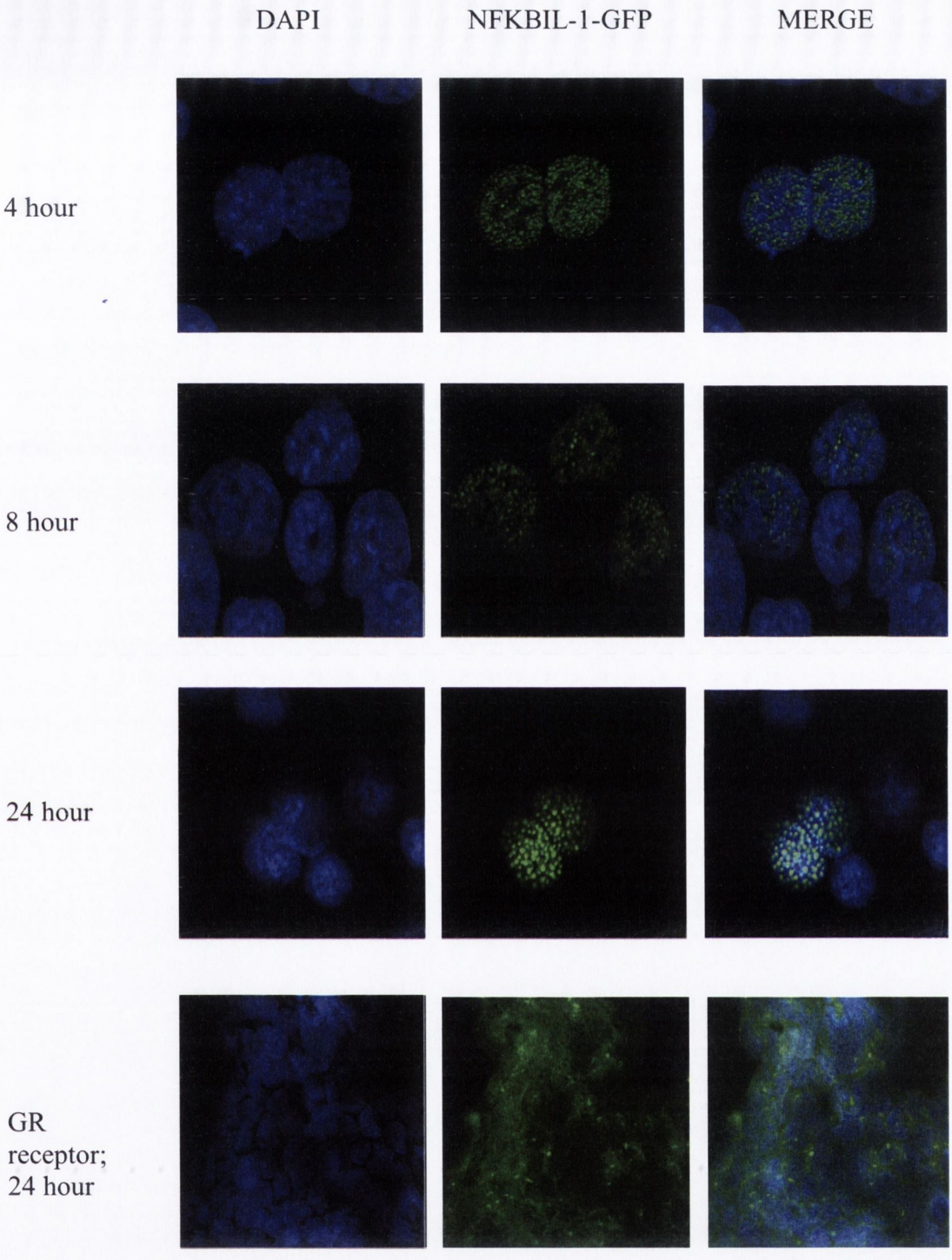
Figure 3.2.2: NFKBIL-1 does not translocate from the nucleus when stimulated with IL-1ß. 1x 10^5 HEK 293 cells were plated per well in 8-chambered glass slide. 24 hours later the cells were transfected with 1µg of NFKBIL-1/well. Cells were incubated for further 24 hours and then stimulated with IL-1ß (10ng/ml) for various time points ranging from 15 minutes to 24 hours. The cells were fixed and stained with DAPI and observed under Con-focal microscope. As a positive control the cells were stimulated for 30 minutes with IL-1ß (10ng/ml) and probed for p65 subunit of NFκB. Translocation of p65 confirmed adequate stimulation. Merged image demonstrates that NFKBIL-1 does not translocate out of the nucleus when stimulated with IL-1ß. Blue: Nucleus; Green: NFKBIL-1-GFP; Red: p65

3.2.2.2 NFKBIL-1 does not translocate in cells stimulated with anti-inflammatory agent Dexamethasone

HEK 293 cells transfected with NFKBIL-1-GFP vector were stimulated with Dexamethasone (10μM) for various time points ranging from 15 minutes to 24 hours. As described before, the cells were fixed and stained with the nuclear stain DAPI and observed under the microscope. Glucocorticoid receptor that binds to dexamethasone and translocates to the nucleus to regulate gene expression was used as a positive control. NFKBIL-1 did not translocate out of the nucleus at any particular time point suggesting that NFKBIL-1 was sequestered within the nucleus (Figure 3.2.3).

3.2.3 NFKBIL-1 does not translocate in cells stimulated with anti-inflammatory agent Dexamethasone (more information on page 119).





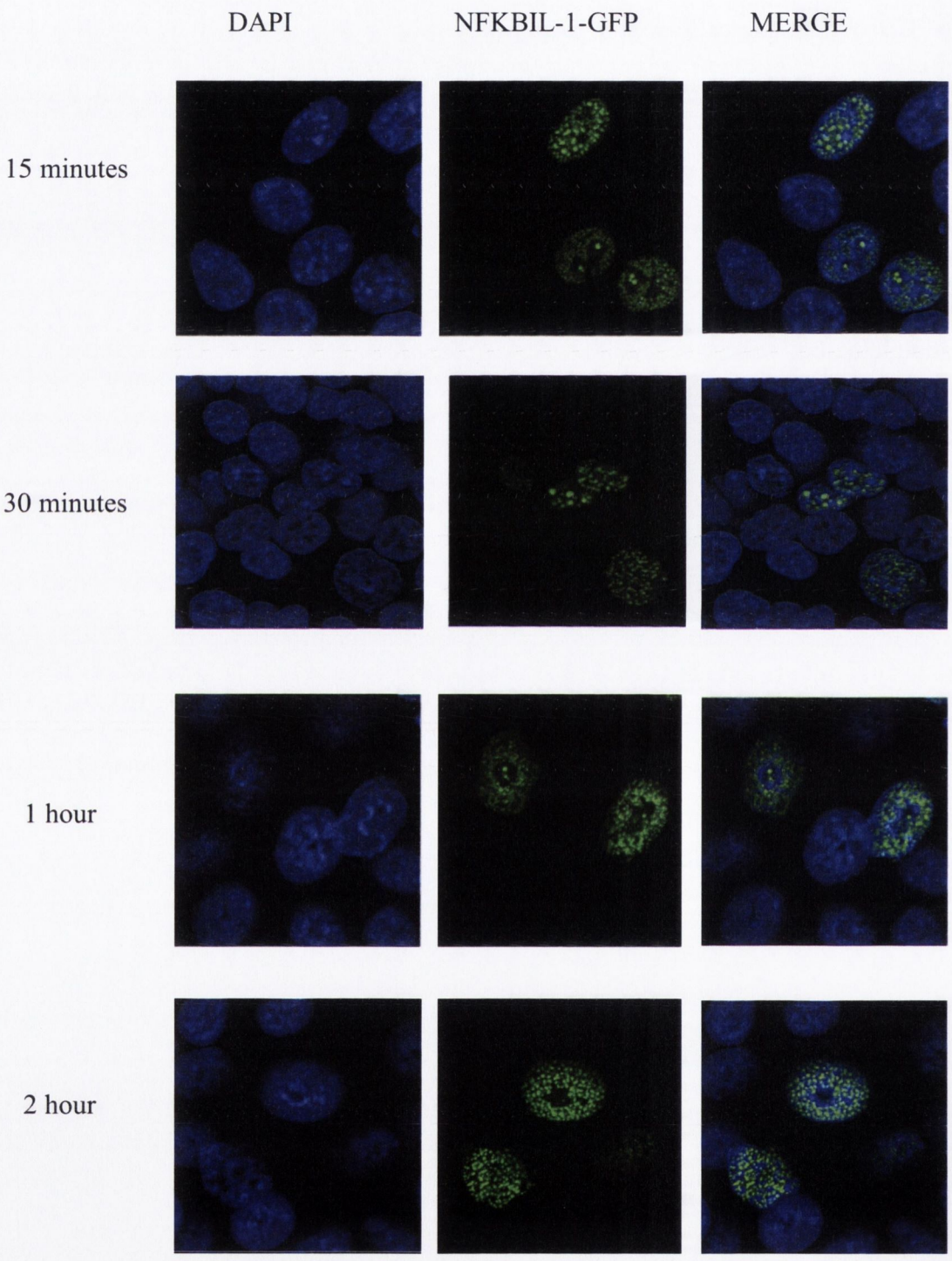
3.2.3 *(Continued):* NFKBIL-1 does not translocate in cells stimulated with anti-inflammatory agent Dexamethasone. As a positive control the cells were stimulated for 24 hours and stained for the glucocorticoid receptor (Bottom).

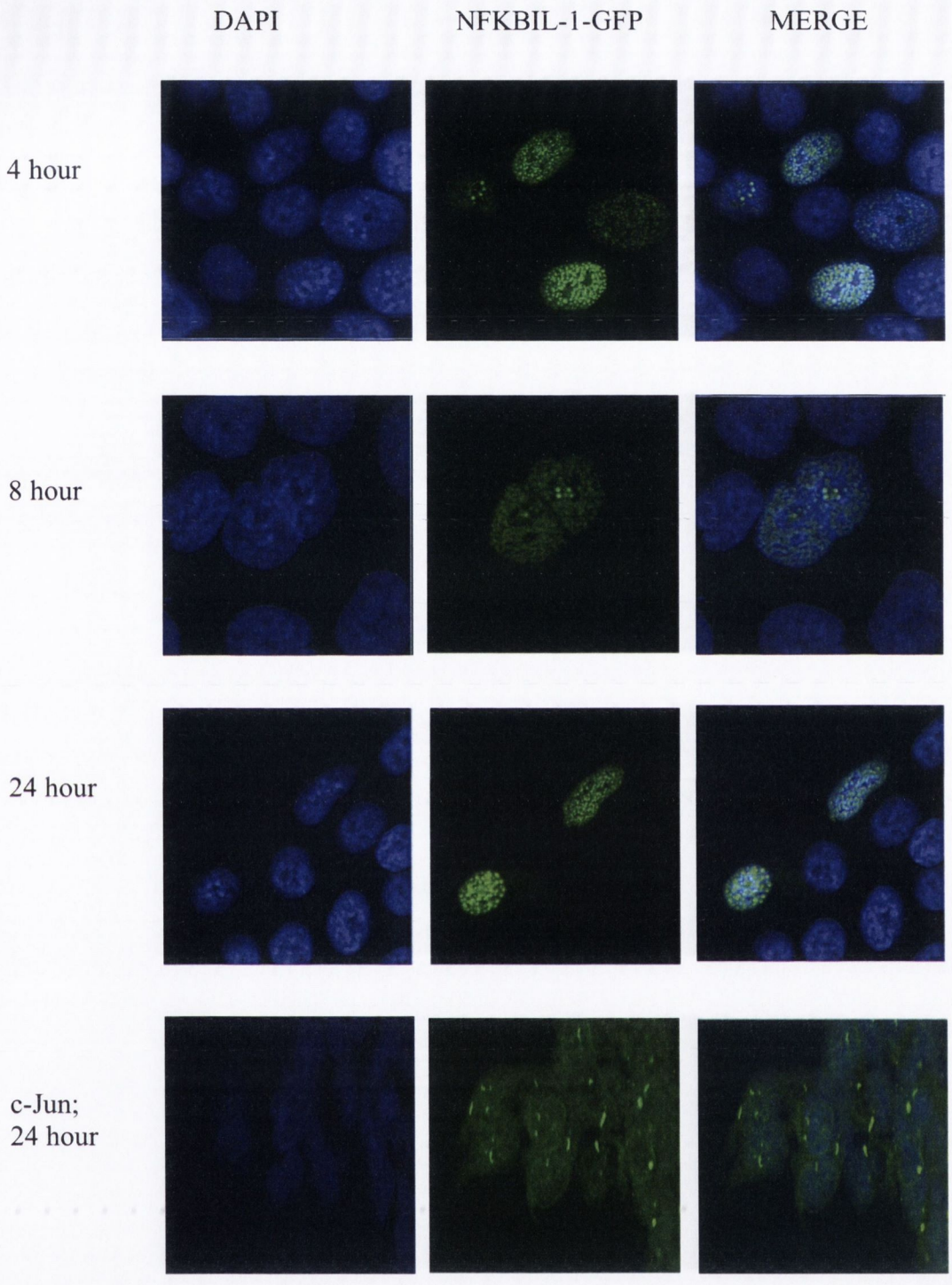
Figure 3.2.3: NFKBIL-1 does not translocate from the nucleus when stimulated with Dexamethasone. 1x 10^5 HEK 293 cells were plated per well in 8-chambered glass slide. 24 hours later the cells were transfected with 1μg of NFKBIL-1/well. Cells were incubated for further 24 hours and then stimulated with Dexamethasone (10μM) for various time points ranging from 15 minutes to 24 hours. The cells were fixed and stained with DAPI and observed under con-focal microscope. As a positive control the cells were stimulated with Dexamethasone for 24 hours and probed for the Glucocorticoid Receptor. Merged image demonstrates that NFKBIL-1 does not translocate out of the nucleus when stimulated with Dexamethasone. Since GR is present in every cell and because of confluency of the cells staining for GR appears indistinct. Blue: Nucleus; Green: NFKBIL-1-GFP; Green: Glucocorticoid Receptor (Bottom panel) (Figure 3.2.4).

3.2.2.3 NFKBIL-1 does not translocate in cells stimulated with PMA

PMA activates AP-1 transcription factor. Since we had previously demonstrated that NFKBIL-1 inhibits AP-1 activity, we investigated if this inhibition of AP-1 occurred within the nucleus or in the cytoplasm of the cell. HEK 293 cells transfected with NFKBIL-1-GFP vector were stimulated with PMA (10ng/ml) for various time points ranging from 15 minutes to 24 hours. Activation by PMA leads to the nuclear translocation of the AP-1 subunits including c-Jun. Hence, as positive control, the stimulated cells were probed for c-Jun. The cells were fixed and stained with the nuclear stain DAPI and observed under the microscope. Again NFKBIL-1 did not translocate out of the nucleus suggesting that regulation of AP-1 by NFKBIL-1 occurred within the nucleus (Figure 3.2.4).

3.2.4 NFKBIL-1 does not translocate in cells stimulated with PMA (more information on page 123).





3.2.4 *(Continued)*: NFKBIL-1 does not translocate in cells stimulated with PMA. As a positive control the cells were stimulated with PMA and probed for the c-Jun subunit of AP-1 (Bottom).

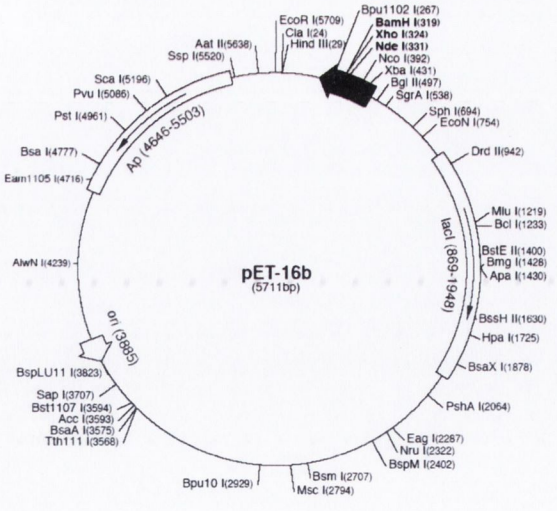
Figure 3.2.4: NFKBIL-1 does not translocate from the nucleus when stimulated with PMA. 1x 10^5 HEK 293 cells were plated per well in 8-chambered glass slide. 24 hours later the cells were transfected with 1μg of NFKBIL-1/well. Cells were incubated for further 24 hours and then stimulated with PMA (10ng/ml) for various time points ranging from 15 minutes to 24 hours. The cells were fixed and stained with DAPI and observed under Con-focal microscope. As a positive control the cells were stimulated with PMA for 24 hours and probed for c-Jun. Merged image demonstrates that NFKBIL-1 does not translocate out of the nucleus when stimulated with PMA. Blue: Nucleus; Green: NFKBIL-1-GFP; bottom panel Green: c-Jun

3.2.3 Characterisation of NFKBIL-1 native protein: synthesis of anti-NFKBIL-1 antibody

To enhance our understanding of NFKBIL-1 function, it was desirable to characterize the function of the native protein. For this we decided to generate an anti-NFKBIL-1 antibody. The first step in this process was to clone the NFKBIL-1 coding sequence into a protein expression cassette pET16b vector. This vector enables controlled expression of the recombinant protein in transformed bacteria.

3.2.3.1 Cloning of *NFKBIL-1* coding sequence into pET expression vector system.

pET16b vector (Novagen) is an expression vector derived from the plasmid pBR322. The multiple cloning sites include *Bam*H I, *Xho* I and *Nde* I. Since there are no *Xho* I or *Nde* I sites within the coding sequence of *NFKBIL-1*, we utilised these two restriction sites for cloning our inserts.



pET-16b sequence landmarks	
T7 promoter	466-482
T7 transcription start	465
His•Tag coding sequence	360-389
Multiple cloning sites (*Nde* I -*Bam*H I)	319-335
T7 terminator	213-259
lacI coding sequence	869-1948
pBR322 origin	3885
bla coding sequence	4646-5503

Figure 3.2.5: Vector map of pET16b expression plasmid. *NFKBIL-1* coding sequences were cloned using the *Xho*1 and *Nde* I restriction sites.

3.2.3.2 PCR amplification of the target sequences

The full-length NFKBIL-1, N- and C- terminal coding sequences were amplified by PCR. NFKBIL-1-GFP, described in section 3.1.2.1, was used as template. PCR primers were designed to contain the restriction sites *Xho1* and *Not1* that were subsequently used to clone the fragments into pET16b. The PCR product was gel extracted and cloned into the pET16b vector (Figure 3.2.6).

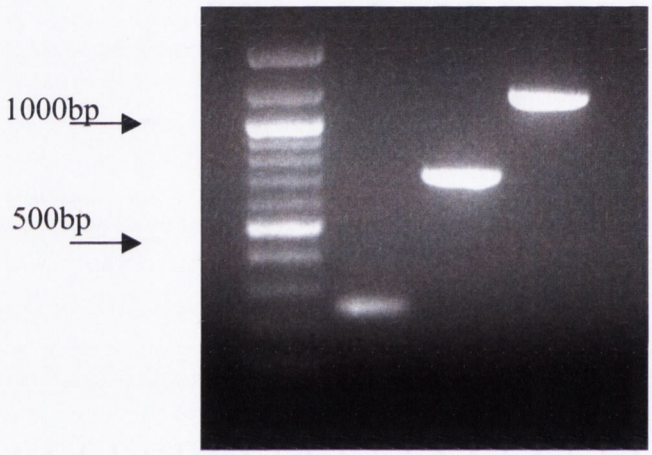


Figure 3.2.6: PCR was preformed to amplify coding sequences for full-length protein, N-terminal and C-terminal NFKBIL-1 peptides. Left to right: 100 bp DNA ladder, N-terminal fragment (size 246 bp), C-terminal fragment (size 780 bp), and full-length NFKBIL-1 fragment (size 1127 bp). The bright bands in the ladder correspond to fragment sizes of 500 bp and 1000 bp.

3.2.3.3 Expression and purification of His-tagged full-length recombinant NFKBIL-1 protein

Although we had successfully cloned all the three fragments i.e full length, N-and C-terminal) we decided to generate antibody against the full-length protein only. For this recombinant pET16b vector with successfully cloned inserts were transformed into bacteria and expressed as recombinant protein (see section 2.8.2 for details regarding the methodology). This protein was isolated and purified using affinity chromatography. Eluted samples from alternative columns were loaded into the bis/acrylamide gel and western blotting was performed to confirm the expression of the recombinant NFKBIL-1 protein. A single band was observed at the expected molecular weight for full-length NFKBIL-1-His tag protein. Maximum amount of recombinant protein was observed in the elution fraction 8 (Figure 3.2.7).

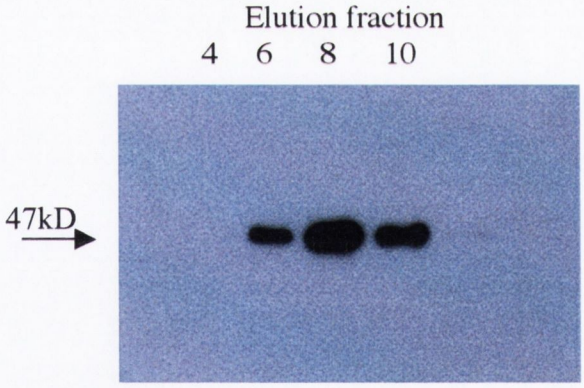


Figure 3.2.7: Recombinant full lengthNFKBIL-1-His protein was isolated, purified and probed on a western immunoblot using an anti-His antibody. Elution fraction 4, 6, 8 and 10 were loaded on the SDS/PAGE gel and Western blotting was performed. Arrow: Recombinant NFKBIL-1 protein

3.2.3.4 Generation of anti-NFKBIL-1 antibody

A polyclonal antibody was produced in a New Zealand White rabbit using subcutaneous immunisation with purified recombinant full length NFKBIL-1 protein in the Bioresource unit in Trinity College Dublin. The protocol followed is described at http://www.tcd.ie/Bioresources/documentsgeneral/antibodies.htm. Before the rabbits were immunised with the recombinant protein, pre-immune sera was collected which could be used as a negative control in future experiments to observe the specificity and sensitivity of the antibody. At the end of the protocol the Bioresource unit collected the immune sera. This serum was aliquoted and stored immediately at -20°C.

3.2.3.5 Testing anti-NFKBIL-1 antibody:

The frozen total serum provided from the immunised mice was thawed gently on ice. A series of Western blots were performed to confirm the presence of anti-NFKBIL-1 antibody. Recombinant full-length NFKBIL-1 protein, used to generate the antibody, was used as a positive control. Various different concentrations of antibody (1:100, 1:500, 1:1000 and 1:5000) were used to probe for the NFKBIL-1 protein. It was observed that 1:1000 dilutions were able to detect the recombinant protein. However, when total lysate from the HEK293 cells was subjected to western blot and probed for NFKBIL-1 using the antibody, non-specific bands were observed. Yet again, various different concentrations of the antibody (1:100, 1:500, 1:1000 and 1:5000) were used for these experiments (data not shown).

Simultaneously immunocytochemistry experiments were performed to identify the specificity of NFKBIL-1 antibody. Briefly $1x10^5$ HEK 293 cells were plated in 8 well chambered slides incubated for 24 hours. The cells were then fixed and probed using the polyclonal anti-NFKBIL-1 anti sera as described in section 3.2.3.5. As a negative control pre-immune serum from the same rabbit was used. Although a strong signal was detected in the cells probed for NFKBIL-1, the specific speckled patter, characteristic of NFKBIL-1 was not observed (3.2.8). This experiment was repeated by fixing the cells with both methanol and paraformaldehyde. As before different dilutions of the antibody were used. This included 1:50, 1:100, 1:1000 and 1:2000. The results suggested that the antibody lacked the required specificity to bind to NFKBIL-1 alone and that it was binding to several other proteins in the cell. This experiment was repeated with several different antibody titers but the staining pattern still remained non-specific. The lack of specificity could result from the recognition,

by the antibody, of several different common domains, such as the ankyrin repeat domains, that is common to NFKBIL-1 and many other proteins. Availability of antibody against the native protein would be essential to characterize the function of the protein.

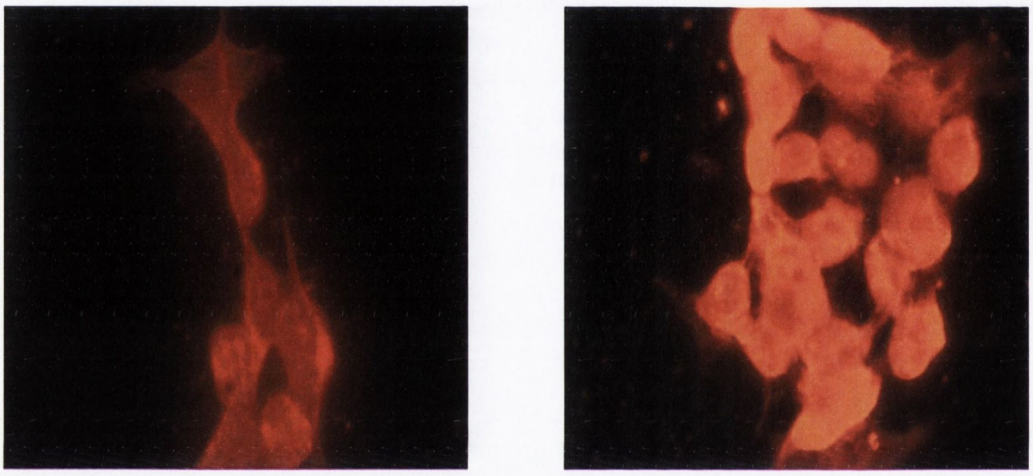


Figure 3.2.8: Anti-NFKBIL-1 antibody binds non-specifically to various proteins in HEK 293 cells. Immunocytochemistry was performed to identify the native NFKBIL-1 protein in HEK 293 cells. Cells were cultured in 8 well chamber slides, fixed and probed with anti-NFKBIL-1 antibody (Right). As a control cells were also probed with pre-immune sera (Left).

Results Section 3
3.3 Identification of proteins interacting with NFKBIL-1

We had previously demonstrated that overexpression of NFKBIL-1 inhibits the activity of NFκB, AP-1 and STAT1. NFKBIL-1 also inhibited TLR mediated NFκB activation and down regulated IL-8 expression. We also observed that when HEK 293 cells were transfected with NFKBIL-1-GFP expression vector and stimulated with various stimuli, there was no translocation of the protein from the nucleus. These findings clearly suggest that the inhibition of these various transcription factors by NFKBIL-1 most likely occurs within the nucleus. The distribution of NFKBIL-1 in specific sub-nuclear structures called speckles provided a further clue that it might constitute part of the transcription machinery or be involved in RNA processing. In order to discover the mechanism by which NFKBIL-1 inhibited NFκB and other transcription factors, we specifically wanted to identify if it interacted directly with the transcription factors, specifically either p65 or p50 subunits of NFκB. To examine this question, we employed immunoprecipitation and immunocytochemistry staining of NFKBIL-1-GFP expressing cells with antibodies against various potential interacting proteins.

3.3.1 Immunoprecipitation assay (IP)

HEK 293 cells were transfected with either NFKBIL-1-GFP or empty vector. Cells were collected and total protein was isolated. We performed immunoprecipitation assay employing an anti-GFP monoclonal antibody following the manufacturers instructions. The immunoprecipitated sample was subjected to western blotting and probed with anti-GFP antibody. Supernatant from the IP assay was included as a

control to check the efficiency of the assay. Our data showed that we were able to successfully perform the IP (Figure 3.3.1). We then extended our study to identify potential proteins interacting with NFKBIL-1. For this the IP samples were subjected to SDS/PAGE gel and stained with Commassie blue. No specific difference could be distinguished between NFKBIL-1 IP sample and eGFP IP sample. In order to optimise this experiment different reaction conditions were employed and the exeperiment was repeated several times. We were still not able to distinguish the differences between the samples.

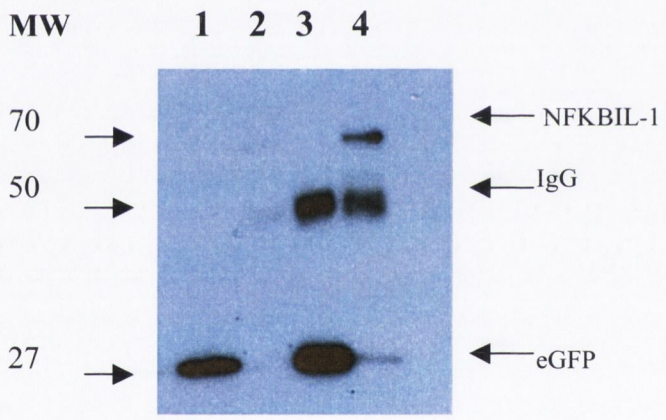


Figure 3.3.1: Immunoprecipitation of NFKBIL-1-GFP protein. Total protein isolated from HEK 293 cells was subjected to immunoprecipitation assay using an anti-GFP antibody. Western blot was performed and the membrane was probed for GFP. From left to right: Lane 1: IP supernatant from empty vector (eGFP) transfected lysate, Lane 2: IP supernatant from NFKBIL-1-GFP vector transfected lysate, Lane 3: IP pellet from empty vector (eGFP) transfected lysate, Lane 4: IP pellet from NFKBIL-1-GFP vector transfected lysate.

3.3.2 NFKBIL-1 does not co-localise with either p65 or p50 subunits of NFκB

As another approach to establishing the mechanism by which NFKBIL-1 exerts its effect over NFκB, we sought evidence of whether it interacts with either the p65 or the p50 subunits of NFκB in the nucleus. Most of the known inhibitors of NFκB act by interacting directly with the subunits of NFκB (described in section 1.3.1.1). Therefore, HEK 293 cells were plated in 8 well-chambered slides and transfected with NFKBIL-1-GFP expression vector. The cells were incubated for further 24 hours and stimulated with IL-1β (10ng/ml) for 2 hours. This was followed by the fixation and permeabilisation of the cells after which the cells were probed for the p65 and p50 subunits of NFκB. The cells were observed using Zeiss LSM 510 con-focal microscope and the image was analysed using the software provided. We did not observe any co-localisation between NFKBIL-1 and the either subunit of NFκB. Furthermore, the scanned image was also analysed using the integral software package that analyses the intensity of one fluorescent protein and compares it with the intensity of second fluorescent protein along a user defined cross-section of the image. If both the proteins co-localise, parallel change in the intensities of the proteins are observed. We did not observe such parallel changes in intensities with either p50 or p65 and NFKBIL-1-GFP, corroborating the visual evidence that these subunits of NFκB did not co-localise with NFKBIL-1.

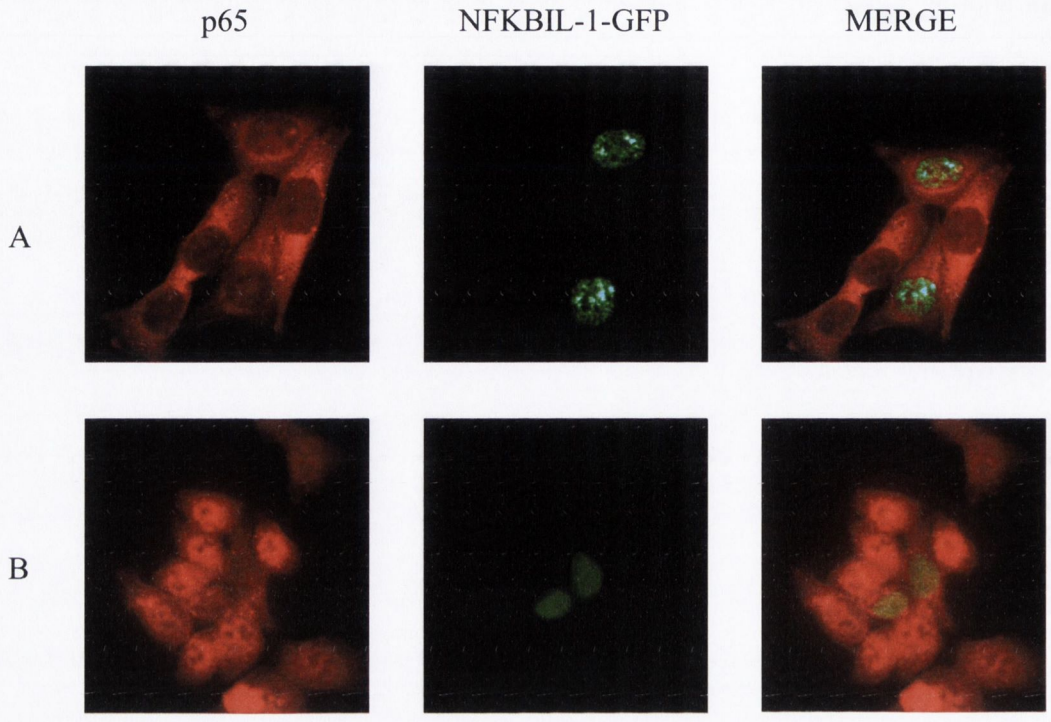


Figure 3.3.2: NFKBIL-1 does not co-localise with p65 subunit of NFκB. HEK 293 cells were plated on 8 well-chambered slides and probed for the p65 subunit of NFκB. Images were scanned using the Zeiss LSM 510 con- focal microscope. Top: Cells were either unstimulated (A) or stimulated with IL-1β (10ng/ml) for 2 hours (B). Merged images show that NFKBIL-1 does not co-localise with p65 in either unstimulated or stimulated cells.

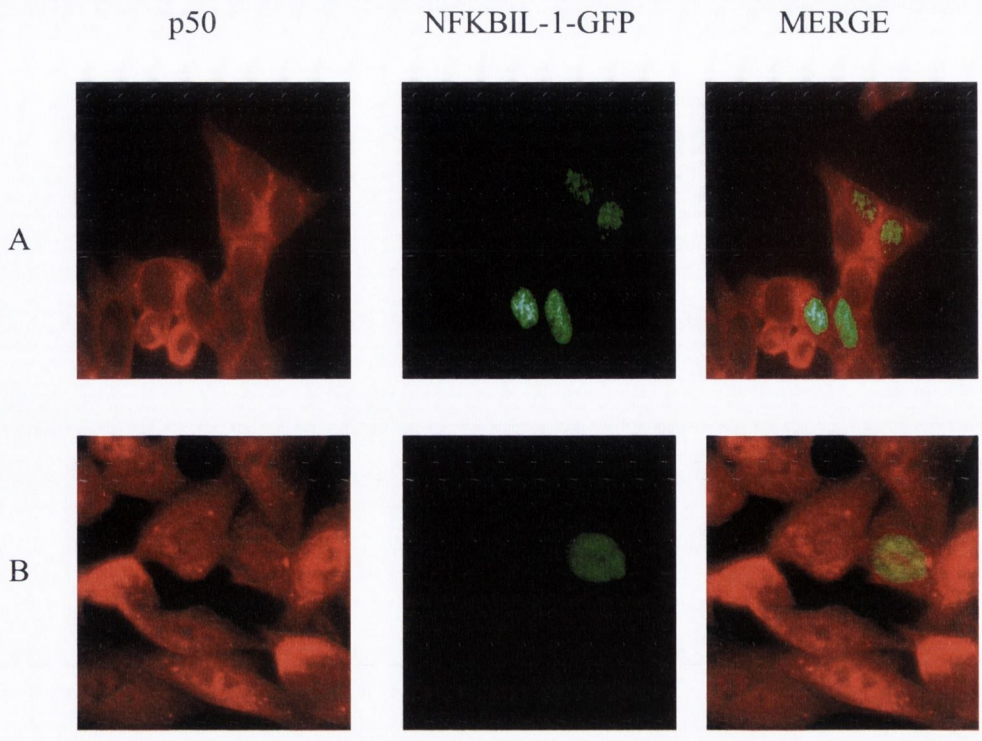


Figure 3.3.3: NFKBIL-1 does not co-localise with p50 subunit of NFκB. HEK 293 cells were plated on 8 well-chambered slides and stained for the p50 subunit of NFκB. Images were scanned using the Zeiss LSM 510 con-focal microscope. Top: Cells were either unstimulated (A) or stimulated with IL-1β (10ng/ml) for 2 hours (B). Again the merged images show that NFKBIL-1 does not co-localise with p50 in either unstimulated or stimulated cells.

3.3.3 NFKBIL-1 co-localises with HDAC3

Acetylation and deacetylation are one of the several known mechanisms for regulating transcription factors. As discussed in section 1.3.1.3, HDAC3 has been previously shown to inhibit NFκB activity (Chen and Greene, 2003; Greene and Chen, 2004). HDAC3 is known to also regulate AP-1 and can potentially regulate other transcription factors (Jin et al., 2002). As such it might represent a regulatory nexus which could be acted upon by other regulatory proteins, and thus potentially NFKBIL-1. Interestingly, HDAC3 is also distributed in nuclear speckles (Chen and Greene, 2003; Greene and Chen, 2004). In the absence of an interaction between NFKBIL-1 and the p65 or p50 subunit, we thus investigated if NFKBIL-1 colocalised with HDAC3. HEK 293 cells were therefore transfected with NFKBIL-1-GFP expression vector and probed for HDAC3. The cells were observed and imaged as before. We observed significant co-localisation between HDAC3 and NFKBIL-1. This is demonstrated by a histogram of signal intensities along a line shown in figure 3.2.12, which shows that the locations of NFKBIL-1 and HDAC3 overlap.

HDAC3 NFKBIL-1-GFP MERGE

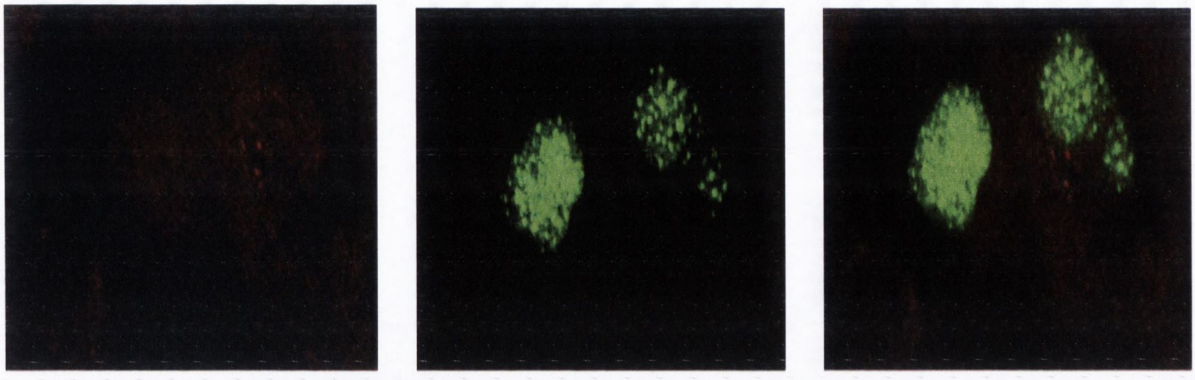
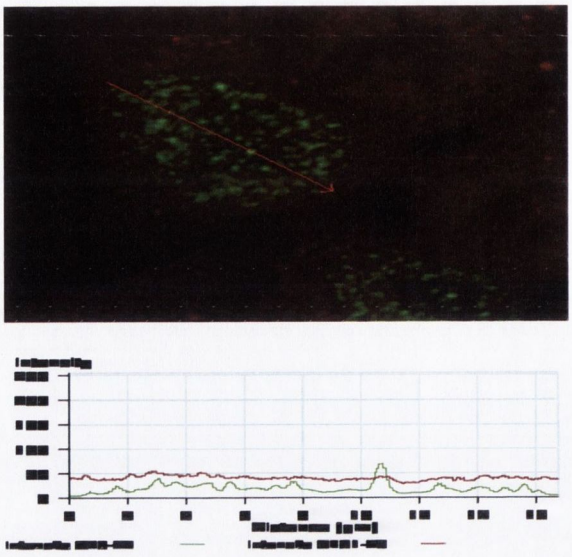
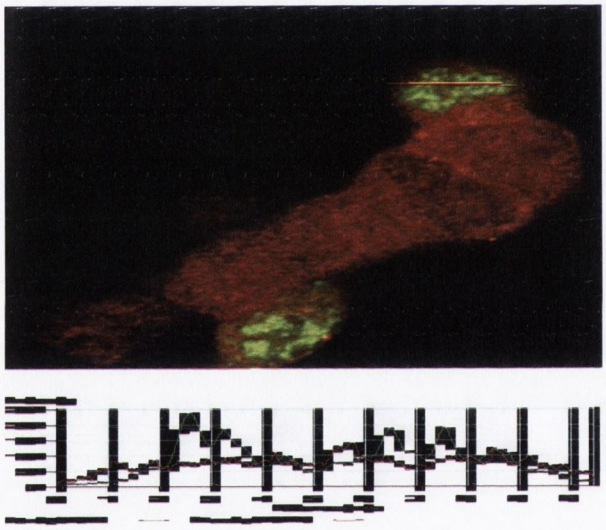


Figure 3.3.4: NFKBIL-1 co-localises with HDAC3. HEK 293 cells were plated on 8 well-chambered slides and stained for HDAC3. Images were scanned using the Zeiss LSM 510 con focal microscope. Top: Merged images show that NFKBIL-1 co-localises with HDAC3.

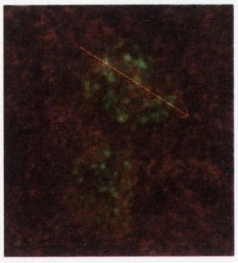
A



B



C



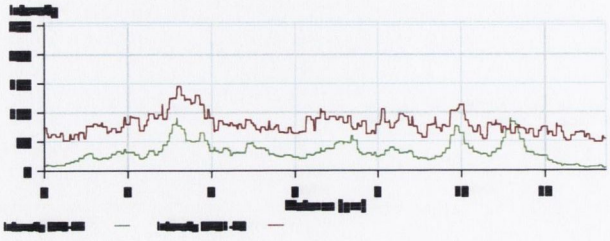


Figure 3.3.4: Parallel changes in fluorescent intensities. As an additional parameter the parallel changes between the red and green fluorescence was quantified from a cut section across the cell and shown as a graph. The software provided to quantify the change in fluorescence performed this. Red arrow marks the cut section. Bottom figure demonstrates the intensity of each protein in parallel. Two proteins that colocalise with each other show similar variation in the pattern of flourescence. A: NFKBIL-1 (green) does not colocalise with p65 subunit (red) of NFKB. B: NFKBIL-1 (green) does not colocalise with p50 subunit (red) of NFKB. C: NFKBIL-1 (green) colocalises with HDAC3 (red).

3.4 Discussion

Inflammation is an essential response closely allied to immunity and initiated by the cell to evade and destroy any external or internal insult or damage. Proper execution of this response requires the coordinated activity of several important transcription factors. NFκB, AP-1 and STAT protein complexes are a few of the many transcription factors that regulate the expression of genes involved in the cell cycle, apoptosis, inflammation and immunity allowing the cell to respond appropriately and adequately to stimuli. The regulation of these transcription factors is critical, as persistent activation could likely lead to sustained inflammation of the tissue, dysregulated cell division and un-coordinated antigenic challenge. Once the threat or damage is removed, the programme being executed by these transcription factors must be terminated and indeed the programmes are likely to be highly nuanced allowing for appropriate levels of response to the particular stimulus. Clearly therefore there is a requirement for a sophisticated system of regulation of these transcription factors.

The novel gene *NFKBIL-1* was first identified as a potential protein encoded within the MHC region. Analysis of its coding sequence illustrated considerable homology between *NFKBIL-1* and the known inhibitors of NFκB. Functional studies by Semple *et al.* have demonstrated that NFKBIL-1 is constitutively expressed within the nucleus in "speckles" (Semple et al., 2002). Other experiments investigating transcription factor binding of the *NFKBIL-1* promoter have shown binding by the E box protein, E47, and USF-1 (Boodhoo et al., 2004).

Our initial studies, using EMSA, showed that NFKBIL-1inhibited both IL-1ß and TNF-α induced binding of NFκB to its target DNA (Daly, J and McManus, R; unpublished data). Our current data confirms, by means of alternative techniques, that NFKBIL-1 is indeed an inhibitor of NFκB activity stimulated by a number of different signalling molecules including both IL-1β and TNF-α (Figure 3.1.1A and Figure 3.1.1B). This finding therefore suggested that NFKBIL-1 regulated NFκB activation at some point in the signal transduction pathway common to both IL-1β and TNF-α.

While a number of different stimuli activate NFκB, recombinant IL-1β and TNF-α proteins are commonly used, and well understood stimuli in the study of NFκB activity. Although IL-1β and TNF-α do not share sequence homology with each other and bind to distinct receptors, the intracellular signalling activated by the two cytokines converges at the adaptor molecules, TNF receptor associated factor (TRAF) 2 and TRAF 6, each of which lead to the activation of NFκB and MAP kinases. Consequently many of the target genes for IL-1β and TNF-α overlap. However the biological effects of the stimulation by both of these signalling molecules are different. For example, in addition to IκB ζ, other genes preferentially induced by IL-1β include Neutrophil gelatinise-associated lipcalin, the growth-related oncogene α homolog, the extracellular metalloprotease MMP-3 and IL-6. On the other hand, complement factor H was upregulated specifically by TNF-α. These observations suggest that stimulus-specific activators and repressors are present that determine the gene expression pattern in response to specific signalling molecules (Cowland et al., 2003; Elias and Lentz, 1990; Katz and Strunk, 1989; MacNaul et al., 1990; Ng et al., 1994; Ohmori and Hamilton, 1994; Tebo et al., 2000).

Recent studies have identified a potent mechanism of activation of NFκB in the innate immune system by a set of novel receptors called Toll like receptors (TLRs). The cytoplasmic domains of the TLR and the IL-1 receptor are homologous and use the same signalling pathways leading to the activation of NFκB. We had previously shown that NFKBIL-1 inhibited IL-1β and TNF-α activation of NFκB. Hence we investigated the role of NFKBIL-1 in TLR mediated signalling pathways. Normal HEK 293 cells lack both TLR2 and TLR4 receptors. Using HEK 293 cell lines stably transfected with TLR2 and TLR4 we performed a κB element driven, luciferase reporter assay. NFKBIL-1 significantly inhibited NFκB activation by these pathways thus establishing the role of NFKBIL-1 in the modulation of immune system responses (Figure 3.1.3A and Figure 3.1.4A). Toll like receptors are critical sensors in the immune system. They initially activate the innate immune system and subsequently, through various cytokines, trigger the adaptive immune response. NFκB is one of the major transcription factors activated by TLRs and is essential for eliciting the appropriate antigenic response. Other IκBs also inhibit TLR induced NFκB activity. Since NFKBIL-1 reduced NFκB activation in response to all stimuli tested here (including TLR2 and 4 specific ligands), it is possible that NFKBIL-1 may act like a generic NFκB inhibitor regardless of the nature of the stimulus. There are many other Toll like receptors that are activated by specific ligands and it will be interesting to see if NFKBIL-1 regulates NFκB activity when cells are exposed to these ligands.

Thus we have established that NFKBIL-1 inhibited NFκB activation in cells stimulated with IL-1β, TNF-α and TLR ligands. It should be noted that NFKBIL-1 also regulated on the basal NFκB activity. Indeed, it appears that NFKBIL-1 regulated

both the basal and stimulated NFκB activity. Since both TNF-α and TLRs also activate other transcription factors via MAPK and IRF pathways, we extended our study to identify the role of NFKBIL-1 in the regulation of other transcription factors. Interestingly, this showed that NFKBIL-1 inhibited both AP-1 and Jak/STAT induced gene expression (Figure 3.1.13 and Figure 3.1.14). Regulation of these two key transcription factors by NFKBIL-1 suggested that it had a far broader role than just being an inhibitor of NFκB and is potentially a broad specificity anti-inflammatory regulatory protein and may function as a transrepressor.

For our initial studies we used the HCT-116 cell line to investigate the function of NFKBIL-1. Since it is an epithelial cell line, one of the advantages of using it is the high transfection efficiency that can be achieved using standard transfection protocols. Since HCT-116 cell line is a tumor cell line we decided to replicate our results in a non-transformed 'normal' cell line. For this we chose the HEK 293 cell line since it has been extensively used to study the regulation of NFκB. Our results demonstrated that the inhibition of NFκB by NFKBIL-1 was common to both cell lines (Figure 3.1.2A and Figure 3.1.2B). The fact that NFKBIL-1 is widely, perhaps ubiquituously, expressed, raises the intriguing possibity that this is an important mechanism in regulating pro-inflammatory stimuli in all cell types throughout the body.

Inhibition of NFκB should, of course, have quantifiable effects on the expression of pro-inflammatory cytokines such as IL-1β and (either directly or indirectly) of anti-inflammatory cytokines such as IL-10. In order to demonstrate that the inhibition of NFκB was altering the activity of a downstream effector molecule important to the

function of the host cells being used in these experiments, the cellular response to IL-1β stimulation was investigated. Randox Evidence Investigator®, a novel multi-analyte Biochip Array platform for discovering the cytokine profile of cells, allows the quantification of 12 different cytokines and growth factors at the same time in the single sample. We observed that IL-8 was the major chemokine expressed in HEK 293 (Figure 3.1.5) and thus this was chosen as a readout to assess the impact of inhibiting NFκB through overexpression of NFKBIL-1 in these cells.

IL-8 is a member of the CXC subfamily of chemokines and is one of the major mediators of the inflammatory response. Many cell types, including monocyte/macrophages, T cells, neutrophils, fibroblasts, endothelial cells, keratinocytes, hepatocytes, chondrocytes, and various tumor cell lines, can produce IL-8 in response to a wide variety of pro-inflammatory stimuli such as exposure to IL-1β, TNF-α, LPS, and viruses (Kunsch and Rosen, 1993). Using an IL-8 promoter driven luciferase vector, we demonstrated that NFKBIL-1 strongly inhibits the expression of the IL-8 promoter (Figure 3.1.6). We went on to show that NFKBIL-1 inhibits IL-8 protein production by ELISA (3.1.7). Thus it appears that NFKBIL-1 activity can exert a real influence on the functioning of these cells and can inhibit their production of proinflammatory chemokines. Since we used Il-1β as the stimulus in these experiments, it is possible that NFKBIL-1 is exerting this effect through inhibiting both NFκB and AP-1 and possibly other transcription factors.

We also wished to investigate the means by which NFKBIL-1 was inhibiting these transcription factors. A number of different studies have revealed the central importance of the ankyrin repeat domains (ARD) to the functioning of IκB family

proteins. It has been convincingly demonstrated that IκB α and IκB ε require the ARD for nuclear translocation; however, the ARD of IκB β is ineffective at translocating the protein to the nucleus. Additionally, there is variability in the relative effectiveness of the different ARDs in promoting nuclear translocation. So, when stimulated, the ARD of IκB-α promotes rapid relocalization of the protein to the nucleus but in contrast, the ARD of IκB ε is less efficient (Lee and Hannink, 2002). Most proteins undergoing cytoplasmic nuclear shuttling require the help of other proteins such as importin α and CRM-1 (Faustino et al., 2007). While the nuclear export of some of the IκBs does require the interaction with CRM-1, it is the ARD-assisted nuclear import function that is particularly interesting. Although ARD motifs are very common, until now it was assumed that this motif is only required for protein-protein interaction. Current data suggests that apart from promoting nuclear translocation, the ankyrin repeats are also essential for inhibition of NFκB. For example, the transcriptional activity of NFκB is inhibited by full-length IκB ζ and even more by a C-terminal construct containing the ankyrin domain (Totzke et al., 2006). Hence we decided to investigate the role of the ARD of NFKBIL-1 in inhibition of NFκB. The N- and C- terminals (as defined by the junction with the ARD) and the alternative splice variant form of NFKBIL-1 were cloned into the GFP plasmid and the luciferase assays repeated (Figure 3.1.10). Our data shows that none of the partial NFKBIL-1 sequences are sufficient to inhibit NFκB or IL-8 expression, while the alternative isoform of the protein was functional in this regard (Figure 3.1.11 and Figure 3.1.12). These data suggest that the ankyrin repeat domains of the NFKBIL-1 protein are critical for the inhibition of NFκB and IL-8 promoter activity. It would be interesting to further subclone the N- and C- terminal NFKBIL-1 coding sequences with ankyrin repeats and in order to attempt to define the minimal protein

structure required to reconstitute inhibitory activity. A comparison between the results from the N- and C- terminal sequences with ankyrin repeats and without ankyrin repeats will provide further insights into the relevance of the ankyrin repeat domains in the function of NFKBIL-1.

The inhibitory effect of NFKBIL-1 appeared to be less potent than IκB α; there may be a number of potential causes for this observation, which was however consistent. One plausible reason is that while IκB α inhibits NFκB at two different levels: first in the cytoplasm by binding and sequestering NFκB subunits and preventing their nuclear translocation and secondly, by binding and exporting out of the nucleus any p65 subunit of NFκB that might be active, NFKBIL-1 must inhibit NFκB activity by means of a different mechanism. In any case it is clear that different inhibitors of NFκB have different characteristics, thus offering potential for nuanced regulation. At a mechanistic level, since IL-1β, TNF-α and the TLR ligands LPS and PAM_2CSK_4 activate the IKK complex via different adaptor proteins, it was evident that NFKBIL-1 must regulate NFκB activation either at the level of this complex or at some step further downstream in the pathway. The fact that NFKBIL-1also inhibits AP-1 and STATs would indicate that it acts at a terminal stage of the activity of these transcription factors, perhaps in the nucleus (discussed below).

In this light, it is worthy of note that all the three transcription factors inhibited by NFKBIL-1 shuttle between the cytoplasm and nucleus. Therefore one mechanism by which NFKBIL-1 might act, may be to promote the export of these transcription factors to the cytoplasm and thus terminate their action. Alternatively, it is possible that they interact at the level of the transcription factor / initiation complex. This

complex consists of many different proteins including core members such as, CREB, CBP/p300, HAT and RNA pol II. Interestingly like NFKBIL-1, RNA pol II and CBP have been observed to be distributed as speckles (described in section 1.6 and results section 2). Thus it is possible that NFKBIL-1 may be part of this general complex and consequently regulate the ability of these transcription factors to promote transcriptional initiation.

SNPs in the *NFKBIL-1* gene have been associated with several different diseases such as Myocardial infarction and rheumatoid arthritis. One of the common underlying themes in these diseases is the contribution of dysregulated inflammatory pathways to the pathogenesis of the disease. In this regard it is interesting to note that the transcription factors NFκB, AP-1 and STAT have all been implicated in the pathogenesis of Rheumatoid Arthritis, Myocardial Infarction and Inflammatory bowel disease (Firestein and Manning, 1999; Frantz et al., 1999; Negoro et al., 2000; Neurath, 1998; Neurath et al., 1998; Shiozawa et al., 1997; Yoshiyama et al., 2001). It is possible that the SNPs in *NFKBIL-1* might influence the protein function by either decreasing the amount of protein available or by decreasing the potency of its action. Indeed, data from this lab (Caraher, E and McManus, R; unpublished data) and others show that the -62 promoter polymorphism is functional and acts to reduce NFKBIL-1 expression on the coeliac disease associated MHC haplotype.

We next decided to extend our studies to identify potential mechanisms of transcription factor regulation by NFKBIL-1. For this, we decided to characterize *NFKBIL-1* gene products. First of all, we investigated the effect of various pro- and anti- inflammatory stimuli on the expression of NFKBIL-1 (Figure 3.2.1). We

observed that NFKBIL-1 was expressed constitutively and in large amounts. Secondly, there was no change in the expression of NFKBIL-1 in cells stimulated with IL-1β when compared to the resting cells. There are a number of potential explanations for this: NFKBIL-1 mRNA may be long lived and thus even though transcription may be decreased (as previous studies using promoter constructs would indicate (Caraher, E and McManus, R; unpublished data) we do not see an appreciable reduction in mRNA levels. It is even possible that NFKBIL-1 mRNA is being stabalized by IL-1β in a negative feedback loop (as is the case with IκB ζ, discussed below) leading to a steady state of expression.

We then investigated if NFKBIL-1 expression was altered in cells stimulated with Dexamethasone. Dexamethasone is a steroidal agent that binds the glucocorticoid receptor (GR) that then dimerises and translocates to the nucleus where it binds to DNA with the GR binding site element (GRE). Glucocorticoids profoundly influence the state of the cell and exert powerful anti-inflammatory effects. Among the many genes whose expression is affected, glucocorticoids up-regulate IκB α mRNA expression in a time dependent manner (Quan et al., 2000) and thus mediate the anti-inflammatory response. The NFKBIL-1 promoter has consensus sequences corresponding to GREs. Indeed, we did observe an increase in the expression of NFKBIL-1 transcript in cells stimulated with Dexamethasone. This is in keeping with an earlier finding in the lab that promoter constructs of the NFKBIL-1 gene are upregulated by Dexamethasone (Caraher, E and McManus, R; unpublished observations). Glucocorticoid-mediated increase in the expression of NFKBIL-1 would be expected to further lead of inhibition of NFκB given our findings. Therefore, it is possible that the very extensive anti-inflammatory effects of

glucocorticoids, apart from their actions on other proteins such as IκB α and a variety of signalling pathways, may also be mediated by NFKBIL-1.

Negative autoregulatory loops provide an effective mechanism for controlling NFκB activity, especially because fast and slow negative feedback loops exist. In contrast to the rapid IκB α expression, IκB ε represents a slower negative feedback regulatory mechanism. However IκB α, IκB ζ and Bcl3 expression are induced rapidly, suggesting that they are part of the fast negative feed back loop. For instance, upon cell stimulation, IκB α is rapidly degraded leading to an immediate, but transient activation of NFκB. In contrast, IκB β persists over a longer time and its degradation causes a more delayed and sustained activation of the transcription factor (Totzke et al., 2006).

Some inhibitors such as IκB α require strong transactivation post stimulation while others such as IκB ζ require sustained mRNA stabilisation. Paradoxically, stability of IκB ζ mRNA was shown by Totzke *et al.* to be specifically increased by stimulation with LPS or IL-1β, but not with TNF-α. However co-stimulation with TNF-α and IL-17 did induce IκB ζ expression suggesting that IκB ζ mRNA expression is regulated by another pathway (Totzke et al., 2006). This demonstrates that many (or perhaps even all) pro-inflammatory stimuli also carry within them the seeds of their own demise in the form of nascent negative regulatory loops

We next wanted to identify the potential mechanism by which NFKBIL-1 inhibited transcription factors. Since our results showed that NFKBIL-1 inhibited IL-1β stimulated NFκB activation and PMA activated AP-1 activity, we wished to

investigate the effects of these and other stimuli on the expression and localisation of NFKBIL-1. HEK 293 cells transfected with NFKBIL-1-GFP were stimulated for various time points ranging from 15 minutes to 24 hours and observed under confocal microscope. The cells were probed for p65 as positive control for stimulations with IL-1β. Anti-c-Jun and anti-GR antibodies were used as positive controls for PMA and Dexamethasone stimulations respectively. We did not observe any translocation of NFKBIL-1 in cells stimulated with IL-1β, Dexamethasone or PMA at any time point (Figure 3.2.2, Figure 3.2.3 and Figure 3.2.4 respectively). Proteins that are imported into the nucleus in general, require the nuclear localisation sequence (NLS). IκB proteins lack a conventional NLS but still are imported into the nucleus. It was demonstrated that the ankyrin repeat domains facilitated the IκB nuclear import (Sachdev et al., 1998) . NFKBIL-1 possesses both ankyrin repeat domains as well as an arginine rich domain known to function as an NLS. Thus it is possible that either or both of these domains promote the nuclear localisation of NFKBIL-1.

The lack of translocation of NFKBIL-1 under various stimulations suggests that the inhibition of NFκB, AP-1 and STAT1, as demonstrated here, occurs within the nucleus, by an unknown mechanism. Taken with the observation that these pathways do not share common elements within the cytoplasm, this lack of translocation indicates that the nexus for their interaction with NFKBIL-1 is most likely to be within the nucleus. Possible mechanisms include interaction of NFKBIL-1 with other general regulators of transcription such as for example, histone acetyl transferases (HATs) or deacetylases (HDACs) giving rise to post-translation modification of some of the subunits of these transcription factors.

The NFKBIL-1-GFP fusion protein, while a useful tool to allow visualisation of the protein within the cell and detection by means of the GFP moiety, may not faithfully reflect the properties of the native NFKBIL-1 protein. In the absence of a commercial antibody against NFKBIL-1, we decided to raise an anti-NFKBIL-1 antibody. For this the full length, N-terminal and C-terminal coding regions of NFKBIL-1 gene were cloned into the pET16b expression vector. We decided initially to raise antibody against the full-length protein only. For this the full-length recombinant NFKBIL-1 protein was isolated, purified, quantified and injected into rabbits as per standard protocols (Figure 3.2.5, Figure 3.2.6 and Figure 3.2.7 respectively). This part of the project was carried out at the Bioresource unit of Trinity College Dublin. In order to test the specificity and sensitivity of the antibody we performed immuno-cytochemistry probing for the native NFKBIL-1 protein (Figure 3.2.8). We observed non-specific staining patterns. From the immunostaining images it appears that staining was very generalised suggesting that although this antibody might be detecting the native NFKBIL-1 protein, its specificity was very broad. Since it is a polyclonal antibody, it is impossible to predict the actual epitope(s) against which the antibody was binding, however it is likely that there are many potential antibody-binding sites reducing the specificity of the antibody. In particular the presence of ankyrin repeats may have allowed the antibody to bind many proteins containing this motif. We had decided to generate antibodies against NFKBIL-1 using synthetic peptides based on unique sequences identified from NFKBIL-1. However shortage of time prevented us from completing this particular project. It would be very helpful to have an antibody against the native NFKBIL-1 protein.

In order to discover the mechanism by which NFKBIL-1 inhibited various transcription factors, we wanted to identify the proteins interacting with NFKBIL-1. For this we first employed the standard immunoprecipitation technique. Using a monoclonal anti-GFP antibody we performed pull down assays of the NFKBIL-1-GFP and eGFP protein from HEK 293 cell total lysates. We were able to successfully isolate the GFP proteins (Figure 3.2.9A). However, when we subsequently performed a SDS/PAGE gel to identify potential NFKBIL-1 interacting patterns, we observed a number of non-specific protein bands (3.2.9B). This was repeated several times with the same results. Further optimisation needs to be done to clean up the IP sample leading to a better outcome.

Although we have previously demonstrated that NFKBIL-1 inhibited the activity of NFκB, AP-1 and STAT1, it is clear that NFKBIL-1 did not migrate from the nucleus under any of the conditions we tested, at any time. It appears therefore to be an exclusively nuclear located protein and if this is true, it follows that regulation of transcription factors by NFKBIL-1 must occur due to events limited to the nucleus.

Well-characterized regulators of NFκB such as IκB α inhibit its activity by not only binding with it in the cytoplasm but also enforce termination of transcriptional activity by binding with NFκB in the nucleus and promoting the translocation of NFκB from the nucleus back to the cytoplasm. Other inhibitors such as Bcl3 bind with p50 subunit of NFκB and decrease the binding of NFκB to DNA. Other known processes by which transcription factors are regulated include addition/removal of phosphate or acetyl groups to the protein. Not only may the transcription factors be modified directly, but enzymes such as HAT catalyse the addition of acetyl moieties to the

histones while HDAC proteins remove the acetyl group. In general, increased levels of histone acetylation are associated with looser binding of histones by DNA, giving rise to a more permissive chromatin structure and increased transcriptional activity. On the other hand, decreased levels of acetylation are associated with repression of gene expression.

Unlike other known inhibitors of NFκB, NFKBIL-1 did not co-localise with either the p65 or the p50 subunit of NFκB (Figure 3.3.2 and Figure 3.3.3). This suggested that NFKBIL-1 might regulate NFκB by another indirect process. Given the range of potential interactions involved in transcription initiation and the lack of interaction of NFKBIL-1 with either p50 or p65, we wanted to explore the possibility that NFKBIL-1 exerted its regulatory influence by interacting with other general regulators of transcription. Histone Deacetylase 3 (HDAC3) has previously been reported to be an important specific regulator of NFκB activity (Chen and Greene, 2003), as well as being capable of regulating transcription generally through histone modification as discussed above. Interestingly in an unrelated study, Djabali *et al.* reported that HDAC3 was distributed ia a nuclear speckle-like pattern (Djabali and Christiano, 2004). Hence we examined whether NFKBIL-1 co-localised with HDAC3. Con-focal microscopy images visually confirmed a high degree of co-localisation of NFKBIL-1 with HDAC3 (Figure 3.3.4). Although we were unable to provide statistical evidence for the significance of this co-localisation we were able to confirm it by using image analysis software where we observed parallel changes in the intensities of NFKBIL-1 and HDAC3 (Figure 3.3.4). While co-localisation of NFKBIL-1 with HDAC3 itself might not suggest an obvious mechanism for the inhibition of various transcription factors by NFKBIL-1 or even direct interaction of HDAC and NFKBIL-1, it seems

likely that NFKBIL-1 might be a part of a bigger complex that regulates transcription factors. It is also possible that NFKBIL-1 can influence, directly or indirectly, the function of HDAC3 and consequently affect the acetylation status of NFκB. STAT1 is also known to be acetylated at Lys410 and Lys413 and acetylation at these residues allows interaction of STAT1 and p65 (Kramer et al., 2006). Interestingly STAT1 and p65 interact synergistically to increase pro-inflammatory gene expression although certain classes of genes are negatively regulated. Both STAT1 and p65 interact with the HAT CBP/p300 and HDAC3 and HDAC1 among others (Ito et al., 2007). Thus intervention at these junctures clearly is a mechanism by which NFKBIL-1 could modulate both of these transcription factors whose signalling pathways do not converge further upstream. In the absence of evidence regarding the effect of acetylation on the activity of AP-1 it is difficult to determine the mechanism for its inhibition by NFKBIL-1. However it has been reported in *Drosophila* that AP-1 can terminate NFκB activity by causing the recruitment of dHDAC1 to specific promoter sites, showing a capacity to interact with at least one HDAC (Kim et al., 2005) and it has been shown that HDAC1 can inhibit AP-1 binding of DNA in pancreatic stellate cells although the manner by which it does this has not been elucidated (Bulow et al., 2007). What is clear therefore is that all of these transcription factors have intimate relationships with members of the acetylation and deacetylation enzyme families, indicating that regulation of acetylation status by NFKBIL-1 - either of histones or the enzymes themselves - could plausibly take place at this point.

In conclusion, our study demonstrated that NFKBIL-1 was localised specifically within the nucleus and did not translocate even when stimulated. We also observed that the anti-inflammatory action of glucocorticoids might be due in part to increased

expression of NFKBIL-1. Further studies identifying other interactions of NFKBIL-1 or the consequences of interactions between NFKBIL-1 and HDAC3 or other HATs and HDACs on transcription factors may give us a better understanding of the exact regulatory mechanism of various transcription factors by NFKBIL-1.

Chapter 4

General Discussion

4.0 General Discussion
4.1 Introduction

Cells reprogram gene expression in response to environmental changes to maintain homeostasis. This cellular homeostasis may be disrupted by numerous factors. Inflammation is a physiologic response that is triggered by tissue damage resulting, for example, from microbial infection, chemical irritation, and/or wounding. This response, initiated by the local release of cytokines and chemokines, terminates with the resolution of inflammation and the return of the cell to its normal state.

Although required for normal homeostasis, inflammation is also a major element in the pathogenesis of both acute and chronic diseases such as Sepsis, Rheumatoid arthritis, Inflammatory Bowel Disease and Myocardial Infarction. All these diseases involve activation of pro-inflammatory proteins. NFκB, AP-1 and STAT proteins are pro-inflammatory transcription factors whose mechanism of action, regulation and contribution to inflammatory process has been the extensively studied. Rheumatoid Arthritis, a systemic disease initiated by immune complexes and complements, cripples patients by progressively destroying cartilage and bone (Weissmann, 2006). In this disease, immune complexes signal *via* the MEK kinase that apart from activating AP-1, also activates NFκB (Firestein and Manning, 1999). Likewise in Inflammatory Bowel Disease, there exists chronic inflammation of the intestinal tract (Hanauer, 2006). Here again there is evidence that suggests the contribution of NFκB and AP-1, leading to dysregulated cytokine production, in the pathogenesis of this condition (Neurath, 1998) and indeed, it has been conclusively demonstrated that the expression of the p65 subunit of NFκB is increased in intestinal biopsy specimens from patients with active Crohn's disease (Schreiber et al., 1998). Meanwhile the role

of the Jak/STAT pathway in acute myocardial infarction was explored by Negoro and colleagues. They reported that inhibition of Jak/STAT pathway activation in the healthy border myocardium increased Bax and caspase-3 activation, which resulted in augmentation of myocyte apoptosis in the damaged zone in acute myocardial infarction (AMI) hearts. These data demonstrated a potential cytoprotective role of the Jak/STAT pathway in AMI (Negoro et al., 2000). It is notable that SNPs in *NFKBIL-1* have been associated with Rheumatoid arthritis, Inflammatory Bowel Disease and Myocardial Infarction. Data from this lab shows a genetic association of *NFKBIL-1* with coeliac disease. Our data, demonstrating that NFKBIL-1 inhibits NFκB, AP-1 and STAT1, suggests that NFKBIL-1 is a novel anti-inflammatory protein of broad specificity. This hypothesis is further strengthened by our observation that Dexamethasone, a known potent anti-inflammatory agent, up regulates the expression of NFKBIL-1. Furthermore, our data that NFKBIL-1 co-localises with HDAC3 provides potential insights into the mechanistic basis for the anti-inflammatory property of NFKBIL-1.

4.2 Nuclear Regulation of Transcription Factors

As discussed, NFKBIL-1 appears to exert control over transcription factors including NFκB. NFκB is a widely expressed transcription factor that regulates the expression of a diverse array of cellular genes that are important in immunity, inflammation and development. Given the complexity of cell signalling and the possibility that slight perturbations of these pathways may be involved in disease pathogenesis, it is crucial to understand how multiple intracellular signalling pathways converge to activate a single transcription factor and conversely how a single pathway can stimulate many

transcription factors. NFκB undergoes rapid nucleocytoplasmic shuttling when activated. In the cytoplasm, NFκB is present as an inactive complex by physically associating with inhibitory molecules. Extensive studies have been performed to address how various stimuli trigger its translocation from the cytoplasm to the nucleus. Seminal works from several laboratories have determined a sequence of biochemical events that result in the ubiquitination dependent degradation of IκB proteins leading to the translocation of NFκB to the nucleus and subsequent modulation of the expression of target genes.

Name of the regulator	Mechanism of Action
Bcl3	**Binds to p50 and p65**
IκB ζ	**Binds to p50 and p65**
UXT	**Binds to p65**
ZAS3	**Competes for κB regulatory elements**
HATs	**Acetylate histones and transcription factors**
HDACs	**Deacetylate histones and transcription factors**
Kinases (IKKα, MSK-1, etc)	**Phosphorylate NFκB subunits**

Table 4.1: Nuclear regulators of NFκB. A number of proteins bind to NFκB subunits and regulate its activity. The acetylation and deacetylation of NFκB subunits regulates the binding of NFκB subunits to DNA while phosphorylation can either promote transactivation or repress it.

The regulation of this diverse array of target genes requires the precise control of NFκB, which like any other biochemical pathway occurs at multiple levels, and is achieved by various mechanisms including post-translational modification and

subcellular compartmentalization as well as by interactions with other cofactors or co repressors. Much less is understood concerning the active regulation and functional interaction of NFκB with other proteins inside the nucleus. Recent progress has shed light on the importance of nuclear events in shaping the strength and duration of the NFκB transcriptional response, which is achieved partly by post-translational modification of the NFκB transcription factor complex or the histones that surround various NFκB target genes. For instance, Sun *et al.* have recently characterized a novel protein UXT and identified it as an essential co-factor for NFκB function. They demonstrated that UXT directly interacts with the p65 subunit of NFκB and suggested that most probably it decreased the amount of nuclear p65 (Sun et al., 2007). Similarly, Hong *et al.* reported another novel protein ZAS3 that inhibited NFκB activity. They showed that ZAS3 interferes with NFκB mediated transcription by competing for κB gene regulatory elements and by repressing transcription. Additionally ZAS3, or most probably an isoform of ZAS3, binds to TRAF2, thereby inhibiting nuclear translocation of p65 (Hong et al., 2003). These novel proteins are part of a group of regulators of NFκB localised within the nucleus and hence may play a significant role in the termination of NFκB activity (Table 4.1).

Regulation of gene expression by NFκB requires the interaction of numerous cofactors within the nucleus. In unstimulated cells NFκB genes are basally repressed by p50 or p52 dimers (Silverman and Maniatis, 2001). For this action the p50 dimers recruit repressor complexes composed of SMRT and HDAC3 or Bcl3, N-CoR and HDAC3 (Baek et al., 2002; Hoberg et al., 2004). SMRT and N-CoR are known to regulate numerous transcription factors besides NFκB (Jepsen and Rosenfeld, 2002). In order to initiate transcription, these protein complexes need to be removed in a

process termed depression. This is achieved by MEKK1-dependent phosphorylation of TAB2, which facilitates the removal of N-CoR and HDAC3 (Baek et al., 2002). Furthermore, in order to initiate signal induced transcription, p65 recruits HAT-associated complexes containing p300, CBP, p/CAF and SRC -1 -2 and -3 proteins (Na et al., 1998b; Sheppard et al., 1999). The recruitment of these proteins requires phosphorylation of p65 within the Rel homology domain. This phosphorylation of p65, catalysed by IKKα, displaces corepressor activity allowing p300 to acetylate p65 and initiate transcription (Hoberg et al., 2006).

HDACs belong to a class of proteins that play a critical role in transcription factor induced gene expression. For example, HDAC1 directly interacts with MyoD basic helix-loop-helix transcription factors along with NFkB, and STAT among others, while HDAC2 converts the YY1 transcription factor from an activator to a repressor (Mal et al., 2001; Yang et al., 1996). Other similar examples of HDAC activity are discussed below.

A potential role for acetylation in the regulation of NFκB mediated transactivation first emerged with the finding that the HDAC inhibitor, TSA, enhances κB-luciferase reporter gene expression in cells stimulated by TNF-α. It was shown that while HDAC1 directly interacts with p65 and may recruit other corepressors directly to p65's-DNA binding site, HDAC3 did not bind with p65 but was tethered to SMRT or N-CoR (Ashburner et al., 2001). However work done by Chen *et al.* further illuminated the important role of acetylation in the regulation of NFκB activity (Chen and Greene, 2003). They showed that p65 is acetylated by p300/CBP and deacetylated specifically by HDAC3 and that the acetylation of the 221 lysine residue of p65

enhanced the DNA binding properties of p65. Additionally, they demonstrated that deacetylation of p65 by HDAC3 not only decreased the affinity of p65 for its binding site on DNA, but also potentiated the interaction of p65 with IκB α, leading to the export of the complex to the cytoplasm. Based on this evidence, the authors suggested that reversible acetylation of intra-nuclear p65 not only regulated the duration of NFκB-mediated transcriptional response but also contributed to the replenishment of the depleted cytoplasmic pool of latent NFκB/IκB α complexes, thereby preparing the cell for any further NFκB inducing signal. Our evidence suggests that NFKBIL-1 co-localises with HDAC3. Thus one possible mechanism by which NFKBIL-1 regulates NFκB could be by modulating the activity of HDAC3 thereby influencing p65 acetylation and NFκB activity.

4.3 Dynamic regulation of transcription factors

Signal activated transcription factors need to terminate their action as soon as the signal is lost. Some stimuli can induce the activation of several transcription factors at the same time, and it would seem beneficial that a common mechanism should exist to quickly terminate their action when appropriate. Apart from this, cells need to have a regulatory system in place to "sense" the change in the intensity of the stimuli and respond rapidly accordingly. It is likely that these interactive regulatory networks are capable of exerting a significant degree of complexity in the control of transcription factor function. An example of how this may be achieved can be seen in the oscillatory nature of gene expression regulation by NFκB (see figure 4.2). Indeed it is becoming increasingly clear that the physiological response to any stimulus involving transcription factors may be either stable or dynamic (oscillatory) and whether a

response is oscillatory or sustained has a big influence on the functional outcome in biological events.

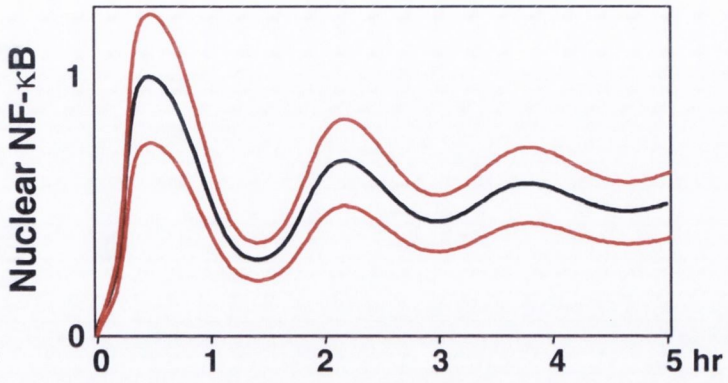


Figure 4.1: Oscillatory movements observed in the cytoplasmic/nuclear translocation of NFκB observed over time after stimulation. This pattern also influences the downstream target gene expression (Nelson et al., 2004).

An oscillatory response moves between a peak and a trough in the amplitude of gene expression and might be executed by, for instance, the binding/release of a transcription factor from cofactors or cyclical alterations to post-translational modifications. IκB α is particularly important in promoting this response pattern, whereas IκB β and IκB ε appear to have a dampening effect on NFκB oscillations (Hoffmann et al., 2002). Further studies reported that, in fact, IκB ε also contributed to the oscillatory response of NFκB but in an antiphase to that of IκB α, leading to an overall dampening of the effect. Therefore the relative abundances of these two inhibitors and their combined temporal expression patterns could cooperatively affect the regulatory activity of NFκB, in a manner that would not be appreciated through the study of either inhibitor alone. It has been suggested that this antiphase response might explain cell-to-cell differences in NFκB induced gene expression since the ratio

of IκB α and IκB ε is cell type dependent (Kearns et al., 2006). The dynamic nature of NFκB regulation was also demonstrated by Nelson *et al.,* who showed that cessation of NFκB activity was independent of p65 nuclear export, but was dependent on rapid, oscillatory phosphorylation/dephosphorylation of NFκB in the nucleus (Nelson et al., 2004). Whatever the mechanism behind these oscillatory movements, it is becoming apparent that they play an important role in the activity of NFκB.

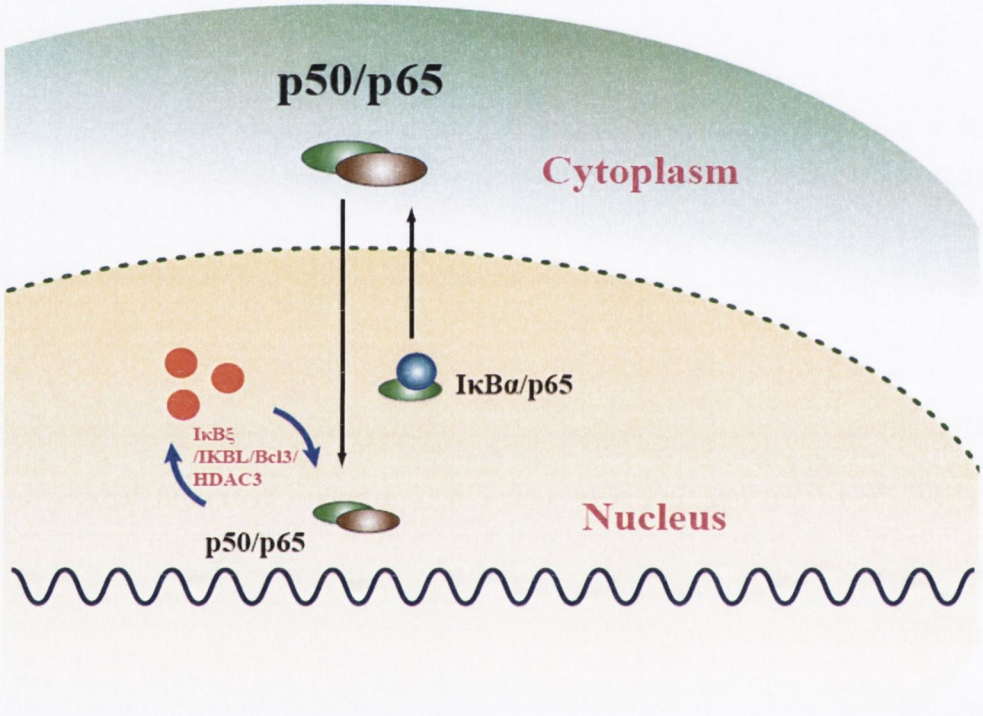


Figure 4.2: Potential Role of Nuclear regulators of NFκB oscillations. Nuclear regulators such as NFKBIL-1 (IKBL) may interact with NFκB and shuttle between the speckles and the target DNA. This movement adds another component to the oscillations seen in NFκB activity due to IκB α mediated translocation of NFκB between cytoplasm and nucleus.

While we were not able to demonstrate the contribution of NFKBIL-1 in this oscillatory action of NFκB, one manner in which NFKBIL-1 may function, could be

to facilitate the oscillatory movements of NFκB as depicted in figure 4.2. Oscillatory responses are not confined to NFκB - STAT proteins have also recently been shown to have such a response pattern (Yoshiura et al., 2007) and it is possible that this is a more widespread phenomenon. Therefore, besides a possible effect that NFKBIL-1 might have on, for example, the acetylation status of these target transcription factors, it the manner by which it does this may include oscillatory mechanisms as outlined above.

4.4 NFKBIL-1: a nuclear chaperone protein?

Studies of the response of cells to changing hormone levels have provided new insights into a potential system for rapid termination of transcription factor activity and preparation of the cell for the next stimulus. Freeman and Yamamoto examined the modulation of hormonal signal and extended their study to explore the regulation of transcription factors (Figure 4.1) (Freeman and Yamamoto, 2002). They reported that over expression of two molecular chaperones, p23 and Hsp90, reduced the activity of AP-1 and NFκB in cells stimulated with PMA and TNF-α respectively (Freeman and Yamamoto, 2001; Freeman and Yamamoto, 2002).

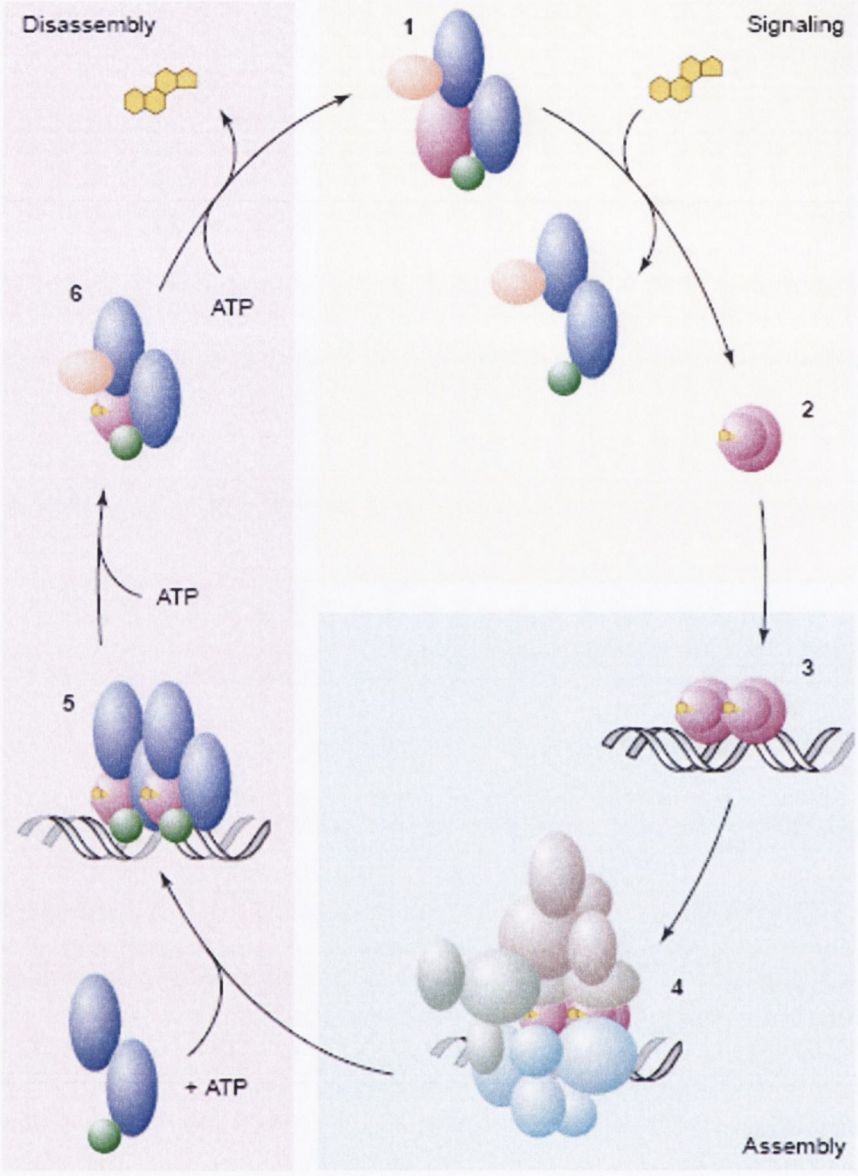


Figure 4.3: Cyclical movement of regulators. From the upper right, when a steroid receptor (purple, top) binds a hormone (yellow), it kicks off chaperones (blue, green, and red). It then settles on DNA and draws in several other proteins (light blue and grey) to form a regulatory complex. Disassembly of this complex is triggered by chaperones using energy provided by ATP, ultimately returning the receptor to its initial state (Freeman and Yamamoto, 2002).

The importance of molecular chaperones has been clearly outlined in circumstances of cellular stress whereby the trafficking of misfolded proteins require chaperoning to execute their function (Loyola and Reinberg, 2003). Examples of molecular

chaperones include heat shock protein family members such as Hsp-27 and Hsp-90. Additional roles for these proteins came to light when it was shown that Hsp-90 binds to heat shock transcription factor (HSF1) and modulates its function (Blake et al., 1995; Giardina and Lis, 1995). Acting at the level of transcription may be a more widespread property of chaperone proteins given the findings of Virbasius *et al.,* who identified a novel protein, bZIP-enhancing factor (BEF), that not only acted as a molecular chaperone but additionally regulated the activity of nuclear transcription factors containing the bZIP DNA binding domain (Virbasius et al., 1999). More recently Jin *et al* showed that Jun dimerization protein-2 (JDP-2), a component of AP-1 transcription complex, was also a chaperone protein that regulated histone acetylation and nucleosome formation. They reported that for this activity, JDP-2 recruited HDAC3 to the promoter region of the target gene (Jin et al., 2006). According to Freeman *et al.,* chaperone proteins such as p23 and hsp90 facilitate the binding and release of transcription factors from the DNA in a cycle of continuous assembly and disassembly of regulatory complexes. Such a cyclical process is an attractive proposition, as it has the advantage of enabling regulators to sense and respond quickly and efficiently to fluctuating stimuli (Freeman and Yamamoto, 2001; Freeman and Yamamoto, 2002).

It is notable that a number of known regulators of NFκB and other transcription factors share some of the characteristics of chaperone proteins and may in fact be chaperones. Bcl3 regulates the transcription activity of not only NFκB but also AP-1 and RXR (Na et al., 1998a; Na et al., 1999). RXR belongs to a family of ligand-dependent transcriptional regulatory proteins that function by binding to specific response elements in the promoter of target genes and is an obligate binding partner

for many nuclear receptors (Mangelsdorf et al., 1995). Bcl3 also interacts with the basal transcription machinery including TFIIA, TFIIB and TBP (Na et al., 1998a) and association with other co-activators such as CBP/p300, TORC3 and SRC-1 has also been demonstrated (Hishiki et al., 2007). CBP and p300 are known to be essential for the activities of many different transcription factors (Na et al., 1998b). Since NFKBIL-1 also has broad specificity in the proteins it regulates, further investigations of its mechanism of action should consider the possibility that it functions similarly to BCL3, as a nuclear chaperone protein that acts as a bridge connecting both transcription factors and the components of the basic transcriptional apparatus.

While the regulation of NFκB was the major focus of our study, we believe our observation that NFKBIL-1 inhibited activity of both AP-1 and STAT1 to be important. AP-1 regulation is thought to occur primarily at the levels of the composition of dimer formation, protein degradation and subcellular localisation. Novel proteins such as JDP-2 have been recently identified that regulate AP-1 (Virbasius et al., 1999). The nuclear regulation of STAT activation is less well understood. However we have shown that by regulating all three, NFKBIL-1 is likely to work at the final stages of transcriptional regulation, perhaps in transcriptional complexes. Thus in this discussion, I have emphasised potential mechanisms of action that may apply to all three transcription factors and possibly more generally.

4.5 Identification of NFKBIL-1 function: recent findings

In a recent study Ewing *et al.* carried out large scale mapping of human protein-protein interactions. They used 338 bait proteins selected on the basis of suspected

diseases and functional associations. Essentially, they used Flag tagged version of these proteins to perform immunoprecipitation and the precipitated protein complexes were then identified by Mass Spectrometry. *NFKBIL-1* was included in the study and some very interesting interactions were identified (Ewing et al., 2007) (See table 4.2). The interaction between NFKBIL-1 and Gemin4 had the highest confidence score. While the exact function of Gemin4 is not known, it has been reported that Gemin4 interacts with the DEAD box protein, Gemin3, the Sm core proteins and some snRNPs molecules, suggesting that it is part of the spliceosomal complex. Another protein listed in table 4.2, Luc7L is known to regulate myogenesis in mice and localizes in the nucleus with a speckled intranuclear distribution. The authors concluded that Luc7L is another potential regulatory factor of splicing. Interestingly, they also observed that the arginine-serine rich domain (RS domain) of the protein was important for the nuclear distribution of Luc71, and that the mutant protein without the RS domain lost the speckled pattern (Kimura et al., 2004). RFXANK has four ankyrin repeats, and plays a critical role in formation of the MHC class II enhanceosome which is required for the recruitment of the essential transcriptional coactivator CIITA to HLA class II promoters (Krawczyk et al. 2005) Interestingly however in the context of NFKBIL-1, it appears that RFXANK may also specifically bind the class II HDACs 4 & 5, which then cause repression of the promoters (Zika and Ting 2005, McKinsey et al. 2006) . The interaction with the lowest confidence score was between NFKBIL-1 and RNAS1. RNA binding protein S1 is part of pre- and post- splicing multiprotein mRNP complexes. This protein is also distributed in nuclear speckles (Loyer et al., 1998) . Since the authors did not confirm these interactions by other techniques, it is difficult to identify which if any of these represent true positive interactions.

Bait gene name*	Prey gene name*	Confidence score**
NFKBIL1	GEMIN4	0.552
NFKBIL1	21439128	0.547
NFKBIL1	MRPS22	0.52
NFKBIL1	RFXANK	0.457
NFKBIL1	LUC7L	0.421
NFKBIL1	PNN	0.409
NFKBIL1	MRPS34	0.389
NFKBIL1	MRPL44	0.387
NFKBIL1	MRPS18B	0.373
NFKBIL1	KRT1B	0.346
NFKBIL1	42657378	0.341
NFKBIL1	FLJ22965	0.334
RNPS1	NFKBIL1	0.233

Table 4.2: Some of the putative protein interactions of NFKBIL-1 as reported by Ewing *et al.* (Ewing et al., 2007).

*Bait and prey gene names provided where available. Numeric values are GenBank gi protein sequence identifiers.

**Confidence score assigned from regression model of peptide and protein identification parameters. Takes either a numeric value between 0 and 1 or is undefined. All prey protein observations are subject to filtering based upon search engine parameters; confidence score equal to 0 therefore implies that the prey protein is present but at the lowest acceptable score. For ease of use, the authors recommend confidence scores greater than 0.3 as high confidence interactions and all of the undefined or less than 0.3 interactions as low confidence.

Our own data showed that NFKBIL-1 co-localises with HDAC3. It is interesting to note that Ewing *et al.* did not detect any explicit interaction between NFKBIL-1 and HDAC3. Even though we have observed these to co-localise, this does not imply that they necessarily interact directly, since complexes such as nuclear speckles contain many proteins. It is also possible that the lack of interaction maybe a technical rather

than a biological effect. For example, differences in the cell line used and the methodology employed for immunoprecipitation might account for the lack of interaction between the two proteins. In an unrelated study, Lehner *et al.* performed a yeast two hybrid study to identify the function of proteins encoded within the MHC III region. They were unable to identify proteins interacting with NFKBIL-1 (Lehner et al., 2004).

More recently Greetham and colleagues reported on the function of NFKBIL-1(Greetham et al., 2007). They found that, while in PBMCs both the full-length and the alternative splice variant isoforms of NFKBIL-1 were expressed in equal amounts, the full-length isoform was preferentially expressed in brain and the splice variant more in muscles, small intestine, placenta and skin. They also observed that that NFKBIL-1 protein was selectively expressed in the rheumatoid epithelial cells but not the synovium suggesting a potential role in the pathogenesis of rheumatoid arthritis. The authors also performed immunoprecipitation experiments and identified several potential interaction candidates in HeLa cells: these were CTP synthase I, elongation factor 1 a and leukophysin. Based on these results they concluded that NFKBIL-1 was involved in mRNA processing.

It is possible that NFKBIL-1 might be involved in mRNA processing of transcription factors such as NFκB, AP-1 or STATs. Indeed, the Leucine zipper domain present in the NFKBIL-1 also suggests that it might be involved in gene regulation by binding to the DNA. In contrast, although defects in mRNA processing have been linked to diseases, very few of these have a strong inflammatory component as is the case with Rheumatoid Arthritis and Inflammatory Bowel disease (Stoilov et al., 2002).

It should be noted that Mintz *et al.* characterized the proteins localized in the interchromatin granule clusters that appear as speckles, but did not find NFKBIL-1 in their sample (Mintz et al., 1999). Interestingly, the authors described the presence within the speckles of several proteins such as Actin and Hsp 70 that are involved in biological processes other than mRNA processing. Our results (and those of others) demonstrate that NFKBIL-1 is a constitutively expressed protein and is present in large amounts; hence it should be a significant component of the speckles. Absence of NFKBIL-1 in the speckles, as reported by Mintz *et al.*, is therefore difficult to explain. Most proteins localised within the speckles require serine-arginine rich region. NFKBIL-1 lacks this particular domain and it is the ankyrin repeat region of NFKBIL-1 that directs it to the speckles (Semple et al 2002). In fact it has been suggested that that the ankyrin repeats serve to perform two actions: nuclear import and localisation into the speckles (Semple et al., 2002). Although the exact mechanism of how the ankyrin repeats facilitate nuclear import is not known, it is possible that NFKBIL-1 might interact with other proteins by its ankyrin repeat domain and thus allowing it to move into the nucleus.

In fact, several transcription factor cofactors, including CBP/p300 and SMRT, are also known to be distributed in speckles (Liao et al., 2003; von Mikecz et al., 2000). SMRT interacts with HDAC3 to repress gene expression (Fischle et al., 2002). Indeed it is possible that NFKBIL-1 might also colocalise with SMRT. Further studies will shed more light on the potential interaction between NFKBIL-1 and other corepressors such as SMRT, N-CoR and HDACs and the mechanism of regulation of transcription factor activity.

Our own attempts to identify proteins interacting with NFKBIL-1 did not yield significant results. Although immunoprecipitation of NFKBIL-1-GFP was successfully performed using a monoclonal anti-GFP antibody, potential problems arose when the same experiment was performed to identify other interacting proteins. In this regard, it should be noted that in the results reported by both Ewing *et al.* and Greetham *et al.,* no common NFKBIL-1 interacting protein were found. It is possible that the potential dynamic movements of NFKBIL-1 in the nucleus might have influenced the experimental outcome in these two studies.

Most of our results regarding the function of NFKBIL-1 are based on the use of the GFP tagged NFKBIL-1 protein. A number of other methods are available to study the translocation process, including immunocytochemistry, organelle separation and biochemistry. Most of these techniques allow us to understand protein biochemistry on a larger scale. On the other hand, the use of green fluorescent protein (GFP) as a tag for shuttling proteins now allows the study of the trafficking of signalling molecules in individual living cells over a long period of time. At the same time, the degree of GFP expression might influence the underlying signalling pathways. It is possible that in a cell where the GFP fusion protein is strongly expressed it might overwhelm the other proteins involved in the pathways and the cascading amplification of the signal might be influenced by the limited amounts of other proteins

Based on our findings we believe it is reasonable to hypothesise that these novel nuclear regulators of NFκB such as Bcl3, IκB ζ and NFKBIL-1 may be viewed as chaperone proteins or components of multi-protein regulatory complexes within the

nucleus, bringing together the NFκB subunits or other transcription factor subunits when required. Likewise, upon reduction or cessation of the stimuli, these proteins might chaperone the removal of NFκB from DNA thus terminating its action. Indeed it is plausible that such a crucial transcription factor as NFκB would need a highly evolved, specific and specialised regulatory system to specifically control its action even while in the nucleus. While a majority of the NFκB subunits might recycle back to the cytoplasm and prepare the cell for the next wave of stimuli, a critical amount of NFκB needs to remain constantly within the nucleus. In this context it is possible that these nuclear regulators sequester this fraction of NFκB and thus deactivate it. The importance of these regulators, their mechanism of action and how they interact to produce an outcome can be a subject of future studies.

4.6 NFKBIL-1 and diseases

Dysregulation of transcription factor activity may contribute to several inflammatory diseases and indeed a variety of other conditions also (see table 4.3). For example, in the pathogenesis of acute inflammatory conditions including acute respiratory distress syndrome, glomerulonephritis, septic shock among others, NFκB plays a major role, while a more complex network of transcription factors are implicated in the causation of chronic diseases such as asthma and atherosclerosis (Abraham, 2003; Aud and Peng, 2006; Barnes and Adcock, 1998; Blaschke et al., 2004; Cho et al., 2004; Haaxma et al., 2003; Kimura et al., 2006; Metzler et al., 2003; Ware and Matthay, 2000).

Acute Inflammation	*Transcription Factors Implicated*	*Chronic Inflammation*	*Transcription Factors Implicated*
Acute Respiratory Distress Syndrome	*NFκB*	*Arthritis*	*NFκB, AP-1 and STATs*
Glomerulonepritis	*NFκB*	*Asthma*	*NFκB, AP-1, GATA3 and STATs*
Septic Shock	*NFκB, STATs, C/EBP-α and HNF-1α*	*Atherosclerosis*	*Egr-1, Heat shock transcription factor-1, PPAR-γ and NFκB*
Vasculitis	*NFκB*	*Pulmonary Fibrosis*	*Nrf2, GATA3*

Table 4.3: Transcription factors implicated in the pathogenesis of various Acute and Chronic Inflammatory conditions. Our data provides evidence that NFKBIL-1 inhibits the activity of NFκB, AP-1 and STAT1 and thus may play a significant role in some of these diseases.

Given that we have shown that NFKBIL-1 regulates activity of NFκB, AP-1 and STAT1, it could therefore play a role in the pathogenesis of several diseases. Indeed, Greetham *et al.* have recently provided evidence for a role for NFKBIL-1 in diseases (Greetham et al., 2007). They showed that NFKBIL-1 was expressed in T cells and macrophages in the rheumatoid synovium. They speculated that NFKBIL-1 might contribute to the pro-inflammatory response in rheumatoid arthritis. Further studies will be required to understand the contribution of NFKBIL-1 in the pathogenesis of rheumatoid arthritis and other inflammatory diseases. As stated earlier, work in this lab has associated genetic variants associated with lower NFKBIL-1 production with coeliac disease and has also shown that increased expression of NFKBIL-1 is significantly associated with poor outcome in sepsis patients, in a pattern highly

correlated to that of IL-10. (M O'Dwyer, T Ryan and R McManus, unpublished observations). Thus real evidence exists to suggest that NFKBIL-1 may be an important regulator of transcriptional activity that significantly affects public health.

4.7 Future prospects

While our data indicates that NFKBIL-1 regulates multiple transcription factors involved in inflammation and immunity, due to time limitations the exact mechanism for the inhibition has not been identified. In addition, the transcription factors studied by us are involved in pro-inflammatory response. Thus, while NFKBIL-1 decreases the activity of pro-inflammatory transcription factors, it is also possible that it increases the activity of anti-inflammatory transcription factors. Nuclear factor-erythroid 2–related factor 2 (Nrf2) is a possible example: this anti-inflammatory mediator regulates a battery of antioxidant and other cytoprotective genes (Thimmulappa et al., 2002) conferring protection against various environmental stresses (Rangasamy et al., 2005). Another important transcription factor is Hypoxia-Inducible Factor 1 (HIF-1). HIF-1 is one of the key regulators of oxygen homeostasis. At the cellular level, this response requires that the transcription factor HIF-1 bind to a hypoxia-responsive element (HRE) in the promoter of target genes to increase their expression (Coleman and Ratcliffe, 2007). Indeed, further studies could be undertaken to identify if NFKBIL-1 regulates the activities of other important transcription factors such as EGR-1, other members of the STAT family and transcription factors that regulate non-immune systems such as HIF-1. These studies will confirm whether NFKBIL-1 specifically functions as an regulator of transcription factors implicated in inflammation and immunology or is a general transrepressor.

In order to understand the function of the native protein it is essential to generate specific antibodies against NFKBIL-1. Although our attempts to generate antibody using the full-length recombinant protein proved futile, it is possible to use short synthetic peptides to generate more specific antibodies. This will enable us to understand the functions and interactions of the native NFKBIL-1 protein with other proteins in the cell.

While we did show that NFKBIL-1 interacts with HDAC3 we can only speculate that NFKBIL-1 might modulate the activity of either HDAC3 directly or the HDAC regulated transcription factors indirectly. In a manner similar to Hsp 90, NFKBIL-1 may also act as novel nuclear chaperone protein influencing the activity of various transcription factors. It is also possible that NFKBIL-1 might interact with other components of NFκB such as p52. Indeed such an interaction might enable these protein dimers to inhibit NFκB transactivation.

Further studies looking at the role of NFKBIL-1 using siRNA studies may provide additional information regarding the effect of loss of function of NFKBIL-1 on the activity of transcription factors. Knockout mouse models, wherein NFKBIL-1 expression is completely abolished, will allow further elucidation of the function of NFKBIL-1.

4.8 Conclusions:

The location of *NFKBIL-1* in the MHC, the association of SNPs in the gene with various inflammatory conditions and our own observations that NFKBIL-1 regulates several important transcription factors provides some useful insight into the function of this novel protein. Transcription factors, including NFκB, AP-1 and STATs, regulate expression of genes involved in immunity and inflammation. The action of NFκB is very critical for the cell and it needs to be regulated dynamically. Since NFκB subunits undergo nucleo-cytoplasmic shuttling, its regulation in the nucleus would also likely require such a mechanism whereby NFκB is chaperoned to/from sites within the nucleus. It is possible that, in a fashion similar to the cytoplasmic inhibitors of NFκB, the nuclear inhibitors of NFκB too sequester the subunits and thus play an important role in the regulation of this transcription factor. A growing number of studies have implicated NFκB dependent pathways in the development of various diseases such as Inflammatory Bowel Disease, Rheumatic Arthritis, atherosclerosis and asthma to name a few. Numerous chemical compounds and other biological agents that inhibit NFκB have been developed or are under development. Nevertheless, because of the ubiquitous presence of NFκB in virtually all cells and its involvement in many different cellular pathways and functions, application of these drugs to inhibit NFκB non-specifically in a certain context might comprise the danger of unwanted side effects (Karin et al., 2004; Tak and Firestein, 2001; Yamamoto and Gaynor, 2001). Therefore, understanding the function of these nuclear chaperones may permit a subtler targeting and manipulation of NFκB molecule. These regulators are downstream participants of the NFκB signalling pathways and may enable a less blunt abolition of NFκB acitivity. Indeed, further studies into these crucial

components involved in the nuclear regulation of NFκB and identification of similar mechanisms in the regulation of other transcription factors will provide additional insights into the action of transcription factors in various diseases raising the prospect of identifying better therapeutic targets.

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