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Crosstalk in NF-κB signaling pathways

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NF-κB transcription factors are critical regulators of immunity, stress responses, apoptosis and differentiation. A variety of stimuli coalesce on NF-κB activation, which can in turn mediate varied transcriptional programs. Consequently, NF-κB-dependent transcription is not only tightly controlled by positive and negative regulatory mechanisms but also closely coordinated with other signaling pathways. This intricate crosstalk is crucial to shaping the diverse biological functions of NF-κB into cell type– and context-specific responses.

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In mammals, there are five members of the transcription factor NF-κB family: RelA (p65), RelB and c-Rel, and the precursor proteins NF-κB1 (p105) and NF-κB2 (p100), which are processed into p50 and p52, respectively. NF-κB transcription factors bind as dimers to κB sites in promoters and enhancers of a variety of genes and induce or repress transcription[1,2]. All NF-κB proteins share a Rel homology domain responsible for DNA binding and dimerization. Bacterial and viral infection, inflammatory cytokines and engagement of antigen receptors all elicit NF-κB activation, which highlights the crucial role of this transcription factor in the orchestration of immunity[3]. The range of NF-κB-inducing stimuli further extends to physical, physiological and oxidative stresses, and its additional functions include regulation of cell differentiation, proliferation and survival[4,5]. As a consequence, dysregulation of NF-κB activity is linked to inflammatory disorders, autoimmune and metabolic diseases, as well as cancer[6–9].

It is thus not surprising that NF-κB activity is tightly controlled at multiple levels by positive and negative regulatory elements. Under resting conditions, NF-κB dimers are bound to inhibitory IκB proteins, which sequester NF-κB complexes in the cytoplasm. Stimulus-induced degradation of IκB proteins is initiated through phosphorylation by the IκB kinase (IKK) complex, which consists of two catalytically active kinases, IKKα and IKKβ, and the regulatory subunit IKKγ (NEMO). Phosphorylated IκB proteins are targeted for ubiquitination and proteasomal degradation, releasing bound NF-κB dimers to translocate to the nucleus (**Fig. 1**). Transcriptional activity of nuclear NF-κB is further regulated by post-translational modifications (PTMs)[2].

Overall, two main NF-κB-activating pathways exist in cells. The canonical pathway is induced by most physiological NF-κB stimuli; for example, signals emanating from cytokine receptors, such as the tumor necrosis factor receptor (TNFR) and interleukin 1 (IL-1) receptor (IL-1R), antigen receptors and pattern-recognition receptors, including Toll-like receptor 4 (TLR4). The canonical pathway is defined as dependent on IKKβ and NEMO and leads mainly to phosphorylation of IκBα and nuclear translocation of mostly p65-containing heterodimers. In contrast, the noncanonical pathway depends on IKKα-mediated phosphorylation of p100 associated with RelB and leads to partial processing of p100 and the generation of p52-RelB complexes. Noncanonical signaling is induced by specific members of the TNF cytokine family, such as CD40 ligand, BAFF and lymphotoxin-β[2].

Since its discovery 25 years ago, NF-κB has served as a model system for inducible transcription and, because of its broad physiological and medical effects, has garnered tremendous research interest. Nevertheless, the diversity of NF-κB function still raises questions about how a limited set of signaling mediators is able to integrate diverse stimuli to achieve a cell type– and stimulus-specific response. However, NF-κB does not exist in isolation, and studies have begun to elucidate how crosstalk with parallel signaling networks shapes the NF-κB response. As activation depends on IκB degradation, the IKK complex is the gatekeeper for NF-κB signaling and, like the NF-κB complexes themselves, represents a critical node for interaction with parallel signaling pathways. There is also considerable conservation of signaling intermediates upstream of the IKK complex, such as the receptor-interacting proteins (RIPs) and TNFR-associated factors (TRAFs) that are critical to IKK activation and also signaling to other pathways. In this review we briefly introduce these important NF-κB signaling nodes and focus on molecular events that allow for both integration and communication with non-NF-κB signaling pathways.

NF-κB signaling nodes: TRAFs

There are seven members of the mammalian TRAF family (TRAF1–TRAF7), defined by the presence of the TRAF domain that mediates binding to receptors and signaling mediators. TRAFs have overlapping roles in innate and adaptive immunity, stress responses and bone metabolism[10]. With the exception of TRAF1, all TRAFs have a RING domain that can potentially function as an E3 ubiquitin ligase, although E3 activities have been definitively demonstrated only for TRAF2 and TRAF6 (ref. 11). Many but not all biological functions of TRAFs are mediated via NF-κB, whereby TRAFs can positively or negatively regulate canonical and noncanonical signaling[10]. In this context, TRAFs can function downstream of multiple receptors, and several receptors can use more than one TRAF for signal transduction. As a result, the contributions of individual TRAFs to specific signaling pathways remain incompletely defined.

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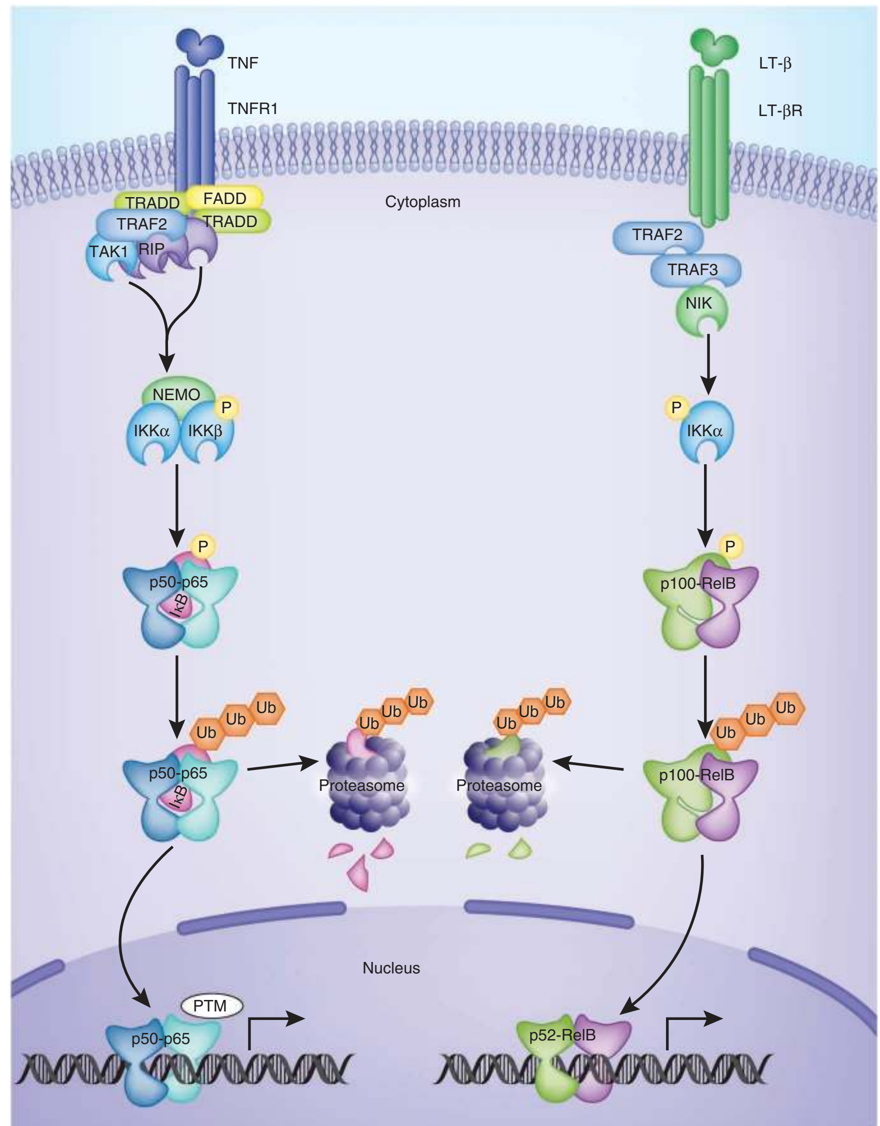

Figure 1 Canonical and noncanonical pathways of NF-κB activation. Under resting conditions, NF-κB dimers are bound to inhibitory IκB proteins, which sequester inactive NF-κB complexes in the cytoplasm. Stimulus-induced degradation of IκB proteins is initiated through phosphorylation by the IκB kinase (IKK) complex, which consists of two catalytically active kinases, IKKα and IKKβ, and the regulatory subunit IKKγ (NEMO). Phosphorylated IκB proteins are targeted for ubiquitination and proteasomal degradation, which thus releases the bound NF-κB dimers so they can translocate to the nucleus. NF-κB signaling is often divided into two types of pathways. The canonical pathway (left) is induced by most physiological NF-κB stimuli and is represented here by TNFR1 signaling. Stimulation of TNFR1 leads to the binding of TRADD, which provides an assembly platform for the recruitment of FADD and TRAF2. TRAF2 associates with RIP1 for IKK activation. In the canonical pathway (right), IκBα is phosphorylated in an IKKβ- and NEMO-dependent manner, which results in the nuclear translocation of mostly p65-containing heterodimers. Transcriptional activity of nuclear NF-κB is further regulated by PTM. In contrast, the noncanonical pathway, induced by certain TNF family cytokines, such as CD40L, BAFF and lymphotoxin-β (LT-β), involves IKKα-mediated phosphorylation of p100 associated with RelB, which leads to partial processing of p100 and the generation of transcriptionally active p52-RelB complexes. IKKα activation and phosphorylation of p100 depends on NIK, which is subject to complex regulation by TRAF3, TRAF2 and additional ubiquitin ligases. LT-βR, receptor for lymphotoxin-β.

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Although TRAFs are also engaged by Nod-like receptors and RIG-I-like receptors, TRAF function has been most thoroughly studied in signaling by members of the Toll–IL-1R and TNFR superfamilies. Stimulation with IL-1 or lipopolysaccharide (LPS) triggers recruitment of the adaptor MyD88, followed by IRAK and TRAF6, which ultimately leads to IKK activation. Stimulation of TNFR1, in contrast, leads to binding of the adaptor TRADD, which provides an assembly platform for recruitment of the adaptor FADD and TRAF2. Whereas binding of FADD leads to caspase activation and apoptosis, TRAF2 (or in the absence of TRAF2, TRAF5)[12] associates with ubiquitin ligases cIAP1 and cIAP2 and the kinase RIP1 for NF-κB activation. RIP1 undergoes linear or Lys63 (K63)-linked ubiquitination, which is thought to recruit and activate kinase TAK1 and IKK complexes. In noncanonical NF-κB signaling, the kinase NIK activates IKKα. Noncanonical signaling is regulated by TRAF3, which associates with NIK under resting conditions and mediates its ubiquitination and proteasomal degradation. After stimulation, TRAF3 itself undergoes degradation (mediated by TRAF2 and cIAP), thereby resulting in stabilization of NIK[13]. NIK acts together with IKKα to induce the phosphorylation and proteasomal processing of p100, thereby leading to the formation of p52-containing NF-κB dimers[14,15] (**Fig. 2a**).

Non–NF-κB-related TRAF activities

TRAFs represent a central point of divergence for activation of the NF-κB and AP-1 transcription factor pathways (**Fig. 2a**). Activation of AP-1 is promoted by the mitogen-activated protein kinases (MAPKs) Jnk, Erk1-Erk2 and p38, and activated AP-1 affects cell survival, apoptosis and stress responses. Activation of MAPKs occurs via a three-tiered phosphorylation cascade: MAPK is activated by MAPK kinase (MAPKK or MEK), which in turn is activated by MAPKK kinase (MAP3K or MEKK). In some pathways, the initial inducer of this cascade is MAP3K kinase (MAP4K)[16]. TRAF2 can associate with both MAP3K and MAP4K (for example, ASK-1 (MAPKKK5), MEKK-1 and GCKR), to initiate AP-1 activation in response to TNF. In IL-1R or TLR signaling, TRAF6 induces activation of TAK1, a MAP3K, to trigger activation of both AP-1 and NF-κB[17,18]. This demonstrates that although activation of NF-κB and AP-1 represent common outcomes of TRAF-dependent signaling, the underlying regulatory mechanisms differ. Use of distinct TRAFs by different receptors thus allows pathway-specific or pathway-overlapping regulation through unique or common TRAF-binding proteins such as TRIP, A20 or I-TRAF (TANK)[19–23].

TRAFs have also been suggested to participate in antiviral responses mediated by both TLRs and RIG-I-like receptors. TLRs recognize viral RNA signals through the adapters TRIF (TLR3) or MyD88 (TLR7 or TLR9) to activate the IKK family proteins IKKε and TBK1. TBK1

or IKKε then phosphorylates the interferon-response factors IRF3 and IRF7, which results in the transcription of genes encoding type I interferons[24]. Activation of IRF3 and IRF7 via MyD88 or TRIF requires TRAF3. Thus, TRAF3-deficient macrophages and plasmacytoid dendritic cells do not produce type I interferons in response to viral RNA[25]. Similarly, activation of IRF3 and IRF7 by RIG-I and the cytosolic receptor Mda5, which respond to cytosolic viral RNA, depends on TRAF3 (refs. 25,26). In support of those functions, TRAF3 interacts with the adaptors MAVS, which mediates RIG-I–Mda5 signaling, as well as TRIF, IRAK1, TBK1 and IKKε. Nevertheless, the mechanism of TBK 1–IKKε activation downstream of TRAF3 remains elusive. TRAF6 has also been shown to interact with IRF7, and TRAF6-deficient fibroblasts have less MyD88-IRF7–triggered interferon reporter activity[27]. As this activity of TRAF6 depends on a functional RING domain and the ubiquitin-conjugating enzyme Ubc13, TRAF6-mediated ubiquitination has been suggested to have a role in IRF7 activation. Furthermore, *in vitro* reconstitution of the RIG-I pathway has suggested that polyubiquitin chains generated by TRAF6 or another E3 ligase, such as RIM25, are bound by RNA-loaded RIG-I, which leads to binding to MAVS and activation

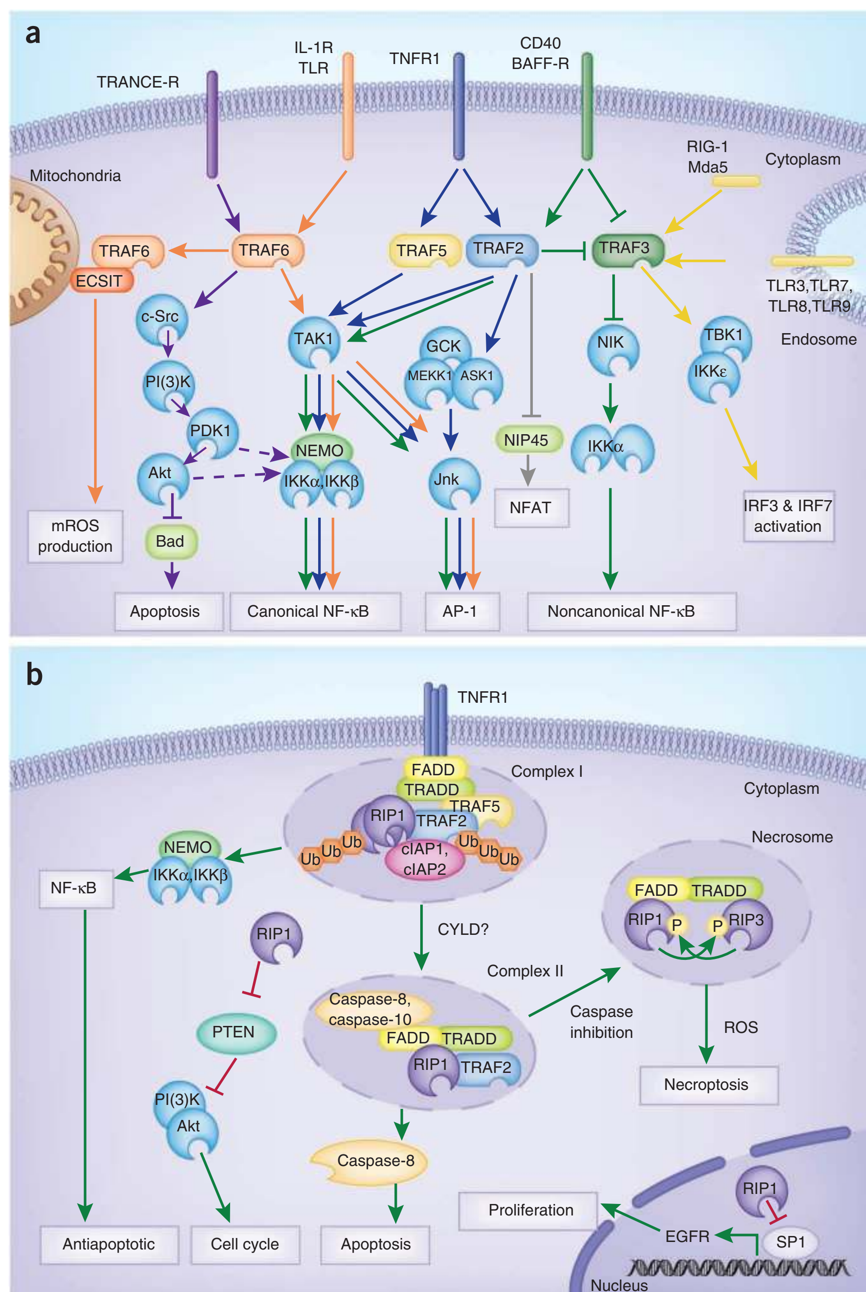

Figure 2 TRAF- and RIP1-dependent signaling pathways. (**a**) TRAF-dependent signaling pathways. TRAFs function downstream of many various receptors and promote the activation of AP-1 and NF-κB transcription factors. Also, several receptors can use more than one TRAF protein for signal transduction, which allows combinatorial specification of signaling outcomes. The function of TRAF2 and TRAF5 is best characterized in TNFR1 signaling, whereas TRAF6 and TRAF3 have been extensively studied in IL-1R or TLR signaling and in noncanonical NF-κB signaling, respectively. Each receptor and the signaling pathway(s) it induces are in a similar color. In addition to its role in noncanonical NF-κB signaling (green), TRAF3 has been demonstrated to be critical for virus-induced activation of IRF3-IRF7 and interferon production (yellow). TRAF2 is involved in signaling downstream of CD40 or the BAFF receptor BAFF-R through the regulation of TRAF3 stability and activation of AP-1 and NF-κB. TRAF2 and TRAF5 mediate canonical activation of NF-κB and AP-1 in response to TNF and other proinflammatory cytokines (blue). Downstream of IL-1R and TLR, this role is exerted by TRAF6 (orange). After engagement of TLR1, TLR2 or TLR4, TRAF6 also translocates to mitochondria, where it binds to ECSIT to induce mitochondrial ROS (mROS) and enhance bacterial killing. In osteoclasts, TRAF6 has also been shown to function in signaling via the TRANCE receptor TRANCE-R by mediating activation of c-Src (purple). In addition, TRAF2 has been shown to inhibit IL-4 and T helper type 2 differentiation of T cells by negatively regulating the NFAT-interacting protein NIP45 (gray). (**b**) RIP1-dependent signaling pathways. Through its involvement in the regulation of survival (Complex I), apoptosis (Complex II) and necroptosis (Necrosome), RIP1 is positioned at the center of cell-fate 'decisions'. After stimulation with TNF, rapid assembly of complex I (containing TRADD, RIP1 and TRAF2) occurs at the receptor, which triggers NF-κB activation through recruitment of the IKK complex. In the course of signal transduction, TRADD-RIP1-TRAF2 dissociates from the receptor, binding FADD and caspases to induce apoptosis. The deubiquitinase CYLD has been demonstrated to promote apoptosis and/or necroptosis by enhancing the RIP1-FADD interaction, which suggests that the ubiquitination status of RIP1 may 'tune' its activity in different pathways. When caspase activation is inhibited, such as during certain viral infections, RIP1 acts with RIP3 to induce necroptosis. RIP1-RIP3 transphosphorylation leads to RIP3-dependent production of ROS, which contributes to necroptotic cell death. Furthermore, RIP1 has been suggested to be involved in activation of the PI(3)K-Akt pathway through NF-κB-independent downregulation of PTEN and to influence EGFR expression through its action as a negative regulator of the transcription factor Sp-1. Bad, Bcl-x_L–Bcl-2–associated death promotor.

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of IRF3 (ref. 28). The relative contribution to this process of TRAFs and other E3 ligases needs further investigation under physiological conditions. Furthermore, it remains unclear whether the requirement for TRAF3 in interferon production likewise depends on its E3 ligase activity. As it would seem to be an important potential mechanism of crosstalk, it also remains important to determine whether TLR-induced interferon production is affected by the TRAF3 degradation that occurs in the course of noncanonical NF-κB signaling.

Regulation of the mitochondrial production of reactive oxygen species (ROS) in the course of an innate immune response has been added to the functional repertoire of TRAFs[29]. TRAF6 associates with ECSIT, an adaptor protein required for embryonic development that has been linked to signaling in the bone-morphogenetic protein and TLR pathways[30]. However, ECSIT can localize to mitochondria, where it is critical for the assembly of complex I of the mitochondrial respiratory chain and respiratory-chain function[31]. Interestingly, in response to engagement of TLR1, TLR2 and TLR4, TRAF6 translocates to mitochondria, where it binds ECSIT, triggering mitochondrial production of ROS. This phenomenon contributes to the clearance of *Salmonella*, as demonstrated by less killing of *Salmonella* by ECSIT-deficient macrophages *in vitro* and the greater susceptibility of ECSIT-deficient mice to intraperitoneal challenge with *Salmonella*[29]. Such findings describe a unique cross-regulation between TLRs and mitochondria and thus place TRAF6 at the interface between innate immune signaling and mitochondrial function.

Crosstalk of TRAFs with several other pathways has been reported, but experimental evidence to explain such crosstalk is rather limited. TRAF6, for example, has been shown to associate with the kinase c-Src and the receptor for the TNF family cytokine TRANCE in a stimulus-dependent manner in dendritic cells and osteoclasts. Furthermore, TRANCE-dependent activation of the kinase Akt depends on the interaction of c-Src with TRAF6 and can be blocked by the expression of dominant-negative TRAF6 (ref. 32). Interestingly, c-Src-deficient mice have osteoclast defects similar to those of mice lacking TRAF6, which supports the idea of a common function for these proteins in osteoclasts[33]. In T cells, TRAF1 and TRAF2 have been described as suppressing T helper type 2 responses because of their negative effect on IL-4 expression, which promotes T helper type 2 differentiation. It seems that both TRAF1 and TRAF2 can mediate this effect through direct binding to NIP45, an NFAT-interacting protein and enhancer of *Il4* transcription, which thereby potentially links TRAFs to the activation of another major transcription factor, NFAT[34,35].

Despite the fact that TRAF function has been studied in many pathways, knowledge about the regulation of TRAF activity still remains limited. Autoubiquitination is frequently discussed; however, direct proof of the importance of ubiquitination in signal transduction is scarce, and obtaining such evidence has probably been hampered by difficulties in mapping the true acceptor site(s). However, autoubiquitination at a distinct lysine residue in TRAF6 has been demonstrated to be critical for signaling by the TNF superfamily member RANKL[36], and the effect of MCPIP1 as a critical mediator of inflammatory signaling has been attributed to its deubiquitinase activity toward ubiquitinated TRAF2, TRAF3 and TRAF6 (ref. 37). Such data support the hypothesis that TRAF ubiquitination is an important factor in TRAF-mediated signaling. Nevertheless, precise genetic experiments, probably involving knock-in site-specific mutation of genes encoding putative ubiquitination sites, will be necessary for unequivocal conclusions about the exact role of ubiquitination in signaling to be drawn.

Additional TRAF PTMs have also been linked to the modification of signaling to NF-κB. Phosphorylation of TRAF2 at Ser11, Ser55 or Thr117, probably through members of the protein kinase C (PKC) family, has been reported to occur in response to TNF[38–40]. Reconstitution of TRAF2-deficient fibroblasts with mutant TRAF2 constructs in which phosphorylation is either abolished or mimicked has suggested that TRAF2 phosphorylation is required for prolonged IKK activation and the expression of a subset of NF-κB target genes (such as *RANTES* and *ICAM1*) but negatively affects prolonged Jnk activation. It is thought that phosphorylation of TRAF2 at Thr117 is a prerequisite for ubiquitination[40]. Although the exact contributions of the various phosphorylation sites remain elusive, physiological importance for TRAF phosphorylation has been suggested by the finding that TRAF2 is constitutively phosphorylated in some cancer cell lines and Hodgkin lymphoma and that constitutive TRAF2 phosphorylation can lead to resistance to stress-induced apoptosis in oncogenic Ras-transformed cells[38].

RIPs

RIPs are a family of seven serine-threonine kinases that are crucial for sensing cellular stress from both extracellular and intracellular sources[41]. RIPs share a homologous kinase domain but also contain unique domains that confer functional specificity. In addition to their involvement in immunity, RIPs are key to the control of cell death[41,42]. Here we will focus mainly on the role of RIP1, which has been studied most extensively.

The RIP1 death domain mediates binding to several death domain–containing receptors (such as Fas (CD95) and TNFR1) and adaptors (TRADD and FADD). In TNF signaling, RIP1 is recruited to TNFR via TRADD and is essential for activation of NF-κB and MAPK. Although the kinase activity of RIP1 is not required for NF-κB signaling, in the absence of RIP1, the IKK complex is recruited to TRAF2 but is not activated, which suggests that RIP1 must have a role beyond simple scaffolding. In this context, K63 or linear ubiquitination of RIP1 may facilitate the recruitment and activation of IKK and TAK1 complexes[43,44]. RIP1 is also involved in NF-κB activation via the TLR3-TRIF pathway and after DNA damage[41].

RIP1 as a complex regulator of cell death

Although RIP1-dependent NF-κB activation leads to the induction of antiapoptotic genes, RIP1 overexpression can trigger apoptosis. An elegant mechanistic explanation for these seemingly contradictory effects has been provided by the assembly of two TNF-induced signaling complexes. Whereas the membrane-associated complex I comprises TRADD, RIP1 and TRAF2 and is responsible for rapid NF-κB dependent expression of genes encoding antiapoptotic molecules, the cytosolic complex II, consisting of TRADD, RIP1, TRAF2, FADD, caspase-8 and caspase-10, is proapoptotic[45]. After TNF stimulation, rapid assembly of complex I occurs at the TNF receptor, triggering NF-κB activation through recruitment of the IKK complex. Within 1 hour, a substantial amount of TRADD-RIP1-TRAF2 dissociates from the receptor, binding FADD and caspases in the cytosol to induce apoptosis. Whether this dissociation is driven by PTMs of complex subunits at the plasma membrane or depends on endocytosis of TNFR1 remains unclear[45,46]. It is thought that complex II is able to trigger apoptosis only when NF-κB activation is blocked or induces insufficient amounts of antiapoptotic proteins such as XIAP or $FLIP_L$. The transformation of complex I into complex II thus represents a checkpoint that ensures the elimination of cells with defective NF-κB signaling. These findings are also particularly interesting because they provide a striking example of how selective signaling depends on the assembly of multiprotein complexes with specific subcellular localization.

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RIP1 and RIP3 have been shown to be involved in necroptosis, a form of programmed cell death. Reconstitution experiments using RIP1-deficient Jurkat human T lymphocytes have demonstrated that FADD binding and the kinase activity of RIP1 are required for RIP1-dependent necroptosis induced by TNFR, Fas and the receptor for the regulatory ligand TRAIL[47]. Interestingly, RIP3 as well as RIP1-RIP3 transphosphorylation are also essential for necroptosis[48,49]. Studies of RIP3-deficient fibroblasts have established that RIP1-RIP3 phosphorylation stabilizes the pro-necroptotic complex and that RIP3-dependent signaling increases the activity of metabolic enzymes that stimulate the production of ROS, which probably contribute to necroptosis, thus linking RIPs to the basal metabolic machinery of the cell[50]. Although necroptosis is generally investigated under rather artificial conditions through the inhibition of caspases and protein synthesis, the inhibition of pathological cell death during cerebral ischemia or myocardial infarction with RIP1-RIP3–inhibiting necrostatins clearly suggests a physiological relevance for this phenomenon[48,51]. During some viral infections, caspase inhibition can also occur, which suggests another scenario in which necroptosis may be physiologically important[52]. Necroptosis differs from apoptosis in that it results in the release of proinflammatory signals (danger-associated molecular patterns), which may augment antiviral immune responses. Notably, studies of RIP3-deficient T cells infected with vaccinia virus have indeed demonstrated that necroptosis is an important cell-death mechanism when apoptosis is hampered by viral inhibitors. That proposal has been confirmed by the detection of extensive liver inflammation in wild-type mice but not in RIP3-deficient mice after infection with vaccinia virus, which clearly demonstrates a role for RIP3 in virus-induced inflammation. As mentioned above, the mechanisms that trigger the conversion of complex I into complex II and the necrosis-signaling complex (the 'necrosome') are not completely clear; however, some mechanisms of crosstalk have been established. Apoptosis, for example, can induce caspase-8-dependent cleavage of RIP1, and cleaved RIP1 acts as a dominant-negative molecule for TNF-induced NF-κB activation[53]. Furthermore, the deubiquitinase CYLD has been reported to act as a positive mediator of TNF-induced apoptosis and necroptosis by promoting RIP1-FADD association[54,55] and triggering degradation of the RIP1-targeting ubiquitin ligases TRAF2 and cIAP1-cIAP2 (refs. 56,57). Thus, the ubiquitin status of RIP1 may 'tune' its activity in different pathways[58]. Ubiquitinated RIP1 would thus 'preferentially' bind NEMO and trigger IKK–NF-κB activation, whereas non-ubiquitinated RIP1 would bind FADD. CYLD would consequently favor FADD-RIP interaction and the induction of necroptosis (**Fig. 2b**).

Interestingly, several studies have also linked RIP1 to NF-κB-independent regulation of cell proliferation and survival. RIP1-deficient mouse embryonic fibroblasts have higher expression of epidermal growth factor receptor (EGFR). That finding has been attributed to RIP1-mediated repression of Sp1, a critical activator of EGFR expression[59]. However, it remains unclear how that finding correlates with an earlier study suggesting a role for RIP1 in facilitating NF-κB activation downstream of EGFR[60]. Also, the broader biological importance of the repression of Sp1 by RIP1, particularly in the context of EGFR signaling in cancer, remains unclear. Furthermore, RIP1 has been described as being involved in activation of the phosphatidylinositol-3-OH kinase (PI(3)K)–Akt pathway through not only NF-κB-dependent negative regulation of expression of the kinase mTOR but also NF-κB-independent downregulation of the PI(3)K antagonist PTEN[61]. In this context, it is notable that both activation of NF-κB by RIP1 and negative regulation of PTEN have been shown to require Sharpin, which regulates linear ubiquitination of RIP1 as part of the HOIP-HOIL ubiquitin chain–assembly complex[44,62,63]. Although the role of RIP1 in these pathways requires further characterization, work so far has positioned RIP1 at the interface between inflammation and cancer and suggests that additional mechanisms must exist to regulate the contribution of RIP1 to the opposing pathways in which it functions.

IKK complex

The IKK complex is activated through the phosphorylation of key serine residues in the T loops of IKKα and IKKβ either by IKK kinase (IKK-K) or by oligomerization-induced autophosphorylation. Regulatory ubiquitination of upstream scaffold proteins and NEMO itself is thought to mediate IKK activation through the recruitment of IKK-Ks or oligomerization of IKK. Once activated, IKK phosphorylates IκB proteins in the conserved destruction box, which leads to their proteasomal degradation[64]. Because IKK activation represents a bottleneck in NF-κB signaling, the IKK complex is the most thoroughly studied point of cross-regulation with non-NF-κB pathways.

Intersection of non-NF-κB pathways at the IKK complex

The MAPKs NIK, NAK, TAK1, MEKK1 and MEKK3, as well as Cot (TPL-2), PKC-θ, PKC-ζ and PKC-λ, can phosphorylate IKKs *in vitro* or upon overexpression. The large number of potential IKK-Ks may reflect the varied stimuli that elicit IKK activation, although a clear role in IKK activation *in vivo* has been established for only some of these kinases[64]. One example of the controversies surrounding IKK-K-dependent activation of the IKK complex is the serine-threonine kinase Akt. Akt, a bona fide oncoprotein, is constitutively active in a variety of cancers and has been suggested to directly phosphorylate IKK in response to TNF[65]. Although that remains a controversial finding, it is consistent with the observation that Akt promotes oncogenesis in a NF-κB-dependent manner[66]. However, in PTEN-deficient prostate cancer cells, Akt seems to control IKK activity indirectly through its downstream effector mTOR[67]. Although silencing of the gene encoding mTOR impairs the phosphorylation of IKK T loops, clear experimental evidence in support of the idea of direct phosphorylation of IKK through mTOR is lacking. As Akt activates mTOR through phosphorylation and inactivation of the tumor suppressor TSC2, such findings are in line with another study that has shown a role for TSC2 in NF-κB activation[68] and potentially place TSC2 upstream of mTOR-IKK activation. Given that Akt and NF-κB are frequently coordinately activated and can be affected by upstream molecules such as PI(3)K and the kinase PDK1, it remains important to elucidate the level(s) at which cross-regulation of these pathways occurs.

In addition to activation of IKK through T-loop phosphorylation, other IKK-complex PTMs probably facilitate crosstalk. A stretch of amino acids in the C terminus of IKKα as well as of IKKβ, called the NEMO-binding domain (NBD), mediates efficient interaction with NEMO, and peptides with this amino acid sequence can be used to inhibit NF-κB activation in a dominant-negative manner[69]. A mutant IKKβ in which two serine residues near the NBD have been replaced with phosphomimetic glutamate residues is no longer stimulated by NEMO or IL-1 (ref. 70). That result suggests an interesting mechanism by which phosphorylation of the NBD might interfere with the phosphorylation of IKK T loops. Although IKKβ itself, as part of a negative feedback loop, and the serine-threonine kinase Plk1 can phosphorylate the NBD sequence *in vitro* and have thus been put forward as candidate kinases, it remains to be investigated under which conditions and through which kinases NBD phosphorylation can occur *in vivo*. Nevertheless, NBD phosphorylation might represent a means of maintaining the IKK complex in a basal state

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or fine tuning its reactivity by influencing the binding of IKK to NEMO. Modification of proteins with O-linked N-acetylglucosamine (O-GlcNAc) monosaccharides is increasingly appreciated as a PTM that can modulate protein function in transcription, translation and the cell cycle[71]. Intriguingly, constitutive O-GlcNAc modification of IKKβ at Ser733, which can also be targeted by negative regulatory phosphorylation, has been observed in mouse and human fibroblasts deficient in the tumor suppressor p53 (ref. 72). O-GlcNAc modification of IKKβ interferes with negative regulatory phosphorylation and can thus lower the signaling threshold required for activation of NF-κB in tumors lacking p53. These and other examples suggest that there could be considerable fine tuning of the NF-κB pathway through PTM of IKK, but carefully designed experiments are needed to clearly delineate the resulting effects.

Because of the important role of NF-κB in the induction of antiapoptotic genes, cross-regulation with apoptotic pathways has been postulated. At the level of the IKK complex, it has been shown that induction of apoptosis in Jurkat cells induces caspase-dependent cleavage of IKKα and NEMO. The resulting cleaved NEMO is signaling deficient and cannot support NF-κB activation[73], which potentially prevents continued expression of proinflammatory molecules in cells destined to die. As IKKα is not essential for the expression of genes encoding antiapoptotic molecules by NF-κB, the relevance of IKKα cleavage in this context remains unclear. Although it is not detected in Jurkat cells, caspase-3 has been shown to cleave IKKβ in HeLa human cervical cancer cells and MCF-7 human breast cancer cells, which results in the abrogation of IKKβ catalytic activity and promotion of TNF-induced killing[74]. Caspase-mediated proteolysis of RIP1, discussed above, as well as of p65 and IκBα, leading to dominant-negative and super-repressor proteins, respectively, has also been reported in various cell lines[53,75,76]. Although all these scenarios support a common idea, the physiological relevance of caspase-mediated cleavage of NF-κB signaling components is unclear. *In vivo*, target specificity might depend on the specific nature and strength of the death signal as well as the cellular context. It also remains unclear whether other processes that induce caspase activation without triggering apoptosis, such as T cell activation or cell-cycle progression, use these mechanisms to modulate NF-κB activation.

Signaling through Notch receptors orchestrates differentiation, proliferation and survival in a context- and cell type–specific manner. Direct NF-κB activation after stimulation with the Notch ligand has been reported[77]. One study has shown that in cervical cells, Notch-1 can activate NF-κB through direct interaction with IKKα[78]. It was therefore proposed that Notch-1 may maintain NF-κB activity in cervical cancer. Additional Notch–NF-κB crosstalk mechanisms include Notch-dependent expression of NF-κB subunits and NF-κB dependent transcriptional regulation of Notch pathway components[79]. In addition, Notch-1 has been demonstrated to interact with p50, but the reported transcriptional outcome seems to be specific to the context and cell type[79]. In summary, the data now available addressing signaling by Notch and NF-κB show intricately linked crosstalk but also underscore deficits in the understanding of this multilayered network. The fact that Notch and NF-κB are commonly activated in several cancers will probably fuel research interest in this area.

NF-κB-independent signals emanating from the IKK complex

IKKs were long thought to be highly specific for IκB proteins. However, evidence has accumulated indicating that IKKs target not only upstream mediators in NF-κB cascades but also proteins unrelated to NF-κB signaling[80]. In the next section we will highlight important NF-κB-independent functions of both IKKα and IKKβ; several detailed reviews have also been published elsewhere about IKK functions in the NF-κB pathway[64,81,82].

IKKβ-mediated signaling

The expanding list of proposed IKKβ substrates is a reflection of its broad role in antiapoptotic, proinflammatory and proliferative pathways (**Fig. 3a**). In addition to augmenting transformation through the induction of inflammation, IKKβ can also directly affect oncogenesis independently of NF-κB through direct targeting of proposed tumor suppressors. For example, it has been shown that phosphorylation of the tumor suppressor Foxo3a by IKKβ triggers its exclusion from the nucleus and degradation, thereby blocking the Foxo3a-dependent transcription of genes encoding molecules that promote cell-cycle arrest and apoptosis[83]. Consistent with that model, the result of treatment of leukemia cells overexpressing Foxo3a with inhibitors of IKK suggests that the effects of IKKβ on Foxo3a are largely inhibitory[84]. IKKβ-mediated phosphorylation of TSC1 has also been reported[85]. In complex with TSC2, TSC1 functions as a repressor of signaling by the small GTPase Rheb and mTOR and thus has tumor-suppressor qualities. IKKβ-mediated phosphorylation interferes with TSC1 function and is required for mTOR activation in response to proinflammatory cytokines such as TNF. Indeed, activated IKKβ correlates with TSC1 phosphorylation and enhanced production of vascular endothelial growth factor in primary breast tumors[85]. These data support a model in which IKKβ-mediated inhibition of tumor suppressors, particularly in the context of a proinflammatory milieu, might promote tumor survival, growth and angiogenesis in a manner that is independent of NF-κB activation.

IKKβ is also proposed to function independently of NF-κB in immune responses. For example, IKKβ has been demonstrated to exert both NF-κB-dependent and NF-κB-independent functions in allergic reactions[86]. In mast cells, after stimulation of the receptor FcεRI, IKKβ is recruited and phosphorylates SNAP-23, a component of the SNARE complex involved in exocytosis[87]. IKKβ-mediated phosphorylation of SNAP-23 promotes assembly of the SNARE complex, mast cell degranulation and anaphylactic reactions. Similarly, IKKβ supports TNF secretion by mast cells, which suggests that in the course of late-phase allergic reactions, IKKβ can regulate TNF secretion at the level of mRNA transcription through NF-κB activation and in an NF-κB-independent manner through regulation of cytokine exocytosis[86]. Although SNAP-23 had been shown to modulate exocytosis, and PKC can phosphorylate SNAP-23 *in vitro*[88,89], the study described above[86] has provided convincing evidence of a critical role for IKKβ in this process *in vivo* and adds an interesting new target to the growing list of IKKβ substrates.

One of the best-characterized NF-κB-independent functions of IKKβ is its effect on MAPK pathways. In unstimulated cells, Cot (TPL-2), the upstream kinase for activation of the MAPKs MKK1-MKK2–Erk1-Erk2, is bound to p105, which acts as an inhibitor of the kinase activity of Cot (TPL-2)[90,91]. IKKβ can induce proteolysis of p105, which leads to the release and activation of Cot (TPL-2); this makes IKKβ and p105 critical for the activation of both NF-κB and MAPK and provides a possible link between the activation of NF-κB and of AP-1 in macrophages[92,93]. NF-κB p105 has also been shown to interact with other non-NF-κB proteins. The coatamer-b subunit protein COPB2, Jnk-interacting leucine zipper protein and ABIN-1 have been identified through the use of large-scale tandem-affinity purification of p105 from HEK293 human embryonic kidney cells[94]. In addition, the helix-loop-helix transcription factor LYL1 (ref. 95) and c-FLIP[96] can bind to p105 when overexpressed. To our knowledge, only the interaction with ABIN-2 has been verified through the use

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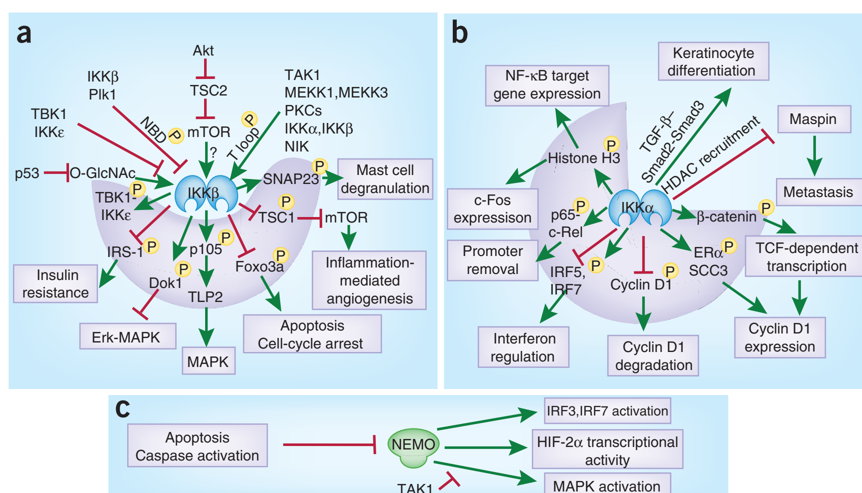

Figure 3 NF-κB-independent functions of IKK complex subunits. (**a**) NF-κB-independent IKKβ signaling. After T-loop phosphorylation, IKKβ activation occurs by trans-autophosphorylation or an IKK kinase (IKK-K). Inhibitory phosphorylation of IKKβ may also occur in certain settings. Additionally, phosphorylation of IKKβ by the IKK-related kinases TBK1 and IKKε has been shown to modulate IKK activation. Phosphorylation of NBD has also been proposed to impede activation of IKKβ. O-GlcNAc modification of IKKβ, which is repressed by p53, may augment IKK activity. IKKβ phosphorylates many substrates in addition to IκB proteins, in general promoting antiapoptotic and proinflammatory processes. Through inhibitory phosphorylation of TSC1 and consequent mTOR activation, IKKβ regulates tumor progression and inflammation-mediated angiogenesis. Other pro-proliferative or antiapoptotic targets include the tumor suppressor Foxo3a, as well as p105 and Dok1, through which IKKβ affects MAPK activation. Phosphorylation of IRS-1 inhibits insulin signal transduction, which affects the development of insulin resistance. Finally, IKKβ phosphorylates the t-SNARE SNAP23 to regulate degranulation in mast cells. (**b**) NF-κB-independent IKKα signaling. Because of its nuclear-localization signal, IKKα can target both cytosolic and nuclear proteins. In the nucleus, IKKα modulates gene expression through histone H3 modification and regulation of the recruitment of histone deacetylase (HDAC). IKKα has also been suggested to directly phosphorylate p65 and c-Rel, triggering their turnover and removal from the promoter to terminate the canonical NF-κB response and limit inflammation. In addition, IKKα is also closely intertwined with the regulation of cyclin D1 through transcriptional as well as post-translational processes and can affect interferon production through context-dependent phosphorylation of IRF5 and IRF7. IKKα also exerts kinase activity-independent functions in development. (**c**) NF-κB-independent NEMO-dependent signaling. Evidence for NF-κB-independent roles of NEMO is more limited than that for IKKα or IKKβ, and for many of the described NF-κB-independent functions of IKKα and IKKβ, it remains unclear whether NEMO is also required. However, the role of NEMO in NF-κB activation in response to DNA damage, in which NEMO translocates to the nucleus and becomes phosphorylated by the kinase ATM, demonstrates that NEMO may function independently of IKKα and IKKβ. NF-κB-independent regulation of the activity IRF3 and IRF7 and of HIF-2α and the activation of MAPKs by NEMO has also been described. Finally, activation of apoptotic pathways counteracts NF-κB signaling via caspase-dependent cleavage of NEMO, which results in a signaling-deficient truncated protein.

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of endogenous proteins and has been attributed physiological significance by the finding that ABIN-2 stability depends on p105 expression[97]. Although ABIN-1 and ABIN-2 bind the ubiquitin-editing enzyme A20 and inhibit NF-κB after being overexpressed in various systems[98], studies of mouse embryonic fibroblasts and macrophages deficient in ABIN-1 or ABIN-2 have failed to demonstrate a clear role for these molecules in NF-κB signaling[99,100]. However, given the alterations in the activation of caspase-8 and Erk in ABIN-1-deficient mouse embryonic fibroblasts and ABIN-2-deficient macrophages, respectively, it is possible that ABIN proteins mediate crosstalk emanating from p105 and the IKK complex. Thus, although most of these suggested p105 interactions clearly require further investigation, the IKKβ-induced degradation of p105 holds the potential to influence many additional pathways. IKKβ can also phosphorylate Dok1, a Ras GAP–associated tyrosine-kinase substrate and inhibitor of cell growth, which leads to the inhibition of Erk1 and Erk2, demonstrating the context dependence of IKKβ-mediated MAPK regulation[101].

In addition to regulating the activity of heterologous kinase pathways, there is growing evidence of crosstalk between IKKα-IKKβ and the closely related IKK family kinases IKKε and TBK1. Studies of mouse fibroblasts deficient in NEMO and TRAF6, in combination with pharmacological inhibition of kinases, have shown that canonical IKKs can phosphorylate and activate TBK1 and IKKε downstream of IL-1, TLR3 and TLR4. In turn, IKK-related kinases are able to phosphorylate the catalytic domains of canonical IKKs, resulting in less IKK-complex kinase activity and lower expression of NF-κB-dependent target genes[102]. Phosphorylation of I-TRAF (TANK) may also be involved in the regulation of canonical IKK activity by IKKε–TBK-1 (refs. 23,103). Such findings clearly demonstrate a role for IKK-related kinases in limiting the activation of IKK and NF-κB and thus reveal an intricate network that balances the activities of IKKs and IKK-related kinases during innate immune responses. TBK1 has also been identified as a critical factor for the survival of cancer cells driven by the proto-oncoprotein K-Ras[104]. Interestingly, gene-set identification in oncogenic K-Ras-driven lung cancer cell lines as well as non–small-cell lung tumors has revealed a correlation between oncogenic Ras and NF-κB gene signatures. Downregulation of TBK1 in the respective cell lines leads to the suppression of NF-κB target genes such as *CCND1*, *BCL2* and *IL8*, whereas interferon-responsive genes are unaffected. Correlating with those findings, knockdown of TBK1 results in less nuclear abundance of c-Rel, whereas expression of a dominant inhibitor of NF-κB leads to cell death. Thus, TBK1-mediated NF-κB signaling is thought to be critical for the survival of K-Ras-driven cancers, which have so far eluded effective clinical targeting, and highlight the necessity of testing the effects of inhibition of TBK1 and NF-κB in these settings. In addition, IKKε has been shown to be overexpressed and activate NF-κB in breast cancer cell lines and a large percentage of patient-derived tumor samples[105]. That finding further supports the idea of a role for the IKK-related kinases in NF-κB-mediated survival signaling in the context of oncogenesis and highlights the idea that NF-κB activation may be driven via different mechanisms in distinct tumor species[106].

Metabolic diseases such as obesity and type 2 diabetes have traditionally been attributed to overnutrition, but chronic low-grade inflammation is now widely recognized as an important factor in their progression. Many studies have indicated the involvement of NF-κB activation in this process, and the production of proinflammatory cytokines such as TNF clearly promotes pathology[107]. In addition, IKKβ can directly phosphorylate the insulin receptor substrate IRS-1, which interferes with the insulin receptor–mediated tyrosine

phosphorylation of IRS-1 (refs. 108–111). As defects in insulin signaling are known to contribute to the development of insulin resistance, IKKβ might thus contribute to the pathology of metabolic diseases in an NF-κB-dependent and NF-κB-independent manner.

IKKα-mediated signaling

Because the role of IKKα in NF-κB signaling was initially ambiguous, substantial effort has been invested in identifying IKKα targets outside the NF-κB pathway. The first alternative IKKα substrate to be discovered was β-catenin. In resting cells, β-catenin is targeted to a multiprotein destruction complex containing glycogen synthase kinase 3β (GSK3β). Phosphorylation of β-catenin by GSK3β leads to its proteasomal degradation. Stimulation by Wnt triggers disassembly of the destruction complex and consequent accumulation and nuclear translocation of β-catenin, which then functions as a coactivator for transcription mediated by members of the TCF-LEF family of transcription factors[112]. IKKα can phosphorylate β-catenin at residues distinct from the phosphorylation sites targeted by GSK3β, leading to its stabilization and induction of TCF-dependent expression of cyclin D1 (refs. 113–115). Interestingly, IKKα can also modulate the abundance of cyclin D1 through phosphorylation and activation of the transcription factor ERα[116,117] or by promoting complex formation between ERα and the steroid receptor coactivator SRC-3 (ref. 116). SRC-3 may be a direct substrate for both IKKα and IKKβ in response to TNF, and phosphorylation of SCR-3 is thought to enhance its import into the nucleus and to be required for the expression of a subset of NF-κB target genes[117]. Other data suggest conflicting roles for IKKα in regulating cyclin D1 expression. IKKα can directly phosphorylate cyclin D1 at Thr286, inducing its degradation. IKKα may also downregulate cyclin D1 by diminishing Erk1-Erk2–dependent transcription of the gene encoding cyclin D1 and increasing proteolysis of cyclin D1 in a p38-dependent process[118,119]. Studies of fibroblasts lacking IKKα[113,120] have failed to clarify the relative contributions of IKKα to the transcriptional and post-translational regulation of cyclin D1. Such findings suggest a complex, context-dependent interplay between IKKα and cyclin D1 (**Fig. 3b**) that clearly needs further investigation.

As discussed above, IRF transcription factors are phosphorylated and activated by the IKK family members TBK1 and IKKε. Interestingly, IRF7 can also be phosphorylated by IKKα, which positions IKKα as a positive regulator in interferon production downstream of TLR7 and TLR9 (refs. 121,122). IRF5 has also been reported to be an IKKα substrate. In contrast to IRF7 phosphorylation, IKKα-dependent phosphorylation of IRF5 is inhibitory, despite the fact that phosphorylation seems to induce the formation of IRF5 dimers[123]. It remains unclear how these seemingly contradictory effects of IKKα activation contribute to antiviral responses *in vivo*.

IKKα contains a nuclear-localization sequence, and several of its substrates are nuclear. One nuclear function ascribed to IKKα is the regulation of chromatin structure through the phosphorylation of histone H3 at Ser10 (H3S10)[124,125]. In immortalized embryonic fibroblasts, IKKα accumulates in the nucleus in response to TNF and associates with chromatin at the promoters of a subset of NF-κB target genes. Here, IKKα promotes H3S10 phosphorylation to facilitate gene expression. Interestingly, IKKα-dependent H3S10 phosphorylation has also been shown to be important for optimal expression of NF-κB-independent genes such as *Fos* after stimulation with EGF[126]. However, in the case of EGF stimulation, the p38-induced kinases MSK-1 and MSK-2 can also phosphorylate H3, and this modification enhances accessibility and thus recruitment of IKKs to certain promoters of genes encoding NF-κB proteins in primary human monocyte-derived dendritic cells[127]. As p38 and IKKs kinases share many common activating stimuli, H3 modification might represent a mechanism through which both pathways can in cooperation or combination shape the specificity of the NF-κB response.

However, the mechanism(s) targeting IKKα or MSK-1- or MSK-2-mediated H3S10 phosphorylation to specific promoters remain(s) elusive. Also, the results obtained with fibroblasts have been put into question by the finding that IKKα-deficient mice as well as IKKα kinase–deficient knock-in mice (IKKα^{AA} mice) do not suffer from liver apoptosis, which challenges the idea of a crucial role for IKKα in TNF-induced NF-κB activation in hepatocytes[128–131]. Instead, mice homozygous for the mutated allele encoding IKKα^{AA} show enhanced inflammation and are more susceptible to LPS-induced septic shock[132]. Mechanistically, it has been suggested that IKKα accelerates the removal of DNA-bound complexes of p65 and c-Rel in macrophages. The contribution of IKKα-mediated phosphorylation of histone H3, p65 and c-Rel to NF-κB activation in different scenarios thus remains unclear. In addition, other chromatin-related functions have been ascribed to IKKα, which are independent of its kinase activity. In keratinocytes, chromatin-bound IKKα promotes expression of the cell cycle–checkpoint gene *14-3-3σ* by shielding histone H3 from Suv39h1-mediated Lys9 trimethylation[133]. Thus, in cells that lack IKKα, 14-3-3σ is lower in abundance and there is more proliferation. This finding suggests that not all functions of IKKα are pro-proliferative and, in agreement with that, IKKα has been shown to act as a tumor suppressor in squamous cell carcinoma[134]. Furthermore, these findings correlate with the described kinase-independent role of IKKα in tooth development and keratinocyte differentiation[135,136], in which IKKα can also function as a cofactor in the pathway of transforming growth factor-β and the signal transducers Smad2 and Smad3 (ref. 137).

Nevertheless, the IKKα targets discussed above reveal considerable involvement of IKKα in the positive regulation of cell proliferation and, consequently, tumorigenesis. This role is also reflected by the crosstalk of IKKα with the tumor suppressor p53. As NF-κB and p53 can be activated by the same stimuli, the integration of both pathways is critical for cell-fate 'decisions'. Competition between NF-κB and p53 for the histone acetyltransferases CBP and p300, which are required for transactivation by both factors, has been suggested as a major determinant in the communication between these two pathways[138,139]. It has been demonstrated that this competition is regulated through IKKα-mediated phosphorylation of CBP, which leads to more histone acetyltransferase activity, as well as augmented binding of CBP to NF-κB than to p53. This results in enhanced expression of NF-κB target genes, whereas p53-dependent transcription is repressed, ultimately promoting cell proliferation[140]. Intriguingly, IKKα has also been linked to metastasis. A greater abundance of phosphorylated nuclear IKKα correlates with the progression of prostate carcinomas and lower expression of maspin, a member of the serpin family of proteins and a tumor suppressor. The underlying mechanism involves recruitment of nuclear IKKα to the promoter of the gene encoding maspin in inflammatory cells that have infiltrated tumors and consequent repression of the expression of this gene through the recruitment of unknown DNA methyltransferases[141].

NEMO-mediated signaling

The first NF-κB- and IKKα-IKKβ-independent function of NEMO was postulated on the basis of the specific interaction between NEMO and HIF-2α[142]. HIF proteins are critical transcriptional regulators of the cellular response to low oxygen and mediate the expression of a variety of genes that help augment oxygen supply and facilitate metabolic adaptation to the hypoxic state. The interaction of NEMO

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with HIF-2α enhances normoxic HIF-2α transcriptional activity. NEMO may augment transcription by promoting the recruitment of CBP (**Fig. 3c**); however, more evidence is needed to substantiate that NEMO does this directly. Furthermore, HIF proteins are negatively regulated by hydroxylation through the HIF asparagine hydroxylase FIH, which interferes with CBP recruitment. It has been shown that IκBα and p105 can also be hydroxylated at conserved asparagine residues in the ankyrin-repeat domains by FIH *in vitro* and *in vivo* in a hypoxia-dependent manner[143]. However, no function in regulating the p65-p50 interaction or NF-κB activation has been demonstrated for this modification, which thus leaves unclear the physiological importance of the hydroxylation of ankyrin repeat domains.

As with IKKα, NF-κB-independent functions in tumorigenesis and antiviral immune responses have been attributed to NEMO. In hepatocarcinoma, TAK1 has been reported to promote carcinogenesis by a process that depends on NEMO but not NF-κB[144]. Whereas NEMO acts as a tumor suppressor in the presence of TAK1 by activating NF-κB, in the absence of TAK1, NEMO acts to promote hepatocyte dysplasia and early carcinogenesis. Although the exact mechanism remains unclear, TAK1 deficiency might enhance NEMO-dependent recruitment of RIP and/or TRAF proteins, resulting in more activation of MKK4-MKK7 and Jnk. Similarly, it has been proposed that sustained DNA damage may promote the activation of Jnk and caspase pathways through continued activation of NEMO and phosphorylation of RIP1 (ref. 145). Also, stimulation with CD40 leads to the formation of a complex containing TRAF2 and TRAF3, Ubc13, cIAP1-cIAP2, NEMO and MEKK1 (ref. 146). NEMO is critical for the assembly of this complex and subsequent activation of MEKK1. Together these findings strongly support the idea of IKK-independent functions for NEMO in MAPK activation. Additionally, in studies of NEMO-deficient fibroblasts, NEMO has been shown to be required for RNA virus–induced activation of IRF3 and IRF7 and consequent interferon production[147]. Here, NEMO functions downstream of the RIG-I–MAVS complex by recruiting TBK1 and IKKε through its interaction with TANK. As interferon production is unaltered in IKKβ-deficient mouse embryonic fibroblasts, impaired IRF activation in NEMO-deficient cells is probably not due to abolished NF-κB activity. Although these contributions of NEMO await further investigation *in vivo*, the findings so far highlight NEMO as a possible interface for the coordinated activation of both NF-κB and IRF transcription factors during viral infection.

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NF-κB complexes

The transcriptional activity of NF-κB is subject to regulation through a variety of PTMs. As these modifications have the potential to modulate the interaction of NF-κB with coactivators, corepressors, IκB proteins and the binding of NF-κB to heterologous transcription factors, they represent an important means of shaping NF-κB-dependent gene programs and are thought to be critical for the integration of non-NF-κB pathways and context-specific tailoring of the transcriptional response. In addition, the formation of NF-κB-containing enhanceosomes, which are supramolecular complexes with heterologous transcription factors, enables complex cross-regulation of transcriptional activities that allows the integration and regulation of non-NF-κB pathways (**Fig. 4**).

Direct modulation of NF-κB function by non-NF-κB pathways

PTMs of NF-κB subunits have been studied most thoroughly for p65 and have been found to be numerous and distinct in their functional outcomes[148]. Phosphorylation of p65 Ser276 by the catalytic subunit of protein kinase A (PKAc), which is bound to cytosolic NF-κB–IκB complexes and is activated after IκB degradation, is one of the key p65 modifications. Phosphorylation of Ser276 is critical for the recruitment of CBP and transcriptional activity of p65 (refs. 149,150). Although PKAc is licensed to phosphorylate p65 Ser276 in the cytosol in response to LPS, TNF induces phosphorylation of p65 Ser276 by MSK-1 or MSK-2 in the nucleus[151]. As MSKs are activated by Erk and p38 in response to various physiological and pathological stimuli, they hold the potential to integrate these pathways in modulating p65 activity.

Additional amino acids in p65 that undergo phosphorylation include Ser311 in the Rel homology domain, by PKCζ; Ser468, by IKKβ, IKKε and GSK3β; and Ser529 and Ser536 in the transactivation domain, by casein kinase II, IKKβ, IKKα, IKKε, TBK1 and the ribosomal subunit kinase RSK1 (refs. 152–157). All these phosphorylation events are proposed to enhance the transcriptional activity of p65. However, relatively little is known about effects of non-NF-κB activating stimuli on these phosphorylation events, even in cases in which the respective kinases are known to be activated by a variety of NF-κB-related as well as NF-κB-unrelated inducers. Notably, IKKβ-mediated phosphorylation of p65 Ser536 has been shown to require PI(3)K-Akt activity, which is elicited by cytokines as well as growth factors and thus represents an emerging node for crosstalk between the NF-κB and PI(3)K-Akt pathways[158]. Phosphorylation of p65 has also been suggested to be targeted by the tumor suppressors p53 and ARF, but with different outcomes. The ARF-activated checkpoint kinase CHK-1 can phosphorylate p65 at Thr505, negatively regulating p65 activity by increasing the recruitment of histone deacetylases and thus repressing the expression of antiapoptotic genes[159]. On the other hand, in studies using overexpression and RNA-mediated interference, p53 has been suggested to trigger phosphorylation of p65 Ser536 through activation of RSK1 to activate p65 transcriptional activity under circumstances in which NF-κB is thought to exert proapoptotic functions[156]. However, as ARF can also activates p53 in a CHK-1-dependent manner, it remains unclear how these seemingly contradictory findings contribute to the coordinated regulation of cell-cycle control and cell survival.

In the nucleus, p65 is also modified by acetylation of at least four different lysine resides (Lys122, Lys218, Lys221 and Lys310), which leads to both the promotion and dampening of transcriptional activity[148,160–162]. Methylation of p65 at lysine residues can also occur, although the functional outcome of this PTM is poorly characterized. The methyltransferase Set9, for example, is suggested to target Lys37 in response to cytokine stimulation and to promote DNA binding by p65 and the expression of certain target genes[163,164]. In contrast, another study has demonstrated that methylation by Set9 promotes termination of the NF-κB response[165]. The observed variations in results may be attributable to the use of different cell lines or nonequivalent efficiency of Set9 knockdown. Reversible methylation of Lys218 and Lys221 driven by the nuclear receptor–binding protein NSD1 is also proposed to regulate transcriptional responses, as demonstrated by the failure of p65 lacking these methylation sites to reconstitute the expression of certain target genes in p65-deficient mouse embryonic fibroblasts[166]. Although so far such modifications have been described in the context of NF-κB-activating stimuli, we are tempted to speculate that non-NF-κB pathways could also trigger PTMs that modulate, or potentially even directly induce, NF-κB-dependent gene-transcription programs.

Synergism with heterologous transcription factors

The interaction of NF-κB dimers or monomers with heterologous transcription factors, either through direct binding or occupancy at adjacent sites on DNA, profoundly influences transcriptional responses.

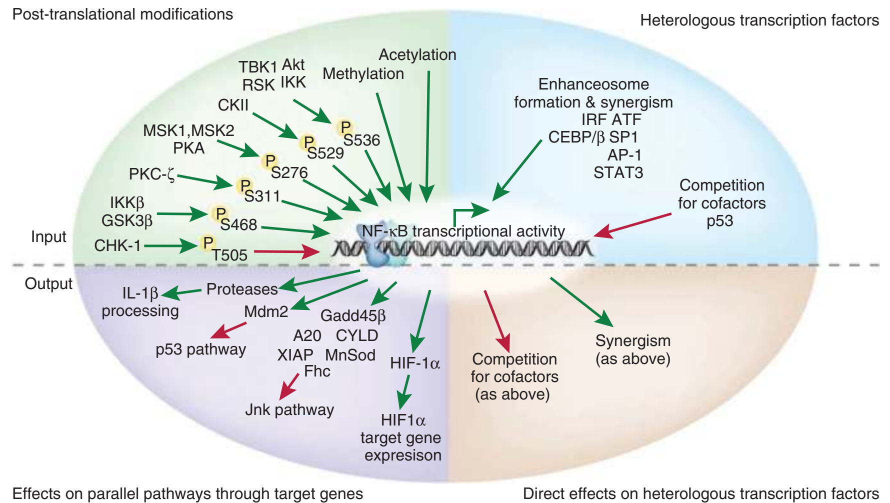

Figure 4 Crosstalk mechanisms involving NF-κB subunits. The transcriptional activity of NF-κB subunits is subject to regulation via a variety of PTMs, including phosphorylation, acetylation and methylation. As PTMs have the potential to modulate the interaction of NF-κB with coactivators, corepressors and IκB proteins, as well as the binding of NF-κB to cooperatively functioning, heterologous transcription factors, they represent a major determinant of selectivity in the induced gene expression signature and are thought to be critical for integration of non-NF-κB pathways and contextual tailoring of the transcriptional response. The formation of NF-κB-containing enhanceosomes at the promoters of target genes requires cooperative action between transcription factors, which facilitates both the integration and regulation of non-NF-κB pathways. NF-κB activity also affects heterologous pathways, such as the Jnk and p53 pathways, through transcriptional regulation of signaling pathway components. Gadd45β, growth-arrest and DNA damage–inducible protein; MnSod, manganese superoxide dismutase; Fhc, ferritin heavy chain; XIAP, X-linked inhibitor of apoptosis protein.

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Crosstalk mechanisms between p65 and IRF3 exemplify these mechanisms. NF-κB p65 and IRF3 can form a stable complex[167] that can be recruited through an interferon-response element (IRE) or a κB site, with the indirectly recruited transcription factor acting as a cofactor to facilitate the activation of transcription. In addition, the IRF dependence of NF-κB target genes can be mediated by an IRE in close proximity to the κB site[168]. Interestingly, glucocorticoid receptors, which after binding their ligand inhibit a subset of NF-κB-dependent transcriptional responses, have been suggested to directly displace IRF3 from p65 (ref. 169). In general, enhanceosome formation involving NF-κB and IRF has been most thoroughly investigated for the enhancer of the gene encoding interferon-β, where assembly of NF-κB, IRF and ATF–c-Jun transcription factors occurs after viral infection[170]. Other inducers of NF-κB do not trigger transcription of this gene, as coordinated activation of all components is required for productive enhanceosome assembly. In this case, transcriptional synergy is conferred by both cooperative DNA binding and recruitment of coactivators. This combinatorial mechanism allows great specificity and selectivity in the induction of gene expression. A new facet has been added to this intricate gene regulatory circuit with the suggestion that p50 homodimers repress a subset of interferon-inducible genes through direct binding to guanine-rich IRE sequences and probably through direct competition with IRF3 (ref. 171). Because they lack this inhibitory mechanism, p50-deficient macrophages activated with LPS show enhanced expression of target genes containing guanine-rich IREs. Interestingly, the binding of p50 to guanine-rich IREs also seems to be involved in conferring signal-specific responses. As a result, in the absence of p50, cooperatively regulated promoters (those that would normally require activation of both IRF3 and NF-κB) are responsive to activation of NF-κB alone. Thus, p50 homodimers act as homeostatic repressors to enforce the stimulus specificity of cooperatively regulated promoters and consequently restrict antiviral responses to the appropriate stimuli. Although this has not been investigated directly, these findings might also be connected to the enhanced interferon-induced antiviral response to influenza infection of immortalized fibroblasts doubly deficient in p65 and p50 (ref. 172) and the resistance of p50-deficient mice to infection with encephalomyocarditis virus[173].

Additional transcription factors for which synergistic interaction with NF-κB has been described are Sp1, AP-1, STAT3 and CEBP/β. Sp1 is a ubiquitous transcription factor that regulates constitutive transcription from many eukaryotic promoters but can also activate or repress stimulus-induced transcription. Sp1-binding elements are frequently located in close proximity to κB sites, and NF-κB and Sp1 have been demonstrated to act together in the induction of several target genes, such as those encoding the integrin ligand ICAM-1 and the cytokine GM-CSF[174–176]. AP-1 transcription factors are dimers composed of members of the Fos and Jun protein families that, like NF-κB, orchestrate gene expression in response to cytokines, growth factors, physiological stresses and infection. NF-κB p65 can interact directly with both c-Jun and c-Fos and can stimulate the binding of AP-1 to DNA and its activation through AP-1 sites. Congruently, c-Jun and c-Fos can promote transactivation of p65 through κB sites even in the absence of AP-1 sites[177]. NF-κB activity has also been demonstrated to regulate subsequent AP-1 activation by promoting the expression of members of the AP-1 family, such as JunB, JunD, B-ATF and c-Fos. In turn, secondary AP-1 activation in pre-B cells can further augment primary NF-κB target gene expression, which demonstrates a critical role for this interaction in mounting an adequate immune response[178,179]. However, NF-κB transcriptional activity may also be paradoxically inhibited by c-Fos in certain situations. Stimuli that increase macrophage cAMP concentrations have been shown to inhibit NF-κB-dependent cytokine production after LPS stimulation. It has been reported that such suppression of the production of TNF and IL-12 is dependent on the phosphorylation of c-Fos by IKKβ and that the resultant stabilization of c-Fos augments this effect[180]. As STAT3 is activated in response to growth, stress and inflammatory stimuli and is critical for the induction of immune-response genes, as well as pro-proliferative and antiapoptotic genes, it is not surprising that the activities of STAT3 and NF-κB are closely intertwined. In this context, direct physical interaction between STAT3 and several NF-κB subunits has been described to result in both transactivation and repression depending on the cellular context and target gene examined. STAT3 and NF-κB can therefore act together to regulate the expression of an overlapping group of target genes (including those encoding PAI-1, Bcl-3 and Bcl-2). Synergism between NF-κB subunits and CEBP/β has similarly been demonstrated for several genes, such as those encoding serum amyloid A2, IL-6 and IL-8, for which the ratio of activated CEBP/β to NF-κB seems to determine a negative or positive outcome for the crosstalk[181,182].

Regulation of parallel pathways via NF-κB target genes

NF-κB activity also influences numerous parallel pathways, a comprehensive description of which is beyond the scope of this review, through induction of target genes. Two prominent examples for which this occurs are the Jnk and p53 pathways, which are affected by the products of NF-κB target genes that counteract their proapoptotic functions. Interference with Jnk activity is achieved through NF-κB-dependent upregulation of genes encoding antioxidant proteins such as ferritin heavy chain or manganese-superoxide dismutase, as ROS help to sustain Jnk activity. In addition, expression of genes encoding inhibitors of Jnk signaling such as GADD45β, A20 and XIAP, is triggered by NF-κB[183]. The stability of p53 is regulated mainly through the E3 ubiquitin ligase MDM2, which induces proteasome-dependent degradation of p53. NF-κB induces *Mdm2* transcription, thereby decreasing p53 abundance[184] and contributing to the survival- and tumor-promoting abilities of NF-κB.

NF-κB is also a critical transcriptional regulator of *Hif1a*, but not *Hif2a*, and IKKβ-mediated basal NF-κB activation is required for the hypoxia-induced accumulation of HIF1-α and the expression of HIF1-α target genes[185]. In addition, NF-κB regulates the expression of genes encoding products that can inhibit either caspase-1 (in macrophages) or other serine proteases (in neutrophils), which are required for the processing of pro-IL-1β. Therefore, an absence of IKKβ results in more plasma IL-1β because of more pro-IL-1β processing. This connection is particularly interesting as it explains the surprising finding that mice with IKKβ-deficient myeloid cells are more susceptible to endotoxin shock[186] and also highlights potential concerns for long-term treatment with IKK inhibitors as anti-inflammatory agents.

Perspectives and implications for understanding NF-κB

There is no question that understanding how the NF-κB pathway influences and is influenced by different signaling pathways will provide crucial insight into the regulation of immune responses. Given that NF-κB signaling is central to a variety of pathological processes and is consequently of considerable pharmaceutical interest, detailed knowledge of the context- and stimulus-specific signaling mechanisms will yield invaluable guidance for the design of targeted therapeutics. For example, as IKK inhibitors are under study for the treatment of both inflammatory diseases and cancers, understanding the full substrate complement and functional outcome of this central kinase complex under physiological as well as pathological conditions remains an important goal. This indicates a need for careful confirmation of the physiological relevance and respective cellular context for the growing number of IKK substrates.

Although tremendous advances have been made in characterizing the role of the IKK complex and NF-κB subunits, as well as upstream modulators such as TRAFs and RIPs, much remains to be learned about how these signaling modules affect non-NF-κB pathways. However, clear genetic proof of crosstalk between pathways is difficult to obtain as it is necessarily obfuscated by deficits in the main pathway in which a given signaling component functions. The same holds true for interpretation of the biological outcomes of deleting signaling molecules that may bridge NF-κB to other pathways. Nevertheless, such approaches are sorely needed to further confirm many of the crosstalk mechanisms discussed in this review. It is our hope that as the NF-κB pathway itself becomes more clearly delineated, there are new opportunities to more precisely test the biological relevance of many of the proposed mechanisms of crosstalk discussed here. Such efforts will require careful analysis of signaling components and the identification of protein interactions or functions that are selectively required only for inter- or intra-pathway functions. As an example, delineation of the divergent functions of RIP1 as scaffolding protein and kinase has allowed the function of this protein in NF-κB and death-signaling pathways to be probed independently. Similar plurality of function has been described for other proteins, yet in many cases, these proposed functions and interactions remain to be rigorously tested through the generation of appropriate genetically modified animal models.

As a major regulator of cell survival, NF-κB is closely involved in carcinogenesis. As discussed here, NF-κB acts together with other important oncogenic signaling pathways, including Ras, p53 and Notch signaling. Although there has been considerable effort to decipher the specific activation mechanisms and contribution of NF-κB to oncogenesis and metastasis, the pathobiological role of crosstalk between NF-κB and other pathways remains poorly defined. Characterization of these crosstalk mechanisms will be invaluable in judging the benefit of combinatorial therapies and in identifying biomarkers that can be used to predict the efficacy of NF-κB inhibition. As systemic blockade of NF-κB will lead to adverse effects, elucidating the pathways that selectively activate and shape NF-κB responses may represent a clinically relevant first step in the design of specific inhibition of NF-κB in malignancy as well as in chronic inflammatory diseases.

Extensive research efforts have been devoted to characterizing the mechanisms that activate NF-κB in various scenarios. Nevertheless, it seems that understanding of how specificity is conferred to NF-κB gene signatures is still just beginning. In addition to the functionally relevant differences between IκB and NF-κB subunits, as well as κB site sequences, parallel signaling pathways clearly contribute to specificity through multiple mechanisms. Especially in the context of dynamic promoter regulation, through either synergism or antagonism of transcription factors, technical and computational advances in combination with detailed genetic and biophysical analyses will probably provide innovative answers to the longstanding question of how NF-κB regulates so many diverse cellular and organismal responses. Consequently, we anticipate exciting new discoveries in the coming years as the tremendous work of the previous quarter of a century is built upon to further improve the understanding of the complex interaction between NF-κB and other key regulators of physiology and pathology.

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