

The physiological functions of IKKs-selective substrate identification and their critical roles in diseases

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ABSTRACT

The nuclear factor κ B (NF- κ B) transcription factors exert central hub functions in multiple physiological processes including immune response, cell survival, proliferation and cytokine production, which has naturally become the core of research almost in all aspects of biomedical science over 30 years. Since both the activation and termination of NF- κ B pathway are tightly regulated, little alteration can lead to excessive inflammatory responses and even result in tissue damage and severe diseases. The inhibitor of nuclear factor kappa-B (I κ B) kinase (IKK) complex is the main regulator of the NF- κ B signaling pathway, they mediate and deliver signals through phosphorylating certain substrates. In recent years, increased proteins have been identified to be targeted by IKK members and the particular modification mechanism becomes clear with the development of detecting techniques and structural biology. In this review, we summarize the known substrates of IKK family members either relevant or irrelevant to NF- κ B signaling, their structures and phosphorylation patterns, and the related physiologic and/or pathologic responses. Understanding the regulation of IKKs on their substrates may be helpful to connect IKKs with specific signaling pathways or physiological phenomena, and is essential for targeting IKKs in clinical research.

Keywords: NF-κB · IKKs · Substrates · Immune response · Disease

Brief introduction of NF-κB pathway and IKK families

The nuclear factor κB (NF- κB) family of transcription factors are structurally homologues that include NF- $\kappa B1$ p50, NF- $\kappa B2$ p52, RELA p65, RELB and c-REL, which exist inactive in the cytoplasm in a complex combined with members of the inhibitor of κB (I κB) family (1, 2). In response to multiple stimuli including numerous mediators in immune systems like tumor necrosis factor (TNF), lipopolysaccharide (LPS), interleukin-1 (IL-1) or T cell activators (3), I κB kinase (IKK) complex mediates NF- κB activating process by directly phosphorylating I κB , targeting it for proteasomal degradation (4). When released into the nucleus, NF- κB proteins regulate

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the expression of a large spectrum of genes encoding transcription factors, cytokines, adhesion molecules and immunoreceptors, thus to be essential for cell proliferation and survival, innate and adapt immune response and inflammation (5).

Signal-induced activation of NF- κ B pathway demands specific phosphorylation of I κ B proteins at Ser32 and Ser36 through the canonical IKK-dependent pathway (6). This IKK complex consists of two catalytic subunits IKK α and IKK β , together with the regulatory subunit NF- κ B essential modulator NEMO (also called IKK γ) (7, 8). The 85 kDa IKK α (previously known as CHUK), containing a protein kinase domain at its N-terminal half, and an elongated α -helical scaffold/dimerization domain (SDD) and a NEMO binding domain (NBD) at its C-terminal, is identified first by yeast two-hybrid screen for NF- κ Binducing kinase (NIK)-interacting proteins in 1997 (9, 10) (**Figure 1**).

By searching for IKK α -related kinases with an expressed sequence tag (EST) on NCBI database, the second

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component of the IKK complex is cloned and designated IKK β (11). Overall, the two subunits are 52% identical, while their kinase domain exhibit 65% identity (12). Both of their kinase domains have two serine residues (Ser176, Ser180 for IKK α and Ser177, Ser181 for IKK β), whose phosphorylation are required for the kinase functions (13, 14). A great structure difference between IKK α and IKK β locates in their ubiquitin-like domain. IKKß contains a key leucine at position 353 which is critical for IKKβinduced NF-kB activation, while deleting the equivalent region on IKK α has no impact on its activities (15). Moreover, there is a putative nuclear localization signal on IKK α but not IKK β , which possibly contributes to the reported functions of IKK α in the nucleus (16). Recent years, diverse combinations of the IKK components have gradually been observed, ΙΚΚα or ΙΚΚβ can respectively form homodimers either with or without the scaffold NEMO, however the predominant IKK complexes remain to be the 1:1:2 ratio combination of IKKα-IKKβ-NEMO (4, 17).

In addition to IKK α and IKK β , it has been reported that there exist two other non-canonical IKK-related kinases, TNF receptor-associated factor (TRAF) family memberassociated NF-kB activator (TANK)-binding kinase 1 (TBK1) and IKKε (IKKi) (18). TBK1 (also called NF-κBactivating kinase NAK), was initially amplified by PCR with primers containing the same sequences to IKKa and IKK β , and was first identified by virtue of its interaction with TANK in a yeast two-hybrid screening (19, 20). IKKE, which shares 64% homology to TBK1, was found by searching for proteins similar to IKKα and IKKβ in a database, and then isolated by using suppression subtractive hybridization technique in 1999 (21). These two kinases contain the same trimodular structure to IKK β and the N-terminal catalytic domain of IKK ϵ has approximate 30% similarity with that of IKK β (22) (Figure 1). In contrast to the constitutive expression of IKK α and IKK β in almost all cell types, the IKK ϵ protein is primarily observed in immune cells, with the highest expression in the spleen (21). TBK1 and IKKE also form homodimers and heterodimers adopting the similar assembly of the canonical IKK subunits, which require a scaffold protein to efficiently target their substrates, whereas TANK, (NAK-associated protein 1) NAP1 and SINTBAD (similar to NAP1 TBK1 adaptor) seem to fulfill this function (23, 24). Using phosphopeptide mapping and site-specific mutagenesis, it has been found that phosphorylation on Ser172 in the kinase activation loop of TBK1 and IKKE should be necessary for the kinase activity (25).

In spite of the sequence similarities and presence in common complexes, IKK α and IKK β or IKK ϵ and TBK1 maintain distinct substrate specificities and largely nonoverlapping functions. Pro-inflammatory stimuli like TNF α and IL-1, or Toll-like receptor (TLR) ligands like LPS mainly activate IKK β and NEMO for canonical NF- κ B pathway and innate immunity, whereas IKK α plays an ineffectual supporting role. In contrast, in response to CD40-ligand or B cellactivating factor (BAFF), IKK α primarily targets p100 for processing and exhibits its kinase activity and function in adaptive immunity and lymphoid organ development (26, 27).

IKK ε and TBK1 were initially described as NF- κ B- and IKK-activating kinases, whereas it is already clear that they are also important mediators of antiviral responses by activating two other transcription factors, interferon regulatory factor 3 and 7 (IRF3 and IRF7) (28-30). Additionally, TBK1 is reported to be involved in neuroinflammation and autoimmunity, and to be more essential than IKK ε in the innate immune response (31).

In recent years, numerous substrates in addition to I κ Bs and various NF- κ B-independent functions of the IKK family have been discovered. These substrates and functions encompass signaling pathways regulating tumorigenesis, inflammation and cell cycle, and provide a bridge for crosstalk between IKK-related signaling cascades with critical diseases. This review focuses on the kinase activities of four IKK members, highlights some of the major substrates targeted by IKKs either NF- κ B pathway-dependent or independent with the emphasis on the exact phosphorylation patterns and regulatory mechanisms. Understanding these post-translation modifications may shed light on exploitative capabilities of IKKs as effective therapeutic targets.

Substrates identification by IKKs and its physiological functions

IKK α /IKK β mainly regulates canonical and alternative pathways of NF- κ B

The pivotal function of the IKK complex is to phosphorylate IkBs, including classical IkB α , IkB β and IkB ϵ , and NFκB precursors p105 and p100 (17) (Figure 2). These IκB proteins share a tandem helical repeat motif called ankyrin repeat domain (ARD), which can functionally bind to the NF-kB dimers (2). A structurally flexible PEST domain exists between their C-terminal and the ARD, and it is abundant in proline (P), glutamic acid (E), serine (S) and threonine (T) (32). Besides, two critical serine residues on the N-terminal of I κ B α , β and ϵ serve as phospho-acceptor sites during canonical NF-KB pathway activation (the exact sites are Ser32 and Ser36 on IkBa, Ser19 and Ser23 on IkBβ, and Ser18 and Ser22 on IkBE) (8, 27, 33). Via adaptors like TRAFs, a variety of membrane receptors activate the canonical NF- κ B pathway. Once phosphorylated by IKK β , IκBs are recognized and ubiquitinated by SCF/β-TrCP E3 ubiquitin ligases, and then go through polyubiquitination and degradation (34, 35). Although IKK β phosphorylates unbound IkBs as well (36), NF-kB-bound IkBs are better substrates for phosphorylation (37). The degradation of IkBs releases the restraint of NF-kBs, the free NF-kBs thus go through nuclear transport and bind promoters of a wide range of target genes to function in numbers of NFκB-related cellular regulations.

As a non-classical IkB molecule, p105 is phosphorylated with the same mechanism as IkB α . But in contrast to the rapidly phosphorylation of IkB α , IKK β phosphorylates p105 with slow kinetics (38). In cells stimulated with NFkB-activating agents, IKK β directly phosphorylates Ser927



Figure 1. The structural comparison of the IKK family members. The principal structural motifs of the four members of IkB kinase (IKK) family are shown, together with amino acid numbers corresponding to the human proteins. ULD, ubiquitin-like domain; SDD, scaffold dimerization domain; LZ, leucine-zipper; HLH, helix-loop-helix; NBD, NEMO-binding domain.

in the PEST domain of p105 causing degradation of p105 via the ubiquitin-dependent proteasome pathway, however not affecting the p105 processing (39, 40). Furthermore, a functional p105 death domain is reported necessary for recruitment of IKK, with an additional requirement of Ser932 phosphorylation on p105 proteolysis (41, 42). In addition, p105 is also found to form a complex with the MEK kinase TPL2 in some inactivated cells. Following the degradation of p105, the released TPL2 activates the pro-proliferative mitogen-activated protein kinase (MAPK) signaling pathway (43). On the other hand, during the non-canonical NF-kB pathway activation, processing of p100 to generate p52 depends only on the IKK α , neither IKK β nor NEMO (44, 45). The processing pathway is triggered by different ligands, such as $LT\beta$ or BAFF, and further activated IKKa can phosphorylate Ser99, 108, 115, 123, 866 and 870 of p100, which leads to ubiquitination of p100 by SCF/ β -TrCP and then partial degradation of its ARD by proteasome (46, 47). Intriguingly, due to the slow activation kinetics of kinase NIK/IKKa, the degradation of p100 is also a relatively slow process. Unlike the classical IkBs which assemble with a single NF-kB dimer, p105 and p100 prefer to integrate two inhibitor proteins with at least two NF-kB molecules (48). Given the situation that p100 and p105 bind over half of cellular RelA, RelB and c-Rel, they could have profound impact on multiple NF-kB-mediated cellular processes (49).

In addition to p105 and p100, the phosphorylation of p65 conducts in the similar signaling pathways. The N-terminal Rel homology region of p65 is responsible for its association with $I\kappa Bs$ and NF- κBs , and also determines its nuclear

localization and DNA binding, while the transcriptional activation domain (TAD) at C-terminal determines its function as an activator of transcription (2). Several serine residues on the p65 TAD have been identified as phosphorylation sites and are targeted by distinct signaling pathways, the most extensively studied are Ser536, 468 and 276 (50). It has been reported that either induced by LPS or TNF, the inactive p65 in the cytoplasm is phosphorylated on Ser536 by IKK β , and is dephosphorylated rapidly in the nucleus (51-53). The Ser536 phosphorylation by IKK β is a key to potentiate p65 transcriptional activity, and it requires prior phosphorylation of IkBa at Ser32 and Ser36 as well as an intact NF- κ B/I κ B α complex (54). Additionally, more recent reports have shown that Ser468 in TAD2 of p65 is also phosphorylated by IKK β , and moreover, IKKα is found capable of phosphorylating p65 and c-Rel, which shows an opposite function compared to IKK β . Directly phosphorylated by IKKa on C-terminal, p65 and c-Rel go through accelerated turnover followed by quick detachment from target genes, resulting in the termination of NF-kB-associated transcription of critical genes (55). These discoveries reveal that selective inhibition of IKK α , which has little impact on $I\kappa B\alpha$, has remarkable effect on upregulating innate immunity by preventing the turnover of p65 and c-Rel, raising the novel therapeutic roles for IKK α inhibitors in the treatment of intricate infections.

Although no catalytic function of NEMO has been reported, this crucial regulatory component of the IKK complex is indispensable for signal-dependent activation of IKK β (56). NEMO is comprised of a C-terminal zinc-finger region, a leucine-zipper and two coiled-coil domains. The N-terminal region of NEMO interacts with



Figure 2. The substrates of IKK α and IKK β and the related functions. In addition to activation of the canonical and non-canonical NF-kB pathways through phosphorylation of the IkBs, IKK α and IKK β are now thought to phosphorylate a number of other substrates. And in turn, other than immune responses, IKK α and IKK β are also important signaling proteins for critical cellular processes associated with several diseases like cancer. For more information see text.

the IKK's NBD which is essential for IKK activation (17). Multiple phosphorylation sites have been identified on NEMO, however the precise model and the underlying molecular mechanism contributing to IKK activation are missing until the phosphorylation of NEMO by IKK β is found (57). *In vitro*, IKK β phosphorylates NEMO at Ser43, Ser68 and Ser85 within IKK binding domain, while only Ser68 phosphorylation exhibits physiological functions *in vivo*. The NEMO phosphorylation at position Ser68, which is located in the interacting region with IKK β , has negative effect on NEMO's dimerization as well as the NEMO-IKK β interaction, suggesting the negative regulatory function of phosphorylated NEMO upon the activation of the IKK complex and NF- κ B (58).

IKKβ phosphorylates tumor suppressors and promotes tumorigenesis

It is worth mentioning that IKK β is a commonly activated

oncogenic kinase in human cancers. It has been reported that IKKβ phosphorylates several tumor suppressor proteins, among which the most remarkable one is p53 (Figure 2). p53 contains the same phosphorylation motif $(D/A) \leq (G/L/D/R) (G/D/R) \times XS$ in its C-terminal as I $\kappa B\alpha$ does, and indeed, IKKB phosphorylates p53 at Ser362 and Ser366 within this motif. This DNA-damaging reagent-induced phosphorylation is recognized by β-TrCP-mediated ubiquitination and leads to proteasomal degradation of p53 (59). The resulting loss of p53 causes enhanced IKK β activity and impairs the glucocorticoidsdependent repression of NF-kB target gene transcription, and increases the rate of aerobic glycolysis (60, 61). Together, these findings suggest a positive-feedback loop exists during the IKK-NF-kB activation driven by glycolysis and provides evidence for the mechanism that tumor cells may damage normal inflammatory regulation in the tumor microenvironment.

Another two tumor suppressor proteins, Forkhead O transcription factor 3 (FOXO3) and tuberous sclerosis complex 1 (TSC1) are also identified as substrates of IKK β . We have known that, FOXO3 can suppress tumor growth and tumor size in breast cancer (62). IKK β , together with AKT/protein kinase B and extracellular regulated protein kinase (ERK) which are commonly activated in human cancers, can phosphorylate FOXO3a in response to insulin stimulation and growth factor. Phosphorylation of FOXO3a by IKK β occurs at Ser644, which triggers ubiquitination and subsequent proteolysis of FOXO3a. Such downregulation of FOXO3a in breast results in cell proliferation and tumorigenesis (63, 64).

A similarly interesting story is that, the TSC1/ TSC2 complex which acts as an inhibitor upstream of mammalian target of rapamycin (mTOR) pathway is also inactivated under the control of IKK β , AKT and ERK. In response to TNF, IL-1 β or a cardiovascular homeostasis controller angiotensin II (Ang II), IKKß mediates Ser487/ Ser511 phosphorylation of TSC1, accompanied by the phosphorylation of TSC2 by AKT and ERK. The phosphorylation results in the destruction of the TSC1/ TSC2 complex, and thus induces the oncogenic mTOR signaling, promotes tumor angiogenesis and ultimately leads to tumor development (65, 66). Furthermore, Ser511 phosphorylation of TSC1 is reported in multiple tumor types associated with vascular endothelial growth factor production, and is closely related to poor clinical outcomes in breast cancer (65).

Notably, in addition to IKK β , substrates regulated by IKK α also have crucial functions in a variety of inflammation-mediated tumorigenesis. The oncoprotein β -catenin and I κ Bs are both phosphorylated at the similar N-terminal serines, and in normal situation regulated by IKK β , β -catenin is subsequently targeted for ubiquitination and followed proteasomal degradation like I κ B (67). However, once phosphorylated by IKK α , β -catenin abundance increases. IKK α could directly phosphorylate β -catenin at Ser33 to stabilize β -catenin signaling, which stimulates the pro-proliferative cyclin D1 promoter. Knockdown of IKK α leads to downregulation of β -catenin expression, therefore results in multiple myeloma cell growth inhibition (68).

The observation of IKK α in the nucleus provides possibilities for its novel functions, the related substrates direct various mechanisms in inflammation and cancers. When it translocates into the nucleus, IKK α phosphorylates Ser2410 of SMRT (silencing mediator of retinoic acid and thyroid hormone receptor) and releases this repressor protein from Notch1 target gene, resulting in increased expression of Notch-dependent genes like *herp2/hrt1* and *hes1*, and the malignant transformation in colorectal cancer (69). Additionally, IKK α phosphorylates FOXA2 at Ser107/Ser111 in the nucleus to suppress its transactivation activity, leading to decreased expression of downstream targets such as NUMB, which act as Notch1 inhibitors, therefore help the proliferation of hepatocytes and tumorigenesis in hepatocellular carcinoma (70). p27/Kip1, the cyclin-dependent kinase inhibitor and a negative regulator during cell cycle G1-S transition, which has been reported involved in ErbB2induced mammary tumorigenesis, is also phosphorylated by IKK α at Ser12, Thr42, Ser175, Ser178 and Ser183. Intriguingly, the adjacent sequences are similar to known IKK phosphorylation sites and seem to be conserved in mammals. Phosphorylated p27 goes through nuclear export or exclusion, thus loses the suppressing effect on tumor-initiating cells (71). Actually according to current literature, the role of IKK α in tumor development differs within tissues. In colorectal, hepatocellular, breast and prostate cancers, IKK α acts as promoter of tumorigenesis, but inversely to be tumor suppressor in skin cancer and lung carcinomas (72).

Herein, dissection of the crosstalk between IKK α / IKK β and such tumor-related proteins provide new understanding of inflammation-related tumorigenesis. In all, inhibitors of IKK α /IKK β , AKT and ERK may work to reactivate tumor suppressors like FOXOs, TSC and p53, therefore developing therapeutic agents of the above multitarget may provide an efficient strategy for antitumor treatment.

IKKα and IKKβ play essential roles in immune response and immunological disorders

Type 1 interferon (IFNs) are a group of polypeptides constituting the first line of host immune defense, and infection by virus or double-strand RNA (dsRNA) leads to the activation of transcription factors of IRF family IRF3, IRF5 and IRF7, key activators of IFN genes and chemokine genes (73). IRF proteins consist of two major domains, a C-terminal activating domain along with an N-terminal DNA binding domain. The C-terminal autoinhibitory region is responsible for their activation in the cytoplasm, phosphorylation of critical serine and threonine residues stimulates the dimerization and nuclear transport, as well as the initiation of transcription (74).

IKKα has been reported critically involved in the TLR-MyD88-TRAF6 pathway-induced IFN production, and IRF5 and IRF7 are direct substrates of IKKα (Figure 2). Intriguingly, phosphorylation of IRF5 by IKKα attenuates transcriptional activation of IFNs (75), while in contrast, phosphorylated IRF7 serves as a positive regulator to manipulate TLR-induced IFN production (76). Multiple C-terminal serine residues of IRF5 are suggested to be phosphorylated by IKK α , phosphorylation then inhibits K63 linked ubiquitination of IRF5 and suppresses transcriptional activation of IFN genes (77). On the other hand, activation of ifna gene expression via IKKaphosphorylating IRF7 suggests that, IKKa could be a potential therapeutic target for certain autoimmune disorders whose IFN-a production is elevated, such as systemic lupus erythematosus (76).

In early-phase allergic response, IKK β participates in and regulates immune response independent of NF- κ B activation. Immunoglobulin E-activated IKK β phosphorylates substrate SNAP23 at Ser120 and Ser95, which in turn initiates membrane fusion and exocytosis of the SNARE complex of SNAP23/syntaxin4/VAMP2 for degranulation of mast cells (78, 79). The following release of LTC4, histamine and multiple biochemical mediators all triggers allergic inflammation which may lead to allergic diseases, for instance asthma, atopic rhinitis and dermatitis. NF- κ B activation is not responsible for mast cell degranulation, however, NF- κ B-dependent IKK β kinase activity induces proinflammatory cytokine secretion which in turn promotes late-phase allergic reactions (79). All of the above findings indicate the central regulator role IKK β plays in allergic reactions.

IKKα and IKKβ are double-edged swords in autophagy

In response to sub-lethal stress like cellular starvation, cells may utilize autophagy, a fundamental cellular process contributing to numbers of physiological functions in eukaryotic cells, to survive from nutrient depletion and maintain basal homeostasis (80). IKK is known to mediate amino acid starvation-induced autophagy via a mechanism independent of NF-kB by phosphorylating the phosphatidylinositol 3-kinase (PI3K) regulatory subunit p85 (Figure 2). When suffering nutrient deprivation, IKK phosphorylates p85 Ser690 at the conserved Srchomology 2 (SH2) domain, which results in reduced SH2phosphotyrosine interaction and subsequent blocking of Akt and mTOR signaling. The inhibition of mTORC1 is reported conducive to the initiation of autophagy (81). Another research suggests that c-Jun-N-terminal kinase 1 and AMP-activated protein kinase are also IKK substrates and phosphorylation on their specific serine/threonine residues could mediate starvation-induced autophagy. Interestingly, this pathway requires mTOR inhibition as well (82). Yet, studies of PTEN-inactive prostate cancer cells demonstrate that IKKa suppresses autophagy via mediating mTOR activation (83). Meanwhile, starvation also upregulates anti-apoptotic gene expression like Birc3 through activation of the NF- κ B signaling (84).

Altogether, these studies show the dual opposite roles of IKK against cellular stress through separate pathways: on the one hand, it could initiate or suppress autophagy in the absence of NF- κ B; on the other hand, IKK regulates anti-apoptotic gene transcription in a NF- κ B-dependent manner.

IKKs mediate insulin resistance in diabetes

Insulin resistance contributes importantly to type 2 diabetes mellitus, while many related factors like free fatty acids or TNF α , are also activators of IKK complex (85). Thus there rises the crosstalk between metabolism and inflammatory signaling in the development of insulin resistance. IKK complex phosphorylates Ser312 on insulin receptor substrate 1 (IRS1), thereby blocking out the IRS1 ability (86) (**Figure 2**). As we know, the metabolic actions of insulin are predominantly mediated by signaling involving the IRS family proteins (87). Furthermore, Ser307 phosphorylation of rat IRS1 has been reported to inhibit insulin stimulation of the MAPK or the PI3K cascades, suggesting an equivalent function of human IRS1 Ser312 phosphorylation (88). Besides, the

probable existence of extra IKK phosphorylation sites on the IRS family proteins implies that they may represent a novel group of substrates for IKK kinases (89).

Furthermore, insulin receptor (IR) is also a direct substrate of TBK1. It has been shown that TBK1 can phosphorylate IR on Ser994 to impair the activity of the receptor, and may negatively regulate insulin signaling and result in insulin resistance (90).

Taken together, these findings place the inhibition of such kinases as promising targets in the treatment of type 2 diabetes and related disorders.

TBK1/IKKε in NF-κB regulation

Like traditional IKK $\alpha/\beta/\gamma$, IKK ε can be induced in response to proinflammatory cytokines such as TNFa, LPS, and phorbol myristate acetate, and Ser172 of IKKE is essential for phosphorylation (21). However, IKKE has different substrate specificity and kinetics from IKKa and IKKβ (91). Activated IKKε specifically phosphorylates Ser36 of IkBa, but not Ser32, therefore it seems that phosphorylation of Ser36 may predispose IkBa towards Ser32 phosphorylation and subsequent degradation (21) (Figure 3). Mouse embryonic fibroblasts lacking IKKE or TBK1 exhibit normal NF-kB activity while they are incapable of inducing numbers of well-characterized NF-kB target genes, whereas phosphorylation of p65 is proposed to explain these defects (92). Quite a few serine residues on p65 have been identified as phosphorylation sites and are targeted by distinct signaling pathways. Besides IKKa and IKKB, upon IL-1 and T cell costimulation, IKKE also phosphorylates Ser468 and 536 of p65 to enhance its transcriptional activity. Ser536phosphorylated p65 is mainly found in the cytosol while Ser468 phosphorylation occurs predominantly in the nucleus (93, 94). A series of NF-kB regulated genes are largely dependent on IKKE-mediated p65 phosphorylation, such as Il6, Vcam1, IP10, Saa3 and Cox2 (95). In addition, as an oncogene expressed in numerous types of cancer cells, IKKE controls the basal/constitutive p65 Ser536 phosphorylation and plays vital roles in cancer cell survival and proliferation (96).

TBK1 and IKKε initiate tumorigenesis through phosphorylating Akt and induce transformation

As the crucial downstream effector in PI3K pathway, Akt is essential in normal cellular physiology like proliferation, survival and growth, while pathological Akt activation contributes to many human cancers (97). A common model for Akt activation is that, phosphatidyl inositol-dependent kinase-1 and mTORC2 separately phosphorylate Akt on its activation loop Thr308 and hydrophobic motif Ser473 (98, 99). Here is a report showing that IKKɛ/TBK1 is sufficient to phosphorylate both the activation loop and hydrophobic motif of Akt protein, and such activation can be induced by several growth factors, for instance platelet derived growth factor and epidermal growth factor (**Figure 3**). Additionally, PI3K signaling is required in this activation process although the IKKɛ/TBK1 activity is irrelevant to PI3K.



Figure 3. The substrates of TBK1 and IKKε and the related functions. Activated IKKε and TANK-binding kinase 1 (TBK1) modify numerous substrates and function in many biological processes, such as immune response, cell proliferation and survival, insulin signaling, and autophagy, some of which result in immune disorders and cancers.

This phosphorylation is required to sustain pathological oncogene-dependent Akt signaling and contributes to primary tumor initiation and development (100). Intriguingly, Akt activation diverges from PI3K in the TRIF (TIR domain-containing adaptor inducing IFN- β)dependent signaling, a cascade responsible for TLR4dependent IRF3 activation. In response to ligand stimulation, TBK1 cooperates with Akt to enhance Ser473 phosphorylation of Akt. In this context, Akt serves as a downstream component of the TRIF/TBK1 pathway to promote activation of IRF3 (101, 102). These discoveries indicate that pathological activation of TBK1 or IKK ϵ promotes tumorigenesis by activating AKT, at least partially so.

Ubiquitin carboxyl-terminal hydrolase CYLD, a member of the deubiquitinating enzyme family specific for K63linked poly-ubiquitins, acts as a tumor suppressor in familial cylindromatosis (103). CYLD suppresses a large number of inflammatory mediators including TRAF2, TRAF6, and NEMO by removing their poly-ubiquitins (104). However, this disruption disappears once CYLD Ser418 phosphorylation is induced by activated IKKs in a NEMO-dependent way, and the efficiency is comparable to the IkB phosphorylation by IKKs. Phosphorylated CYLD then inactivates its TRAF2 de-ubiquitination activity, promoting expression (105). Not long after, IKK ϵ is also identified to phosphorylate CYLD on Ser418 and seems much more efficient than IKK α or IKK β . Same as IKKs, IKK ϵ -mediated phosphorylation of CYLD suppresses CYLD activity and increases NF- κ B activation, and moreover, is necessary for IKK ϵ to fully induce transformation (106). These findings connect the oncogene IKK ϵ and the tumor suppressor CYLD, spell out the regulation of NF- κ B on cell transformation and lead to an increased understanding of how they function in oncogenesis.

TBK1 and IKKε regulate innate immunity by regulating IRF3 and IRF7 activity

In addition to IKK α , IKK ϵ /TBK1 is also involved in IFN- β induction through activation of IRF3 and IRF7 by direct phosphorylation on their C-terminal regulatory domain (107) (**Figure 3**). In the first step, TLR3 or TLR4 ligands like LPS and dsRNA recruit TRIF, which then connects to the TBK1/IKK ϵ complex through TRAF3 for subsequent IRF3 phosphorylation (28). However, TBK1/

IKKε do not seem to be required in TLR7-, TLR8- or TLR9-related IRF7 phosphorylation (108). There are three clusters of phospho-acceptor sites on IRF3, Ser385/ Ser386 (cluster1), Ser396/Ser398 (cluster2), and Ser402/ Ser404/Ser405 (cluster3). It has been shown that cluster2 and cluster3 are the first sites targeted by IKKε or TBK1 and then prime the targeting of cluster1, sequential phosphorylation is indispensable for the complete unfolding and full activation of IRF3 (109).

Besides TLR-dependent pathway, innate immune responses to viral pathogens or dsDNA separately rely on the melanoma differentiation-associated gene 5 (MDA5) and retinoid acid-inducible gene I (RIG-I) or DNAdependent activator of IFN-regulatory factors to trigger TBK1/IKKE-mediated IRF3 and IRF7 phosphorylation and IFN gene expression (110). The two caspase recruitment domains (CARDs) on N-terminal of both MDA5 and RIG-I are required for signal transmitting to the downstream CARD-containing adaptor protein VISA (111), while VISA has been demonstrated to be responsible for the activation of IRF3 (112). Researchers designate an uncharacterized protein as MITA, who is phosphorylated on Ser358 by TBK1 during viral infection, TBK1 and IRF3 are then recruited to VISA through this activated scaffold protein. Therefore, TBK1-mediated phosphorylation of MITA is closely associated to virustriggered IRF3 activation (113). Intriguingly during dsDNA stimulation, endoplasmic reticulum-resident adaptor protein STING acts as another scaffold to recruit IRF3 for TBK1-mediated IRF3 activation (114).

In addition to extra substrates, crystal structure reveals a potential trans-autoactivation domain of TBK1 existing to support the fully autoactivation and maintain the normal function of TBK1 (115). In this situation, glycogen synthase kinase 3β (GSK3 β) physically associated with TBK1, promoting the prerequisite dimerization or oligomerization of TBK1 for its Ser172 autophosphorylation, and leading to virus-triggered induction of IRF3, IFN- β as well as antiviral response (116).

TBK1 and IKKε-mediating STAT family phosphorylation crosslinks innate immunity and oncogenic pathways

Type I IFNs, like many other cytokines, utilize the canonical JAK (Janus kinase) - STAT (signal transducers and activators of transcription) pathway to function in innate immunity (117). Phosphorylation of STAT1 at Ser708 by IKK ε has been reported to disrupt STAT1 homodimerization, thereby enlarging the STAT1 pool for optimally assembling the crucial IFN-I transcription factor complex ISGF3, which subsequently results in the shift of GAS-driven type I IFN-induced gene expression to ISRE-driven gene expression (**Figure 3**). This well-characterized process implies a role of IKK ε in manipulating and balancing the IFN-I and IFN-II signaling pathways (118).

In addition, viral infections or cytosolic nucleic acids activate TBK1 to phosphorylate STAT6 at Ser407 or STAT3 at Ser754 in the transactivation domain, in a STING-dependent manner. However, phosphorylated STAT6 induces chemokines for recruiting immune cells to defend against viral infection, such as CCL2, CCL20 and CCL26, whereas phosphorylated STAT3 restricts its activity to respond to cytosolic DNA for target gene expression (119, 120). Given the well-established roles of STAT3 and STING in carcinogenesis and disease progression, these findings may shed light on the crosstalk between STAT-driven oncogenic signaling cascade and innate immune responses.

Phosphorylation of receptors by TBK1 contributes to selective autophagy

Canonical IKK members have been previously shown to control autophagy initiation, here it is reported that autophagic process is controlled by TBK1 (121). Autophagy receptors such as SQSTM1 (p62), optineurin (OPTN), NDP52 and NBR1, are involved in selective autophagy, whose function is to link ubiquitin cargoes to autophagosomal membranes as the ubiquitin signaling decoders (122). As the most closely related protein to NEMO, OPTN was identified as a substrate of TBK1 in two-hybrid screens (123) (Figure 3). TBK1 binds and phosphorylates OPTN on Ser177, Ser473 and Ser513. Ser177 phosphorylation in OPTN is known for ATG8 recruitment, while the dual phosphorylation of Ser473 and Ser513 activates polyubiquitin chain binding of OPTN, followed by the facilitated activation of TBK1 in vivo, as well as OPTN retention on damaged mitochondria, and mitophagy (124, 125). Within a positive feedback loop, ubiquitinated Salmonella or mitochondria recruits TBK1 and facilitates its clustering and activation, which in turn phosphorylates OPTN (126).

Earlier studies have suggested that SQSTM1 is recruited to mitochondria clusters to induce autophagosome of damaged mitochondria (127). However, subsequent observations have indicated that SQSTM1 mediates the aggregation of dysfunctional mitochondria through polymerization, but not for itself (128). Activated TBK1 phosphorylates SQSTM1 on Ser403, which is indispensable for its role in autophagic clearance and autophagosomal engulfment of polyubiquitinated mitochondria (129). Altogether, TBK1-mediated phosphorylation of autophagy receptors profoundly affects the regulation of TBK1 and selective autophagy pathway via a self-reinforcing positive feedback mechanism.

Due to its essential contribution to the suppression of excessive reactive oxygen species accumulation(130) and its participation in cellular death and senescence(131), autophagy is assumed to be a potent tumor-suppressive mechanism. It is worth further investigation that whether the IKK-dependent autophagy contributes to their tumor-suppressive function.

IKKε phosphorylates c-Jun to promote rheumatoid arthritis (RA)

Rheumatoid arthritis (RA) is a chronic autoimmune disease which is closely related to the transcription factor

NF- κ B (132). Surprisingly in fibroblast-like synoviocytes, c-Jun, a well-known regulator of matrix metalloproteinase (MMP) gene and activator of antiviral program (133), is identified as an efficient downstream target of IKK ϵ (**Figure 3**). Upon stimulation of IL-1 and TNF α , IKK ϵ immunoprecipitates rapidly and efficiently phosphorylate c-Jun, although the specific residues are still missing. The induction of MMP is significantly increased, which plays a key role in joint destruction in arthrtis (134). These intriguing data suggest IKK ϵ as a novel and potential therapeutic target in RA which links innate immunity, extracellular matrix destruction and cell recruitment.

Conclusions and perspectives

During the past three decades, research has gained great insight into the composition, activation, regulation and function of IKK members. The substrate spectrum of the IKKs exhibits far more than IkBs and precursors, which includes some other components in the NF-kB pathway and lots of extraneous proteins. While some excellent reviews focus on the activation and NF-kB-independent functions of IKK members (135), and non-conventional roles of specific NF-kB elements and the connection with other signaling pathways (84), in this review we have revised some of the well characterized substrates regulated by IKKs and emphasized the exact phosphorylation sites and modification patterns as well as their related function *in vivo*.

Despite the increasing identification of IKK-related substrates with diverse biological functions mediated by their phosphorylation, there still remains an important unanswered question: how substrate specificity is exactly achieved. Hoffmann et al. have demonstrated that NEMO functions as a scaffold targeting IKK to IkBs specifically and reduces alternate substrate phosphorylation within the inflammatory pathway (136). It offers us the idea that there must exist other scaffolds that may target IKKs towards alternate substrates and functions. Such scaffolds may totally replace NEMO and represent a new pathway determinant, or they may connect to IKKs together with NEMO and provide the crosstalk of inflammation with the other pleiotropic functions of IKKs. On the other hand, proteomic and immunofluorescence studies imply that TBK1 may use localization as a key specificity determinant, since its interactions with various adaptor proteins lead to different subcellular localizations and direct TBK1 towards diverse downstream pathways (137). To this end, co-recruitment and co-localization of kinases and substrates both offer to drive IKK substrate selection, and understanding the mechanism of IKKs' substrate specificity in different signaling axes may be critical for targeting IKKs in numerous diseases.

As our understanding of IKKs in mammalian physiology and pathophysiology is continuously developing, the IKK members involved human diseases like cancers and immune disorders also increase, however actually the pharmacological intervention targeting IKKs is still limited in clinical application. IKK β has been by far the major focus in developing selective inhibitors for therapy,

and in fact inhibitors targeting IKKB and NEMO have already been developed and put into preclinical studies, and inhibitors of TBK1 and IKKE also have been tested in several cell studies and animal models. However, due to the unwanted toxicity by interfering with systemic NF- κ B activities, none of them receive clinical approval (138), which get us thinking what kind of IKK inhibitors could make ideal drugs. The most important point is that it should only specifically intervene the given diseases or cancers with minimal effects on fundamental NF-KB functions in normal tissues. The numerous roles of IKKs in multiple pathways bring most of the complexity, because in different cells requiring NF-kB with diverse purposes, the outcomes vary. Secondly, the wanted inhibitors should be able to specifically and efficiently recognize its targeted IKK member, since the catalytic domains of four IKKs have significant similarity. Also, the dose, delivery method and delivery schedule which are related to clinical trials should all be taken into consideration. As for designing small molecule inhibitors, the structural differences on catalytic domains of IKKs and the exact phosphorylation sites of non-overlapping substrates targeted by different IKK members which are highlighted in this review may give a clue. Moreover, through comparing the effects of kinase inhibitors versus small molecule inhibitors, we could make it clear which functions are kinase-dependent, and the others perhaps are kinase-independent like scaffolding roles.

Regardless of the difficulties in the development of effective inhibitors targeting IKK members, the notable roles of IKKs in multiple signaling pathways and pathological processes we have presented in this review still indicate that these proteins are potential therapeutic targets for various diseases and malignancies. Additionally, with the developing structural and biophysical techniques, combined with the newly arisen single-cell computational studies, the latest research may shed light on the unsolved problems mentioned above.

Conflict of interest

The authors declare that they have no conflict of interest.

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