Expanding the View of IKK: New Substrates and New Biology

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The inhibitor of kappa B kinase (IKK) family consists of IKKα, IKKβ, and the IKK-related kinases TBK1 and IKKε. These kinases are considered master regulators of inflammation and innate immunity via their control of the transcription factors NF-κB, IRF3, and IRF7. Novel phosphorylated substrates have been attributed to these kinases, a subset of which is not directly related to either inflammation or innate immunity. These findings have greatly expanded the perspectives on the biological activities of these kinases. In this review we highlight some of the novel substrates for this kinase family and discuss the biological implications of these phosphorylation events.

IKK Family Kinases Have Pleiotropic Functions

The **inhibitor of kappa B kinase (IKK)** (see Glossary) family, considered master regulators of inflammation, was initially characterized when IKKβ was found to be the kinase responsible for the inducible phosphorylation of IκBα, which in turn leads to its ubiquitination and proteasomal degradation. Upon degradation, IκBα can no longer inhibit the **NF-κB** heterodimer p50-p65/RelA, allowing it to accumulate in the nucleus and drive transcription of NF-κB target genes [1–7]. In addition to IKKβ, the homologous kinase IKKα was found to be in the canonical IKK complex, while two other IKK-related kinases, **TBK1 and IKKε**, were found to be involved in innate immunity [8,9] (described in later sections). While roles for IKK in the NF-κB pathway have been extensively studied (for further review see [1–3]), it is now appreciated that IKK phosphorylates proteins not directly involved in NF-κB-mediated transcription and these substrates will be the focus of this review (Figure 1 and Table 1, Key Table) [10,11]. A recent search of Pubmed and PhosphoSite revealed approximately 50 phosphorylated substrates attributed to IKKα/β and many of these have been identified recently. These novel substrates implicate IKK in biological processes, including cell growth, metabolism, **apoptosis**, cell cycle, and cell migration and invasion. Since IKK is important in a variety of diseases (including cancer, pathogen infection, heart disease, obesity, and diabetes), analyzing the ability of IKK to control the phosphorylation of these substrates will be critical for a better understanding of these diseases.

NF-κB-Independent IKKα and IKKβ Substrates

The canonical IKK complex consists of two protein kinase subunits, IKKα and IKKβ, as well as a scaffold and substrate specificity factor known as IKKγ or NF-κB essential modulator (NEMO) [10,12]. The canonical IKK complex is activated by inflammatory cytokines, by activated pathogen-associated molecules, downstream of oncogenic factors (including mutant RAS), and by other signaling pathways [1]. In addition to the canonical IKK complex, a 'noncanonical' IKK complex, comprised of IKKα homodimers, activates the p52-RelB heterodimeric NF-κB transcription factor downstream of the **NF-κB-Inducing Kinase (NIK)** in response to stimuli such as CD-40 or RANK ligand [13] (Figure 1). IKKα and IKKβ are activated by phosphorylation of their activation loops, either by an upstream kinase, such as **TAK1** or MEKK3, or by trans-autocatalytic phosphorylation [10]. For other signals, such as in response to platelet-derived

Highlights

The IKK family is most well studied in the context of control of inflammatory gene expression, particularly in the regulation of NF-κB and IRF transcription factors.

While the major targets of IKK in regulation of NF-κB and IRF signaling have been extensively studied, IKK family members are now known to phosphorylate proteins not related to NF-κB- or IRF-dependent transcription mechanisms. Recently, a variety of novel IKK family-dependent phosphorylation events have been described. This aspect of IKK function, in our opinion, is currently underappreciated and is critical for understanding the biology of these kinases and understanding effects of IKK family-directed inhibitors.

The nuances of these regulatory mechanisms are especially important since aberrant regulation of IKK family kinases has been implicated in a wide range of human diseases, including cancer, obesity, diabetes, heart disease, and pathogen infection.

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growth factor, Akt phosphorylates IKKα, which is associated with the induction of IKK activity (although this mode of activation is likely cell-type specific) [14,15].

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While IKKα and IKKβ have similar activities and biological roles, their functions are not redundant, as evidenced by different phenotypes in the respective knockout mice [16]. In addition to their partial redundancy, the fact that IKKα and IKKβ function together in the same complex and the fact that IKKα and IKKβ crossregulate their activity [7] create challenges in parsing their respective roles (Box 1). For this review, we attribute phosphorylation sites to the kinase that the authors used in their respective manuscripts (if both kinases were assigned, we use 'IKKα/β').

Phosphorylation of a substrate protein by IKKα/β can have different effects. Frequently, phosphorylation by IKKα/β targets the protein for ubiquitination and proteasomal degradation, as is the case for IκBα. Other IKKα/β substrates that are targeted for proteasomal degradation upon phosphorylation include the mRNA stabilizing factor HuR [17], the ribonuclease Regnase-1 [18], the apoptotic modulator PUMA [19], the transcription factor **Foxo3a** [20], the guanine nucleotide exchange factor RAPGEF2 [21], and the cell cycle regulator **Cyclin D1** [22]. In select

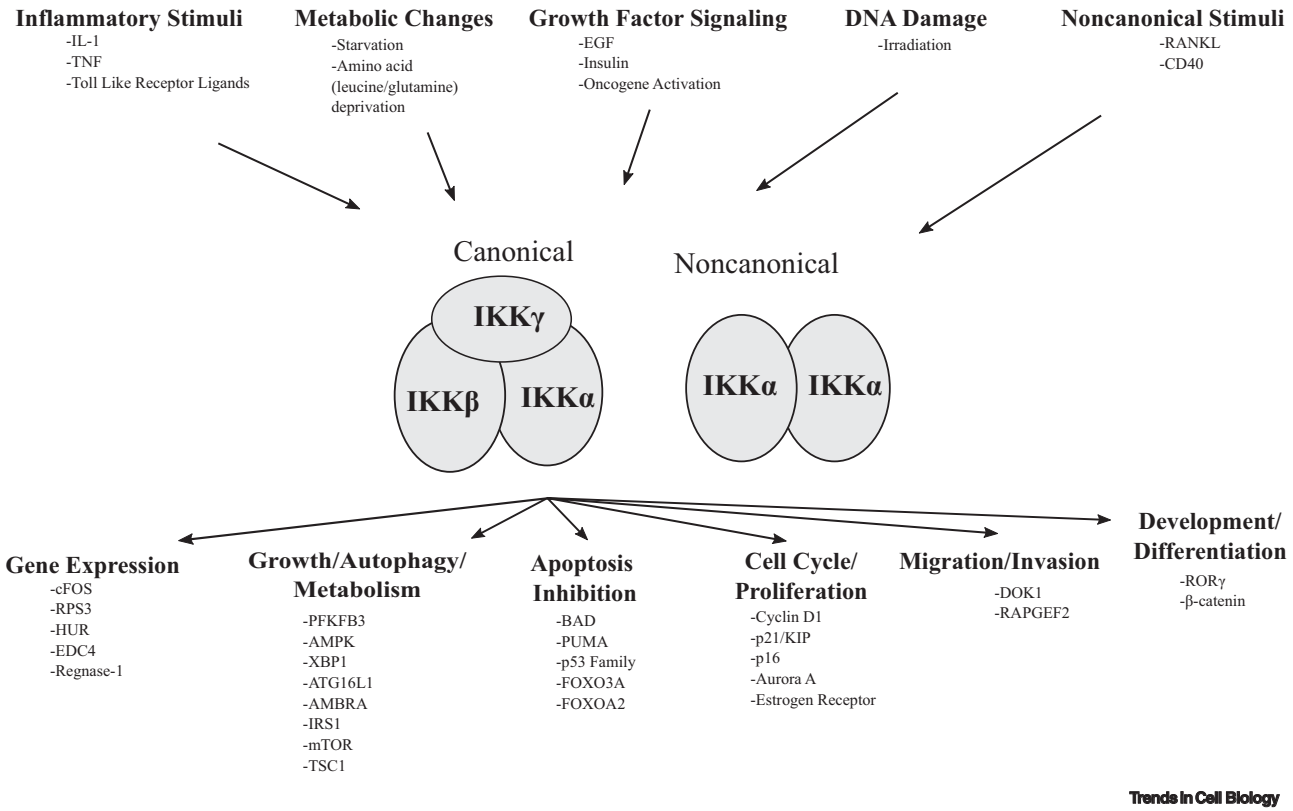

Figure 1. IKKα and IKKβ Are Pleiotropic Kinases. IKKα and IKKβ are primarily found in two distinct complexes. The canonical IKK complex consists of IKKα, IKKβ, and IKKγ (also known as NEMO) and the noncanonical complex consists of IKKα homodimers. The canonical complex was originally described to be activated by proinflammatory stimuli (interleukin-1β, tumor necrosis factor-α, and Toll-like receptor ligands) and the noncanonical complex by stimuli such as RANKL and CD40. Other stimuli, including changes in the metabolic state (such as leucine [44] or glutamine deprivation [26]) are also known to promote IKK activity. While originally described to activate canonical and noncanonical NF-κB, IKKα and IKKβ are now known to regulate a wide variety of biological processes through phosphorylation of non-NF-κB-related substrates, including regulation of apoptosis, gene expression, growth, and metabolism. Abbreviations: CD40, cluster of differentiation 40; IKK, inhibitor of kappa B kinase; IL-1β, interleukin 1β; NEMO, NF-κB essential modulator; NF-κB, nuclear factor kappa B; RANKL, receptor activator of NF-κB ligand; TNF-α, tumor necrosis factor alpha.

cases, such as for the **autophagy** protein Atg16L1 [23], phosphorylation by IKKα/β increases the stability of the target protein. IKKα/β can regulate the nuclear localization of its target, such as for the ribosomal protein RPS3 [24] or the transcription factor Foxo3a [20], and can regulate the enzymatic activity of its target, such as for the energy sensor **AMPK** [25] and the glycolysis regulator PFKFB3 [26] .

IKKα/β Promote NF-κB-Dependent Gene Expression

Upon stimulation by proinflammatory signals (e.g., TNF-α, IL-1β, and LPS), IKK promotes the expression of NF-κB-dependent genes. In addition to components of the NF-κB system, IKKα and IKKβ phosphorylate other transcription factors to modify promoter selection or activity of NF-κB and thus can fine-tune the signal for a given context. Examples of IKK substrates that act in conjunction with NF-κB to modulate gene expression include the transcription factor c-Fos [27] and the ribosomal protein RPSS3 [24].

IKKα/β also regulate gene expression through phosphorylation of RNA binding proteins that affect mRNA stability. IKKα phosphorylates HuR [17] and IKKβ phosphorylates EDC4 and Regnase-1 to regulate the stability of numerous mRNAs, especially cytokine-encoding transcripts [18,28].

IKK Regulation of Cell Growth, Autophagy, and Metabolism

Dynamically adapting to changes in the metabolic state is critical for the survival and optimal functioning of both individual cells and entire organisms. Broadly, cells sense and respond to changes in metabolic states by two mechanisms: direct binding of metabolites to sensors encoded within cells and, in multicellular organisms, by responding to extracellular cues. Examples of energy of sensors include the Ragulator and GATOR amino acid sensors and the ATP/ADP sensor AMPK [29–32]. Examples of extracellular signals include hormones and growth factors, such as insulin and epidermal growth factor (EGF). A key downstream effector of these extracellular signals is the PI3K/Akt pathway [33,34].

The metabolite sensors and the PI3K/Akt pathways exert their effects partly by regulating the mechanistic target of rapamycin (mTOR), a master switch between catabolism and anabolism [35]. When mTOR is activated, it promotes catabolic processes and inhibition of mTOR potently induces autophagy [35].

It has long been recognized that inflammation can lead to altered metabolic states. For instance, the inflammatory cytokine TNF-α, a potent IKK agonist, was also known as 'cachexin' because it induced an extreme catabolic wasting in cancer [36]. Since IKK is an important regulator of inflammation, it follows that IKK plays a critical role in regulating metabolism. Indeed early studies indicated that IKK induces insulin resistance *in vivo*, demonstrating that IKK plays a role in the crosstalk between inflammation and metabolism [37,38].

Mechanistically, IKK regulates metabolism, in part through NF-κB, and this has been reviewed elsewhere [39–41]. However, it is now appreciated that IKKα and IKKβ can modify growth and autophagy signals by direct phosphorylation of components of mTOR complexes, sensors of energy status that regulate mTOR (e.g., AMPK [25], XBP1 [42]), transducers of extracellular signals to mTOR (e.g., TSC1 [43], PI3K [44], IRS1 [45]), downstream effectors of mTOR, such as autophagy machinery (e.g., ATG16L1 [46], AMBRA1 [47]), and metabolic enzymes (PFKFB3 [26]).

In some contexts, IKK phosphorylation events promote the Akt/mTOR pathway and in other contexts inhibit Akt/mTOR [48]. To promote mTOR activity in response to acute TNF-α

Glossary

AMPK: AMP-activated protein kinase plays a key role in cellular energy homeostasis and the regulation of autophagy.

Apoptosis: a well-studied cell death mechanism, mediated by caspases. Proapoptotic factors, such as Bad, promote cell death and antiapoptotic factors, such as Bcl-2, suppress this response.

Autophagy: a regulated cellular mechanism that removes damaged and unnecessary cellular components via degradation and recycling.

Cyclin D1: a cyclin that is a regulatory subunit of the cyclin-dependent kinases CDK4 and CDK6, which are required for G1/S transition in the cell cycle.

Foxo3a: a member of the FOXO family of forkhead transcription factors that controls processes such as cell proliferation, apoptosis, and cell cycle progression.

Inhibitor of kappa B kinase (IKK): kinases involved in promoting NF-κB activity. Canonical IKK is comprised of IKKα, IKKβ, and NEMO (NF-κB essential modulator, a scaffold protein without kinase activity). Noncanonical IKK is a dimer of IKKα which regulates the noncanonical NF-κB pathway. The review provides a focus on activities of IKK that are not associated with regulation of NF-κB.

IRF3, IRF7: the transcription factors interferon regulatory factors 3 and 7 are activated in the innate immune response to promote transcription of type I interferon genes. Their activation occurs via TBK1 activation downstream of activated STING and MAVS.

NF-κB: a family of transcription factors (described in the text) involved in inflammation and immunity, but also associated with control of cell survival, cell proliferation, and other processes. NF-κB is dysregulated in a variety of diseases, including cancer.

NF-κB-Inducing Kinase (NIK): controls noncanonical NF-κB activation via the phosphorylation and activation of IKKα.

Optineurin: an autophagy receptor that shuttles cargo from the cytosol to the autophagosome and is related to the canonical IKK subunit NEMO.

TAK1: transforming growth factor-β activated kinase 1 (TAK1) is a member of the mitogen-activated protein three kinase (MAP3K) family and is also known as MAP3K7. In the canonical NF-κB

stimulation, IKKβ phosphorylates TSC1, a negative regulator of mTORC1 [43], and IKKα activated downstream of Akt directly phosphorylates mTOR [49]. In other contexts, IKKα/β phosphorylates substrates linked with inhibition of Akt/mTOR. IKKβ induces resistance to insulin-induced Akt activation through phosphorylation of IRS-1 [45] and IKKα/β promotes feedback inhibition of Akt in response to cell starvation by phosphorylating the p85 subunit of PI3K [44].

The fact that IKK can promote or inhibit mTOR may seem contradictory but could explain observations that inflammation, and correspondingly IKK, mediates diseases that manifest as extreme anabolism (e.g., cancer and obesity) and other diseases that manifest as extreme catabolism (e.g., cachexia). Understanding how IKK activity is directed towards procatabolic versus proanabolic substrates is an open area of investigation, but may involve NEMO [12] (see Outstanding Questions).

IKK Promotes Survival in Response to Metabolic Stress

IKK promotes cell survival during metabolic stress by phosphorylating substrates that favor energy conservation and recycling of metabolites. Cells sense energetic stress via the kinase AMPK, a critical sensor of the ATP/ADP balance in cells that promotes autophagy and other energy conserving processes when activated. IKKβ was shown to phosphorylate AMPK at its activating residue in response to cytokines (TNF-α and IL-1β) and in cancer cells and this promotes cancer cell survival in response to energetic stress induced by phenformin [25]. These experiments were performed in cells lacking the tumor suppressor LKB1, the major activating kinase for AMPK, and future studies should determine whether this can occur in contexts where LKB1 is expressed. This was also interesting as TAK1, which is also an activating kinase for IKK, was previously shown to activate AMPK in response to cytokine stimulation [32]. The study on IKK and AMPK [25] now implicates IKK downstream of TAK1 in cytokine-induced AMPK activation. In response to glutamine deprivation, IKKβ favors survival by phosphorylating PFKFB3, an enzyme that converts fructose-6-phosphate to fructose-2,6-bisphosphate, to inhibit aerobic glycolysis and conserve TCA cycle intermediates for reactive oxygen species clearance [26]. IKKα stabilizes the autophagy protein ATG16L1 through phosphorylation and phosphorylates AMBRA1 to promote mitophagy [46,47]. In addition to situations of nutrient depletion, IKKβ was also shown to improve glucose homeostasis during metabolic stress induced by excessive caloric intake by phosphorylating XBP1, a transcription factor that promotes the expression of genes involved in endoplasmic reticulum stress and homeostasis [42].

IKK Suppresses Apoptosis

Promoting the transcription of antiapoptotic proteins is a well-established function of IKKβ within the canonical NF-κB pathway [1,50]. To complement this role, IKKα/β also inhibits the activity of the proapoptotic proteins Bad, by phosphorylation of murine Ser26 [51] (a site not conserved in humans) and PUMA at Ser10 [19]. Both of these phosphorylation sites were described in the context of TNF-α-induced signaling and it will be important to determine whether these sites are phosphorylated in diseases, such as cancer, where IKK is constitutively activated and where inhibition of apoptosis is an important oncogenic mechanism.

IKKα and IKKβ also inhibit proapoptotic gene expression by phosphorylating and suppressing proapoptotic transcription factors, including forkhead family transcription factors [20,52] and p53 and its homologs, p63 and p73 [53–55]. We note that IKK regulation of p53 and its homologs is complex and not entirely understood and while in some contexts IKK may promote p53 activity, IKK is generally thought to suppress p53 family proapoptotic transcriptional activity. IKKα also phosphorylates the transcription coactivator CBP to switch its binding preference from p53 to NF-κB, which promotes antiapoptotic gene expression [56,57].

pathway, it phosphorylates IKKβ to activate its activity towards IκBα and other substrates.

TBK1 and IKKε: members of the IKK family that have been heavily studied in the innate immune response pathway. New substrates for TBK1/IKKε and regulation of distinct cellular processes are described in the review.

Key Table

Table 1. List of Noncanonical IKKα/β Substrates Highlighted in this Review

Biological process	Kinase	Gene	Species	Site(s)	Treatments *in vitro*	Animal models	Effect	Refs
Gene expression	IKKβ	c-FOS	Human	S308	LPS, cAMP		Stabilizes protein, promotes inflammatory cytokine expression	[27]
	IKKβ	RPS3	Human	S209	TNF-α	*E. coli* piglet Infection	Nuclear localization, NF-κB specifier	[24]
	IKKα	HuR	Human	S304	Glycolysis inhibition, glucose deprivation		Destabilizes the protein	[17]
	IKKβ	EDC4	Human	Multi	TNF-α, IL-1β, irradiation		Enhances formation of P-bodies	[28]
	IKKβ	Regnase-1	Mouse	S439	TNF-α, IL-1β, LPS		Promotes degradation of Regnase-1	[18]
Growth/autophagy/ metabolism	IKKα	mTOR	Human	S1415	Insulin		Modulates raptor binding, activates mTOR	[49]
	IKKβ	TSC1	Human	S511, S487	TNF-α, angiotensin II	Breast cancer xenograft	Destabilize TSC1, activates mTORC1	[43]
	IKKα/β	p85/PI3K	Mouse	S690	Starvation, LPS, TNA-α	Fasted mice	Inhibits Akt	[44]
	IKKα/β	IRS-1	Human	S307, S312	TNF-α, calyuclin A		Inhibits Akt	[45]
	IKKα	Atg16L1	Mouse	S278	TNF-α	DSS-induced colitis	Prevents caspase cleavage of Atg16L1	[23]
	IKKβ	AMPK	Human	T172	IL-1β, TNF-α		Promotes AMPK activity	[25]
	IKKβ	PFKFB3	Human	S269	Glutamine deprivation	Fibrosarcoma xenograft	Inhibits activity, metabolic adaptation to glutamine starvation	[26]
	IKKβ	XBP1	Mouse	S148		Obese mice	Stabilizes and increases activity of XBP	[42]
	IKKα	AMBRA	Human	S1043	Ischemia		Promotes mitophagy	[47]
Apoptosis	IKKβ	FOXO3a	Human	S644	TNF-α	Breast cancer xenograft	Induces degradation, mimics Akt activation, nuclear localization	[20]
	IKKα	FoxA2	Human	S107, S111	TNF-α	Liver cancer xenograft	Suppresses transactivation activity	[52]
	IKKβ	Bad	Mouse	S26	TNF-α	TNF-α-induced mortality	Inhibits BAD activity	[51]
	IKKα/β	PUMA	Human	S10	Serum, IL-3		Destabilizes PUMA	[19]
	IKKβ	p53	Human	S363, S366	Doxorubicin		Destabilizes p53	[53]
Cell Cycle/Proliferation	IKKα	Cyclin D1	Human	T286	Serum		Subcellular localization, stabilizes protein	[22]
	IKKα	p21/KIP	Mouse	S175		Breast cancer orthotopic xenograft	Stimulates nuclear export	[59]
	IKKα	Aurora A	Human	T288			Promotes activity	[61]
	IKKα	ER-α	Human	S118	Estradiol		Promotes ER-dependent transcription	[58]
Migration/invasion	IKKβ	RAPGEF2	Human	S1254	HGF	Breast cancer metastasis (zebrafish)	Promotes degradation leading to migration/invasion	[21]
	IKKβ	DOK1	Human	Multiple	TNF-α, IL-1β, IR		Inhibits activity	[62]

Table 1. (continued)

Biological process	Kinase	Gene	Species	Site(s)	Treatments *in vitro*	Animal models	Effect	Refs
Development/differentiation	IKKα	ROR-γ	Mouse	S376	Th17 differentiation media		Promotes ROR-γ-dependent gene expression	[63]
	IKKα/β	Beta-catenin	Human	Multiple	TNF-α		IKKα inhibits and IKKβ promotes beta-catenin transcription	[64]

IKK Promotes Proliferation through Cell Cycle Progression

The role of IKK in cell cycle progression is most well-described in the context of cancer, where aberrant IKK activation contributes to cancer proliferation. In breast cancer, IKKα phosphorylates the cell cycle regulator Cyclin D1 [22] and the estrogen receptor-α [58] to enhance the expression of genes with estrogen response elements, including Cyclin D1. IKKα also phosphorylates p27/Kip, a tumor suppressor, in breast cancer to promote its nuclear exclusion in self-renewing cell populations and this nuclear exclusion allows for proliferation of this important population of cells [59]. IKKα, but not IKKβ, phosphorylates Aurora A to promote its activity and to drive progression through M-phase [60,61]. IKKβ also promotes proliferation by phosphorylating and inhibiting Dok1, a receptor tyrosine kinase-associated protein that promotes feedback inhibition of proliferation induced by receptor tyrosine kinase activation [62].

Other Substrates

IKK phosphorylates the Retinoic Acid Related orphan receptor gamma (RORγ) to regulate Th17 differentiation [63]. IKKα and IKKβ both phosphorylate β-catenin, but have opposing effects on beta catenin-mediated gene transcription, offering an example of where canonical and non-canonical IKK complexes may oppose each other [64]. IKK promotes invasion and migration by targeting RAPGEF2, a guanine exchange factor that negatively regulates the promigratory protein Rap1, for degradation and phosphorylation of DOK1 [21,62].

The IKK-Related Kinases TBK1 and IKKε

TBK1 and IKKε are members of the IKK family, best known for their critical roles in innate immunity. TBK1/IKKε are activated in response to the engagement of pattern recognition receptors (PRRs) and promote the expression of genes to prevent pathogen replication by phosphorylating or controlling key transcription factors (**IRF3**, **IRF7**, STAT1, and p65/RelA) [8,9]. Most critically, TBK1 and IKKε were initially identified as the kinases primarily responsible for expression of type I interferons (IFNs), secreted proteins that orchestrate broad antiviral and antipathogen gene expression programs [8,9]. As with IKKα/β, distinguishing between the roles of TBK1 and IKKε can be a challenge. A number of publications refer to 'TBK1/IKKε' activity without distinguishing whether both kinases are involved in a particular mechanism. In terms of their regulation, both TBK1 and IKKε are activated by activation loop phosphorylation (S172) and are brought into proximity to their substrates to induce target phosphorylation [8,9]. An additional layer of regulation exists for IKKε, expression of which is inducible, whereas TBK1 is constitutively expressed in most cell types [65]. While these two kinases can have redundant activities, there are cases of substrate specificity. In a recent example focused on VHL-deficient renal cancer, knockdown of TBK1, but not IKKε, inhibited oncogenesis (Figure 2) [66]. p62/SQSTM1 phosphorylation (Box 2), which was shown to be important for TBK1 oncogenic activity in this setting, is controlled by TBK1 and not IKKε in renal cancer cells, as shown in cell-free assays.

TBK1 and IKKε in Innate Immunity

As with IKKα/β, the view of the functions of TBK1/IKKε has expanded with the discovery of novel substrates (Table 2). Within the innate immune response, several transcription factors

Box 1. Defining IKK Substrates

Identifying *bona fide* IKK substrates is challenging. Several early studies using phosphorylation of combinatorial peptide libraries *in vitro* predicted that IKKα/β have a preference towards leucine at the +1 position relative to the target serine or threonine [106]. However, unlike many other kinases, the surrounding amino acid sequence is not a strict requirement for IKK phosphorylation. Indeed, we found that only around half of the substrates currently attributed to IKKα/β match the prediction of having a leucine at the +1 position relative to the target serine. Absent a preferred sequence motif, IKK substrates are often discovered using hypothesis-driven or proteomic approaches. Generally, a substrate is defined as an IKK target if IKK phosphorylates the protein in cell-free kinase assays, IKK inhibition decreases the phosphorylation *in vivo*, and if IKK agonists (often TNF-α and IL-1β) promote phosphorylation of the site. Where IKKβ is mostly cytoplasmic, IKKα can be found in the nucleus as well as in the cytoplasm, thus IKKα is known to phosphorylate nuclear proteins whereas IKKβ does not. We note that a significant portion of phosphorylation sites described in this review have only recently been attributed to IKK (or TBK1/IKKε) and thus the level of evidence supporting the role of IKK in regulating each site is limited (see Outstanding Questions).

(other than the classic substrates mentioned earlier), including STAT3 [67] and STAT6 [68], have been shown to be TBK1 substrates. TBK1/IKKε also phosphorylate proteins to relieve negative regulation of innate immune gene expression. For example, IKKε promotes lysosomal degradation of yes-associated protein (YAP), a negative regulator of IRF3 dimerization and activation, by phosphorylation of YAP S403 [69], and IKKε phosphorylates fas-associated factor 1 (FAF1)

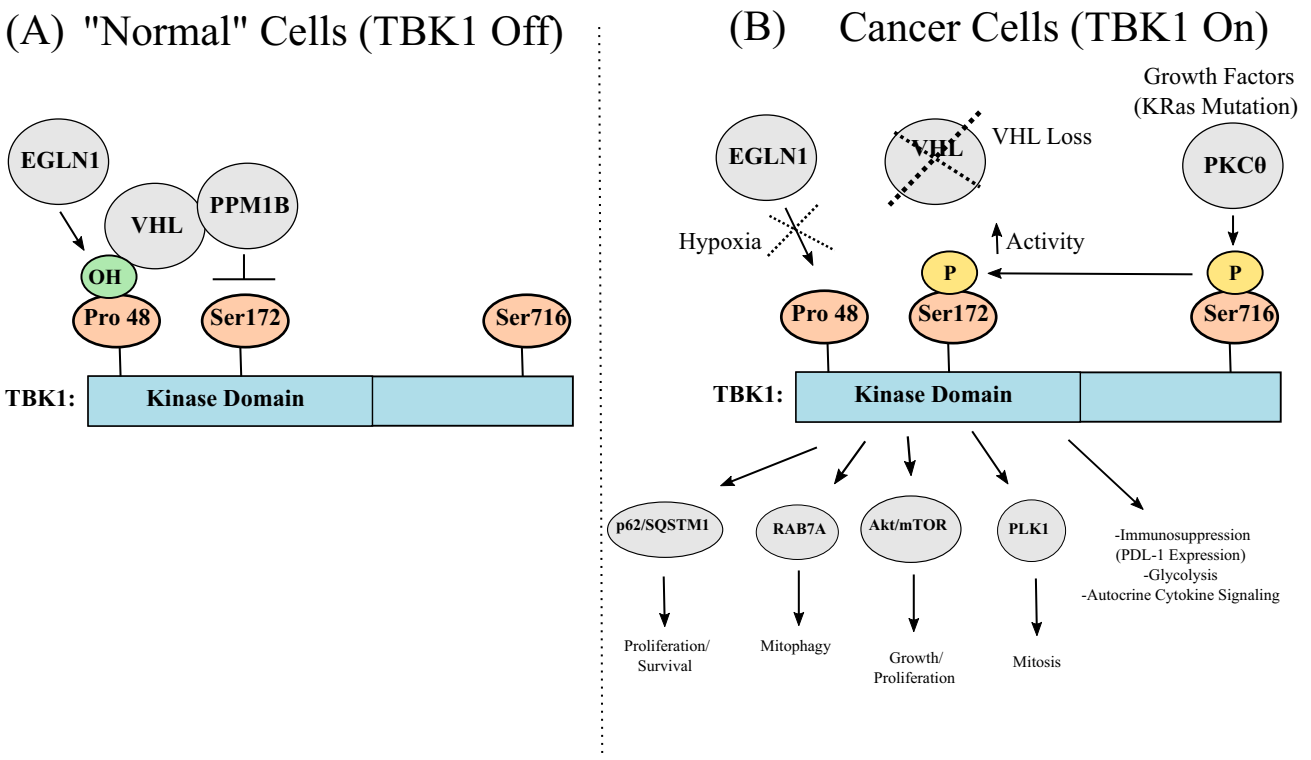

Figure 2. TBK1 in Cancer. While TBK1 has been described as playing a role in cancer, the molecular mechanisms of how TBK1 is activated to promote cancer have only recently begun to be appreciated. In a recent manuscript, it was shown that in renal cells expressing the tumor suppressor VHL, EGLN1 hydroxylates proline 48 on TBK1. This hydroxylation leads to the association of TBK1 with VHL, where the phosphatase PPM1B promotes dephosphorylation of TBK1 serine 172, the active site serine associated with TBK1 kinase activity (A). Under hypoxic conditions, or in renal cancers with loss of VHL, TBK1 is no longer inhibited and phosphorylates p62/SQSTM1 to promote the oncogenic phenotype [66]. In some cancer cells, growth factors and oncogenic mutations (such as KRas) lead PKCθ to phosphorylate TBK1 serine 716, which in turn promotes TBK1-mediated auto-phosphorylation of serine 172 [109]. Relieving a negative repression on TBK1 and promoting active site phosphorylation lead to constitutive TBK1 activity, which in turn promotes the phosphorylation of various pro-oncogenic substrates, including: p62/SQSTM1, RAB7A, Akt, p70S6K, and PLK1 (B). Abbreviations: EGLN1, Egl nine homolog 1; PKCθ, protein kinase C theta; PLK1, Polo-like kinase 1, PPM1B, protein phosphatase 1b; RAB7A, Ras-related protein Rab7a; SQSTM1, sequestome 1; TBK1, TANK binding kinase 1; VHL, von Hippel Lindau.

Box 2. TBK1 and IKKε in Cancer

There are numerous reports regarding the involvement of TBK1 and IKKε in cancer (see Durand *et al*. [107]). An early study linked loss of TBK1 with synthetic lethality in certain KRAS+ cancer cell lines [108] and a recent study showed that TBK1 is important for KRas-induced tumor growth using a genetically engineered mouse model [109]. TBK1 and potentially IKKε phosphorylate RAB7 at S72 [82,110]. In breast cancer cells this led to STING stabilization and enhanced innate immune gene expression. These cells were hyper-responsive to STING agonists, which enhanced cytotoxicity and recruitment of T cells *in vitro*.

As noted in the main text, a recent report demonstrated that VHL-loss in renal cancer activates TBK1 and that TBK1 is important for the oncogenic phenotype in VHL-deficient ccRCC [66]. This group identified S366 on p62/SQSTM1 as a TBK1 phosphorylation site and demonstrated that this phosphorylation event is important for stabilization of p62 and cancer cell proliferation/survival (this phosphorylation site is distinct from the site on p62 shown to be involved in autophagy/mitophagy) (Figure 2).

Table 2. IKKε and TBK1 Substrates Highlighted in This Review

Biological process	Kinase	Gene	Species	Site(s)	Treatments *in vitro*	Animal models	Effect	Refs
Innate immunity	TBK1	STAT3	Human	S754	dsDNA, STING agonist		Inhibits STAT3-dependent transcription	[67]
	TBK1	STAT6	Human	S407	Sendai virus	Viral infection	Promotes STAT6 transcription	[68]
	TBK1/IKKε	YAP	Human	S419	Sendai virus		Targets YAP for lysosomal degradation	[69,111]
	IKKε	FAF1	Human	S556	Viral infection	Viral infection	Promotes FAF1 degradation	[70]
Autophagy/mitophagy/ bacteriophagy	TBK1	p62/SQSTM1	Human	S403, S366	IL-1β, mycobacteria		Promotes autophagy, required for cancers with VHL loss	[66]
	TBK1	Syntaxin 17	Human	S202	Starvation		Controls assembly of pre-autophagosomal structures	[72]
	TBK1	Optineurin	Human	S473, S513	Mitochondrial uncoupling		Promotes ubiquitin binding	[79]
	TBK1	LC3C	Human	S93, S96	Mitochondrial uncoupling		Inhibits cleavage by ATG4	[73]
	TBK1	GABARAP-L2	Human	S87/88	Mitochondrial uncoupling		Inhibits cleavage by ATG4	[73]
	TBK1	SMRC8	Human	S402, T796	mTOR inhibition		Promotes autophagosome formation	[74]
	TBK1/IKKε	RAB7A	Human	S72	Mitochondrial uncoupling, STING agonists		Promotes mitophagy, promotes cytokine expression	[82,110]
mTOR	TBK1/IKKε	mTOR	Human	S2159	Poly (I:C), LPS, EGF		Promotes activity	[91]
	TBK1	Raptor	Human	S877	LPS	LPS (spleen)	Limits mTORC1, regulates cell size	[87]
	TBK1	p70S6K	Human	T421/S424	Amino acids		Promotes activity	[92]
	TBK1/IKKε	Akt	Human	S473, T308	KRas transformation, insulin, EGF		Promotes activity	[88,89]
Energy homeostasis	TBK1	AMPKα1	Human	S459, S476	TNF-α	High-fat diet	Inhibits activity	[96]
	TBK1	Exo84	Rat	Multiple	Insulin		Inhibits interaction with RalA, promotes Glut4 translocation	[99]
Mitosis	TBK1	PLK1	Human	T210	Nocodazole		Promotes mitosis	[103]
	TBK1	CEP170	Human	Multiple	Nocodazole		Promotes mitosis	[104]
	TBK1	NUMA	Human	Multiple	Nocodazole		Promotes mitosis	[104]

S556 to overcome the negative effect of FAF1 on MAVS (a pathogenic RNA sensor) [70]. Notably, TBK1 (and likely IKKε) has highly cell type-specific functions in innate immunity. While TBK1 promotes type I IFN expression downstream of PRRs in most cell types, in dendritic cells TBK1 is dispensable for IRF3 activation and paradoxically limits type I IFN expression [71].

TBK1 and Autophagy: Roles for p62 and Optineurin

While promoting gene transcription as part of an innate immune response, TBK1 also promotes pathogen clearance by regulating selective autophagic degradation of intracellular pathogens. A recent series of publications demonstrated that TBK1 promotes the formation of autophagosomes by phosphorylating Syntaxin 17, SMRC8, LC3C, and GABARAP-L2 [72–74]. To promote selective autophagy in response to the proinflammatory cytokine IL-1β, TBK1 phosphorylates p62 at S403 in the ubiquitin-associated domain, which promotes binding with K48- and K63-linked ubiquitin chains, leading to autophagosome maturation and autophagic clearance of intracellular mycobacteria [75]. Phosphorylation of **optineurin** (Ser177), an autophagy receptor, by TBK1 was reported to promote selective autophagy of cytosolic *Salmonella* through enhanced LC3 binding [76]. While the assembly of ubiquitin chains on mitochondria drives the recruitment of autophagy adapter proteins, it also leads to the activation of TBK1.

The mechanisms by which TBK1 induces degradation of intracellular bacteria are also used to target organelles for degradation, particularly damaged mitochondria, through a process called mitophagy. This may not be surprising, as mitochondria are thought to have evolved from bacteria. However, there are important differences in the way that damaged mitochondria and bacteria are sensed by TBK1, which may prevent an inappropriate trigger of innate immune gene expression during mitophagy. Damaged mitochondria are sensed by the kinase PINK1 and the ubiquitin ligase PARKIN. For damaged mitochondria, PINK1 drives PARKIN to the outer membrane of mitochondria to facilitate ubiquitination of key resident proteins [77,78]. These ubiquitinated proteins are shuttled to the lysosome for degradation by autophagy receptors optineurin, NDP52, TAXBP1, and p62. These autophagy receptors have significant affinity for the ubiquitinated mitochondrial proteins when phosphorylated by TBK1 [79]. (IKKβ can also regulate optineurin but this phosphorylation site is most often associated with TBK1 [80,81]). Additionally, TBK1 phosphorylates RAB7A at Ser72 to promote mitophagy and this response requires PARKIN [82].

Similar to its effects on mitochondria, TBK1 also promotes degradation of STING (which is activated downstream of the cytosolic DNA sensor cGAS) as a feedback mechanism to limit IFN production, using a similar mechanism of p62 phosphorylation to promote its binding to ubiquitinated STING [83,84].

TBK1 and mTORC1

In addition to directly regulating the process of autophagy, TBK1 also regulates mTORC1, a major regulator of autophagy (see earlier text). Several studies have reported that TBK1 regulates mTORC1, although some studies indicate positive regulation while others indicate a negative regulation. In analyzing mechanisms whereby bone marrow cells promote cancer cell dormancy, one study found that association of osteoblasts with prostate cancer cells induced TBK1 expression [85]. In this context, TBK1 interacts with and inhibits mTOR, which in turn induces cell cycle arrest of prostate cancer cells, suggesting that TBK1 promotes cancer cell dormancy in the bone marrow niche. In a mouse model of chronic immune stimulation, it was found that TBK1 inactivates mTORC1, although the mechanism was not established [86]. In a study to determine the mechanism whereby TBK1 inhibits mTORC1, it was shown that TBK1 phosphorylates Raptor, the key regulatory subunit of mTORC1, at S877 and suppresses mTORC1 activity [87]. In that study, TBK1 did not phosphorylate mTOR using mTOR-Raptor immunoprecipitates in cell-free kinase assays.

In contrast to the studies mentioned earlier, other studies implicate TBK1 in the positive regulation of mTORC1. Both TBK1 and IKKε have been reported to directly phosphorylate and activate Akt in cancer cells and in response to growth factors [88,89]. TBK1-mediated regulation of Akt was also shown to be involved in Toll receptor ligand-induced glycolysis in dendritic cells [90]. Since Akt activates mTORC1, TBK1 could promote mTORC1 through this pathway. A recent publication indicated that TBK1 can directly phosphorylate mTOR at S2159 [91]. TBK1 activated downstream of TLR3 and TLR4 agonists, as well as EGF (but not insulin), promoted mTORC1 activity. This work indicates that mTORC1 functions with TBK1 to promote IRF3 activation and IFN production. Future experiments could determine if TBK1 activation of mTORC1 in innate immune signaling fails to suppress autophagy, given the importance of autophagy in pathogen clearance. In starved cancer cells stimulated with amino acids, TBK1 also phosphorylates the mTORC1 effector p70S6K1 [92]. These findings were performed in different cell types (cancer cells, mouse embryonic fibroblasts, macrophages) and under different conditions (e.g., different starvation protocols and TBK1 agonists). Exploring factors that govern whether TBK1/IKKε promote or inhibit mTOR are an important future research direction (Table 2 and see Outstanding Questions).

TBK1 and IKKε in Energy Homeostasis

Given the roles for TBK1/IKKε in regulating autophagy and the mTOR pathway, which are important regulators of metabolism, it follows that these kinases also play an important role in regulating organismal energy homeostasis. In high fat diet-fed mice, IKKε knockout suppressed obesity and the associated chronic inflammation in fat and the liver, along with insulin resistance [93]. Mechanistically, this phenotype was linked to IKKε-mediated suppression of UCP1 [93], phosphorylation of PDE3B [94], and recruitment of proinflammatory macrophages by regulation of chemokine expression. Similar results were observed with TBK1, which is activated in adipose tissue of high-fat diet mice [95], such that adipocyte-specific knockout of TBK1 led to resistance to HFD-induced obesity and increased energy expenditure [96]. This phenotype was linked to TBK1-mediated inhibition of AMPK (a target of the antidiabetic drug metformin) by directly phosphorylating AMPKα at S459/47. Given the recent findings that IKKβ positively regulates AMPK activity, this could represent an aspect of the crosstalk between IKKβ and TBK1 [25,97]. Unlike IKKε knockout, adipocyte TBK1 knockout led to increased inflammation and insulin resistance. The increased inflammation could be due to the fact that TBK1, but not IKKε, can suppress inflammation and NF-κB activity (noncanonical) via the induced degradation of NIK via S862 phosphorylation and the fact that AMPK suppresses NF-κB by promoting ULK-dependent TBK1 activity [96,98]. The increased insulin resistance may be explained by a study where TBK1, but not IKKε, was also found to promote glucose uptake by promoting GLUT4 translocation in adipocytes [99].

These findings on the roles of TBK1/IKKε in energy balance led to a clinical trial with the TBK1/IKKε inhibitor amlexanox in obese patients with type 2 diabetes, which demonstrated that amlexanox lowered blood sugar [100]. It is critical in interpreting these studies to note that amlexanox inhibits other proteins, including Grk5 and PDE4b, which alter both inflammatory and metabolic signaling [101,102]. The recent studies comparing IKKε and TBK1 knockout phenotypes also suggest that IKKε-specific inhibitors may be a way to suppress obesity without inducing insulin resistance and inflammation.

TBK1 and Mitosis

In addition to the biological processes mentioned earlier, several publications have also implicated TBK1/IKKε in cell cycle regulation. To elucidate the mechanism of TBK1 dependency in several KRas mutant cancer cell lines, phospho-proteomics using TBK1 knockdown cells identified a number of TBK1-dependent phosphorylation changes, including that on polo-like

kinase 1 (PLK1), which was also shown be phosphorylated *in vitro* by TBK1. Phosphorylation of TBK1 at S172 was shown to be highest in mitosis, along with phosphorylation of PLK1 (T210) [103]. Another study found that TBK1 associates with the centrosome and is necessary for microtubule dynamics and completion of mitosis. TBK1 was found to bind and phosphorylate two centrosome-associated proteins, namely CEP170 and NUMA [104]. PINK1/PARKIN activation of TBK1 in mitophagy leads to removal of TBK1 from centrosomes and a block on mitosis [105]. In that study, cells with TBK1 knockout exhibited defects in the cell cycle and increased polyploidy. Collectively, these findings demonstrate that TBK1 can regulate mitosis, particularly in cancer cells, and future work will be needed to determine whether TBK1 also regulates mitosis in nononcogenic settings.

Concluding Remarks and Future Perspectives

Recently, interesting novel phosphorylation substrates have been linked to the IKK family which has greatly expanded the biological landscape of these kinases. These NF-κB-unrelated IKK family-dependent phosphorylation events may serve as mechanisms to promote a permissive environment for NF-κB regulation, or to modify the NF-κB signal, depending on the cellular context. However, many of the substrates described earlier give IKK functions well beyond the NF-κB regulatory pathway. This also applies to the IKK-related kinases TBK1 and IKKε, which have been linked with novel phosphorylation substrates and biological functions. It will be critical to assess the roles that IKK family members play in regulating phosphorylation-dependent substrates in different settings and diseases in order to fully appreciate the function of these pleiotropic kinases. In this regard, while some of the studies described earlier generated phosphorylation site-specific antibodies, these typically are not widely available to the research community. Broad availability of these antibodies would allow researchers to elucidate the effects of manipulating each IKK family member genetically (such as using mouse knockouts or CRISPR lines) or pharmacologically (in particular, using inhibitors specific to each IKK family member) to better understand the contribution of each kinase to the regulation of the phosphorylation sites described. Additionally, *in vivo* mutations of a particular phosphorylation site (for those not already studied) would allow for further validation and corresponding elucidation of the role of phosphorylation of substrates in physiological settings (see Outstanding Questions). Despite these concerns, it is clear that the IKK family of kinases drive an impressive breadth of critical biological and disease-related mechanisms.

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Outstanding Questions

How is IKK regulated by nutrient changes? While IKK activation mechanisms in response to cytokines and TLR ligands are well established, much less is known about the way IKK activity is regulated by changes in nutrient status. Serum and other growth signals, total nutrient deprivation, and the withdrawal of the individual amino acids leucine or glutamine are associated with the induction of IKK, which is poorly understood.

How can the same kinase promote different phosphorylation events in different contexts? The fact that IKK can target different phosphorylation sites depending on the context suggests that there are context-specific mechanisms to direct IKK activity towards different substrates. For the canonical IKK complex, certain substrate recognition likely occurs through NEMO. TBK1 and IKKε are often brought into proximity with their substrates by adaptor molecules such as STING or MAVS. More studies need to be performed to identify substrate-specifying mechanisms for the IKK family.

Could IKK activation be a way to activate autophagy and mTOR at the same time? Could this be why chronic inflammation promotes a permissive state for oncogenic transformation? There are several situations where mTOR activation and autophagy are required simultaneously. For example, autophagy is important for certain cancers, which also require mTOR, a potent suppressor of autophagy.

When in evolution did functions of IKK and TBK1 evolve? Is it possible that IKK proteins evolved to regulate autophagy (and other pathways) before they evolved to regulate NF-κB and immunity (given that autophagy is a much more ancient pathway)? The fact that IKK family kinases regulate more than just inflammation raises interesting evolutionary questions. One hint may be that NEMO and the autophagy receptor optineurin are homologous.

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