nature portfolio

Peer Review File

Molecular mechanism of IKK catalytic dimer docking to NF-κB substrates

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The authors in this paper have identified a binding motif in the PEST domain of IkBa which binds to the distal end of the
dimeric SDD domain of IKKa or IKKb. PEST domain has been shown previously to function as an exosite for IKKb to recruit
IkBa during its phosphorylation (Xu. G, et al, Nature. 2011 Apr 21;472(7343):325-30). This docking motif binds
to the groove of the dimeric SDD domain in both IKKa and IKKb, which explains the observation by (Xu.G et al) that
deletion of SDD domain in IKKb renders the C-terminal phosphorylation of IkBa instead of N-terminal phosphorylation on S32S36 by this truncated KD-ULD construct of IKKb. Because the docking motif binding to SDD domains mask the phosphorylatoin sites in the PEST domain of IkBa. Similar docking motif has been identified in p100 or IRF7, which are substrates of IKKa and TBK1 in the cells respectively. Interestingly, IKKb binds specifically to the docking motif in IkBa, while IKKa binds to that in both IkBa and P100. The docking motif binding mode has been modeled to the structure of IKKb by molecular docking. It is highly recommended to be published in Nature Communications with the following revisions:

Major revision:

The measured binding affinity between the docking motif and IKKa (KD = 9 μM) is significantly higher as compared to IKKb
(KD = 40 μM). However, in the cells, IKKb contributes mostly to the phosphorylation of IkBa in the canonical pathway to activate
P50-RelA. In an in vitro kinase assay (Burke, J.R,. JBC Vol. 274, No. 51, Issue of December 17, pp. 36146–36152, 1999),
IKKb is much more active in phosphorylating IkBa than IKKa; And an recombinant IKKa/IKKb heterodimer is more active in phosphorylating IkBa than either IKKb or IKKa homodimer (Huynh, Q.K. JBC Vol. 275, No. 34, Issue of August 25, pp. 25883–25891, 2000).

Does this imply that the docking motif-IKK binding negatively regulate the IkBa phosphorylation by IKKa,
or weaker binding to IKKb increases the turnover rate of the phosphorylated IkBa substrate to be released from IKKb
to increases IKKb catalytic efficiency?

Minor revision:

Line 177, “This is in agreement with binding data from Hu and coworkers 11, “

to “This is in agreement with binding data from Xu and coworkers 11,”

Reviewer #2:
Remarks to the Author:
This is an extremely thorough demonstration of a novel function for a motif at the C-terminus of IkBa. The function of this "PEST extension" has been a longstanding question in the field. The authors demonstrate convincingly that the motif binds to IKKa and IKKb with micromolar affinity and that a peptide corresponding to the same sequence functions in cells to disrupt IKK phosphorylation of IkBa and subsequent NFkB signaling. I really liked that the authors report everything they tried to figure out the structure of the peptide-IKK interaction. This is a difficult problem and taken together their data are convincing. I also like how they found a similar motif in several other IKK substrates. In terms of the methods, all of the biophysical measurements are rigorously performed, and they got what they could out of the structural work. Their thorough characterization of the motif sequence by mutagenesis and the added bonus of showing that phosphorylation of the key tyrosine residue disrupts binding were really nice also.
I do not have any recommendation for changes to this really well-done manuscript.

Reviewer #3:
Remarks to the Author:
Li et al reports on a new Short Linear Motif (SLiM) that appears to be functionally important in NF-kB signaling. The YDDphi-x-phi motif was first identified in IkBalpha in its presumably disordered C-terminal region and the authors nicely show the importance of binding between this IkBalpha region and IKK dimers in classical in vitro pull-down experiments or kinase assays, quantitative ITC measurements, in a cell-based PCA assay, and in functionally relevant cell culture systems by monitoring biological outputs such IKK phosphorylation-dependent degradation of IkBalpha and the subsequent change in NF-kB’s nucleo-cytoplasmic shuttling. Moreover, it is also shown that the YDDphi-x-phi SLiM in IkBalpha is a phosphoswitch since its tyrosine phosphorylation eliminates its binding capacity to IKK dimers.
The discovery and characterization of the IKK exosite mediated binding to a central component of the NF-kB pathway, namely to IkBalpha, which is regulated by a

phosphodegron located in its N-terminal region is in good agreement with earlier observations and complements the mechanistic insights that we had on this IKK controlled system. The data and the mechanistic model for example nicely explains why IKK dimerization is required for IkBalpha phosphorylation, degradation and hence for efficient signaling output. The exosite on the IKK dimers is mapped to the so-called helical scaffold dimerization domain (SDD) region of IKKalpha/beta, which is distinct to the kinase domain and it serves an important regulatory role in IKK signaling, by cross-linking mass spectometry (CLMS) and mutational analysis in vitro and in cell-based tests. Unfortunately, a good crystallographic model of an IKK dimer/YDDphi-x-phi peptide is missing, albeit the authors report on the structure of the IKKbeta dimer crystallized in the presence of a 21-amino-acid long IkBalpha peptide, however the electron density for the peptide could not be found or interpreted. Despite the low quality of the diffraction data (low resolution, highly anisotropic diffraction, low completeness) the authors use their crystallographic model as the starting model for CLMS data guided HADDOCK modeling (albeit higher resolution IKK crystallographic dimer models are available in the PDB) and they present a structural model of the protein-peptide complex in which the peptide binds in an extended conformation at the interface of the SDD region from the two IKKbeta protomers.
Overall the presented structural model for the complex makes sense and is novel, however in my opinion it falls short in providing a reliable structural basis for IKK dimer/YDDphi-x-phi motif peptides in general. The author would need to characterize IKK exosite/peptide motif structure more carefully to be able to draw those general conclusions that go beyond the relevance of this interaction for IKKbeta dimer-IkBalpha binding. The manuscript is well-written, the methods are described in sufficient detail, the experiments are well-executed and are presented well. The outcome is original and the presented IKK catalytic domain docking of NF-kB substrates, as the authors point out, indeed resemble to the interaction system of AKAPs with the regulatory subunit dimer of PKA. However, the structural details of this IKK exosite mediated interaction is not sufficiently explored and for a broader impact of this work the characterization of YDDphi-x-phi motif containing in silico hits (76!) will be required (see major comments).

Major comments:

1) The best HADDOCK model is presented on Fig. 5C (and also made available as a PDB file as Supplementary Data 1). Based on this model the side-chain of C308 fits into a small hydrophobic pocket. Despite to this, as shown in Fig. 1e and also quantitatively confirmed on Fig. 2 or Table 1, the C308F or L mutants bind equally well compared to wild-type and even better to the IKKbeta dimer. If the binding pose of the peptide in the HADDOCK model is correct how can the authors explain this discrepancy? What do the authors think about the opening/closing of the “scissor-like” SDD region of the IKK dimer in general? Could this have some relevance here?

2) The last section in the Results (starting at line 361) aims to broaden the scope of the original finding about YDDphi-x-phi motif in general. A proteome-wide bioinformatic analysis using SLIMSearch detected 76 matches with this sequence pattern. This analysis, as it stands now, is highly problematic. Supplementary Table 2 lists all these identified motifs but the authors fail to discuss that most of the hits occur in structured protein regions, their IUPRED score is really low and are predicted to be part of secondary structural elements, which would be totally incompatible with that binding mode that they propose for the IKKbeta dimer/ IkBalpha peptide complex. Hence the diagram shown in Fig. 6A about the enrichment of "hits" in different pathways is also not appropriate (probably they all appear enriched because of the same set of handful proteins; but it would be good to list these explicitly to be able to judge this properly). The author tested the motif from p100 which turned out to be indeed positive, while the motif in IRF7 seems to be negative and there is a superfluously long discussion on the latter (see line 384-405). I believe that the authors need to carefully test several other candidate sequences and establish a more precise consensus and a better understanding of the structural basis of this new linear motif governed interaction system, moreover test at least some of them in full length protein context to prove functional relevance, to be able to make claims on YDDphi-x-phi motif and IKK dimer binding from a more general standpoint.

Minor comments:

1) The C308L mutant displays slightly lower entalphy gain but more importantly a lot smaller entropic cost upon binding in Table 1, hence better equilibrium binding must come from a greatly decreased entropic cost for the mutant. How the authors explain this unusual finding if the peptide is mostly in random coil in solution and modeled in that extended conformation as shown in the best HADDOCK model (see Supplementary fig. 6 and major comment 1)?

2) it is not clear what the sequence logo on fig. 6B shows exactly. In the legends it says that "Sequence logo calculated from the YDDFxF sequences from IkBa, IkBb, p100 and IRF7 and showing the position-specific frequency of each amino acid composing the motif". Do the authors mean the orthologs of all these proteins from some species? (since based on the alignment of the sequences shown above the logo is odd)

3) at line 372, the figure panel call-out should be Fig. 6B, instead of Fig. 6A

4) at line 1047 the figure panel call-out is wrong: Fig. 1D is needed instead of Fig 1C

5) at line 1284 ref 33 looks funny

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):
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Answer: We thank the reviewer for these positive comments and in particular for pointing to the information described in these early kinetic activity studies. We comment on these studies in the new version of the manuscript (lines 431-439).

Major revision

Reviewer: The measured binding affinity between the docking motif and IKKa (KD = 9 μM) is significantly higher as compared to IKKb(KD = 40 μM). However, in the cells, IKKb contributes mostly to the phosphorylation of IkBa in the canonical pathway to activateP50-RelA. In an in vitro kinase assay (Burke, J.R,. JBC Vol. 274, No. 51, Issue of December 17, pp. 36146–36152, 1999), IKKb is much more active in phosphorylating IkBa than IKKa; And an recombinant IKKa/IKKb heterodimer is more active in phosphorylating IkBa than either IKKb or IKKa homodimer (Huynh, Q.K. JBC Vol. 275, No. 34, Issue of August 25, pp. 25883–25891, 2000).

Answer: In the light of the studies mentioned by the reviewer and our results, we propose that the high activity of the **IKKα/IKKβ heterodimer derives from the combination of the kinetic efficiency of the IKKβ kinase domain (KD) with the higher docking affinity of the IKKα SDD domain in a single species.** The data in support of this model are:
1/ *Burke et al. 1999* show that the IKKβ homodimer phosphorylates much more efficiently **a peptide** form the N-terminal region **of IκBα** (residue 26-42) than the IKKα homodimer. This is due to the higher selectivity of the IKKβ KD domain for the DpSGxxpS/T phosphorylation motif.
2/ *Burke et al. 1999* also report that IKKβ homodimer kinase activity is moderately increased when the substrate is **full-length IκBα** instead of the IκBα N-ter peptide (10-fold decrease in Km, 2-fold increase in Vmax). This increase is linked to the presence of the docking motif within the C-terminal region of IκBα and is in agreement with our results shown in Supplementary Fig. 2A (compare wt IκBα and IκBα YD/SS mutant).
3/ In a previous report (Burke JR *et al* JBC, vol. 273, pp.12041, 1998), the same authors show that an IKKα/IKKβ heterodimer immunoprecipitated from Hela cells displays an even higher kinase activity for **full-length IκBα** (2500-fold decrease in Km, 10-fold increase in Vmax, for full-length IκBα against IκBα N-ter peptide). In contrast, *Huynh, Q.K. et al. 2000* find that IKKα/IKKβ heterodimer and IKKβ homodimer have very similar catalytic efficiencies for the **IκBα N-ter peptide** (kcat/KM 17.33 and 47.5 respectively). Together, these data indicate that the IKKα subunit enhances catalytic activity only if the docking motif is present on the IκBα substrate.
4/ As mentioned by the reviewer, the IKKα homodimer has higher affinity for the docking motif as compared to the IKKβ homodimer. Although we were unable to perform ITC experiments for the IKKα/IKKβ heterodimer (lines 163-164 of new manuscript), based on the band intensities in the pull-down experiments we think that the affinity of the IKKα/IKKβ heterodimer for the docking motif might be at least as high as that of the IKKα homodimer for the same motif.

Rewiewer: Does this imply that the docking motif-IKK binding negatively regulate the IkBa phosphorylation by IKKa…?
Answer: We can rule out this possibility since IKKα displays very poor kinase activity for the DpSGxxpS/T phosphorylation motif of IκBα.
Rewiewer: …or weaker binding to IKKb increases the turnover rate of the phosphorylated IkBa substrate to be released from IKKb to increases IKKb catalytic efficiency?
Answer: Results from our kinase experiments of Supplementary Fig. S2A show that the higher affinity IκBα C308L mutant is phosphorylated at higher levels compared to wt IκBα. Hence, a reinforced docking interaction seems to enhance kinase activity, at least for the binding affinity range we are looking at here. Indeed, the K_D value for the homodimer IKKβ/IκBα pep C308L interaction is very similar to the K_D for the homodimer IKKα/IκBα pep wt interaction (see Table 1).

Minor revision:

Reviewer: Line 177, "This is in agreement with binding data from Hu and coworkers 11, " to "This is in agreement with binding data from Xu and coworkers 11,"
Answer: we have corrected this mistake.

Reviewer #2 (Remarks to the Author):
…..

Answer: We are deeply grateful to the reviewer for recognizing our contribution in this field. These comments represent a great encouragement for us to pursue future studies in this field.

Reviewer #3 (Remarks to the Author):
….

Answer: We thank the reviewer for the careful reading of the manuscript, the positive comments and also for the constructive criticism, which has helped us to improve the quality of our work. In particular, the experiments related to major comment 2 have led to the identification of a novel partner of IKK in NF-κB signaling.

Major comment 1:

Reviewer : The best HADDOCK model is presented on Fig. 5C (and also made available as a PDB file as Supplementary Data 1). Based on this model the side-chain of C308 fits into a small hydrophobic pocket. Despite to this, as shown in Fig. 1e and also quantitatively confirmed on Fig. 2 or Table 1, the C308F or L mutants bind equally well compared to wild-type and even better to the IKKbeta dimer. If the binding pose of the peptide in the HADDOCK model is correct how can the authors explain this discrepancy? What do the authors think about the opening/closing of the "scissor-like" SDD region of the IKK dimer in general? Could this have some relevance here?
Answer: To address the issue raised by the reviewer on the size of the pocket interacting with C308 of the peptide, we have used our best HADDOCK model presented in the manuscript and PROT-ON (a computational tool that uses Fold-X and EvoEF1 - Kosaca M et al. Frontiers in Molecular Biosciences, DOI 10.3389/fmolb.2023.1063971, http://proton.tools.ibg.edu.tr:8001/new-run) to predict the effects of mutations within the peptide. Results are reported in the heatmap below (Fig. 1) and show that **mutations of C308** (C13 according to the peptide numbering) **into Phe, Met and Leu are associated** with negative

ΔΔG scores and therefore **with an increase in binding affinity**. This indicates that this binding pose is in principle compatible with a leucine at position 308 of IκBα.

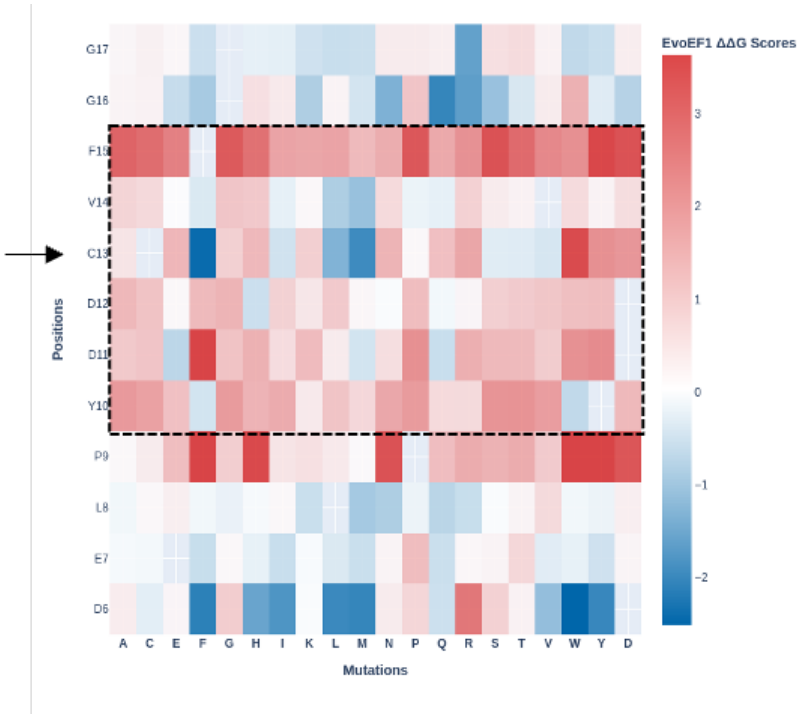

Fig 1. Predicted effects of peptide mutations calculated from the best model reported in the manuscript. The hatched box indicates the YDDΦxΦ motif residues. Residues E2-E5 and Q18-L22 of the peptide are not shown because of flexiblitly or lack of cross-linking data.

To explore the point on dimer flexibility raised by the reviewer, we have repeated HADDOCK calculations using (i) the more 'open' C-D dimer observed in the asymmetric unit of the crystal obtained from an IKKβ-IκBα pep complex sample (see Supp. Fig. S4C of the manuscript), (ii) an IκBα pep comprising the C308L mutation and (iii) the same set of CLMS distance restraints. The best model from this calculation is very similar to the one presented in the manuscript, with differences observed mainly at the level of side-chain orientation (see Fig. 2 below).

Hence, **dimer opening does not appear to have a major effect on the structure of the YDDΦxΦ binding groove**. As previously pointed out, dimer opening mostly impacts the inter-subunit distance between the KD-SDD regions (see Fig 3 C in Polley *et al.* PloS Biology 2013). However, this opening-closing dynamics may induce some sort of "breathing" of this groove, favoring subtle main- and side-chain rearrangements that would allow for adapting to sequence variations in the docking motif.
The results from these qualitative analyses are provided here for the reviewer. We prefer not to show them in the manuscript to avoid over-interpretation of our structural model.

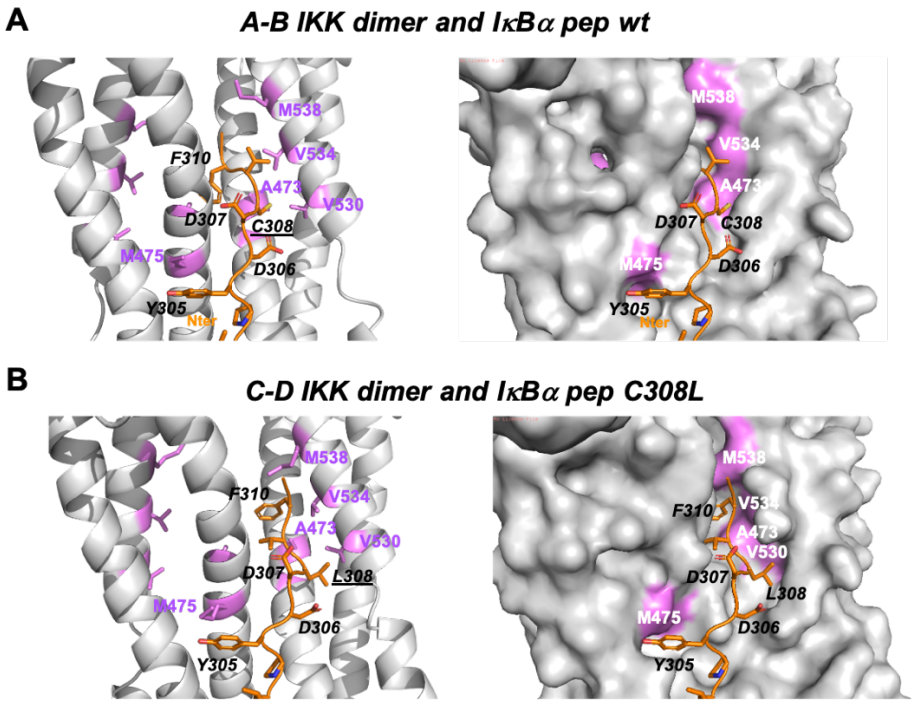

Fig. 2. The YDDΦxΦ binding groove in the models calculated using the 'closed' A-B IKKβ dimer and IκBα pep wt (A) and the 'open' C-D IKKβ dimer and IκBα pep C308L (B). The two structures are shown in the same orientation. Interface IKKβ hydrophobic residues are shown in pink (purple labels, left, and white labels, right). The IκBα pep is shown in orange. The conserved YDDΦxΦ residues are labeled black.

Major comment 2

Reviewer: The last section in the Results (starting at line 361) aims to broaden the scope of the original finding about YDDphi-x-phi motif in general. A proteome-wide bioinformatic analysis using SLIMSearch detected 76 matches with this sequence pattern. This analysis, as it stands now, is highly problematic. Supplementary Table 2 lists all these identified motifs but the authors fail to discuss that most of the hits occur in structured protein regions, their IUPRED score is really low and are predicted to be part of secondary structural elements, which would be totally incompatible with that binding mode that they propose for the IKKbeta dimer/ IkBalpha peptide complex. Hence the diagram shown in Fig. 6A about the enrichment of "hits" in different pathways is also not appropriate (probably they all appear enriched because of the same set of handful proteins; but it would be good to list these explicitly to be able to judge this properly).

Answer : The reviewer is right. In the new version of the manuscript motif matches have been filtered based on accessibility (see new Supplementary Data 4). As a result, we now have 27 matches with an accessible motif. We have also removed the diagram of former Fig. 6A.

Reviewer: The author tested the motif from p100 which turned out to be indeed positive, while the motif in IRF7 seems to be negative and there is a superfluously long discussion on the latter (see line 384-405).

Answer : We have reduced the text describing the negative results on IRF7 (lines 382-393 of the new manuscript).

Reviewer: I believe that the authors need to carefully test several other candidate sequences and establish a more precise consensus and a better understanding of the structural basis of this new linear motif governed interaction system, moreover test at least some of them in full length protein context to prove functional relevance, to be able to make claims on YDDphi-x-phi motif and IKK dimer binding from a more general standpoint.

Answer : We have followed the reviewer's recommendations and selected 7 candidate motif peptides for binding experiments. The criteria for the selection were the following:

(i) accessibility of the motif. Five of the selected hits are in intrinsically disordered regions (IDR), two in loops within domains;

(ii) functional relations to signaling;

(iii) availability of cDNAs for the full-length proteins in DNASU or ADDGENE repositories.

The motif peptides were cloned as fusions to MBP and tested against recombinant purified IKKα and IKKβ homodimers in two independent pull-down experiments. Only one peptide, namely BANK1 pep, interacts with IKKα, while none interact with IKKβ (new Supplementary Fig. 11). Based on these results, we suppose that certain amino acids at specific positions, e.g. the x position within the motif or positions flanking the motif, would block IKK binding. However, such amino acids are difficult to precisely identify from negative datasets of this size. The sequence Logo has been updated with the BANK1 orthologous sequences (Fig. 6H of the new manuscript).

The interaction between the full-length IKKa and BANK1 proteins has been validated *in vivo* by the GPCA assay. Furthermore, mutation of the Y_1 and D_3 motif positions into Ser residues (YD/SS) within BANK1 reduces the interaction with IKKα (new Fig. 6G). Please note that for the expression blot of Fig. 6G (right panel) we used a polyclonal anti-gluc antibody from Invitrogen that is not the one used in Figs. 1D, Fig. 6E and Supp. Fig. 10D (not sold any more). This new antibody gives a better signal for the G2-fusion proteins.

BANK1 is a scaffold protein, which functions downstream of the BCR receptor and associates to the TRAF6/MyD88 complex in B-cells. These results provide additional support to the view that the YDDΦxΦ motif mainly plays a role in NF-kB signaling processes. They also confirm a broader selectivity for IKKα as compared to IKKβ.

To conclude, we show that bioinformatic searches based on the YDDΦxΦ motif can predict IKK binders. The predictive power will certainly increase with a better definition of the consensus, which can only be derived from high-resolution structural analyses and/or larger datasets from peptide arrays, as pointed out by the reviewer.

<u>*Minor comment 1:*</u>

Reviewer: The C308L mutant displays slightly lower entalphy gain but more importantly a lot smaller entropic cost upon binding in Table 1, hence better equilibrium binding must come from a greatly decreased entropic cost for the mutant. How the authors explain this unusual finding if the peptide is mostly in random coil in solution and modeled in that extended conformation as shown in the best HADDOCK model (see Supplementary fig. 6 and major comment 1)?

Answer : The referee raises a legitimate question to which we can only propose the following tentative answer related to water molecules being dislodged upon ligand binding. We may suppose as a first approximation that such a bound molecule may be compared to a water molecule bound to ice and going back into the bulk during ice melting at $T_0 = 273.15$ K. Within this comparison, there is no reason for the positive enthapy of ice melting to be a good approximation for the positive enthalpy of dislodging water from the ligand cavity. However, the positive entropic term has to be a good approximation. For ice melting $\Delta_m H \approx 1.4$ kcal mol^{-1} and $\Delta_m S = \Delta_m H/T_0 \approx 5.2$ cal mol^{-1} K^{-1}. At $T \approx 300$ K this implies $T\,\Delta_m S \approx 1.6$ kcal mol^{-1} K^{-1}.

In the case of IKKα, we see that two additional water molecules being dislodged would explain quite well the observed positive variation of entropy (3.6 against 3.2 kcal mol^{-1} K^{-1}) from the wt to the C308L peptide, but would give only a correct order of magnitude for the variation of enthalpy (1.7 against 2.8 kcal mol^{-1}). In the case of IKKβ, the variations of entropy and enthalpy would rather be compatible with three water molecules (5.2 against 4.8 kcal mol^{-1} K^{-1} and 3.6 against 4.2 kcal mol^{-1}).
For sure, this is only speculation. Unfortunately, the resolution of our structure is insufficient to locate water molecules.

Minor comment 2

Reviewer: it is not clear what the sequence logo on fig. 6B shows exactly. In the legends it says that "Sequence logo calculated from the YDDFxF sequences from IkBa, IkBb, p100 and IRF7 and showing the position-specific frequency of each amino acid composing the motif". Do the authors mean the orthologs of all these proteins from some species? (since based on the alignment of the sequences shown above the logo is odd).
Answer: Yes, indeed, the sequence logo is based on the orthologous peptides of these proteins. We have clarified this in the new version of the manuscript (see legend of new Fig. 6H).

Other minor comments.
3) at line 372, the figure panel call-out should be Fig. 6B, instead of Fig. 6A
4) at line 1047 the figure panel call-out is wrong: Fig. 1D is needed instead of Fig 1C
5) at line 1284 ref 33 looks funny
Answer. We have corrected these mistakes.

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
All my questions have been addressed in the revision. The revised manuscript is recommended for publication in NATURE COMMUNICATIONS.

Reviewer #3:
Remarks to the Author:
The authors gave a fair response for my questions/comments and greatly improved the manuscript regarding their bioinformatics based hit list. I recommend publication in Nature Communications.