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

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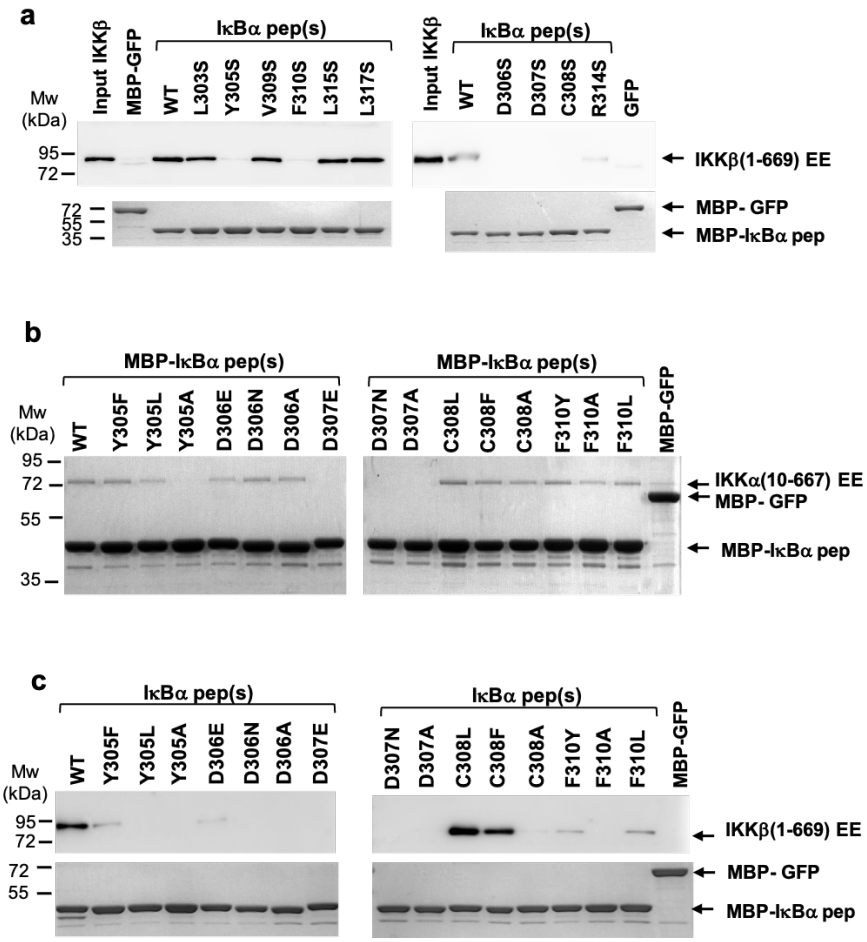
Supplementary Information:

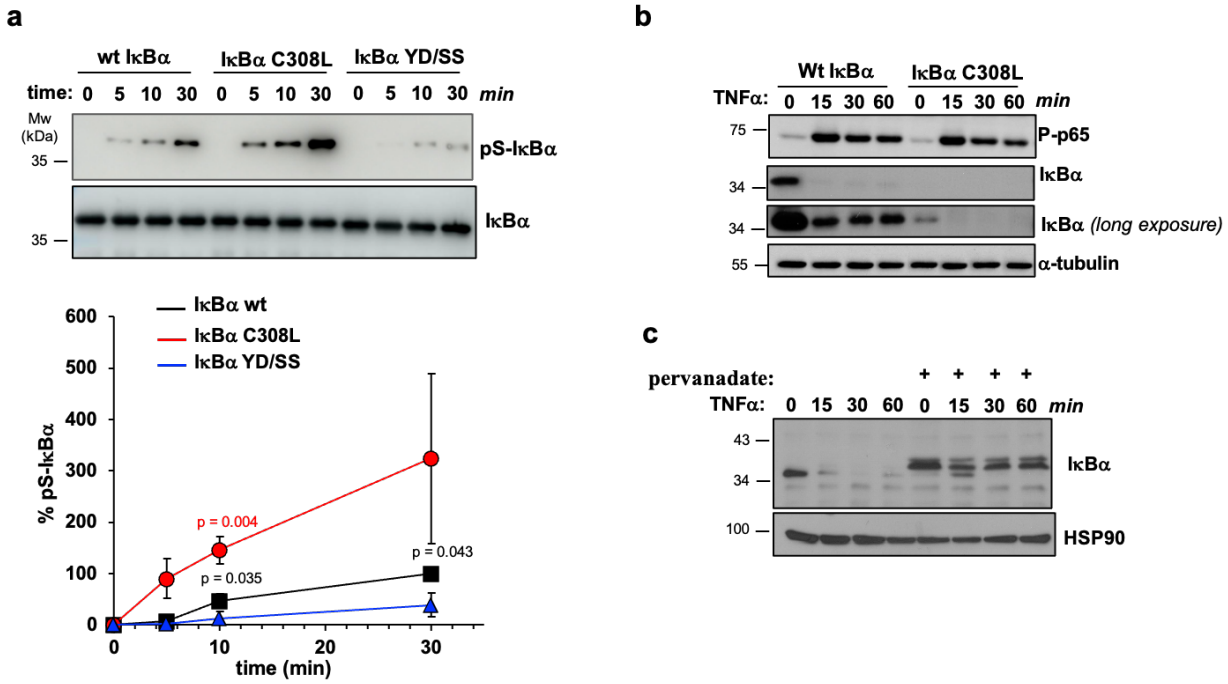
Supplementary Figures 1 to 12

Supplementary Table 1

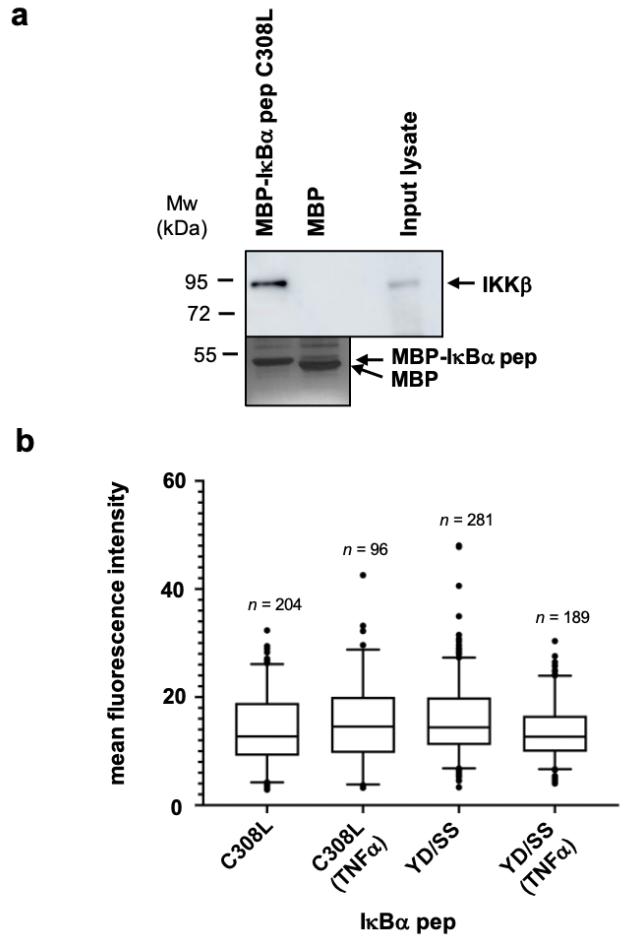
Original gel images for Supplementary Figures



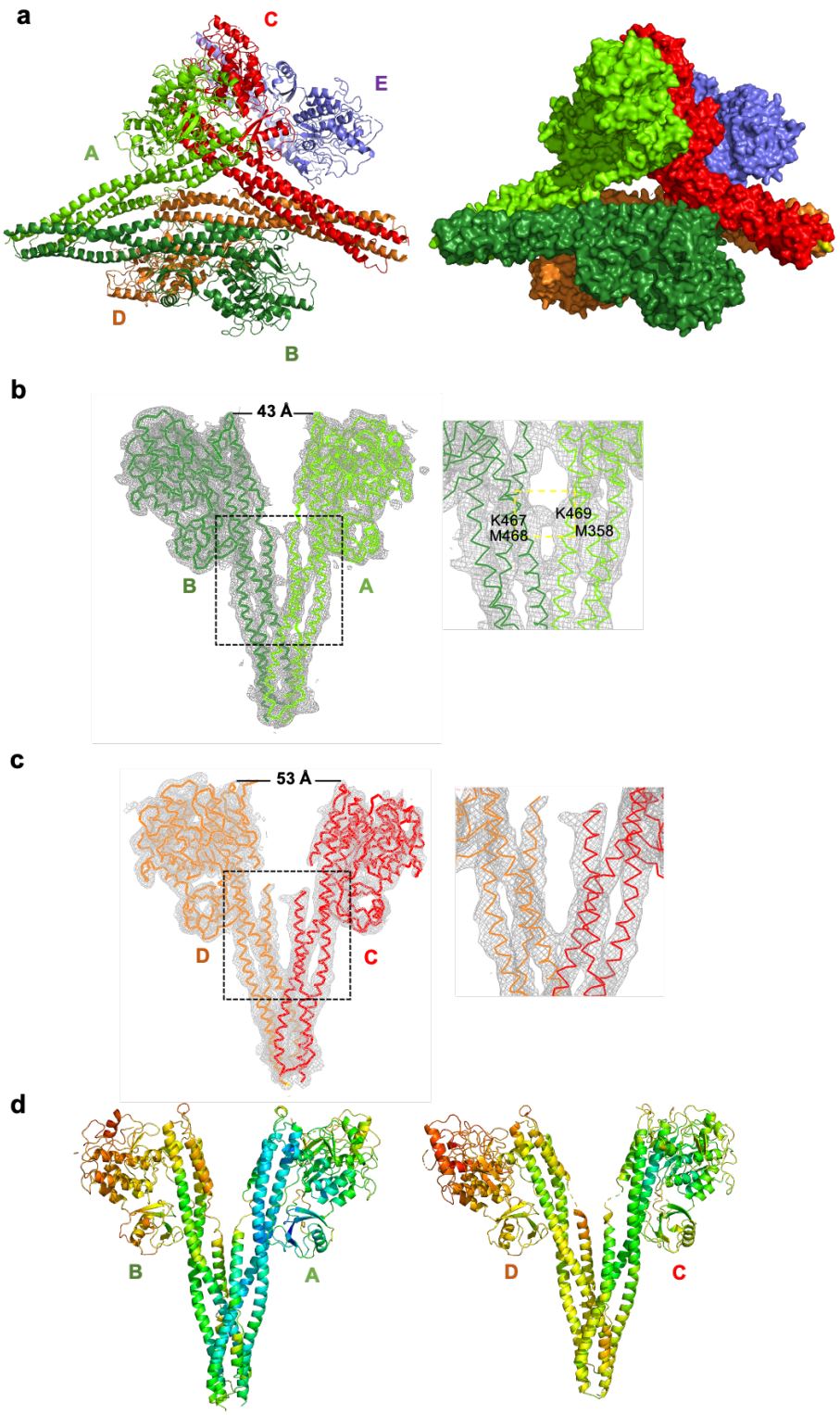
Supplementary Fig. 1. Pulldown analyses of MBP-IκBα pep(s) mutants. **a** Interactions of the 6xHis-IKKβ(1-669) EE with MBP-IκBα pep(s) constructs bearing the indicated serine substitutions. These results were confirmed with a second independent pulldown experiment. **b-c** Representative images of the pulldown experiments on the interactions between 6xHis-IKKα(10-667) EE or 6xHis-IKKβ(1-669) EE homodimer samples and MBP-IκBα pep(s) constructs containing single amino acid substitutions at the conserved position of the YDDΦxΦ consensus. See also Fig. 1e of main text for quantifications. **a-c** All pulldown samples were migrated on a 10% SDS-PAGE gel. 6xHis-IKKβ(1-669) EE was detected by Western blotting (anti-His antibody), whereas 6xHis-IKKα(10-669) EE and MBP-IκBα pep constructs were detected by Coomassie staining. Original uncropped images are provided at the end of this file.



Supplementary Fig. 2. Activity and functional analyses. **a** *In vitro* kinase activity experiments using purified 6xHis-IKKβ(1-669) EE (0.5 nM) and full-length IκBα wild type (wt), C308L and YD/SS proteins (1 μM). Reaction samples were incubated at 30 °C, aliquots taken at the indicated times and analyzed by Western blotting using an antibody specific for Ser32/Ser36 phosphorylation of IκBα (pS-IκBα), and an anti-His antibody for 6xHis-IκBα detection. Samples were run onto two separate 10% SDS-PAGE gels for detection of total IκBα and pS-IκBα. (*Top panel*) Representative Western blot images. (*Lower panel*) Quantification of pS-IκBα levels normalized to IκBα at 30 min. The data (mean +/- SD) are averages of three independent kinase activity experiments. The indicated *P*-values (p) are obtained from a two-tailed unpaired *t*-test, $n = 3$ biological triplicates. Black: differences between IκBα YD/SS and IκBα wt; red: differences between IκBα C308L/F and IκBα YD/SS. **b** *In cellulo* degradation of IκBα C308L in MEFs KO for IκBα, IκBβ, IκBε. Wt IκBα is shown for comparison. See also legend of Fig. 3b of main text for experimental conditions. Note that IκBα C308L is only detectable at long exposures times, indicating low expression levels. **c** Western blot showing pervanadate inhibition of IκBα degradation. HEK293T cells were incubated or not with pervanadate (1 mM) for 30 minutes and stimulated with TNFα (10 ng/ml). Aliquots were taken at the indicated time points after TNFα stimulation. Source data are provided as a Source Data file. Original uncropped images are provided at the end of this file.

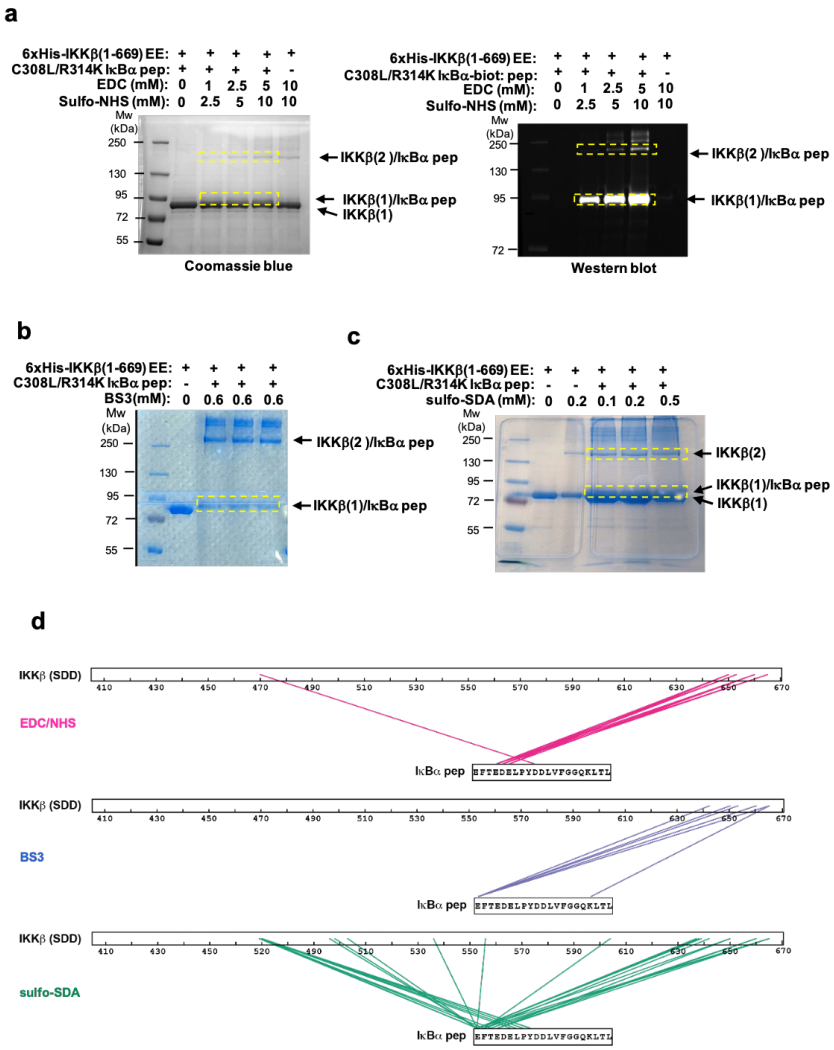


Supplementary Fig. 3. Inhibition of IKK activity by IκBα pep C308L. **a** Pulldown analysis of the interaction between the MBP-IκBα pep C308L peptide and endogenous IKKβ. MBP-IκBα pep C308L was expressed in *E. coli*, coupled to amylose resin and incubated with clarified extracts of HEK293T cells. Pulldown samples were migrated on two separate 10% SDS-PAGE gels. Endogenous IKKβ was detected by Western blotting using an anti-IKKβ antibody, whereas IκBα by Coomassie staining. MBP was used a negative control. This result was confirmed in a second independent pulldown experiment. **b** Mean fluorescence intensities of nuclear of mScarlet-E3-IκBα pep fusions in MRC5 cells for the four conditions of the experiment shown in Fig. 4c of the main text. The data are displayed in a box-and-whisker representation showing the median in the center line, the 75/25 percentiles at the boxes, the 5/95 percentiles at the whiskers and the extreme values to the minimum and maximum of the raw fluorescence data. *n*: number of cells analyzed. See also legend of Fig. 4b-c of the main text. Source data are provided as a Source Data file. Original uncropped images are provided at the end of this file.

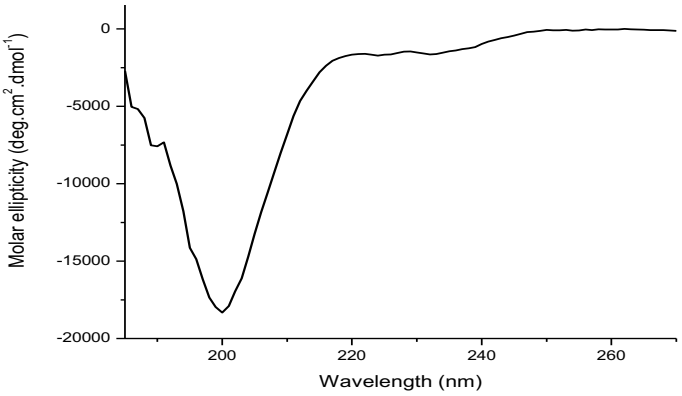


Supplementary Fig. 4. X-ray structure of the IKKβ homodimer. **a** The asymmetric unit of the crystal showing IKKβ protomers A-E in the ribbon (*left*) and surface (*right*) representations. A-B and C-D dimers stack onto each other through the V-shape and antiparallel interfaces described by *Polley et al.* [12]. Protomer E forms a homodimer with a molecule in the next unit

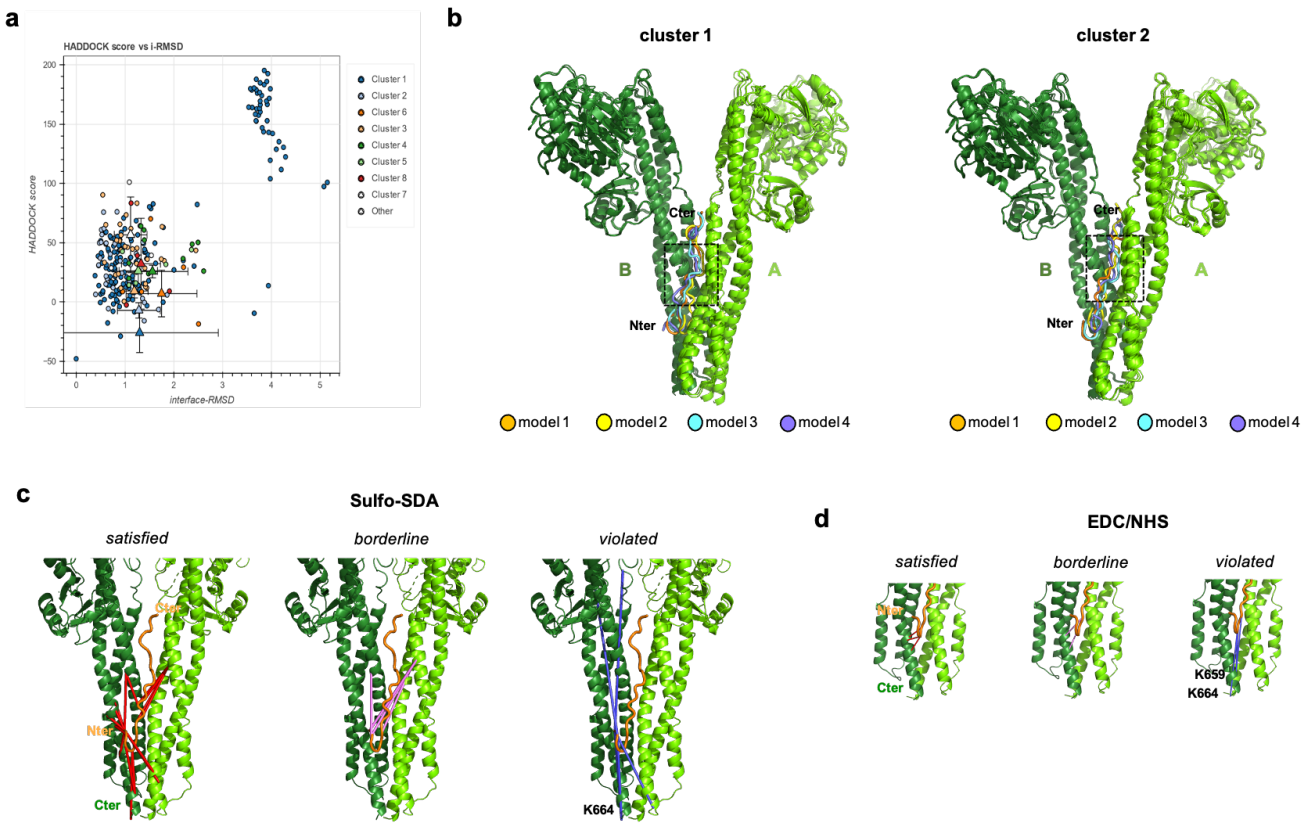
cell. **b-c** (*Left panels*) Molecular views showing the direct electron density map (plotted at 1σ) for dimers A-B and C-D. Boxes indicate the position of the right hand-side views. The distance between residues Asp580 in the dimer are reported (Cα-Cα). The A-B Dimer displays a closed conformation as compared to the C-D dimer. (*Right panel*s) Enlarged views of the region binding to the IκBα pep according to the CLMS data. Yellow dashed lines: lobe of extra electron density in the proximity of K469 of the A-B dimer. This lobe is not visible in the C-D dimer. **d** Crystallographic average B-factors displayed on structures of the A-B (*left panel*) and C-D (*right panel*) homodimers with color code ranging from blue (low B-fractor) to red (high B-factor).



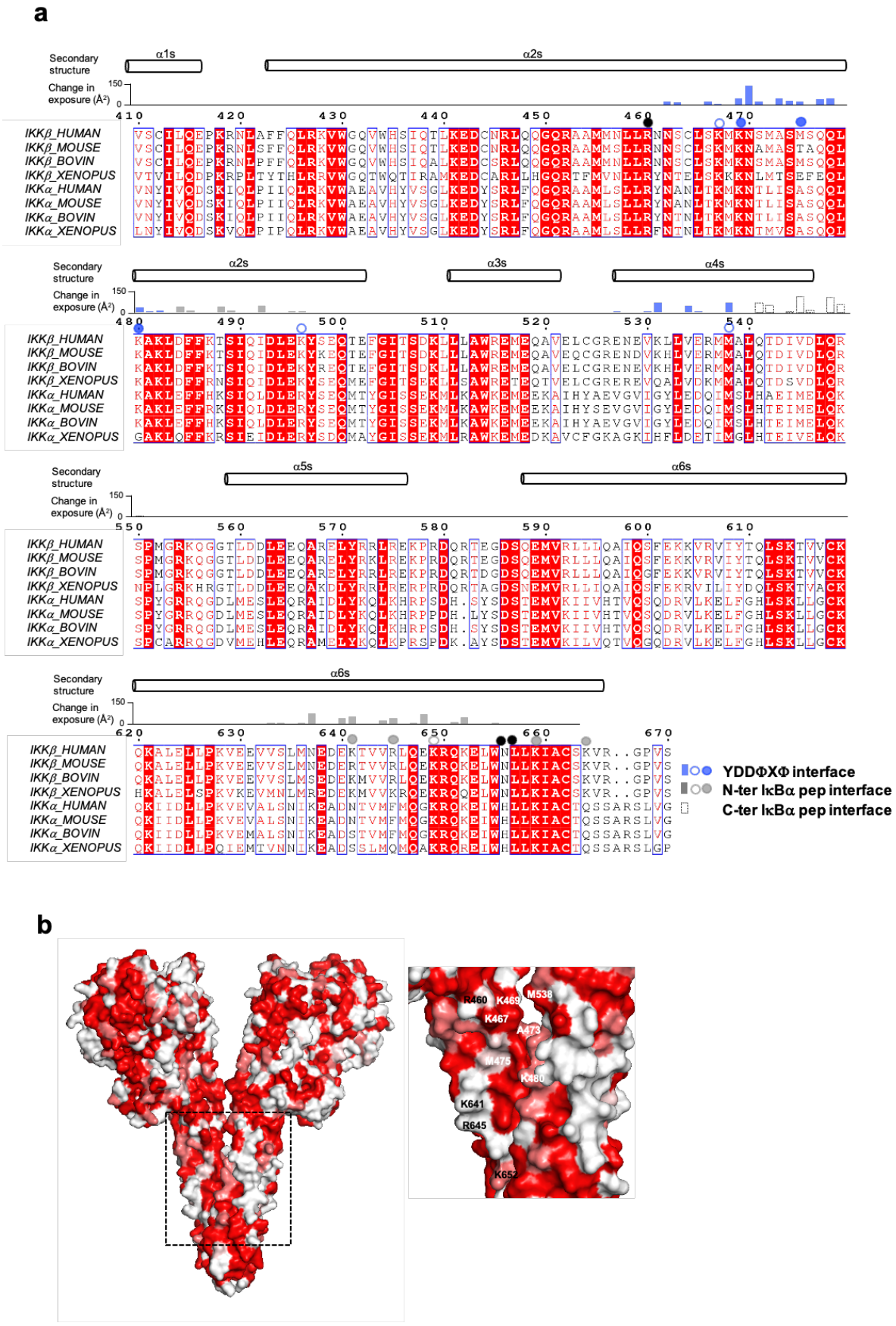
Supplementary Fig. 5. Cross-linking of the IKKβ/IκBα pep complex. Purified 6xHis-IKKβ(1-699) EE protein was mixed to a 10-fold excess of the synthetic C308L/R314K IκBα pep. After cross-linking samples were migrated on an 8% SDS-PAGE gel. Yellow dashed lines indicate protein bands analyzed by MS, consisting of either a monomer of IKKβ (IKKβ(1)) or a crossed-linked dimer of IKKβ (IKKβ(2)), in both cases conjugated to IκBα pep. **a** Cross-linking with EDC/sulfo-NHS at the indicated concentrations. (*Left panel*) Coomassie stained gel. (*Right panel*) Western blot analysis using the biotinylated peptide variant (C308L/R314K IκBα-biot pep) and streptavidin conjugated with Alexa Fluor™ 680 for evaluation of cross-linking efficiency. **b-c** Cross-linking with BS3 and sulfo-SDA at the indicated concentrations (Coomassie stained gels). **d** IKKβ-peptide cross-links identified by MS analysis in conditions of EDC/sulfo-NHS *(upper panel*, magenta lines), BS3 (*middle panel*, purple lines) and sulfo-SDA (*lower panel*, green lines). All the links identified map to the SDD domain of IKKβ (residues 410-667).



Supplementary Fig. 6. Far UV circular dichroism spectrum of synthetic peptide IκBα pep. The spectrum was acquired at T=25 °C using a Jasco J-815 instrument. The peptide (IκBα pep C308L at 1.25 mM concentration) was resuspended in 20 mM Hepes pH 8.0. The spectrum is consistent with the following contribution: 5% α-helix, 11,3 % β-sheet, 9.3 % turn and 74.2 % random coil.

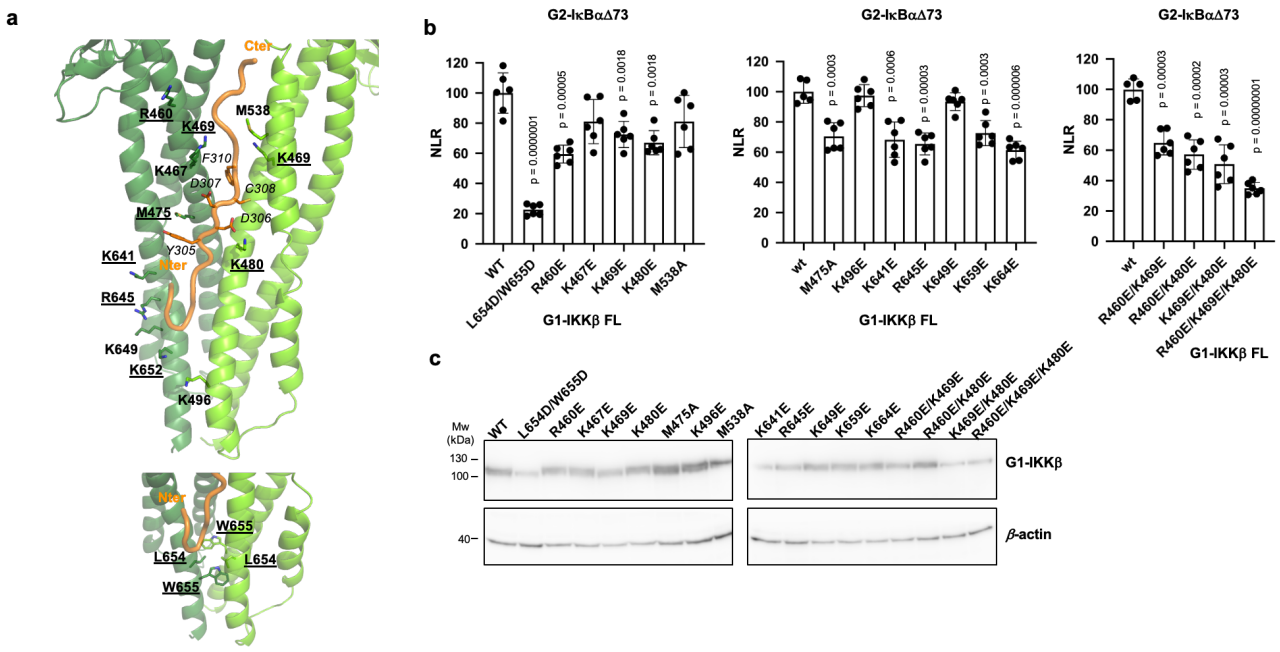


Supplementary Fig. 7. Integrative modelling of the IKKβ/ IκBα pep complex using x-ray diffraction and CLMS data. **a** Score convergence for the accepted clusters. The HADDOCK score plotted as a function of interface root-mean-square-deviation (RMSD) with a cutoff of 5 Å. Circles: individual structures; triangles: cluster averages. Error bars: SD values. The mean within-cluster interface RMSD of the best scoring cluster (cluster 1) is 1.3 Å. **b** Structural alignment of the four best scoring models for the two best scoring clusters (cluster 1 and cluster 2). The IKKβ/IκBα pep complexes are oriented and colored as in Fig. 5a except for the peptide backbone, which is color coded for the different models are indicated. The hatched box marks the position of the YDDΦxΦ motif. **c-d** Unique CLMS distance restraints used for the docking calculations mapped on the final model. **c** Sulfo-SDA derived restraints. Red lines: satisfied links with a Cβ-Cβ distance </= 22.5 Å; pink lines: borderline links with a Cβ-Cβ distance 22.5 - 27.5 Å; purple lines: violated links with Cβ-Cβ distance > 22.5 Å. **d** EDC/sulfo-NHS derived restraints. Red lines: satisfied links with a Cβ-Cβ distance </= 7.0 Å; pink lines: borderline links with a Cβ-Cβ distance 7.0 - 9.5 Å; purple lines: violated links with Cβ-Cβ distance > 9.5 Å.

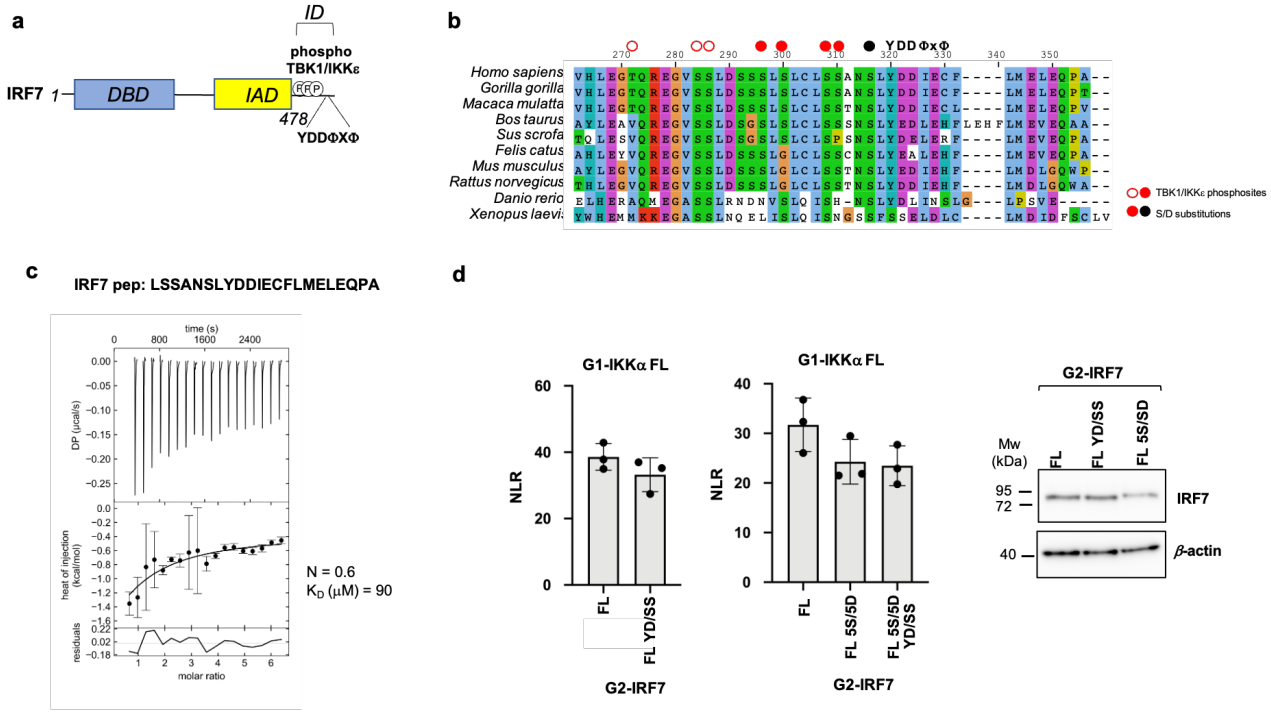


Supplementary Fig. 8. Residue conservation within the YDDΦxΦ binding groove. **a** Sequence alignment of the SDD domains of IKKβ and IKKα proteins (residues 410-664). Residue

numbering on top of the alignment refers to the human IKKβ protein. The histogram on top of the alignment reports on the burial of residues at the interface with IκBα pep. The "change of exposure" value corresponds to the change in the solvent accessible surface (SASA) in the presence or absence of the peptide. SASA values were calculated using PyMOL and were summed over protomers A and B. Circles indicate residues mutated in this study. Filled circles: mutations decreasing binding to IκBα. Open circles: mutations having no impact on IκBα binding (see also Supplementary Fig. 9b). Purple histograms/circles: residues contacting the YDDΦxΦ motif; gray histograms/circles: residues contacting the N-terminal region of IκBα pep; white hatched histograms: residues contacting the C-terminal region of IκBα pep, which is poorly defined in the different models of cluster 1 due to lack of CLMS restraints (see Supplementary Fig. 5c); black circles: other residues. **c** (*Left panel*) Residue conservation mapped on the IKKβ homodimer. The dashed box marks the IκBα pep binding region reported in the right panel. Red: absolute conservation; salmon: conservation of amino acid physico-chemical properties; light pink: partial conservation. (*Right panel*) View of the YDDΦxΦ binding groove. Molecular views in the left and right panel are shown in the same orientations as in Fig. 5 of the main text.



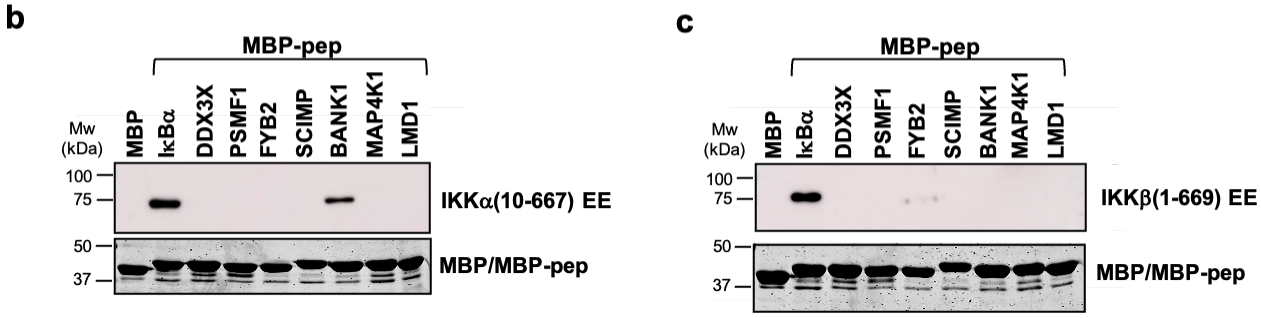
Supplementary Fig. 9. Mutagenesis of IKKβ residues at the IκBα pep and dimer interfaces. **a** Views of the IKKβ/IκBα pep interface (*upper panel*) and IKKβ dimerization (*lower panel*) regions of the complex. The side chains of mutated residues of IKKβ are displayed (black bold labels). Residues whose mutagenesis leads to decreased binding are indicated (underlined labels). **b** GPCA analysis of the interactions between mutants of full-length IKKβ and IκBαΔ73 fused to the G1 and G2 fragments of the luciferase, respectively. For each interaction data (mean +/- SD) are derived from two GPCA datasets, with each dataset comprising three independent transfection experiments of the same interaction. The data are normalized to the average value of the wt IKKβ/IκBαΔ73 interaction in each dataset. *P*-values (p) are derived using a two-tailed unpaired *t*-test, $n = 6$ biological replicates and report on the differences with the wt IKKβ/IκBαΔ73 interaction. **c** Western blot analysis of the expression levels of G1- IKKβ mutants in HEK293T cells. See also legend of Fig. 1c of the main text. Source data are provided as a Source Data file. Original uncropped images are provided at the end of this file.



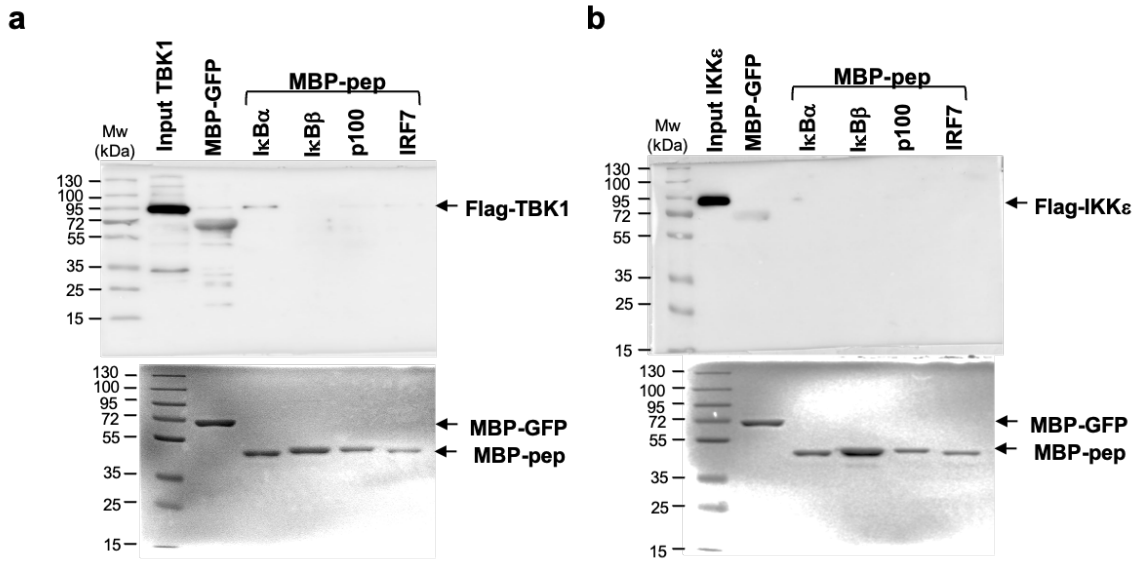
Supplementary Fig. 10. Interaction between IKKα and IRF7. **a** Schematic representation of IRF7 domain architecture. DBD: DNA binding domain (residues 10-127); IAD: IRF association domain (residues 237-423); disordered C-terminal region containing the the YDDΦxΦ motif and adjacent TBK1/IKKε phosphorylation cluster. **b** Sequence alignment of the IRF7 C-terminal region (residues 458-503). Ser/Thr residues targeted by TBK1/IKKε according to Caillaud *et al.* [36] and tenOever *et al.* [37] are indicated by red circles. The YDDΦxΦ motif is indicated in bold. Residues substituted with aspartic acid in the IRF7 5S/5D construct are indicated by filled circles. **c** ITC binding isotherm for the IKKα/IRF7 pep interaction, characterized by low affinity and weakly exothermic binding. A global fit based on two independent datasets was attempted but due to high experimental error it did not converge. Error bars correspond to the RMSD of the fitted curve and experimental values. The tentative estimate of the binding constant is indicated on the right-hand side. **d** (*Left and middle panels*) Representative GPCA datasets of the interactions between full-length wt G1-IKKα and G2-IRF7 constructs. G2-IRF7 FL YD/SS: G2-IRF7 FL Y502S/D504S; G2-IRF7 FL 5S/5D: G2-IRF7 FL S477D/S479D/S483D/S484D/S487D; G2-IRF7 FL 5S/5D YD/SS: G2-IRF7 FL S477D/S479D/S483D/S484D/S487D/Y502S/D504S. (*Right panel*) Expression levels of G2-IRF7 proteins in HEK293T cells. See also legend of Fig. 1c of the main text. Source data are provided as a Source Data file. Original uncropped images are provided at the end of this file.

a

IκBα (297-317)	EFTFDELP**YDDC** V**F**GGQRLTL
DDX3X (155-174)	NTGI NFEK**YDD I** P**V**EATGNN
PSMF1 (257-271)	NPDH LPPPG**YDDMYL**
FYB2 (579-599)	LKSQEVI I **YDDV**D**L**SEKESKD
SCIMP (123-143)	SY I EPEDD**YDDV**E **I** PANTEKA
BANK1 (476-495)	GSESSEDQ**YDDL**Y **V**FI PGAD
MAP4K1 (373-393)	LSESSDDD**YDDV**D **I** PTPAEDT
LIMD1 (1-16)	MDK**YDDL**G **L**EASKFI E



Supplementary Fig. 11. IKKα and IKKβ binding to YDDΦxΦ motif peptides derived from proteins identified using SLIMSearch [35]. **a** Motif peptide sequences from *human* DDX3X, PSMF1, FYB2, SCIMP, BANK1, MAP4K1 and LIMD1 proteins used in the pulldown experiments shown in (b). **b** Pulldown analyses of the interactions between recombinant 6xHis-IKKα(10-667) EE (*left panel*) or 6xHis-IKKβ(1-669) EE (*right panel*) proteins and MBP-peptide fusions. IKKα and IKKβ were detected by Western blot (anti-His antibody) and MBP-peptides by Coomassie staining. These results were reproduced in a second independent experiment. See also legend of Fig. 1b. Original uncropped images are provided at the end of this file.



Supplementary Fig. 12. Pulldown analyses of TBK1 (**a**) or IKKε (**b**) and YDDΦxΦ motif peptides from IκBα, IκBβ, p100 and IRF7. Cleared cellular lysates of HEK23T overexpressing Flag-TBK1 or Flag-IKKε proteins were incubated with MBP-YDDΦxΦ peptides coupled to amylose resin. Pulldown samples were processed as described in the Methods section. TBK1 and IKKε were detected by Western blot (anti-Flag antibody) and MBP-peptides and negative control MBP-GFP by Coomassie staining. These results were confirmed with a second independent experiment. See also legend of Fig. 6b.

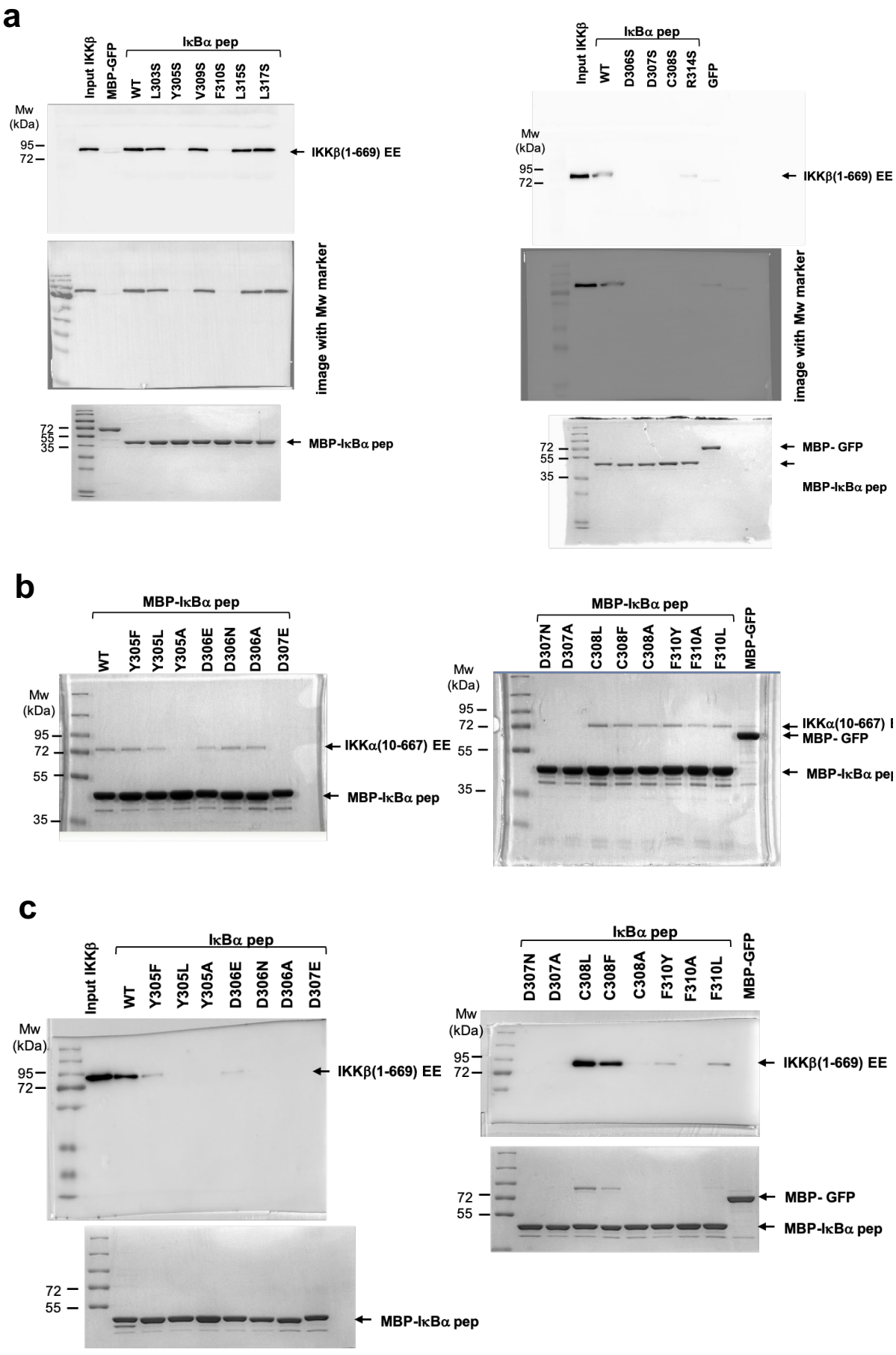
	8OMV
Data collection	
Source	Proxima 2A, SOLEIL
Detector	Dectris EIGER X 9M
Wavelength (Å)	0.98
Temperature (K)	100
Resolution range (Å)	63.25 - 4.16 (4.31 - 4.16)
Space group	C 1 2 1
Unit cell	
a, b, c (Å)	226.29, 136.80, 204.36
α, β, γ (°)	90, 91.453, 90
Total reflections	329672 (32729)
Unique reflections	46834 (93)
Multiplicity	7.0 (7.1)
Completeness (%)	43.62 (2.02)
{I/σ(I)}	3.71 (0.11)
Wilson B-factor (Å²)	154.8
R_{merge}	0.2766 (12.62)
R_{meas}	0.2996 (13.61)
R_{pim}	0.1137 (5.059)
$CC_{1/2}$	0.993 (-0.0095)
CC*	0.998 (-0.138)
Anisotropic truncation	
Ellipsoidal resolution (Å) (direction)	3.98 (0.973 a* - 0.229 c*)
	6.81 (b*)
	5.532 (0.254 a* + 0.967 c*)
Best / worst diffraction limits (Å)	4.16 (4.42 – 4.16) / 7.52
Unique reflections (ellipsoidal)	20490 (1025)
Mean I/sigma(I) (ellipsoidal)	7.5 (1.2)
R_{meas} (ellipsoidal)	0.206 (2.731)
R_{pim} (ellipsoidal)	0.079 (1.070)
CC/2 (ellipsoidal)	0.980 (0.244)
Ellipsoidal completeness (%)	88.1 (61.8)
Refinement	
Reflections used in refinement	20489 (351)
Reflections used for R_{free}	1018 (11)
R_{work}	0.257 (0.348)
R_{free}	0.299 (0.416)
CC(work)	0.857 (0.365)
CC(free)	0.809 (0.554)
Number of non-hydrogen atoms	25125
macromolecules	25125
ligands	0
solvent	0

Protein residues	3111
RMSD bond lengths (Å)	0.002
RMSD bond angles (°)	
Ramachandran plot	0.57
Favoured regions (%)	95.62
Additionally allowed regions (%)	4.28
Outlier regions (%)	0.1
Rotamer outliers (%)	0
Clashscore	8.92
Average B-factor (Å^2)	304
macromolecules	304

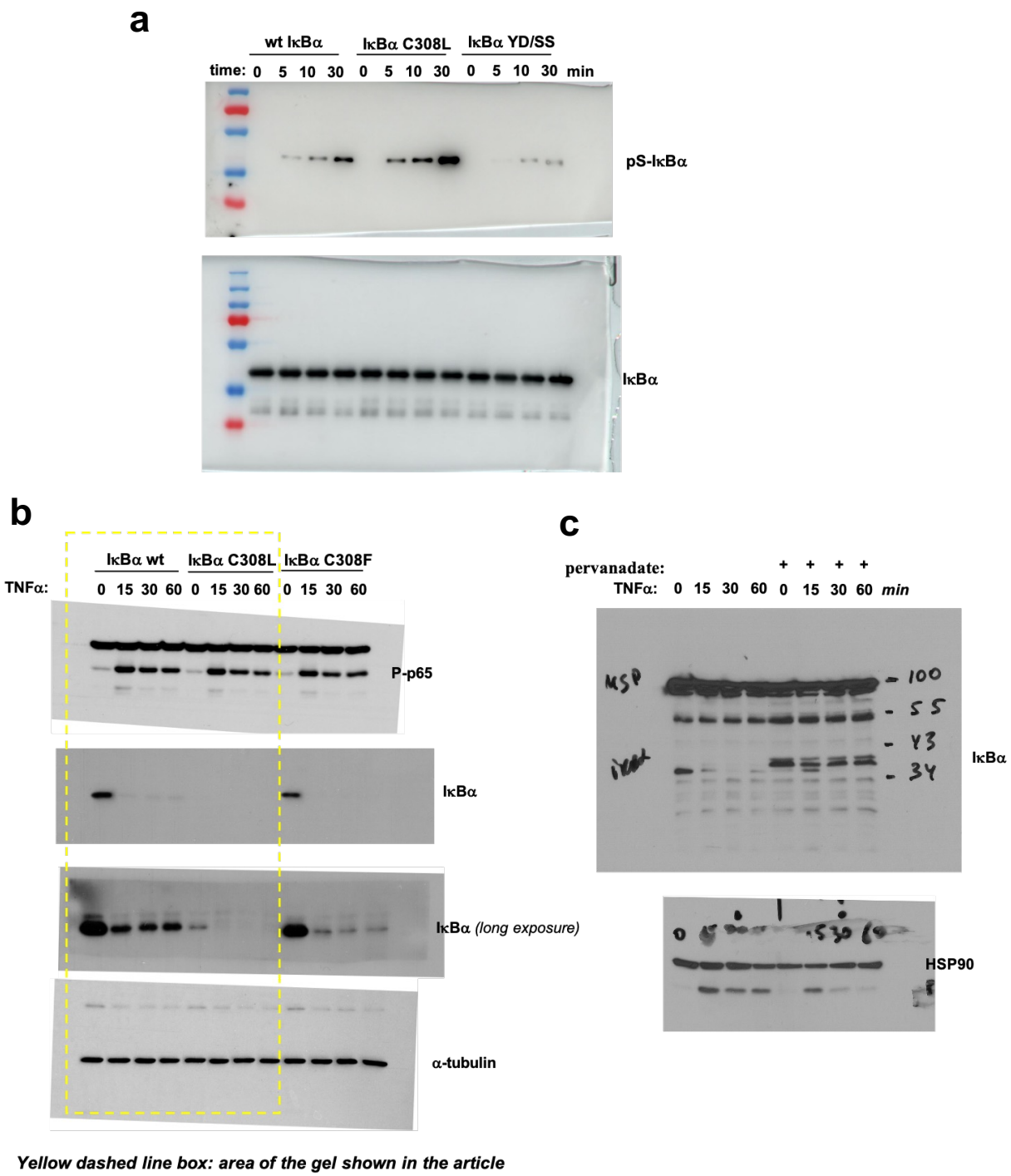
Supplementary Table 1. X-ray data collection and refinement statistics. Values in parentheses are for the highest resolution shell.

Original gel images for Supplementary Figures

Supplementary Figure 1



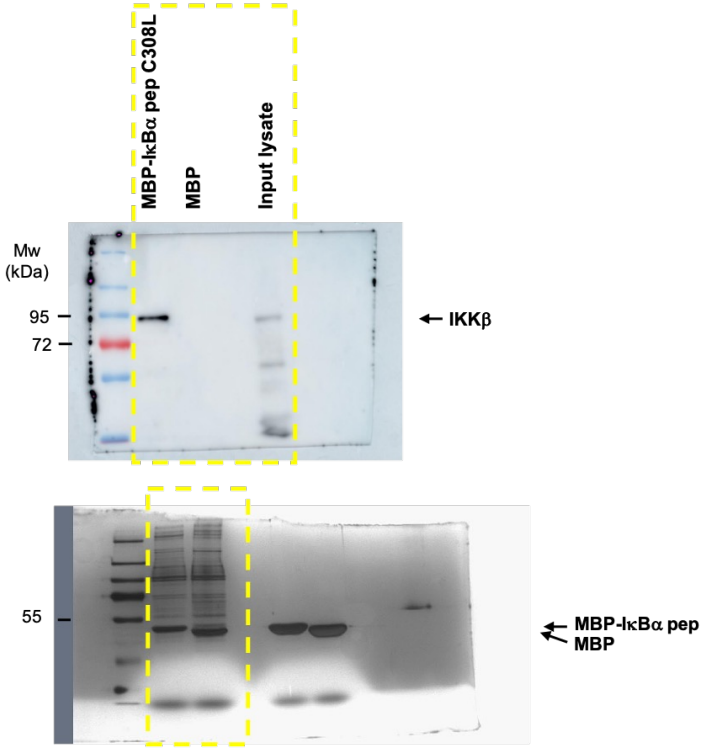
Supplementary Figure 2



Yellow dashed line box: area of the gel shown in the article

Supplementary Figure 3

a



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Supplementary Figure 9

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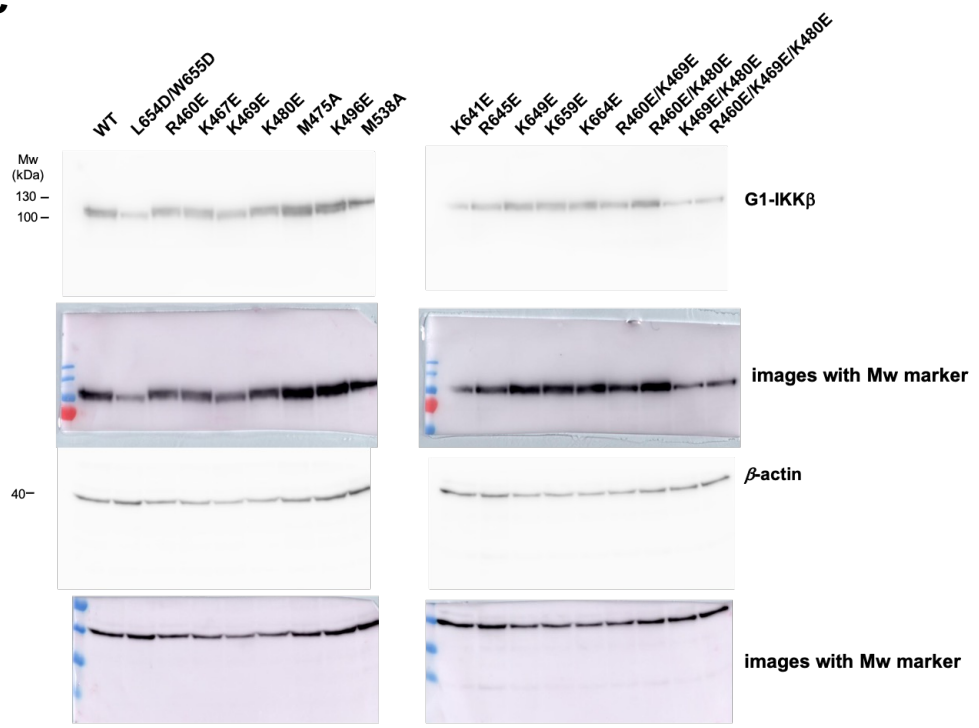

Supplementary Figure 10

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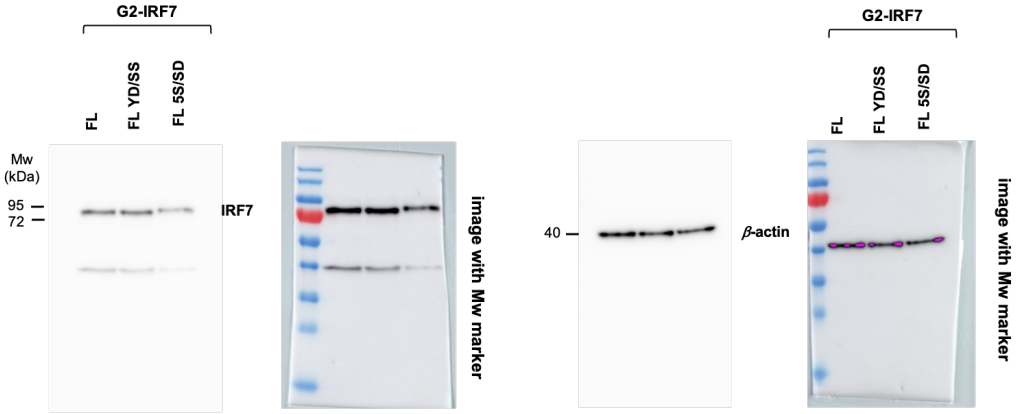

Supplementary Figure 11