

## REVIEW

# Nuclear transcription factor- $\kappa$ B as a target for cancer drug development

A Garg and BB Aggarwal

Cytokine Research Laboratory, Department of Bioimmunotherapy, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

**Nuclear factor kappa B (NF- $\kappa$ B) is a family of inducible transcription factors found virtually ubiquitously in all cells. Since its discovery by Sen and Baltimore in 1986, much has been discovered about its mechanisms of activation, its target genes, and its function in a variety of human diseases including those related to inflammation, asthma, atherosclerosis, AIDS, septic shock, arthritis, and cancer. Due to its role in a wide variety of diseases, NF- $\kappa$ B has become one of the major targets for drug development. Here, we review our current knowledge of NF- $\kappa$ B, the possible mechanisms of its activation, its potential role in cancer, and various strategies being employed to target the NF- $\kappa$ B signaling pathway for cancer drug development.**

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## Introduction

Transcription is an important regulatory event in the pathway leading to gene expression. Transcription factors regulate transcription by binding to specific sequences present within the promoter, enhancer, or other regulatory regions of DNA. Hundreds of transcription factors with functionally separable domains, essential for DNA-binding and activation, have been identified and characterized in several organisms.<sup>1</sup> One such transcription factor, NF- $\kappa$ B, has been the subject of intense study based on the implications of its role as a key mediator of a wide variety of cellular responses.<sup>2</sup>

## Nuclear Factor $\kappa$ B

NF- $\kappa$ B was first identified in the nuclei of mature B lymphocytes as a transcription factor that binds an 11-bp DNA sequence in the  $\kappa$ -light chain enhancer GGGACTTCC.<sup>3</sup> Mammalian cells have five distinct NF- $\kappa$ B subunits based on a highly conserved 300 amino acid dimerization domain called the rel homology domain, which is required for binding DNA and mediating the transcription of over 180 target genes. These subunits may be classified into two functional groups, one containing the NF- $\kappa$ B1 (p105/p50) and NF- $\kappa$ B2 (p100/p52) subunits and the other containing the RelA (p65), Rel B, and c-Rel subunits. Members of the second group share a carboxy-terminal transactivation domain usually required within the Rel/NF- $\kappa$ B structure to promote transcription. Members of the first group exist as precursor subunits p105 and p100 (105000 and 100000 daltons), which contain a series of five to seven ankyrin repeat domains that mask the nuclear

localization signals (NLS) within the rel homology domain. They must be cleaved to the active p50 and p52 subunits (from p105 and p100, respectively) before allowing the translocation of the NF- $\kappa$ B complex from the cytoplasm to the nucleus, where transcription takes place.

Other inhibitory subunits that utilize a similar ankyrin repeat domain (but do not contain the rel homology domain of the five subunits above) include I $\kappa$ B $\alpha$  (most common), I $\kappa$ B $\beta$ , I $\kappa$ B $\gamma$  (derived from the C-terminal of p100), I $\kappa$ B- $\epsilon$ , Bcl-3, pp40 (chicken homologue), and avian swine fever virus protein p28.2. More recently, another I $\kappa$ B-like subunit called I $\kappa$ B- $\zeta$ , with six ankyrin repeat domains, was discovered and was found to retain the NF- $\kappa$ B proteins in the nucleus instead of the cytoplasm.<sup>4</sup>

There are several different structural combinations of subunits in the cytoplasm that are called NF- $\kappa$ B, with the most common heterodimer consisting of a Rel A subunit (p65), a NF- $\kappa$ B1 subunit (p105/p50), and the I $\kappa$ B $\alpha$  inhibitory subunit.<sup>5</sup> On activation, degradation of I $\kappa$ B $\alpha$  exposes nuclear localization signals (NLS) on the p50-p65 heterodimer, leading to nuclear translocation and binding to a specific sequence in the DNA, which in turn results in gene transcription. This pathway is well conserved, both in structure and function, from *Drosophila* to humans.<sup>6</sup>

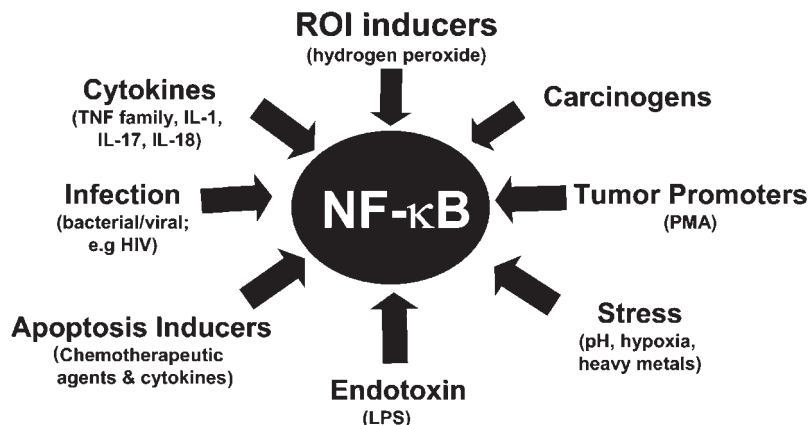
## How is NF- $\kappa$ B activated?

A lot has been learned about NF- $\kappa$ B activation in the last decade. Cellular responses to a wide variety of diverse stimuli have been identified, and have shown to lead to the activation of NF- $\kappa$ B (see Figure 1). These stimuli reveal that NF- $\kappa$ B is a common pathway for cellular adaptation to stress.<sup>2,7,8</sup> The stimuli include inflammatory cytokines, immune-related stress such as bacterial infection of *S. aureus*<sup>9</sup> and their products such as lipopolysaccharide<sup>3</sup> (or LPS), viruses such as HIV-1<sup>10</sup> and their products such as hemagglutinin of the flu virus,<sup>11</sup> physiologic stress such as ischemia,<sup>12,13</sup> physical stress such as UV irradiation,<sup>14</sup> environmental hazards such as cigarette smoke,<sup>15</sup> many therapeutic drugs such as taxol<sup>16</sup> or haloperidol,<sup>17</sup> apoptotic mediators such as anti-Fas,<sup>18</sup> growth factors such as insulin,<sup>19</sup> physiologic mediators such as angiotensin II<sup>20</sup> or PAF,<sup>21,22</sup> oxidative stress such as exposure to hydrogen peroxide,<sup>23</sup> and many more (see Figure 1).

Depending on the stimulus, the mechanism of activation involves overlapping and nonoverlapping steps. Among all the stimuli, perhaps the most is known about the mechanism by which TNF activates NF- $\kappa$ B. This pathway involves the interaction of the ligand with its receptor at the cell surface (TNFR), which then recruits a protein called TNF receptor-associated death domain (TRADD). This protein binds to TNF receptor-associated factor (TRAF)-2, which activates receptor-interacting protein (RIP). RIP interacts with mitogen-activated protein kinase kinase kinase 3 (MEKK3) to phosphorylate and

Correspondence: BB Aggarwal, Cytokine Research Laboratory, Department of Bioimmunotherapy, Box 143, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, Texas 77030, USA; Fax: 713-794-1613

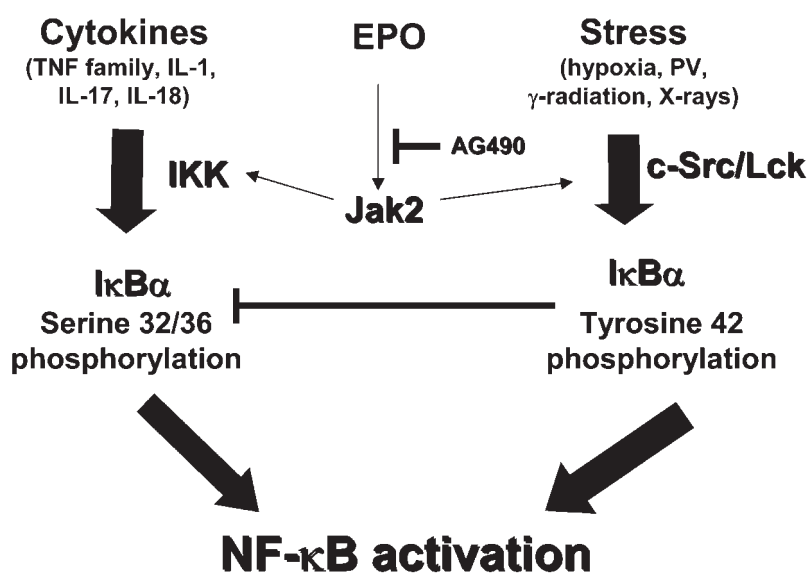
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**Figure 1** Activation of NF- $\kappa$ B by various agents reactive oxygen intermediates (ROIs), cytokines, infections, apoptotic inducers, endotoxin, physical stress, tumor promoters, and carcinogens.

activate the  $\text{I}\kappa\text{B}\alpha$  kinase complex (IKK).<sup>60</sup> The IKK complex phosphorylates  $\text{I}\kappa\text{B}\alpha$  at serines 32 and 36, which leads to ubiquitination at lysines 21 and 22, and this leads to the degradation of  $\text{I}\kappa\text{B}\alpha$  by the 26S proteasome, resulting in the translocation of NF- $\kappa$ B to the nucleus, where it binds to its consensus sequence (5'-GGGACTTTC-3') and activates gene expression. It should be noted, however, that other mechanisms of NF- $\kappa$ B activation that do not require  $\text{I}\kappa\text{B}\alpha$  degradation have been identified, including those induced in response to such stimuli as oxidative stress and X-rays<sup>24,25</sup> (Figure 2). These mechanisms, although not precisely understood, likely involve tyrosine phosphorylation of  $\text{I}\kappa\text{B}\alpha$  instead of the traditional serine phosphorylation that causes its subsequent degradation.<sup>26,27</sup> Interestingly, it was recently found that erythropoietin (EPO) activates NF- $\kappa$ B through phosphorylation of tyrosine and serine residues of  $\text{I}\kappa\text{B}\alpha$  and this is mediated through Janus kinase-2<sup>28</sup> (JAK2) (see Figure 2), the only protein tyrosine kinase thus far implicated in the activation of NF- $\kappa$ B (see below).

The mechanism that activates the IKK complex is considered a common pathway for a number of different activation pathways (see below) and has been the subject of intense study.<sup>29</sup> An IKK complex consists of three subunits including IKK $\alpha$ , IKK $\beta$ , and IKK $\gamma$  (also called NEMO). IKK $\beta$  is an inducible catalytic subunit that phosphorylates  $\text{I}\kappa\text{B}\alpha$  at serine 32 and 36 and causes the subsequent degradation of  $\text{I}\kappa\text{B}\alpha$ , leading to the activation of NF- $\kappa$ B. Physiologic roles of IKK $\beta$  via gene deletion studies have shown IKK $\beta$  to be integral in liver development and protection of T cells from TNF- $\alpha$ -induced apoptosis.<sup>30,31</sup> IKK $\alpha$  has recently been shown to be involved in the activation of NF- $\kappa$ B via an  $\text{I}\kappa\text{B}\alpha$ -independent pathway that involves the direct phosphorylation of NF- $\kappa$ B2 (p100 precursor) in response to upstream kinases.<sup>32</sup> Gene deletion studies of IKK $\alpha$  have shown that it plays an unexpected role in skin and skeletal development.<sup>33,34</sup> IKK $\gamma$  (aka NEMO or IKKAP1) is a regulatory subunit without intrinsic kinase activity and was found to play an integral role in the activation of NF- $\kappa$ B as well via modulation of  $\text{I}\kappa\text{B}\alpha$  degra-



**Figure 2** Various mechanisms leading to NF- $\kappa$ B activation. Depending on the inducing agents, NF- $\kappa$ B activation may follow different pathways. In response to pro-inflammatory cytokines, NF- $\kappa$ B activation follows IKK activation and subsequent  $\text{I}\kappa\text{B}\alpha$  phosphorylation at serine 32/36. In response to physical stress such as hypoxia and X-rays, c-Src/Lck is activated and causes the subsequent  $\text{I}\kappa\text{B}\alpha$  phosphorylation at tyrosine 42 which leads to NF- $\kappa$ B activation. Interestingly, the latter route inhibits the serine 32/36 phosphorylation of  $\text{I}\kappa\text{B}\alpha$  in response to TNF. Also, both pathways may be activated in response to erythropoietin (EPO) via the JAK2 kinase that can activate both IKK and C-Src/Lck.

dation pathways.<sup>35,36</sup> Gene deletion studies (X-linked) reveal that IKK $\gamma$  is imperative for male survival in mice and important in lymphocyte development and persistence.<sup>37</sup> The novel IKK-related kinase called IKK $\epsilon$ /IKKi is an LPS and PMA inducible kinase whose role in NF- $\kappa$ B activation is less well defined although likely involves the unique preferential phosphorylation of only serine 36 (and not serine 32) on I $\kappa$ B $\alpha$ . Its mechanism may involve interaction with the TRAF interacting protein/TRAF family member-associated NF- $\kappa$ B activation pathway as well as interactions with unidentified upstream and downstream kinases.<sup>38–40</sup> Additional research will clearly be needed to elucidate its precise mechanism.

Extensive research over the last few years indicates that NF- $\kappa$ B activation is highly complex and may involve as many as 20 different protein kinases (see Table 1). These kinases may form a cascade, and different cascades may be formed depending on the NF- $\kappa$ B activator. For instance, IKK can undergo phosphorylation by either NIK, MEKK or AKT. Although several signaling proteins and protein kinases have been recently identified that mediate IKK activation, the exact role of these kinases and their placement within the activation cascade is controversial. The activation of some of these kinases may be specific to cell type and to the stimulus employed to activate NF- $\kappa$ B.<sup>81</sup> For instance, NIK, while found critical for NF- $\kappa$ B activation by LT and CD40L, was found to have no role in TNF-induced NF- $\kappa$ B activation.

### Lessons learned from NF- $\kappa$ B gene deletion

In the past 7 years, mouse models with a deletion of one or more of the genes that code for specific Rel/NF- $\kappa$ B proteins (termed 'knockout mice') have provided a valuable insight into the function and relevance of various NF- $\kappa$ B gene products. Overall, individual knockouts have caused either mild to severe immune-related deficiencies (eg p105/p50, p100/p52, Rel A, Rel C, I $\kappa$ B $\alpha$ ), liver apoptosis (Rel A), or various other developmental abnormalities (eg I $\kappa$ B $\alpha$ , IKK). When p105/p50 is knocked out, there are functional defects in the immune system despite an otherwise normal development and phenotype.<sup>82</sup> More specifically, p105/p50 is essential for the survival of non-activated B cells but not essential for all B cell-activated pathways.<sup>77,83,84</sup> For example, p50-deficient mice are susceptible to *L. monocytogenes* and *S. pneumoniae* infections and do not proliferate in response to LPS but do respond to *Haemophilus influenzae* and *Escherichia coli*.<sup>77</sup> Knocking out the Rel A subunit causes embryonic lethality as a result of fetal liver cell apoptosis and granulopoiesis.<sup>85</sup> This implicates Rel A in cell survival, specifically in response to the cytotoxic effects of TNF- $\alpha$  via induction of I $\kappa$ B $\alpha$ . Also, Rel A has been shown to be important in induced lymphocyte proliferation and isotype switching but not basal transcription.<sup>86</sup>

C-rel knockout mice show normal development but B and T

**Table 1** Protein kinases implicated in activation of NF $\kappa$ B

NF- $\kappa$ B activating kinases	Ligand(s)	Refs
I $\kappa$ B kinase $\alpha$ (IKK $\alpha$ )	Proinflammatory cytokines (eg TNF, IL-1, IL-6), LPS	42–45
I $\kappa$ B kinase $\beta$ (IKK $\beta$ )	Proinflammatory cytokines (eg TNF, IL-1, IL-6), LPS	42–45
NEMO/I $\kappa$ B kinase $\gamma$ (IKK $\gamma$ )	Proinflammatory cytokines (eg TNF, IL-1, IL-6), LPS	35, 36
I $\kappa$ B kinase i (IKKi/IKK $\epsilon$ )	TNF, PMA	38, 39
NF- $\kappa$ B inducing kinase (NIK)	TNF, CD95, and IL-1	41
NF- $\kappa$ B activating kinase (NAK; also called T2K, TBK1 or TRAF2 interacting kinase)	Phorbol esters, growth factors	61–63
Phosphatidylinositol 3 protein kinase (PI-3K)	IL-1	64
Protein kinase B $\alpha$ (Akt 1)	TNF, TCR/CD28	53, 65
Hematopoietic protein kinase-1 (HPK-1)	Unknown	67
Protein kinase C-alpha (PKC- $\alpha$ )	TPA, TNF, Bimp1, Bcl10/MALT1	48–50
Protein kinase C-beta (PKC- $\beta$ )	IgM receptor, Bimp1, Bcl10/MALT1	49, 51
Protein kinase C-theta (PKC- $\tau$ )	CD3-CD28 (TCR/CD28), Bimp1, Bcl10/MALT1	49, 52, 53
Protein kinase C-delta (PKC- $\delta$ )	TNF $\alpha$	54
Protein kinase C-epsilon (PKC- $\epsilon$ )	Phorbol ester, Bimp1, Bcl10/MALT1	49, 55
Atypical protein kinase C (aPKC)	TNF, ras p21	46, 47
Mitogen activated protein kinase kinase kinase 1, 2 (MEKK1,2)	Proinflammatory cytokines	59, 43
Mitogen activated protein kinase kinase kinase 3 (MEKK3)	TNF, other proinflammatory cytokines	43, 60
TPL-2/Cot kinase	TNF	58
Ribosomal protein S6 kinase (pp90rsk)	<i>p. aeruginosa</i>	56
Raf-1 kinase	Serum growth factors, phorbol ester and PTK oncogenes	70, 71
RNA-dependent protein kinase (PKR)	dsRNA	57
TGF- $\beta$ activated kinase-1 (TAK1/MAPKKK)	XIAP, TGF- $\beta$	68, 69
Mixed lineage kinase (MLK3)	CD3/CD28	66
p21 activated kinase (PAK1)	p21, LPS	72
Bruton's tyrosine kinase (BTK)	B-cell antigen receptor	73
Janus kinase-2 (JAK2)	Erythropoietin	28
Protein kinase A (PKA)	IL-1, LPS, many others	3, 74, 75
IL-1 receptor-associated kinase-1 (IRAK-1)	IL-1	76, 77
IL-1 receptor-associated kinase-2 (IRAK-2)	IL-1, LPS	78
IL-1 receptor-associated kinase-M (IRAK-M)	IL-1, LPS	78
P56 lck	Ceramide	79, 80

<sup>a</sup>Demonstrated in neutrophils. Type of kinase activated may vary with cell type.

cell deficiencies.<sup>87</sup> Specifically, c-rel-deficient B cells cannot proliferate in response to immunogens due to a cell cycle block at G1 and more prevalent activation-induced apoptosis due to a failure to upregulate A1 (homologue of Bcl-2), a pro-survival protein.<sup>78,88</sup> C-rel has also been shown to cause a tissue-specific deficiency of various cytokines and growth factors in T cells and macrophages affecting both innate and humoral immune responses in the host.<sup>89–92</sup> Mice deficient in the NF- $\kappa$ B2 gene (p100/p52) mainly have defects in lymph node and splenic architecture although development is normal.<sup>93,94</sup> This leads to antigen presentation impairment from accessory cells such as dendritic cells and macrophages but does not affect B or T cells directly.<sup>95</sup> Knocking out the major inhibitory subunit I $\kappa$ B $\alpha$  produces severe runting (one-third of normal weight) despite normal development, death by day 8 of life due to widespread dermatitis and granulocytosis, scaly appearing skin with significant sloughing, extensive post-natal granulopoiesis, small spleen size caused by depletion of cells of erythroid and lymphoid lineages (not myeloid though), and elevated levels of NF- $\kappa$ B in hematopoietic tissues and some NF- $\kappa$ B-dependent target genes (implying that additional transcriptional factors are involved).<sup>90,96,97</sup>

Recently, IKK $\alpha$  and IKK $\beta$  knockouts have demonstrated that IKK $\beta$  is the major subunit involved in NF- $\kappa$ B activation in response to a majority of stimuli (ie pro-inflammatory cytokines).<sup>98,99</sup> Gene deletion studies have also revealed that IKK $\alpha$  plays little role in NF- $\kappa$ B activation but has proven to play an unexpected role in skin and skeletal development.<sup>29,33</sup>

### Relevance of NF- $\kappa$ B to cancer

NF- $\kappa$ B is an ideal target for anticancer drug development for several reasons (see Figure 3). Cancer is a hyperproliferative disorder that involves transformation, initiation, promotion, angiogenesis, invasion, and metastasis. The diversity of its clinical presentation, aggressiveness, and current treatment strategies imply an equally diverse number of potential targets in the molecular pathways leading to its formation. NF- $\kappa$ B activation participates at multiple steps in these pathways shown below and its suppression may lead to the suppression of cancer development. First, NF- $\kappa$ B mediates the expression of genes that are involved in tumor promotion, angiogenesis, and metastasis.<sup>100,101</sup> Second, it has been shown that NF- $\kappa$ B is activated by hypoxia and acidic pH, both indigenous to the

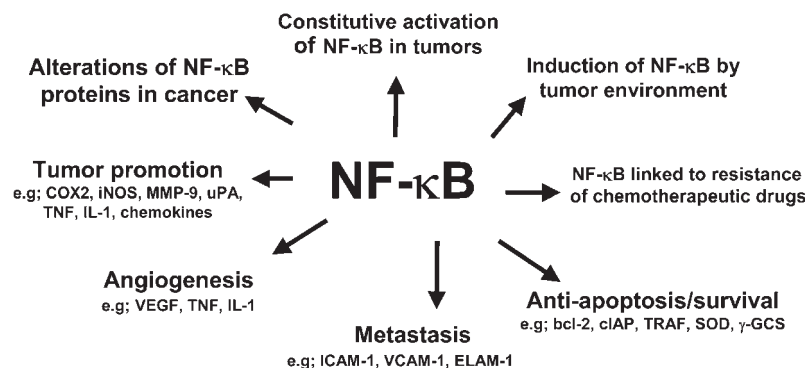
tumor microenvironment.<sup>102</sup> Third, the activation of NF- $\kappa$ B blocks apoptosis and promotes cell proliferation.<sup>103–106</sup> Fourth, several tumor types show a persistent constitutive nuclear activation of NF- $\kappa$ B. Fifth, NF- $\kappa$ B activation has been shown to induce resistance to various chemotherapeutic agents.<sup>107,108</sup> Sixth, NF- $\kappa$ B gene products can be oncogenic when aberrantly expressed and are implicated in a number of tumor types.

### NF- $\kappa$ B and tumor promotion

The development of cancer is generally categorized into three stages: tumor initiation, tumor promotion, and tumor metastasis. Besides regulating a number of genes involved in prolonged cell survival (see above), NF- $\kappa$ B regulates many genes involved in the promotion of cancer (ie clonal expansion, growth, diversification, angiogenesis, adhesion, extravasation, degradation of extracellular matrix, etc). For example, NF- $\kappa$ B may regulate the production of prostaglandins via the pro-inflammatory gene cyclooxygenase-2 (COX2), which has been shown to be overexpressed in a variety of cancers including colorectal cancer and mesothelioma.<sup>109–111</sup> Similar studies have been found for many other pro-inflammatory genes regulated by NF- $\kappa$ B including tumor necrosis factor<sup>112</sup> (TNF), interleukin-1<sup>113</sup> (IL-1), inducible NO-synthase<sup>114</sup> (iNOS), matrix metalloproteinase<sup>115</sup> (MMP-9), urokinase-type plasminogen activator<sup>116</sup> (uPA), and many other chemokines.<sup>117–119</sup>

### Role of NF- $\kappa$ B in angiogenesis

Tumor cells, just like normal cells, need oxygen to survive and thus can be a limiting factor to progression of tumors. Vascularization of tumors requires the release of angiogenic growth factors (eg VEGF, MCP-1) from tumor cells and/or inflammatory cells such as macrophages and neutrophils or in response to pro-inflammatory cytokines (eg TNF).<sup>120–122</sup> NF- $\kappa$ B regulates the expression of such growth factors and cytokines (VEGF, TNF, MCP-1) necessary for angiogenesis providing another pathway for which inhibition of NF- $\kappa$ B may be justified in anti-cancer therapy.<sup>123–126</sup>



**Figure 3** Potential mechanisms by which NF- $\kappa$ B activation can cause development of cancer. First, it regulates the transcription of many genes that are involved in tumor promotion, angiogenesis, metastasis, and increased cell survival. Second, NF- $\kappa$ B may be induced by the tumor microenvironment (eg hypoxia) and cause perpetuating tumorigenesis. Third, its activation is linked to increased resistance of tumors to chemotherapeutic drugs and radiation therapy. Fourth, the constitutive activation of NF- $\kappa$ B is present in a variety of tumors. Finally, aberrant expression of the NF- $\kappa$ B proteins themselves have shown to be linked with the development of cancer.

## NF- $\kappa$ B and metastasis

The metastasis of cancer requires the migration of cancerous cells both into and out of the vessel walls that transport them to other parts of the body. The ability to cross vessel walls is mediated by specific molecules that are expressed in response to a number of signals from inflammatory cells, tumor cells, etc. Among those special molecules are ICAM-1, ELAM-1, and VCAM-1, all of which have been shown to be expressed in response to NF- $\kappa$ B activation.<sup>127–129</sup>

## The induction of NF- $\kappa$ B by the tumor microenvironment

The stress of fluctuation in blood flow in the microenvironment of solid tumors and the resultant intermittent hypoxia has been shown to activate NF- $\kappa$ B.<sup>130</sup> Since oxygen is needed for a tumor to grow, the tumor must secrete chemotactic signals such as growth factors and cytokines in order to induce neovascularization.<sup>131</sup> Many of these growth factors and necessary signals for tumor progression (see below) are target genes of NF- $\kappa$ B and utilize its activation for their transcription. Once again, the cellular signals and precise mechanism of activation have not been elucidated, but the pathway of NF- $\kappa$ B activation by hypoxia is somewhat unique. The more 'traditional' pathway of activation in response to stimuli (eg TNF- $\alpha$ , IL-1) via I $\kappa$ B inhibitor phosphorylation, ubiquitination and degradation requires the phosphorylation of serines on I $\kappa$ B (see above), but Koong *et al*<sup>130</sup> have shown that hypoxia stimulates the phosphorylation of tyrosine groups instead. The addition of this pathway leading to NF- $\kappa$ B activation adds to the belief that NF- $\kappa$ B may involve several more pathways that are as yet undiscovered.

## NF- $\kappa$ B in apoptosis, cell proliferation, and tumor initiation

In 1996, three separate reports appeared implicating NF- $\kappa$ B in the inhibition of apoptosis.<sup>104,132,133</sup> Since then, a flurry of reports, mostly supporting the original conclusions, have confirmed NF- $\kappa$ B's role as a mediator of inhibition of apoptosis in many cell types. Tumor initiation begins with the prolonged survival of a cell, and so, given its role in apoptosis, NF- $\kappa$ B has obvious implications for cancer.

An anti-apoptotic role of NF- $\kappa$ B has been linked to T cell lymphoma, osteoclasts,<sup>134</sup> melanoma,<sup>135</sup> pancreatic cancer,<sup>136</sup> bladder cancer,<sup>137</sup> and breast cancer.<sup>138</sup> Cell types (not necessarily oncogenes) that display an anti-apoptotic role for NF- $\kappa$ B include B cells,<sup>139,140</sup> T cells,<sup>141,142</sup> granulocytes,<sup>143</sup> macrophages,<sup>144</sup> neuronal cells,<sup>145,146</sup> and smooth muscle cells.<sup>147</sup>

Although rare, there are systems in which NF- $\kappa$ B has been shown to play a pro-apoptotic role in addition to its more common anti-apoptotic role. Examples of its pro-apoptotic effects in cells include those found in B cells,<sup>148</sup> T cells,<sup>149,150</sup> neuronal cells,<sup>151,152</sup> and endothelial cells.<sup>153</sup>

The opposing effects of NF- $\kappa$ B are thought to be cell type specific and/or dependent on the inducing signal (eg IL-1, TNF- $\alpha$ , and UV radiation). Different activation pathways of NF- $\kappa$ B may cause the expression of proteins that promote apoptosis (eg Fas, c-myc, p53, I $\kappa$ B $\alpha$ ) or inhibit apoptosis (eg TRAF2, IAP proteins, Bcl-2-like proteins).<sup>154–156</sup> In addition, NF- $\kappa$ B activation variably controls the regulation of cell cycle

proteins (eg cyclin D1 and CDK2 kinase)<sup>157–159</sup> and the interaction with various cellular components (eg p300 and p53) that promote or induce apoptosis<sup>160,161</sup> (see Figure 3).

## The constitutive activation of NF- $\kappa$ B in cancer

Another potential mechanism through which NF- $\kappa$ B could play a role in tumorigenesis involves its constitutive activation. As explained above, the activation of NF- $\kappa$ B occurs as it is transported from the cytoplasm to the nucleus upon degradation of the inhibitory subunit. In the nucleus it binds to specific  $\kappa$ B sites on the DNA and mediates the expression of a number of genes involved in the cellular response to various stresses. Thus, when NF- $\kappa$ B is found persistently in the nucleus, it is referred to as constitutive activation. Constitutive activation is shown in a wide variety of tumor types<sup>162–184</sup> (see Table 2) including those tumors induced in animal models. A higher level of NF- $\kappa$ B binding activity was found in 86% of nuclear extracts from mammary tumors that were induced in rats vs normal rat mammary glands and higher levels of NF- $\kappa$ B were found in estrogen receptor-negative breast cancer cell lines in rats and humans which correlated with tumorigenesis. Also, increased expression of NF- $\kappa$ B was found in papillary, anaplastic, and follicular thyroid cancer cell lines vs normal cells. Furthermore, the inhibition of p65 in these cancer cells led to a decrease in *c-myc* expression and a decrease in growth. Finally, constitutive activation was found in 83% of human pancreatic cancer cell lines. Taken together, these results, along with other experiments cited in Table 3, suggest a strong correlation between NF- $\kappa$ B expression and tumor formation, making the inhibition of NF- $\kappa$ B a valid therapeutic frontier. It should be noted, however, that constitutive activation of NF- $\kappa$ B is not limited exclusively to tumors as evidenced by the existence of normal cells which show constitutive activation.

The precise role of constitutive activation in tumors is not known but has been linked to resistance to apoptosis in human cutaneous T cell lymphoma cells.<sup>105</sup> It is tempting to believe that a similar mechanism accounts for the progression of all tumors that constitutively express NF- $\kappa$ B, but such a link has yet to be clearly identified. Normally, activation of NF- $\kappa$ B has been shown to be stimulus dependent, such that some

**Table 2** Tumors that express constitutively active NF- $\kappa$ B

Tumor type	Refs
B cell lymphoma	162, 163
Hodgkin's disease	164–166
T cell lymphoma	167, 105
Adult T cell leukemia	168
Acute lymphoblastic leukemia	169
Breast	138, 106
Liver	170–172
Thyroid	173
Pancreas	174, 175
Prostate	176, 177
Melanoma	178
Head and neck SCC	179
Colon	180
Multiple myeloma	181
Ovarian cancer	182
Bladder cancer	137
Lung carcinoma	183, 184

**Table 3** Tumors with altered NF- $\kappa$ B proteins

NF- $\kappa$ B protein	Cancer	Refs
c-Rel	Diffuse lymphomas with large cell component	191–193
	Thymic B cell lymphoma, follicular large cell lymphoma	190, 184, 194
	Follicular lymphoma	195
	Human diffuse lymphoma cell line	195
	Non-small cell lung carcinoma	184
Rel A	Squamous head and neck carcinoma	188
	Adenocarcinomas of breast and stomach	133, 108
	Thyroid carcinoma cell line	173
	Multiple myeloma	190
NF- $\kappa$ B1 (p105/p50)	Non-small cell lung carcinoma	184
	Acute lymphoblastic leukemias	196
	T cell acute leukemias	197
	Non-small cell lung carcinoma	182, 189
NF- $\kappa$ B2 (p100/p52)	Colon, prostate, breast, brain, bone cell lines	189
	B cell non-Hodgkin's lymphoma	198–202
	Chronic lymphocytic leukemia, multiple myelomas, cutaneous T cell lymphomas	106, 184, 203
I $\kappa$ B $\alpha$	Breast cancer, colon cancer	
	Hodgkin's lymphoma	166, 204, 205

stimuli such as TNF- $\alpha$  cause its activation in certain cells whereas other stimuli such as IL-1 or hypoxia cause its activation in other cells. While many NF- $\kappa$ B stimuli have been identified, the stimulus responsible for constitutive activation of NF- $\kappa$ B in most cell types is not understood. Cells that express constitutively activated NF- $\kappa$ B are resistant to various chemotherapeutic agents.

#### NF- $\kappa$ B activation in chemotherapy and radiation therapy

The activation of NF- $\kappa$ B has been linked to cellular resistance of chemotherapeutic drugs and radiation treatment, making the development of anti-NF- $\kappa$ B drugs all the more promising.<sup>132</sup> Antagonizing NF- $\kappa$ B activity has also been shown to increase the efficacy of chemotherapeutic agents and radiation in some tumor cell lines.

In the case of radiotherapy, which has been a valuable tool in the treatment of several cancers including lymphomas and leukemias, NF- $\kappa$ B has been shown to be activated in response to therapy in tumor cell lines. Fibrosarcoma cells expressing genetically manipulated I $\kappa$ B $\alpha$  (an inhibitors of NF- $\kappa$ B not sensitive to phosphorylation, 'super repressor', see below) are more sensitive to radiation-induced apoptosis than in controls.<sup>132</sup> In addition, glioblastoma cell lines (A172, M054) that express the super repressor are more susceptible to radiation treatment.<sup>185</sup> While the precise mechanism leading to induction of radiation resistance is not clear, these examples make the development of inhibitors to NF- $\kappa$ B more promising.

Likewise, the precise mechanism for NF- $\kappa$ B's involvement in chemotherapeutic efficacy is not clear, but its enhanced activity has been shown to be linked to decreased apoptosis via expression of the anti-apoptotic gene A1/Bfl-1 and enhanced expression of the multiple-drug resistance gene product or MDR gene (prevents the intracellular accumulation of toxic drugs such as those used in chemotherapy), both likely factors in the progression of tumors.<sup>108,186</sup>

#### Alterations of NF- $\kappa$ B proteins in cancer

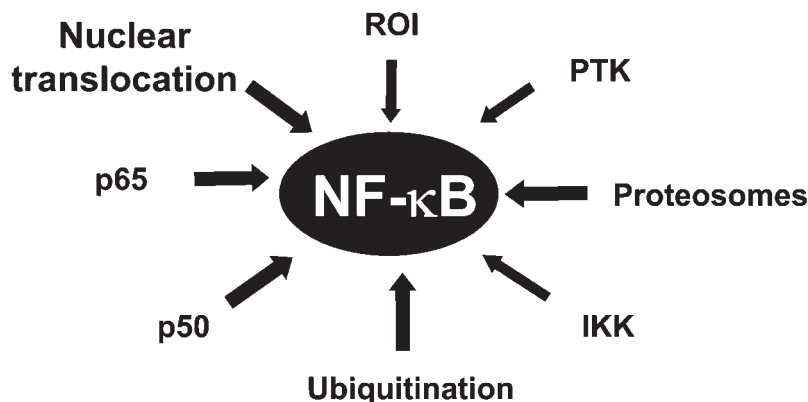
While the expression of a large number of genes involved in the development of cancer are regulated by NF- $\kappa$ B, the genes that code for individual NF- $\kappa$ B proteins themselves have also been implicated in the development of several types of cancers, both hematopoietic and solid tumors. These genes are expressed aberrantly, ie amplification of gene on chromosome, rearrangement, overexpression, substitution, mutation, truncation, etc.<sup>187</sup> Alterations in NF- $\kappa$ B proteins have been found in a wide variety of tumors (see Table 3). Thus, the altered expression of the NF- $\kappa$ B gene products may play a critical role in tumorigenesis.

#### Strategies to block NF- $\kappa$ B activation

Several strategies have been employed to block the activation of NF- $\kappa$ B. A wide variety of compounds (both natural and synthetic) have been screened for their ability to suppress NF- $\kappa$ B. These compounds block NF- $\kappa$ B activation through multiple mechanisms by intercepting various steps leading to NF- $\kappa$ B activation (see Figure 4). How specific some of these inhibitors are and whether they block other signaling pathways<sup>206</sup> remains to be elucidated. The following gives examples for each of the strategies shown to block NF- $\kappa$ B activation in response to one or more stimuli.

#### Block binding of NF- $\kappa$ B to the DNA

The most direct strategy for blocking activation of NF- $\kappa$ B is to block its binding to specific  $\kappa$ B sites on DNA. This is assessed by assaying the amount of NF- $\kappa$ B protein that is able to bind to DNA after administration of inhibitor. One mechanism is the use of a transcription factor decoy (TFD) peptide, called double-stranded oligodeoxynucleotide (ODN), that binds the same complementary region of specific DNA sites, competitively inhibiting NF- $\kappa$ B binding.<sup>207</sup> Other examples of inhibi-



**Figure 4** Potential target for inhibition in the pathway leading to NF- $\kappa$ B activation. These include the prevention of ROI formation, the inhibition of protein tyrosine kinases, proteasome inhibitors, the inhibition of IKK stimulation, the inhibition of ubiquitination of I $\kappa$ B $\alpha$ , the inhibition of direct activation via p50 and p65, and the blocking of nuclear translocation of the active complex to prevent DNA binding and transcription.

tors that block DNA binding of NF- $\kappa$ B either directly or indirectly include atrial natriuretic peptide<sup>208</sup> (ANP), IL-4,<sup>209</sup> metals such as chromium, cadmium, gold, mercury, and zinc,<sup>210,211</sup> ribavirin,<sup>212</sup> vascular endothelial growth factor<sup>213,214</sup> (VEGF), caffeine acid phenylether ester<sup>215</sup> (CAPE), and vasoactive intestinal peptide<sup>216</sup> (VIP).

#### Block proteasome from degrading I $\kappa$ B inhibitory subunit

A more upstream strategy for blocking the activation of NF- $\kappa$ B is by affecting the inhibitory pathways in NF- $\kappa$ B activation. Proteasome inhibitors block the 26S proteasome necessary to degrade the I $\kappa$ B inhibitory subunit after its phosphorylation and ubiquitination in the cytoplasm and thus its release from the NF- $\kappa$ B complex.<sup>217,218</sup> Examples of these include peptide aldehydes such as ALLnL, LLM, Z-LLnV, and Z-LLL,<sup>111,119,220</sup> lactacystine,<sup>221</sup> PS-341,<sup>222</sup> ubiquitin ligase inhibitors,<sup>223</sup> and cyclosporin A.<sup>224</sup> Other I $\kappa$ B degradation inhibitors, whose mechanisms are unknown, include capsaicin,<sup>225</sup> core protein hepatitis C virus,<sup>226</sup> fungal gliotoxin,<sup>227</sup> IL-13,<sup>228</sup> and pervanate.<sup>229</sup>

#### Block phosphorylation of I $\kappa$ B

Because phosphorylation of I $\kappa$ B $\alpha$  is critical for NF- $\kappa$ B activation, compounds that block this phosphorylation prevent I $\kappa$ B $\alpha$ 's ubiquitination and further degradation. Examples include aspirin,<sup>230,231</sup> (sodium salicylate), ibuprofen,<sup>232</sup> nitric oxide,<sup>233–235</sup> prostoglandin A1,<sup>236</sup> sanguinarine,<sup>237</sup> and YopJ (encoded by *Yersinia pseudotuberculosis*).<sup>238</sup> Recently, 4-hydroxy-2-nonenal, a lipid peroxidation product, has been shown to block phosphorylation by direct inhibition of IKK.<sup>239</sup> Also, a novel peptide that selectively blocks the association of IKK- $\gamma$  (NEMO) with the rest of the IKK complex has been shown to inhibit NF- $\kappa$ B activation in response to pro-inflammatory cytokines in mice while preserving basal NF- $\kappa$ B activity.<sup>240</sup>

#### Up-regulate inhibitory subunit

Up-regulating the amount of I $\kappa$ B $\alpha$  is another strategy employed by various compounds to inhibit NF- $\kappa$ B activation.

Examples include beta-amyloid (found in Alzheimer's),<sup>145</sup> glucocorticoids such as dexamethasone or prednisone,<sup>241,242</sup> IL-10,<sup>243,244</sup> and IL-13.<sup>228</sup>

#### Antioxidants

Various antioxidants have also been shown to inhibit NF- $\kappa$ B activation in response to a variety of stimulants (ie TNF- $\alpha$ , IL-1, phorbol ester, LPS, UV) through diverse and largely unknown mechanisms.<sup>245–247</sup> Examples include disulfiram,<sup>248</sup> curcumin,<sup>249</sup> glutathione,<sup>215,250</sup> and vitamin C.<sup>251</sup>

#### Block nuclear translocation

Another approach to inhibiting NF- $\kappa$ B activation is to use cell-permeable peptides that block the nuclear localization of the NF- $\kappa$ B complex. The mechanism works by mimicking the sequence of p50 responsible for transporting the NF- $\kappa$ B complex from the cytoplasm to the nucleus to block the normal import machinery.<sup>252</sup> Examples of these include SN-50<sup>253</sup> and *o,o'*-bismyrystoyl thiamine disulfide.<sup>254</sup>

#### Suppression of NF- $\kappa$ B by gene transfer

Another strategy to block the activation of NF- $\kappa$ B is through the transfer of genes that code for proteins shown to suppress NF- $\kappa$ B activation. The most direct target is the I $\kappa$ B $\alpha$  gene. This entails the modification of I $\kappa$ B $\alpha$  at the specific phosphorylation sites (ser 32 and 36 switched with ala) and ubiquitination sites (lys 21 and 22 switched with arg) to prevent its degradation. This 'superrepressor' keeps the NF- $\kappa$ B complex in the cytoplasm indefinitely.<sup>255–257</sup> Recently, a nonphosphorylatable form of I $\kappa$ B $\alpha$  was shown to inhibit osteoclastogenesis and block bone resorption when injected into bone marrow macrophages.<sup>258</sup>

Another potential target for gene transfer has recently been shown to be HDAC3, a histone deacetylase that acts directly upon nuclear Rel A (part of the combined active p50/Rel A complex) enabling its association with I $\kappa$ B $\alpha$  and its subsequent export from the nucleus. Expression of HDAC3 in TNF- $\alpha$ -stimulated HeLa cells repressed both NF- $\kappa$ B DNA-binding and levels of Rel A with a corresponding increase in

inactive cytoplasmic I $\kappa$ B $\alpha$ /NF- $\kappa$ B complexes.<sup>259</sup> This mechanism was shown to control the duration of NF- $\kappa$ B activation and thus may be a potential weapon against constitutive NF- $\kappa$ B activation.

Finally, the presence of pro-apoptotic cellular proteins have been shown to inhibit the anti-apoptotic function of NF- $\kappa$ B as described earlier, serving as potential targets for gene transfer. Erg-1, a transcription factor that is activated by similar stimuli as NF- $\kappa$ B, has recently been shown to block NF- $\kappa$ B activation both *in vitro* and *in vivo*. Erg-1 dimerizes with the p65 (Rel A) subunit of NF- $\kappa$ B via a specific zinc-finger domain and prevents NF- $\kappa$ B from binding to its promoter regions on DNA.<sup>260,261</sup> The RAI (Rel A-associated inhibitor) gene also encodes a protein that associates with p65 (Rel A) and inhibits the anti-apoptotic activity of NF- $\kappa$ B. RAI shares a homologous region with 53BP2, a protein involved in apoptosis regulation.<sup>262</sup> Par-4 (prostate apoptosis response-4) is another recently identified inhibitor of NF- $\kappa$ B at the level of IKK and activator of the Fas apoptotic pathway.<sup>263,264</sup> In addition, Par-4 inhibits Bcl-2, a well known anti-apoptotic oncogene.<sup>265</sup> E2F-1 is another transcription factor whose expression has been correlated with increased apoptotic activity via mechanisms including the inhibition of NF- $\kappa$ B. E2F-1 promotes cell cycle progression and its aberrant expression is present in most tumors.<sup>266</sup> Finally, p53 (the classic gatekeeper of cell cycle progression) and cyclin E-cdk2 may inhibit the anti-apoptotic action of NF- $\kappa$ B via the transcriptional co-activator protein CRB/p300.<sup>267–270</sup> Before NF- $\kappa$ B binds to its promoter regions on DNA, it is acetylated by CRB/p300 which maintains its presence in the nucleus. P53 and cyclin E-cdk2 compete for the finite CRB/p300 complexes and prevent its interaction with NF- $\kappa$ B. It should be noted, however, that the classic antagonism between the pro-apoptotic p53 and the anti-apoptotic NF- $\kappa$ B has been the subject of debate after p53 was found to activate NF- $\kappa$ B and correlate with the ability of p53 to induce apoptosis.<sup>271</sup>

#### *Further genetic manipulation of proteins involved in the activation cascade*

It has been possible to block the activation of NF- $\kappa$ B by manipulating the genes that encode proteins (eg TRAF2, TRAF6, I-TRAF, NIK, MEKK1, and IKK) found directly in the known activation pathways.<sup>206</sup> TRAF2 (TNF receptor-associated factor) and TRAF6 interact with TNF receptors and serve as adapters for the activation of NF- $\kappa$ B. Dominant negative mutants of TRAF2 and TRAF6 have been shown to repress NF- $\kappa$ B activity in response to TNF- $\alpha$  and IL-1, respectively.<sup>272,273</sup> I-TRAF interacts with TRAF2, and its overexpression inhibits TRAF2 activation of NF- $\kappa$ B.<sup>274</sup> NIK (NF- $\kappa$ B-inducing kinase) is induced by several proteins in the activation pathway, including TRAF-2 and TRAF-6, and activates IKK $\alpha$  (as does MEKK1). Therefore it has been shown that a dominant-negative mutant of NIK (and MEKK1) represses NF- $\kappa$ B activation.<sup>41,275,276</sup> The IKKs (I $\kappa$ B kinases, alpha and beta) phosphorylate I $\kappa$ B $\alpha$  to subsequently cause its ubiquitination and ultimate degradation. Thus, alterations in the ATP-binding site of the IKK complex (IKK $\beta$  more so than IKK $\alpha$ ) or its activation loop have been shown to block the activation of NF- $\kappa$ B as well.<sup>42–44,277–279</sup>

#### *Physiologic consequence of NF- $\kappa$ B inhibition and future direction*

The inhibition of NF- $\kappa$ B with the methods illustrated above represents a theoretical approach to the more complicated

issue of creating drug therapies that are effective in preventing or attenuating tumorigenesis. So far, several agents have been shown to utilize the modulation and/or inhibition of NF- $\kappa$ B to carry out some part of their therapeutic purpose such as glucocorticoids, nonsteroidal anti-inflammatory agents (NSAID), vitamin E, curcumin, thiols, cyclosporin, rifampicin, dithiocarbamates, methotrexate, thalidomide, leflunomide and various fungal and bacterial metabolites.<sup>280</sup> Could these agents and a number of others mediate an anti-carcinogenic role? It is too early to tell because our knowledge of their precise mechanisms of action, specificity, and even toxicity with respect to NF- $\kappa$ B is still incomplete. We do know, however, that many of these drugs influence the NF- $\kappa$ B pathway among others, and the interactions between these different pathways *in vivo* may play a role in their characteristic effects.

While we show that NF- $\kappa$ B is potentially capable of causing much distress, the elements in its pathways that we have targeted for inhibition above (eg ubiquitin ligase inhibitors, proteasome inhibitors, I- $\kappa$ B $\alpha$  super repressors, etc) may be valuable parts for other pathways that are required for the normal functioning of the body. For example, the turnover of the outer layers of skin requires the function of a specific ubiquitin ligase complex, SCF $\beta$ -TrCP, which is also part of the traditional activation cascade for NF- $\kappa$ B.<sup>281</sup> Ironically, the consequences of its inhibition include oncogenesis. Furthermore, the complete inhibition of NF- $\kappa$ B via genetic knockout may produce severe toxicity as evidenced from mice studies using dominant-negative Rel A among others<sup>282</sup> (see above 'Lessons to be learned from NF- $\kappa$ B knockouts'). These Rel A-deficient mice die during embryogenesis due to pathologic apoptosis of the liver. Other gene knockouts that involve the NF- $\kappa$ B complex and its inhibitors have shown us that its complete deficiency likely results in severe immunodeficiencies and accelerated apoptosis.

Thus it is clear that for effective drug development, targeting NF- $\kappa$ B must focus on its partial and specific inhibition with respect to toxicity. This level of specificity is achievable given our current progress, as in the example of glucocorticoids,<sup>283</sup> which target the inhibition of NF- $\kappa$ B in lymphoid cells making it the most effective, although not ideal anti-asthmatic therapy.

#### **Conclusion**

From this description, it is clear that during the last decade there have been major developments in our understanding of how NF- $\kappa$ B is activated and also how it may contribute to the development of cancer. Activation of NF- $\kappa$ B is emerging as one of the major mechanisms of tumor cell resistance to cytokines and chemotherapeutic agents. Until now most of our knowledge about NF- $\kappa$ B and its role in cellular physiology has been based on *in vitro* experimentation. Future *in vivo* studies may demonstrate the true importance of NF- $\kappa$ B and allow that knowledge to be used in clinical medicine. The development of specific inhibitors that can block NF- $\kappa$ B activation will have great potential in improving cancer therapy.

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