Structure and ubiquitination-dependent activation of Tank-Binding Kinase 1

Daqi Tu1,3,†, Zehua Zhu2,†, Alicia Y. Zhou2,4, Cai-hong Yun1,3, Kyung-Eun Lee5, Angela V. Toms1,3, Yiqun Li1, Gavin P. Dunn2, Edmond Chan2, Tran Thai2, Shenghong Yang2, Scott B. Ficarro1,3, Jarrod A. Marto1,3, Hyesung Jeon5, William C. Hahn2,4, David A. Barbie2,4,* and Michael J. Eck1,3,*

1Department of Cancer Biology, Dana-Farber Cancer Institute, 450 Brookline Ave. Boston, MA 02215 USA
2Medical Oncology, Dana-Farber Cancer Institute, 450 Brookline Ave. Boston, MA 02215 USA
3Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115 USA
4Broad Institute of Harvard and MIT, Cambridge, MA 02142, USA
5Biomedical Research Center, Korea Institute of Science and Technology, Seoul, Korea

Summary

Upon stimulation by pathogen-associated inflammatory signals, the atypical IκB kinase TBK1 induces type-I interferon expression and modulates NF-κB signaling. Here we describe the 2.4 Å-resolution crystal structure of nearly full-length TBK1 in complex with specific inhibitors. The structure reveals a novel dimeric assembly, created by an extensive network of interactions among the kinase, ubiquitin-like (ULD) and scaffold/dimerization (SDD) domains. An intact TBK1 dimer undergoes K63-linked polyubiquitination on Lysine 30 and Lysine 401, and these modifications are required for TBK1 activity. The ubiquitination sites and dimer contacts are conserved in the close homolog IKKε, but not in the canonical IκB kinase IKKβ, which assembles in an unrelated manner. The multidomain architecture of TBK1 provides a structural platform for integrating ubiquitination with kinase activation and IRF3 phosphorylation. The structure of TBK1 will facilitate studies of the atypical IκB kinases in normal and disease physiology and will further development of more specific inhibitors that may be useful as anti-cancer or anti-inflammatory agents.

Introduction

The NF-κB transcription factors are central regulators of innate immunity, inflammation, cell proliferation and apoptosis (Dolcet et al., 2005; Karin, 2006). Their activity is tightly regulated through the control of the IκB kinase (IKK) family of proteins. The canonical IKK complex consists of the catalytically active IKKα and IKKβ subunits as well as the regulatory subunit, IKKγ/NEMO (Hayden and Ghosh, 2004). In response to stimuli such as

© 2013 The Authors, Published by Elsevier Inc. All rights reserved.
†To whom correspondence should be addressed: Michael J. Eck eck@red.dfci.harvard.edu or David A. Barbie dbarbie@partners.org.
*These authors contributed equally to this work

Publisher’s Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
cytokines, non-degradative Lys63(K63)-linked and linear (Met1)-linked polyubiquitination of NEMO results in the activation of the IKK\(\alpha\) and IKK\(\beta\) kinases (Bianchi and Meier, 2009; Tang et al., 2003; Zhou et al., 2004). These activated kinases phosphorylate the inhibitor of NF-\(\kappa\)B (I\(\kappa\)B) proteins, resulting in their degradative Lys48(K48)-linked polyubiquitination and subsequent proteasome-mediated degradation. Upon degradation of the I\(\kappa\)B proteins, the NF-\(\kappa\)B dimers translocate into the nucleus and activate the transcription of effector genes that mediate immune and inflammatory responses and regulate cell survival (Hacker and Karin, 2006).

In addition to the IKK\(\alpha\) and IKK\(\beta\) kinases, two closely related serine-threonine kinases, Tank-binding kinase (TBK1) and inhibitor of \(\kappa\)B kinase \(\varepsilon\) (IKK\(\varepsilon\)) play key distinct roles in innate immune responses to viral infection and other pathogen-associated inflammatory stimuli by inducing type-I interferon expression and modulating NF-\(\kappa\)B signaling (Bonnard et al., 2000; Peters et al., 2000; Pomerantz and Baltimore, 1999; Shimada et al., 1999). TBK1 and IKK\(\varepsilon\) are found together in a complex and share several binding partners including TANK (Chariot et al., 2002; Goncalves et al., 2011), which facilitates inter-regulation of the canonical IKKs (Clark et al., 2011b). TBK1 is constitutively expressed and TBK1-deficient mice exhibit embryonic lethality due to widespread hepatic apoptosis, a phenotype that closely resembles IKK\(\beta\)-deficient mice (Bonnard et al., 2000; Li et al., 1999).

By contrast, the expression of IKK\(\varepsilon\) is inducible and largely immune cell-specific, reflected in the observation that IKK\(\varepsilon\)-deficient mice are viable, but hypersensitive to viral infection (Tenoever et al., 2007). IKK\(\varepsilon\)-deficient mice are also less prone to diet-induced obesity and inflammation (Chiang et al., 2009).

Upon activation by Toll-like receptors (TLRs) or cytoplasmic RIG-1 like receptors (RLRs), TBK1 and IKK\(\varepsilon\) stimulate type I interferon production via direct phosphorylation of transcription factors IRF3 and IRF7 (Chau et al., 2008). TLR-mediated activation of TBK1 involves TRIF or MYD88-dependent pathways, while engagement of RLRs activates the mitochondrial adaptor MAVS, which facilitates TBK1/IKK\(\varepsilon\) mediated activation of IRF3/7 and NF-\(\kappa\)B. Recently, the adaptor protein STING was found to play an essential role in the signaling response to cytoplasmic dsDNA, promoting TBK1-specific activation of IRF3 as well as STAT6 (Chen et al., 2011; Ishikawa and Barber, 2008). TBK1 also plays a role in mediating autophagy in response to intracellular bacterial pathogens (Radtke et al., 2007; Thurston et al., 2009; Wild et al., 2011). Thus, TBK1 and IKK\(\varepsilon\) play essential roles in both antiviral and antibacterial innate immunity.

In addition to their role in innate immunity, TBK1 and IKK\(\varepsilon\) contribute directly to cell transformation (Shen and Hahn, 2011). IKK\(\varepsilon\) is a breast cancer oncogene amplified in 30% of breast cancers. In these cancers, IKK\(\varepsilon\)-mediated activation of NF-\(\kappa\)B signaling is required for transformation, at least in part through phosphorylation of the tumor suppressor CYLD (Boehm et al., 2007; Hutti et al., 2009) and TRAF2 (Shen et al., 2012). In cancers dependent on KRAS-signaling, RALB-mediated activation of TBK1 promotes cell survival (Barbie et al., 2009; Chien et al., 2006; Xie et al., 2011). These observations implicate these two serine-threonine kinases as potential therapeutic targets in cancer.

Although the IKK-related kinases exhibit partial homology to the IKK\(\alpha\) and IKK\(\beta\) kinases, these kinases play distinct roles in both normal and malignant physiology. Indeed, the kinase domain of TBK1 shares only \approx 35% sequence identity with that of the canonical IKKs and the SDD domain is quite divergent with only \approx 10% identity over 250 residues. To understand the biochemical differences among these major immune-signaling kinases, we have undertaken structural and biochemical studies and describe a high-resolution crystal structure of nearly full-length TBK1 in complex with small molecule inhibitors MRT67307 (Clark et al., 2011a) and BX795 (Clark et al., 2009). The structure reveals a novel dimeric...
assembly that is stabilized by an extensive network of interactions among the kinase, ubiquitin-like (ULD) and scaffold/dimerization (SDD) domains. The contacts that stabilize the dimer are largely conserved in the close homolog IKKe, but not in the canonical IkB kinase IKKβ, which dimerizes in an unrelated manner (Xu et al., 2011). In addition, the SDD domain in TBK1 adopts a very different conformation from that recently observed in the corresponding domain of IKKβ (Xu et al., 2011). We find that TBK1 undergoes K63-linked ubiquitination at sites in the kinase domain (Lys30) and SDD domain (Lys401). These modifications require an intact dimer, and are in turn required for TBK1 activation. Our data provide a structural foundation for understanding TBK1 regulation and for further dissection of the functional differences observed between the canonical IkB kinases and the IKK-related kinases TBK1 and IKKe.

Results
Structure of TBK1
We crystallized and determined the structure of TBK1 (residues 1–657) in complex with inhibitors MRT67303 and BX795 at resolutions of 2.4Å and 2.5Å, respectively (see Supplementary Table S1 for crystallographic details). In addition, we refined a third structure crystallized under different buffer conditions at a resolution of 3.3 Å. To obtain protein in a homogeneous, non-phosphorylated state amenable to crystallization, we expressed the kinase-dead D135N mutant and truncated the C-terminal domain (CTD, Figure 1A). The crystal structure reveals an elongated dimer with a central stalk formed by the two SDD domains, which run roughly parallel to each other along the length of the dimer. The kinase and ULD domains interact with each other and with the SDD domain to form a globular head at one end of the stalk (Figure 1B). As discussed below, the kinase and ULD domains also contribute to dimerization via multiple contacts with the SDD domain of the opposite subunit in the dimer. The SDD domain resembles a triple-helical coiled-coil and organizes the structure as a whole (Figure 1B, Supplementary Figure S1B). Two of the SDD helices are continuous along the entire length of the domain; the first spans residues 408–481(α1) and the third spans residues 577–651(α3). The intervening segment is mostly helical (α2a, α2b, and α2c), but is interrupted by two loop segments that appear to accommodate the overall superhelical twist of the domain. The ULD and kinase domains associate primarily with the first helix in the SDD (Figure 1C, E). The interactions of the ULD domain are particularly extensive and largely hydrophobic. The saddle-shaped surface formed by strands β1-β5 in the ULD domain arch across the SDD at approximately its midpoint, centered on Gly442. Phe380 in the ULD domain packs against Gly442, which is conserved in both IKKs and IKK-related kinases (Supplementary Figure S1A). Additional ULD/SDD interactions are illustrated in Figure 1C. The linker connecting the ULD and SDD domain adopts an extended conformation and packs in a hydrophobic groove between the first and third helices in the SDD (Figure 1D). The extensive hydrophobic contact among the ULD and SDD domains and the intervening linker suggest that these elements comprise a fixed structural unit that is unlikely to dissociate or rearrange in the context of TBK1 recruitment and activation. The kinase C-lobe is also well anchored but largely by polar interactions with the SDD (Figure 1E) and ULD domains (Figure 1F). Structures have recently been reported for the isolated ULD (Li et al., 2012) and kinase domains of TBK1 (Ma et al., 2012), and for a fragment containing both the kinase and ULD domains (Ma et al., 2012). Comparison with these structures reveals essentially identical interdomain orientations, indicating that the kinase/ULD interaction is well-defined irrespective of scaffolding interactions with the SDD (Supplementary Figures S1C, D).

The TBK1 kinase domain exhibits the conserved protein kinase architecture consisting of N- and C-terminal lobes with the active site cleft at the interface. In the present structures, the kinase domain adopts an inactive conformation, with the C-helix and key residue Glu55
displaced from the active site (Figures 2A, S1C). The loop that connects strand β3 to the C-helix is partially disordered. Rotations of the C-helix are a regulatory feature of many kinases, and a conserved glutamic acid in this helix makes a key salt bridge interaction with an active site lysine residue when the C-helix pivots into the inward, active position. In some kinases, the non-phosphorylated activation loop plays a role in maintaining the outward, inactive position of the C-helix. This appears not to be the case in TBK1, as the activation loop is largely disordered in the present structure. The most N-terminal portion of the activation loop, the DFG motif, is well resolved, but the remainder of the loop (residues 160–175) is not visible in the electron density map. TBK1 is activated by phosphorylation of Ser172 in the activation loop, and comparison with the recently described structure of the Ser172-phosphorylated kinase domain shows that the activation loop is indeed organized by phosphorylation (Ma et al., 2012), and that Ser172 phosphorylation also promotes the active position of the C-helix (Supplementary Figure 1C).

Inhibitor binding

The compound BX795 was developed as an inhibitor of the kinase PDK1 (Feldman et al., 2005) and was later discovered to potently inhibit TBK1 (Clark et al., 2009). The related compound MRT67307 was developed as a more specific inhibitor of TBK1 (IC$_{50}$ = 19 nM) (Clark et al., 2011a). These compounds share a common anilinopyrimidine core and bind in a similar manner, with the pyrimidine and aniline nitrogen atoms hydrogen bonding with the backbone amide and carbonyl groups, respectively, of Cys89 in the “hinge” segment at the edge of the ATP-binding cleft (Figure 2B, Supplemental Figure S2). The pyrimidine and aniline rings are roughly co-planar, and in contact with Met142 and Gly92 on the floor of the nucleotide binding cleft. In MRT67307, the methylmorpholino substituent contacts Phe88 in the hinge, while the cyclobutyl group located on the pyrimidine 4-substituent packs between P-loop residues Gly18 and Val23 and Asp157 in the DFG motif (Figure 2B). The lack of activity of MRT67307 on the canonical IKKs appears to stem from several structural differences within the binding site, in particular Phe88 in TBK1 is replaced with a tyrosine in IKKβ (which would interfere with the observed orientation of the morpholino group), and Thr156 is replaced with isoleucine (which would eliminate a water-mediated hydrogen bond with the carbonyl in the inhibitor).

TBK1 dimer contacts are conserved and required for signaling

The dimeric architecture of TBK1 appears to be critically important for its function and is created by conserved interactions involving each of its domains (Figure 3A, B). The crystallographic asymmetric unit contains a single copy of the TBK1 polypeptide chain, and the dimer lies on a two-fold symmetry axis in the P3$_2$121 space group. Examination of the crystal lattice reveals a second possible dimer relationship in which the subunits could associate via a “head to head” contact between the kinase domains to yield an extremely elongated dimer (Supplementary Figure S3A). Visualization of the purified TBK1 protein by negative stain electron microscopy confirms the side-by-side dimer interpretation described here (Supplementary Figures S3B and S3C). Analysis of the oligomeric state of TBK1 in solution using SEC-MALS showed that both the full-length protein (residues 1–729) and the crystallized construct are dimeric (Supplementary Figure S3D). Thus the truncated CTD region, which contains the binding site for TANK and other adaptors, is not required for the structural integrity of the dimer.

The dimer is stabilized by contacts between the ULD and kinase domain with the SDD of the contralateral subunit (Figure 3A). Although there is little direct contact between the SDD domains in the 2.4 and 2.5Å (form I) structures described here, we observe considerable interaction in an alternate crystal form obtained using 2-methyl-2,4-pentanediol (MPD) rather than polyethylene glycol as a crystallization agent (form II crystals, Supplementary
Figure S3E,F). The ULD domain contributes its “EGR” sequence (residues 355–357) to the dimer interface. In each of the ULD domains, Glu355 forms a salt bridge with Arg444 and also hydrogen bonds with the indole nitrogen of Trp445 in the SDD of the opposite subunit. Gly356 packs against the dimer-related Gly356 residue, and Arg357 forms a salt bridge with Asp452 in the contralateral SDD (Figure 3A).

Both the N- and C-lobes of the kinase domain participate in dimer contacts with the SDD of the opposite subunit (Figure 3A). In the N-lobe, a salt-bridge is formed between Asp33 in the kinase and Lys589 in the SDD. In the C-lobe, the β7-β8 turn contacts the SDD of the opposing subunit. While the dimer interactions do not block the kinase active site, they do appear to “clamp” the relative orientations of its N- and C-lobes. Because kinase activity is thought to require flexibility between the lobes, this arrangement is suggestive of an autoinhibited state of TBK1. Thus we measured the specific activity of dimeric TBK1, including both the full-length protein and a 1–657 construct (Supplementary Table S2). Both are highly active in vitro, with specific activity similar to that of the isolated kinase domain (residues 1–310), which is monomeric. The dimer interaction does not block activation loop auto-phosphorylation in trans, and the Ser172-phosphorylated protein is also dimeric (Supplementary Figure S3D). These data indicate that TBK1 activation does not involve dissociation of the dimer. Furthermore, the similar N- and C-lobe orientations observed in the activated kinase domain (Supplementary Figure S1C) suggests that the dimer interactions of the kinase domain proper could be largely maintained upon activation.

Mapping of the evolutionary variation among vertebrate TBK1 and IKKe sequences onto the surface of the protein shows that the dimer contacts are generally well conserved, indicating that the dimer is functionally significant and that IKKe is very similarly organized (Figure 3B). Residues involved in the N-lobe contact are particularly highly conserved. We note also that the N-lobe contact on the SDD is part of a more extensive conserved surface that wraps onto the opposite face of the SDD; the conservation of this surface is striking in contrast to the remainder of the exposed surface of the SDD (Figure 3B, right panel). We tested the importance of the dimer interaction surfaces for TBK1 activation and signaling (Figure 3C). In the ULD dimer interface, the E355A, R357A, and E355A/R357A double mutation abrogated phosphorylation of TBK1 on Ser172 as well as phosphorylation of IRF3. Mutations in the kinase/SDD dimer interface also impaired TBK1 activation and IRF3 phosphorylation (D33A in the kinase domain; R547D and K589D in the SDD domain). By contrast, mutation of kinase residue K251, which is not involved in dimer formation, was without effect. Mutations in each of the dimer contacts also impaired activation of both interferon-stimulated response element (ISRE) and NF-κB-activated luciferase reporters (Figure 3D). Moreover, expression of these mutants failed to induce expression of the direct IRF3 target gene IFNB1, in addition to RANTES (Figure 3D). We also tested the dimer interface mutants for their ability to form dimers in vitro. The R547D and E355A/R357A mutants were largely monomeric in solution (Figure 3E). However, the D33A and E355A single point mutations were not sufficient to disrupt the dimer in vitro, at least at the relatively high protein concentrations required for analysis by multi-angle light scattering. We further found that the R547D and E355A/R357A mutants impaired dimer formation in a cellular context as well, as assessed by co-immunoprecipitation of Flag- and V5-tagged constructs expressed in HEK293T cells (Figure 3F).

The larger conserved surface on the end of the SDD (including residues Tyr577, Glu580 and Ile582) also appears to be required for efficient IRF3 phosphorylation and expression of IFNB1 and RANTES mRNA (Figure 3G). Strikingly, however, mutation of these residues does not impair TBK1 phosphorylation (Figure 3G). These residues are not involved in dimerization or other interdomain contacts; thus, it will be of interest to test directly the role of these surfaces in recognition of TBK1 binding partners and substrates.
TBK1 and IKKβ differ in their mode of dimerization and quaternary organization

TBK1 and IKKβ are both dimers composed of sequential kinase, ULD, linker and SDD domains, but the sequence and conformations of their SDD domains are markedly divergent. Thus their mode of dimerization and overall quaternary organization is quite different. Interestingly, superposition of the ULD domains of TBK1 and IKKβ shows that both interact with the first long helix in the SDD domain, centered on a glycine residue that is conserved in primary sequence (Figure 4A). This superposition supports the notion that the entire domain assembly of both TBK1 and IKKβ share a common evolutionary origin, and it also highlights the structural divergence in the SDD domains. Despite the observation that both share a “down-up-down” topology of the three mostly helical segments, their conformation is quite different and the domains overall cannot be superimposed along their entire length (Supplementary Figure S4). In IKKβ, the end of the final helical segment in the SDD mediates dimerization (Xu et al., 2011), while in TBK1 it is the end of the first helical segment that contributes to the SDD/SDD interface in the form II crystals (Supplementary Figure S3F, S4A). The divergence in the SDD domains begets larger differences in other domain interactions; the ULD domains in IKKβ extend away from the SDD domain of the opposite subunit, while in TBK1 they bridge between the two SDD domains (Figure 4B).

K63-linked polyubiquitination of TBK1 on Lys30 and Lys401 is required for TBK1 activation and signaling

In the accompanying manuscript, Zhou et al. show that K63-linked polyubiquitination of IKKε at Lys30 and Lys401 is required for its catalytic activation. Mutation of Lys30 and Lys401 blocks IKKε kinase function, NF-κB activation and transformation. These modified lysine residues are conserved in TBK1 (with identical residue numbers, Figure S1A). Additionally, K63-linked polyubiquitination is required for viral activation of IRF3 (Zeng et al., 2009) and TBK1 associates with polyubiquitin-binding proteins including optineurin (Gleason et al., 2011). Thus we determined whether TBK1 is similarly modified by K63-polyubiquitination. We co-transfected HEK293T cells with GST-TBK1 and either wild type HA-epitope tagged ubiquitin or a HA-tagged mutant ubiquitin that forms only K63-linked chains. When we isolated GST immune complexes and looked for HA-epitope tagged ubiquitin, we confirmed that TBK1 is also modified by K63-linked ubiquitination (Figure 5A). This ubiquitination does not require TBK1 activity, as WT and a kinase-dead mutant TBK1 construct were similarly modified (Figure 5B). Individual K30R and K401R point mutations exhibited decreased TBK1 ubiquitination, and we failed to observe ubiquitination of the TBK1 K30R/K401R double mutant (Figure 5C).

We next examined the effect of dimer-disrupting mutations on TBK1 ubiquitination. The E355A/R357D, R547D and D33A mutants exhibited markedly decreased K63-linked polyubiquitination in HEK293T cells (Figure 5D). In contrast, the E355A and H459E/N474A mutants that retained the ability to form dimers (Figure 3E) were still ubiquitinated. We also found that the D33A mutant showed markedly decreased ubiquitination, despite retaining the ability to form dimers in solution. We note that Asp33 is proximal to the Lys30 ubiquitination site, thus this mutation may interfere with ubiquitination by affecting recognition of the modification site.

We next tested the effect of point mutations in the ubiquitination sites on TBK1-driven gene expression and on TBK1 activation. While the individual TBK1 K30R and K401R mutants induced IFNB1 and RANTES mRNA levels to a similar degree as WT TBK1, the TBK1 K30R/K401R mutant failed to induce IFNB1 and RANTES mRNA levels (Figure 6A). Addition of the “phosphomimetic” S172E mutation did not bypass the requirement for ubiquitination, but this mutation has been previously shown to be ineffective in activating TBK1 (Kishore et al., 2002). Consistent with their effects on IFNB1 and RANTES.
expression, we observed that the single point mutants retained constitutive phosphorylation of Ser172, whereas the K30R/K401R double mutant was not phosphorylated, even when expressed at similar levels (Figure 6B). It is unlikely that the K30R and K401R mutations affect folding or stability of TBK1, as both residues are exposed on the surface of the dimer (Figure 6C). In addition, we note that the individual K30R and K401R mutants retained activity and at least some degree of K63-linked polyubiquitination and also supported TBK1 phosphorylation (Figure 5C). We further observed that while dimerization and K63-polyubiquitination mutants exhibited impaired TBK1 activation and signaling in cells, these mutants do not abolish TBK1 kinase activity in vitro (Supplementary Figure S5).

To determine the requirements for dimerization and ubiquitination in a physiologic setting, we reconstituted TBK1−/− mouse embryo fibroblasts (MEFs) with wild type or mutant TBK1 constructs and tested the effect of poly I:C stimulation on interferon-β expression (Figure 6D). Stimulation increased IFNB1 mRNA levels in cells reconstituted with wild type TBK1 at 2 and 4 hours, as expected. This response was ablated in cells reconstituted with dimerization defective TBK1 mutants E355A/R357A and R547D, and in the K30R/K401R polyubiquitination mutant (Figure 6D). We conclude that like IKKe (Zhou et al. accompanying manuscript), TBK1 undergoes K63-linked polyubiquitination on conserved Lysines 30 and 401. Polyubiquitination on at least one of these two sites and dimer formation are required for phosphorylation of Ser172 in the kinase activation loop and regulation of IRF-responsive genes.

**Discussion**

TBK1 is engaged by a diversity of stimuli, yet how it is activated in these contexts remains incompletely characterized. In the setting of cytosolic dsDNA, STING acts as a scaffold that recruits IRF3 and TBK1 to its C-terminal tail (Tanaka and Chen, 2012). Oligomerization of STING and ER localization upon cytosolic DNA detection recruits both TBK1 and IRF3, promotes TBK1 Ser172 phosphorylation, and thus facilitates specific activation of IRF3. A similar mechanism may facilitate phosphorylation of STAT6 by TBK1 in the setting of antiviral innate immunity (Chen et al., 2011). Recent work has also demonstrated that viral infection and RLR-mediated MAVS activation induces large functional aggregates on the mitochondrial membrane that involve K63-linked polyubiquitination and recruitment of TRAF2 and TRAF6 (Hou et al., 2011). Recruitment of NEMO to these MAVS complexes may bind TANK and TBK1 to promote its activation (Zeng et al., 2009). A similar model was proposed for MYD88-dependent TBK1 activation that occurs downstream of IL1 or specific TLRs (Clark et al., 2011b). In this setting K63-linked polyubiquitin chains formed by TRAF6 are proposed to recruit a complex including NEMO and TANK that links TAK1 and the canonical IKK complex to TBK1/IKKε Ser172 phosphorylation and activation.

In addition to these recruitment models, direct K63-linked polyubiquitination of TBK1 by TRAF3, NDRP1, or MIB1 E3 ligases may be involved in its innate-immune mediated activation of TBK1 (Li et al., 2011; Parvatiyar et al., 2010; Wang et al., 2009). CYLD-mediated deubiquitination of TBK1 has also been shown to inhibit the TBK1-driven IRF3 response (Friedman et al., 2008). A recent report identifies Lys69, Lys154 and Lys372 as K63-linked polyubiquitination sites in TBK1 (Wang et al., 2012). From a structural perspective, two of these sites appear unlikely to represent regulatory modification sites. Lys154 forms key structural interactions, and is near-universally conserved among all protein kinases. Mutation or modification of this residue can be expected to disrupt the fold of the kinase domain. Lys372 is among the most variable residues in vertebrate TBK1 and IKKε sequences, and access to this residue appears to be impeded in the TBK1 dimer. Lys69, however, is relatively exposed for modification and lies near the Lys30 and Lys401 sites we have identified. In the accompanying manuscript, Zhou et al. have dissected the
essential role of K63-linked polyubiquitination in the regulation of IKKε in the context of both cell transformation and immune signaling. Interestingly, the two essential residues for IKKε K63-linked polyubiquitination, Lys30 and Lys401, are conserved with TBK1. We demonstrate here that K63-linked polyubiquitination of TBK1 at Lys30 and Lys401 is essential for its downstream activity. Our structural work reveals that these two residues are found on opposing faces of the TBK1 monomers but are in close proximity in the context of the catalytically active TBK1 dimer (Figure 6C). Furthermore, mutation of both sites disrupts signaling to IFN-β following TBK1 overexpression in HEK293T cells, or upon poly I:C stimulation in TBK1-null MEFs. TBK1 Ser172 phosphorylation is also disrupted in the K30R/K401R mutant, suggesting that ubiquitination is directly required for TBK1 activation in a cellular milieu.

The mechanistic role of K63-linked ubiquitination at these sites remains to be fully elucidated. The active sites of the two kinase domains lie on opposite faces of the TBK1 dimer, and therefore the isolated dimer cannot autoactivate. Thus K63-linked ubiquitination may regulate recruitment of another kinase that promotes Ser172 phosphorylation, and it may promote assembly of TBK1 into higher order complexes that allow its autoactivation in trans. In support of this model, NEMO has been shown to bind to polyubiquitinated TBK1 via its ubiquitin binding domains (Wang et al., 2012). Furthermore, TBK1 binds the NEMO-related protein optineurin as well as adaptor NDP52, both of which recognize K63-linked polyubiquitinated proteins (Morton et al., 2008; Thurston et al., 2009; Wild et al., 2011). Mutation of the ubiquitin recognition domain of optineurin impairs activation of TBK1 in response to the TLR agonists LPS and polyI:C (Gleason et al., 2011). While a polyubiquitin-dependent recruitment or clustering mechanism appears to be an essential component of TBK1 activation, we cannot rule out the possibility that ubiquitination could also play an allosteric role by releasing or modifying intramolecular contacts of the kinase N- and C-lobes to promote TBK1 activation.

Taken together, these observations reveal a multi-step mechanism for TBK1 activation in which K63-linked polyubiquitination is a pre-requisite for kinase activation by Ser172-phosphorylation in cells. The dimeric, multidomain architecture of TBK1 is critical for orchestrating these regulatory events, and for subsequent phosphorylation of IRF3. Dimer interface mutations in the kinase (D33A), ULD (E355A/R357A) or SDD domains (R547D) block K63-linked polyubiquitination, and thus also prevent Ser172 phosphorylation. Our biophysical characterization of the monomeric proteins shows that they are not simply misfolded, and we find that even the isolated (monomeric) kinase domain retains kinase activity in vitro. It is unclear whether kinase activation is the only role of polyubiquitination, because the S172E phosphomimetic mutant does not effectively activate TBK1 (Kishore et al., 2002). In addition to its roles in mediating dimer formation and as a site of polyubiquitination, the SDD domain is also required for proper substrate phosphorylation in cells – the conserved surface surrounding Glu580 is required for IRF3 phosphorylation and reporter activation, despite the fact that it is dispensable for TBK1 activation per se. The SDD is not required for efficient IRF3 phosphorylation in vitro, as the isolated kinase domain and intact TBK1 phosphorylate IRF3 with similar kinetics (Ma et al., 2012). We speculate that this surface may be a site of interaction with a substrate-recruiting adapter such as STING (Tanaka and Chen, 2012).

The conserved Glu580 surface of the TBK1 SDD domain, as well as its dimer contacts and polyubiquitination sites are preserved in IKKε, but not in the IKKα and IKKβ. Furthermore, the SDD- and ULD- mediated contacts with the N- and C-lobes of the kinase are not found in the IKKβ structure, which dimerizes in an essentially unrelated manner. Although the canonical IκB kinases and the IKK-related kinases TBK1 and IKKε share some homology, the dramatic structural divergence between these classes of IKKs reflects their different
biology and regulation. Indeed, although TBK1 and IKKε were discovered vis-à-vis their intersections with NF-κB signaling, it is increasingly clear that many of their major functions are quite distinct from this pathway and from those of canonical IKKs. The present data provide a structural foundation for understanding the regulation and interactions of both TBK1 and IKKε, and for further dissection of participation of TBK1 in diverse processes including viral clearance, autophagy (Weidberg and Elazar, 2011; Wild et al., 2011), and survival of KRAS-dependent cancers (Barbie et al., 2009; Ou et al., 2011; Xie et al., 2011). Additionally, the structures described here will facilitate the development of small molecule inhibitors that may be useful as anti-cancer or anti-inflammatory agents.

**Experimental Procedures**

**Expression Constructs**

The pCMV6-XL5-TBK1-WT and pCMV6-XL5-TBK1-K38M constructs were purchased from Origene and PCR subcloned using Gateway technology into the pLEX980 vector. Sequencing of pLEX980-TBK1-WT and pLEX980-TBK1-K38M confirmed that these sequences were correct. TBK1 mutants of the pLEX980-TBK1-WT construct were generated using standard PCR mutagenesis, Gateway cloned back into the pLEX980 or the closely related pLEX304 vector, and sequence verified. HA-ubiquitin and HA-Ub K63-only constructs were used as described (Abbott et al., 2004; Boehm et al., 2007).

**Protein Expression, purification and crystallization**

Constructs spanning residues 1–310, 1–657 or full length (1–729) of human TBK1 bearing the wild-type sequence or D135N mutation were expressed as 6xHis plus glutathione-S-transferase (GST) fusion proteins in Hi5 insect cells using the pTriEx transfer vector (Novagen) and BacVector-3000 Baculoviral DNA (Novagen). Details of purification by Nickel-NTA and glutathione sepharose chromatography are given in Supplemental Methods online. The TBK1(1–657) D135N protein was concentrated to 8 mg/ml for, for crystallization. MRT67307 or BX-795 was added to the TBK1 protein solution to 0.25 mM immediately prior to crystallization. Crystals of form I were obtained in 4% (w/v) PEG8000 and 100 mM Tris pH 8.7. Crystals of form II grew in 5% MPD and 100 mM Hepes pH 7.5. The SeMet TBK1 protein was crystallized at 7 mg/ml with BX795 added to 0.5 mM in hanging drops over a reservoir containing 5% PEG6000, 2.5% MPD, 100 mM Hepes pH 7.5, and 10 mM TCEP.

**Structure determination**

The structure was determined by SAD phasing with a 3.65 Å SeMet derivate dataset collected on the NE-CAT beamline at Argonne National Laboratory. The TBK1/BX795 structure was refined to a final R-value of 19.7% (Rfree=25.5%) at 2.5Å resolution, and the TBK1/MRT67307 to a final R-value of 19.9% (Rfree=23.6%) at 2.4 Å resolution. The form II crystal structure was refined to an R-value of 22% (Rfree=27.9%) at 3.3Å resolution. Crystallographic data collection and refinement statistics are presented in Supplementary Table S1, and further details of structure determination and refinement are provided in Supplemental Methods online.

**In-vitro enzyme kinetics and other biophysical studies**

Kinase assays were carried out in triplicate using the ATP/NADH coupled assay system in a 96-well format as described (Yun et al., 2007). Further details and experimental procedures for IP kinase assays, in vitro phosphorylation of kinase-dead TBK1 proteins, SEC-MALS, and Electron Microscopy are provided in Supplemental Methods online.
**Reporter Assays and Quantitative Reverse-Transcriptase PCR**

HEK293T cells were seeded in 6 well plates at $7 \times 10^5$ cells/well, and reverse transfected using Fugene 6 Reagent. Cells were transfected with 1µg pLEX980-EGFP, pLEX980-TBK1-WT, TBK1-K38M, or the indicated TBK1 mutants together with 500 ng NFκB(1) Firefly luciferase reporter vector (Panomics) or 500 ng ISRE Firefly luciferase reporter vector (Panomics), and 500 ng pRL-TK Renilla luciferase control vector. At 24 h post-transfection cells were trypsinized and seeded at a density of 2500 cells/well in 384-well plates, with 64 replicate wells per condition. At 48 h post-transfection, luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega) or RNA was prepared for QRT-PCR, as described in Supplemental Methods.

**Immunoblotting/Antibodies**

Lysates were prepared 60 h following transient transfection of HEK293T cells with TBK1 constructs using standard RIPA buffer. Immunoblotting was performed as previously described\(^3\). Antibodies were obtained from Cell Signaling Technology (anti-TBK1 #3013, anti-phospho-TBK1 Ser172 #5843),Santa Cruz (anti-IRF3 #sc-9082), Millipore (anti-phospho-IRF3 Ser 396), and HA (Clone12C5) (Boehringer Mannheim). Anti-V5 affinity gel sepharose was obtained from Sigma-Aldrich Glutathione affinity gel sepharose was obtained from GE Healthcare. Membranes were developed using both the Odyssey Infrared Imaging System (LICOR) as well as traditional ECL-based detection.

**Dimer Immunoprecipitation**

N-terminal FLAG and C-terminal V5-tagged pLEX-TBK1-WT, pLEX-TBK1-E355A/R357A, and pLEX-TBK1-R547D constructs were transiently transfected into HEK-293T cells and 48h later cells were lysed in RIPA buffer. FLAG immunoprecipitation was performed in RIPA buffer using FLAG conjugated agarose beads (Sigma). Immunoblotting of whole cell extracts and immunoprecipitated material was performed with a mouse anti-FLAG antibody (Sigma) or mouse anti-V5 antibody (Invitrogen).

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

We thank Dr. Natalia Shapiro and Sir Philip Cohen, MRC Protein Phosphorylation Unit, University of Dundee, Scotland, UK for synthesizing and sharing the MRT67307 used this study. We thank Nathanael Gray for helpful discussions and the NE-CAT beamline staff at the Advanced Photon Source, Argonne National Labs, for assistance with data collection and processing. This work was supported in part by grants from the U.S. NIH PO1 CA154303 (MJE, WCH), R01 CA080942 (MJE), and R01 GM071834 (MJE), and RO1 CA080942 (MJE). D.A.B. is supported by a V Scholar Grant from the V Foundation for Cancer Research. W.C.H. and M.J.E. are consultants for Novartis Pharmaceuticals.

Crystallographic coordinates and structure factors were deposited in the Protein Data Bank with accession codes 4IM0 (MRT67307 complex), 4IM2 (BX795 complex) and 4IM3 (form II structure).

**References**

Abbott DW, Wilkins A, Asara JM, Cantley LC. The Crohn's disease protein, NOD2, requires RIP2 in order to induce ubiquitylation of a novel site on NEMO. Curr Biol. 2004; 14:2217–2227. [PubMed: 15620648]

Ashkenazy H, Erez E, Martz E, Pupko T, Ben-Tal N. ConSurf 2010: calculating evolutionary conservation in sequence and structure of proteins and nucleic acids. Nucleic Acids Res. 2010; 38:W529–W533. [PubMed: 20478830]
Barbie DA, Tamayo P, Boehm JS, Kim SY, Moody SE, Dunn IF, Schinzel AC, Sandy P, Meylan E, Scholl C, et al. Systematic RNA interference reveals that oncogenic KRAS-driven cancers require TBK1. Nature. 2009; 462:108–112. [PubMed: 19847166]

Bianchi K, Meier P. A tangled web of ubiquitin chains: breaking news in TNF-R1 signaling. Mol Cell. 2009; 36:736–742. [PubMed: 20050583]

Boehm JS, Zhao JJ, Yao J, Kim SY, Firestein R, Dunn IF, Sjostrom SK, Garraway LA, Weremowicz S, Richardson AL, et al. Integrative genomic approaches identify IKBKE as a breast cancer oncogene. Cell. 2007; 129:1065–1079. [PubMed: 17574021]

Bonnard M, Mirtsos C, Suzuki S, Graham K, Huang J, Ng M, Itie A, Wakeham A, Shahinian A, Henzel WJ, et al. Deficiency of T2K leads to apoptotic liver degeneration and impaired NF-kappaB-dependent gene transcription. EMBO J. 2000; 19:4976–4985. [PubMed: 10990461]

Chariot A, Leonardi A, Muller J, Bonif M, Brown K, Siebenlist U. Association of the adaptor TANK with the I kappa B kinase (IKK) regulator NEMO connects IKK complexes with IKK epsilon and TBK1 kinases. J Biol Chem. 2002; 277:37029–37036. [PubMed: 12133833]

Chau TL, Gioia R, Gatot JS, Patrascu F, Carpentier I, Chapelle JP, O'Neill L, Beyeart R, Piette J, Chariot A. Are the IKKs and IKK-related kinases TBK1 and IKK-epsilon similarly activated? Trends Biochem Sci. 2008; 33:171–180. [PubMed: 18353649]

Chen H, Sun H, You F, Sun W, Zhou X, Chen L, Yang J, Wang Y, Tang H, Guan Y, et al. Activation of STAT6 by STING is critical for antiviral innate immunity. Cell. 2011; 147:436–446. [PubMed: 22000020]

Chiang SH, Bazuine M, Lumeng CN, Geletka LM, Mowers J, White NM, Ma JT, Zhou J, Qi N, Westcott D, et al. The protein kinase IKKepsilon regulates energy balance in obese mice. Cell. 2009; 138:961–975. [PubMed: 19737522]

Chien Y, Kim S, Buneister R, Loo YM, Kwon SW, Johnson CL, Balakireva MG, Romeo Y, Kopelovich L, Gale M Jr, et al. RalB GTPase-mediated activation of the IkappaB family kinase TBK1 couples innate immune signaling to tumor cell survival. Cell. 2006; 127:157–170. [PubMed: 17018283]

Clark K, Peggie M, Plater L, Sorcek RJ, Young ER, Madwed JB, Hough J, McIver EG, Cohen P. Novel cross-talk within the IKK family controls innate immunity. Biochem J. 2011a; 434:93–104. [PubMed: 21138416]

Clark K, Plater L, Peggie M, Cohen P. Use of the pharmacological inhibitor BX795 to study the regulation and physiological roles of TBK1 and IkappaB kinase epsilon: a distinct upstream kinase mediates Ser-172 phosphorylation and activation. J Biol Chem. 2009; 284:14136–14146. [PubMed: 19307177]

Clark K, Takeuchi O, Akira S, Cohen P. The TRAF-associated protein TANK facilitates cross-talk within the IkappaB kinase family during Toll-like receptor signaling. Proc Natl Acad Sci U S A. 2011b; 108:17093–17098. [PubMed: 21949249]

Dolcet X, Llobet D, Pallares J, Matias-Guiu X. NF-kB in development and progression of human cancer. Virchows Archiv: an international journal of pathology. 2005; 446:475–482. [PubMed: 15856292]

Feldman RI, Wu JM, Polokoff MA, Kochanny MJ, Dinter H, Zhu D, Biroc SL, Aliche B, Bryant J, Yuan S, et al. Novel small molecule inhibitors of 3-phosphoinositidedependent kinase-1. J Biol Chem. 2005; 280:19867–19874. [PubMed: 15772071]

Friedman CS, O'Donnell MA, Legarda-Addison D, Ng A, Cardenas WB, Yount JS, Moran TM, Basler CF, Komuro A, Horvath CM, et al. The tumour suppressor CYLD is a negative regulator of RIG-I-mediated antiviral response. EMBO reports. 2008; 9:930–936. [PubMed: 18636086]

Gleason CE, Ordureau A, Gourlay R, Arthur JS, Cohen P. Polyubiquitin binding to optineurin is required for optimal activation of TANK-binding kinase 1 and production of interferon beta. J Biol Chem. 2011; 286:35663–35674. [PubMed: 21862579]

Goncalves A, Burckstummer T, Dixit V, Scheicher R, Gorna MW, Karayel E, Sugar C, Stukalov A, Berg T, Kralovics R, et al. Functional dissection of the TBK1 molecular network. PLoS One. 2011; 6:e23971. [PubMed: 21931631]

Hacker H, Karin M. Regulation and function of IKK and IKK-related kinases. Sci STKE. 2006; 2006:re13. [PubMed: 17047224]
Hayden MS, Ghosh S. Signaling to NF-kappaB. Genes Dev. 2004; 18:2195–2224. [PubMed: 15371334]

Hou F, Sun L, Zheng H, Skaug B, Jiang QX, Chen ZJ. MAVS forms functional prion-like aggregates to activate and propagate antiviral innate immune response. Cell. 2011; 146:448–461. [PubMed: 21782231]

Hutti JE, Shen RR, Abbott DW, Zhou AY, Sprott KM, Asara JM, Hahn WC, Cantley LC. Phosphorylation of the tumor suppressor CYLD by the breast cancer oncogene IKKepsilon promotes cell transformation. Mol Cell. 2009; 34:461–472. [PubMed: 19481526]

Ishikawa H, Barber GN. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. Nature. 2008; 455:674–678. [PubMed: 18724357]

Karin M. Nuclear factor-kappaB in cancer development and progression. Nature. 2006; 441:431–436. [PubMed: 16724054]

Kishore N, Huynh QK, Mathialagan S, Hall T, Rouw S, Creely D, Lange G, Caroll J, Reitz B, Donnelly A, et al. IKK-i and TBK-1 are enzymatically distinct from the homologous enzyme IKK-2: comparative analysis of recombinant human IKK-i, TBK-1, and IKK-2. J Biol Chem. 2002; 277:13840–13847. [PubMed: 11839743]

Li J, Li J, Miyahira A, Sun J, Liu Y, Cheng G, Liang H. Crystal structure of the ubiquitin-like domain of human TBK1. Protein & cell. 2012; 3:383–391. [PubMed: 22610919]

Li Q, Van Antwerp D, Mercurio F, Lee KF, Verma IM. Severe liver degeneration in mice lacking the IkappaB kinase 2 gene. Science. 1999; 284:321–325. [PubMed: 10195897]

Li S, Wang L, Berman M, Kong YY, Dorf ME. Mapping a Dynamic Innate Immunity Protein Interaction Network Regulating Type I Interferon Production. Immunity. 2011

Ma X, Helgason E, Phung QT, Quan CL, Iyer RS, Lee MW, Bowman KK, Starosvaskin MA, Dueber EC. Molecular basis of Tank-binding kinase 1 activation by transautophosphorylation. Proc Natl Acad Sci U S A. 2012; 109:9378–9383. [PubMed: 22619329]

Morton S, Hesslin L, Peggie M, Cohen P. Enhanced binding of TBK1 by an optineurin mutant that causes a familial form of primary open angle glaucoma. FEBS Lett. 2008; 582:997–1002. [PubMed: 18307994]

Ou YH, Torres M, Ram R, Formstecher E, Roland C, Cheng T, Brekken R, Wurz R, Tasker A, Polverino T, et al. TBK1 directly engages Akt/PKB survival signaling to support oncogenic transformation. Mol Cell. 2011; 41:458–470. [PubMed: 21329883]

Parvatiyar K, Barber GN, Harhaj EW. TAX1BP1 and A20 inhibit antiviral signaling by targeting TBK1-IKKi kinases. J Biol Chem. 2010; 285:14999–15009. [PubMed: 20304918]

Peters RT, Liao SM, Maniatis T. IKKepsilon is part of a novel PMA-inducible IkappaB kinase complex. Mol Cell. 2000; 5:513–522. [PubMed: 10882136]

Pomerantz JL, Baltimore D. NF-kappaB activation by a signaling complex containing TRAF2, TANK and TBK1, a novel IKK-related kinase. EMBO J. 1999; 18:6694–6704. [PubMed: 10581243]

Radtkle AL, Delbridge LM, Balachandran S, Barber GN, O'Riordan MX. TBK1 protects vacuolar integrity during intracellular bacterial infection. PLoS Pathog. 2007; 3:e29. [PubMed: 17335348]

Shen RR, Hahn WC. Emerging roles for the non-canonical IKKs in cancer. Oncogene. 2011; 30:631–641. [PubMed: 21042276]

Shen RR, Zhou AY, Kim E, Lim E, Habellhah H, Hahn WC. IKK{varepsilon} phosphorylates TRAF2 to promote mammary epithelial cell transformation. Mol Cell Biol. 2012

Shimada T, Kawai T, Takeda K, Matsumoto M, Inoue J, Kanamaru A, Akira S. IKK-i, a novel lipopolysaccharide-inducible kinase that is related to IkappaB kinases. Int Immunol. 1999; 11:1357–1362. [PubMed: 10421793]

Tang ED, Wang CY, Xiong Y, Guan KL. A role for NF-kappaB essential modifier/IkappaB kinase-gamma (NEMO/IKKgamma) ubiquitination in the activation of the IkappaB kinase complex by tumor necrosis factor-alpha. J Biol Chem. 2003; 278:37297–37305. [PubMed: 12867425]

Teneoever BR, Ng SL, Chua MA, McWhirter SM, Garcia-Sastre A, Maniatis T. Multiple functions of the IKK-related kinase IKKepsilon in interferon-mediated antiviral immunity. Science. 2007; 315:1274–1278. [PubMed: 17332413]
Thurston TL, Ryzhakov G, Bloor S, von Muhlinen N, Randow F. The TBK1 adaptor and autophagy receptor NDP52 restricts the proliferation of ubiquitin-coated bacteria. Nat Immunol. 2009; 10:1215–1221. [PubMed: 19820708]

Wang C, Chen T, Zhang J, Yang M, Li N, Xu X, Cao X. The E3 ubiquitin ligase Nrdp1 'preferentially' promotes TLR-mediated production of type I interferon. Nat Immunol. 2009; 10:744–752. [PubMed: 19483718]

Wang L, Li S, Dorf ME. NEMO Binds Ubiquitinated TANK-Binding Kinase 1 (TBK1) to Regulate Innate Immune Responses to RNA Viruses. PLoS One. 2012; 7:e43756. [PubMed: 23028469]

Weidberg H, Elazar Z. TBK1 Mediates Crosstalk Between the Innate Immune Response and Autophagy. Sci Signal. 2011; 4:pe39. [PubMed: 21868362]

Wild P, Farhan H, McEwan DG, Wagner S, Rogov VV, Brady NR, Richter B, Korac J, Waidmann O, Choudhary C, et al. Phosphorylation of the autophagy receptor optineurin restricts Salmonella growth. Science. 2011; 333:228–233. [PubMed: 21617041]

Xie X, Zhang D, Zhao B, Lu MK, You M, Condorelli G, Wang CY, Guan KL. IkappaB kinase epsilon and TANK-binding kinase 1 activate AKT by direct phosphorylation. Proc Natl Acad Sci U S A. 2011; 108:6474–6479. [PubMed: 21464307]

Xu G, Lo YC, Li Q, Napolitano G, Wu X, Jiang X, Dreano M, Karin M, Wu H. Crystal structure of inhibitor of kappaB kinase beta. Nature. 2011; 472:325–330. [PubMed: 21423167]

Yun CH, Boggon TJ, Li Y, Woo MS, Greulich H, Meyerson M, Eck MJ. Structures of lung cancer-derived EGFR mutants and inhibitor complexes: mechanism of activation and insights into differential inhibitor sensitivity. Cancer Cell. 2007; 11:217–227. [PubMed: 17349580]

Zeng W, Xu M, Liu S, Sun L, Chen ZJ. Key role of Ubc5 and lysine-63 polyubiquitination in viral activation of IRF3. Mol Cell. 2009; 36:315–325. [PubMed: 19854139]

Zhou H, Wertz I, O'Rourke K, Ultsch M, Seshagiri S, Eby M, Xiao W, Dixit VM. Bcl10 activates the NF-kappaB pathway through ubiquitination of NEMO. Nature. 2004; 427:167–171. [PubMed: 14695475]
Highlights

- TBK1 forms a dimer structurally distinct from IKKβ
- Crystal structure of TBK1 with inhibitor MRT67307 reveals basis for specificity versus canonical IKKs
- TBK1 is K63 polyubiquitinated on Lys30 in the kinase domain and Lys401 in the SDD domain
- K63-linked ubiquitination of dimerized TBK1 is required for kinase activation and signaling
Figure 1. TBK1 structure and interdomain interactions
(A) The domain structure of TBK1 includes the kinase domain (KD), ubiquitin-like domain (ULD), scaffold and dimerization domain (SDD) and C-terminal domain (CTD). The linker connecting the ULD and SDD domains is colored magenta and all domains are colored as in the structural representations presented here. (B) Overall structure of the TBK1 dimer in complex with MRT67307. The domains of the second subunit in the dimer are colored dark blue (kinase), orange (ULD) and tan (SDD). The compound is shown in a stick representation (yellow). (C) Interaction of the ULD with the helix α1 in the SDD. Each of the five β-strands of the ULD contributes to the mostly hydrophobic interface. (D) The linker segment (pink) that connects the ULD and SDD domains packs into the SDD (green.)
surface) via mostly hydrophobic interactions. (E) The largely polar interface between the kinase and SDD domains includes hydrogen bonds between Tyr564 and Glu100, Lys416 and Glu99, and Arg427 and the carbonyl of Ser266. (F) The ULD associates with the Clobe of the kinase domain. Tyr325 in the ULD hydrogen bonds with Glu109 in the kinase domain, and Lys323 in the ULD is positioned to make favorable electrostatic interactions with Glu109. See also supplementary Figure S1.
Figure 2. Structure of the kinase domain and interactions with MRT67307
(A) The kinase adopts an inactive conformation with helix αC rotated out of the active site. The activation loop is disordered (dashed line) beyond the DFG motif (red). (B) Detailed view of inhibitor interactions. MRT67307 forms dual hydrogen bonds with the amide and carbonyl groups of Cys89, as well as a water-mediated hydrogen bond with Thr156. Electron density for the compound and for the structure of the BX795 complex are shown in Supplementary Figure S2.
Figure 3. Structure, conservation and function of the TBK1 dimer interface

(A) Oblique view of the TBK1 dimer interface, highlighting interactions between the kinase and ULD domains of one subunit and the SDD domain of the opposite subunit in the dimer (tan). Hydrogen bonds that stabilize the dimer are indicated by dashed lines. (B) Conservation among 43 vertebrate TBK1 and IKKe sequences mapped onto the surface of TBK1. The inward, dimer-forming surface of TBK1 subunit is shown on the left, the outward-facing surface on the right. Contact points in the dimer interface are well-conserved (yellow circles). Note the highly conserved surface on the upper end of the SDD (surrounding Glu580). Analyses were performed with the CONSURF server (Ashkenazy et al., 2010). (C) Expression of WT or mutant TBK1 in HEK-293T cells following transient
transfection of the indicated dimer contact mutants. TBK1 protein levels, Ser172 phosphorylation, as well as total and Ser396 phospho-IRF3 were analyzed by immunoblotting 60 h post-transfection. The K38M mutant is a kinase-inactive positive control, K251A is a negative control mutation remote from the dimer interface. QRT-PCR for TBK1 mRNA expression was performed 48 h post-transfection. (D) EGFP control-normalized values for mRNA levels of IFNB1 and RANTES (upper panel) or ISRE and NF-κB luciferase reporter activity (lower panels) 48 h post-transfection of WT or mutant TBK1 constructs. Reporter activity was measured in tandem with a control renilla luciferase vector to which values were standardized. (E) Size exclusion chromatography-multi-angle light scattering (SEC-MALS) analysis of TBK1 dimer interface mutants. Purified TBK1 dimer interface mutants and wild type protein are analyzed on a Superdex 200 gel filtration column coupled to a multi-angle light scattering detector. All proteins analyzed contain the D135N catalytic site mutation because it can be expressed abundantly. The elution profiles as measured by refractive index are shown. The labeled horizontal traces indicate the measured molar mass, ~146.3 KDa for TBK1 wild type (expected molar mass of a dimer is 152.2 KDa) and ~ 86.9 KDa for E355A/R357A and ~ 95.1 KD for R547D. D33A, E355A and H459E/N474A elute at the same position as the wild type protein, and their molar masses are also similar. (F) Immunoblot of whole cell extracts (WCE, lower panels) or FLAG IP (upper panels) following transient transfection of FLAG- and V5-tagged TBK1-WT, TBK1-E355A/R357A, or TBK1-R547D constructs as indicated. Arrow indicates the specific band representing V5-TBK1. (G) Conserved SDD patch mutants were expressed in 293T cells and analyzed for total and phospho-TBK1 levels, total and phospho-IRF3 levels, TBK1 mRNA levels, as well as IFNB1 and RANTES expression. See also Supplementary Figure S2 for further analysis of the dimer.
Figure 4. Comparison with the structure of IKKβ

(A) Superposition of TBK1 with IKKβ (magenta). Structures are superimposed using the ULD, which highlights the general similarity in the manner in which the ULD anchors the kinase domains (KD) to the SDD in both proteins. This structural alignment also superimposes a glycine residue (black dot) in the ULD/SDD interface (Gly442 in TBK1 and Gly450 in IKKβ) that is conserved in both proteins and occurs in the same position in their primary sequences, despite the overall divergence in their SDD domains (see also Supplementary Figure S4E, F). Note also that the orientation of the kinase domains in the two structures is quite different relative to the ULD and SDD domains. (B) Surface views of TBK1 (top panels) and IKKβ (lower panels), with corresponding domains of IKKβ colored as in TBK1. In TBK1, the ULD domains bridge between dimer-related SDD domains, while in IKKβ they extend away from the opposite SDD domain. Likewise, the kinase domains in IKKβ are differently oriented and do not form dimer contacts. The IKKβ structure is drawn from PDB code 3QA8 (Xu et al., 2011).
Figure 5. TBK1 is modified by K63-linked polyubiquitin chains
(A) TBK1 is K63-linked polyubiquitinated. HA-tagged wild type or K63-only ubiquitin were cotransfected with GST-TBK1 into HEK293T cells. GST immune complexes (TBK1) were isolated followed by immunoblotting with the indicated antibodies. 5% of the whole cell lysate was loaded for comparison (input). (B) Wild type and kinase dead TBK1 are ubiquitinated equally. HA-tagged K63-only ubiquitin were cotransfected with wild type (WT) and kinase dead (KD) GST-TBK1 into HEK293T cells. GST immune complexes (TBK1) were isolated followed by immunoblotting with the indicated antibodies. 5% of the whole cell lysate was loaded for comparison (input). (C) TBK1 undergoes K63-linked polyubiquitination at Lys30 and Lys401. HEK293T cells were transfected as indicated. V5 immune complexes (TBK1) were isolated followed by immunoblotting with the indicated antibodies. 5% of the whole cell lysate was loaded for comparison (input). (D) TBK1 dimer-deficient mutants do not undergo K63-linked ubiquitination. HEK293T cells were transfected as indicated. V5 immune complexes (TBK1) were isolated followed by immunoblotting with the indicated antibodies. 5% of the whole cell lysate was loaded for comparison (input).
Figure 6. K63-linked polyubiquitination is required for TBK1-induced gene expression and kinase activation

(A) mRNA levels of INFβ1 and RANTES were measured using qRT-PCR 30 h following transient transfection of the indicated ubiquitination site mutants and normalized to control EGFP-transfected cells. (B) Immunoblot showing total TBK1 and Ser172 phospho-TBK1 levels 48 h post-transfection of the indicated constructs. (C) Locations of Lys30 and Lys401 ubiquitination mapped on TBK1 structure. One TBK1 monomer is in green, the other in cyan. Note that Lys401 from monomer “a” is close to Lys30 of monomer “b” across the dimer interface. (D) qRT-PCR measurement of INFβ1 and TBK1 mRNA following TBK1 reconstitution in TBK1−/− MEFs and stimulation with polyIC. Cells were nucleofected with...
EGFP control or the indicated TBK1 constructs, and 24h later were stimulated with 100 µg/ml polyIC for 0h, 2h, or 4h.