

Crystal structure of inhibitor of κ B kinase β

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Inhibitor of κ B (I κ B) kinase (IKK) phosphorylates I κ B proteins, leading to their degradation and the liberation of nuclear factor κ B for gene transcription. Here we report the crystal structure of IKK β in complex with an inhibitor, at a resolution of 3.6 Å. The structure reveals a trimodular architecture comprising the kinase domain, a ubiquitin-like domain (ULD) and an elongated, α -helical scaffold/dimerization domain (SDD). Unexpectedly, the predicted leucine zipper and helix-loop-helix motifs do not form these structures but are part of the SDD. The ULD and SDD mediate a critical interaction with I κ B α that restricts substrate specificity, and the ULD is also required for catalytic activity. The SDD mediates IKK β dimerization, but dimerization per se is not important for maintaining IKK β activity and instead is required for IKK β activation. Other IKK family members, IKK α , TBK1 and IKK-i, may have a similar trimodular architecture and function.

Nuclear factor κ B (NF- κ B) transcription factors are master regulators of inflammatory, immune and apoptotic responses^{1,2}. In the canonical pathway, NF- κ B dimers are held in the cytoplasm through binding to I κ B proteins, which mask their nuclear localization signals. When cells are stimulated by NF- κ B inducers, I κ B proteins are phosphorylated by the Ser/Thr-specific IKK, a modification that triggers their Lys-48-linked polyubiquitination and subsequent proteasomal degradation². Freed NF- κ B dimers enter the nucleus to regulate gene transcription². In the non-canonical pathway, activated IKK phosphorylates the I κ B-like domain in the NF- κ B family member NF- κ B2 (p49/p100)³ (NFKB2 in humans). NF- κ B signalling pathways are associated with a vast number of human diseases including inflammatory disorders and cancer, which renders IKK a potentially important therapeutic target⁴ (see, for example, <http://www.nf-kb.org>).

IKK was originally purified from HeLa cells as a multiprotein complex that contains the kinase subunits IKK α (CHUK) and/or IKK β (IKKBK), and the regulatory protein NEMO^{5–11} (IKK γ , or IKBK γ). IKK α and IKK β both contain an amino-terminal kinase domain (KD), predicted leucine zipper (LZ) and helix-loop-helix (HLH) domains, and a carboxy-terminal NEMO-binding domain (Fig. 1a). IKK β seems to have an additional ULD following the KD, which is not predicted in the corresponding region of IKK α ². The IKK-related kinases TBK1 (NAK) and IKK-i (IKK- ϵ , or IKBKE) seem to have a similar domain organization¹². Whereas IKK β mediates activation of the canonical NF- κ B pathway in response to pro-inflammatory stimuli, IKK α has an indispensable role in non-canonical NF- κ B signalling by phosphorylating NF- κ B2 (p49/p100). Protein kinase assays suggest that IKK β accounts for nearly all of the catalytic activity of the IKK holoenzyme towards I κ B α ^{3,13} (NFKBIA).

The activation loop in both the IKK α and the IKK β KD contains the mitogen-activated protein (MAP)-kinase consensus motif SXXXS, where X is any amino acid^{6–8,10} (Ser 177 and Ser 181 in human IKK β). Some MAP-kinase family members, such as TGF- β -activated kinase 1 (MAP3K7, previously TAK1) and NF- κ B-inducing kinase (MAP3K14, or NIK), were shown to phosphorylate IKK β ^{3,14,15}. IKK α and IKK β can also undergo autophosphorylation and activation as a result of overexpression or signal-dependent NEMO clustering^{10,16}. Ala substitutions of the activation-loop Ser residues prevent IKK activation whereas the phosphomimetic, double

Glu mutation S177E/S181E (EE) of IKK β renders it constitutively active^{7,13}.

Trimodular arrangement of IKK β

We determined the crystal structure of *Xenopus laevis* IKK β (ikkbk) EE (residues 4–675; Fig. 1a) in complex with either inhibitor Cmpd1 or inhibitor Cmpd2 (Supplementary Fig. 1) at resolutions of 4.0 and 3.6 Å in the *I*₄22 and *P*1 space groups, respectively (Supplementary Tables 1 and 2 and Supplementary Fig. 2). Eight IKK β molecules in *P*1 and the single molecule in *I*₄22 are highly similar to each other (Supplementary Fig. 3 and Supplementary Table 3) and show conserved dimerization (see below). Our structural description will use the first dimer (chains A and B) in *P*1. The sequences of human and *Xenopus* IKK β (henceforth hIKK β and xIKK β , respectively) have 74% identity with no gaps within the region of the structure; residue numbers designated for xIKK β are also true for hIKK β .

The IKK β dimer structure resembles a pair of shears and has the overall dimensions of approximately 100 Å × 130 Å × 60 Å (Fig. 1b, c). It contains the KD (residues 16–307), the ULD (310–394) and the SDD (410–666) (Fig. 1a and Supplementary Fig. 4). The KD and the ULD form the 'handle' of the shears, and the SDD is the 'blade'. Both the KD and the ULD intimately associate with the SDD, suggesting that the KD does not function independently. Instead, the structure indicates that IKK β is an integral trimodular unit.

The IKK β KD in complex with either Cmpd1 or Cmpd2 has the typical bilobal kinase fold¹⁷. Although it has only 21.1% sequence identity with human ubiquitin, the ULD of IKK β indeed has the ubiquitin fold (Fig. 1d). A major difference is the presence of an eight-residue insertion (373–380) that forms part of the loop connecting β -strands β 4' and β 5' in the ULD, which is mostly disordered. The hydrophobic surface patch of ubiquitin¹⁸, which is often recognized by ubiquitin-binding proteins, is conserved in the ULD, with residues Val 318, Leu 354 and Leu 389 equivalent to Leu 8, Ile 44 and Val 70 of ubiquitin (Supplementary Fig. 5).

The SDD comprises six α -helices (α 1s– α 6s), of which α 2s and α 6s contain 70 and 77 residues, respectively. They twist together with a stretch of three shorter helices between them to fold as an elongated structural ensemble. The C lobe of the KD sits on the N-terminal end

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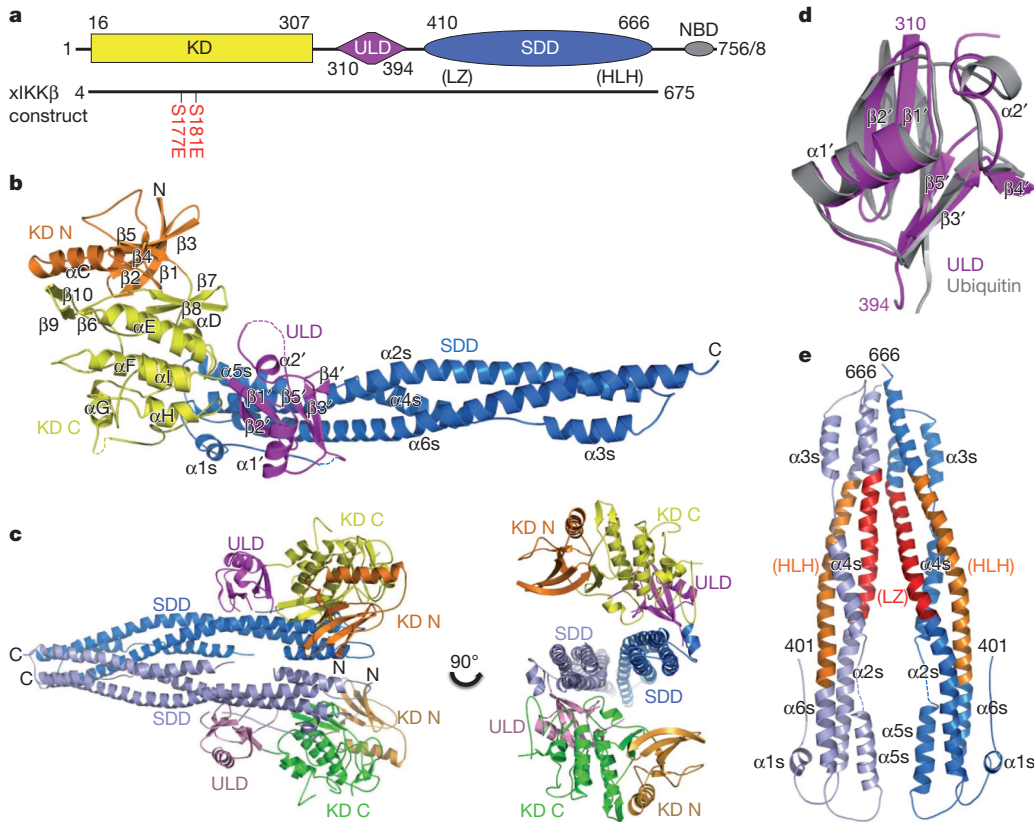


Figure 1 | Structure of xIKK β . **a**, Linear representation of IKK β showing the boundaries for the KD, the ULD and the SDD. Sequences of hIKK β and xIKK β are of 756 and 758 residues, respectively, and differ only at the most C-terminal region. The crystallized xIKK β construct is shown. The previously designated LZ and HLH regions are shown in parentheses. **b**, Ribbon diagram of an xIKK β protomer in the P1 crystal form. The N and C termini, KD N lobe (orange) and C lobe (yellow), ULD (magenta) and SDD (blue) are labelled. Secondary structural elements are labelled, with those in the ULD indicated with a prime and those in the SDD indicated with an 's'. **c**, Ribbon diagram of an xIKK β dimer. **d**, Superposition of the ULD (magenta) with ubiquitin (grey). **e**, Ribbon diagram of an SDD dimer, showing locations of the previously designated LZ (red) and HLH (orange) regions.

of the SDD, and the ULD binds close to the middle of the SDD. Unexpectedly, formerly designated LZ (458–485) and HLH domains^{7,8,10} (605–644) do not exist as such in the structure and are both part of the SDD (Fig. 1e). Most of the hydrophobic residues in the predicted LZ point inwards and are not available for mediating dimerization as previously proposed.

Structure of inhibitor-bound IKK β KD

The inhibitor binds to the IKK β KD at the hinge loop connecting the N and C lobes, a region that recognizes the adenine in ATP^{19,20} and is often used for inhibitor binding^{21–23} (Fig. 2a and Supplementary Fig. 6). The KD conformation is incompatible with that of an active kinase^{17,24,25} (Fig. 2b, c). The activation segment, which begins from Asp 166–Leu 167–Gly 168 as the conserved DFG triad and extends to Ala 190–Pro 191–Glu 192 in the conserved APE motif, is fully ordered, including

phosphomimetic residues Glu 177 and Glu 181 (Fig. 2b). However, the C-terminal anchor of the activation segment, including the APE motif, is completely out of place, with the result that essential interactions with the catalytic centre are lost (Fig. 2c). The gross conformation of the N-terminal anchor of the activation segment is preserved, with β 9 paired with the β 6 strand that precedes the catalytic loop. Part of the activation loop (175–177) contains an additional β -strand (β 10), which sits between the lobes to form a three-stranded β -sheet with β 9 and β 6. The α C helix is tilted up and outwards (Fig. 2c) to make room for β 10, which also disrupts its productive interactions with the DFG motif in active kinase structures.

Interactions among the KD, ULD and SDD

The KD, ULD and SDD interact mutually, with the ULD–SDD interaction being the most extensive, followed by the KD–SDD and KD–ULD

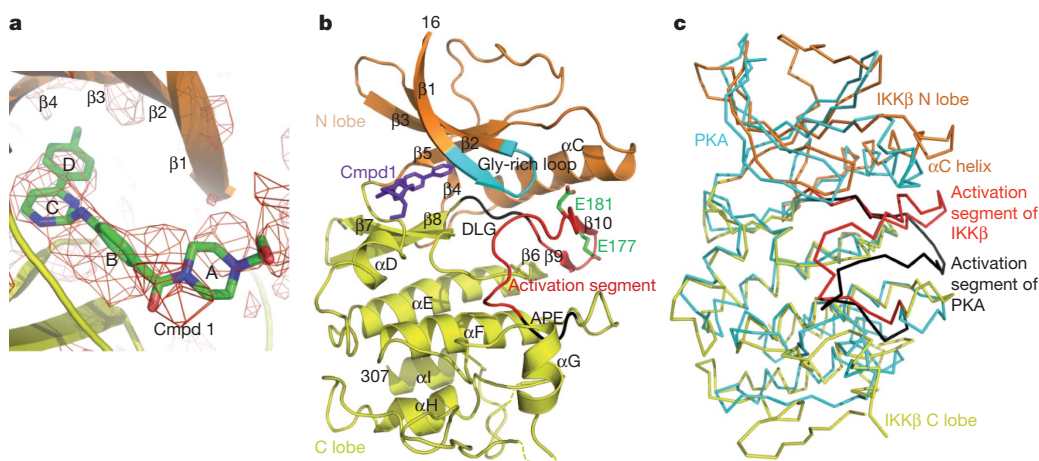


Figure 2 | Inhibitor-bound xIKK β kinase domain. **a**, $F_o - F_c$ electron density map for Cmpd1 in the I4₁₂₂ structure, contoured at 2.0 σ . Carbon, nitrogen and oxygen atoms are shown in green, blue and red, respectively. The four ring structures in Cmpd1 are labelled A, B, C and D, respectively. **b**, Structure of the xIKK β KD. Gly-rich loop, cyan; activation segment, red (except that the DLG and APE sequences are in black); Cmpd1, purple. Side chains of phosphomimetic residues Glu 177 and Glu 181 are shown. **c**, Superposition of xIKK β (orange and yellow) and protein kinase A (PKA, cyan; Protein Data Bank ID, 1ATP). The activation segments of xIKK β and PKA are shown in red and black, respectively.

interactions. The ULD binds close to the middle of the SDD, contacting helices $\alpha 2s$ and $\alpha 6s$ (Fig. 3a). The interaction buries $\sim 700 \text{ \AA}^2$ of surface area per partner. There are substantial hydrophobic contributions, supplemented by electrostatic interactions. Residues Met 315, Met 317, Leu 354, Ile 387, Leu 389 and Phe 390 on one side of the ULD pack against Leu 612, Tyr 609, Leu 447 and the main chain of $\alpha 2s$ of the SDD to form the central hydrophobic core of the interface. This hydrophobic patch of the ULD is immediately adjacent to and overlaps the conserved hydrophobic patch in the ubiquitin fold. Electrostatic interactions are observed between Glu 352 and Lys 619 and between Lys 394 and Asp 610. Additional interfacial residues include Ser 319 and Ser 357 of the ULD and Thr 453, Gln 449 and Val 616 of the SDD.

Consistent with an important role of the ULD in interacting with the SDD, mutations in residues that are not at the interface, P347A, L361A and Q351A, had minimal effects on NF- κ B activation²⁶. In contrast, mutation in a residue within a ULD surface loop that contacts the SDD, G358A (Fig. 3a), affected IKK β -induced NF- κ B activity²⁶. It was also shown that Leu 353 is required for IKK β activity²⁶; however, Leu 353 is buried in the ULD core and the L353A mutation may have disrupted IKK β structural integrity. Double substitutions of hIKK α and hIKK β , which are equivalent to I608R/Y609P of the SDD of α IKK β , did not affect their interaction with wild-type IKK β but negatively impacted kinase activity¹⁰; Ile 608 is buried in the SDD core and Tyr 609 directly interacts with the ULD (Fig. 3a).

Like the ULD, the KD also makes contact with the N terminal end of the SDD (Fig. 3b), burying a surface area of $\sim 650 \text{ \AA}^2$ of each interface. The binding forces are mainly van der Waals in nature. Limited hydrophobic interactions are observed between residues Ala 252 and Val 253 of the KD and Tyr 423 of the SDD, and between Phe 111 of the KD and the hydrophobic portions of Arg 572, Arg 575 and Glu 576 of $\alpha 5s$ of the SDD. The KD is linked to the ULD through a two-amino-acid linker between αI of the KD and $\beta 1'$ of the ULD (Fig. 3c), burying only $\sim 350 \text{ \AA}^2$ of surface area. Side-chain contacts between Leu 311 of the ULD and Ile 268 of the KD are observed, and together form the small hydrophobic patch at the KD-ULD junction

consisting also of Leu 269 and Ile 306 of the KD and Leu 309 of the linker. Together with the ionic interaction between Asp 373 of the ULD and Arg 123 of αE of the KD, these interactions may confer rigidity to the KD-ULD junction despite the small buried surface area.

Structural comparison with other kinase structures revealed a similarity between the locations of the SDD and ULD and those of several known docking sites for substrates and regulatory proteins²⁷. In the crystal structure of the Ser/Thr kinase SRPK1 in complex with a docking motif in its substrate, ASF/SF2²⁷ (SRSF1), the peptide motif interacts with the kinase at the location of the SDD (Fig. 3d). In the structure of the TAK1 KD fused with the C-terminal region of its binding protein, TAB1²⁸, TAB1 interacts with the C lobe of the kinase at a position analogous to both the SDD and the ULD, presumably to activate the kinase (Fig. 3d).

ULD-SDD binds the I κ B α C-terminal region

Previous studies have suggested that the ULD in TBK1 and IKK-i is involved in substrate recognition because its deletion impaired activity of the respective kinases and a glutathione *S*-transferase (GST)-ULD fusion protein interacted with the specific substrate, IRF3 or IRF7²⁹. Because ULD deletion in IKK β also abolished its activity²⁶, we proposed that the ULD may recognize its specific substrate, I κ B α . However, we were surprised to find that GST-I κ B α (1-317 and 54-317) pulled down only a minute amount of the ULD (Fig. 4a, lanes 9 and 13) and GST alone did not pull down any (Fig. 4a, lane 4), suggesting that the interaction of I κ B α with the ULD is specific, but very weak. In contrast, GST-I κ B α robustly pulled down full-length IKK β or IKK β lacking C-terminal NEMO-binding domain (Fig. 4a, lanes 15 and 16).

I κ B α has an N-terminal region (1-54) that contains cognate phosphoacceptor sites at Ser 32 and S 36, followed by a C-terminal region (55-317) that contains multiple ankyrin repeats and the PEST region^{30,31}. Strikingly, the N-terminal region of I κ B α did not pull down any IKK β constructs (Fig. 4a, lanes 5-8), and the C-terminal region of I κ B α interacted robustly with full-length IKK β as well as its ULD-SDD region (Fig. 4a, lanes 9-12), and very weakly with the ULD alone (Fig. 4a, lane 9). Further mapping on I κ B α showed that both

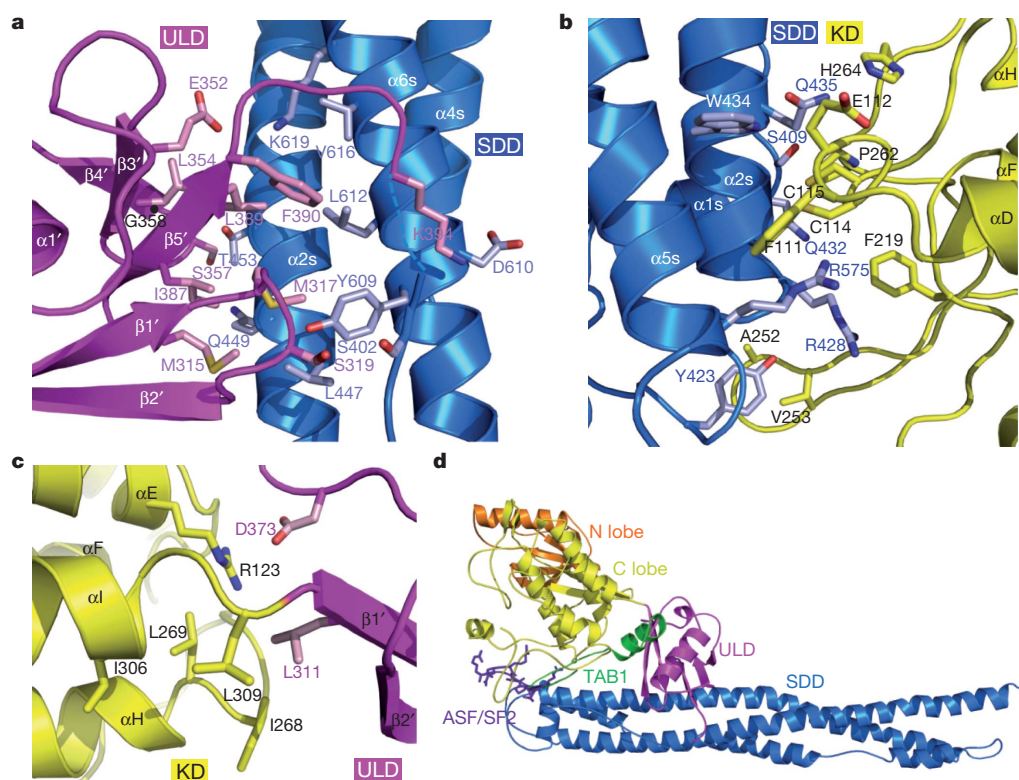


Figure 3 | Interactions among the KD, ULD and SDD. **a**, Interaction between the ULD (magenta) and SDD (blue). Important interfacial side chains are shown with nitrogen atoms in blue, oxygen atoms in red, sulphur atoms in orange and carbon atoms in either pink (ULD) or light blue (SDD). Gly 358 is marked with a black ball on the main chain. **b**, Interaction between the KD (yellow) and SDD (blue). **c**, Interaction between the KD (yellow) and ULD (magenta). **d**, Locations of the TAB1 peptide (green ribbon; PDB ID, 2EVA) and the ASF/SF2 peptide (purple stick model; PDB ID, 1WBP) relative to the IKK β structure after superposition of the corresponding kinase domains.

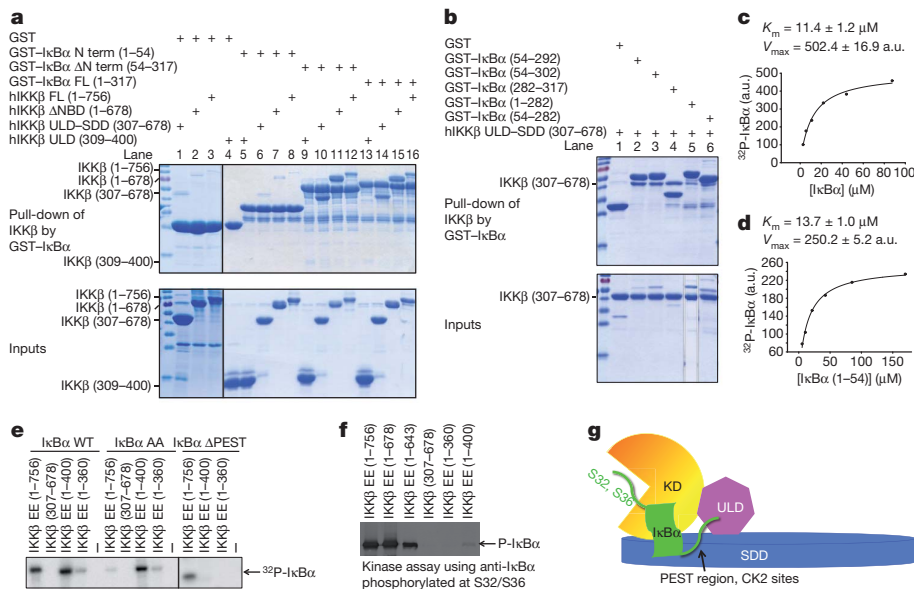


Figure 4 | ULD-SDD restricts IKK β specificity and ULD is required for catalytic activity.

a, b, Pull-down of hIKK β constructs using GST-I κ B α constructs, showing the reciprocal interaction between ULD-SDD of IKK β and the C-terminal region of I κ B α containing ankyrin repeats and the PEST region. FL, full length. **c, d**, Measurement of K_m and the relative V_{max} of IKK β against full-length I κ B α (**c**) and the N-terminal region of I κ B α (1–54) (**d**). a.u., arbitrary units. **e**, Kinase assay of purified hIKK β proteins against I κ B α , its S32A/S36A mutant (AA) and its PEST-deletion construct (Δ PEST, 1–282) using [γ - 32 P]ATP. WT, wild type. **f**, Kinase assay of purified hIKK β proteins using antibody against I κ B α phosphorylated at Ser 32 and Ser 36. **g**, Interaction between the SDD of IKK β and the C-terminal region of I κ B α may position the N-terminal cognate phosphorylation sites of I κ B α at the active site of IKK β . CK2, casein kinase 2.

ankyrin repeats (1–282 or 54–282) and the PEST region (282–317) interacted with IKK β ULD-SDD (Fig. 4b, lanes 4–6). The extent of pull-down suggests that the PEST region contributes more to IKK β interaction than do the ankyrin repeats. Despite trying multiple constructs, we could not obtain soluble protein for IKK β SDD to test its interaction with I κ B α . However, the pull-down data suggest a dominant role for SDD in I κ B α interaction. In any case, it is clear that the mutual interaction between IKK β and I κ B α is mediated by their ULD-SDD and C-terminal domains, respectively.

ULD-SDD limits specificity and the ULD aids catalysis

The specific interaction between ULD-SDD of IKK β and I κ B α suggests that the Michaelis constant, K_m , of phosphorylation by IKK β might be lower for full-length I κ B α than for its N-terminal region (1–54) alone. We performed kinase kinetics analysis of IKK β EE against the two different substrates. Unexpectedly, the measured apparent K_m values were 11.4 and 13.7 μ M for full-length I κ B α and the N-terminal region alone, respectively (Fig. 4c, d), suggesting that binding at the SDD, an exosite, does not alter the K_m of the reaction drastically. This could be due to opposing effects of the SDD-I κ B α interaction, which increases substrate interaction but slows down product dissociation. The relative maximum velocity, V_{max} , values were 502.4 and 250.2, respectively, suggesting that the SDD-I κ B α interaction moderately enhances catalysis.

Casein kinase 2 phosphorylates the C-terminal PEST region of I κ B α around residues 283–299^{30,31}. To determine whether the SDD-I κ B α interaction restricts substrate specificity, we compared the kinase activity of purified IKK β EE proteins against either I κ B α or its S32A/S36A mutant (AA) using [γ - 32 P]ATP (Fig. 4e). Although the KD-ULD (1–400) construct gave rise to a small amount of protein, it produced robust phosphorylation of wild-type I κ B α , comparable to full-length IKK β (1–756), suggesting that it is catalytically competent. Remarkably, KD-ULD effectively phosphorylated the AA mutant, which, in contrast, was a poor substrate for full-length IKK β . The C-terminal PEST region seemed to harbour the major sites of phosphorylation by KD-ULD because a construct lacking PEST (1–282) was not significantly phosphorylated by KD-ULD but was phosphorylated by full-length IKK β (Fig. 4e). In addition, when I κ B α phosphorylation was detected by antibody against I κ B α phosphorylated at Ser 32/Ser 36, only very weak phosphorylation was seen for the KD-ULD construct in comparison with full-length IKK β (Fig. 4f). These experiments suggest that although the KD-ULD construct is active, it possesses an altered substrate specificity causing preferential

phosphorylation of the C-terminal PEST sequence, consistent with a previous observation³². Hence, ULD-SDD seems to position I κ B α specifically such that only its N-terminal region is accessible to the IKK β catalytic pocket (Fig. 4g).

We expressed three kinase domain constructs, 1–301, 1–310 and 1–360, in insect cells and obtained only small amount of protein for the 1–360 construct. Kinase assay showed that IKK β EE (1–360) had very low residual activity against I κ B α or its AA mutant (Fig. 4e), suggesting that KD activity is compromised in the absence of the ULD. Without an isolated KD structure, we cannot deduce the molecular mechanism by which the ULD acts. However, in analogy to TAK1 activation by TAB1²⁸ (Fig. 3d), it may be speculated that this KD-ULD interaction allosterically potentiates kinase activity. Further kinase assay using antibody against I κ B α phosphorylated at Ser 32/Ser 36 showed no detectable activity of the KD alone (Fig. 4f), confirming that the low residual activity may also be directed towards the C-terminal PEST region, similar to KD-ULD. Therefore, whereas ULD-SDD is critical for IKK β specificity, ULD is required for its catalytic activity.

SDD mediates IKK β dimerization

Full-length hIKK β (1–756) and its KD-ULD-SDD region (1–678) are dimers in solution as determined by gel-filtration chromatography (Fig. 5a). In the P1 and I4_{1,22} crystals, two conserved dimerization interfaces exist, one mediated by the SDD and the other mediated by the KD. Because hIKK β (1–643) that truncates into the SDD is a monomer in solution (Fig. 5a), SDD-mediated dimerization (Fig. 5b) is probably of greater importance than KD-mediated dimerization.

SDD in an IKK β dimer does not form extensive interactions along the entire length dimension of the helical bundle. Rather, interactions are mostly localized at the end of the bundle (Fig. 5b), distal from the KD and ULD and burying $\sim 1,000 \text{ \AA}^2$ of surface area of each monomer. The interaction is mostly hydrophobic. Residues that contribute significantly to dimerization include Gln 478, Lys 482, Phe 485, Ile 492, Lys 496, Ile 505, Gln 651, Leu 654, Trp 655, Leu 658 and Ile 660, with residues Leu 654, Trp 655 and Leu 658 from helix α 6s burying the most surface area (Fig. 5b). This dimerization interface is entirely unexpected as it was predicted earlier that the LZ mediates IKK β dimerization. The structure now explains that failed dimerization of the LZ-defective L462S/L469S mutant of IKK α ¹⁰ is due to the structural role of Leu 469, whose equivalent residue of IKK β , Met 472, is buried in the SDD core. Superposition of four IKK β dimers in P1

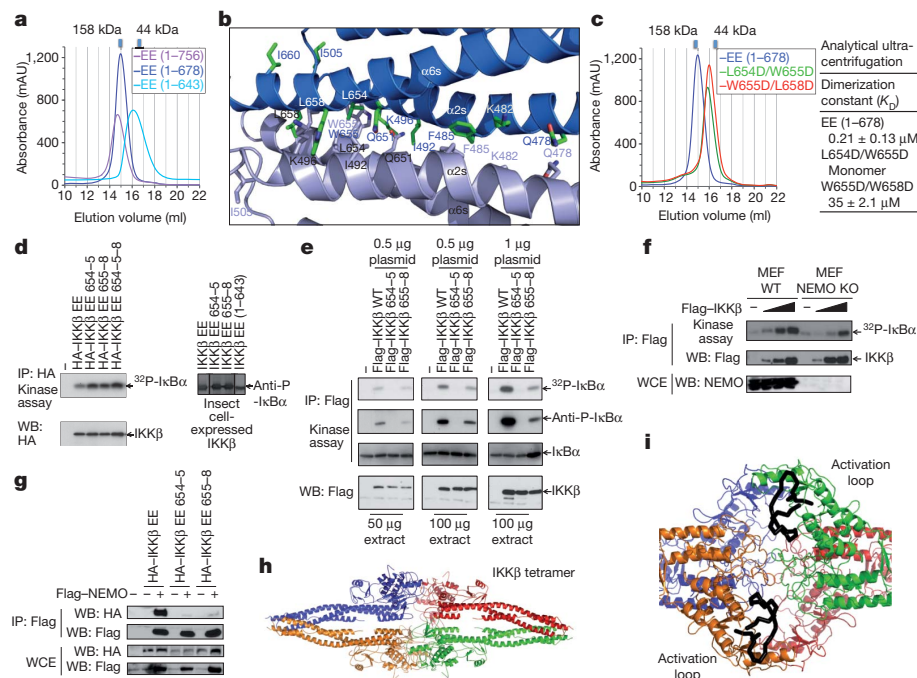


Figure 5 | Dimerization is critical for IKK β activation but not for its activity. **a**, Gel filtration profiles of various hIKK β constructs showing that those containing the KD–ULD–SDD region (1–756, 1–678) are dimeric and that a further truncated construct (1–643) is monomeric. mAU, milliabsorbance unit. **b**, The dimerization interface of xIKK β . **c**, Structure-based mutations disrupt hIKK β dimerization as shown by gel filtration and analytical ultracentrifugation. **d**, Kinase activity of HEK293T-cell-transfected or insect-cell-purified hIKK β EE and dimerization-defective mutants L654D/W655D (654–655), W655D/L658D (655–658) and L654D/W655D/L658D (654–655–658). **e**, Autoactivation of HEK293T-cell-transfected wild-type hIKK β and its dimerization-defective mutants. **f**, Transfection of hIKK β into wild-type and NEMO $^{-/-}$ mouse embryonic fibroblasts (MEFs), showing reduced IKK β activation in the absence of NEMO. **g**, Dimerization mutants of IKK β showing reduced interaction with NEMO. **h**, Tetramer of xIKK β in the P1 structure. **i**, Close-up view of the tetramer interface, showing that the activation loops of neighbouring protomers (black) face each other.

and the single IKK β dimer in *I4*₂₂ shows that IKK β dimers are conserved (Supplementary Fig. 7). In *I4*₂₂, the distal part of the SDD is not visible, owing to a lack of crystal packing in this region and dynamic disorder, not degradation (Supplementary Fig. 8).

To confirm the observed interface in IKK β dimerization, we performed structure-based mutagenesis on residues that bury the most surface area, Leu 654, Trp 655 and Leu 658. These residues are not within the predicted LZ region. Two purified double mutants, L654D/W655D and W655D/L658D, failed to dimerize, as shown by the considerable shift in gel filtration positions (Fig. 5c). Furthermore, equilibrium analytical ultracentrifugation experiments showed that wild-type IKK β is indeed a dimer whereas one of the IKK β mutants is a monomer and the other has a 167-fold weaker dimerization affinity (Fig. 5c).

Dimerization in IKK β activation but not activity

The geometry of the IKK β dimer with KDs facing away from each other suggests that each IKK β protomer is independent in its kinase activity. To confirm this, we transfected HEK293T cells with hIKK β EE mutants, L654D/W655D (654–655), W655D/L658D (655–658) and L654D/W655D/L658D (654–655–658), and found that all mutants had robust kinase activity (Fig. 5d). Dimerization mutants expressed in insect cell and purified showed the same results. In addition, a purified IKK β construct with truncation into the SDD (1–643) that is monomeric in solution (Fig. 5a) was active in phosphorylating I κ B α (Fig. 5d).

Previous studies have shown that IKK β can undergo *trans*-autophosphorylation and activation on transfection^{13,16}. This autophosphorylation and phosphorylation by TAK1 probably both contribute to IKK β activation on cell stimulation. To determine whether the observed dimerization interface is critical for this activation, we tested dimerization mutations in IKK β activation on overexpression in HEK293T cells (Fig. 5e). Whereas wild-type IKK β was robustly activated, the L654D/W655D and W655D/L658D mutants completely and partly lost this activation, respectively, in a manner that correlates with the degree of impairment in dimerization (Fig. 5c, e). Overexpression of IKK β in NEMO-deficient mouse embryonic fibroblasts resulted in its activation, but to a lesser extent than in wild-type mouse embryonic fibroblasts (Fig. 5f). We found that IKK β dimerization mutants failed to interact with NEMO efficiently (Fig. 5g), perhaps owing to reduced

avidity for oligomeric NEMO. Therefore, although IKK β kinase activity does not depend on dimerization once its activation loop is phosphorylated, IKK β activation requires dimerization and is probably enhanced by interaction with NEMO.

Discussion

The IKK β structure presented here predicts that IKK α , TBK1 and IKK-i all have an overall structural organization that comprises KD, ULD and SDD. Although a ULD was not predicted in IKK α , conservation of buried residues in this region and of the ULD–SDD interface suggests that IKK α also has this domain (Supplementary Fig. 4). The ULD and SDD probably have the same structural role but may have evolved additional, differential functions in the individual kinases. SDD-mediated dimerization may also be conserved. In particular, residues at the observed IKK β dimerization interface are highly conserved in IKK α (Supplementary Fig. 4), explaining how IKK α and IKK β can form both homo- and heterodimers^{8,13}. Given our structure of IKK β and the previously determined structures of NEMO fragments^{33–37}, we may speculate that the full IKK complex is also a dimer, or a dimer of dimers with a molecular mass of around 270 or 540 kD. The apparent 700–900-kD molecular mass of the IKK holoenzyme on gel filtration^{5–10} may be due to the elongated nature of NEMO and the complex (Supplementary Fig. 9).

Because the conserved IKK β dimer structure does not place the KDs close to each other for *trans*-autophosphorylation, we wondered whether higher-order oligomerization, which is probably enhanced by NEMO and its ability to bind ubiquitin^{33,34}, is responsible for this autoactivation. In both *P1* and *I4*₂₂, IKK β exists as dimers of dimers (Fig. 5h and Supplementary Fig. 10). In particular, active sites of two neighbouring protomers in the tetramer face each other such that an activation loop from one protomer might reach into the active site of the other (Fig. 5i), which may mimic an autophosphorylation state.

The ULD–SDD region of IKK β directly interacts with the C-terminal portion of I κ B α . This interaction may serve several purposes. First, it probably orients I κ B α such that its N-terminal cognate phosphorylation sites are presented to the KD active site (Fig. 4g). Without this interaction, IKK β preferentially phosphorylates the C-terminal PEST motif of I κ B α . Second, the interaction seems to enhance IKK β activity. Third, phosphorylation at the PEST by casein kinase 2 or other kinases may regulate I κ B α interaction with IKK β

and hence affect phosphorylation at the cognate sites. Fourth, the interaction may allow concerted phosphorylation at both Ser 32 and Ser 36 of I κ B α without intervening dissociation. In MAP-kinase cascades that involve dual phosphorylations, specific docking interactions occur between the kinases and their substrates^{27,38,39}. The β -catenin protein in the Wnt signalling pathway contains the same dual-phosphorylated destruction box motif as I κ B α (ref. 40). Consistent with this, β -catenin is brought to the responsible kinase, GSK-3, by means of the adaptor protein axin, allowing both specificity and concerted phosphorylation^{41,42}. Therefore, in a general statement that structure serves function, the IKK β structure seems to fit its function as the I κ B α kinase perfectly, being poised to turn on the important NF- κ B pathway specifically, efficiently and concertedly in response to cellular physiology.

METHODS SUMMARY

Xenopus laevis IKK β was expressed in insect cells and purified to homogeneity using nickel affinity chromatography, ion exchange and gel filtration chromatography. We crystallized the P1 and I4,22 forms at 4 °C in polyethylene glycol 6000 and potassium/sodium phosphate, respectively. The structure was determined by multiwavelength anomalous diffraction of the selenomethionyl protein.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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- Hayden, M. S. & Ghosh, S. Shared principles in NF- κ B signaling. *Cell* **132**, 344–362 (2008).
- Vallabhapuram, S. & Karin, M. Regulation and function of NF- κ B transcription factors in the immune system. *Annu. Rev. Immunol.* **27**, 693–733 (2009).
- Scheidereit, C. I κ B kinase complexes: gateways to NF- κ B activation and transcription. *Oncogene* **25**, 6685–6705 (2006).
- Karin, M. Nuclear factor- κ B in cancer development and progression. *Nature* **441**, 431–436 (2006).
- Chen, Z. J., Parent, L. & Maniatis, T. Site-specific phosphorylation of I κ B α by a novel ubiquitination-dependent protein kinase activity. *Cell* **84**, 853–862 (1996).
- DiDonato, J. A. *et al.* A cytokine-responsive I κ B kinase that activates the transcription factor NF- κ B. *Nature* **388**, 548–554 (1997).
- Mercurio, F. *et al.* IKK-1 and IKK-2: cytokine-activated I κ B kinases essential for NF- κ B activation. *Science* **278**, 860–866 (1997).
- Woronicz, J. D. *et al.* I κ B kinase- β : NF- κ B activation and complex formation with I κ B kinase- α and NIK. *Science* **278**, 866–870 (1997).
- Yamaoka, S. *et al.* Complementation cloning of NEMO, a component of the I κ B kinase complex essential for NF- κ B activation. *Cell* **93**, 1231–1240 (1998).
- Zandi, E. *et al.* The I κ B kinase complex (IKK) contains two kinase subunits, IKK α and IKK β , necessary for I κ B phosphorylation and NF- κ B activation. *Cell* **91**, 243–252 (1997).
- Rothwarf, D. M., Zandi, E., Natoli, G. & Karin, M. IKK- γ is an essential regulatory subunit of the I κ B kinase complex. *Nature* **395**, 297–300 (1998).
- Hacker, H. & Karin, M. Regulation and function of IKK and IKK-related kinases. *Sci. STKE* **2006**, re13 (2006).
- Zandi, E., Chen, Y. & Karin, M. Direct phosphorylation of I κ B by IKK α and IKK β : discrimination between free and NF- κ B-bound substrate. *Science* **281**, 1360–1363 (1998).
- Sato, S. *et al.* Essential function for the kinase TAK1 in innate and adaptive immune responses. *Nature Immunol.* **6**, 1087–1095 (2005).
- Liu, H. H., Xie, M., Schneider, M. D. & Chen, Z. J. Essential role of TAK1 in thymocyte development and activation. *Proc. Natl Acad. Sci. USA* **103**, 11677–11682 (2006).
- Tang, E. D. *et al.* Roles for homotypic interactions and transautophosphorylation in I κ B kinase (IKK β) activation. *J. Biol. Chem.* **278**, 38566–38570 (2003); erratum **278**, 49661 (2003).
- Knighton, D. R. *et al.* Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science* **253**, 407–414 (1991).
- Dikic, I., Wakatsuki, S. & Walters, K. J. Ubiquitin-binding domains — from structures to functions. *Nature Rev. Mol. Cell Biol.* **10**, 659–671 (2009).
- Zheng, J. *et al.* 2.2 Å refined crystal structure of the catalytic subunit of cAMP-dependent protein kinase complexed with MnATP and a peptide inhibitor. *Acta Crystallogr. D* **49**, 362–365 (1993).
- Bossemeyer, D. *et al.* Phosphotransferase and substrate binding mechanism of the cAMP-dependent protein kinase catalytic subunit from porcine heart as deduced from the 2.0 Å structure of the complex with Mn²⁺ adenylyl imidodiphosphate and inhibitor peptide PKI(5–24). *EMBO J.* **12**, 849–859 (1993).
- Xu, R. M., Carmel, G., Kuret, J. & Cheng, X. Structural basis for selectivity of the isoquinoline sulfonamide family of protein kinase inhibitors. *Proc. Natl Acad. Sci. USA* **93**, 6308–6313 (1996).
- Sicheri, F., Moarefi, I. & Kuriyan, J. Crystal structure of the Src family tyrosine kinase Hck. *Nature* **385**, 602–609 (1997).
- Noble, M. E., Endicott, J. A. & Johnson, L. N. Protein kinase inhibitors: insights into drug design from structure. *Science* **303**, 1800–1805 (2004).
- Nolen, B., Taylor, S. & Ghosh, G. Regulation of protein kinases; controlling activity through activation segment conformation. *Mol. Cell* **15**, 661–675 (2004).
- Jeffrey, P. D. *et al.* Mechanism of CDK activation revealed by the structure of a cyclinA-CDK2 complex. *Nature* **376**, 313–320 (1995).
- May, M. J. *et al.* A novel ubiquitin-like domain in I κ B kinase β is required for functional activity of the kinase. *J. Biol. Chem.* **279**, 45528–45539 (2004).
- Goldsmith, E. J. *et al.* Substrate and docking interactions in serine/threonine protein kinases. *Chem. Rev.* **107**, 5065–5081 (2007).
- Brown, K. *et al.* Structural basis for the interaction of TAK1 kinase with its activating protein TAB1. *J. Mol. Biol.* **354**, 1013–1020 (2005).
- Ikeda, F. *et al.* Involvement of the ubiquitin-like domain of TBK1/IKK-i kinases in regulation of IFN-inducible genes. *EMBO J.* **26**, 3451–3462 (2007).
- Kato, T. Jr, Delhase, M., Hoffmann, A. & Karin, M. CK2 is a C-terminal I κ B kinase responsible for NF- κ B activation during the UV response. *Mol. Cell* **12**, 829–839 (2003).
- Barroga, C. F., Stevenson, J. K., Schwarz, E. M. & Verma, I. M. Constitutive phosphorylation of I κ B α by casein kinase II. *Proc. Natl Acad. Sci. USA* **92**, 7637–7641 (1995).
- Shaul, J. D., Farina, A. & Huxford, T. The human IKK β subunit kinase domain displays CK2-like phosphorylation specificity. *Biochem. Biophys. Res. Commun.* **374**, 592–597 (2008).
- Lo, Y. C. *et al.* Structural basis for recognition of diubiquitins by NEMO. *Mol. Cell* **33**, 602–615 (2009).
- Rahighi, S. *et al.* Specific recognition of linear ubiquitin chains by NEMO is important for NF- κ B activation. *Cell* **136**, 1098–1109 (2009).
- Rushe, M. *et al.* Structure of a NEMO/IKK-associating domain reveals architecture of the interaction site. *Structure* **16**, 798–808 (2008).
- Bagn eris, C. *et al.* Crystal structure of a vFlip-IKK γ complex: insights into viral activation of the IKK signalosome. *Mol. Cell* **30**, 620–631 (2008).
- Cordier, F. *et al.* Solution structure of NEMO zinc finger and impact of an anhidrotic ectodermal dysplasia with immunodeficiency-related point mutation. *J. Mol. Biol.* **377**, 1419–1432 (2008).
- Remenyi, A., Good, M. C. & Lim, W. A. Docking interactions in protein kinase and phosphatase networks. *Curr. Opin. Struct. Biol.* **16**, 676–685 (2006).
- Kallunki, T., Deng, T., Hibi, M. & Karin, M. c-Jun can recruit JNK to phosphorylate dimerization partners via specific docking interactions. *Cell* **87**, 929–939 (1996).
- Wu, G. *et al.* Structure of a β -TrCP1-Skp1- β -catenin complex: destruction motif binding and lysine specificity of the SCF(β -TrCP1) ubiquitin ligase. *Mol. Cell* **11**, 1445–1456 (2003).
- Ikeda, S. *et al.* Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK-3 β and beta-catenin and promotes GSK-3 β -dependent phosphorylation of β -catenin. *EMBO J.* **17**, 1371–1384 (1998).
- Hart, M. J. *et al.* Downregulation of β -catenin by human Axin and its association with the APC tumor suppressor, β -catenin and GSK3 β . *Curr. Biol.* **8**, 573–581 (1998).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions G.X. cloned, expressed, purified, crystallized and determined the crystal structure of α IKK β and performed experiments to determine K_m . Y.-C.L. cloned, expressed, purified and crystallized hIKK β and performed pull-down experiments and kinase assays using phospho-I κ B α antibody. Q.L. expressed the hIKK β mutants in insect cells. G.N. and X.W. performed transfection, immunoprecipitation and kinase assays and M.K. supervised these experiments. H.W. supervised the project. G.X. and H.W. made the figures and wrote the manuscript.

Author Information Atomic coordinates and structure factors have been deposited in the Protein Data Bank under accession codes 3QA8 and 3QAD. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to H.W. (haowu@med.cornell.edu).

METHODS

Protein expression and purification. To elucidate the molecular basis of IKK β function, we expressed IKK β from a number of species using baculovirus-mediated insect cell expression. The xIKK β sequence in the NCBI database starts at a Met residue that is equivalent to Met 17 of both the hIKK β and the mouse IKK β (mIKK β) sequence. Translation of the DNA sequence preceding the ATG codon of Met 17 revealed sequences that are almost identical to residues 9–16 of hIKK β and mIKK β . These were taken as part of the xIKK β sequence and residues 1–8 were taken from the corresponding mIKK β sequence. This reconstructed xIKK β sequence has the same residue numbering as the hIKK β sequence until after the SDD.

Various constructs of IKK β wild type and the phosphomimetic S177E/S181E mutant were designed with an N-terminal polyhistidine tag and a tobacco etch virus protease-cutting site between the tag and the protein. Recombinant IKK β baculoviruses were made in DH10BAC cells, amplified and used to infect Hi5 insect cells in serum-free media (Invitrogen). The cells were cultured in suspension and harvested 48 h after infection. The recombinant proteins were purified by nickel affinity chromatography, anion exchange and gel filtration chromatography. For crystallization, the polyhistidine tag was cleaved by the tobacco etch virus protease during protein purification.

All I κ B α proteins were expressed in *Escherichia coli* using pET28a, pGEX4T3 and pET-SUMO vectors and purified by their respective affinity tags. For kinase assays, the SUMO tag was cleaved from I κ B α proteins. His-ULD and His-ULD-SDD of hIKK β were also expressed in *E. coli* using the pET28a vector.

Crystallization and data collection. Unlike many protein kinases, the IKK β KD cannot be recombinantly expressed as a well-behaved biochemical entity for structural studies. In addition, after mapping a compact region by limited proteolysis, IKK β was still refractory to crystallization, both alone and in the presence of various ATP analogues. To overcome this obstacle, we used several IKK β inhibitors, including Cmpd1 and Cmpd2 (Supplementary Fig. 1), which were identified against the S177E/S181E (EE) mutant, in co-crystallization. A hIKK β EE construct (1–678) lacking only the C-terminal NBD did crystallize; however, these crystals only diffracted to a resolution of ~ 7.5 Å. Searching IKK β orthologues that may give better crystals led to success in crystallizing the analogous region of xIKK β EE (4–675; Fig. 1a).

The xIKK β (S177E/S181E) protein containing residues 4–675 was concentrated by ultrafiltration (Amicon) to about 15 mg ml $^{-1}$ in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl and 10 mM dithiothreitol (DTT). It was mixed with an inhibitor compound in a 1:2 molar ratio before crystallization. Cmpd1 is 4-((4-(4-chlorophenyl)pyrimidin-2-yl)amino)phenyl(4-(2-hydroxyethyl)piperazin-1-yl)methanone and Cmpd2 is 1-(4-(4-((4-(pyridin-4-ylsulfonyl)phenyl)pyrimidin-2-yl)amino)benzoyl)piperazin-1-yl)ethanone. The P1 crystals were grown using hanging-drop vapour diffusion at 4 °C by mixing equal volumes of the purified protein and the crystallization condition of 100 mM *N*-(2-acetamido)iminodiacetic acid at pH 6.5, 10% (w/v) polyethylene glycol 6000, 50 mM Li $_2$ SO $_4$, 300 mM NaCl and 10 mM DTT. The I $_4$ 22 crystals were grown at 4 °C with well solution containing 1.8 M K/Na phosphate at pH 5.6 and 10 mM DTT. For data collection, all crystals were flash frozen in the respective crystallization conditions supplemented with 25% (v/v) ethylene glycol. Diffraction data were collected at the 24ID-C beam line of the Advanced Photon Source. Multiwavelength anomalous diffraction (MAD) data on heavy-atom derivative crystals or selenomethionyl crystals were collected near the respective absorption edges. All diffraction data were processed using the HKL2000 suite⁴³ and their statistics are shown in Supplementary Table 1 and Supplementary Table 2.

Structure determination, refinement and analysis. The initial xIKK β crystals grew in the P1 space group in the presence of the inhibitor Cmpd1 or Cmpd2 and diffracted to a resolution of 3.6 Å. Selenomethionyl crystals were obtained, but we failed to locate the large number of expected selenium sites. Among the extensive heavy-atom searches, an ytterbium-derivative was obtained, with eight well-defined sites, which probably correspond to eight IKK β molecules in the asymmetric unit. However, the electron density map calculated from a three-wavelength ytterbium-anomalous diffraction data set was insufficient for tracing, and phase combination with the selenomethionyl data set could not be performed, owing to non-isomorphism.

The structure determination was eventually successful in the alternative crystal form, I $_4$ 22, which contains one molecule of IKK β in complex with Cmpd1 and diffracted to a resolution of 4.0 Å, using MAD of the selenomethionyl crystals (Supplementary Tables 1 and 2 and Supplementary Fig. 2). Twelve selenium sites were determined using the program SHELXD⁴⁴ and refined with the program MLPHARE in the CCP4 suite⁴⁵. MAD phases were calculated at a resolution of 4.0 Å with data from I $_4$ 22 crystals using the program SHARP⁴⁶. A few cycles of model building and refinement were carried out with the program O⁴⁷ and REFMAC with TLS parameterization⁴⁸. The I $_4$ 22 crystals contain one monomer in the asymmetric unit and 80% solvent when calculated with the entire IKK β construct and 84% solvent when considering only the ordered part of the structure. The inhibitor Cmpd1 has density in the MAD experimental map and the $F_o - F_c$

omit map. The Dundee PRODRG2 Server was used to generate topology and restraint files of the compound for refinement. The refined model contains residues 16–236, 243–286, 290–376, 384–394, 401–475 and 528–637 and Cmpd1.

The structure of the P1 form was determined by molecular replacement using the refined model of the I $_4$ 22 crystal form as the search model, in which eight molecules were located. Selenium sites of the single-wavelength anomalous diffraction data set of a selenomethionyl crystal in the P1 space group was calculated by difference Fourier analysis and used to verify residue registration in the P1 structure. Refinement in the P1 structure was conducted at a resolution of 3.6 Å and incorporated tight non-crystallographic symmetry restraints (root mean squared deviation of 0.02 Å in atom positions). After several rounds of refinement at a resolution of 3.6 Å, new electron densities appeared in the P1 crystal form to complete the model building. Although Cmpd2 was in the crystallization condition, it did not have clear density and was not included in the refinement. The refined model of the P1 crystal form contains four IKK β dimers in the asymmetric unit. Three of the dimers encompass residues 16–236, 243–286, 290–376, 384–394, 401–551 and 559–666. One dimer contains the same residues as the structure in I $_4$ 22. The structures were analysed using the CCP4 suite⁴⁵ and the Dali server⁴⁹, and the figures were made using PYMOL⁵⁰.

GST pull-down. The tagged proteins were first purified with glutathione or Ni-NTA beads and their expression levels were assessed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Beads containing estimated equivalent quantities of the tagged proteins were mixed with the cell lysates or the purified versions of the interaction partners. The mixtures were incubated at room temperature (20 °C) for 1 h with rotation. After centrifugation, the supernatants were removed. The beads were then washed twice, eluted and subjected to SDS–PAGE analysis. All pull-down experiments were repeated two to four times with consistency.

Kinase assays using anti-phospho-I κ B α antibody. The hIKK β proteins (0.1 μ g μ l $^{-1}$) were incubated with recombinant I κ B α (1 μ g μ l $^{-1}$) in 50 mM Tris-HCl at pH 8.0, 100 mM NaCl, 10 mM MgCl $_2$ and 2 mM DTT for 30 min at 30 °C. SDS–PAGE sample buffer was used to terminate the reactions. The products were separated on 15% SDS–PAGE and transferred to PVDF membranes. Anti-phospho-I κ B α antibody (Cell Signaling Technology) was used to detect phospho-I κ B α .

Determination of K_m of hIKK β for I κ B α (1–54) and full-length I κ B α . To derive the K_m of hIKK β for full-length I κ B α , kinase assays were performed at substrate concentrations of 2.8, 5.3, 10.6, 21.3, 42.5 and 85 μ M. Similarly, to derive the K_m of hIKK β for I κ B α (1–54), kinase assays were performed at substrate concentrations of 5.3, 10.6, 21.3, 42.5, 85 and 170 μ M. A time course of the kinase reactions was first performed to select a hIKK β amount and a time point within which the reactions are linear with time. The final selected reactions contain 10 ng baculovirus-expressed hIKK β , 100 mM cold ATP and 1 μ l [γ - 32 P]ATP (3,000 Ci mmol $^{-1}$, 1 mCi per 100 μ l) in 25 μ l of reaction buffer containing 50 mM Tris-HCl at pH 8.0, 100 mM NaCl, 10 mM MgCl $_2$ and 2 mM DTT. The phosphotransfer reaction was allowed to proceed for 10 min at 30 °C and quenched with SDS–PAGE sample buffer. The products were separated on 15% SDS–PAGE and subjected to autoradiography. The relative amounts of phosphorylated I κ B α were quantified using IMAGEJ, plotted against total I κ B α concentrations and fitted using nonlinear regression to the Michaelis–Menten equation to obtain K_m using SIGMAPLOT.

Transfection, immunoprecipitation and kinase assay. The constructs Flag-hIKK β EE and its truncation mutants; HA-hIKK β EE and its dimerization mutants L654D/W655D, W655D/L658D and L654D/W655D/L658D; and Flag-hIKK β and its dimerization mutants L654D/W655D and W655D/L658D were generated in the vector pcDNA3 by conventional PCR. All IKK β constructs were transfected in HEK293T cells with Lipofectamine 2000 (Invitrogen). After 24 h, cell extracts were immunoprecipitated with anti-Flag antibodies bound to agarose beads (M2, Sigma) or anti-HA bound to agarose beads (Sigma). IKK β kinase assays were essentially done as previously described^{6,13}. Briefly, immunoprecipitates were incubated with 2 μ M full-length I κ B α (1–317) in 20 mM HEPES at pH 7.5, 10 mM MgCl $_2$, 20 mM β -glycerophosphate, 10 mM PNPP, 50 mM Na $_3$ VO $_4$, 1 mM DTT, 20 mM ATP, and 1–10-mCi [γ - 32 P]ATP at 30 °C for 30 min, and subjected to SDS–PAGE and autoradiography. Immunoblotting was performed using anti-Flag (Sigma), anti-HA (Sigma) or anti-IKK β antibodies (Upstate, 05-535).

Equilibrium analytical ultracentrifugation measurements. Experiments were performed in a Beckman XL-A/I analytical ultracentrifuge (Beckman-Coulter), using six-cell centre pieces with straight walls, a 12-mm path length and sapphire windows. Samples were kept and diluted in 50 mM Tris-HCl at pH 8.0 and 300 mM NaCl. Samples from wild-type protein were diluted to 6.9, 4.5 and 2.4 μ M, mutant L654D/W655D to 7.4, 4.8 and 2.6 μ M and mutant W655D/L658D to 4.9, 3.2 and 1.7 μ M for channels A, B and C, respectively. Dilution buffer was used as blank. All samples were run at 4 °C at 9,000 r.p.m. (5,900g; held for 20 h then scanned four times at 1-h intervals), 11,000 r.p.m. (8,800g; held for 10 h then scanned four times at 1-h intervals), 14,000 r.p.m. (14,300g; held for

10 h then scanned four times at 1-h intervals) and 17,000 r.p.m. (21,000g; held for 10 h then scanned four times at 1-h intervals). Detection was by ultraviolet absorption at 280 nm. Solvent density and the protein partial specific volume at each temperature were determined using the program SEDNTERP (Alliance Protein Laboratories). For calculation of K_D and the apparent molecular weight, all useful data were used in a global fit, using the program HETEROANALYSIS, obtained from University of Connecticut (<http://www.biotech.uconn.edu/auf>).

43. Otwinowski, Z. & Minor, W. Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol.* **276**, 307–326 (1997).
44. Schneider, T. R. & Sheldrick, G. M. Substructure solution with SHELXD. *Acta Crystallogr. D* **58**, 1772–1779 (2002).
45. Collaborative Computational Project, Number 4. The CCP4 suite: programs for protein crystallography. *Acta Crystallogr. D* **50**, 760–763 (1994).
46. Bricogne, G. *et al.* Generation, representation and flow of phase information in structure determination: recent developments in and around SHARP 2.0. *Acta Crystallogr. D* **59**, 2023–2030 (2003).
47. Jones, T. A., Zou, J.-Y., Cowan, S. W. & Kjeldgaard, M. Improved methods for building models in electron density maps and the location of errors in those models. *Acta Crystallogr. A* **47**, 110–119 (1991).
48. Winn, M. D., Murshudov, G. N. & Papiz, M. Z. Macromolecular TLS refinement in REFMAC at moderate resolutions. *Methods Enzymol.* **374**, 300–321 (2003).
49. Holm, L. & Sander, C. Dali: a network tool for protein structure comparison. *Trends Biochem. Sci.* **20**, 478–480 (1995).
50. DeLano, W. L. PyMOL Molecular Viewer (<http://www.pymol.org>) (2002).