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Emerging roles for the non-canonical IKKs in cancer

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Abstract

The IKK-related kinases TBK1 and IKKΣ play essential roles as regulators of innate immunity by modulating interferon and NF-κB signaling. Recent work has also implicated these non-canonical IKKs in malignant transformation. IKKΣ is amplified in approximately 30% of breast cancers and transforms cells through the activation of NF-κB signaling. TBK1 participates in RalB-mediated inflammatory responses and cell survival and is essential for the survival of non-small cell lung cancers driven by oncogenic *KRAS*. The delineation of target substrates and downstream activities for TBK1 and IKKΣ has begun to define their role(s) in promoting tumorigenesis. In this review, we will highlight the mechanisms by which IKKΣ and TBK1 orchestrate pathways involved in inflammation and cancer.

Introduction

The NF-κB pathway is a pivotal regulator of several important physiological functions including the inflammatory immune response, proliferation, cell survival and cell invasion. These activities are well-described hallmarks of cancer, and NF-κB activation has been observed in a wide range of tumors (Baud and Karin, 2009; Dolcet *et al.*, 2005; Prasad *et al.*, 2010), leading some to suggest that NF-κB serves as a bridge between inflammation and cancer (Baldwin, 2001; Karin, 2006). Activation of NF-κB in cancer arises either from extrinsic signals in the tumor microenvironment or from intrinsic dysregulation of the pathway within the tumor. In either case, several different components of the NF-κB signaling cascade may contribute to this activity in particular cancers.

Five NF-κB members exist in mammals including RelA (p65), RelB, c-Rel, p50/p105 (NF-κB1) and p52/p100 (NF-κB2). While RelA, RelB, and cRel are synthesized as mature forms, p105 and p100 are synthesized as longer precursor proteins that are cleaved to form the mature p50 and p52 proteins. This processing is necessary for p50 and p52 to function as transcription factors (Dolcet *et al.*, 2005). Distinct NF-κB complexes are formed from combinations of homo- and heterodimers of these family members (Bonizzi and Karin, 2004). NF-κB complexes are retained in the cytoplasm by a family of NF-κB-binding proteins known as the inhibitors of NF-κB (IκBs). A variety of inflammatory stimulants initiate the induction of NF-κB and trigger activation of the IκB Kinase (IKK) complex, which is composed of the catalytic kinases IKKα and IKKβ, and the regulatory NF-κB essential modifier, (NEMO, or IKKγ) (Perkins, 2007). One important function of the IKK complex is to mark IκB for phosphorylation and ubiquitination, which in turn, leads to proteasomal degradation. This activity facilitates the release and accumulation NF-κB dimers in the nucleus where a transcriptional program involving many target genes related to immune response are activated. While the classical NF-κB pathway primarily involves the activation of RelA and p50 and is strictly facilitated by the IKK complex, an alternative pathway is active in B cells where NF-κB inducing kinase (NIK) promotes the activation of RelB and p52/p100 complexes (Ghosh and Hayden, 2008). Genetic experiments have demonstrated that IKKβ is the principal IKK for the canonical NF-κB pathway, whereas

IKKα plays a more dominant role in the non-canonical pathway (Bonizzi and Karin, 2004; Pasparakis *et al.*, 2006; Perkins, 2007). In addition to the conventional IKKs, a related pair of non-canonical kinases, IKKΣ (IKKi, encoded by *IKBKE*) and TBK1 (NAK), have been identified as important mediators of both inflammatory and oncogenic signaling.

IKKε and TBK1 function in inflammation

IKKΣ was simultaneously identified in a subtractive hybridization screen as a LPS-inducible gene and as a PMA-inducible protein with a kinase domain that is 27% identical to IKKα and IKKβ (Peters *et al.*, 2000; Shimada *et al.*, 1999). Concurrently, TBK1, which exhibits 49% identity and 65% similarity to IKKΣ was identified as an interaction partner with the scaffolding molecule, TRAF-associated NF-κB activator (TANK) (Pomerantz and Baltimore, 1999). Both IKKΣ and TBK1 are comprised of an N-terminal kinase domain, an ubiquitin-like domain, a C-terminal LZ and a HLH motif (Figure 1). Despite their similarity in structure, TBK1 and IKKΣ exhibit differential expression patterns. TBK1, like IKKα and IKKβ, is ubiquitously expressed (Shimada *et al.*, 1999). In contrast, IKKΣ expression is restricted to particular tissue compartments, with highest levels detected in lymphoid tissues, peripheral blood lymphocytes and the pancreas (Shimada *et al.*, 1999). Various epithelial derived cell lines also exhibit IKKΣ expression (Bibeau-Poirier *et al.*, 2006; Gravel and Servant, 2005; Honda *et al.*, 2005; Shimada *et al.*, 1999). Mitogenic stimulation with LPS and TNFα can also induce IKKΣ and TBK1 expression in a NF-κB dependent manner (Hemmi *et al.*, 2004; Kravchenko *et al.*, 2003; Shimada *et al.*, 1999). With these partially overlapping characteristics, IKKΣ and TBK1 are functionally more similar to each other than the canonical IKKs (Clement *et al.*, 2008).

The non-canonical IKKs coordinate the interferon response

IKKΣ and TBK1 are critical inducers of interferon signaling in response to viral infection (Fitzgerald *et al.*, 2003; Sharma *et al.*, 2003). Following activation of toll-like receptors (TLR) via viral components, IKKΣ and TBK1 assemble with TRAF3 and TANK to phosphorylate interferon regulatory factors (IRF) 3, 5, and 7 at multiple serine and threonine residues (Caillaud *et al.*, 2005; Cheng *et al.*, 2006; McWhirter *et al.*, 2004; Mori *et al.*, 2004; Pomerantz and Baltimore, 1999). This activity allows for heterodimerization and nuclear translocation of the IRFs and induction of proinflammatory and antiviral genes, including type I interferon (Lin *et al.*, 1998; Sato *et al.*, 2000). TLR-independent mechanisms also activate IKKΣ and TBK1 to induce the interferon response. In this scenario, viral dsRNA and dsDNA initiate signaling through intracellular RNA and DNA sensors such as RIG-I, MDA-5 and DAI (Andrejeva *et al.*, 2004; Kawai *et al.*, 2005; Meylan *et al.*, 2005; Seth *et al.*, 2005; Takaoka *et al.*, 2007; Yoneyama *et al.*, 2004). IFNβ also activates a TLR-independent pathway by stimulating IKKε phosphorylation of STAT1 to facilitate binding with ISGF3, a complex that serves as the transcriptional machinery important for activating a subset of interferon response genes (Tenoever *et al.*, 2007). Moreover, engagement of both TLR-dependent and -independent pathways recruits additional scaffolding molecules including FADD, TRADD, MAVS, NAP1, HSP90, and SINTBAD necessary for IKKΣ and TBK1-mediated interferon activation (Balachandran *et al.*, 2004; Gatot *et al.*, 2007; Guo and Cheng, 2007; Hacker et al., 2006; Michallet et al., 2008; Oganesyan *et al.*, 2006; Rothe *et al.*, 1996; Ryzhakov and Randow, 2007; Yang *et al.*, 2006). Thus, IKKΣ and TBK1 form several protein complexes, the composition of which is dependent on the type of cellular stimuli. Ultimately, these signaling complexes share a role in activating interferon responses required to induce the antiviral response.

The ability of IKKΣ and TBK1 to activate the interferon response is also dependent on posttranslational modifications by proteasome independent Lys-63 linked ubiquitin chains (Figure 2). Both IKKΣ and TBK1 have an ubiquitin like domain (ULD), and Lys-63

ubiquitination of both kinases are promoted by TRAF3, which itself is Lys-63 ubiquitinated. Disruption of this activity by TAXBP1 and the deubiquitinase A20 ablates the interferon response (Ikeda *et al.*, 2007; Parvatiyar et al., 2010). Both TBK1 and IKKΣ also mediate ubiquitination of TANK by an unknown E3 ligase (Gatot *et al.*, 2007). Although TANK further serves as a phosphorylation target of TBK1 and IKKΣ, TANK ubiquitination seems to occur independently of this kinase activity. The mechanism by which TANK ubiquitination contributes the activation of IKKΣ and TBK1 is unknown. Lys -63 ubiquitination also plays a role in the negative regulation of IKKε and TBK1 induced responses. For example, during by RNA viruses, Lys-63 ubiquitination of MAVS is essential for the recruitment of IKKΣ and leads to the inhibition of antiviral and NF-κB induced inflammatory genes (Paz *et al.*, 2009). Ultimately, these modifications will likely dictate a dynamic system of regulating IKKΣ and TBK1-mediated function in both inflammation and cancer.

The non-canonical IKKs function as NF-κB effectors

Although TBK1 and IKKΣ are not a part of the classical IKKα/β/γ signaling complex, these kinases were originally characterized as activators of NF-κB and target multiple NF-κB members and effectors. Both IKK-related kinases phosphorylate IκBα at, one of the two serine residues typically targeted on IκBα. Although IKKε phosphorylates Ser^{36}, TBK1 phosphorylates Ser^{32} (Pomerantz and Baltimore, 1999; Shimada *et al.*, 1999; Tojima *et al.*, 2000). IKKε overexpression reduces IκBα levels suggesting that phosphorylation at a single residue may result in increased IκBα turnover. However, it remains possible that the IKK-related kinases phosphorylate a canonical IKK that in turn leads to IκBα turnover (Boehm *et al.*, 2007; Eddy *et al.*, 2005). The canonical NF-κB, RelA/p65, is another substrate for IKKΣ and TBK1, and phosphorylation of RelA at serine 536 by these kinases occurs at a basal level independently of extracellular stimuli (Buss *et al.*, 2004; Fujita *et al.*, 2003). cRel is also phosphorylated by both IKK-related kinases. This activity is sufficient to promote nuclear translocation; however, it does not modulate downstream NF-κB activity (Harris *et al.*, 2006). TANK plays a similar role in IKKε/TBK1-mediated NF-κB activation as it does in interferon activation and acts as both a substrate and critical adaptor molecule for these kinases. Furthermore, in most cells, autophosphorylation of TBK1 and IKKΣ is readily detected. However, it has also been suggested that other kinases may phosphorylate the IKK-related kinases (Gatot *et al.*, 2007; Ikeda *et al.*, 2007). The significance of these phosphorylation events remains unclear.

Although closely related, TBK1 and IKKΣ also phosphorylate distinct substrates and may activate NF-κB through different mechanisms (Table 1). For example, TBK1 is the only of the two IKK-related kinases to phosphorylate and activate IKKβ, suggesting that TBK1 may act preferentially on canonical NF-κB signaling (Tojima *et al.*, 2000). On the other hand, in stimulated T cells, IKKΣ targets a second residue on p65/RelA, serine 468. Although phosphorylation at this site occurs in addition to the serine 536 residue that is phosphorylated by both IKK-related kinases, it is not clear whether this event occurs in other cellular contexts (Mattioli *et al.*, 2006). IKKΣ also associates with p100/p52 in a ternary complex with p65 following TNF induction and this interaction facilitates transactivation of p52 dependent genes (Wietek *et al.*, 2006). Moreover, recent experiments indicate that IKKΣ can associate with the chromatin of promoters of specific NF-κB target genes (Moreno *et al.*, 2010).

Activation of NF-κB by the IKK-related kinases occurs in response to specific stimuli. PMA-induced NF-κB activation is dependent on IKKε (Peters *et al.*, 2000). Similarly, induction of NF-κB via the T cell receptor (TCR) involves TBK1 (Tojima *et al.*, 2000). In contrast, IKKε or TBK1 are not required for TNF- or IL-1-induced NF-κB activation (Peters *et al.*, 2000; Pomerantz and Baltimore, 1999). The role of the IKK-related kinases in NF-κB

activation is not well understood and appears to be highly dependent on specific cellular stimuli. This area has been extensively reviewed elsewhere (Chau *et al.*, 2008; Clement *et al.*, 2008; Hacker *et al.*, 2006). Ultimately, the multiple modes of IKKΣ and TBK1-mediated NF-κB activation highlight both independent and synergistic relationships that are likely dictated by cellular and signal-induced contexts.

In consonance with these biochemical observations, genetically engineered mice lacking either *Tbk1* or *Ikbke* exhibit distinct phenotypes. *Tbk1* deficient animals are embryonic lethal and die at E14.5 due to extensive fetal liver degeneration, a phenotype that is also observed in IKKγ, IKKβ and RelA deficient animals (Bonnard *et al.*, 2000; Hemmi *et al.*, 2004). By contrast, *Ikbke* deficient animals are viable, but are impaired in initiating a productive IFN-β response (Bonnard *et al.*, 2000; Hemmi *et al.*, 2004). Interestingly, NF-κB activation in either *Tbk1* or *Ikbke* single and double knockout models is predominantly normal, apart from minimal defects in the induction of select NF-κB target genes. These models demonstrate that although the non-canonical IKKs are sufficient but not essential for NF-κB activation, IKKΣ and TBK1 play a key role in the induction of interferon signaling.

Extrinsic vs. Intrinsic NF-κB activation in Cancer

In addition to a role in facilitating inflammatory responses to foreign agents, the IKK-related kinases were recently recognized as NF-κB effectors that contribute to tumorigenesis and thus represent a link between NF-κB-mediated inflammation and cancer.

The contribution of NF-κB to inflammation-associated cancer has been studied extensively, and inhibition of the canonical pathway in mouse models of inflammation-associated cancer demonstrates that NF-κB is often essential for cancer initiation and progression (Greten *et al.*, 2004; Karin, 2006; Lawrence *et al.*, 2005; Pikarsky *et al.*, 2004). For example, deletion of IKKβ in either epithelial or myeloid cells of a mouse model of colitis-associated cancer dramatically decreases tumor incidence by nearly 80% (Greten *et al.*, 2004). Anti-TNFα treatment and introduction of the NF-κB super repressor in a model of inflammatory hepatocellular carcinoma also induces apoptosis and inhibition of tumor progression (Pikarsky *et al.*, 2004). These observations underscore how malignant cells are critically dependent on the inflammatory microenvironment for their survival. However, it is difficult to assess whether there are additional cell autonomous contributions of NF-κB activation in inflammation-associated cancer models, and it is likely that both cell autonomous and non-cell autonomous functions are involved.

While much focus of NF-κB activation has revolved around inflammation-associated cancer, this pathway plays a direct cell autonomous role in transformation and tumorigenesis. Activation of NF-κB in tumor cells is frequently observed in both solid tumors and hematologic malignancies (Basseres and Baldwin, 2006; Baud and Karin, 2009; Dolcet *et al.*, 2005). Until recently, it was assumed that this activation is the result of immune stimuli originating from the microenvironment. However, somatic mutations of components in the NF-κB pathway were recently described and provide an alternative means of NF-κB activation in tumors. For example, mutations in NF-κB family members *NFKB1*(encoding p105/p50) and *NFKB2* (encoding p100/p52) as well as genes whose protein products regulate the IKK complex such as *CIAP1*, *CIAP2*, *CYLD*, *NIK*, and *TRAF3* are associated with the pathogenesis of multiple myeloma (Annunziata *et al.*, 2007; Baud and Karin, 2009; Keats *et al.*, 2007). The application of targeted sequencing analyses in other lymphoid malignancies including diffuse large B cell lymphoma and marginal zone lymphomas has led to the identification of mutations in additional regulators of the NF-κB pathway including *A20, CARD11, TRAF2, TRAF5, TAK1, and RANK* (Compagno *et al.*, 2009; Novak *et al.*, 2009). Furthermore, a more recent genomic analysis of somatic copy number

alterations across a large subset of 3131 human cancer tissues and cell lines found amplifications of major NF-κB regulators including *TRAF6*, *IKBKB*, *IKBKG*, *IRAK1* and *RIPK1* (Beroukhim *et al.*, 2010). Importantly, all of these genes were significantly amplified in epithelial cancers, suggesting that alterations of NF-κB effectors are far more frequent than previously appreciated.

In addition to direct mutations in the NF-κB pathway, NF-κB activation is observed in tumors harboring mutations in numerous oncogenes including HRAS, ERB2, PI3K, Bcr-Abl, the viral oncoprotein TAX, Vav-Pim2, BRAF, MUC1, and BMI-1 (Basseres and Baldwin, 2006; Biswas *et al.*, 2004; Finco *et al.*, 1997; Gustin *et al.*, 2004; Hammerman *et al.*, 2004; Ikenoue *et al.*, 2003; Li *et al.*, 2010; Pianetti *et al.*, 2001; Reuther *et al.*, 1998; Xiao *et al.*, 2001) Although activation of the NF-κB pathway has been documented in each of these contexts, the mechanism by which these oncogenes activate NF-κB signaling and the role of such signaling in tumorigenesis remains incompletely understood. However, H-RAS^{V12}–induced transformation depends on NF-κB activity (Mayo *et al.*, 1997), and recent work in genetically engineered mouse models demonstrated that NF-κB activity was required for the development of lung adenocarcinomas driven by oncogenic KRAS in the setting of p53 loss (Basseres *et al.*, 2010; Meylan *et al.*, 2009). Other recent studies suggest that the non-canonical IKKs, IKKε and TBK1 may play key roles in the cell autonomous activation of NF-κB in cancer.

IKKΣ in cancer

A potential role for IKKΣ in breast cancer was first shown by Sonenshein and her colleagues who demonstrated that IKKε was constitutively expressed in MDA-MB-231 and Hs578T breast cancer cells lines, 4 of 6 breast patient carcinomas, and carcinogen-induced mouse mammary tumors (Eddy *et al.*, 2005). In this study, tumors and mammary glands derived from a casein kinase 2 catalytic subunit alpha (CK2α) transgenic mouse model of mammary adenocarcinoma exhibit higher levels of IKKε. Likewise, overexpression of CK2 in MCF10F cells also increased IKKε expression. These results suggested that CK2 might play an important role in regulating IKKΣ. In addition, expression of the kinase-inactive form of IKKΣ in breast cancer cells reduced levels of two NF-κB target genes, Cyclin D1 and RelB, as well as anchorage-independent growth and invasion in matrigel (Eddy *et al.*, 2005). Adli *et al.* also found elevated IKKε expression in prostate and breast cancer cell lines. In this work, IKKε was found to activate NF-κB at a basal level by phosphorylating serine 536, and inhibition of this activity significantly suppressed cancer cell proliferation (Adli and Baldwin, 2006). Together, these studies provided the first evidence of IKKε involvement in breast cancer.

More recently, IKKΣ was identified as a breast oncogene through the intersection of three complementary genomic approaches. First, Boehm *et al.* developed an experimental model of cell transformation that was dependent on both MEK and AKT signaling. By screening kinases that could replace AKT and induce tumorigenesis, we found that IKKΣ and CSNK1E were able to transform such cells (Boehm *et al.*, 2007). In parallel, loss of function screens to identify kinases that were essential for the proliferation and survival of breast cancer cell lines revealed that both IKKΣ and CSNK1E were required for the survival of breast cancer cell lines. Of these two genes, *IKBKE* is amplified in 8 of 49 (16.3%) breast cancer cell lines and in over 30% of primary breast tumors (Boehm *et al.*, 2007). FISH analysis further confirmed increased copy number of *IKBKE* by up to 10 additional copies is the consequence of these amplification events. Taken together, these studies suggested that IKKε was a breast cancer oncogene.

IKKε activates both interferon and NF-κB signaling, and overexpression of IKKε in mammary epithelial cells induced both interferon regulated genes and several NF-κB target genes including *MMP9*, *BCL2*, *CIAP1* and *CIAP2*. In agreement with these observations, primary breast carcinomas in which IKKΣ expression was elevated showed nuclear localization of the NF-κB factor cREL. Many cancer cells with increased levels of IKKΣ also exhibit increased levels of serine 536 phosphorylated p65. Strikingly, expression of the NF-κB super-repressor in IKKΣ-transformed cells blocked IKKε– induced cell transformation. In contrast, suppression of IRF3 and IRF7 via shRNA failed to alter the transformation phenotype, indicating that the role of IKKΣ in mediating inflammatory interferon responses is unrelated to its function in cancer (Boehm *et al.*, 2007).

Recent observations demonstrate that IKKΣ also functions to protect cells from DNA damage-induced death, and the underlying mechanism for this response is mediated by IKKΣ sumoylation (Renner *et al.*, 2010). Following genotoxic stress, IKKΣ translocates to the nucleus and induces phosphorylation of the promyelocytic leukemia tumor suppressor PML. This activity results in the sequestration of IKKΣ in PML nuclear bodies. Multiple nuclear targets including p65 are then phosphorylated following critical sumoylation of IKKΣ by the SUMO ligase TOPORS. Ultimately, these events facilitate an NF-κB mediated antiapoptotic response to DNA damage (Renner *et al.*, 2010). These observations identify a novel function for IKKΣ in promoting cell survival and suggest that IKKΣ acts in parallel to the p53 proapoptotic pathway.

Although *IKBKE* copy number gain does not correlate with estrogen receptor (ER) or HER2/Neu status, Guo et al. demonstrated that IKKε phosphorylates estrogen receptor alpha (ERα). In ER-positive cells, this activity is necessary for activation of Cyclin D1. However, IKKε is also sufficient to activate Cyclin D1 in ER-negative cells. (Boehm *et al.*, 2007; Guo *et al.*, 2010). Furthermore, although overexpression of IKKε promotes tamoxifen resistance, suppression of IKKε sensitizes cells to tamoxifen-induced death (Guo *et al.*, 2010). These findings highlight potential crosstalk between NF-κB dependent and independent pathways in IKKε transformed breast cells.

In addition to breast cancer, IKKε also appears to have a role in ovarian cancer. A recent study showed that IKKε is overexpressed in 63 of 95 ovarian carcinomas, and elevated IKKε levels were associated with poor prognosis (Guo *et al.*, 2009). Notably, 76.5 % of breast cell lines and 47.6% of breast carcinomas exhibit increased expression of IKKΣ in the absence of 1q32 copy number changes. Furthermore, recent deep sequencing of a set of human cancers including, breast, ovarian, lung and prostate tumors identified two IKKε mutations, G417D and W445S, in lung adenocarcinomas (Kan *et al.*, 2010). The function of these mutations, which do not map to any previously described domains of IKKε, remains unclear (Figure 1). Taken together, these observations suggest that IKKΣ expression may be dysregulated through multiple mechanisms in various cancer types.

TBK1 in Cancer

TBK1 is highly expressed in lung, breast and colon cancers and a recent TBK1 mutation, P675L, was recently identified in lung adenocarcinoma (Barbie *et al.*, 2009; Kan *et al.*, 2010; Korherr *et al.*, 2006) (Figure 1). RalB is a key factor that mediates TBK1 activation in cancer (Bodemann and White, 2008). The Ras-like RalB proteins function downstream of RAL-GEF (Ras-like- guanine nucleotide exchange factor) to initiate a variety of regulatory processes associated with oncogenesis (Bodemann and White, 2008). The activation of RalB by Ral-GEF is necessary for RAS-induced transformation (Lim *et al.*, 2006; Rangarajan *et al.*, 2004; Yoneyama *et al.*, 2004). Downstream of Ral-GEF, RalB-mediated activation of TBK1 promotes TBK1 assembly with the exocyst complex, which contains several core

secretory proteins, namely Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84. TBK1 is able to participate in the exocyst complex through its direct interaction with Sec5 and further facilitates transformation through the phosphorylation of Sec5 (Camonis and White, 2005; Chien *et al.*, 2006). This cascade is not only a critical function in transformation, but is also essential for mediating the host defense to viral infection. Importantly, suppression of either TBK1 or Sec5 was shown to induce apoptosis in Ras-transformed cells, and expression of oncogenic alleles of KRAS induced cell death in TBK1 deficient murine embryonic fibroblasts (Chien *et al.*, 2006).

In recent work, TBK1 was identified as a gene whose expression was selectively required in *KRAS*-dependent cancer cell lines (Barbie *et al.*, 2009). Suppression of TBK1 affects the survival of *KRAS*-dependent cells, while having little to no effect in cells where *KRAS* is not essential. Transcriptional profiling using gene set enrichment analysis in *KRAS*-transformed cells revealed the enrichment of several NF-κB activation signatures. Expression of the NF-κB super repressor also selectively induced apoptosis in *KRAS*-dependent lung cell lines. In these cells, TBK1 promoted degradation of IκBα and induced expression of the survival factor BCL-xL, which in part sustains the survival of *KRAS*-driven tumors (Barbie *et al.*, 2009).

In addition, a recent study identified another novel function for TBK1 as an inducer of angiogenesis. Korherr et al. screened a genome-wide cDNA library in HEK293 cells for genes that regulate vascularization. Supernatants isolated from growth factor-producing HEK293 cells were evaluated for the ability to induce proliferation of HUVEC endothelial cells. TBK1 was found to act as a trigger in stimulating a secretion program consisting of endothelial growth factors RANTES and IL-8, as well as other proangiogenic factors such as CXCL10, CXCL11, and IFN-β. Moreover, several cancer cell lines with upregulated TBK1 exhibit a similar autocrine makeup (Korherr *et al.*, 2006).

New and old substrates of IKKΣ and TBK1 in cancer

The initial characterization of IKKΣ as a breast cancer oncogene indicated that its intrinsic kinase activity was essential to activate NF-κB and transform cells, but the substrates and effectors responsible for these phenotypes remained undefined. Both TBK1 and IKKΣ appear to have many substrates that modulate NF-κB (Figure 2) in response to extracellular stimuli, namely p65, cREL, and IκBα. However, engagement of these targets by the non-canonical IKKs is different than what has been observed for the other IKKs that lead to NF-κB activation. For example, it is uncertain how phosphorylation of one of the two required sites on IκBα is sufficient to activate NF-κB. Similarly, although IKKΣ and TBK1 can phosphorylate cREL, this modification fails to promote significant NF-κB activation (Harris *et al.*, 2006).

To identify IKKΣ and TBK1 substrates, Hutti and her colleagues developed and used an unbiased peptide screening method coupled with bioinformatic analyses to identify a potential substrate recognition motif. In this approach, a collection of 198 biotinylated peptide libraries was employed to perform simultaneous kinase assays with recombinant IKKε or TBK1 and radiolabeled ATP. Each library contains mixtures of degenerate peptides with two fixed residues, a serine at the central position and another natural amino acid at one additional position neighboring the serine. The relative IKKε or TBK1 preference for each amino acid at each position was determined after capture of the biotinylated peptides on a streptavidin-coated membrane. The resulting IKKε/TBK1 substrate recognition motif was used to identify potential substrates through the proteomic search engine Scansite (Hutti *et al.*, 2009).

The IKKΣ and TBK1 phosphorylation motif consists of a central serine that is surrounded by a hydrophobic residue at the +1 position relative to the phosphorylation site, an aromatic residue at the −2 position and bulky hydrophobic residues at the +3 position (x-Y/F/P-x-pS-L/I-x-Y/W/F-x). Parallel analysis of IKKΣ and TBK1 using the scanning peptide library screen independently identified the same target motif for each kinase (Hutti *et al.*, 2009). *In vitro* kinase assays demonstrate that IKKΣ efficiently targets peptides with the identified IKKΣ recognition motif. In contrast, the observed activity of IKKΣ toward a peptide representing the IκBα target sequence was much less robust, suggesting that IκBα is unlikely to be a preferred substrate of IKKΣ (Hutti *et al.*, 2009).

Bioinformatic analysis of the IKKΣ recognition motif identified many potential IKKΣ substrates. Interestingly, the top scoring candidates consisted of many NF-κB regulators including the known IKKΣ/TBK1 substrate TANK. Additional putative substrates appear to be involved in ubiquitination. Although further functional validation of these substrates is necessary, these findings suggest that IKKΣ and TBK1 function in a network of pathways that converge to activate NF-κB (Hutti *et al.*, 2009).

The proteomic/ bioinformatic approach taken by Hutti *et al.* further identified the familial tumor suppressor *CYLD* as an IKKΣ substrate that was essential for transformation. CYLD is a deubiquitinase that attenuates NF-κB signaling by targeting various effectors in the pathway (Brummelkamp *et al.*, 2003; Kovalenko *et al.*, 2003; Trompouki *et al.*, 2003). IKKΣ phosphorylates CYLD at serine 418 and disruption of this activity substantially hinders both IKKΣ-induced NF-κB activation and tumorigenicity. Although CYLD expression is necessary for IKKΣ–mediated transformation, it is likely that other substrates also facilitate IKKΣ function in cancer (Hutti *et al.*, 2009).

Several other cancer-related substrates have also been described for IKKΣ/TBK1. As previously discussed, IKKΣ phosphorylates estrogen receptor alpha (ERα) through a direct interaction. This activity occurs on serine 167 and promotes ERα transactivation and induction of target genes such as Cyclin D1 (Guo *et al.*, 2010). STAT1 is another substrate of IKKΣ discussed above that typically functions in mediating the interferon response, but may play an additional role in cancer progression (Tenoever *et al.*, 2007). STAT proteins, including STAT1, STAT3 and STAT5, are critical for the regulation of several genes that function in antiviral immunity and also act as oncogenic transcription factors in various malignancies (Bowman *et al.*, 2000). STAT1 expression is constitutively active in many breast cancer cell lines, but also has a paradoxical role in promoting apoptosis in tumor cells (Kim and Lee, 2007; Yarilina *et al.*, 2008). In addition to the STAT proteins, IRF3, IRF5 and IRF7 also behave as tumor suppressors and facilitate anti-tumorigenic effects in various cancers. However, since suppression of these factors in IKKε-transformed cells fails to decrease tumorigenic potential, they likely play a minimal role in the IKKε oncogenic pathway.

Conclusions

IKKΣ and TBK1 appear to behave as integrators of signals induced not only by proinflammatory stimuli but also by oncogenes and tumor suppressors (Figure 2). Through the phosphorylation and modulation of several NF-κB effectors and interferon regulatory factors, the IKK-related kinases primarily function as inflammatory regulators. More recently, IKKε was identified as a breast oncogene that requires NF-κB activation to mediate transformation, and TBK1 was demonstrated to be essential for the survival of *KRAS*-dependent non-small cell lung cancers. The discovery of IKKε and TBK1 as oncogenic kinases that are intricately associated with Ras–mediated transformation suggests that these non-canonical IKK regulators are subverted in cancer cells.

While recent studies have defined CYLD as an important IKKΣ substrate that contributes to transformation, it is not known whether other targets are involved in the IKKΣ oncogenic pathway. Likewise, it will be important to define the critical substrates for TBK1 in *KRAS*-dependent lung cancers. It is clear for IKKΣ that kinase activity contributes to its transforming activity.

The finding that both TBK1 and IKKΣ preferentially phosphorylate the same consensus motif, and for the most part share the same substrates, raises the question as to how these kinases act differently in cell transformation (Hutti *et al.*, 2009). One possibility is that IKKΣ and TBK1 may be differentially regulated. Negative feedback mechanisms involving CYLD and A20 have been shown to control both TBK1 and IKKΣ activity (Zhang *et al.*, 2008). However, other negative regulators such as the phosphatase SHP2 and the NOD-like protein NLRC5 primarily affect TBK1 and not IKKε activity (An *et al.*, 2006; Cui *et al.*, 2010; Lin *et al.*, 2006). In addition, the participation of IKKΣ and TBK1 in different protein complexes that are modified by Lys-63 linked ubiquitin chains proposes that the regulation, stability and activation of complex formation could contribute to differential oncogenic signaling. Another explanation for the differential roles of the non-canonical IKKs in cancer is the growing list of substrates that are distinct for IKKε and TBK1 (Table 1). The subtle but disparate regulation and activity of the non-canonical IKKs would likely influence divergent activities of TBK1 and IKKε in cancer.

Current work suggests that IKKΣ and TBK1 participate in both cell autonomous and cell non-autonomous functions in inflammation and cancer (Figure 2). The non-canonical IKKs may augment inflammatory signaling in addition to its intrinsic role of promoting cell survival. Integration of these pathways may explain the high levels of NF-κB activation observed in aggressive inflammatory breast cancer (Lerebours *et al.*, 2008; Van Laere *et al.*, 2006). The recent finding that IKKΣ regulates fat metabolism adds another potential mechanism by which IKKε may contribute to transformation (Chiang *et al.*, 2009). Both IKK-related kinases increase the production of IL-6 and TNF, two inflammatory cytokines associated with insulin resistance and the induction of oncogenic STAT3 signaling (Chiang *et al.*, 2009; Park *et al.*, 2010; Peant *et al.*, 2009). These findings begin to suggest crosstalk between pathways involving inflammation, insulin resistance, and cellular transformation.

Several drug discovery efforts have focused on the development of NF-κB inhibitors for various malignancies (Karin *et al.*, 2004; Lee and Hung, 2008). Although there is some concern that prolonged NF-κB inhibition will lead to unacceptable side effects in inflammatory disease, such inhibitors could be used transiently to treat cancer patients. Surprisingly, IKKβ inhibitors increase the production of IL-1β and other inflammatory cytokines that are normally suppressed by NF-κB, and treatment with these agents results in an adverse exacerbation of inflammation (Greten *et al.*, 2007). The non-canonical IKKs may thus serve as more attractive candidates for therapeutic development in cancer. Although further work is necessary to decipher whether these non-canonical IKKs are therapeutic targets in certain malignancies, the finding that these non-canonical IKKs play important roles in the maintenance of epithelial tumors suggests that targeting the NF-κB pathway may be an attractive strategy in a wide range of epithelial cancers.

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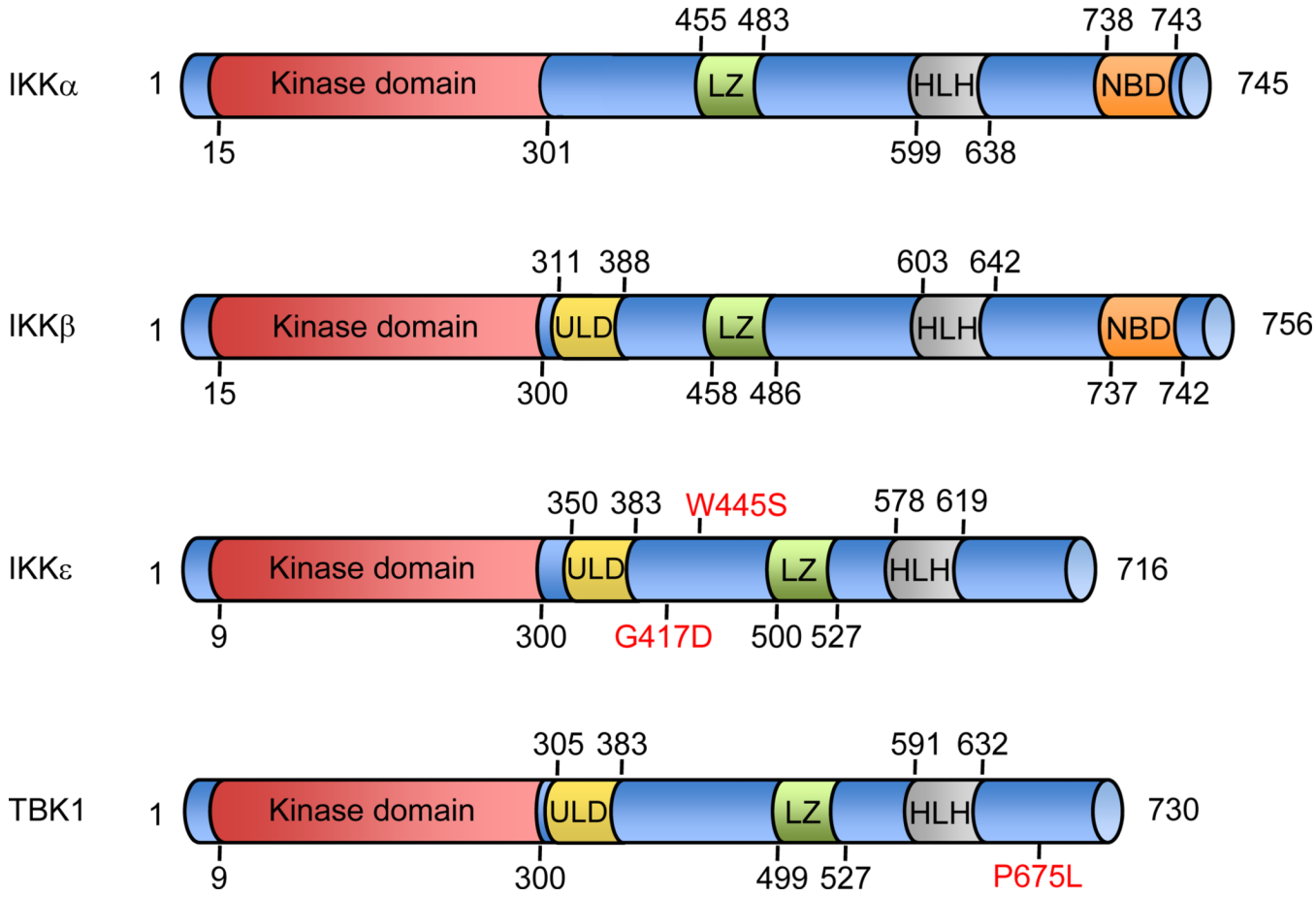

Figure 1. Structural comparison of the classical and non-canonical IKKs

The major domains of each IKK kinase are depicted with amino-acid numbers that correspond to the human proteins. The kinase domain of IKKε exhibits 27% and 24% identity to IKKα and IKKβ respectively, and TBK1 shares 49% identity and 65% similarity to IKKε. Somatic mutations of IKKε and TBK1 recently identified in lung adenocarcinomas are marked in red. ULD, ubiquitin-like domain; LZ, leucine zipper; HLH, helix-loop-helix; NB, NEMO-binding domain (Hiscott *et al.*, 2006; Kan *et al.*, 2010; May *et al.*, 2004; Perkins, 2007).

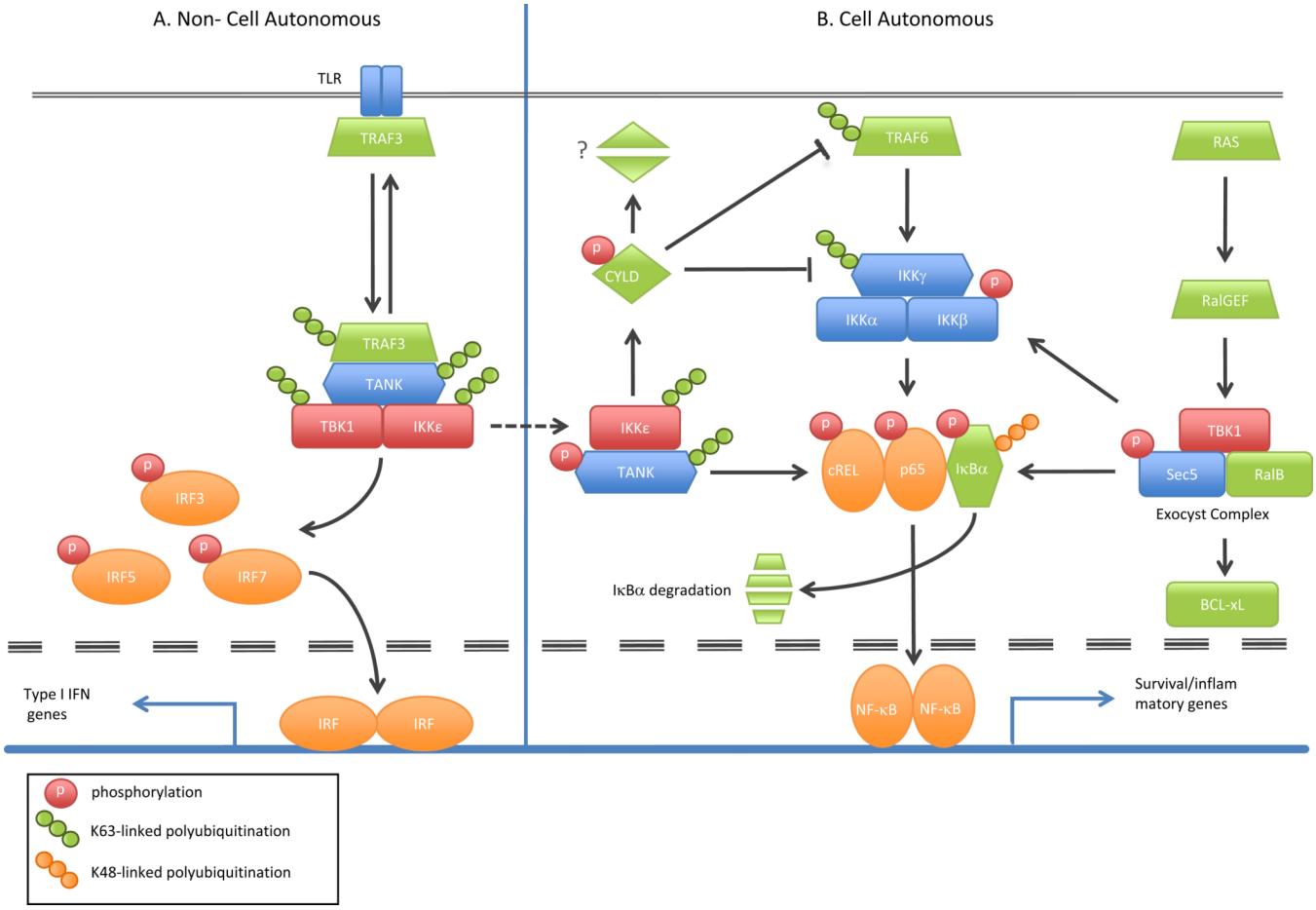

Figure 2. Cell autonomous and non-cell autonomous roles of IKKε and TBK1 in inflammation and cancer

(A) Engagement of toll-like receptors (TLRs) initiates Lys-63 linked ubiquitination of TRAF3 and recruitment of IKKε and TBK1 through the adaptor molecule TANK. This recruitment promotes Lys-63 linked ubiquitination and activation of IKKε/TBK1. TANK is modified by both ubiquitination and phosphorylation through IKKε/TBK1, although it is unclear how these activities are related. The TANK/IKKε/TBK1 complex can then target interferon response factors (IRF3, IRF5, and IRF7) for phosphorylation and promote translocation to the nucleus where IFN target genes are activated. This cascade primarily defines the non-cell autonomous functions of the IKK-related kinases. In parallel to the interferon pathways, IKKε and TBK1 also phosphorylate many NF-κB effectors such as IκBα, p65 and cREL in response to cellular stimuli. This activity facilitates NF-κB nuclear translocation as a homo- or hetero- dimer and induces numerous inflammatory and survival-related target genes. (B) The IKKε/TBK1 oncogenic pathways function in a cell autonomous manner and are predominantly driven by aberrant levels of IKKε/TBK1. In this context, oncogenic RAS activates RalGEF, which in turn recruits the exocyst complex consisting of TBK1, RalB and Sec5, amongst other secretory proteins. TBK1 mediates phosphorylation of multiple effectors including Sec5, IKKβ, and IκBα that ultimately lead to the activation of both BCL-xL and NF-κB. Similarly, aberrant levels of IKKε in cancer also promote NF-κB activation through the phosphorylation of CYLD. CYLD negatively regulates NF-κB signaling by deubiquitinating Lys-63 linked ubiquitin chains on TRAF6 and IKKγ. It is hypothesized that IKKε-mediated phosphorylation of CYLD leads to either its degradation or sequestration, thereby inactivating its negative role on the NF-kB signaling cascade.

Table 1

Differential substrates of the non-canonical IKKs

Substrates	Targeted region	Phosphorylation site	Suggested function	References
IKKε specific substrates				
RelA/p65	TAD	Ser 468	NF-kB activation	Matioli et al.
p100/p52	-	not phosphorylated	Transactivation via p65	Wietek et al.
STAT1	C-Terminal	Ser 708	ISGF3 stability	Tenover et al.
CYLD	Central	Serine 418	NF-kB activation	Hutti et al.
ERα	N-Terminal, AF-1	Serine 167	ERα Transactivation	Guo et al.
TBK1 specific substrates				
Sec5	Ral Binding domain	Unknown	Interferon induction	Chien et al.
IKK	Activation loop	Ser 177/181	IKK activation	Tojima et al.
DDX3X	DEAD and helicase domains	Ser 181/183/240/269/429/442/456/520, Thr 438	Interferon induction	Soulat et al.
IR (Insulin Receptor)	C-Terminal	Ser 994	insulin resistance	Munoz et al.