

SUPPLEMENTARY INFORMATION

Distinct pseudokinase domain conformations underlie divergent activation mechanisms among vertebrate MLKL orthologs

Davies et al.

Supplementary Table 1. X-ray crystallography data collection and refinement statistics

	Rat MLKL (179-464)	Horse MLKL (188-475)
Data collection		
Space group	P 6 ₁ 2 2	C 2
Cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	190.0, 190.0, 77.79	155.5, 124.8, 81.05
α , β , γ (°)	90, 90, 120	90, 112.7, 90
Resolution (Å)	47.5 – 2.19 (2.27 – 2.19)	40.1 – 2.75 (2.84 – 2.75)
R-merge	0.1024 (1.24)	0.1914 (1.548)
<i>I</i> / σ <i>I</i>	24.83 (3.04)	8.79 (0.99)
Completeness (%)	100 (98)	99 (90)
Redundancy	20.2 (20.3)	6.9 (6.2)
Refinement		
Reflections used in refinement	42667 (4080)	36830 (3346)
R-work	0.1852 (0.2348)	0.1834 (0.3138)
R-free	0.2161 (0.2913)	0.2379 (0.3601)
No. non-hydrogen atoms	4300	6645
Protein	4112	6585
Ligand	6	12
Water	182	48
Average B-factor	51.33	59.53
Protein	51.21	59.44
Ligand	91.83	65.59
Water	52.50	45.13
R.M.S. deviations		
Bond lengths (Å)	0.01	0.01
Bond angles (°)	1.02	1.38

Statistics for the highest-resolution shell are shown in parentheses.

SUPPLEMENTARY FIGURES

Supplementary Figure 1. Amino acid sequence alignments for MLKL orthologs examined in this work.

Sequence alignment of orthologs with Linnaean names on the left and common names on the right. Residues conserved with identity (dark green) and high similarity (light green) are highlighted in the alignment. Regions equivalent to catalytic motifs on canonical kinases are coloured red, and residues of the α C helix (dark blue) and activation loop (light blue) are highlighted. The position of the horse β 3- α C loop helix is indicated in bright blue, and the residues equivalent to RIPK3 binding residue (F373 in mouse) are highlighted in yellow. The domain architecture of mouse MLKL (PDB:4BTF) is illustrated above the alignment: lines are loops, cylinders are helices and arrows β -sheets. Underlined, bolded residues are RIPK3 substrates in mouse and human MLKL. Residue numbering corresponds to the mouse MLKL sequence.

Four-helix Bundle

	1	α1	α2	α3	62
<i>M. musculus</i>	M D K L G Q I K L G Q L I Y E Q C E K	K M K Y C R K Q C Q R L G N R V H G L L Q P L Q R L Q A Q G K K N L P D D - I T A A L G			Mouse
<i>R. norvegicus</i>	M D K L G Q I K L G Q L I Y D Q C E K	K M K Y C R K Q C R R L R N R V H G L L Q P L Q R L Q A Q E K K S L P S - D I T A A L G			Rat
<i>H. sapiens</i>	M E N L K H I I T L G Q V I H K R C E E M K Y C K K Q C R R L G H R V L G L I K P L E M L Q D Q G K R S V P S E K L T A M N				Human
<i>E. caballus</i>	M D K L G Q I T S L G Q I I Y K Q C N E M K Y C Q K Q C Q R L G Q R V N G L L Q P L Q M L Q T E G G R S L P D - K I I N A L D				Horse
<i>S. scrofa</i>	M D H L G R I I S L G K L I Y N Q C E E M K Y C Q K Q C W R L G N R V Q G L L Q P L Q M L R D Q G E R N L S P E - I T A L S				Pig
<i>G. gallus</i>	M D I I E K V F S I A H A I H S Q F E H V K C K C H Q C Q R L V E R I H I L E P V R V L Q A Q P S W R I S H - H E E Q M L T				Chicken
<i>S. punctatus</i>	M E I I E K V K V L S V A Q V I Y T Q W E Q V K G C K F Q F Q R L I T R I Q I L L R P V E T L Q A O S A K H I S P A - V E K T W				Tuatara
<i>X. tropicalis</i>	M E I I L G N V L E I A Q T I Y N L C D Q A S S N K R Q C S R L K K R I Q I L L M A E K L K K Q P E K S G - - - E L K I V L R				Frog
<i>G. aculeatus</i>	M E I I V K E I T S I A K K I Y S L V E K V K A N K K R S Q R L G L R V K A L D D L V R S I K - - - K E Q S G R C K E V K K C L T				Stickleback

63 α4 125

<i>M. musculus</i>	R F D E V L K E A N Q Q I E K F S K K S H I W K F V S V G N D K I L F H E V N E K L R D V W E E L L L L L Q V Y H W N T V S D
<i>R. norvegicus</i>	R F E D V L K E A R Q Q I E K F S Q K S N I W K F A T V G S D K I L F H E V N E K L R D I W E E L L L L L Q V D Q W K S D S E
<i>H. sapiens</i>	R F K A A L E E A N G E I E K F S N R S N I C R F L T A S Q D K I L F K D V N R K L S D V W K E L S L L L Q V E Q R M P V S P
<i>E. caballus</i>	R F H A V L Q E A Q G R I H E F S N K P S I L K R T A G H E K I L F S E V N E R L R D V W E E F S L Q L Q V D Q R I L L S S
<i>S. scrofa</i>	R F Q A V L E E A K E K I D K F S K K S N I Q K F L R A G H D R I L F S G V N K R L R D V W E E F S L Q L Q V D Q R M H I S R
<i>G. gallus</i>	K L L Q A L G E A Q K L V T K Y S Q T S W I Q K F L S A R S S G E E F V W V W N R S L E D I A Q G L S L L L Q A E Q K Q A L L E
<i>S. punctatus</i>	K L L G T L A Q A Q E L L M R K Y C H Q N W L Q K F M N A G N S A E E F T L L N E H L G D A A Q G L S L L L Q A E Q K Q F F L E
<i>X. tropicalis</i>	E M Q L T L R N A K S W V L K Y S N Q G W W M K I I K A N G I K E E F D L I N D R L K D A A D D I S V M L A M E H R E L L L K
<i>G. aculeatus</i>	E L S C T L T T A K E L I Q S Y T E A T W V K R L V K V G S H K D E F N C V N D R L T D A F Q N L S G A L Q V E Q S I V Q H R

Brace region

	126	Brace helix 1	Brace helix 2	172
<i>M. musculus</i>	V S Q P A S W Q Q E D R Q D A E E D G - - - - - N E N M K V I L M Q L Q I S V E E I N K T L K Q C S L - - - - - K	Mouse		
<i>R. norvegicus</i>	V S Q P A S W Q Q E D Q Q D A E E D G - - - - - N E N M K V I L V K L Q I S M E E I K E A L K Q C S L - - - - - K	Rat		
<i>H. sapiens</i>	I S Q G A S W A Q E D Q Q D A E E D R R A F Q M L R - - - R D N E K I E A S L R R L E I N M K E I K E T L R Q Y L P - - - - - K	Human		
<i>E. caballus</i>	I R Q K A S W Q Q E D Q Q D A E E D R K V F Q K L E - - - S G D E I V E A S L R K L E I S I E E T R R I V R Q Y L Q - - - - - K	Horse		
<i>S. scrofa</i>	I S P G V S W H Q E D Q Q D A E E D K R V I Q G L R - - - R E N A V I E T L L Q K L E N T T R E T I E T V R S L M Q - S D A H K	Pig		
<i>G. gallus</i>	A F Q A K I C R Q D A E D L R D K A F L D Q V I A S T K E P E D A W E I Q M G S Q C M E S K V D W M Q G L K - - - - - K	Chicken		
<i>S. punctatus</i>	A C E K V T W S K E D G Q D A R A D R A T L E E L L T K T K D A V N N V H G E V K H V G S K V D N V E N K M D Q V L Q M M K D	Tuatara		
<i>X. tropicalis</i>	F F D E N K R M R Q S Q R D I E E D M S E L K E H L N R M S E Y P V I L N I H N V F I V S S G T R T P R - - - L - - - - - Frog			
<i>G. aculeatus</i>	V F E L V S R M K E D E E D R A E D A E L K T R Q T D K V G - - - L L E Y M Q E Q Q R D M E I L K N N V E K I L M -- G M A Q	Stickleback		

Pseudokinase domain

	173	β1	β2	β3	β3-αC loop	αC	232
<i>M. musculus</i>	P T Q E I P Q D L - - - Q I K E I P K E H L G P - P W T K L K T S K M S T I Y R G E Y H R S P P V T K V F N N P Q A E S V G I V	Mouse					
<i>R. norvegicus</i>	P M Q E V P Q D F - - - Q V K E I P K E D L G Y - P W T K L K T N K L S T I Y R G E Y Y H R S P P V T K V F N N P Q A E S V G I V	Rat					
<i>H. sapiens</i>	C M Q E I P Q E - - - Q I K E I K K E Q L S G S P W I L L R E N E V S T L Y K G E Y Y H R S P P V T K V F N N P Q A E S V G I V	Human					
<i>E. caballus</i>	P M K E I P Q D - - - Q I K E I K K E E L S G W D G I P L T K G E F S T L Y K G E Y Y H R S P P V T K V F N K L Q A G S I A I V	Horse					
<i>S. scrofa</i>	P K K G L P E E - - - K I K E I K K E E L P K A D W V L L R K N E C S T L Y E G K Y Y H K S P P V A K V F S K S Q A R S I X I V	Pig					
<i>G. gallus</i>	- K V N V G K R E - - - D I T E I E R S Q L T - - - F H R H L Q D T E R Y D L Y E G E Y L K Y P V A K T F K R P L T T D T V K V	Chicken					
<i>S. punctatus</i>	L Q G A M R R D S M E I T E I E R E H L T K T L W T L L M E N K N H T L Y K G E Y Y H K S P P V A K V Y R P N V I T C T Q T V	Tuatara					
<i>X. tropicalis</i>	- - - - - L C T E I K S E D L I W G S L V M E R P N F D L Y K G Q Q Y Y R S P V A K V L K G Q P T E N D E Q I	Frog					
<i>G. aculeatus</i>	P S I T D Q Q I R - - - N I K H H E L K Y D D - - - V K K P F M V T A N T E V Y K G E F N G F P V A K R Y V D P L N I S Q S E L	Stickleback					

233 β4 293

	233	β4	β5	Hinge	αD		293
<i>M. musculus</i>	R F T F N D E I K T M M K K F D S P N I L R I F G I C I D Q T V K P - P E F S I V M E Y C E L G T L R E L L D R E K D - L T M S	Mouse					
<i>R. norvegicus</i>	R L T F N E E I K T M M K K F D S P N I L R I F G I C I D Q T V K P - P E F S I V M E Y C E L G T L R E L L D R D K D - L T M S	Rat					
<i>H. sapiens</i>	R Q T F N K E I K T M M K K F D S P N I L R I F G I C I D E T V T P - P Q F S I V M E Y C E L G T L R E L L D R E K D - L T L G	Human					
<i>E. caballus</i>	R H T F N N E I K T M M K K F D S P N I L R I F G I C I D E T V T P - P Q F S I V M E Y C E L G T L R E L L D K D K D - L I I F A	Horse					
<i>S. scrofa</i>	R T F N N E I K T M M K K F D S P N I L R I F G I C I D E T V T P - P Q L S I V M E Y C E L G T L R E V L D K E K D - L A L A	Pig					
<i>G. gallus</i>	R D I F E K E I Q T L K K F E S P N I L L R M Y G I C I D E E K D G I - P C F S I V M E Y C K H G T L T K Q Q H - L S W E	Chicken					
<i>S. punctatus</i>	R E I F K K E V E A L K Q F E S P H I L L R M Y G I C I D E S G L S - P C F S I I M E Y C E K G T L T R D V L T K E P S - L T W K	Tuatara					
<i>X. tropicalis</i>	R E I F N S E V K T M M K K Y E C V N I L L R I F G I C I D K A K S E - P C Y Y M V T E F C E K G T L T G E L L S R E K N - L S W N	Frog					
<i>G. aculeatus</i>	Q K I F D K E V E T M R R F E S P N I L L R M F G I C V Q S G P G T S P R F L I I M E Y C E K G S L R D V L D S D W A A L S W P	Stickleback					

294 αE 353

	294	αE	β6	β7	activation loop		353
<i>M. musculus</i>	V R S L L V L R A A R G L Y R L H H S E T - - - L H R N I S S S S F L V A G G Y Q V K L A G F E L S K T Q N S S I S R T A K S T	Mouse					
<i>R. norvegicus</i>	V R S L L V L R A A R G L Y R L H H S G T - - - L H R N I S S S S F L V A G G Y Q V K L A G F E L S K T Q N S S I S R A T K S T	Rat					
<i>H. sapiens</i>	K R M V L V L G A A R G L Y R L H H S E A P - - - E L H R G K I R S S N F L V T Q G Y Q V K L A G F E L S K T Q N S S I S R A T K S T	Human					
<i>E. caballus</i>	L R I V L V L Q A A K G L Y R L H H S E A P - - - E L H R N I S S S T S F L V T D G Y Q V K L A G F E L S K T Q N S S I S R G T K G K	Horse					
<i>S. scrofa</i>	L R I V L V L G A A R G L Y R L H H S E T P A E L H R N I S S S T S F L V T K D Y N V K L A G F E L S K T Q N S S I S R H T R G K	Pig					
<i>G. gallus</i>	I R M A L G A M R A G L Y R L H Q T E E K S R L H G C I C S S K F L V A G D Y C V K L S G F E L S K T Q N S S I K R N F V S K K	Chicken					
<i>S. punctatus</i>	I R T Q M A L H A S I G L Y R L H K T G E K P K V H G C I N S S S K F L V T D G Y G V V K L T G F E L C E T E S S I K R N F K E R	Tuatara					
<i>X. tropicalis</i>	Q R I H M T L D A A R A L Y R L H H T E T K P M L H G S L S S S K F L V D G T Y C L K L S G F E L S K T E S S M R R Q P K A V	Frog					
<i>G. aculeatus</i>	T R A R M C R D A A R G L Y R L H Q T E E K S K V H G C L N S S N K F L V A E G Y T V K L G G F E L A K T E T S L R K T R R K E	Stickleback					

354 F373 413 αH 464

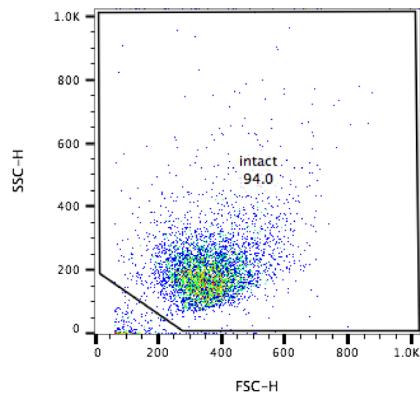
	354	F373	αF	αG	413	αH	αI	464
<i>M. musculus</i>	K A E R S S S T I Y V S P E R L K N P F C L Y D I K A E I Y S F G I V L W E I A T G K I P F E G C D - - - S K K I R E L V A	Mouse						
<i>R. norvegicus</i>	K A E R F S S T A Y V S P E R L Q D P F C K Y D T K A E I Y S F G I V L W E I A T G K I P F E G C D - - - S E K I Y E L V A	Rat						
<i>H. sapiens</i>	K T D R V K S T A Y L S P Q E L E D V F Y Q D V K S E I Y S F G I V L W E I A T G D I P F Q G C N - - - S E K I R K L V A	Human						
<i>E. caballus</i>	E A E R V I S A A Y I S P E R L E N V Y R K Y D I K A E I Y S F G I V L W E I A T G K V P F E G F D - - - S K Q I Y Q R V V	Horse						
<i>S. scrofa</i>	E A E K A S S A A Y I S P Q I L N N V Y H K Y D I K A E I Y S F G I V L W E I A T G K I P F E G C G - - - S K E I H Q L V V	Pig						
<i>G. gallus</i>	N F S Q V C M L A Y I A P E N L M D V Y Y P Y K R P C E I Y S F G I V L W E I A T S K I P F E G C P - - - L Q E I R D K I C	Chicken						
<i>S. punctatus</i>	K Q M E V P A S A Y I I S P Q G L A S L Y H K Y D I P S E I Y S F G I V L W E I V T S K I P F A G C T - - - S Q E I Y Q K V C	Tuatara						
<i>X. tropicalis</i>	K Q K Q G T D W Y I A P E T M Q N I N - A Y D Q K S E I Y S L G V V F Y E I A S G R I P L Q D L S G A D T N P T D M Y Q N L	Frog						
<i>G. aculeatus</i>	S I S S - - - L C Y T S P E M L N D V N L N Y S K E S E I Y S I G I V M W E I A S G R R P F D G W S - - - N K E I N N K V C	Stickleback						

Supplementary Figure 2. An example of the flow cytometry gating strategy used throughout this study.

Intact cells were gated as shown in (a). Subsequently, viability was measured based on propidium iodide (PI)-uptake (b), where PI uptake identified dead cells. This strategy has been previously reported¹.

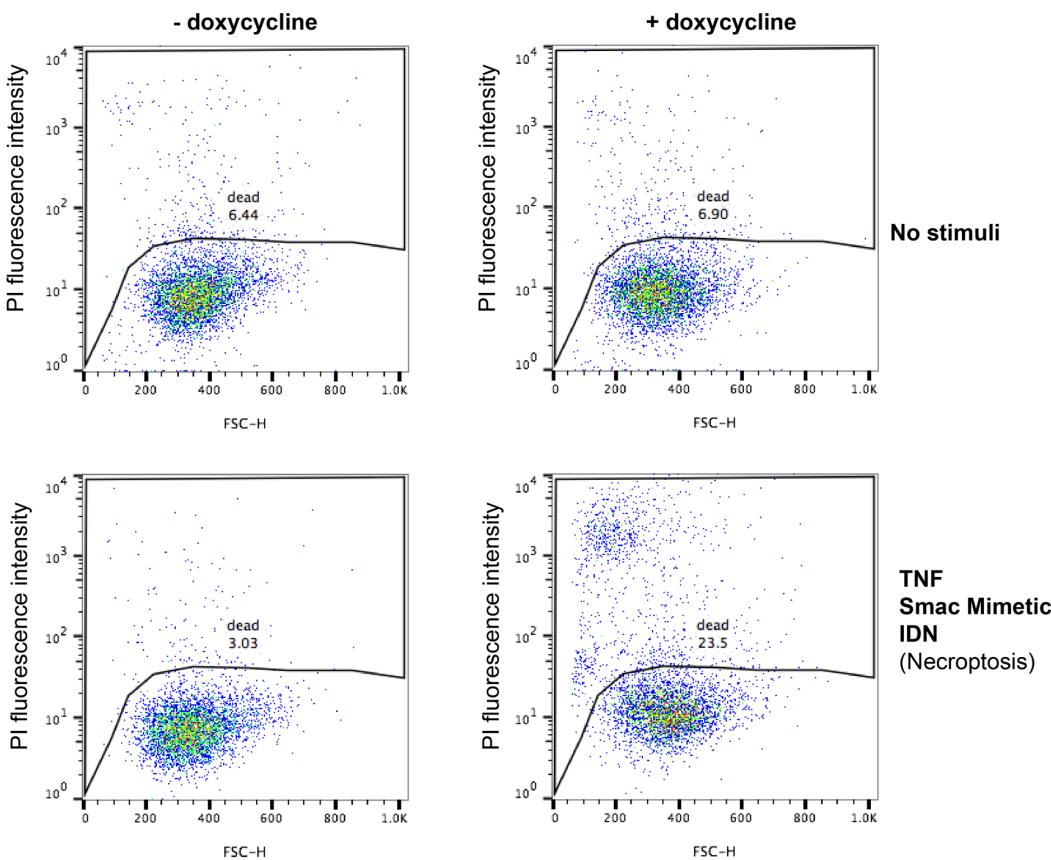
a Gate intact cells

e.g. *Mllt^{-/-}* MDF + Horse MLKL
uninduced, untreated



b Gate PI positive cells

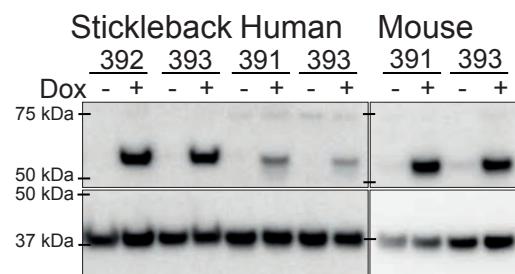
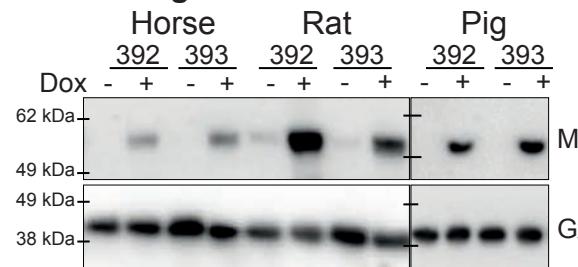
e.g. *Mllt^{-/-}* MDF + Horse MLKL



Supplementary Figure 3. Western blot validation of MLKL ortholog and mutant expression in *Mlkl*^{-/-} mouse dermal fibroblasts (MDF) and *MLKL*^{-/-} U937 human cells.

(a) Doxycycline-induced expression of mouse, human, horse, rat, tuatara, chicken, stickleback, pig and frog MLKL in *Mlkl*^{-/-} MDF cells was validated by western blot using the anti-MLKL mAb (clone 3H1), via a C-terminally fused FLAG tag or using another anti-MLKL mAb (clone 5C4) for pig MLKL. Exogenes were expressed in MDF lines derived from biologically-independent *Mlkl*^{-/-} mice (denoted 391, 392 and 393). GAPDH blots were used as loading controls. (b) Expression of orthologs in *MLKL*^{-/-} human U937 cells was monitored by western blot as for MDF cells in (a). Note tuatara MLKL expression was not detectable in U937 cells. (c, d) Expression of horse and mouse MLKL mutants in *Mlkl*^{-/-} MDF cells was monitored by western blot as described in (a). (e) Rat S345D mutant MLKL expression in *Mlkl*^{-/-} MDF and *MLKL*^{-/-} U937 cells was validated with the pan-MLKL mAb (3H1 clone) by western blot. The positions of molecular weight markers are shown on the left in each panel. The shown western blots are representative of 2 independent repeats. Uncropped blots are provided in a Source Data file.

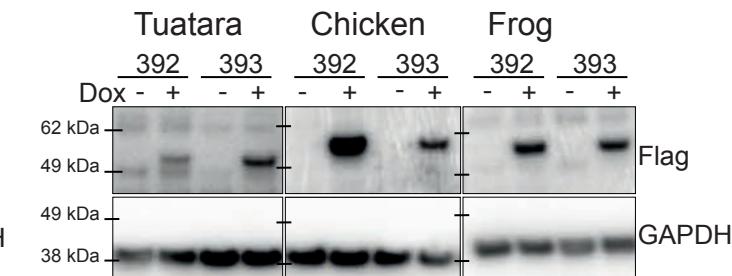
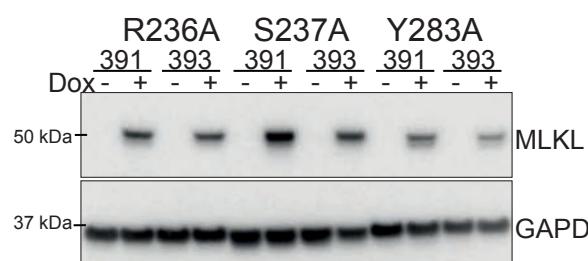
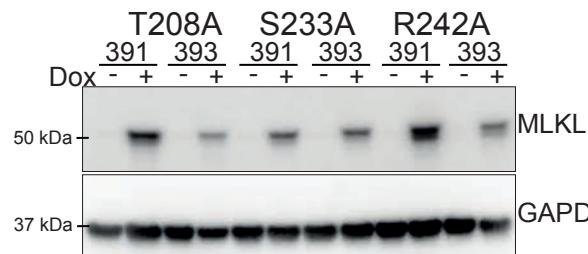
a Orthologs in *Mlk1*^{-/-} MDF cells



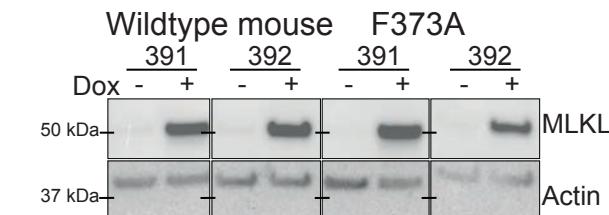
b Orthologs in *MLKL*^{-/-} U937 cells



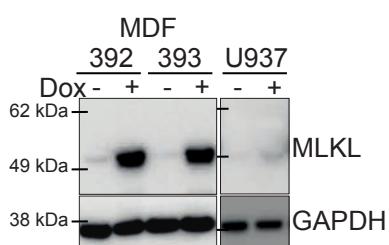
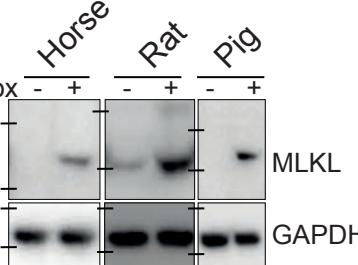
c Horse MLKL mutants *Mlkl*^{-/-} MDF cells



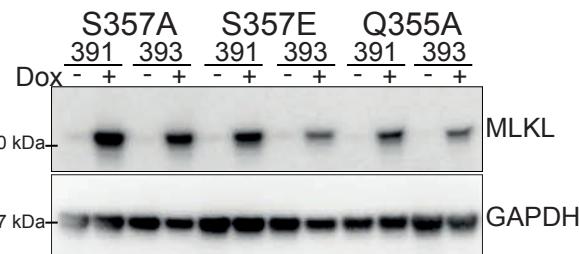
d Mouse MLKL F373A in *Mlk1*^{-/-} MDF cells



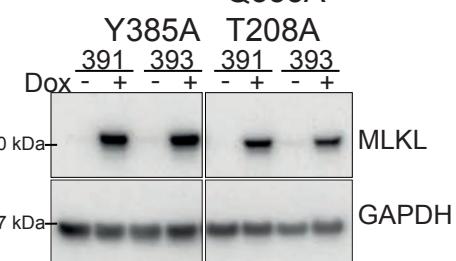
e Rat S345D mutant



T356A- T356E-

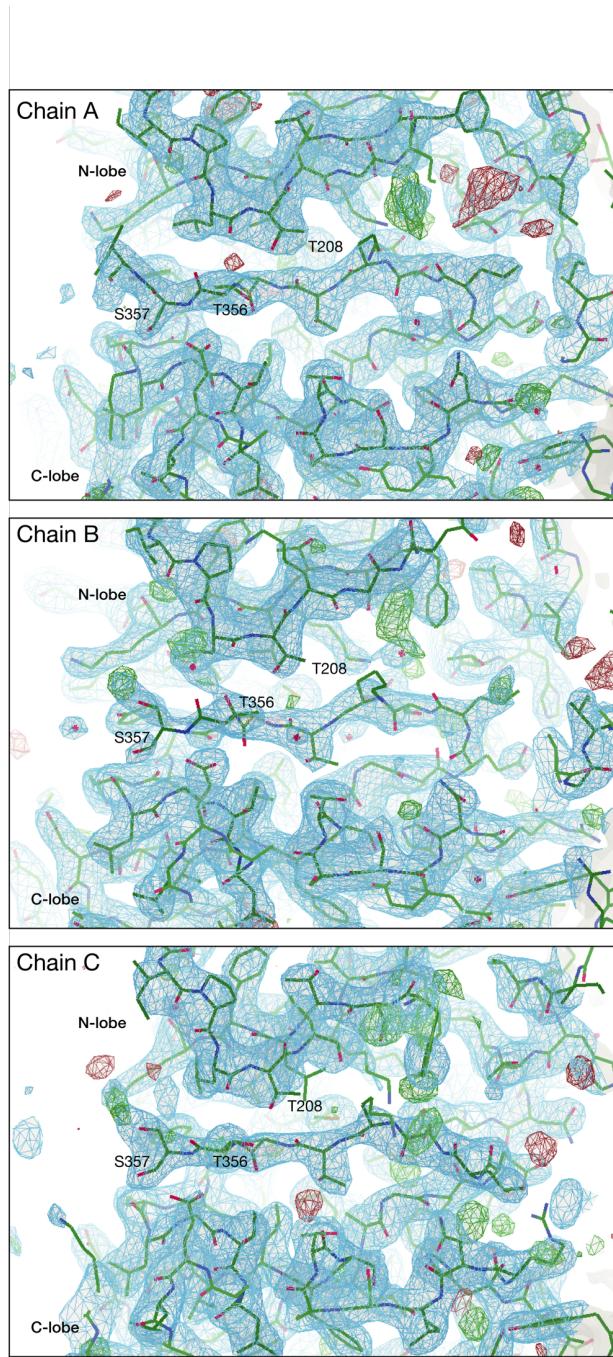


Q355A-



Supplementary Figure 4. Electron density maps for the novel activation loop conformation of horse MLKL.

Electron density maps for the activation loop and surrounding regions of the horse MLKL pseudokinase structure for all three chains of the asymmetric unit. The blue mesh shows the σ weighted 2Fo-Fc refined density, and the red and green mesh show the difference density map, Fo-Fc. Bonds are depicted as lines; green for carbon atoms, red for oxygen and blue for nitrogen. Thr356 and Ser357, the activation loop residues equivalent to those targeted by RIPK3 in human MLKL, and the N- and C-lobes, are labelled. The 2Fo-Fc density was contoured at 1.0 σ and Fo-Fc density contoured at 3.5 σ . Maps were visualised in Coot².



Supplementary References

1. Murphy JM, *et al.* The pseudokinase MLKL mediates necroptosis via a molecular switch mechanism. *Immunity* **39**, 443-453 (2013).
2. Emsley P, Lohkamp B, Scott WG, Cowtan K. Features and development of Coot. *Acta Crystallogr D Biol Crystallogr* **66**, 486-501 (2010).