The IκB kinase complex in NF-κB regulation and beyond.

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Abstract

The IκB kinase (IKK) complex is the signal integration hub for NF-κB activation. Composed of two serine-threonine kinases (IKKα and IKKβ) and the regulatory subunit NEMO (also known as IKKγ), the IKK complex integrates signals from all NF-κB activating stimuli to catalyze the phosphorylation of various IκB and NF-κB proteins, as well as of other substrates. Since the discovery of the IKK complex components about 15 years ago, tremendous progress has been made in the understanding of the IKK architecture and its integration into signaling networks. In addition to the control of NF-κB, IKK subunits mediate the crosstalk with other pathways, thereby extending the complexity of their biological function. This review summarizes recent advances in IKK biology and focuses on emerging aspects of IKK structure, regulation and function.

Glossary

aa: amino acid

ABINs: A20-binding inhibitor of nuclear factor-κB proteins) and NEMO (nuclear factor-κB essential modulator

BAFF: Tumor necrosis factor (ligand) superfamily, member 13b

β-TrCP: β-transducin repeat containing E3 ubiquitin protein ligase

CBM: CARMA1 BCL10 MALT1

CBP: CREB-Binding-Protein

DARPin: Designed ankyrin repeat protein

DD: Death domains

Dok1: Docking protein 1

DUB: Deubiquitinase

CARD: Caspase activation and recruitment domains

CYLD: Cylindromatosis

EDA-ID: Ectodermal dysplasia with immunodeficiency

FLICE: FADD-like interleukin-1 beta-converting enzyme

FOXO3a: Forkhead box O3

HLH: Helix-loop-helix

HTLV1: Human T-lymphotropic virus 1

IκB: Inhibitor of nuclear factor-κB

IL1/IL-1R: Interleukin-1/interleukin-1 receptor

IRF: Interferon regulatory factor

IRS-1: Insulin receptor substrate 1

JNK: Jun N-terminal kinase

LUBAC: Linear ubiquitin chain assembly complex

LZ: Leucine zipper

MAPK: Mitogen-activated protein kinase

MAVS: Mitochondrial antiviral-signaling protein

MEKK3: MAPK∕ERK kinase kinase 3

mTOR: Mammalian (also: mechanistic) target of rapamycin

NCOA3: Nuclear receptor coactivator 3

NBD: NEMO binding domain

NEMO: NF-κB essential modifier

NF-κB: Nuclear factor-κB

NLS: Nuclear localization sequence

OTU: Ovarian tumor domain

OTULIN: OTU DUB with linear linkage specificity

PARP-1: Poly-ADP-polymerase-1

PI3K: Phosphatidylinositide 3-kinases

PIAS1: Protein inhibitor of activated STAT, 1

RHIM: RIP homotypic interaction motifs (RHIMs)

S6K1: Ribosomal S6 protein kinase 1

SMRT: Silencing mediator for retinoid or thyroid-hormone receptors

SNAP23: Synaptosomal-associated protein, 23kDa

TAB1/2: TAK1 binding protein 1/2

TAK1: TGF-β-activated kinase 1

TBK1: TANK-binding kinase 1

TIR: Toll IL-1R (TIR) domain

TNFR: Tumor necrosis factor receptor

TNFRSF: Tumor necrosis factor receptor superfamily

TLR: Toll-like receptor

TRAF: TNF receptor associated factor

TSC1: Tuberous sclerosis 1

UBAN: Ubiquitin binding in ABINs

ULD: Ubiquitin-like domain

Introduction

NF-κB is an inducible transcription factor that coordinates specific gene expression programs to impact the regulation of multiple physiological functions. The most important and evolutionarily conserved role of NF-κB is as mediator of the immune and inflammatory response. However, in addition, NF-κB pathways contribute to cell adhesion, differentiation, proliferation, autophagy, senescence and protection against apoptosis. In line with its multi-layered physiological functions, deregulated NF-κB activity is found in a number of disease states, including cancer, arthritis, chronic inflammation, asthma, neurodegenerative diseases, and heart disease (1, 2).

The NF-κB family consists of NFKB1 (p50/p105), NFKB2 (p52/p100), RelA (p65), c-Rel and RelB, which form various homo- and heterodimers. In resting cells, NF-κB dimers are sequestered in the cytoplasm through interaction with IκB proteins (IκBα, IκBβ and IκBε) or the precursor proteins p100 and p105. Induction of NF-κB depends on the phosphorylation of IκBs on critical serine residues. As a consequence, IκBs are ubiquitinated by the E3 ubiquitin ligase SCF$^{\beta TrCP}$ and degraded by the proteasome, which in turn allows the nuclear translocation of NF-κB heterodimers (Fig. 1) (3, 4). NF-κB precursors are - constitutively or in a stimulus dependent manner - processed by the proteasome to produce the mature transcription factors p50 and p52 (5). As the small IκBs, p105 can undergo signal dependent phosphorylation and degradation, which frees p50 homodimers, leading to the formation of Bcl-3:p50 complexes (6).

The discovery of the phosphorylation-dependent NF-κB activation mechanism initiated the search for an IκB kinase (IKK). An initial biochemical study described a high molecular weight kinase complex of approximately 700 kDa, which required non-degradative ubiquitination for its activity (7). However, the identity of the kinase was not revealed. The serine/threonine kinases IKKα (85 kDa; also known as IKK1 or CHUK) and IKKβ (87 kDa; also known as IKK2), were then identified as the two catalytic components of the IKK complex, which form dimers and are able to phosphorylate IκBs *in vitro* (8-13). As a third component, NEMO (48-kDa; also known as IKKγ, IKKAP1 or Fip-3), was identified by complementation of an NF-κB unresponsive cell line and by sequencing of IKK-associated polypeptides (14, 15). NEMO is a regulatory non-enzymatic scaffold protein. Gene ablation studies of the IKK subunits then revealed that NF-κB activation is achieved alternatively through the mechanistically different canonical and non-canonical pathways.

Inflammatory cytokines, radiation, stress signals and pathogenic assaults evoke the rapid canonical pathway and generally involve ubiquitin mediated complex formation of signaling molecules, ultimately resulting in phosphorylation of two serine residues located in the IKKα and β activation loops, similar to the activation of many other kinases (Fig. 1 and 2). Canonical signaling strictly depends on NEMO, while the catalytic subunits seem to be more redundant (16).

The non-canonical pathway is activated by a restricted group of stimuli, such as the TNF family members lymphotoxin-α/β, BAFF or CD40L, which trigger posttranslational stabilization of NF-κB interacting kinase (NIK) (Fig. 2). Crystal structural analysis showed that the kinase domain of NIK adopts an intrinsically active conformation, so that downstream targets can be phosphorylated without requiring an additional phosphorylation step (17). In unstimulated cells, a TRAF-cIAP complex catalyzes K48-linked ubiquitination of NIK, leading to constitutive NIK degradation. Ligand binding induces recruitment of the TRAF-cIAP complex to the receptor, whereupon TRAF2-mediated, K63-linked ubiquitination of cIAP1∕2 switches its K48 ubiquitin ligase activity from NIK to TRAF3. The ensuing TRAF3 degradation destabilizes the TRAF-cIAP complex and enables the accumulation of newly synthesized NIK (18, 19). NIK is thought to phosphorylate IKKα, which in turn phosphorylates and marks p100 for C-terminal processing to generate p52-containing complexes, primarily p52/RelB. The non-canonical pathway is characterized by a distinctive slower kinetics, requires neither IKKβ nor NEMO and plays a critical role in the development of lymphoid organs (20). Attenuation of the non-canonical NF-κB pathway is controlled through the DUB OTUD7B, which deubiquitinates TRAF3 in a K48-directed manner (21).

The substrate spectrum of the IKK complex is not restricted to IκBs and precursors, but includes a wide range of NF-κB pathway inherent (such as RelA) and extraneous (such as p53 and TCS1) substrates (Table 1). Thus, the IKK complex is both a signaling hub for NF-κB activation and an interface for crosstalk between NF-κB activating pathways and other physiological processes (16, 22).

Despite the steady increase in IKK related publications, important questions still remain unanswered (Sidebar A). How a signaling cascade mechanistically induces the activation of the IKK complex, how IKK activity is turned off and how substrate specificity is achieved has to be resolved. Recent genetic, biochemical and structural data have shed new light on IKK structure, regulation and function.

IKK complex - structures and composition

IKK-mediated activation of NF-κB is differentially regulated by IKKα and IKKβ. Both kinases have high sequence homology (approximately 50 % identity), and contain an N-terminal kinase domain, a dimerization domain and a C-terminal NEMO-binding domain (NBD) (16, 22, 23) (Fig. 3A), although only IKKα has a predicted NLS (24). Kinase activity critically depends on lysine 44 and phosphorylated T-loop serines - S176 and 180 for IKKα, S177 and 181 for IKKβ (9-12, 25). IKKβ contains a conserved ubiquitin-like domain (ULD), which is critical for its catalytic activation (26). Much-anticipated 3D structural information was obtained in the last few years that allowed further elucidation of IKK structure-function relationships (Table 2).

IKK structures reveal complex intra- and inter-molecular interactions

As a major breakthrough, the long-awaited crystal structures of IKKβ were recently presented, revealing the domain organization of this catalytic IKK subunit and shedding new light on protein-protein interactions, mechanisms of activation and the mechanism of substrate recognition (27-29). IKKβ has a trimodular structure, comprising the N-terminal kinase domain (KD), a central ULD and an elongated α-helical scaffold/dimerization domain (SDD) at the C-terminus (Fig. 3B and Table 2). Neither of the previously predicted LZ and HLH motifs form as these residues are part of the SDD. Notably, the IKK-related kinase TBK-1, which is essential for the induction of type I interferon (IFN), has the same trimodular structure (30-32). Both IKKβ and TBK1 form stable homodimers through the SDD domain. A comparison of the IKKβ and TBK1 structures further revealed that the overall dimer conformation differs, depending on the activation status. Dimers of inactive kinases show a more compact geometry, resembling closed shears (29, 31), while activated kinases adopt a more open conformation (27, 28). A detailed inspection of IKKβ and TBK1 kinase domains revealed that the activation loop, when unphosphorylated, adopts conformations that are incompatible with protein substrate binding, explaining the essential role of T-loop phosphorylation at S177/S181 for IKKβ and S172 for TBK1 kinase activity (27, 31, 33). The previously predicted ULD is a critical element of IKKβ, required for kinase activity and, together with the SDD, involved in the exact positioning of the kinase substrate IκBα. Most likely, IκBα is recruited to IKKβ through NEMO, which ensures specific substrate

recognition (see also below) (34). The striking interdependence of the three domains is reflected by their extensive intramolecular interactions (28, 29). Intriguingly, the sequence and conformation of the SDD significantly differ between IKKβ and TBK1 (31, 32). The dimerization interface of TBK1 is almost twice as large as that of IKKβ, resulting in a stimulus independent compact composition and a TBK1-specific overall domain organization. These structural differences might reflect specific activation requirements. IKKβ activation strictly depends on NEMO, whereas TBK1 activation requires K63-linked polyubiquitination of the conserved Lys30 and Lys401 residues (22, 32). In fact, structure-directed mutagenesis revealed that SDD-mediated kinase dimerization of IKKβ is required for NEMO binding and the kinase activation process, but not for enzymatic activity once its activation loop is phosphorylated (29). Likewise, dimer-disrupting mutations on TBK1 had markedly decreased K63-linked polyubiquitination (32).

How signal transmission results in phosphorylation of the IκB kinase T-loop is still a matter of debate. The existence of IKK kinases (IKKKs) and/or IKK trans-autophosphorylation are both feasible scenarios (3, 23). In the closed dimer structure of inactive IKKβ, the active sites of the two neighboring KDs cannot interact with each other. However, activated IKKβ dimers seem to switch to a more open V-shaped conformation, allowing oligomerization and KD-KD interactions, with the potential to facilitate *trans*-autophosphorylation (28, 29). Of note, how the structure and relative position of the KDs in IKKα-IKKβ heterodimers, which are more relevant *in vivo*, may differ, remains to be solved.

Structural insights into NEMO

Important structural information has been obtained for NEMO. On the basis of biochemical assays, NEMO had been predicted to be a mainly α-helical protein containing two coiled-coil domains (CC), a LZ and a C-terminal zinc-finger (ZF) region (Fig. 3A) (22, 23). In addition, NEMO has a minimal oligomerization domain (MOD) and an N-terminal dimerization domain that enable the formation of higher order oligomers (35, 36). Finally, the kinase-binding domain (KBD) was determined by deletion analysis to aa 44 -111 of NEMO (37) . The ZF seems to be required for efficient IκBα binding and might direct IκBα to the ULD/SDD of IKKβ (34).

Although a crystal structure for full length NEMO is still missing, the structures of several NEMO fragments have been resolved (Fig 3C and Table 2) (37-41). The X-ray structure of the NEMO-IKKβ interface revealed an asymmetrical four-helix bundle, composed of a

parallel NEMO dimer. Each NEMO molecule forms a crescent-shaped α-helix, which associates with mainly α-helical IKKβ peptide mononomers via hydrogen bonding and hydrophobic interactions (37). An X-ray structure of the central region, associated with a fragment of the viral IKK activating protein vFLIP, showed that the coil-coil domain encompasses aa 192 – 252 (38) (Fig. 3C).

NEMO has a central role in polyubiquitin mediated IKK activation, as it specifically recognizes polyubiquitins through the CC2 – LZ region domain and becomes itself ubiquitinated. Although the type of ubiquitination is a matter of debate (discussed below) there is ample evidence that both events are important for IKK activation (42, 43). Mutations in the NEMO ubiquitin binding region (UBAN, also known as NUB, NOA or CoZi), which impair ubiquitin binding and NF-κB activation, have been identified in patients suffering from ectodermal dysplasia with immunodeficiency (EDA-ID) (44). The crystal structures of different NEMO fragments including the UBAN domain revealed a dimer, which contains two coil-coil domains representing CC2 and LZ, respectively (40, 41, 45). These NEMO fragments were shown to bind to linear, M1-linked di-ubiquitin with higher affinity than K63- or K11-linked di-ubiquitins (41, 45-47). However, a larger NEMO fragment containing the UBAN domain together with the C-terminal ZF - another *bona fide* ubiquitin-binding domain - had an increased affinity for K63-linked polyubiquitin (39, 48, 49). *In vitro* binding studies demonstrated a high preference of NEMO in solution for M1-linked ubiquitin oligomers, while immobilization enhances the affinity towards K63-linked ubiquitin (50). Competition analyses indicated that NEMO functions as a high affinity receptor for M1-linked ubiquitin chains and a low affinity receptor for different types of long lysine-linked ubiquitin chains (51). The possible existence of M1-K63-mixed-linkage ubiquitin polymers may pose a new problem (52). Taken together, these findings indicate that different types of polyubiquitin are able to bind to NEMO and contribute to IKK activation. Whether the differences in binding affinities determined in *in vitro* studies with NEMO fragments and di-ubiquitins are physiologically relevant and how they impact IKK activation remains to be determined.

Oligomeric composition of the IKK complex

The apparent molecular weight of the IKK complex in gel filtration chromatography is around 700 to 900 kDa. Although numerous proteins have been proposed to interact with IKK-components (23), co-immunoprecipitation studies with S^{35} labeled proteins and size exclusion chromatography analysis with recombinant proteins clearly indicate a tripartite IKK

composition (53-55). However, the exact stoichiometry of IKKα, β and NEMO in the IKK complex remains an open question.

Crystallographic and quantitative analyses of the binding interactions between N-terminal NEMO and C-terminal IKK fragments suggest that IKKβ dimers would interact with NEMO dimers (37, 56). Such a model is supported by the recent crystal structures of IKKβ (see above), and a similar situation is anticipated for IKKβ:IKKα heterodimers. Chemical cross-linking and equilibrium sedimentation analyses of NEMO suggested a tetrameric oligomerization (dimers of dimers), depending on a C-terminal coiled-coil minimal oligomerization domain (MOD) and subsequent dimerization of the dimers with their N-terminal sequences (36). Tetrameric NEMO could sequester four kinase molecules, yielding an $IKK\alpha_2 IKK\beta_2 NEMO_4$ stoichiometry. Such a higher-order oligomerization could provide the basis for an IKK *trans*-autophosphorylation mechanism (as discussed before). Conformational changes in the scaffold induced by polyubiquitin binding to NEMO could bring the catalytic domains of two kinase dimers into proximity. Such a model is supported by the recent finding that IKKβ dimers reversibly oligomerize in solution and the active kinase forms higher order oligomers in the crystal (28, 29).

Taken together, tremendous progress has been made in understanding the structures of the IKK components, which provides the basis to finally understand the dynamic architecture of the IKK complex. Notably, although the tripartite IKK structure is perhaps the most abundant form, other IKK complexes might exist. In fact, co-expression experiments and *in vitro* studies have shown that NEMO can interact with IKK1 or IKK2 homodimers (54, 55). It is a temping assumption that different complex compositions might be required for tissue specific or stimulus specific NF-κB dependent and independent signaling events. Likewise, NIK-dependent activation of the non-canonical NF-κB pathway was proposed to occur through phosphorylation of IKKα homodimers (57). However, whether specific IKK complexes with distinct oligomeric compositions and functions exist in cells remains to be demonstrated.

IKK activation and inhibition

IKKs are activated by a plethora of agents and conditions, including extracellular ligands that bind membrane receptors, such as TNFR, TLR, or IL-1R intracellular stress, such as DNA damage and reactive oxygen species, as well as the recognition of intracellular pathogens mediated by the NOD and RIG-I-like (NLR) family of proteins (Fig. 2). The activated

receptor structures nucleate dynamic regulatory networks, where protein phosphorylation, non-degradative ubiquitination, adapter protein interactions and most likely higher order oligomerization events all contribute to IKK activation (Fig. 1 and 2). Moreover, canonical and non-canonical NF-κB signaling pathways can be activated by human oncogenic viruses, including the human T cell leukemia virus type 1, the Kaposi sarcoma-associated herpesvirus, and the Epstein-Bar virus (58). Recent findings indicate that the virus encoded oncoproteins either use components of the IKK upstream signaling network or directly act on the IKK complex to activate NF-κB (59, 60).

IKK phosphorylation

How signal transmission results in the phosphorylation of the IκB kinase T-loop is still an important unsolved question. In analogy to other signaling pathways, IKK kinases (IKKKs) have been suggested to mediate it. A prominent example is TAK1, which is also engaged in the JNK pathway. TAK1, together with the adaptor proteins TAB1 and TAB2, was found to act as TRAF6-regulated IKK activator in cell-free assays (61, 62). TAB2 can recruit TAK1 to K63-linked polyubiquitin chains of upstream regulators which most likely cause induced-proximity driven IKKβ phosphorylation. However, TAK1 is not an essential general IKKK, but rather a regulatory module with a stimulus and cell-type specific impact on IKK activation (16, 42). MEKK3 was proposed as another potential IKKK, as the kinase can phosphorylate IKK *in vitro* and NF-κB activation is reduced in MEKK3 deficient cells in response to TNF, IL-1 or TLR stimulation (63, 64). IL-1-induced NF-κB activation has been proposed to involve MEKK3 in addition to TAK1 (65, 66). However, IKK subunits could also be activated by trans-autophosphorylation, as previously proposed (67), instead of through an IKKK. This latter possibility is supported by recent structural and composition analyses, as discussed above. Trans-autophosphorylation and IKKK-dependent phosphorylation could even operate successively or in parallel to reach maximum kinase activation (Fig. 1).

Ubiquitin dependent signaling

The activation of the IKKs appears to depend on the induced proximity resulting from the dense organization of signaling complexes and on binding of adapter proteins, such as NEMO or TAB proteins. Non-degradative polyubiquitination, a crucial prerequisite of IKK complex activation, triggers both processes (Fig. 1) (42).

TRAF6 was identified as the first ubiquitin E3 ligase that could - together with Ubc13 and Uev1A - catalyze K63-linked auto-ubiquitination and subsequently trigger IKK activation

(61). TRAF6 is involved in a wide variety of NF-κB stimulating signaling pathways, including those triggered by IL-1R, TLR, TCR, RIG-I-like receptor and DNA double strand breaks (42, 43). In the case of IL-1 signaling, it has been demonstrated that TRAF6 enzymatic activity but not auto-ubiquitination, is required for NF-κB activation (68). In fact, TRAF6 has been shown not only to undergo self-ubiquitination, but also to mediate K63-linked polyubiquitination of several pathway components, such as IRAK1, MALT1 and TAK1 (42, 43). Furthermore, TRAF6 has been proposed to generate free, unanchored K63-linked polyubiquitin which would act as docking platform in the IKK activation process (69). Additional K63-specific E3 ligases involved in specific NF-κB signaling cascades have been identified, such as TRAF2/5, pellino proteins or TRIM25 in the TNFR, IL1R/TLR and RIG-I pathways, respectively. Likewise, there is a growing list of proposed NF-κB pathway regulators that are substrates of inducible K63 ubiquitination, such as Bcl10, NOD2, RIP1, RIP2 and ELKS (42, 43).

In contrast to IL1β, TNFα signaling does not depend on K63-linked ubiquitination, indicating that alternative forms of non-degradative polyubiquitination are important in this pathway (70). Accordingly, linear, M1-linked ubiquitination of NEMO and RIP1, which is catalyzed by the LUBAC complex, has been shown to be important for NF-κB activation (71, 72). This E3 complex, consisting of HOIL1, HOIP and SHARPIN, is recruited to the TNFR1 signaling complex in a TRADD, TRAF2 and cIAP1/2 dependent manner. Furthermore, LUBAC mediated M1-linked ubiquitination contributes to IL1R, CD40, TACI, parkin and DNA damage mediated NF-κB activation, but is dispensable for B-cell receptor-mediated signaling (43, 73-75). The physiological relevance of LUBAC was first demonstrated in mice with chronic proliferation dermatitis (Cpdm), which results from a spontaneous null mutation in the *Sharpin* gene. These mice mimic part of a phenotype of patients suffering X-linked hyper IGM syndrome and hypohydrotic ectodermal dysplasia, which is caused by NEMO mutations, and Cpdm-derived cells display attenuated TNF, CD40 ligand and IL1β signaling (71, 76, 77). Biallelic inactivating mutations in *HOIL1* have been identified in patients suffering an inherited disorder with immunodeficiency, autoinflammation and amylopectinosis (78). HOIL1 deficiency and subsequent LUBAC destabilization resulted in impaired NF-κB-mediated IL1β responses, which differed depending on the tissue (78). Thus, the ubiquitin linkage type seems not only to confer stimulus-specific, but also cell type-specific restraints in NF-κB activation.

There is accumulating evidence that alternative and even hybrid ubiquitin linkages play a role in NF-κB signaling, thereby increasing the complexity of ubiquitin-mediated processes (43). In *vitro assays* have demonstrated that the E3 ligases cIAP1 and TRIM23 can catalyze K6-linked or K27-linked polyubiquitination of NEMO, respectively (79, 80). Modification of RIP1 seems to be a special case, because TNFα treatment induces modification with degradative K48 as well as with non-degradative M1-, K63-, and K11-linked ubiquitin chains (47, 71, 81-83). The fact that only one ubiquitin acceptor site (K377) has been determined until now (81), raises the question, of whether TNFα induces distinct populations of modified RIP1 or, alternatively, the modification of RIP1 with a poly-ubiquitin chain containing mixed linkages. Indeed, an analogous process has been recently reported for IRAK1, IRAK4 and MYD88, which are modified with K63/M1-linked hybrid ubiquitin chains in IL1R or TLR stimulated cells (52). In addition to polyubiquitin, monoubiquitination of proteins has a functional impact (84). In fact, a modified NEMO species 8 kDa larger, which most likely indicates a mono-ubiquitinated form, is generated in cells stimulated with various agents (68, 72, 85, 86). K285 could be determined as the acceptor site using mass spectrometry and mutational analysis (68, 85). The same site, in addition to K309, was determined as acceptor for M1-linked ubiquitination (72). Site-specific ubiquitination of NEMO was shown to be crucial for NF-κB activation in both cell culture and in a mouse model (68, 72, 85, 87). Thus, IKK activation coincides with two parallel or sequentially occurring modifications of NEMO. As an appealing possibility, NEMO ubiquitination and NEMO-dependent ubiquitin binding might trigger and stabilize stimulus-induced, conformational changes to facilitate *trans*-autophosphorylation of the kinases. The Hsp90-Cdc37 chaperone complex, which transiently interacts with IKK, could have a supportive function in this process (53).

NF-κB-inducing lymphogenic virus proteins, such as Kaposi's sarcoma associated herpes virus encoded vFLIP, have been recently shown to bind NEMO directly. This would probably induce conformational changes that would allow bypass of the ubiquitin-dependent signaling cascade (38, 60). In addition to ubiquitination, post translational modifications with ubiquitin-like proteins - such as genotoxic stress induced, PIASy-mediated SUMOylation of NEMO - are critically involved in signal transmission and NF-κB activation (73). Based on our current knowledge, modifications with monoubiquitin, non-degradative polyubiquitins and ubiquitin-like proteins can be concluded to have a crucial role in coordinating appropriate protein-protein interactions within specific NF-κB signaling pathways. However, many details of the non-degradative actions of ubiquitin and ubiquitin-like proteins remain to be discovered.

Protein-protein interactions and higher order oligomerization

A typical early event in NF-κB signaling is the receptor-mediated recruitment of adapter proteins that contain protein-protein interaction domains, such as DDs, CARDs, RHIMs and TIRs. These adapters have the potential to form higher-order signaling platforms (3, 88). X-ray structure analyses showed that the TLR/IL-1R signaling molecules MyD88, IRAK4 and IRAK2 associate in helical assemblies, dependent on the DDs of the individual proteins (89). Likewise, virus induced RIG-I catalyzes the prion-like aggregation of MAVS that depends on CARD interaction. Oligomeric MAVS interacts with TRAF6 and TRAF2, resulting in IKK and TBK1 activation (90). TRAF6 itself can also oligomerize to form a distinct network structure mediated by C-terminal trimerization and N-terminal dimerization with the ubiquitin-conjugating enzyme Ubc13 (91). As a consequence, the increase in local concentration and proximity could promote TRAF6 auto-ubiquitination and downstream signaling. Lastly, activated IKKs have the potential to form higher-order oligomers, which might trigger rapid signal amplification by *trans*-autophosphorylation (28). The weak interactions between IKK kinases observed *in vitro* could be further stabilized by the interaction with clustered upstream signal components and/or through polyubiquitin scaffolds.

Taken together, oligomerization appears to occur at all levels of canonical signaling cascades, in which multiple signaling oligomers combine to perform multiple reactions simultaneously and efficiently. The clustering of signaling molecules is an attractive mechanism that could provide temporal and spatial control of signal transmission and might help to increase the signal-to-noise ratio (92). Therefore, a focus of future research should be to prove whether higher-order assemblies of signaling molecules are a general feature of canonical and even non-canonical NF-κB signaling.

Control of transience and attenuation of IKK activity by feedback regulation

To maintain the transient nature of NF-κB activity, signal transmission is controlled by various levels of negative feedback mechanisms, including direct regulation of IKK as well as negative feedback mechanisms that affect upstream signaling components (Figure 2). Intrinsic attenuation of IKK has been proposed to involve IKKβ-mediated autophosphorylation of c-terminal serines, either close to the HLH or in and around the NEMO binding domain, as well as phosphorylation of NEMO S68 (67, 93, 94). Such events might disrupt the domain interactions of IKK subunits. Moreover, IKKβ can terminate TCR signaling by phosphorylation of the pathway component BCL10 (95, 96). Dephosphorylation of serines in

the activation loops of IKKs by phosphatases PP2A and PP2C has also been suggested as another direct inhibitory mechanism, as reviewed earlier (16, 23). An important step in IKK activation is the binding of the NEMO UBAN domain to polyubiquitin chains. This interaction can be disrupted by optineurin, a negative regulator of TNFα-induced NF-κB activation, which contains a similar UBAN domain and competes with NEMO for binding to polyubiquitin (97). Mutations of optineurin in amyotrophic lateral sclerosis interfere with the inhibitory effect towards NEMO, and thus exaggerate NF-κB activation (98). Another NEMO-dependent feedback mechanism has recently been described: p47 (also known as NSFL1C) —which is major adaptor of the cytosolic triple-A ATPase p97— binds to polyubiquitinated NEMO and induces its lysosomal degradation, resulting in reduced IKK activity (99).

Non-degradative polyubiquitination of signaling molecules by various E3 ligases plays a key role in IKK activation. A series of DUBs, which cleave specific linkage types, counteract these activities and terminate or attenuate the signaling process. The K63-directed DUBs A20 and CYLD have been show to de-ubiquitinate RIP1, TRAF6, RIP2 and MALT1. USP21 and K11-specific Cezanne have been implicated in the removal of poly-ubiquitin from RIP1 (for review see (100-103). A recently identified DUB known as OTULIN or Gumby, which is specific for M1-linked ubiquitin, has been shown to interact with the LUBAC component HOIP, decreasing M1-linked ubiquitination and attenuating NF-κB activity. OTULIN-depleted cells spontaneously accumulate M1-linked ubiquitin chains on LUBAC components and, upon TNFR1 or NOD2 stimulation, on RIPK1 and RIPK2, respectively (104-106). The non-canonical NF-κB pathway is attenuated by the DUB OTUD7B, which deubiquitinates TRAF3, thereby counteracting its degradation and preventing NIK mediated p100 processing (21).

SUMOylation of NEMO is a critical step in the response to DNA double strand breaks. Amongst the Sentrin/SUMO-specific proteases (SENPs), SENP2 has been shown to specifically deSUMOylate NEMO and attenuate NF-κB activation. Since NF-κB promotes SENP2 expression, a negative feedback loop is established (107).

IKK functions

Although the tripartite structure of the IKK complex suggests common physiological roles for IKKα, IKKβ and NEMO, the phenotypes of the single knockouts suggest they have common, but also distinct functions. IKKβ deficient mice are embryonically lethal and die, like p65

deficient mice, at embryonic day 13, primarily due to TNF-induced liver apoptosis. These results underscore the importance of IKKβ in canonical NF-κB signaling (108, 109). However, subsequent studies with IKKβ deficient cells indicate that the absolute requirement for IKKβ in canonical NF-κB signaling depends on the stimulus. IKKα can substitute IKKβ function at least in the case of IL-1R signaling (110).

IKKα deficient mice can survive for a month after birth, but suffer from striking morphological defects, such as markedly hyperplasic epidermis (111). Follow up studies determined that IKKα is a major cofactor in a TGFbeta-Smad2/3 signaling pathway that is required for cell cycle exit and induction of terminal differentiation of keratinocytes (112). Accordingly, IKKα has been shown to be a critical suppressor of skin cancer in humans and mice (113, 114). In addition to its specific function in TGF-β mediated keratinocyte differentiation, IKKα is a crucial regulator of non-canonical NF-κB signaling, required for B cell maturation and formation of secondary lymphoid organs (57). It is also involved in the maturation of dendritic cells and pancreatic homeostasis (115, 116).

Genetic depletion of the scaffold protein NEMO results in a complete loss of canonical signaling and mutant embryos die at E12.5-E13.0 from severe liver damage due to massive apoptosis (22). NEMO exists either as a component of the IKK complex or as an unbound form that shuttles between cytoplasm and nucleus. In response to genotoxic stress, NEMO undergoes sequential post-translational modifications and has a central role in a dual, PARP-1/PIASy and ATM dependent signaling pathway that links the cellular DNA damage response to NF-κB (117).

The key function of the IKK complex is to phosphorylate IκBs and the NF-κB precursors p105 and p100 (23). In addition, IKKs directly modulate the function of RelA and c-Rel. IKKβ dependent phosphorylation of p65 on Ser536 enhances its transactivation potential, while IKKα mediated c-terminal phosphorylation of p65 and c-Rel attenuates their activity (118, 119). However, IKKα has been shown to accumulate in the nucleus in a stimulus dependent manner and to phosphorylate chromatin components (Table 1), implicating a more far-ranging spectrum of biological functions (16). There is ample evidence that IKK activity is not restricted to NF-κB dependent pathways but can also mediate cross-talk with other signaling cascades, such as mTOR and MAPK pathways (1, 16, 120). Hence, numerous additional kinase substrates have been identified, which link IKK activity to a variety of biological functions including immune responses and transcriptional regulation and chromatin remodeling (Table 1).

IκB kinase dependent but NF-κB independent signaling events have been shown to influence various cell fate decision processes. The signal transducer for Wnt dependent cell proliferation, β-catenin, was described as one of the first alternative IKK substrates. IKKβ-mediated phosphorylation of β-catenin was proposed to induce its ubiquitin dependent degradation, whereas phosphorylation by IKKα stabilizes β-catenin expression and induces β-catenin-dependent cyclin D1 transcription (121-123). Indeed, IKKα knockdown in multiple myeloma cells did not inhibit NF-κB activation but correlated with impaired β-catenin expression and significant growth inhibition (124). However, other studies showed IKKα–mediated cyclin D1 phosphorylation and degradation as well as NF-κB dependent transcriptional regulation of cyclin D1, indicating a complex IKK/NF-κB dependent regulation of cyclin D1 (125-127). IKKs not only affect cell proliferation but also cell survival pathways, in an NF-κB-independent manner. IKKβ phosphorylates the BH3-only protein BAD at serine-26 (Ser26), which primes it for inactivation and suppresses TNFα-induced apoptosis (128). In response to oxidative stress, however, IKKβ mediates pro-apoptotic functions through activation of p85 S6K1 (129). The IKK complex also has a direct role in the induction of autophagy (130, 131). Nutrient depletion induces IKK dependent phosphorylation of the p85 subunit of PI3K, thereby blocking Akt and mTOR inhibition (132). This crosstalk may contribute to the induction of autophagic genes, such as Beclin 1 (130). However, studies in PTEN-inactive prostate cancer cells indicate that IKKα functions as a mediator of mTOR activation, which in turn suppresses autophagy (133). Thus, it will be important to elucidate the physiological signaling events that are associated with IKK dependent regulation of autophagy in the respective settings.

Notably, both IKKα and IKKβ have crucial NF-κB dependent and independent functions in various oncogenic scenarios, which are often correlated with inflammation-mediated tumorigenesis. Analysis of prostate cancer mouse models indicates that tumor-infiltrating T cells and macrophages express RANKL, which in turn induces nuclear IKKα activation and subsequent transcriptional inhibition of the tumor suppressor Maspin (also known as serpin B5). The amount of active nuclear IKKα in mouse and human prostate cancer correlates with reduced Maspin expression and with metastatic progression (134). Nuclear IKKα activity, induced through a NIK dependent pathway, is also involved in ErbB2-induced mammary tumorigenesis (135). Activated IKKα phosphorylates the cyclin-dependent kinase inhibitor p27/Kip1 and stimulates its nuclear export or exclusion, which in turn correlates with the expansion of tumor-initiating cells. Notably, nuclear IKKα expression in human breast cancer is inversely correlated with nuclear p27 abundance and metastasis-free survival (136).

Colorectal tumors also have active IKKα in the nucleus, in this case concomitant with derepressed SMRT repressor (also known as NCoR2), which is aberrantly localized in the cytoplasm (137). IKKα phosphorylates SMRT, which is part of a multisubunit repressor complex that includes histone deacetylases (138). IKKα-mediated phosphorylation induces the exchange of corepressor for coactivator complexes on chromatin and potentiates the acetylation of RelA/p65 by p300, inducing full transcriptional activity (139). A truncated p45-IKKα variant has been recently identified the major IKKα form present in the nucleus of colorectal cancer cells. This truncated IKKα is generated by cathepsin activity, which is increased in these cells, and is constitutively active (140). The role of IKKα in tumor development is dependent on the tissue, it can act as promoter of tumorigenesis in breast and prostate cancer, but is a tumor suppressor in lung carcinomas (134-136, 141) and skin cancer (113, 114).

Cellular stress responses, which might serve as anti-tumor barriers, activate two major pathways: NF-κB and p53. The regulation of both pathways shares many similar features and many studies have suggested that crosstalk exists between them. Both transcription factors could compete for a limiting pool of the transcriptional co-activator CBP, as both require such interaction to maximize their activities (1). IKKα has been shown to phosphorylate CBP at S1382 and S1386, thereby switching its binding preference from p53 to NF-κB (142). CBP phosphorylation status and IKKα activation are directly correlated in several tumor cell lines, suggesting that IKKα mediated p53/NF-κB cross-regulation may be a critical factor for cell proliferation and tumor growth (142). The p53/NF-κB crosstalk is not restricted to transcriptional regulation, but extends to cytoplasmic IKK function and regulation. IKK and subsequent NF-κB activities are increased in p53-deficient cells, thereby promoting aerobic glycolysis, which cancer cells typically use as energy source (Warburg effect). The catalytic activity of IKKβ is boosted through an O-linked-N-acetyl-glucosamine modification, establishing a positive feedback regulation from increased glucose metabolism. p53 was also suggested to restrict IKK activation through the suppression of aerobic glycolysis (143, 144), and IKK seems to regulate p53 protein levels through direct phosphorylation at S362 and S366, which leads to its β-TrCP dependent degradation (145), further emphasizing the crosstalk between these two pathways.

Other tumor suppressors, which can be regulated by IKKβ are the TSC1/2 complex and the transcription factor FOXO3a. In breast cancer cells, TNFα-induced IKKβ phosphorylates TSC1 at S487 and S511, resulting in disruption of the TSC1/2 tumor suppressor complex and

consequently activation of the mTOR pathway. Tumor models expressing a TSC1 phosphomimetic mutant showed enhanced angiogenesis and tumor growth. In agreement with this, activated IKKβ is associated with TSC1 phosphorylation and VEGF production in different tumor types and correlates with poor clinical outcome of breast cancer patients (146). The expression of the transcription factor FOXO3a is inversely correlated with that of IKKβ in human breast tumor specimens and positively correlated with the survival rate in breast cancer. This is in line with the observation that IKKβ can phosphorylate FOXO3a and trigger its ubiquitination mediated proteasomal degradation (147). Similarly, pharmacological inhibition of IKK in leukemic cells (AML; T-ALL) was shown to restore FOXO3a expression, correlating with impaired cell proliferation and induced apoptosis (148, 149). Taken together, a number of IKK substrates have been identified, which also have revealed the enormous complexity of IKK function in (patho)biology (Table 1). In many cases, the detailed regulatory mechanisms are still elusive and there are questions regarding substrate specificity and the nature of the signaling outcome depending on the stimulus, tissue and environment. Moreover, cell-type specific variations in IKK expression levels and altered IKK complex composition might contribute to specific functions.

Concluding remarks and perspectives

Our understanding of the impact that IKK/NF-κB signaling has in mammalian physiology and pathophysiology is continuously growing, as the number of diseases with an involvement of the IKK/NF-κB system steadily increases. IKK/NF-κB signaling has also been shown to be one of the key mediators in aging (150). As an example, hypothalamic programming of systemic ageing in mice depends on IKKβ and NF-κB (151). Intriguingly, IKKα and IKKβ also trigger a wide variety of NF-κB independent signaling events, which control various physiological functions and impact disease states like cancer and diabetes (Table 1). In parallel to the growing insight into its physiological relevance, enormous progress has been made in the mechanistic understanding of IKK/NF-κB regulation. Signal transmission to IKK is controlled by a complex network of distinct regulatory modules, many of which are not essential, but most likely assure a tailored response to individual stimuli or cell type restrains, or modulate the strength and duration of signaling. Nevertheless, there are major open questions, ensuring many interesting years to come in the field (see Sidebar A).

The recently reported IKKβ crystal structures mark an important step in the understanding of IKK function. Further insight can be anticipated from the complete structures of the

remaining subunits and of the holo-complex, as well as from studies of the composition and stimulus-dependent dynamics of the latter.

Ubiquitin-dependent processes are undoubtedly the major driving force of IKK activation. An outstanding issue in this regard is the precise validation of putative ubiquitination targets, which can be addressed through mass spectrometry and genetic complementation experiments, as recently demonstrated for NEMO (see above). In addition to the qualitative determination of ubiquitin-dependent processes, also quantitative analyses may aid to understand the individual impact of the different regulatory modules on IKK regulation. So far, IKK research has been focused on K63-linked and M1-linked ubiquitin chains. However, analysis of *Saccharomyces cerevisiae* revealed that ubiquitin linkages at all seven lysine residues contribute to an unexpected diversity in polyubiquitin chain topology and function (152). The biological relevance of these "unconventional" ubiquitin chains is underscored by the fact that individual DUBs have evolved distinct ubiquitin linkage specificities (153). *In vitro* studies have demonstrated that the proteasome can cleave polyubiquitin chains with diverse linkages with preferences following the order K48 > K6, K11, K33 > K27, K29, K63 (154). Thus, unconventional polyubiquitins could provide properties that are important for the temporal control of NF-κB signaling.

Although the NF-κB/IKK pathway is the target of long-standing and intense research, its pharmacological intervention in human disease is still very limited. Given that IKK is involved in numerous physiological processes, it will be important to target the specific upstream modules that control IKK activation in response to particular signals or conditions. However, the design of such a pathway-tailored therapy requires a complete and detailed knowledge of the mechanisms that control IKK activation.

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Conflict of interest

The authors declare that they have no conflict of interest.

Sidebar A: In need of answers

i. The structures of activated and resting IKK holo-complexes will provide important insights into the activation mechanism. Another important question is if different cell types - for example in adult or embryonic tissues - contain different subpopulations of IKK complexes with distinct compositions.

ii. How the IKK activation loop phosphorylation is mechanistically achieved, remains unclear. What are the functions of NEMO ubiquitination and ubiquitin binding in this process? How does the Hsp90/Cdc37 complex influence kinase activity?

iii. How are the specificities of IKK for its different substrates - of the NF-κB pathway or others - determined? What is the role of IKKα in the tripartite complex? Why is IKKα required in the non-canonical pathway?

iv. How is IKK activity regulated by diverse types of ubiquitin chains?

v. How dynamic is the IKK complex? How is the cytoplasmic IKK complex activated by modified NEMO after its nuclear export? Why do NEMO and IKKα, but not IKKβ, appear in the nucleus? How is IKKα recruited to specific gene loci?

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Figure1: Prototypic canonical NF-κB pathway. The activation of a cell surface receptor induces the recruitment and oligomerization of adapter proteins and E3 ligases such as TRAF6 or LUBAC, which in turn synthesize non-degradative Ub chains (K11, K63, and M1) attached to various adaptor proteins, or to themselves. Subsequently, TAK1 and IKK complexes are recruited to upstream ubiquitin-polymers via ubiquitin binding domains. After IKK complex recruitment, NEMO is modified with poly- and mono-ubiquitin, and IKKs are phosphorylated in their T-loop either by TAK1 or through trans-autophosphorylation. IKK activation results in phosphorylation of IκB and p65. IκB phosphorylation leads to its ubiquitin-mediated proteasomal degradation, enabling NF-κB dimers translocation to the nucleus, where they bind to DNA and induce transcription. In addition, IKKs phosphorylate a variety of other substrates, resulting in crosstalk with other, NF-κB independent regulatory systems in the cytoplasm and the nucleus.

Figure 2: Flow chart of the IKK signaling network. Cell-surface-receptors and stress response pathways trigger a dynamic regulatory network involving protein phosphorylation, non-degradative ubiquitination, adapter protein interactions and higher order oligomerization events to activate the IKK complex. Signaling is inhibited by deubiquitinases, phosphatases and IKK-mediated negative feedback regulation (coloured text). Enzymatic IKK activation, modulated by Hsp90-Cdc37 activity, may be achieved by trans-autophosphorylation and the action of IKK kinases (IKKKs). Viral proteins, such as Kaposi's Sarcoma-Associated Herpesvirus vFLIP, have been shown to activate the IKK complex through direct interaction. The IKK complex preferentially phosphorylates IκBs and NF-κBs but has also many other substrates. A subset of the TNF superfamily receptors (TNFSFR) activates the non-canonical pathway, which depends on NIK and selectively on IKKα and results in p100 processing.

Figure 3: The structure of IKKs. (**A**) Domain organization. (**B**) Crystal structure of *Xenopus* IKKβ. IKKβ was found as a dimer of dimers, a conformation with the potential to facilitate trans-autophosphorylation (29). (**C**) Crystal structures of NEMO fragments: N-terminal kinase-binding domain (HLX1) of NEMO in complex with IKKβNBD, HLX2 in complex with vFLIP, CC2-LZ in complex with linear di-Ub, ZF (37, 38, 40, 41, 49) (all crystal structures are from the database of the NCBI structure group; http://www.ncbi.nlm.nih.gov/Structure/index.shtml).

Table 1: Substrates and function of IKKα and β

IKKα dependent

Substrate	Affected molecular process	Biological function	References
Transcriptional regulation			
β-catenin	Interferes with ubiquitination mediated degradation and increases β-catenin-dependent transcription	Cell cycle regulation/cancer	(121-124)
CBP	Enhances NF-κB-dependent transcription	NF-κB dep. transcription ↑	(142)
c-Rel	Accelerates c-Rel turnover	NF-κB dep. transcription↓	(118)
ERα	Enhances estrogen receptor-mediated gene activation	Hormone response	(155)
IRF5	Inhibits TLR mediated interferon production	Inflammatory response	(156)
IRF7	Enhances TLR-mediated interferon production	Immune response	(157)
NCOA3	Enhances nuclear hormone receptor-mediated gene activation	Steroid hormone response	(155)
NF-κB2/p100	Induces processing of p100 into p52	NF-κB dep. transcription ↑	(57)
PIAS1	Represses NF-κB-dependent transcription	Restriction of inflammation	(158)
RelA/p65	Accelerates RelA turnover	NF-κB gene expression ↓	(118)
Unknown	Cofactor in TGFβ-Smad2/3 signaling	Keratinocyte differentiation	(112)
Unknown	Required for efficient induction of mTOR	Autophagy/growth control	(133)
Chromatin regulation			
Histone H3	Enhances NF-κB-dependent transcription	NF-κB dep. transcription ↑	(159-161)
SMRT	De-represses NF-κB target genes	NF-κB dep. transcription ↑	(138, 139)
Cell fate decision			
Cyclin D1	Triggers cyclin D1 degradation	Cell cycle regulation	(127)
p27/Kip1	Stimulates nuclear export of p27	Cell cycle regulation/cancer	(136)
Signaling			
Unknown	Suppress the serine protease inhibitor Maspin	Metastasis	(134)

IKKβ dependent

Substrate	Affected molecular process	Biological function	References
Transcriptional regulation			
FOXO3a	Promotes degradation of FOXO3a	Growth control/cancer	(147)
IκBs	Induces proteasomal degradation of IκBs	NF-κB dep. transcription ↑	(23)
NCOA3	Enhances NCOA3 nuclear import	Hormone response	(162)
NF-κB1/p105	Induces p105 proteolysis, resulting in Bcl-3-p50 Complex formation and TPL-2 activation	NF-κB dep. transcription ↑ and inflammatory response	(6, 163, 164)
NF-κB p65	enhances transcriptional activity	NF-κB dep. transcription ↑	(119)
Cell fate decision			
Bad	Primes Bad for inactivation	Cell survival	(128)
p53	Induces proteasomal degradation	Growth control/cancer	(145)
p85/PI3K	Interferes with Akt and mTOR inhibition after nutrient depletion	Autophagy/growth control	(132)
p85/S6K1	Activation of p85 S6K1upon oxidative stress	Apoptosis	(129)
Signaling			
14-3-3β	Dislocates TTP/14-3-3β from AU-rich elements	mRNA stability	(165)
Aurora A	Induces proteasomal degradation	Genome integrity	(166)
Bcl10	Attenuates TCR signaling	IKK feedback regulation	(95, 96)
β-catenin	Inhibits the β-catenin-dependent transcription	Cell cycle regulation	(123)
CARMA1	Facilitates CBM complex formation	IKK feedback regulation	(167)
CYLD	Inhibits deubiquitinase activity	IKK feedback regulation	(168)
Dok1	Inhibits Erk1 and ERK2 activation	Cell motility	(169)
IRS-1	Inhibits insulin signaling	Diabetes	(170)
NEMO	Modulates IKK structure	Feedback regulation	(93)
SNAP-23	Promotes exocytosis in mast cells	Immune response	(171)
TSC1	Activates the mTOR pathway and VEGF production	Growth control/cancer	(146)

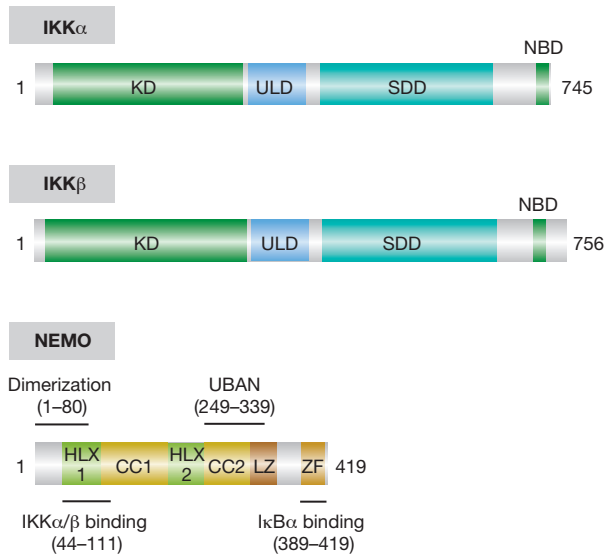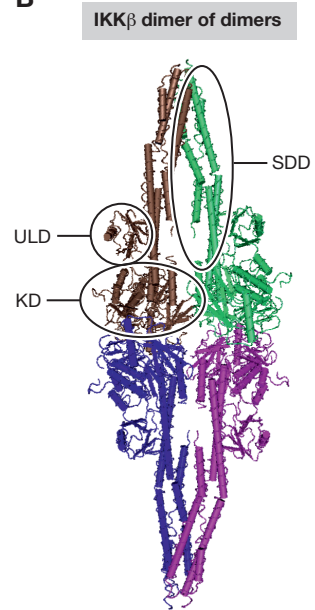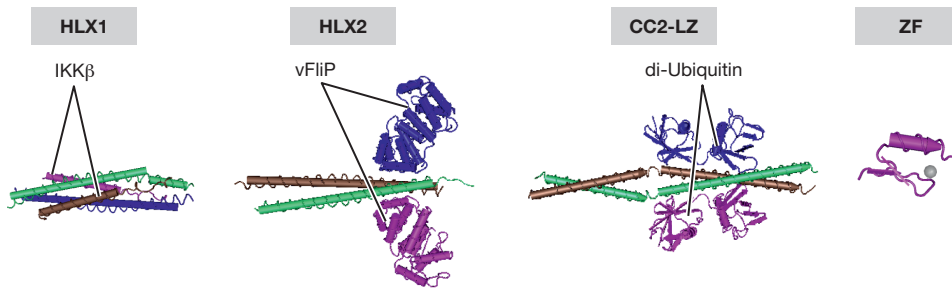**Table 2: Structural analysis of IKK subunits and their relatives**

Protein	Origin	Details	Resolution	Structural features	Ref
IKKβ (aa 4–675)	Xenopus laevis	Complex with inhibitors Cmpd1 or Cmpd2	3.6A°	KD + ULD + SDD; dimer ("a pair of shears")	(29)
IKKβ (aa 11–669)	human	Constitutively active mutant (S177E/S181E)	4 A°	see above; open conformations; permit higher order oligomerization	(28)
His-IKKβ (aa 1-664; HIS tagged)	human	Partially phosphorylated; bound to the staurosporine analog K252a	2.8 Å	see above; comparison of active and inactive kinase domains	(27)
NEMO (aa 44-111)	human	Hybrid complex containing NEMO 44–111 and IKK α/β peptides	2.25 Å	elongated and atypical parallel four-helix bundle	(37)
NEMO (aa 150-272)	human	Hybrid complex containing NEMO 150-272 and vFLIP 1–178	3.2A°	coiled-coil dimer	(38)
NEMO (aa 251-337 His-tagged)	mouse	Hybrid complex containing NEMO 251-337 and DARPin 1D5	2.95 Å	coiled-coil dimer	(40)
NEMO (aa 254–337)	human	NEMO 254-337 alone and in complex with di-ubiquitin	2.8/2.7 Å	coiled-coil dimer	(41)
NEMO (ZF-WT and ZF-C417F)	human	Solution structures determined by NMR	-	WT and mutant ZFs adopt a global ββα structure and bind zinc with comparable affinities; mutation causes instability	(49)
TBK1 (1–657; ΔCTD)	human	A: Complex with inhibitors MRT67303 and BX795 B: D135N mutant (kinase dead)	2.4/2.5 Å and 3,3 Å	KD + ULD + SDD; Dimer; extensive interactions between different domains	(32)
TBK1 (1–657; ΔCTD)	human	Complex of S172A mutant (inactive) or phosphorylated D135N mutant (active) with MRT67307/215 and BX795	2.6/4.0 Å	See above; Activation rearranges KD into an active conformation and maintains overall structure.	(31)

PRELIMINARY

Cell surface receptors
- Cytokines (TNFR, IL-1R)
- Antigens (BCR, TCR)
- Pattern recognition (TLR, NLR)

Stress response
- DSB
- Peptidoglycan (-> NLR)
- Viral RNA (-> RIG-I)

Non-canonical signalling
TNFSFR (BAFF-R, CD27, CD40, FN14, LTβR, RANK)

Oligomerisation
- MyD88/IRAK2/4
- MAVS
- TRAF6/Ubc13

Ubiquitination
- K11 chains
- K63 chains
- M1 chains

DUBs
- A20
- Cezanne
- CYLD
- OTULIN

IKKKs
- TAK1
- MEKK3

Phosphatases
- PP2A
- PP2C

- NIK
- cIAPs/TRAFs

Viral activation
e.g. vFLIP

Modulation
Hsp90/Cdc37

IKK
- NEMO ubiquitination
- NEMO dependent ubiquitin binding
- Trans-autophosphorylation

- Post-inductive attenuation
  (e.g. phosphorylation of NBD)

IKKα

Negative feedback

Chromatin regulators

Other transcription factors

Other signaling molecules

- IκBs
- p105
- p65
- c-Rel

p100

PRELIMINARY

A

IKKα

1 [KD | ULD | SDD] NBD 745

IKKβ

1 [KD | ULD | SDD] NBD 756

NEMO

Dimerization (1–80)   UBAN (249–339)

1 [HLX1 | CC1 | HLX2 | CC2 | LZ | ZF] 419

IKKα/β binding (44–111)   IκBα binding (389–419)

B

IKKβ dimer of dimers

SDD

ULD

KD

C NEMO fragments

| HLX1 | HLX2 | CC2-LZ | ZF |

IKKβ   vFliP   di-Ubiquitin

PRELIMINARY