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Supplementary Information for

Identification of MLKL membrane translocation as a checkpoint in necroptotic cell death using Monobodies

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Supplementary Information Text

Materials and Methods

Recombinant protein expression and purification
Recombinant human MLKL 4HB NTR (residues 2-154) and monobodies were expressed in *E. coli* BL21-Codon Plus (DE3)-RIL cells as TEV protease-cleavable GST (pGEX-2T-TEV) and 6xHis (pPROEX Htb, Invitrogen) fusions, respectively. Expression and purification were performed using established methods (1-3). Briefly, cells were grown in Super Broth containing 100 μg/mL ampicillin, shaking to OD600 of 0.8 at 37°C before the temperature was reduced to 18°C, protein expression induced with 0.5 mM IPTG, with shaking for a further 16 h. Cell pellets were lysed by sonication and debris eliminated by centrifugation before incubation of the supernatant with Ni-NTA resin (HisTag, Roche) for 6xHis tagged proteins or glutathione agarose beads (UBPBio) for GST tagged proteins at 4°C for 1 h. 6xHis tagged proteins were eluted in 0.2 M NaCl, 20 mM HEPES pH 7.5, 5% v/v glycerol containing 250 mM imidazole, cleaved with TEV protease at 25°C for 2 h, dialyzed extensively, and subjected to further Ni-NTA chromatography. GST fusions were cleaved with TEV protease on resin at 4°C for 12 h, before further Ni-NTA chromatography and the supernatant concentrated by centrifugal ultrafiltration (Vivaspin), for a Superdex-200 10/300GL size exclusion column (GE Healthcare) with elution in 0.2 M NaCl, 20 mM HEPES pH 7.5, 5% v/v glycerol. Fractions containing the protein of interest were deduced from reducing SDS-PAGE (Biorad) with SafeStain (ThermoFisher) detection, pooled and concentrated by centrifugal ultrafiltration. The concentration was estimated by A280, the protein aliquoted, snap frozen in liquid nitrogen and stored at –80°C until required.

For monobody screening, bait proteins were expressed with a C-terminally fused flexible penta-Ser linker and AviTag (ASSSSSGLNDIFEAQKIEWHE) and enzymatically biotinylated using recombinant BirA. A human MLKL NTR (residues 2-154) AviTag fusion (synthesized by Bioneer, South Korea) was expressed in *E. coli* BL21-Codon Plus (DE3)-RIL (N-terminus; residues 2-154 as a TEV-cleavable N-terminal GST fusion via pGEX-2T-TEV). Recombinant mouse MLKL NTR (residues 1-158) C-terminally fused to the AviTag was expressed as an N-terminal TEV protease-cleavable His_6-NusA fusion, and purified as previously described for mouse MLKL (1-158) (4). Mouse MLKL NTR-AviTag was expressed and purified as per His_6-tagged proteins above, except for selection using 50 μg/mL kanamycin during expression. Human MLKL (full-length as a TEV-cleavable GST fusion via pFastBac GST; pseudokinase domain, residues 190-471 as a TEV-cleavable N-terminal 6xHis fusion via pFastBac Htb) C-terminally fused to an AviTag were expressed in Sf21 insect cells using the Bac-to-Bac system, as described previously for untagged versions (1-3). Proteins were purified using the methods established for their non-AviTagged counterparts and were eluted from either a Superdex-200 10/300GL or HiLoad 16/60 size exclusion column (GE Healthcare) in 0.2 M NaCl, 20 mM HEPES pH 7.5, 5% v/v glycerol. Fractions containing purified protein were ascertained from reducing SDS-PAGE (Biorad) with SafeStain (ThermoFisher) detection, and fractions pooled and concentrated using centrifugal ultrafiltration (Vivaspin). Protein concentration was estimated using A280, and protein was aliquoted, snap frozen in liquid nitrogen and stored at –80°C until required.

Enzymatic biotinylation
BirA was expressed and purified from *E. coli* BL21-Codon Plus (DE3)-RIL cells as a Trx fusion bearing a non-cleavable N-terminal 6xHis tag via the pET32a vector. Cells were grown in Super Broth containing 100 μg/mL ampicillin, shaking to OD600 of 0.8 at 37°C before the temperature was reduced to 18°C, protein expression induced with 0.5 mM IPTG, with shaking for a further 16 h. Cell pellets were resuspended in 0.2 M NaCl, 20 mM HEPES pH 7.5, 5% v/v glycerol containing 5 mM imidazole, 0.5 mM BondBreaker TCEP (Pierce) and 100 mM PMSF, and lysed by sonication. Cell debris was eliminated by centrifugation and the supernatant incubated with Ni-NTA resin (HisTag, Roche) at 4°C for 1 h. Beads were pelleted by centrifugation and washed with lysis buffer (twice) and 0.2 M NaCl, 20 mM HEPES pH 7.5, 5% v/v glycerol containing 35 mM imidazole (twice), before protein was eluted 0.2 M NaCl, 20 mM HEPES pH 7.5, 5% v/v glycerol containing 250 mM

imidazole. The eluate was concentrated by centrifugal ultrafiltration (Vivaspin), applied to a Superdex-200 10/300GL size exclusion column (GE Healthcare) and eluted in 0.2 M NaCl, 20 mM HEPES pH 7.5, 5% v/v glycerol. Fractions containing BirA were deduced from SDS-PAGE with SafeStain detection, pooled and concentration by centrifugal ultrafiltration. The concentration was estimated by A280, protein aliquoted, snap frozen in liquid nitrogen and stored at –80°C until required.

Biotinylation reactions of human MLKL domains (~75 μM) detagged with His-TEV protease were assembled with 50 mM bicine (pH 8.3), 10 mM Mg-acetate, 10 mM ATP, 0.5 mM biotin and 1μM His-BirA and reacted for 45min in a 30°C water bath. His-TEV protease and His-BirA were subsequently eliminated by incubation for 15min with Ni-NTA resin (HisTag, Roche) and the supernatant concentrated, applied to Superdex-200 10/300GL column (GE Healthcare) and biotinylated protein eluted in 0.2 M NaCl, 20 mM HEPES pH 7.5, 5% v/v glycerol. Fractions containing the protein of interest were identified by reducing SDS-PAGE (Biorad) with SafeStain (ThermoFisher) detection and concentrated using centrifugal ultrafiltration (Vivapin), aliquoted, snap frozen in liquid nitrogen and stored at –80°C until required for monobody screening.

Monobody development

Biotinylated bait proteins were immobilized by binding to Streptavidin MagneSphere Paramagnetic Particles (Promega) and used to screen monobody phage-display libraries, as described previously (5, 6). After four rounds of phage selection with target concentrations of 100 nM, 100 nM, 50 nM and 50 nM, the sorted pools were subcloned into a yeast display library following recombination of 5' and 3' fragments to increase library diversity as described previously (9) before two further rounds of screening with target concentrations of 100 nM and 25 nM using yeast display. Isolated clones were validated for target binding using yeast display, as described previously (5, 6). Specific binding to the MLKL targets was tested using yeast display binding in the presence of HEK293T cell lysate at a protein concentration of 3.3 mg/ml as determined using the Pierce BCA protein assay kit (Thermo Fisher Scientific).

Protein crystallization and structure determination

Recombinant human MLKL (2-154) was cleaved from Glutathione resin (UBPBio) by incubation with TEV protease (2h, 25°C) and mixed with Ni-NTA resin (HisTag, Roche) to remove TEV protease. 6xHis-Mb33 was isolated using Ni-NTA resin, eluted in 0.2 M NaCl, 20 mM HEPES pH 7.5, 5% v/v glycerol, 250mM imidazole, cleaved with TEV protease (2h, 25°C), dialysed into 0.2 M NaCl, 20 mM HEPES pH 7.5, 5% v/v glycerol before TEV protease and uncleaved contaminants were eliminated by mixing with Ni-NTA resin (Roche). The Mb33 supernatant was mixed at a 1.5-2 fold molar excess to MLKL (2-154) and concentrated by 5000 MWCO Vivaspin to 4.7 mg/mL. Crystals grew in 25% PEG-MME 550, 0.1 M MES pH 6.5, 0.01 M Zinc acetate at 20°C. Single crystals were flash cooled in liquid nitrogen and X-ray diffraction data collected at the Australian Synchrotron MX2 beamline (7). Data were indexed, integrated in XDS then merged and scaled in aimless (8, 9). Phases were solved by molecular replacement in phaser (10) using: PDB 6D0J as a template monobody structure (into which the Mb33 loop sequences were modelled using the Sculpt function in Phenix (11); and residues 2-118 of mouse MLKL (PDB 4BTF) as a search model. Manual model building and phase refinement were performed using iterative real-space and reciprocal-space refinement in Coot and phenix.refine, respectively (12, 13). The coordinates have been deposited in the Protein Data Bank as 6UX8.

Expression constructs

Monobody sequences were amplified by PCR from library clone templates and ligated into a derivative of the doxycycline-inducible, puromycin-selectable vector, pF TRE3G PGK puro (2, 14, 15), encoding an N-terminal FLAG and C-terminal GFP sequence (synthesized by ATUM, CA). Mutations were introduced into a human MLKL DNA template (from DNA2.0, CA) using oligonucleotide-directed overlap PCR or were synthesized by ATUM (CA) and subcloned into pF TRE3G PGK puro as BamHI-EcoRI fragments. Insert sequences were verified by Sanger sequencing (Micromon DNA Sequencing Facility, VIC, Australia). Vector DNA was co-transfected

into HEK293T cells with pVSVg and pCMV ΔR8.2 helper plasmids to generate lentiviral particles, as described previously (14, 15).

Cell Death assays

The human histiocytic lymphoma U937 (and their *MLKL*$^{-/-}$ counterparts), human colorectal adenocarcinoma HT29 and mouse dermal fibroblast (MDF) cell lines were cultured in human tonicity RPMI medium (in-house), DMEM and DMEM (Gibco), respectively, supplemented with 8% v/v fetal calf serum (FCS; Sigma), with puromycin (5 μg/mL; StemCell Technologies) added for lines stably transduced with inducible MLKL or monobody constructs, as before (1, 16). Routine testing confirmed cell lines to be mycoplasma-negative. U937 and MDF cells were plated to 5 x 10^4 cells/well in 96- and 24-well plates, respectively. Cells were allowed to settle 24 h prior to treatment with doxycycline (20 ng/mL) for 3 h to induce expression of the relevant monobody or MLKL construct prior to stimulation. HT29 cells were assayed analogously, but plated at 1 x 10^5 cells/well in 24 well plates and allowed to adhere to the plate for 48 h prior before doxycycline (20 ng/mL) for 3 h induction of exogene. Cells were then treated with TNF (100 ng/mL) and the Smac-mimetic Compound A (500 nM) (TS) to induce apoptosis, or TS in the presence of the pan-caspase inhibitor IDN-6556 (10 μM) to induce necroptosis. Cell death quantified by propidium iodide (PI; 1 μg/mL) uptake using flow cytometry 24 h post-stimulation, as previously (1, 16). Wildtype and mutant human MLKL constructs were introduced into 2-3 independent clones *MLKL*$^{-/-}$ U937 cells (except Q135A, which was only introduced into one *MLKL*$^{-/-}$ U937 clone) and each line typically assayed ≥2 times. Combined data are presented as mean ± S.E.M..

Western Blot and Blue Native PAGE

Cells seeded into 96-well plates (MDFs at 5 × 10^4 cells/well; HT29 and U937 at 1 × 10^5 cells/well) were induced overnight with 20ng/mL doxycycline and harvested using 2X SDS Laemmli lysis buffer, sonicated, boiled at 100°C for 5 min, then resolved by 4–15% Tris-Glycine gel (Biorad). After transfer to PVDF, membranes were blocked with 5% skim milk, and then probed with antibodies as indicated.

For Blue Native PAGE, wild-type HT29 cells expressing monobody variants were seeded into six-well plates at 1 × 10^6 cells/well at least 18h before treatment. Expression of monobody constructs was induced with 10 ng/ml doxycycline for 3 h, then treated with TSI (7h), or left untreated, as indicated. Cells were fractionated into cytoplasmic and membrane fractions as previously described (2, 14). In brief, initial cell permeabilization used a buffer containing 0.025% digitonin (BIOSYNTH, Staad, Switzerland), 2 μM N-ethyl maleimide, phosphatase and protease inhibitors. Crude membrane and cytosolic fractions were separated via centrifugation, and then adjusted to a final concentration of 1% w/v digitonin. Fractions were resolved by 4–16% Bis-Tris Native PAGE gel (ThermoFisher), then transferred to PVDF for western blot analyses.

Immunoprecipitation for western blot

HT29 cells seeded into 10cm plates at 2.5 x10^6 cells/plate were induced overnight with 20 ng/mL doxycycline and treated with TNF (100 ng/mL), Smac-mimetic (Compound A; 500 nM) and pan-caspase inhibitor IDN-6556 (10 μM) (TSI) for 3 hours. Cells were harvested in lysis buffer (50 mM Tris-HCl pH 7.4, 1% (v/v) Triton X-100, 150 mM NaCl, 1 mM EDTA, 2 mM Sodium Vanadate, 10 mM NaF, 1 mM PMSF, Complete protease inhibitor tablet (Roche)) and supernatants were mixed with Anti-FLAG M2 Affinity Gel (Millipore). Beads were washed in lysis buffer and proteins harvested using 2X SDS lysis buffer, boiled at 100 ºC for 5 min, then resolved by 4-15% Tris-Glycine gel (Biorad). After transfer to nitrocellulose, membranes were probed with antibodies as indicated.

Immunoprecipitation for mass spectrometry

HT29 cells seeded into 15cm plates at 10^7 cells/plate were induced overnight with 20 ng/mL doxycycline and treated with TNF (100 ng/mL), Smac-mimetic (Compound A; 500 nM) and pan-caspase inhibitor IDN-6556 (10 μM) (TSI) for 3 hours. 3x15 cm plates per condition were harvested in lysis buffer (50 mM Tris-HCl pH 7.4, 1 % (v/v) Triton X-100, 150 mM NaCl, 1 mM EDTA, 2 mM Sodium Vanadate, 10 mM NaF, 1 mM PMSF, Complete protease inhibitor tablet (Roche)) and

supernatants were mixed with Anti-FLAG M2 Affinity Gel (Millipore). Beads were washed in lysis buffer and proteins eluted with FLAG peptide (2X 0.5 mg/ml elutions and 1x 1mg/mL elution).

Reagents and antibodies
Primary antibodies used in this study were: rat anti-MLKL (clone 3H1, produced in-house (15); 1:4000 dilution; available as MABC604, EMD Millipore, Billerica, MA, USA), rabbit anti-MLKL phospho-S358 (AB187091, Abcam; 1:4000 for western blots), mouse anti-Actin (A-1987, Sigma-Aldrich, St Louis, MO, USA; 1:4000), mouse anti-FLAG M2 horseradish peroxidase (HRP) (A8592, Sigma; 1:3000), mouse anti-VDAC1 (MABN504, Merck Millipore; 1:500), rabbit anti-GAPDH (2118, Cell Signaling Technology; 1:1000), rabbit anti-GFP (2956, Cell Signaling Technology; 1:2000). Recombinant hTNF-Fc, produced in-house, and the Smac mimetic, Compound A, have been previously described (17, 18). The pan-caspase inhibitor, IDN-6556/emricasan, was provided by Tetralogic Pharmaceuticals. Necrosulfonamide (NSA) was purchased from Merck Millipore.

Analysis of Monobody immunoprecipitates by mass spectrometry
Eluates were resuspended in 6 M Urea, 10 mM TCEP and 100 mM Tris-HCl pH 7.0 and subjected to protein digestion using FASP (filter aided sample preparation) before lyophilisation using a SpeedVac AES 1010 (Savant, Thermofisher) (19). Samples were analyzed on a nanoElute UHPLC (plug-in V1.0.10.4; Bruker, Germany) coupled to a timsTOF Pro (Bruker) mass spectrometer equipped with a CaptiveSpray source. Peptides were resuspended in 2% ACN, 1% formic acid (FA) separated on a 25 cm X 75 µm analytical column, 1.6 µm C18 beads with a packed emitter tip (IonOpticks, Australia). The column temperature was maintained at 50 °C using an integrated column oven (Sonation GmbH, Germany). The column was equilibrated using 4 column volumes before loading sample in 100% buffer A (99.9% MilliQ water, 0.1% FA) (Both steps performed at 980bar). Samples were separated at 400 nL/min using a gradient from 2% to 17% buffer B (99.9% ACN, 0.1% FA; 55 min), 17% to 25% buffer B (21 min) before ramping to 35% buffer B (13 min), ramp to 85% buffer B (3 min) and sustained for 10 min. The timsTOF Pro (Bruker) was operated in PASEF mode using Compass Hystar 5.0.36.0. Settings were as follows: Mass Range 100 to 1700m/z, 1/K0 Start 0.6 V·s/cm^2 End 1.6 V·s/cm^2, Ramp time 109.9ms, Lock Duty Cycle to 100%, Capillary Voltage 1600V, Dry Gas 3 L/min, Dry Temp 180 °C, PASEF settings: 10 MS/MS scans (total cycle time 1.26 sec), charge range 0-5, active exclusion for 0.4 min, Scheduling Target intensity 20000, Intensity threshold 2500, CID collision energy 42eV.

All raw files were analyzed by MaxQuant v1.6.10.43 software using the integrated Andromeda search engine. Experiment type was set as TIMS-DDA with no modification to default settings. Data was searched against the human Uniprot Reference Proteome with isoforms (downloaded March 2019) and a separate reverse decoy database using a strict trypsin specificity allowing up to 2 missed cleavages. The minimum required peptide length was set to 7 amino acids. Modifications: Carbamidomethylation of Cys was set as a fixed modification, while N-terminal acetylation of proteins and oxidation of Met were set as variable modifications. First search peptide tolerance was set at 70 ppm and main search set at 20 ppm (other settings left as default). Matching between runs and LFQ quantitation was turned on. Maximum peptide mass [Da] was set at 8000. All other settings in group or global parameters were left as default.

Further analysis was performed using a custom pipeline developed in R (3.6.1), which utilizes the LFQ intensity values in the MaxQuant output file proteinGroups.txt. Proteins not found in at least 50% of the replicates in one group were removed. Missing values were imputed using a random normal distribution of values with the mean set at mean of the real distribution of values minus 1.8 s.d., and a s.d. of 0.3 times the s.d. of the distribution of the measured intensities. The probability of differential site modification expression between groups was calculated using the Limma R package (3.4.2). Probability values were corrected for multiple testing using Benjamini–Hochberg method.

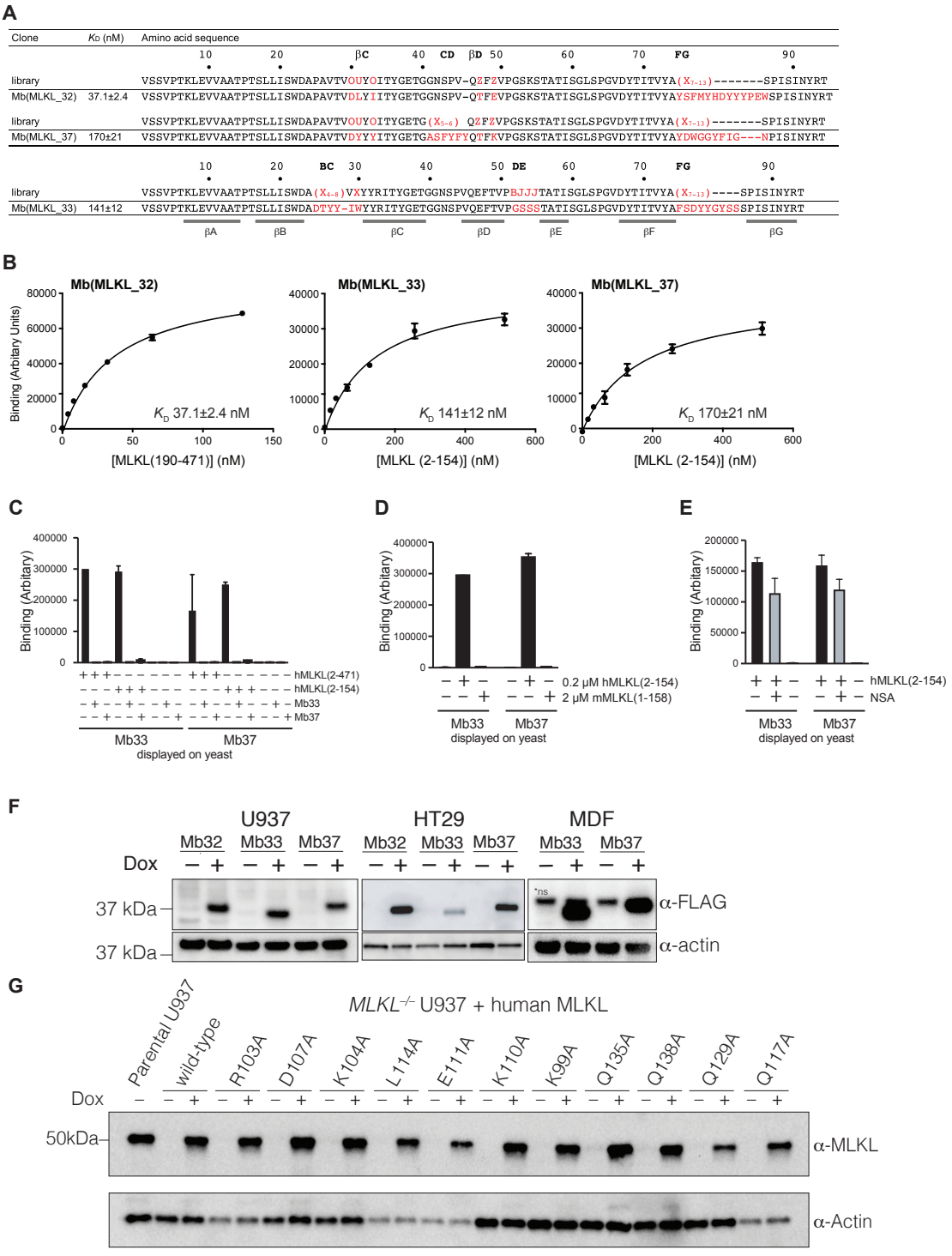

Fig. S1. Development of monobodies; validation of antibodies and construct expression.

(A) Monobody library designs and developed clones. Adapted from Figure 2 in ref. (20). Diversified positions in the “side and loop” library and the “loop only” library are shown in red. "X" denotes a mixture of 30% Tyr, 15% Ser, 10% Gly, 5% Phe, 5% Trp and 2.5% each of all the other amino acids except for Cys; "B", a mixture of Gly, Ser and Tyr; "J", a mixture of Ser and Tyr; "O", a mixture

of Asn, Asp, His, Ile, Leu, Phe, Tyr and Val; "U", a mixture of His, Leu, Phe and Tyr; "Z", a mixture of Ala, Glu, Lys and Thr. The amino acid sequences for the MLKL-binding monobodies are shown in the same manner. Mb(MLKL_33) contains an additional mutation V29L, probably introduced due to an DNA replication error.

(B) Binding titration of the developed monobody clones for their respective target, as measured using yeast display. The K_D values and the errors are the mean and standard deviation from triplicate measurements.

(C) Binding of Mb33 and Mb37 displayed on yeast to human MLKL(2-471) or human MLKL(2-154) in the presence or absence of purified Mb33 or Mb37 protein. The loss of binding with the soluble proteins indicate that Mb33 and Mb37 bind to an overlapping epitope in human MLKL.

(D) Binding of Mb33 and Mb37 displayed on yeast to 0.2 μM human MLKL(2-154) or 2 μM mouse MLKL(1-158).

(E) Effect of NSA modification of human MLKL(2-154) on binding to Mb33 or Mb37. The MLKL sample was reacted with a 10-fold molar excess of NSA in the presence of 0.2 mM TCEP (as previously described (21)) prior to the binding measurements.

(F) The expression of FLAG-Mb32-GFP, FLAG-Mb33-GFP and FLAG-Mb37-GFP in U937, HT29 and MDF cells following doxycycline (Dox)-induction was detected via FLAG western blot. Actin blot used as a loading control.

(G) Expression of wild-type or Ala mutant human MLKL constructs in *MLKL*$^{-/-}$ U937 cells was verified by western blot using the 3H1 anti-MLKL antibody following 8 h of dox induction. Actin blot used as a loading control.

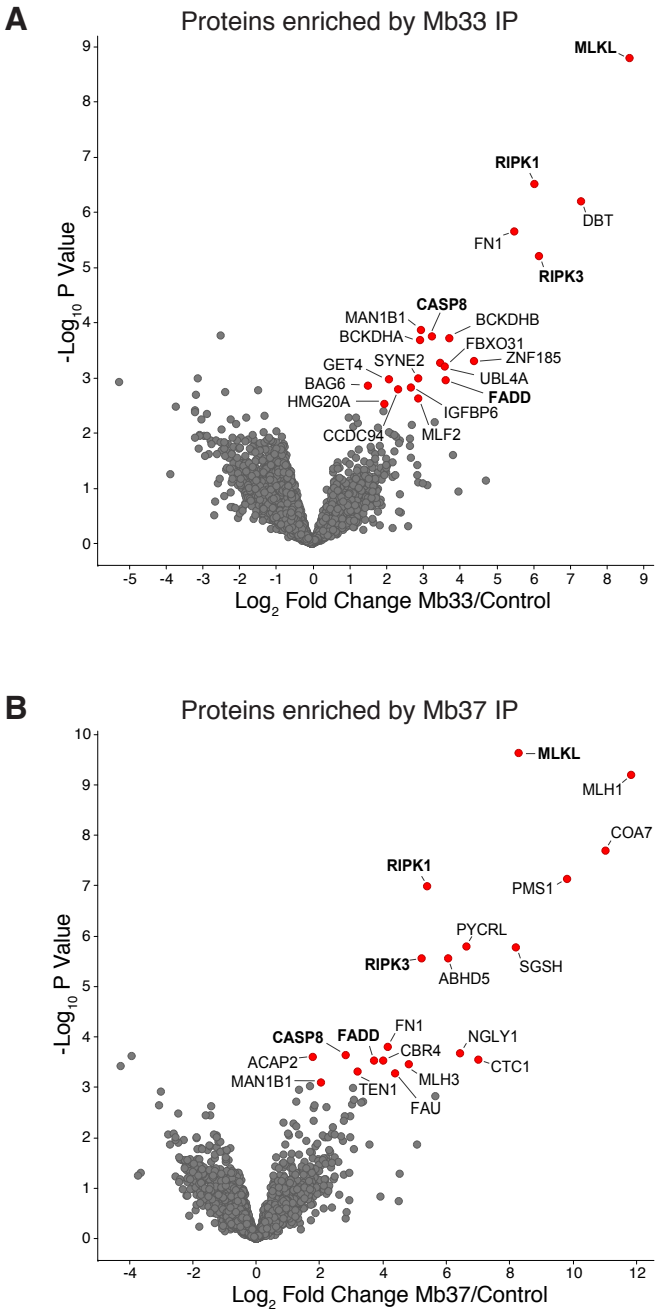

Fig. S2. Mb33 and Mb37 specifically enrich endogenous human MLKL from HT29 cells. Cells were treated with Doxycycline to induce expression of FLAG-tagged monobodies before stimulation with TSI and cell lysis. Monobodies and interacting proteins were enriched by immunoprecipitation using the FLAG tag. Figures demonstrate Log_2 fold change volcano plots of protein enrichment in cells expressing (+Doxycycline) either Mb33 (A) or Mb37 (B) compared to the no expression (–Doxycycline) controls. MLKL was the most significantly enriched protein for both monobodies. The top 20 proteins enriched by either monobody are labelled and highlighted in red. P values calculated using Limma (n=4). MLKL and known necrosomal proteins are highlighted in bold text.

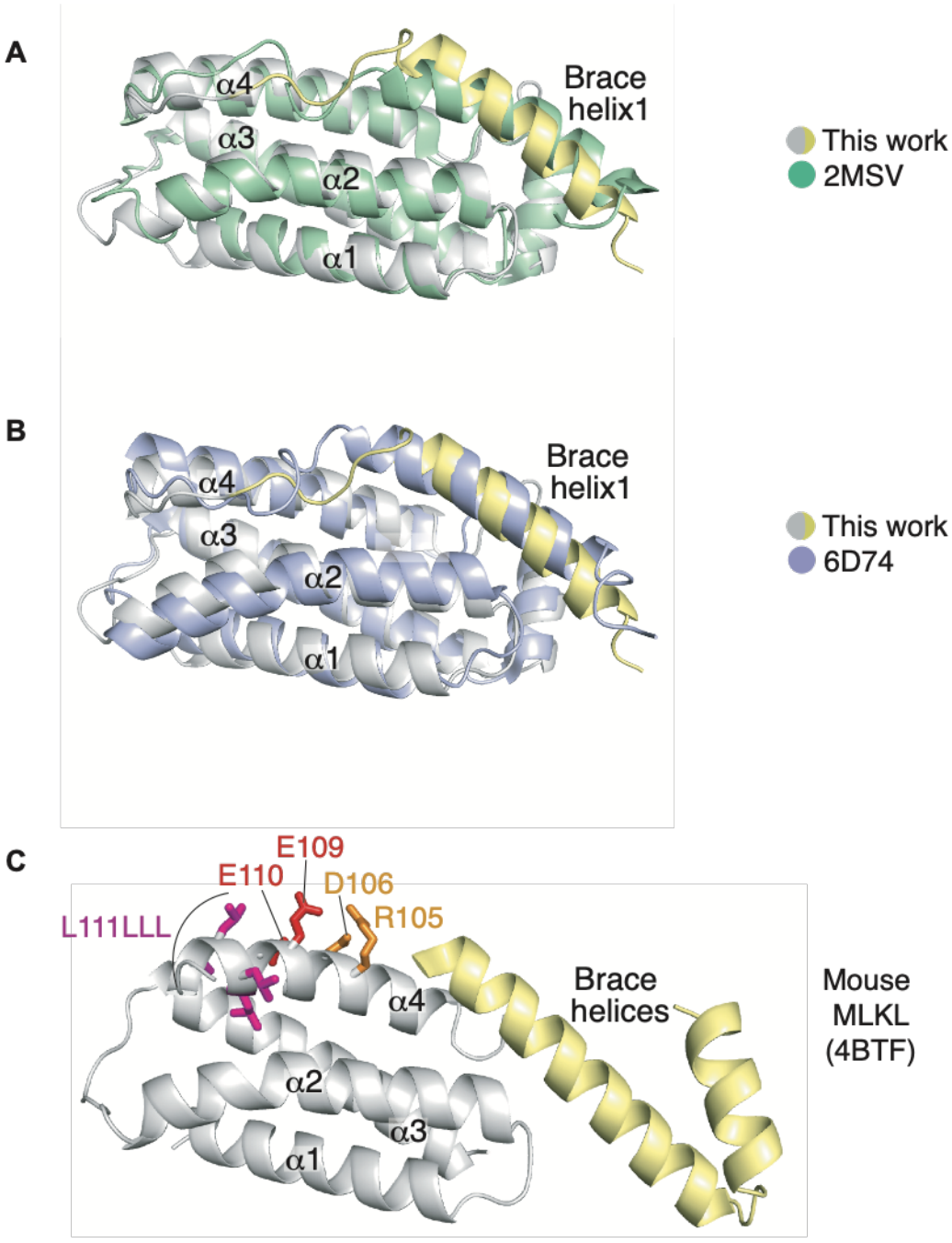

Fig. S3. Comparison of human MLKL structure from the current work with earlier structures

The human MLKL N-terminal region structure reported herein was superimposed with the NMR structures, 2MSV (21) (A) and 6D74 (22) (B) with respective r.m.s.d. of 1.68 and 2.00 Å across Cα atoms. (C) The mouse MLKL counterpart structure was drawn from the mouse MLKL full length structure (4BTF; (15)). Sticks show the mouse MLKL 4HB domain α4 helix residues that disrupted necroptotic signaling when Ala substituted: R105A/D106A, E109A/E110A, L111A/L112A/L113A/L114A (14).

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