SUPPLEMENTAL FIGURE LEGENDS

Fig.S1. Alignment and structures of ubiquitin, IKKβ and Homer3. (A) We previously identified the ULD in IKKβ as a region similar to the UbL domains identified using the PROSITE Ubiquitin_2 profile described at http://ca.expasy.org/prosite/PS50053. Alignment of the IKKβ ULD between Leu^{307} and Met^{384} in human IKKβ with the sequence of human ubiquitin was performed using MacVector software (MacVector, Inc. Cary, NC). The secondary structures of the ULD and ubiquitin were determined using the PSIPRED protein secondary structure prediction server (University College London) at http://bioinf.cs.ucl.ac.uk/psipred/. The positions of Leu^{353} in the ULD and Ile^{44} in ubiquitin are shown in red and blue letters respectively. Ile^{44} is positioned within the hydrophobic patch of ubiquitin required for its ability to associate with its binding partners and mutation of this residue ablates this function of Ubiquitin. We showed previously that Leu^{353} is required for IKKβ function and a similar residue in the IKK-related kinase TBK/IKK-*i* that contains a ULD also regulates the function of that kinase. As shown, the secondary structure of Ubiquitin is a ββαββαβ sequence whereas the IKKβ ULD forms a ββααββ structure. Notably, Leu^{353} in IKKβ exists in an α-helix whereas in ubiquitin and this is a β-sheet. These differences in secondary structure of the ULD and ubiquitin may impart distinct binding-characteristics on these domains for separate interaction partners. **(B)** Structures of IKKβ and Homer3 (LZ, Leucine Zipper; H, Helix-loop-Helix; N, NEMO-binding domain) showing the ULD and mutants used in this study

Fig.S2. Homer3 co-fractionates with the IKK complex. (A) Jurkat S100 lysates were fractionated by gel filtration chromatography (Superdex 200 HR10/30) then fractions were immunoblotted using the antibodies indicated. The relative molecular weights of low and high M*r* calibration standards are shown above the appropriate fractions. **(B)** Jurkat S100 lysates were fractionated using a MonoQ HR 5/5 anion exchange column eluted with a gradient of 50-500mM NaCl. Fractions were immunoblotted using the antibodies indicated.

Fig.S3. Homer3 associates with IKKβ. Lysates from HEK293 cells transfected with FLAG-IKKβ together with either empty Xpress vector or Xpress-Homer3 (H3) were immunoprecipitated with anti-Xpress. Immunoprecipitates and pre-IP extracts from each sample were immunoblotted (IB) using anti-Xpress or anti-FLAG as shown.

Fig.S4. IKKβ phosphorylates Homer3. (A) HEK293 cells were transfected with Xpress-Homer 3 or FLAG-Homer3 either alone or together with FLAG-IKKβ as indicated. Extracts were immunoblotted (IB) with anti-FLAG or anti-Homer3 as indicated. The asterisk labels the up-shifted Homer3 band. **(B)** HEK293 cells were transfected with Myc-Homer3 either alone (*lane 1*) or together with FLAG-IKKβ (*lanes 2 and 3*) in the absence or presence of λ-PPase. Extracts were immunoblotted (IB) with anti-FLAG or anti-Myc as shown. The upshifted band is absent in lane 3 demonstrating that it is a phosphorylated version of Homer3 (H3-P) **(C)** Extracts from HEK293 cells transfected with Myc-Homer3 either alone or together with FLAG-IKKβ, FLAG-IKKβ^{K44M} or FLAG-IKKβ^{L353A} were immunoblotted (IB) using anti-Myc or anti-FLAG

as shown**. (D)** To determine whether the catalytic activity of IKKβ is necessary for its interaction with Homer3 we transfected HEK293 cells with Myc-Homer3 either alone or together with FLAG-IKKβ (1 or 0.5 μg/ml as indicated) or FLAG-IKKβ^{K44M}. Extracts were IP-ed with anti-FLAG then IP and pre-IP samples were immunoblotted (IB) using anti-Myc or anti-FLAG. Homer3 precipitated with FLAG-IKKβ^{K44M} demonstrating that the interaction does not require catalytically active IKKβ.

Fig.S5. Homer3 binds via its CC/LZ with the IKKβ ULD. Our binding studies lead us to propose this model for Homer3 binding via its CC/LZ domain to the IKKβ ULD. In this conformation the EVH1 domain remains free to associate with separate proteins. NEMO binds the NEMO-binding domain (NBD) and IKKα interacts with the LZ (HLH, Helix-loop-Helix; CD, Catalytic Domain).

Fig.S6. Homer3 and NEMO colocalize at the immune synapse. (A) Clone E6.1 Jurkats were either unconjugated (*top*) or conjugated with mouse anti-CD3 and mouse anti-CD28 coated beads (*bottom*) then stained with anti-NEMO (red) or anti-Homer3 (green). Colocalized fluorescence is yellow in overlays (scale bar, 5 μm). Fluorescence of antibody-coated beads is due to detection of the bead-bound antibodies by the secondary antibody used for mouse anti-NEMO. The percentage of NEMO colocalized with Homer3 **(B)** or Homer3 with NEMO **(C)** in unconjugated and conjugated cells is shown (*$p < 0.05$).

Fig.S7. Homer3 over expression activates NF-κB. (A) HEK 293 cells were transiently transfected with the NF-κB-dependent firefly luciferase reporter construct pBIIx-luc and a control β-actin promoter driven *renilla* luciferase vector together with either empty vector (pFLAG-CMV; Control) or increasing concentrations (1, 10 and 100ng) of FLAG-tagged Homer1, Homer2 or Homer3. Cell lysates were immunoblotted using anti-FLAG, and NF-κB activity was determined by dual luciferase assay. **(B)** HEK293 cells were transiently transfected with the luciferase constructs described in **(A)** and either empty vector (pFLAG-CMV; *first lane*) or vectors encoding FLAG-Homer3, FLAG-IKKα(K44M) or FLAG-IKKβ(K44M) as indicated (+). NF-κB activity was determined by dual luciferase assay and lysates were immunoblotted using anti-FLAG. **(C)** HEK293 cells were transiently transfected with the luciferase constructs described in **(A)** and either empty vector (pFLAG-CMV; Control) or constructs encoding FLAG-Homer3 (wild type; WT) or the Homer3 Coiled-Coil / Leucine Zipper (CC/LZ) domain. Protein levels in lysates were detected by immunoblotting using anti-FLAG, and NF-κB activity was determined by dual luciferase assay.

Fig.S8. NF-κB activation is intact in the absence of Homer3. (A) Splenic T cells purified from wild-type (WT) or Homer3$^{-/-}$ mice were incubated with anti-CD3 and anti-CD28 from the times indicated then nuclear extracts were prepared for EMSA. Assays were performed using either a consensus NF-κB binding site probe *(upper panel)* or an Oct1 probe as a loading control *(lower panel)*. **(B and C)** Splenic T cells purified from WT or Homer2/3 double knock-out (DKO) mice were incubated with either TNF or PMA

/ Ionomycin (P/I) **(B)**; or anti-CD3 and anti-CD28 **(C)** from the times indicated. EMSAs were performed on nuclear lysates using either an NF-κB or Oct1 probe as indicated.

Fig.S9. Homer3 associates with IKKβ in the absence of NEMO. (A) Lysates of unstimulated 3T8, 8321 and 8321N cells were immunoblotted using either anti-NEMO or anti-β-actin as indicated. **(B)** 3T8, 8321 and 8321N cells were stained with anti-Homer3 (green) or anti-IKKβ (red) and analyzed by laser-scanning confocal microscopy (scale bar, 5 μm). Co-localized Homer3 and IKKβ appear yellow in the overlay panels and DIC images of the stained cells are shown (*top*). **(C)** The percentage of Homer3 associated with IKKβ (H3:IKKβ) and the percentage of IKKβ associated with Homer3 (IKKβ:H3) in 3T8, 8321 and 8321N cells was calculated using Volocity software.

Fig.S10. Amounts of IKKβ associated with Homer3 are unchanged in the absence of NEMO. The percentage of co-localization in unconjugated cells (*open bars*) and cells conjugated with anti-CD3 / anti-CD28 coated beads (*solid bars*) is shown. Quantitative analysis of the areas occupied and percentage of IKKβ colocalized with Homer3 in 3T8, 8321 and 8321N was performed using Volocity software.

Fig.S11. Homer3 regulates F-actin dynamics in T cells. (A) 3T8 Jurkat cells were transfected for 28 hours with control (C-siRNA) or Homer3-specific FITC-labeled siRNA (H3-siRNA) then lysates were immunoblotted using either anti-Homer3 or anti-tubulin as a loading control. **(B)** 3T8 Jurkats transfected for 28 hours with control (*black line, grey shading*) or Homer3 FITC-labeled siRNA (*green line, unshaded*) were

incubated with the TCR cross-linking IgM antibody C305 (0.25 μg/ml) for the times indicated and then stained for FACS using AF647-linked phalloidin.

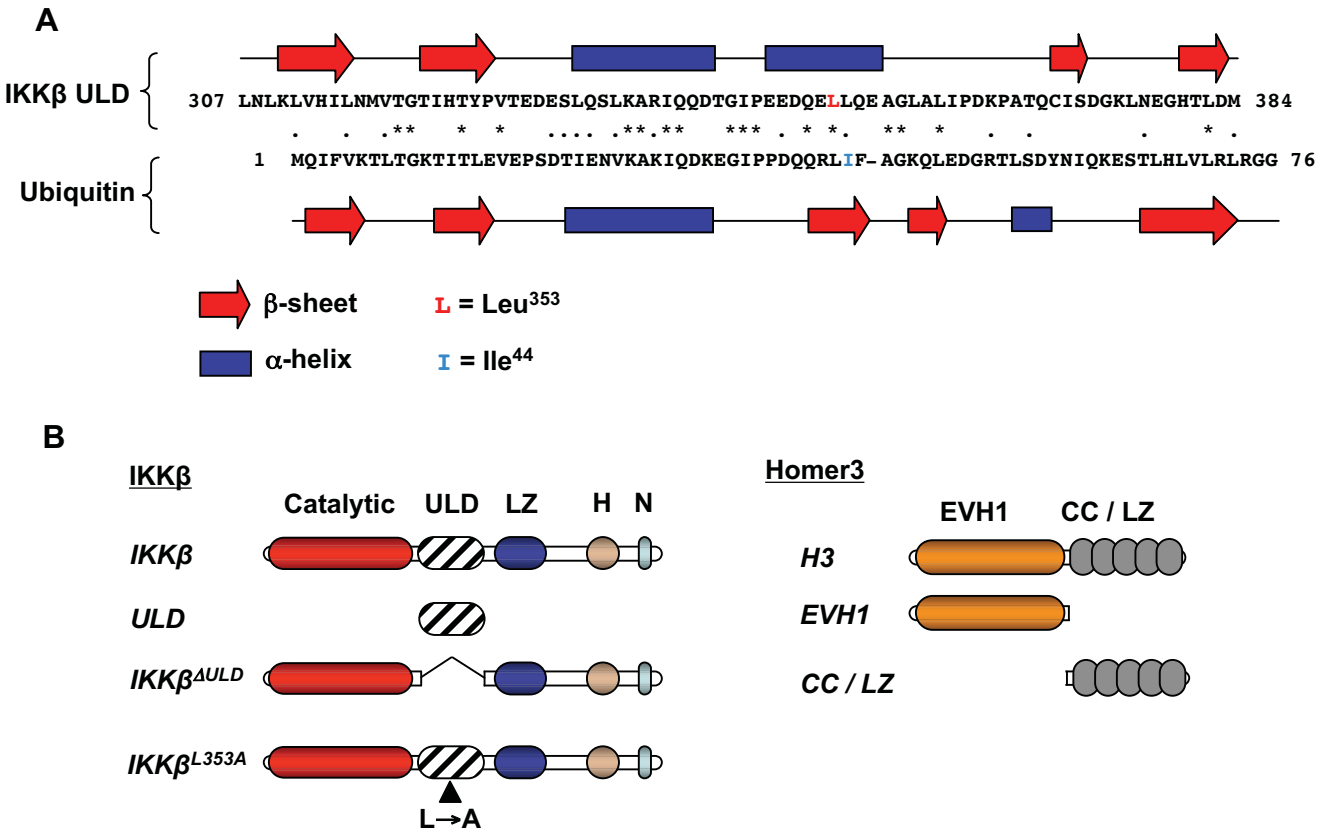
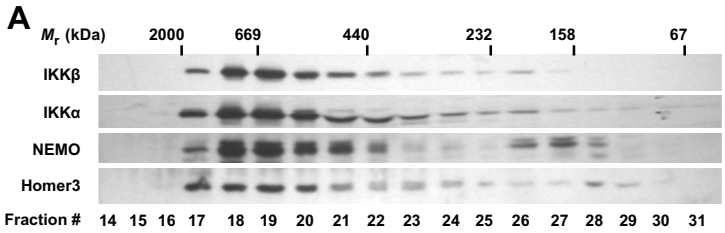


Fig.S1



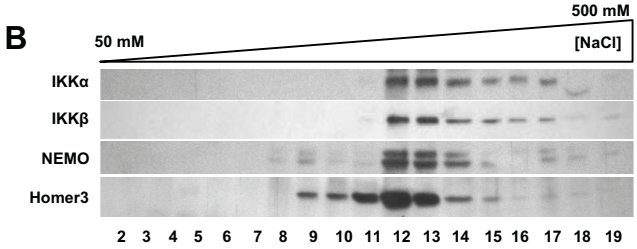
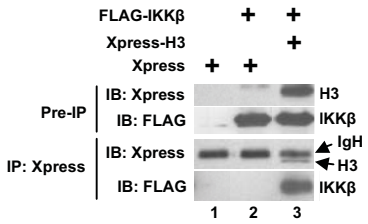


Fig.S2



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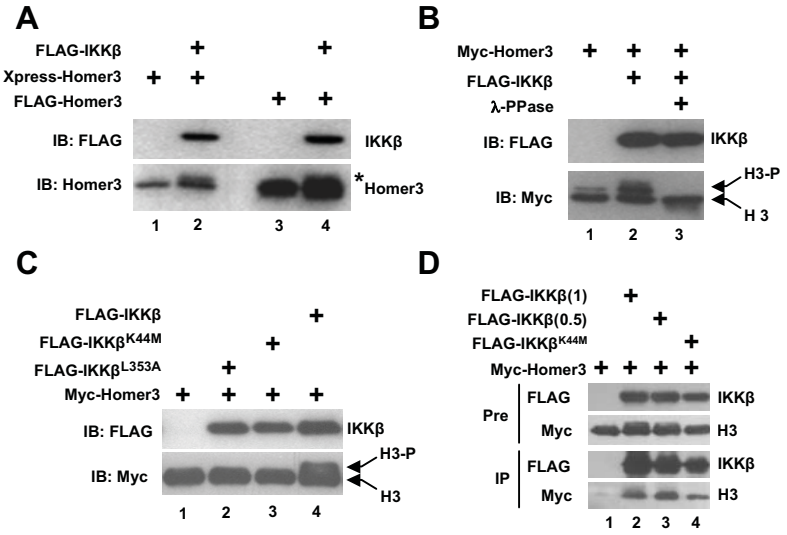

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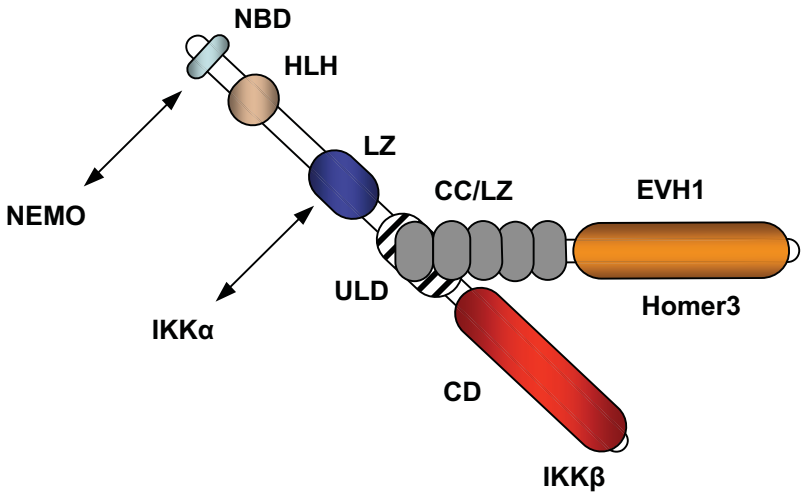

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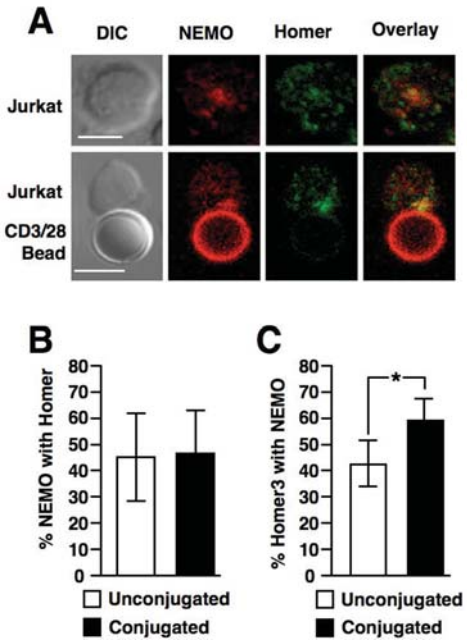

Fig. S6

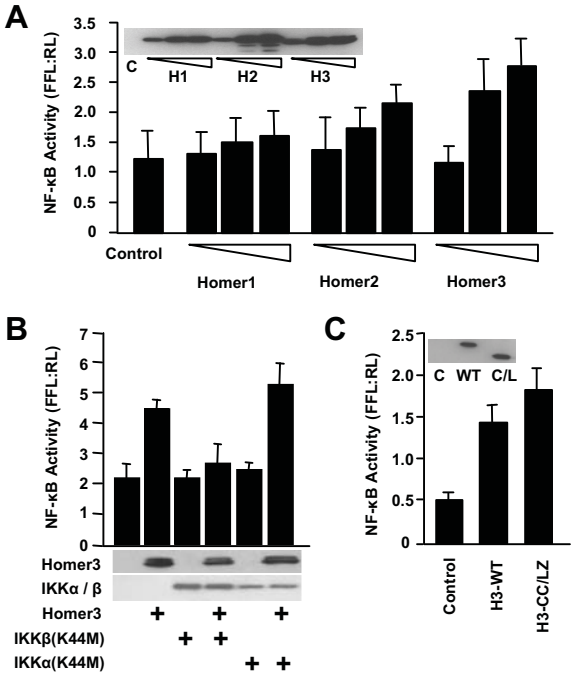

Fig.S7

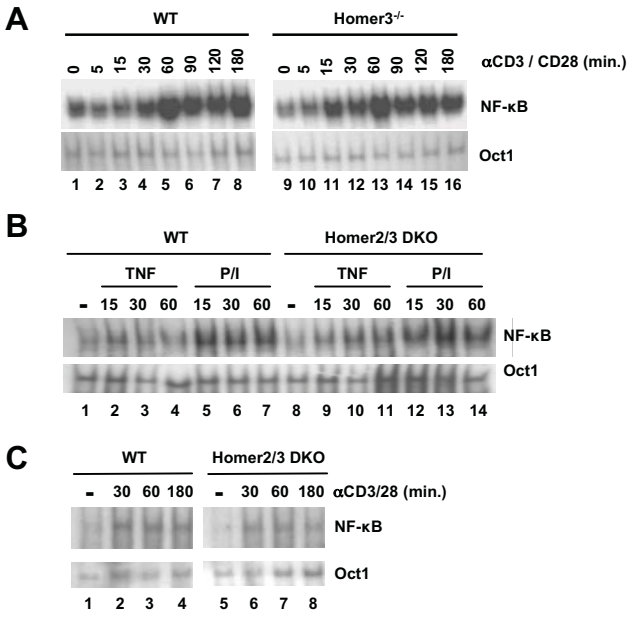

Fig.S8

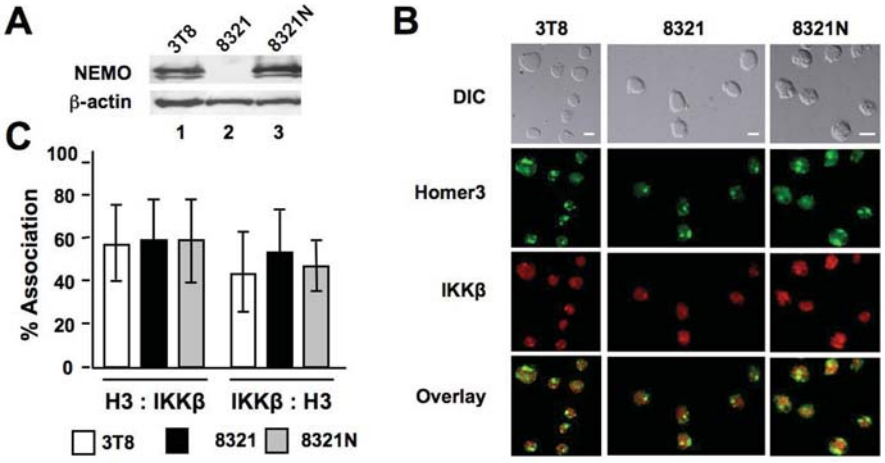

Fig.S9

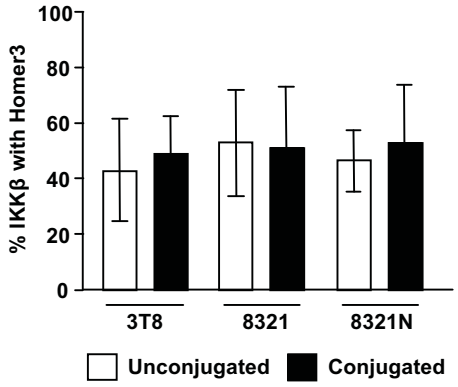

Fig.S10

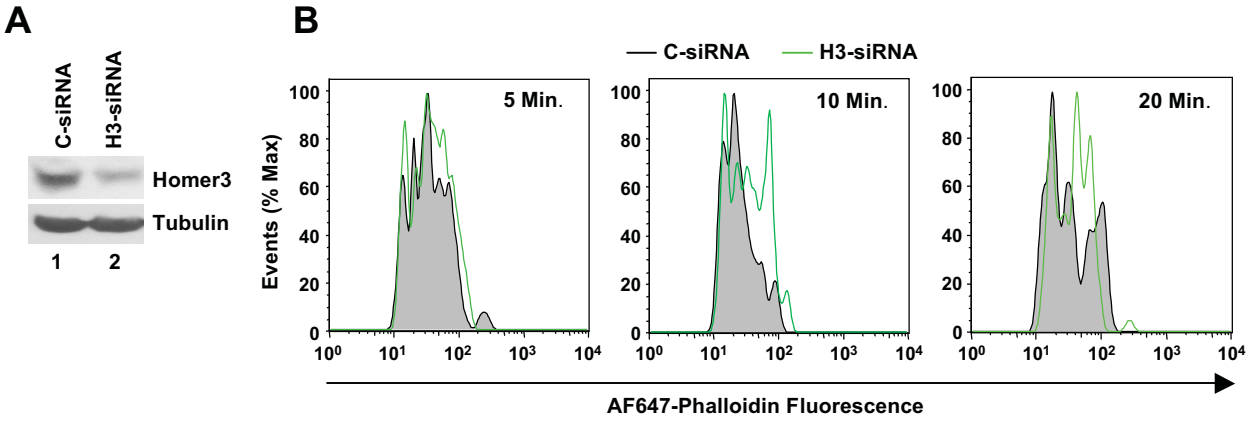

Fig.S11