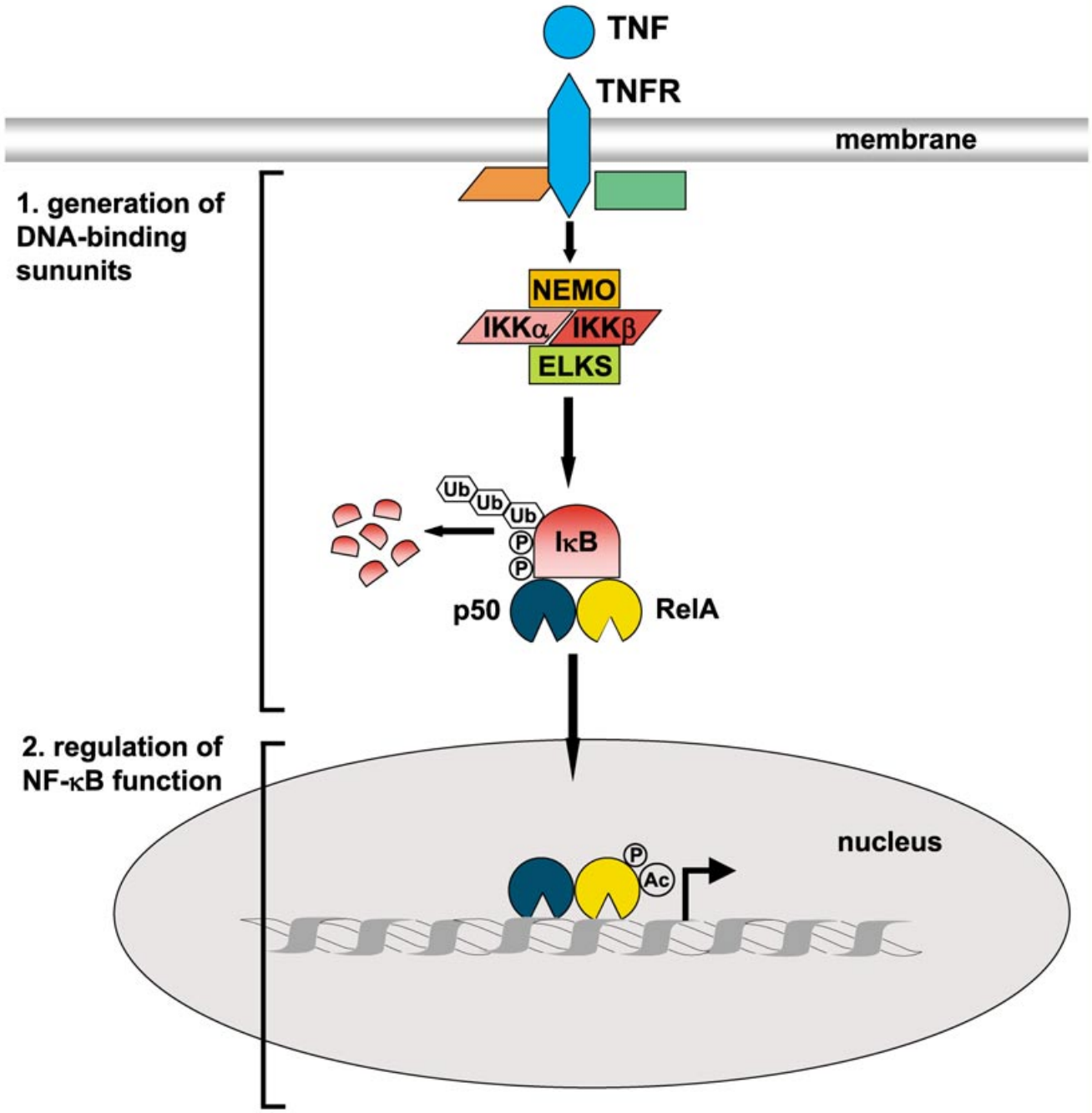

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NF-κB: A Multifaceted Transcription Factor Regulated at Several Levels

M. Lienhard Schmitz,*[a] Ivan Mattioli,[a] Holger Buss,[b] and Michael Kracht*[b]

NF-κB is a generic name for an evolutionarily conserved transcription-factor system that contributes to the mounting of an effective immune response but is also involved in the regulation of cell proliferation, development, and apoptosis. The implication of NF-κB in central biological processes and its extraordinary connectivity to other signaling pathways raise a need for highly controlled regulation of NF-κB activity at several levels. While all NF-κB activation pathways share a central and critical proteasome-mediated step that leads to the degradation of inhibitory proteins and the release of DNA-binding subunits, there is evidence for a downstream level of NF-κB regulation that employs several mechanisms. These include promoter-specific exchange of dimers and modification of the transactivating p65 subunit by phosphorylation, acetylation, ubiquitination, or prolyl isomerization. The signaling pathways and enzymes controlling this second level of regulation and their potential use as therapeutic targets for the treatment of NF-κB-associated pathologies are discussed here.

1. The NF-κB Transcription Factor Family

The NF-κB signaling pathway was developed early in evolution and can already be found in Drosophila and molluscs.[1] In Drosophila, NF-κB-like transcription factors are activated by the Toll and Imd (immune deficiency) pathways in order to combat infections. The Imd signaling pathway is triggered after infection by Gram-negative bacteria, while the Toll receptor can function as a cytokine receptor that is activated in response to fungal infections.[2] The function of NF-κB for the immune response and also the components of the signaling pathway have been evolutionarily conserved in mammals. Five different NF-κB DNA-binding subunits share an N-terminal NF-κB/Rel homology domain (RHD) that mediates DNA binding, dimerization, nuclear translocation, and interaction with the inhibitory IκB proteins.[3] The domain architecture of the NF-κB DNA-binding subunits and the IκB proteins is schematically shown in Figure 1. The NF-κB family members p65 (RelA), RelB, and c-Rel contain C-terminal transactivation domains (TADs) that trigger target gene transcription. The strongest gene activation is mediated by p65, which contains two potent transactivation domains within its C terminus.[4] The other two family members, p50 and p52, are produced as large precursor proteins (p105 and p100, respectively). While p50 is generated by constitutive processing of p105, the cleavage of p100 to p52 is a regulated event that employs phosphorylation and ubiquitination steps.[5] The p50 and p52 subunits are transcriptionally inactive but can induce gene expression when they form heterodimers with p65, c-Rel, or RelB. Homodimers composed of p50/p50 or p52/p52 subunits can repress transcription.[4] On the other hand, a complex between p52 and the coactivating Bcl-3 oncoprotein activates transcription.[6] While the most abundant and prototypical form of NF-κB is considered to be a heterodimer between p50 and p65, slowly activated dimers such as p52/RelB can replace the rapidly activated p50/p65 heterodimers depending on the promoter context.[7] NF-κB is a regulated transcription factor, since the DNA-binding subunits are retained in the cytoplasm upon association with inhibitory IκB proteins in most cell types.[8] These inhibitory proteins share a number of protein/protein interaction domains called ankyrin repeats and form a large genetic family with eight known mammalian members: IκBα, IκBβ, IκBε, IκBγ, IκBNS,

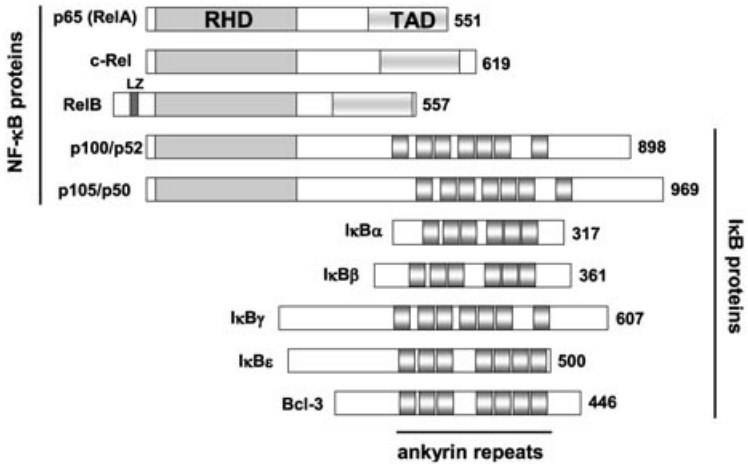

Figure 1. *Architecture of NF-κB and IκB proteins. The NF-κB DNA-binding subunits share an N-terminal RHD. RelB bears a leucine zipper (LZ) in its N terminus and three of the subunits contain one or several C-terminal TADs. All IκB proteins share six or seven ankyrin repeats.*

[a] *Dr. M. L. Schmitz, I. Mattioli*
University of Bern, Department of Chemistry and Biochemistry
Freiestraße 3, 3012 Bern (Switzerland)
Fax: (+41) 31631-4887
E-mail: lienhardschmitz@ibc.unibe.ch

[b] *Dr. H. Buss, Prof. Dr. M. Kracht*
Institute of Pharmacology, Medical School Hannover
Carl-Neuberg Straße 1, 30625 Hannover (Germany)
Fax: (+49) 511-532-4081
E-mail: kracht.michael@mhhannover.de

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M. Lienhard Schmitz was born in 1961 in Tübingen, Germany, and studied biology in Göttingen and Freiburg im Breisgau. He did his PhD thesis under the supervision of Prof. G. Feix in Freiburg im Breisgau and started to become interested in NF-κB when joining the group of Prof. P. A. Baeuerle as a postdoctoral researcher in Martinsried. After his habilitation at the University of Freiburg, he worked as a group leader at the German Cancer Research Center in Heidelberg. Since 2002, he has been a faculty member at the Department for Chemistry and Biochemistry at the University of Bern, Switzerland.

Ivan Mattioli was born in 1977 in St. Gallen, Switzerland, and studied biology at the University of Bern. He is currently a third year PhD student in the laboratory of M.L.S.

Michael Kracht was born in 1962 in Braunschweig, Germany, and studied medicine in Hannover. During his studies, he completed an MD thesis on signal transduction in T-lymphocytes in the laboratory of Prof. M. Szamel in Hannover. After working for two years in a surgical department, he rejoined Prof. Szamel's group as a postdoctoral researcher and started to work on IL-1 signaling, an area that he also pursued as a postdoctoroal researcher in Prof. J. Saklatvala's laboratory in Cambridge, UK. Since 1995, he has been a group leader in the Department of Pharmacology, Medical School Hannover.

Holger Buss was born in 1973 in Aurich, Germany. He studied biochemistry in Hannover and has just completed his PhD thesis in the laboratory of M.K.

Bcl-3, and the p100 and p105 precursor proteins. Crystal structures have been obtained for most of the DNA-binding subunits, either alone or in association with IκB proteins. The DNA-binding subunits display a unique butterfly-shaped structure for the RHDs, which is composed of β strands arranged in a pattern similar to immunoglobulin domains. The IκBβ protein caps the nuclear localization sequences (NLSs) of p50 and also of p65, while IκBα only masks the NLSs of p65.[9,10] These structural data are consistent with biochemical data that identify IκBβ as a truly cytoplasmic protein that prevents NF-κB nuclear entry by masking the NLS domains of both DNA-binding subunits. In contrast, a minor fraction of IκBα is also found in the nucleus and accordingly inhibition of nuclear export by leptomycin B results in nuclear accumulation of IκBα.[11] As the IκBα gene is an NF-κB target, increased synthesis of IκBα shuts down NF-κB-induced gene expression by IκBα-mediated nuclear export of the DNA-binding subunits. Thus, NF-κB activation is terminated by a negative feedback mechanism.[12,13]

2. NF-κB Activation Pathways

A large variety of stimuli can lead to NF-κB activation and most of them represent stressful or precarious conditions. These range from general adverse signals such as pervanadate, γ radiation, and DNA damage to cytokine and immune receptors. To date, three major pathways mediating NF-κB activation have been identified, the so-called canonical and noncanonical pathways and the DNA-damage-induced NF-κB pathway. The past decade has witnessed remarkable progress in our understanding of these signaling cascades.[3,14] All NF-κB activating pathways have in common the fact that they lead to the generation of DNA-binding dimers, as will be discussed in the following section. Further downstream there is a second level of NF-κB regulation, which controls NF-κB-dependent transactivation and the duration and amplitude of NF-κB signaling.[15] These two levels of regulation are schematically displayed in Figure 2.

3. Generation of DNA-binding dimers

3.1. Canonical NF-κB activation

The critical event of this pathway is the activation of the IκB kinase (IKK) complex which ultimately phosphorylates IκBα at serines 32 and 36. This phosphorylation is the prerequisite for the subsequent polyubiquitination by a specific ubiquitin ligase belonging to the SCF (Skp-1/Cul/F box) family. Recognition of N-terminally phosphorylated IκB is mediated by β-TrCP, a protein containing an F box and WD repeat.[16] The ubiquitin-marked IκB proteins are rapidly degraded by the proteasome, thereby allowing nuclear entry, DNA binding, and transcriptional activity of NF-κB. Many of the signaling proteins involved in these very early receptor-proximal events are specific for each pathway. The detailed description of the tumor necrosis factor (TNF), inteleukin 1 (IL-1), T cell receptor, and Toll receptor mediated signaling steps are beyond the scope of this manuscript and can be found in some recent reviews.[17–19]

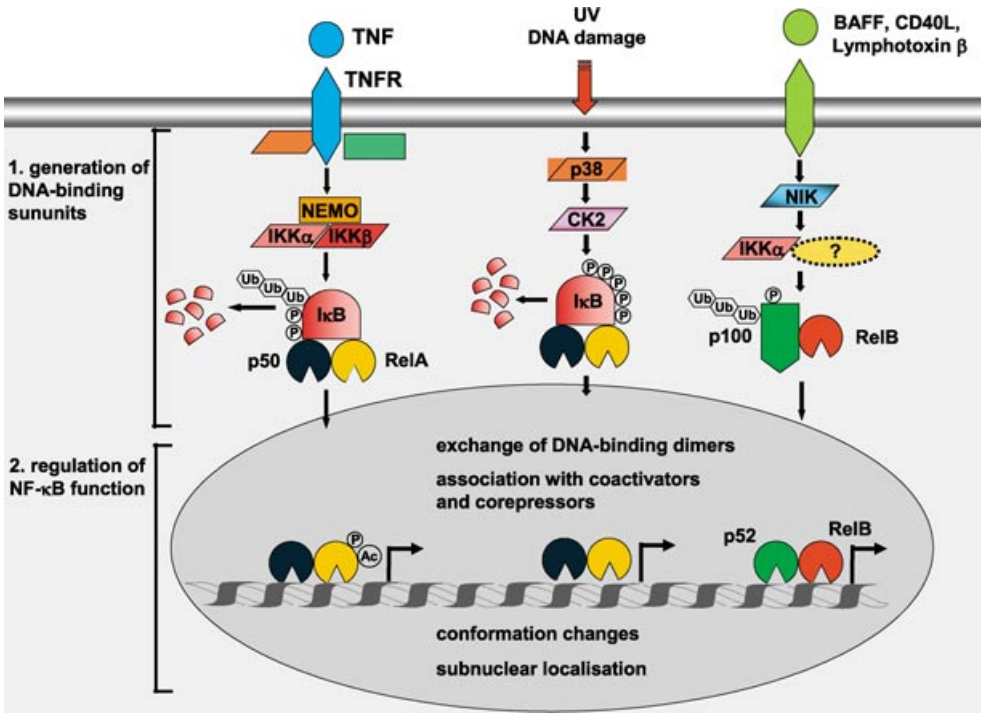

Figure 2. *The two levels of NF-κB activation. While the canonical, DNA-damage-induced and noncanonical NF-κB activation pathways share a critical step that leads to the proteasome-dependent generation of DNA-binding subunits (level 1), the activity of this transcription factor is regulated by further mechanisms within the nucleus (level 2). The shapes of the indicated proteins illustrate a functional category according to the Alliance for Cellular Signalling convention (*http://www.signaling-gateway.org*). P = Phosphorylation sites, Ub = ubiquitination sites.*

These signaling pathways funnel into the common and decisive step, which is the activation of the IKK complex. The IKK complex is formed by three core subunits: the kinases IKKα and IKKβ and the noncatalytic, regulatory NEMO protein.[20] IKKα and IKKβ share an N-terminal catalytic domain, a central leucine zipper, and a C-terminal helix–loop–helix motif that mediates direct interaction between the kinases. The activation of both IKKs is not fully understood but is known to depend on their ability to dimerize. Gene-disruption experiments have shown that the canonical pathway depends on the presence of IKKβ or NEMO.[3] While IKKβ has its major role in the cytoplasm by phosphorylating IκB, IKKα is also found in the nucleus where it is recruited to the promoter regions of NF-κB-regulated genes, phosphorylates histone H3 on serine 10, and thus contributes to the stimulation of gene expression.[21,22] The phosphorylation of serine 10 of histone H3 can also be mediated by further kinases, including RSK1, MSK1, and MSK2, and the relative contribution of each kinase to this process remains to be determined. The different functions of IKKα and IKKβ are also revealed by the analysis of knockout mice.[23–26] Selective ablation of IKKβ prevents NF-κB activation by known proinflammatory stimuli including TNFα, IL-1, and lipopolysaccharide (LPS),[23,26] while IKKα knockouts show no defects in these signaling pathways,[24,27] a result suggesting that despite their close proximity within the IKK signaling complex, both kinases have very different functions.

The activation of the IKK complex requires phosphorylation within the activation loop. IKK phosphorylation may be mediated by transautophosphorylation induced by conformational changes or alternatively by upstream-acting kinases. Many of the proposed IKK-activating kinases, including NIK, MEKK1, and Cot/Tpl-2, have not passed the knockout test[20] and, thus far, only MEKK3 has been identified as an essential component for TNFα-induced IKK activation in fibroblasts.[28] The related kinase MEKK2 forms a complex with IKK/IκB/p65 and promotes a second, delayed phase of NF-κB activation, thus regulating the proinflammatory cytokine-induced biphasic NF-κB activation.[29] Knockout experiments revealed a tissue-specific role of PKCξ for IKK activation in the lung but not in embryonic fibroblasts.[30] Another way IKK activation may be achieved is by the ubiquitination of TRAF6, which occurs in response to IL-1 stimulation.[31] In this pathway, a MAPKKK called TAK1 is brought into proximity with TRAF6 through the adapter protein TAB2 or the homologous protein TAB3.[32] Stimulation of cells with TNFα or IL-1 leads to the mutual interaction and ubiquitination of the TRAF6 and TAB proteins, presumably imposing a conformational change that triggers IKK activation.

3.2. Noncanonical NF-κB activation

Although IKKα$^{-/-}$ mice exhibit normal NF-κB activation in response to TNFα, a more detailed analysis of these animals allowed the detection of the noncanonical pathway that occurs in response to a distinct class of stimuli, particularly in B cells. The activation of this IκB-independent, noncanonical pathway results in the release of p52/RelB[33,34] and p50/RelB dimers.[35] This signaling cascade does not employ the IKK complex and can also occur in the absence of NEMO or IKKβ.[33] Noncanoni-

cal signaling leads to NIK- and IKKα-dependent processing of the p100 protein, which results in the release of p52. Overexpression of NIK fails to induce processing of p100 in the absence of IKKα, a result suggesting that NIK acts upstream from IKKα.[33] The existence of IKKα/NIK complexes in intact cells has not been formally proven, but this idea is supported by the fact that a yeast two-hybrid screen revealed IKKα as an NIK interaction partner[36] and NIK is known to phosphorylate IKKα at serine 176.[37] Noncanonical NF-κB signaling in B cells is triggered by several signals, including the TNF family member BAFF, which serves as a fundamental survival factor for B cells, and lymphotoxin β (LTβ), which is important for the organization and development of lymphoid tissues.[38] LTβ also triggers activation of the classical IKK complex and subsequent phosphorylation of IκBα and p65, thereby showing that it employs canonical and noncanonical pathways. Canonical NF-κB activation rapidly induces the synthesis of IκBα, which then in turn leads to the removal of p65 from its cognate DNA. The slower, noncanonical pathway elicits p100 processing and p52 generation, thus inducing a delayed but sustained activation of primarily RelB-containing NF-κB dimers.[35,39,40] Accordingly, LTβ stimulation results in the activation of two subsets of NF-κB target genes through the canonical or noncanonical pathways.[40] BAFF-induced B cell survival and development requires the specific BAFF receptors, NIK and p100, as revealed by an in vitro B cell differentiation model.[41] Also the TNF receptor family member CD40 also triggers, in addition to the canonical, the noncanonical pathway and induces the processing of p100 into p52. Depending on the pathway employed, p52 can associate with various members of the NF-κB/Rel family. In splenic B cells, the p100 precursor is bound by RelB to liberate a transcriptionally active p52/RelB heterodimer after p100 processing. As cytoplasmic p52 is also found in a complex with p65, activation of the canonical pathway leads to the release of a p52/p65 heterodimer.[39] The constantly growing list of inducers employing the noncanonical pathway includes LPS and the Epstein–Barr virus (EBV) encoded latent infection membrane protein 1 (LMP1).[42,43]

3.3. DNA-damage-induced NF-κB activation

While the noncanonical and canonical NF-κB activation pathways are IKK dependent, these kinases are not required for NF-κB activation in response to DNA damage.[44] The DNA-damaging agent doxorubicin induces proteasome-mediated IκBα degradation in mouse embryonic fibroblasts (MEFs) devoid of both IKKα and IKKβ genes. This degradation does not require the PEST domain or phosphorylation of serines 32 and 36 of IκBα.[44] UV-induced NF-κB activation employs a p38 mitogen-activated protein kinase (MAPK) dependent mechanism that leads to the activation of CK2 and the massive phosphorylation of IκBα at a cluster of serine residues contained in its C terminus.[45] The components of the signaling cascade discussed in this section are just emerging; genetic and pharmacological evidence suggests the involvement of DNA-dependent protein kinase (DNA-PK) and *Ataxia telangiectasia* mutated (ATM) kinase for this activation path.[46] These two kinases promote the sequential activation of the kinase $p90^{rsk}$, which can phosphorylate IκBα at serine 32 and thus induce effective IκBα degradation.[47]

4. Functions of NF-κB

This transcription factor has been implicated in a variety of functions, including innate immunity, which builds up a first line of defence against invading pathogens. During this process, NF-κB-mediated transcription of cytokines, chemokines, antimicrobial peptides, and specific enzymes helps to fight against invading hostile bacteria and fungi.[48] Acquired immunity also employs NF-κB-dependent functions, which contribute to secondary lymphoid organ development and the maturation and activation of immune cells, including B cells and T cells.[49,50] NF-κB also contributes to the control of cell proliferation by its regulatory effect on cell-cycle regulators including cyclin D and cyclin E.[49,51] The role of NF-κB for the control of cell proliferation is seen in B cells from c-$Rel^{-/-}$ or c-$Rel^{-/-}p105^{-/-}$ double-knockout mice, which show a defective proliferative response to mitogenic stimulation and concomitant G1 phase arrest.[49,52] The role of NF-κB in development is not easy to reconcile, as some defects observed in knockout animals (for example, the role of NF-κB during B cell maturation) may instead be explained by its antiapoptotic function,[49,53] due to induced expression of antiapoptotic genes including Bcl-xL, NR13, and Bfl-1 A1.[54] The role of IKKα for development relies on protein-kinase-dependent and -independent functions. A kinase-independent activity is required for differentiation of epidermal keratinocytes, while kinase activity is required for lymphoid organogenesis and mammary gland development.[55] There is also evidence for an NF-κB-independent contribution of IKKα in tooth development by the Notch/Wnt pathway.[56] Thus, the NF-κB system undoubtedly has a variety of unexpected functions in embryonic development of epidermal tissues and limbs, while for the adult organism it is not an oversimplification to state that, in the majority of cases, NF-κB activity is switched on in response to tissue damage, whereby the majority of target genes are switched on as part of an adjustment program serving to cope with this endangering situation. The important role of NF-κB for these central processes implicates the dysregulation of this transcription factor in several ailments, including inflammatory diseases and cancer. Thus, the activation pathways used by this transcription factor and compounds interfering with its activation are a focal point of intense research. Studies have used microarray experiments in order to identify target genes in genetically altered cells lacking NF-κB activity due to the deletion of components of the NF-κB system[25] or displaying a constitutively activated NF-κB pathway.[57] All of these studies confirmed the involvement of NF-κB in the regulation of already known target genes, including chemokines, cytokines, and apoptotic regulators, but also revealed many novel NF-κB target genes. Furthermore, by scanning all p65 binding sites across human chromosome 22, Martone and collegues showed that NF-κB binding is not restricted to promoter regions and occurs at consensus- and nonconsensus sites with equal frequency.[58] Hence, our picture

of NF-κB-mediated functions remains incomplete until all binding sites and target genes are identified. Unraveling the mechanisms of target gene activation is a formidable task but it will undoubtedly extend our knowledge of NF-κB functions.

5. Regulation of NF-κB p65 by Postranslational Modifications

Mice deficient in the protein kinases GSK3β,[59] TBK1/NAK,[60] NIK,[61] and PKCζ[30] show normal phosphorylation and degradation of IκB but impaired activation of NF-κB-dependent target genes. Furthermore, there is increasing evidence from biochemical and genetic experiments that strongly suggests an essential role of phosphorylations, acetylations, and probably also further modifications for the function of NF-κB. The complexity of this regulation is best exemplified by recent studies investigating the signaling pathways responsible for phosphorylation of p65. Hereby, individual phosphorylation sites are targeted either by a single or by several kinases. Examples of the latter situation are seen for phosphorylation of serines 276 or 536. Finally, the most important and least resolved question relates to the mechanisms by which these phosphorylations contribute to the overall activity of NF-κB. The NF-κB p65 phosphorylation sites mapped thus far and the involved kinases are summarized in Figure 3.

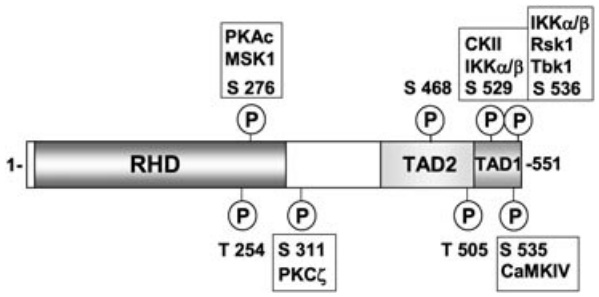

Figure 3. *Summary of inducible phosphorylation sites within NF-κB p65. The positions of the phosphorylation sites and the implicated kinases are given.*

5.1 Phosphorylation of p65 at serine 276

Serine 276 is perhaps the best characterized phosphorylation site of p65 NF-κB. Reconstitution of mutant p65 proteins into p65-deficient fibroblasts in which either serine 276, serine 529, or serine 536 were replaced by alanine revealed that only the S276A mutant showed an impaired TNF-induced expression of the NF-κB target gene IL-6. Furthermore, these cells lost NF-κB-dependent protection against TNF-induced apoptosis.[62] Detailed experiments addressing the role of serine 276 phosphorylation showed that unphosphorylated p65 interacts with histone deacetylase-1 (HDAC-1) while phosphorylation promotes the interaction of p65 with the coactivating acetylase CBP/p300, as revealed by in vitro and in vivo experiments.[63,64] While Zhong et al. identified the catalytic subunit of PKA, PKAc, as the serine 276 kinase,[63,64] others found that neither PKAc, nor IKKβ, IKKi/IKKε, or TBK1, phosphorylated a recombinant GST-p65 (1–305) fusion protein, as shown by in vitro kinase experiments.[62] Interestingly, another study identified MSK1 as a serine 276 kinase. MSK1 phosphorylates p65 at serine 276 in vivo and in vitro and TNFα stimulation triggers the interaction of MSK1 with p65. Both MSK1 and p65 that has been phosphorylated at serine 276 are recruited to the endogenous IL-6 promoter. Furthermore, TNF-induced phosphorylation at serine 276 is impaired in fibroblasts deficient for MSK1 and the closely related kinase MSK2.[65] At present, therefore, the available evidence suggests that there are at least two serine 276 kinases, PKAc and MSK1. While the involvement of PKAc was revealed from experiments in LPS-stimulated cells,[66] the contribution of MSK-1 was detected in TNF-treated cells;[65] this raises the possibility that different serine 276 kinases are used to activate p65 at individual gene promoters in a stimulus-specific manner. MSK1 also phosphorylates histone H3, a fact raising the intriguing possibility that one kinase can modulate the activity of a transcription factor and of the surrounding chromatin at the same time.[65]

5.2. Phosphorylation of p65 at serine 311

Phosphorylation in the p65 RHD is required for p65 transactivation mediated by PKCζ.[67] PKCζ-deficient fibroblasts show normal activation of IKK and nuclear translocation of p65 but reduced NF-κB DNA-binding activity in vitro.[30] PKCζ phosphorylates the RHD and serine 311 was identified as the PKCζ phosphoacceptor site in vitro by a combination of tryptic peptide mapping and mutagenesis.[68] A phosphospecific antibody revealed that serine 311 in endogenous p65 is phosphorylated in response to TNF in vivo, while this phosphorylation is lost in PKCζ$^{-/-}$ cells.[68] However, a reconstitution experiment of the S311A mutant in p65$^{-/-}$ fibroblasts revealed normal DNA binding in vitro. Further experiments indicate that serine 311 phosphorylation promotes the interaction of p65 with CBP and the recruitment of CBP and RNA polymerase II to the IL-6 promoter.[68] A thorough assessment of the relative contribution of serine 311 phosphorylation to p65 NF-κB function with respect to the other TNF-induced phosphorylation sites will require comparative reconstitution experiments in p65$^{-/-}$ cells.

5.3. Phosphorylation of p65 at serine 529

This site has been mapped by the Baldwin group and is phosphorylated by casein kinase II (CKII).[69] CKII is constitutively active in most cells, but its ability to phosphorylate p65 is blocked when p65 is associated with IκBα. Degradation of IκBα enables inducible phosphorylation of p65 at serine 529. This site can also be phosphorylated in vitro by the Tax-activated IKK complex.[70] Further experiments with reconstituted p65$^{-/-}$ cells revealed only a minor role of serine 529 in transactivation, as it only contributes to achieve the Tax-induced maximal transcriptional response.[70] However, in other experimental settings, serine 529 does not contribute to p65 activity in response to IKKα, IKKβ, or TNF.[71,72] Collectively, current evidence suggests only a minor role for serine 529 phosphorylation in

p65 function. In analogy to serines 276, 311, and 536, an antibody recognizing this phosphorylation site on the endogenous p65 protein would be a valuable tool for the discovery of specific NF-κB stimuli or target genes that require serine 529 phosphorylation.

5.4. Phosphorylation of p65 at serine 536

Regulated phosphorylation of serine 536 within the C-terminal TAD1 of p65 was originally found by Sakurai and colleagues who searched for kinase activities in extracts of TNF-stimulated cells that would phosphorylate various truncated recombinant p65 proteins in vitro.[73] This group also identified IKKβ as the p65 TAD kinase.[73] Since then, several groups have confirmed that both IKKs directly phosphorylate the C-terminal TAD of p65.[70,71,74,75] Within the C-terminal TAD, as shown by mutational analysis of GST-p65 fusion proteins[71] or by mass spectrometry analysis of p65 peptides,[74] recombinant or overexpressed IKKs phosphorylate serine 536 in vitro, with IKKβ being somewhat more efficient. Importantly, the availability of an antibody recognizing the phosphorylated serine 536 within the endogenous p65 protein has reconfirmed that this site is phosphorylated in vivo in response to TNF, LPS, T-cell costimulation, lymphotoxin β, or phorbol ester/ionomycin.[71,76,77] Two studies have investigated the signaling pathways leading to p65 serine 536 phosphorylation. Sakurai et al. identified TRAF2, TRAF5, TAK1, and IKKα/β as important mediators for TNFα-induced serine 536 phosphorylation,[71] while induction of this phosphorylation by T-cell costimulation depends on Cot (Tpl2), RIP, PKCθ, NIK, and IKKβ.[77] NF-κB serine 536 phosphorylation is prevented by the overexpression of a phosphorylation-deficient IκBα mutant,[77,78] a result raising the possibility that IκBα phosphorylation is a prerequisite for p65 phosphorylation. In mouse fibroblasts deficient for either IKKα or IKKβ the IL-1- or TNF-stimulated IKK complex isolated by immunoprecipitation with an antibody against the NEMO subunit did not phosphorylate a p65 C-terminal fragment; this supports the view that both IKKs are required for phosphorylation of the p65 TAD.[75] In another study, the LPS- and TNF-induced phosphorylation of endogenous p65 at serine 536 is unaffected in IKKα$^{-/-}$ fibroblasts, while the LPS-induced but not the TNFα-induced phosphorylation is lost in IKKβ$^{-/-}$ cells.[72] Confusingly though, a recent study employing the same IKKα- or IKKβ-deficient fibroblasts showed that the TNFα-induced NEMO-containing IKK complex isolated from these cells is still capable of phosphorylating a recombinant full-length p65 protein.[70] This may either suggest that IKKα and IKKβ can mutually compensate their function for p65 phosphorylation or indicate the involvement of further kinases. Two of these further candidate kinases have recently been identified: Immunoprecipitated RSK1 phosphorylates the p65 TAD and recombinant RSK1 phosphorylates serine 536 in vitro. RSK1 is a downstream effector of the tumor suppressor p53, which activates NF-κB in response to DNA damage. Suppression of endogenous RSK1 by small interfering RNA (siRNA) or blocking of RSK1 activation by the inhibitory compound U0126 significantly reduces serine 536 phosphorylation induced by the DNA-damaging agents doxorubicin and etoposide. Also, the protein kinase TBK1 phosphorylates serine 536 of p65 in response to TNF.[74,79] TBK1 is an IKK-related kinase but is not a component of the IKK complex. Knockout studies showed that this kinase is not required for IκBα phosphorylation and generation of the NF-κB DNA-binding dimer but it is required for transactivation of specific NF-κB target genes.[60] It will be interesting to see whether the list of serine 536 kinases can be extended. In fractionated cell extracts we have found an additional, as yet unidentifed, protein kinase activity that is stimulated by IL-1 and phosphorylates serine 536.[78] Serine 536 phosphorylation can be detected in the cytoplasm and in the nucleus,[71,72,77] but it is unclear if this is due to shuttling of phosphorylated p65 or can be explained by the existence of cytoplasmic and nuclear serine 536 protein kinases. Despite its wide occurrence, the function of serine 536 phosphorylation for p65 activity is largely elusive. Expression of a p65 S536A mutant in a p65$^{-/-}$ background revealed that serine 536 is dispensable for TNF-mediated IL-6 gene induction,[62] but on the other hand it is required for TNF- or LPS-induced activation of a NF-κB reporter gene.[70,72] It is likely that serine 536 phosphorylation mediates contact of p65 with coactivators, corepressors, or components of the basal transcriptional machinery such as TBP and TAFs. However, no such mechanism has been described to date.

5.5. Further p65 TAD kinases and phosphorylation sites

Calmodulin-dependent protein kinase IV associates with p65 and phosphorylates the C terminus of p65 at serine 535.[80] The same study revealed that a phosphomimetic mutation where serine 535 was replaced by glutamic acid exhibited a marked increase in NF-κB-dependent transcription. Also, recombinant GSK-3β phosphorylates the p65 C terminus in vitro, but the phosphorylation site remains to be determined.[81] Evidence from transfection experiments also suggests a role for Akt in p65-mediated transactivation and phosphorylation.[75,82–84] However, the relevance of PI3 kinase (PI3K) and Akt for IL-1- or TNF-induced IKK and NF-κB activation is controversial[85,86] and neither PI3K nor Akt directly phosphorylates p65. Furthermore, the phosphorylation of endogenous p65 at serine 536 is not inhibited by the PI3 kinase inhibitor LY294002[72] or by wortmannin[78] in response to LPS or TNF, respectively. It was also suggested that Akt targets the transactivation function of NF-κB by activating p38 and IKKβ. Akt and the transcriptional coactivators CBP/p300 synergize in the activation of the p65 transactivation domain, a synergy that is blocked by p38 inhibitors.[83] As it is presently not clear whether the effects of PI3K or Akt on NF-κB are direct or indirect, it is difficult to place these two kinases into a scheme of phosphorylation-dependent regulation of p65 activity. Threonine phosphorylation of p65 is involved in p14^{ARF}-mediated p65-dependent transactivation. A candidate site for this effect is threonine 505 as an alanine mutant of this site is less active, but the involved kinase has not yet been described.[87] Also, serine 468, which is contained within the TAD2, can be inducibly phosphorylated in response to T-cell costimulation or IL-1 (I.M., M.L.S., H.B., M.K.,

unpublished data), but the responsible kinase and the functional consequences await elucidation.

5.6. Phosphorylation of p65 at threonine 254 regulates association with the peptidyl-prolyl isomerase Pin1 and controls p65 ubiquitination

There is recent evidence for regulation of p65 stability by phosphorylation-mediated association with Pin1.[88] Cytokine-induced p65 threonine 254 phosphorylation by an unknown kinase stabilizes the interaction of p65 with Pin1, a peptidyl-prolyl isomerase that binds and isomerizes specific phosphorylated serine or threonine residues that precede proline. Binding of Pin1 inhibits the p65 interaction with IκBα, enhances p65 nuclear localization, and increases p65 stability. This increased stability is due to decreased proteasomal degradation of ubiquitinated p65. The ubiquitination involves the E3 ubiquitin ligase suppressor of cytokine signaling 1 (SOCS-1) and the E2 ubiquitin conjugase UbcH5a. Pin1 negatively regulates SOCS-1-mediated ubiquitination and thus stabilizes p65, thereby resulting in enhanced nuclear accumulation and an increase in NF-κB-dependent gene expression. Accordingly, in Pin1-deficient cell lines steady-state levels of p65 are reduced, as is the activity of p65 in response to IL-1, TNF, or LPS. This mechanism provides an explanation of how NF-κB may be constitutively activated in many tumors, as it is known that PIN-1 is overexpressed in many tumors while SOCS-1 is frequently downregulated.[88,89] Therefore, these data also suggest a causal role of constitutive NF-κB activation for malignant disease.

5.7. Acetylation of p65

Histone acetyl transferase (HAT) activity can regulate gene expression at different levels. On the one hand, HATs acetylate histones at their N-terminal tails; this leads to a more accessible and thus more transcriptionally active chromatin structure.[90] On the other hand, HATs can acetylate several transcription factors including p53, GATA-1, GATA-2, MyoD, B-Myb, TFIIEb, and E2F.[91,92] Acetylation of these transcription factors may affect stability, DNA affinity, interactions with binding partners, and transcriptional activity.

The NF-κB p65/p50 heterodimer, but not the p50 homodimer, recruits a coactivator complex that has similarities to that recruited by nuclear receptors.[93] This coactivator complex consists of several proteins, including the HATs CBP and its homologue p300 and the p300/CBP-associated factor PCAF. Acetylation of p65 was first detected in vivo by [^{3}H]-acetate radiolabeling of overexpressed p65.[94] Further studies demonstrated that acetylation of endogenous p65 is induced by stimuli like TNFα or PMA.[94,95] CBP and p300 have both been identified as associating with p65 at its RHD and C-terminal transactivation domains. As a functional consequence, overexpression of CBP/p300 enhances the transactivation potential of p65.[93,96,97] CBP/p300 acetylates cotransfected p65, as detected with antibodies recognizing acetylated lysines. Overexpression of E1F, a specific inhibitor of p300/CBP activity, or a p300 protein with mutations in the acetyl transferase domain prevent acetylation of p65.[98]

Several groups reported prolonged NF-κB-dependent transcription in the presence of deacetylase inhibitors such as trichostatin A or sodium butyrate, a result that may be explained by several mechanisms.[98–100] One mechanism is through the lower binding affinity of acetylated p65 and p50 to newly synthesized IκBα. Inhibition of deacetylase activity therefore reduces the contribution of IκBα to the termination of the transcriptional response. Another mechanism could be due to the reduced level of IκBα observed in TNFα-stimulated cells in the presence of HDAC inhibitors. This reduction correlates with prolonged IKKβ activity and ongoing proteasome-dependent digestion of IκBα.[101] Several acetylated lysines of p65 have been mapped and their function has been tested.[95,98] Charge-conserving lysine to arginine mutations have been made to prevent acetylation of specific residues. Lysines 218 and 221 are highly conserved in all human NF-κB DNA-binding subunits, while lysine 310 is uniquely present in p65. The three lysine residues are also evolutionarily conserved in various species, except that lysine 310 is not preserved in chickens (Figure 4). p65 K221R homodimers display a diminished DNA-binding activity, whereas K218R and K310R mutants show unchanged affinity to their cognate DNA. The K310R mutation impairs the transactivation function of p65, while replacement of lysine 221 by arginine only slightly reduces transactivation.

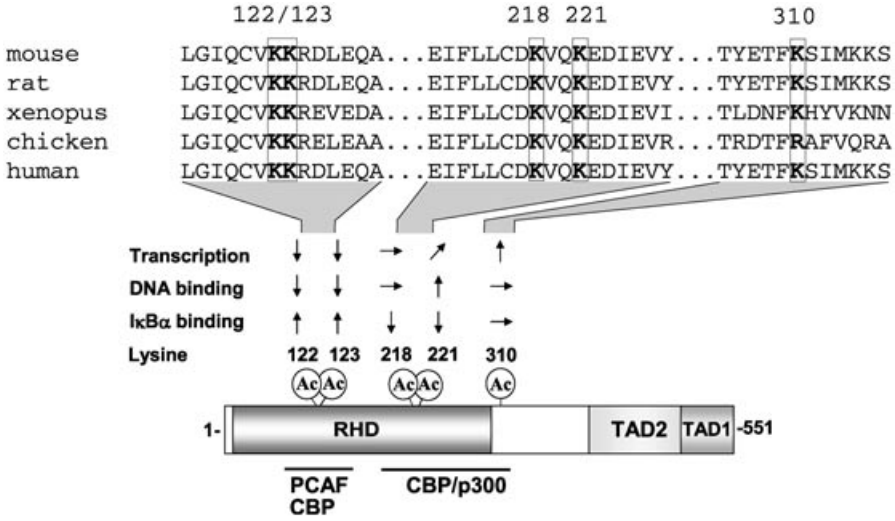

Figure 4. Summary of inducible acetylation sites within NF-κB p65. The stimulatory, inhibitory, or absent effects on transcription and binding to IκB or DNA are indicated by arrows. Ac = Acetylation sites.

In contrast, the function of a K218R mutant is comparable to wild-type p65. Acetylation of K221 and possibly K218 regulates binding of NF-κB to IκB. A further study allowed the identification of two additional residues, lysines 122 and 123, as inducible acetylation sites.[95] A mutant where these two lysine residues were changed to arginine residues shows enhanced transcriptional activity and DNA-binding affinity, while the binding affinity to IκB is impaired. These effects are opposed to those descibed for the acetylation sites at lysines 218, 221, and 310, as summarized in Figure 4. Therefore, the functional consequences of p65 acetylation may be differentially regulated for individual lysine residues. PCAF selectively acetylates p65 at lysines 122 and 123, while p300/CBP also acetylates lysines 218, 221, and 310.

The regulation of selective p65 acetylation is not well characterized. CBP/p300-mediated acetylation is regulated by the accessibility to its substrate rather than by induction of acetyl transferase enzyme activity. An example for a regulation of the interaction between p65 and CBP/p300 is provided by serine 276 phosphorylation.[102] CBP/p300 associates with p65 at two sites. An N-terminal domain of CBP/p300 interacts with the C-terminal region of unphosphorylated p65, and a second domain only interacts with p65 phosphorylated on serine 276. The C-terminal region of unphosphorylated p65 masks its N terminus and therefore prevents the accessibility of CBP/p300 to both sites.[64] Phosphorylation of p65 serine 276 weakens the interaction between the C and N termini of p65 and creates an additional site for interaction with CBP/p300. Another inducible phosphorylation site regulating the recruitment of CBP/p300 to p65 is serine 311 of p65.[68] Mutation of this amino acid to alanine abrogates the interaction of p65 with CBP/p300. The acetylation status of NF-κB can also be regulated by deacetylases. Deacetylation is thought to play a role in the termination of transcriptional activity by enhancing the binding affinity of NF-κB to IκBα, which serves to terminate NF-κB-dependent gene expression. Several histone deacetylases (HDACs), including HDAC-1, HDAC-2, and HDAC-3, have been described as associating with and deacetylating p65.[64,99] HDACs are corepressors of NF-κB, as overexpression of HDACs has a negative effect on transcriptional activity of NF-κB.[98,99] As p65 is transiently phosphorylated after stimulation, the dephosphorylation might promote HDAC recruitment and thus deacetylation, thereby contributing to the shut-down of the transcriptional response.

A major question concerning the regulation of acetylation remains to be answered. How do the different inducible phosphorylation sites control the interaction of p65 with p300/CBP or the HDACs? In addition to the described relevance of p65 serines 276 and 311, several other inducible phosphorylation sites may also participate in the control of HAT and HDAC recruitment. The incoherent effects of p65 acetylation raise the possibility that different acetylation sites are selectively involved in the transcription of different genes with distinct promoter regions.

6. The NF-κB System as a Target for Drug Development

Constitutively active, dysregulated NF-κB activity is frequently found in inflammatory diseases and also in certain cancers.[103,104] Intriguingly, many antiinflammatory drugs that are currently used, including glucocorticoids, aspirin, and salicylates, also impair the function of NF-κB,[105] thereby putting the NF-κB system in the center of an intense effort in drug screening and development. The NF-κB signaling system offers many ways of interference at several levels. However, inhibition of proximal steps of NF-κB activation such as receptor activation or ubiquitination and proteasome-mediated degradation of IκB, bears the risk of global cellular effects and high toxicity for the organism. These strategies would also have only a reduced specificity for NF-κB. As the activation of the IKK complex is the critical event for the induction of NF-κB signaling, the pharmaceutical industry has undertaken a major effort for the development of IKK inhibitors.[106] These drug-screening programs have resulted in the development of more than a dozen small-molecule IKKβ inhibitors, which do not affect the closely related IKKα enzyme. While some of the compounds, including SC-514, reversibly compete with ATP,[107] others, such as BMS-345541, function as ATP noncompetitive inhibitors by binding to an allosteric site of IKKβ.[108] IKKβ inhibitors interfere with the induced production of NF-κB target genes, including IL-6 and IL-8, and some of them, including BMS-345541 and a ureido-thiophenecarboxamide derivative, show promising results in animal arthritis models.[109,110] On the other hand, it should be noted that IKKβ inhibitors may have a high risk of toxicity when applied for longer periods, as mice lacking IKKβ show embryonic lethality and liver degeneration.[23,26] In addition, mice deficient for the expression of IKKβ in enterocytes show a reduced acute inflammation response caused by gut ischemia-reperfusion, but they also show enhanced apoptotic damage of the reperfused mucosa, a result adding a further note of caution to the concept of IKK inhibition.[23,111] For practical reasons, inhibition of NF-κB DNA binding, for example, by the introduction of DNA decoys or siRNA-mediated interference with NF-κB signaling, remains limited to cell-culture experiments and currently has no therapeutic applications. Therefore, enzymes regulating the distal steps of NF-κB activation and affecting only distinct subsets of NF-κB target genes may be interesting candidates for the development of inhibitors usable for longer periods.

7. Outlook

In the recent years the previous "simple" scheme of NF-κB activation by IκB degradation has dramatically changed. NF-κB is a multisite phosphorylated, acetylated, and ubiquitinated protein. Many unexpected proteins, as discovered by gene deletion, contribute to or are essential for NF-κB function. The main question is how NF-κB modifications are translated into gene-regulatory responses. Phosphorylation, acetylation, ubiquitination, and prolyl isomerization can regulate NF-κB half life, nuclear export, and exchange of dimers or may modulate the

interaction of NF-κB with other transcription factors, corepressors, coactivators, and components of the basal transcriptional machinery in many ways at individual gene promoters. The challenge is to follow all these fascinating mechanisms and, at the same time, to integrate them into a comprehensive view of the NF-κB signaling pathway. To address these questions it will be helpful to generate knockin mice expressing p65 variants with single or several modified sites and to study their phenotype and regulation. This experimental strategy is possible as all of the phosphorylation and acetylation sites featured in this review also occur in the murine p65 protein. It is also likely that the newly discovered mechanisms of NF-κB regulation may yield novel strategies to selectively modulate NF-κB target genes in many diseases ranging from chronic inflammation to cancer.

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