

# Mechanistic Studies of MLKL Mediated Cell Death

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## ***Abstract***

Necroptosis is a form of inflammatory programmed cell death. Thought to have evolved to combat pathogen infection, necroptosis can be triggered via signalling through pattern recognition receptors or cytokines from the TNF family. The final stages of necroptosis signalling are characterised by the kinase RIPK3 oligomerising with various RHIM domain containing proteins, which may assemble together into large functional amyloid complexes. Within these complexes RIPK3 is thought to auto-phosphorylate and then recruit and phosphorylate the final known effector of the pathway Mixed Lineage Kinase domain-Like protein (MLKL). Once the pseudokinase MLKL is phosphorylated, it transforms from an inert cytoplasmic monomer, to an oligomeric membrane associated killer.

The means by which phosphorylation of MLKL's pseudokinase domain by RIPK3 acts as a switch to activate the protein is unknown, as is the stoichiometry of the oligomer. Once MLKL reaches the plasma membrane, it disrupts the membrane by an unknown mechanism and this is thought to be the final blow in cell death. The study of MLKL as the final effector protein of necroptosis has also been hampered by differences between the mouse and human protein, the details of which are only emerging.

This thesis details three complementary studies each aimed to elucidate the molecular details of a particular step in MLKL's mechanism of action. Chapter 3 examines the compatibility between MLKL orthologues from different species. Through functional and structural characterisation of MLKL orthologues stringent requirements for RIPK3 compatibility were found, as well as new evidence for the molecular switch mechanism. Chapter 4 describes the identification of specific residues of the MLKL second brace helix that are essential for the formation of MLKL oligomers, and that the second brace helix is essential for communicating regulatory signals through the protein. Chapter 5 has the first study of MLKL associating with Giant Unilamellar Vesicles using confocal microscopy, and the first exploration of MLKL's effect on membranes through neutron reflectometry. Together the separate studies constitute an in-depth exploration of MLKL's entire mechanism of action, and a comprehensive examination of the differences between mouse and human MLKL.



## ***Declaration***

This is to certify that:

- i) this thesis comprises only my original work, except where acknowledged in the preface
- ii) due acknowledgement has been made in the text to all other material used,
- iii) the thesis is less than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Katherine Anne Davies

## **Preface**

The majority of this thesis comprises my own original work. Some experiments were carried out in collaboration with others, whose contribution I acknowledge here:

- James Murphy and Sam Young cloned, or assisted with the cloning of many constructs used in this thesis (detailed in Appendix I)
- Emma Petrie and Dianne Coursier purified the horse pseudokinase protein used in crystallisation trials, and for thermal shift assays (Chapter 3)
- Richard Birkinshaw collected data on the horse pseudokinase domain crystal that was used to solve the structure (Chapter 3)
- Cheree Fitzgibbon purified the rat MLKL pseudokinase protein used in crystallisation trials and thermal shift assays, and purified the full-length rat protein used in SAXS analysis (Chapter 3)
- James Murphy assisted with data collection and analysis for the rat and mouse MLKL SAXS structures (Chapter 3, 4)
- I made some of the double alanine mutant cell lines in MDFs (Chapter 4) in my Honours year
- Maria Tanzer generated all the MLKL chimera cell lines in Chapter 4, except the following which I made, h4HB-hBrace-mPsk, m4HB-mBrace-hPsk and m4HB-hBrace-hPsk in *Ripk3*<sup>-/-</sup> *Mlkl*<sup>-/-</sup> MDFs and m4HB-hBrace-mPsk in *Mlkl*<sup>-/-</sup> MDFs
- Maria Tanzer contributed repeats to chimera death assays in Chapter 4, 29% of data shown (34/117 TSQ repeats), and also performed expression blots for some of the chimera cell lines (9/14 blots shown)
- Emma Petrie also contributed repeats for chimera death assay data in Chapter 4, 17% of data shown (20/117 TSQ repeats)
- Jan Steinkuehler made the phase separated vesicles in Chapter 5, and assisted with confocal microscopy experiments
- Lung-Yu Liang made the recombinant E351K human MLKL protein visualised by CryoEM in Chapter 5
- Wilson Wong assisted with using the electron microscope to take images for Chapter 5
- Eric Hanssen took the Liposome only control image in Figure 5.3 of Chapter 5, during my Honours year, and an image from this data set was also presented in my Honours thesis

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The contributions by others to my PhD experience has been immense, and to thank everyone properly I would need to write another chapter, which I am not going to do at this point. So, to anyone who has known me, and been a part of my life for the last three years I am thanking you, even if it's not spelled out here.

Without further ado, I would like to thank my amazing PhD supervisors, Peter Czabotar, James Murphy and Emma Petrie. I feel extremely privileged to have been able to work with the three of you for the last four years during my PhD and Honours, and I can't imagine any better team to go through this PhD with. Thank you for being so generous with your time, your knowledge and also your great humour and support. Almost without fail, our fortnightly meetings left me feeling lighter than I did going into them, thanks to your insightful feedback, and also owing greatly to your jovial natures and ability to find the bright side to everything. I also thank you for the free-range I have been given during my PhD, which has been one of the most enjoyable and rewarding aspects of the experience for me.

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I would also like to acknowledge the Walter and Eliza Hall Institute, which has been a phenomenal organisation to work within these past four years. The calibre of science performed by my colleagues has been awe-inspiring and a constant source of inspiration. Furthermore, the WEHI culture has also made it an absolute pleasure to work there, and the level of organisational support is amazing.

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## ***List of Abbreviations***

|               |                                                                |
|---------------|----------------------------------------------------------------|
| 4HB           | Four Helix Bundle (MLKL)                                       |
| ATP           | Adenosine Triphosphate                                         |
| AUC           | Analytical Ultracentrifugation                                 |
| $\beta$ ME    | $\beta$ -Mercaptoethanol                                       |
| BN-PAGE       | Blue Native-Polyacrylamide Gel Electrophoresis                 |
| BSA           | Bovine Serum Albumin                                           |
| cFLIP         | Cellular FLICE-Inhibitory Protein                              |
| CHAPS         | 3-[(3-Cholamidopropyl)Dimethylammonio]-1-Propanesulfonate      |
| cIAP          | Cellular Inhibitor of Apoptosis                                |
| CryoEM        | Cryo-electron Microscopy                                       |
| DAMPs         | Damage Associated Molecular Patterns                           |
| DMSO          | Dimethyl sulfoxide                                             |
| Dox           | Doxycycline                                                    |
| EDTA          | Ethylenediamine tetraacetic acid                               |
| FADD          | Fas-Associated Protein with Death Domain                       |
| GST           | Glutathione S-Transferase                                      |
| GUV           | Giant Unilamellar Vesicle                                      |
| hMLKL         | Human MLKL                                                     |
| IDN           | IDN-6556/Emricasan                                             |
| IPTG          | Isopropyl $\beta$ -D-1thiogalactopyranoside                    |
| LUV           | Large Unilamellar Vesicle                                      |
| MDF           | Mouse Dermal Fibroblast                                        |
| MEF           | Mouse Embryonic Fibroblast                                     |
| MLKL          | Mixed Lineage Kinase Domain-Like                               |
| mMLKL         | Mouse MLKL                                                     |
| MW            | Molecular Weight                                               |
| Nec-1         | Necrostatin-1                                                  |
| NF $\kappa$ B | Nuclear Factor Kappa-light-chain-enhancer of activated B cells |
| NMR           | Nuclear Magnetic Resonance                                     |
| NOE           | Nuclear Overhauser Effect                                      |

|       |                                       |
|-------|---------------------------------------|
| PAGE  | Polyacrylamide Gel Electrophoresis    |
| PBS   | Phosphate Buffered Saline             |
| PCR   | Polymerase Chain Reaction             |
| PEG   | Polyethylene Glycol                   |
| PI    | Propidium Iodide                      |
| PIP   | Phosphatidylinositol Phosphate        |
| PMSF  | Phenylmethanesulfonyl Fluoride        |
| PRR   | Pattern Recognition Receptor          |
| PsK   | Pseudokinase                          |
| RHIM  | RIP Homotypic Interaction Motif       |
| RIPK1 | Receptor Interacting Protein Kinase 1 |
| RIPK3 | Receptor Interacting Protein Kinase 3 |
| SAXS  | Small Angle X-ray Scattering          |
| SBP   | Streptavidin Binding Protein          |
| SD    | Standard Deviation                    |
| SDS   | Sodium Dodecyl Sulphate               |
| SEC   | Size Exclusion Chromatography         |
| SEM   | Standard Error of the Mean            |
| SLD   | Scattering Length Density             |
| TCEP  | Tris(2-Carboxyethyl)Phosphine         |
| TEV   | Tobacco Etch Virus                    |
| TLR   | Toll-Like Receptor                    |
| TNF   | Tumour Necrosis Factor                |
| TNFR1 | Tumour Necrosis Factor Receptor 1     |
| TRADD | TNFR1-Associated Death Domain         |
| TS    | TNF + Smac-mimetic                    |
| TSI   | TNF + Smac-mimetic + IDN              |
| vFLIP | Viral FLICE-Inhibitory Protein        |

## **1.0 Introduction**

### *1.1 The significance of necroptosis in health and disease*

Necroptosis is a form of regulated, but highly inflammatory cell death, characterised by its caspase independence, and death via plasma membrane disruption. The pathway exists among a plethora of mechanisms that multicellular organisms have evolved to die (Gudipaty et al., 2018, Ashkenazi and Salvesen, 2014). The most well-known form of programmed cell death, apoptosis, was first described in the early 1970s (Kerr et al., 1972). Apoptosis is an immunologically silent form of cell death, characterised by caspase mediated dismantling of the cell, membrane blebbing, and formation of apoptotic bodies which are decorated with ‘find me’ and ‘eat me’ signals, that facilitate removal of cell remains by macrophages (Hengartner, 2000, Strasser et al., 2000). This form of cell death has a role in embryonic development, homeostasis and also in some pathologies, and nearly fifty years on, apoptosis is still the subject of active research. The first evidence for programmed necrosis was only published in the late 1980’s (Laster et al., 1988). Previous to this publication, cell death via the necrotic morphology of cell swelling and lysis, was only known to be triggered by mechanical or chemical trauma to the cell, not a result of regulated cell signalling. It took until 2005, for the term necroptosis – a combination between necrosis (for the morphology) and apoptosis (to signify a regulated pathway) to be coined (Degterev et al., 2005). In 2020, the study of necroptosis is in full swing. Due to its relatively recent discovery, many molecular details of the pathway remain to be fully characterised.

Necroptosis is thought to have evolved to combat pathogen infection, in particular viruses (Silke and Hartland, 2013, Riegler et al., 2019, Nailwal and Chan, 2019, Petrie et al., 2019b). The lytic nature of necroptosis (Vanden Berghe et al., 2010) results in release of intracellular contents to the extracellular fluid, and these are recognised by the innate immune system as Damage Associated Molecular Patterns (DAMPs) (Kaczmarek et al., 2013, Murakami et al., 2014, Murai et al., 2018) which play a key role in activating the innate immune system (Roh and Sohn, 2018, Seong and Matzinger, 2004). Animal models have shown the importance of necroptosis, and

necroptosis mediators for controlling or clearing pathogen infection (Cho et al., 2009, Riegler et al., 2019, Wang et al., 2014b, Nogusa et al., 2016). Furthermore, the repertoire of proteins that viruses and bacteria carry in order to subvert necroptotic cell death is strong evidence for its significance to innate immunity (Petrie et al., 2019b, Upton et al., 2010, Pearson et al., 2017, Pearson et al., 2013, Li et al., 2013, Upton et al., 2008, Omoto et al., 2015, Guo et al., 2015). Therefore, having a functional necroptotic pathway is likely to be important for human health.

Conversely, the inflammation caused by necroptosis, in the form of DAMP release and activation of the immune system, can be harmful (Rickard et al., 2014b, Roh and Sohn, 2018). Necroptosis has been implicated in an increasing number of inflammatory human pathologies (see Table 1) through a variety of techniques, including genetic depletion of necroptosis effectors such as RIPK3 and MLKL, use of chemical inhibitors of necroptosis, staining for necroptosis markers in pathology sections and more. Additionally, a recent study has identified missense mutations in the necroptosis effector protein MLKL, present in up to 10% of the human population, that are enriched in populations with certain autoimmune diseases (Hildebrand et al., 2019). A summary of a subset of diseases that necroptosis is implicated in, and the corresponding studies, is shown in Table 1.

**Table 1.1 Necroptosis is implicated in multiple pathologies**

| <b>Pathology</b>                     | <b>Reference</b>                                            |
|--------------------------------------|-------------------------------------------------------------|
| Acute Pancreatitis                   | (He et al., 2009, Zhang et al., 2009, Louhimo et al., 2016) |
| Amyotrophic lateral sclerosis        | (Re et al, 2014)                                            |
| Aplastic anaemia                     | (Chen et al, 2014)                                          |
| Arthrosclerosis                      | (Karunakaran et al., 2016)                                  |
| Crohn's disease                      | (Günther et al, 2011)                                       |
| Drug induced liver injury            | (Wang et al., 2014a)                                        |
| Ethanol-induced liver disease        | (Roychowdhury et al, 2013)                                  |
| Heart Disease                        | (Zhe-Wei et al., 2018)                                      |
| Inflammatory bowel disease           | (Pierdomenico et al, 2014)                                  |
| Ischemia reperfusion injury (heart)  | (Luedde et al., 2014)                                       |
| Ischemia reperfusion injury (kidney) | (Linkermann et al., 2012, Muller et al., 2017)              |
| Ischemic reperfusion injury (liver)  | (Ni et al., 2019)                                           |
| Multiple Sclerosis                   | (Ofengeim et al., 2015)                                     |
| Osteoarthritis                       | (Riegger and Brenner, 2019)                                 |
| Parkinson's disease                  | (Lin et al., 2019)                                          |
| Respiratory distress                 | (Pan et al., 2016)                                          |
| Retinal detachment                   | (Murakami et al., 2014, Gao et al., 2014)                   |
| Systemic inflammation                | (Duprez et al., 2011)                                       |
| Systemic lupus erythematosus         | (Fan et el, 2014)                                           |
| Ulcerative colitis                   | (Wu et al., 2019)                                           |

The studies in Table 1 support the notion that execution of necroptosis is a culprit in human pathology. However, like with other forms of programmed cell death, such as apoptosis, a reduced ability to undergo necroptosis may provide cancer cells with a selective advantage (Hanahan and Weinberg, 2000). Low necroptosis effector protein expression has been shown to be a poor prognosis marker in multiple cancers (Sun et al., 2019, Li et al., 2017, He et al., 2013). Delivery of mRNA encoding the effector protein MLKL, or the recombinant MLKL protein itself to cancer cells, has recently even been tested as a cancer therapy (Van Hoecke et al., 2019, Van Hoecke et al.,



2018). These studies collectively provide compelling evidence for the importance of necroptosis in human health and disease, and underlines the importance of studying this cell death pathway.

The following literature review encompasses the discovery of necroptosis, the identification of and role of key players in signalling, and a thorough review of the biophysical characterisation of the final effector protein MLKL. At the end of the literature review is a summary of the main objectives of my PhD, which aims to better characterise the molecular details of this pathway, to firstly build understanding from a basic science perspective, but equally importantly, to provide knowledge that can aid therapeutic strategies to target necroptosis.

### *1.2 A historical view: discovery of the pathway and its main players*

In 1988, Laster et al. were the first to report that the protein Tumour Necrosis Factor (TNF) could induce two forms of cell death in mouse L-M cells (Laster et al., 1988). One form of death was apoptosis, that was already understood to result from molecular signalling. The second form of cell death resembled necrosis, as some L-M cells were observed to swell and burst. Necrosis had previously been observed after mechanical or chemical injury to cells, therefore the authors hypothesised that TNF might rupture the cell membrane, although they noted that this was unusual as the cell death phenotype was cell type specific. The observation of TNF induced necrosis was repeated in 1995 in another murine cell line (Fiers et al., 1995). In the late 1990s, activation of Fas, a death receptor related to the TNF Receptor (TNFR1), was shown to induce both apoptotic and necrotic morphology cell death (Vercammen et al., 1998b), and co-treatment of cells with pan-caspase inhibitors and Fas Ligand (FasL), promoted the necrotic phenotype (Vercammen et al., 1998a). Together, these studies produced the first evidence that a regulated necrosis pathway existed.

### *Receptor Interacting Protein Kinases*

Following these early publications, the signalling mechanisms behind programmed necrosis began to be discovered. In 2000, Receptor-Interacting Protein Kinase-1 (RIPK1) was shown to be essential for the necrotic morphology cell death in Jurkat

cells treated with FasL and z-VAD-fmk, a pan caspase inhibitor (Holler et al., 2000). In 2005, a small molecule inhibitor Necrostatin 1 (Nec-1), was found to inhibit the newly coined 'necroptosis' pathway, and inhibition of necroptosis was first shown to be therapeutically important in a mouse model of stroke (Degterev et al., 2005). Three years later RIPK1 was found to be the target of Nec-1 (Degterev et al., 2008). In 2009, three papers independently found Receptor-Interacting Protein Kinase-3 (RIPK3) to be an essential player downstream of RIPK1 in the necroptosis pathway (Cho et al., 2009, He et al., 2009, Zhang et al., 2009).

#### *A role for MLKL in necroptosis is discovered*

In 2012, two groups used different approaches to identify the Mixed Lineage domain-Like protein (MLKL) as an effector of necroptosis, acting downstream of RIPK3 (Sun et al., 2012, Zhao et al., 2012). MLKL is a pseudokinase protein that had no previously assigned function. Sun et al. (2012) performed a small molecule screen against cells undergoing necroptosis, and identified necrosulfonamide (NSA) as a necroptosis inhibitor that functioned by covalently modifying human MLKL. Zhao et al. (2012) used an shRNA screen of kinases and kinase-like domains in HT-29 cells to search for essential effectors of necroptosis, and found that knockdown of *MLKL* prevented TSZ (TNF, Smac-mimetic and z-VAD-fmk treatment, discussed below) induced necroptosis. Both papers found RIPK3 was MLKL's upstream activator and that various kinase-dead RIPK3 mutants could not bind to or activate MLKL. Sun et al. (2012) identified residues T357 and S358 on human MLKL as the sites of RIPK3 phosphorylation, and showed that blocking MLKL phosphorylation prevented necroptosis from occurring. Therefore, by 2012, the proteins that are still recognised as the central players in necroptosis, RIPK3 and MLKL, had been revealed.

To understand how necroptosis is triggered in cells *in vivo*, and also how necroptosis is recapitulated *in vitro*, knowledge of the signalling mechanisms that induce necroptosis is required. As described, many of the early papers used TNF stimulation to induce necroptosis, and it is TNF induced necroptotic signalling that is the most studied and best understood. More recently, Pattern Recognition Receptors (PRRs) of the innate immune system have also been found to induce necroptosis. These

signalling mechanisms are discussed below in Sections 1.3 and 1.4, and are graphically summarised in Figure 1.1.

### *1.3 TNF receptor necroptosis signalling*

TNF ligation to its receptor TNFR1, induces formation of three distinct signalling complexes, each vying for a different outcome for the cell (described in Figure 1.1). Which path the cell will take is dependent on multiple factors, not all yet well understood, but include cell type, presence of pathogens and relative abundance and activity of effector proteins (Dannappel et al., 2014, Rickard et al., 2014a, Fritsch et al., 2019, Lin et al., 2016, Cook et al., 2014).

#### *Complex I*

At the membrane, upon ligation of TNF to its receptor, signalling through Complex I begins (Micheau and Tschopp, 2003). The death domain of TNFR1 recruits TRADD (Hsu et al., 1995), which subsequently recruits RIPK1 and TRAF2, both through their death domains. Within this complex, RIPK1, as well as other Complex I components, are heavily ubiquitinated; with K63 ubiquitin chains due to the activity of cellular Inhibitors of Apoptosis (cIAPs) (Varfolomeev et al., 2008) and linear ubiquitin chains from the LUBAC complex (Haas et al., 2009). The K63 ubiquitination on RIPK1 serves as a scaffold to recruit other factors to the complex, such as TAK1 and TAK2, and kinase I $\kappa$ K (Ea et al., 2006, Wu et al., 2006). Within Complex I, I $\kappa$ K is phosphorylated, which leads to the phosphorylation of I $\kappa$ B, and its removal by the proteasome, leaving transcription factor NF $\kappa$ B free to translocate to the nucleus (Scheidereit, 2006, Traenckner et al., 1995). NF $\kappa$ B promotes transcription of a range of pro-survival factors, including cellular FLICE-inhibitory protein (cFLIP) (Micheau et al., 2001, Kreuz et al., 2001), cytokines and the deubiquitinase enzyme A20 (Lee et al., 2000), which reduces signalling through Complex I by removing the ubiquitin chains in a negative feedback loop (Wertz et al., 2004), thereby preventing excess inflammation driven by cytokine production.

### *Complex IIa*

Concurrent with signalling through Complex I, is the formation of a secondary signalling complex, Complex IIa, the core of which is formed by RIPK1 and FADD, which associate through their death domains (Micheau and Tschopp, 2003). Inhibition of cIAP mediated ubiquitination of RIPK1 can promote formation of this complex (Bertrand et al., 2008, Vince et al., 2007). FADD can recruit caspase-8 (Muzio et al., 1996), which if left unchecked, will homo-dimerise and autocatalytically activate (Oberst et al., 2010) and induce apoptosis by cleavage of pro-caspase-3 (Stennicke et al., 1998), or Bid to tBid (Li et al., 1998). However, cells do not always undergo apoptosis in response to TNF signalling. This is due to the work of Complex I, that produces cFLIP. cFLIP can heterodimerise with caspase-8 at Complex IIa and dull proteolytic activity of caspase-8 (Oberst et al., 2011, Micheau et al., 2001, Irmeler et al., 1997). In this heterodimer, caspase-8 is thought to maintain low level function and cleaves the Receptor Homotypic Interacting Motif (RHIM) containing domain off RIPK1, an essential step in inhibiting the formation of a third deadly complex (Feng et al., 2007, Lin et al., 1999) (as reviewed in (Mocarski et al., 2011)).

### *Complex IIb*

Caspase inhibition has long been known to be a driver of programmed necrosis, (Vercammen et al., 1998a), and it is now clear that the activity of caspase-8 functions as a key determinant in cells' choice to undergo apoptosis or necroptosis (Mocarski et al., 2011). One way caspase activity can be modulated is through viral effector proteins, that have evolved to thwart apoptosis, to allow viral replication inside host cells (Benedict, 2003). For example, vFLIP is a viral analogue of cFLIP (Hu et al., 1997). Like cFLIP it can heterodimerise with caspase-8 and prevent apoptosis from occurring through Complex IIa. vFLIP however, completely inhibits caspase-8 activity (Hu et al., 1997), and prevents cleavage of the RHIM domain from RIPK1 in Complex IIa. This leads to the formation of a third signalling complex, termed Complex IIb or the "necrosome" (Cho et al., 2009).

The essential components of the necrosome are RIPK1 and RIPK3. They assemble together into a large functional amyloid complex (Mompeán et al., 2018, Li et al.,

2012), via their homotypic RHIM domains (Sun et al., 2002a)). In this complex both RIPK1 and RIPK3 are phosphorylated (Cho et al., 2009). RIPK3 is thought to auto-phosphorylate itself in this complex and can then bind and phosphorylate MLKL, the executioner of necroptosis (Sun et al., 2012, Zhao et al., 2012). Once MLKL is phosphorylated it oligomerises and translocates to the plasma membrane, which it disrupts, resulting in cell death via necroptosis.

#### *Replication of TNF signalling in the lab*

In the lab we use specific drugs to manipulate signalling through the TNF induced complexes, to force specific cell death outcomes, such as apoptosis or necroptosis. To stimulate apoptosis in TNF responsive cell lines, cells are treated with recombinant TNF, to initiate signalling, and a Smac-mimetic (compound A), that inhibits cIAPs, thereby preventing the ubiquitination of RIPK1 at Complex I and inhibiting signalling through it. This combination of drugs (called TS) causes the cell to signal predominantly through Complex IIa and die via apoptosis. This is used as a control in cell death assays, to ensure TNF signalling is functioning as expected in cells. To induce necroptosis, a pan-caspase inhibitor, such as z-VAD-fmk or QVD-OPh, or selective caspase-8 inhibitor such as IDN-6556 (Brumatti et al., 2016) is added alongside TS stimulus. This caspase inhibitor inhibits caspase-8 at Complex IIa, allowing RIPK1 to bind RIPK3 and induce formation of the necrosome, leading to necroptosis. A combination of TNF, Smac-mimetic and IDN (TSI) is the necroptotic stimulus most commonly used throughout this thesis.

### *1.4 Pattern Recognition Receptor signalling*

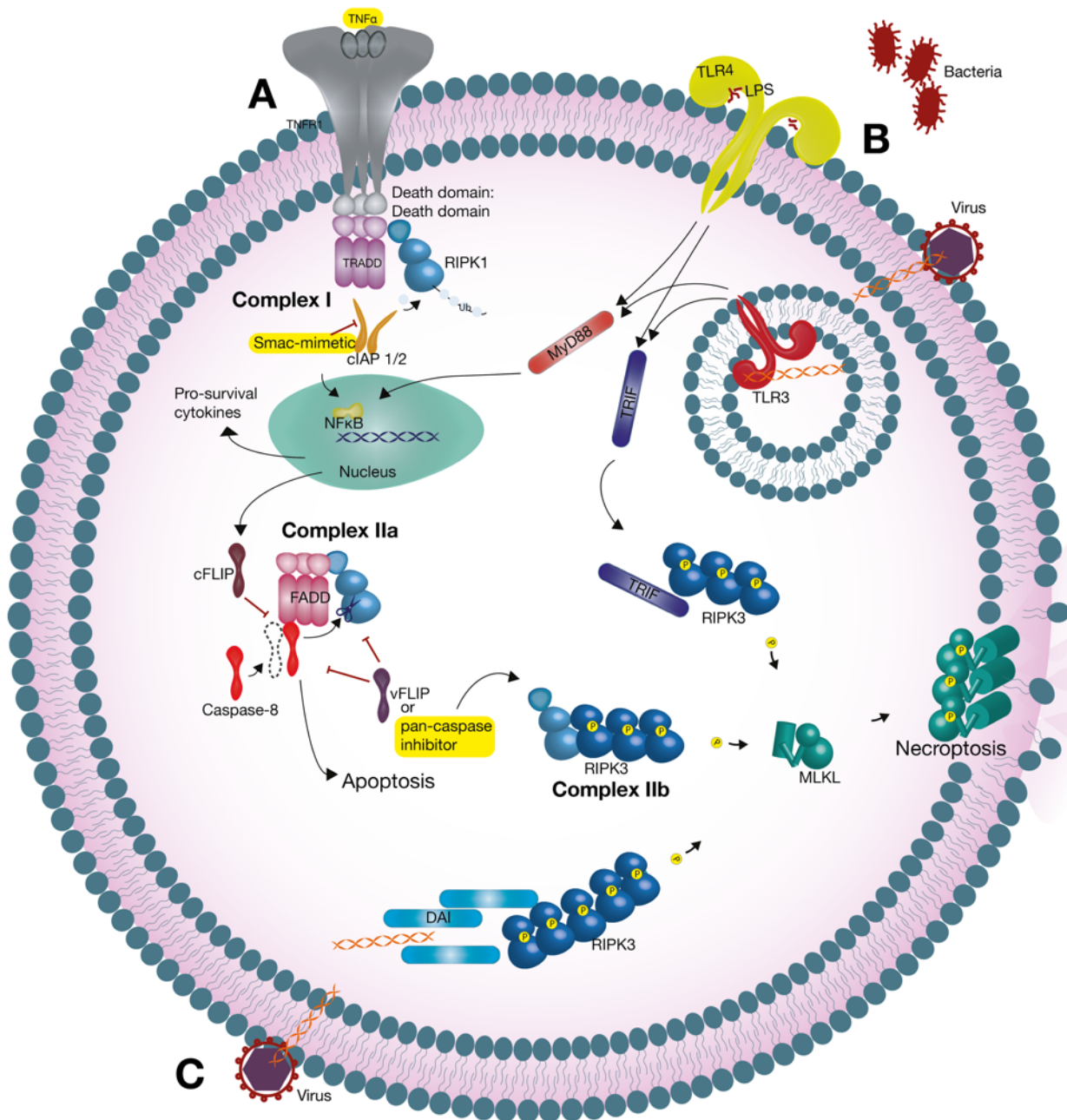
#### *Toll-Like Receptors (TLRs)*

TLRs can also induce necroptosis through their own signalling, or through intersection with the TNF pathway (Kaiser et al., 2013, He et al., 2011). Using genetic models, He et al. (2011) were able to show that signalling through both TLR3 (viral DNA sensor) and TLR4 (LPS sensor) could induce necroptosis in bone marrow derived macrophages, and this was dependent on TRIF and RIPK3. Later studies increased the panel of TLRs that could induce necroptosis to include TLR2 (senses peptidoglycan), TLR5 (flagellin) and TLR9 (unmethylated CpG DNA sensor) (Kumar et

al., 2011, Kaiser et al., 2013). They showed that TLR-3 and 4 signalling can induce necroptosis directly through TRIF, which uses its RHIM domain to bind to RIPK3, leading to RIPK3 autophosphorylation and MLKL recruitment, in an analogous manner to signalling through the necrosome. The TLRs could also signal through adaptor protein MyD88, which results in NF $\kappa$ B translocation to the nucleus, thereby potentially intersecting with TNF signalling (Kaiser et al., 2013).

### *DAI*

DAI is an intracellular pattern recognition receptor, that recognises viral nucleic acids (Maelfait et al., 2017). It contains a RHIM domain, and can interact with both RIPK1 and RIPK3 (Upton et al., 2008). Studies relying predominantly on a mouse cytomegalovirus (MCMV) model have shown that once DAI is activated by binding viral DNA/RNA, it can bind to RIPK3 through RHIM interactions, and promote RIPK3 phosphorylation (Upton et al., 2012, Maelfait et al., 2017). This leads to RIPK3 association with MLKL and subsequent induction of necroptosis (Ingram et al., 2019, Upton et al., 2012) (reviewed in (Upton and Kaiser, 2017)). Further complexity has been added to this story with studies suggesting that DAI dependent necroptosis is not dependent on the presence of viral DNA (Ingram et al., 2019, Lin et al., 2016). Three papers found that DAI is responsible for triggering DAI:RIPK3 mediated necroptosis in either *Ripk1*<sup>-/-</sup> or RIPK1 RHIM mutant mice (Ingram et al., 2019, Newton et al., 2016, Lin et al., 2016). The most recent study has suggested that under the steady state RIPK1 sequesters RIPK3 away from interaction with DAI, and in the absence of RIPK1, DAI is the trigger for RIPK3 dependent necroptosis (Ingram et al., 2019).



**Figure 1.1 Necroptosis signalling showing the interplay between pathways**

**A** Signalling begins with ligation of TNF to TNFR1. Following the arrows, the main players in Complex I (pro-survival signalling), Complex IIa (apoptosis signalling) and Complex IIb (necroptosis signalling) are highlighted. At Complex IIb RIPK3 phosphorylates MLKL leading to necroptosis. Points in the pathway that are targeted by drugs (such as Smac-mimetics and caspase inhibitors) to reconstitute necroptotic signalling in cells, are highlighted in yellow. **B** TLR signalling can induce necroptosis when stimulated by viral or bacterial antigens. TLR3 and 4 can activate TRIF, which oligomerises with RIPK3 using its RHIM domain to induce necroptosis, or integrate with TNF signalling through activation of adaptor MyD88, leading to NFκB translocation to the nucleus. **C** DAI is a viral nucleic acid sensor, it can directly complex with RIPK3 via its RHIM domains, leading to RIPK3 autophosphorylation and MLKL activation, as with the other pathways.

## 1.5 MLKL the killer protein

The focus of this literature review thus far has been on the events upstream of activation of the terminal necroptosis effector, MLKL. MLKL's effector function relies on a series of steps that transform it from an inert cytoplasmic monomer, to a membrane associated, oligomeric killer. The following sections summarise what has been learnt about MLKL's role in necroptosis since its discovery as an effector of the pathway in 2012. These studies form the foundation of my own research into the details of MLKL's molecular mechanism of action described in this thesis.

### *MLKL the pseudokinase*

MLKL is a pseudokinase protein, meaning that although it shares some sequence homology with kinases and adopts a typical kinase fold, it does not have the required amino acid residues at essential catalytic motifs to allow catalysis of phosphotransfer reactions (Murphy et al., 2014b) (Figure 1.2).

| Canonical Kinase | VAIK | HRD | DFG |
|------------------|------|-----|-----|
| Human MLKL       | VAIK | HGK | GFE |
| Mouse MLKL       | VTIK | HRN | GFE |

**Figure 1.2 MLKL's catalytic motifs compared to a kinase**

Three important catalytic motifs of a canonical kinase are shown, with the catalytic residues highlighted in red. Human and mouse MLKL have divergent sequence at these catalytic motifs. They retain the K of the VAIK motif, which is responsible for positioning the ATP phosphate groups during catalysis (Carrera et al., 1993), but do not have either conserved aspartates from the HRD motif (D; a key catalytic residue) or the DFG, (D; coordinates cofactor  $Mg^{2+}$ ) (Knighton et al., 1991).

Many of the important regulatory regions and motifs in MLKL fall within the pseudokinase domain however, and are reliant upon conserved structural motifs from kinases such as the  $\alpha$ C helix, the activation loop and the regulatory spine (R-spine). In a conventional kinase, the  $\alpha$ C helix is the main helix in the  $\beta$ -sheet dominated N-lobe. The position of the  $\alpha$ C is a key modulator of kinase activity, and active kinases have a conserved salt bridge interaction between a glutamate residue from the  $\alpha$ C and



the catalytic lysine of the VAIK motif (Huse and Kuriyan, 2002) (Figure 1.2). The activation loop helix is also a key modulator of kinase activity and is itself a site of phosphorylation, allowing kinases to be activated by upstream kinases, as in a kinase cascade (Huse and Kuriyan, 2002, Johnson et al., 1996). Kinases also share a motif known as the regulatory spine (R-spine) that determines structural rigidity in the kinase domain and can correspond to whether it is active or inactive (Kornev et al., 2006). The R-spine comprises stacked hydrophobic residues down the centre of the kinase domain, and these residues are conserved in MLKL (Murphy et al., 2014a). Like a kinase, MLKL retains ATP binding ability and its activity is modulated by changes to the described motifs, and so MLKL is classified to be a molecular switch type of pseudokinase (Murphy et al., 2014b).

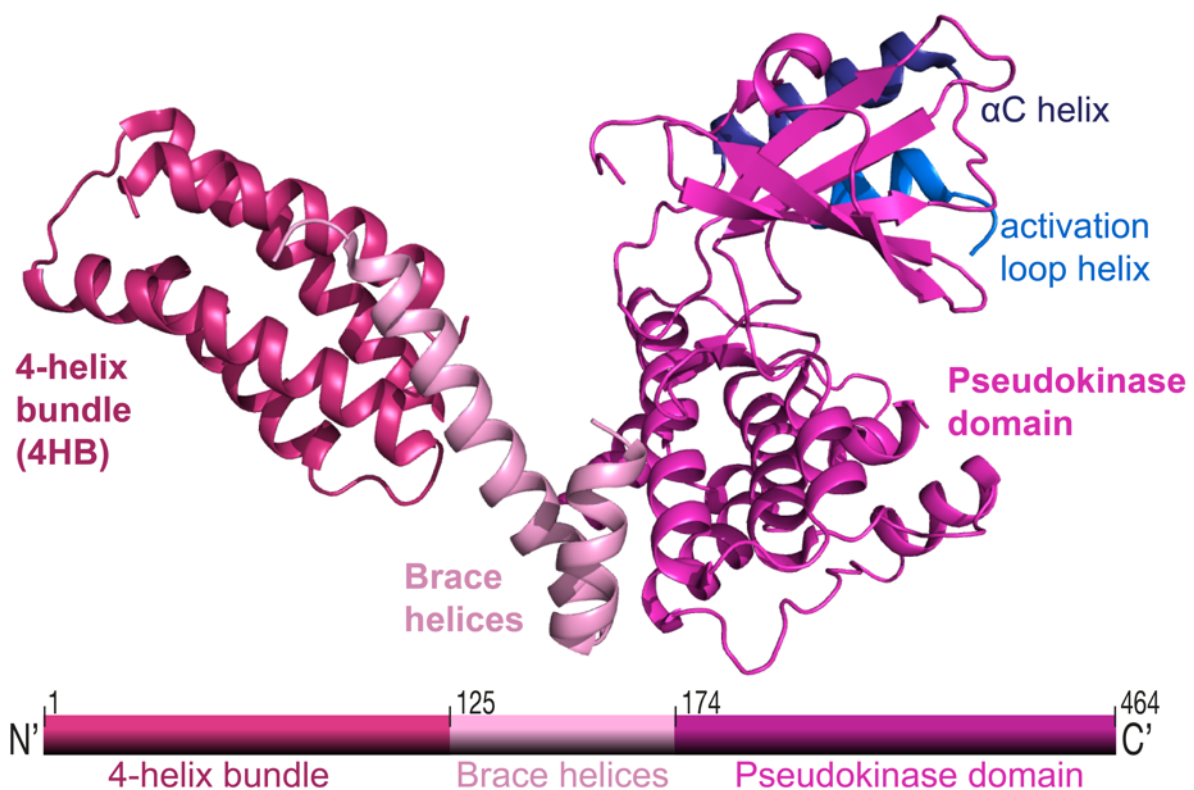
#### *MLKL interspecies differences*

In discussion of MLKL's general mechanism of action it is important to first preface that so far, the study of MLKL has been carried out almost interchangeably on the mouse and human MLKL counterparts. However, emerging evidence suggests that human and mouse MLKL may differ quite markedly in many of the details at each step of their molecular mechanism of action (Petrie et al., 2019a). For instance, the human MLKL interaction with upstream effector RIPK3 is theorised to play a more substantial role in human MLKL activation than the reciprocal interaction between mouse MLKL and mouse RIPK3 (Petrie et al., 2018). Furthermore, the mouse and human proteins cannot bind to the other species' RIPK3 (Chen et al., 2013, Sun et al., 2012). At the point of oligomerisation, mouse and human MLKL may vary in the stoichiometry of their oligomer (Petrie et al., 2018, Hildebrand et al., 2014, Davies et al., 2018). Furthermore, mouse MLKL seems to be intrinsically more poised to kill in cells, with a substantial repertoire of autoactive (those that kill upon expression without necroptotic stimulus) mutants and truncation constructs being described (Murphy et al., 2013, Hildebrand et al., 2014, Tanzer et al., 2016). The various mutants and truncation constructs of human MLKL however show a diminished propensity to induce stimulus independent cell death (Petrie et al., 2018, Tanzer et al., 2016). In light of these differences, in the following description of MLKL's mechanism of action

it is highlighted whether mouse or human MLKL was studied, as are any points of species differences.

#### *MLKL domain architecture*

MLKL has an unusual arrangement of domains, that can be observed in the crystal structure of full-length mouse MLKL (Figure 1.3) (Murphy et al., 2013). Through biochemical and cell biology approaches, distinct roles have been assigned to each of the domains, although the precise way the domains communicate remains to be unravelled.



**Figure 1.3 Structure of full-length mouse MLKL**

The structure of full-length mouse MLKL is shown (PDB:4BTF, (Murphy et al., 2013)), with the 4HB, brace helices and pseudokinase domain labelled, with the corresponding residue boundaries. The  $\alpha$ C helix is shown in dark blue, and the activation loop helix is shown in light blue.

The N-terminal domain of MLKL contains four  $\alpha$ -helices (5 in the human NMR structure (Su et al., 2014)), and is termed the four-helix bundle (4HB) (Figure 1.3). These helices are the site of MLKL's membrane interaction motifs, and are rich in positively charged residues thought to enable MLKL binding to phosphatidyl inositol phosphate lipids (PIPs) (Quarato et al., 2016, Dondelinger et al., 2014). The 4HB

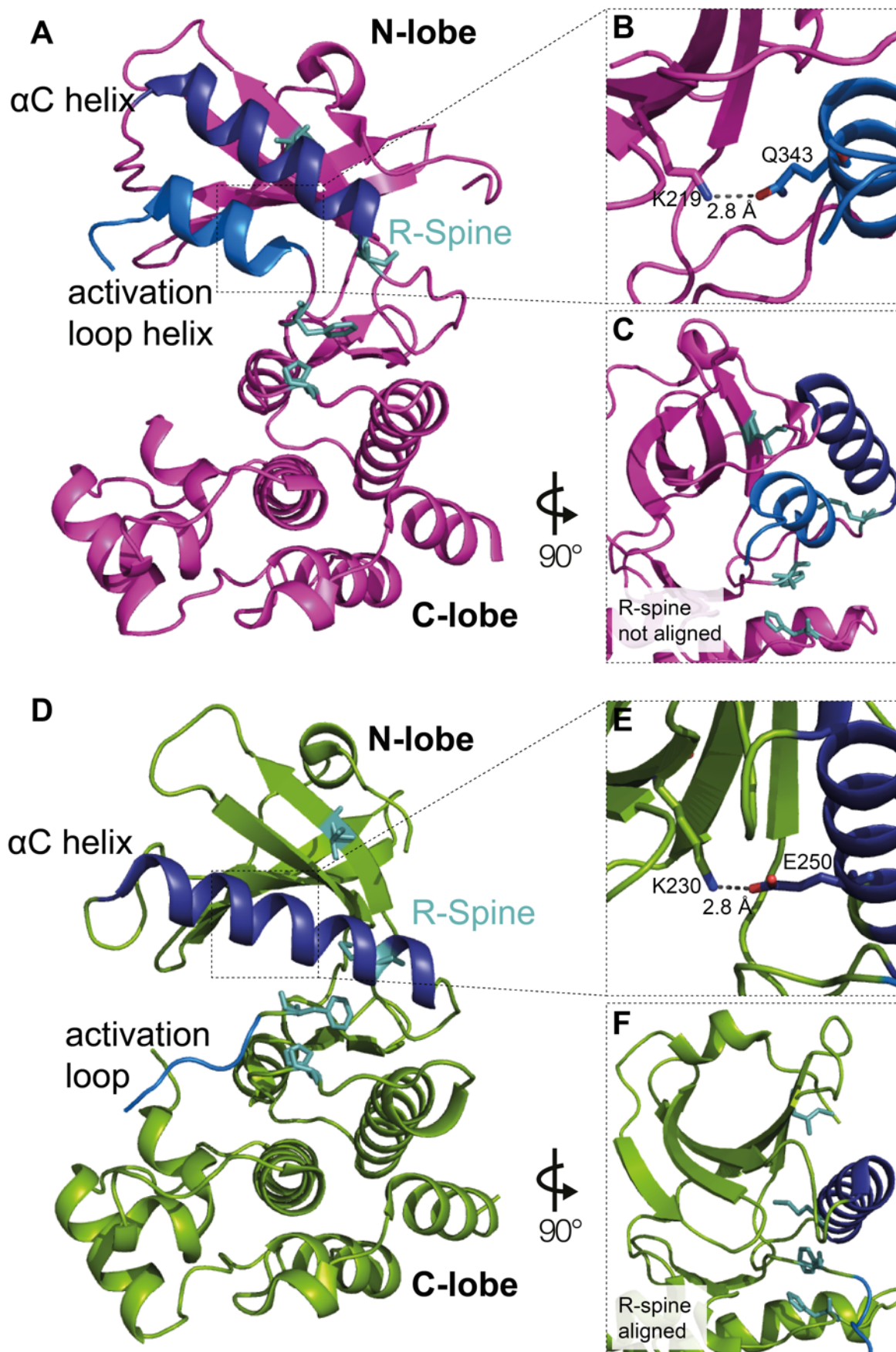
domain is regarded as the executioner domain because mutations to conserved charged residues in this region abrogates necroptosis in cells (Hildebrand et al., 2014, Quarato et al., 2016), and expression of the mouse 4HB alone can constitutively cause cell death in mouse cells (Hildebrand et al., 2014). The 4HB alone or 4HB plus the brace region can permeabilise liposomes *in vitro* (Su et al., 2014, Tanzer et al., 2016). This region of MLKL does not share any homologues in animals, however it was found to share sequence similarities with the HELO proteins found in fungus and plants (Daskalov et al., 2016, Mahdi et al., 2019), that also permeabilise cell membranes, and this is thought to be an example of convergent evolution.

The 4HB domain is followed by two helices termed the 'brace region'. These helices play an essential role in MLKL oligomerisation (Cai et al., 2014, Quarato et al., 2016, Davies et al., 2018), and communication of signals from the C-terminal pseudokinase domain to the 4HB domain (Davies et al., 2018, McNamara et al., 2019).

The pseudokinase domain is at the C-terminus of MLKL, and has the fold of a canonical kinase. When expressed alone in cells, the pseudokinase domain of MLKL cannot induce necroptosis, even when cells are stimulated, and in mouse MLKL it's deletion is sufficient to allow constructs comprising the 4HB and brace to cause constitutive death (Hildebrand et al., 2014). It is therefore thought to play a regulatory role in restraining the 4HB in the steady state (Hildebrand et al., 2014). The pseudokinase domains of mouse and human MLKL retain ATP binding ability, and mutation of Lys from the VAIK motif (responsible for coordinating ATP in active kinases) (Carrera et al., 1993) abolishes this binding (Murphy et al., 2013, Murphy et al., 2014a). Interestingly the pseudokinase domain cannot bind ATP in the presence of magnesium, supporting the idea that the structure of the pseudokinase has diverged from active kinases (Murphy et al., 2014a). The pseudokinase domain of MLKL is also the main site of binding for activator protein RIPK3, and contains the main target site of RIPK3 phosphorylation (Sun et al., 2012), discussed further below.

*Differences between the mouse and human pseudokinase domains*

Multiple structures of both the human (Xie et al., 2013, Murphy et al., 2014a, Petrie et al., 2018) and mouse (Xie et al., 2013, Murphy et al., 2013) pseudokinase domain have been solved. Whilst the structures of human and mouse have been solved in different space groups, the structures of each species' pseudokinase domain remain consistent, and indeed the two species' structures remain consistently different to one another (Figure 1.4).



**Figure 1.4 Mouse versus human MLKL pseudokinase domain**

**A** The mouse MLKL pseudokinase domain (pink; PDB:4BTF). The mouse pseudokinase activation loop forms an atypical helix (light blue), which displaces the  $\alpha$ C helix (deep blue). The residues that comprise the mouse MLKL R-spine are shown in teal. **B** The hydrogen bond interaction between the activation-loop helix residue Q343, and K219 of the VTIK motif. **C** The hydrophobic residues of the R-Spine in mouse MLKL do not stack, and the R-spine is unaligned. **D** The human MLKL pseudokinase domain (green; PDB:4MWI). The human activation loop is highlighted in light blue, and the  $\alpha$ C helix in deep blue. The residues that comprise the human MLKL R-spine are shown in teal. **E** The salt-bridge interaction between the  $\alpha$ C helix residue E250, and K230 of the VAIK motif. **F** The hydrophobic residues of the R-spine in human MLKL stack, and are aligned.

The activation loop of the mouse MLKL pseudokinase domain forms an atypical helix, which takes the position that the  $\alpha$ C helix would adopt in a typical kinase (Figure 1.4). The human MLKL pseudokinase structure forms a more typical kinase fold with a largely disordered activation loop (Figure 1.4 A, D). Due to the positioning of the  $\alpha$ C, the two species exhibit distinct hydrogen bonding interactions. In human MLKL, E250 from the  $\alpha$ C forms a salt bridge interaction with K230 of the VAIK motif, in the manner of an active kinase (Huse and Kuriyan, 2002). In mouse MLKL, this important interaction has been substituted with Q343 from the activation loop helix, which binds to K219 from mouse's VTIK motif (Figure 1.2, Figure 1.4 B, E). This hydrogen bond is of great importance to mouse MLKL's regulation, as disruption of the interaction through mutations Q343A, or K219A induced autoactivity of the MLKL protein in mouse cells (Murphy et al., 2013). Interestingly the equivalent mutation made to human MLKL (K230M) does not induce autoactivity (Petrie et al., 2018).

Mouse MLKL has a disordered R-spine, a conformation that is usually indicative of a kinase in its inactive conformation (Kornev et al., 2006) (Figure 1.4C). In contrast, the human MLKL pseudokinase domain has an intact R-spine, and overall resembles the conformation of an active kinase (Figure 1.4 D,F). The mouse and human pseudokinase domains share a sequence identity of 69.3%, and an open question in the field is whether mouse and human MLKL can adopt the conformation of the other species (Czabotar and Murphy, 2015).

## 1.6 MLKL activation

### *MLKL:RIPK3 binding, species differences*

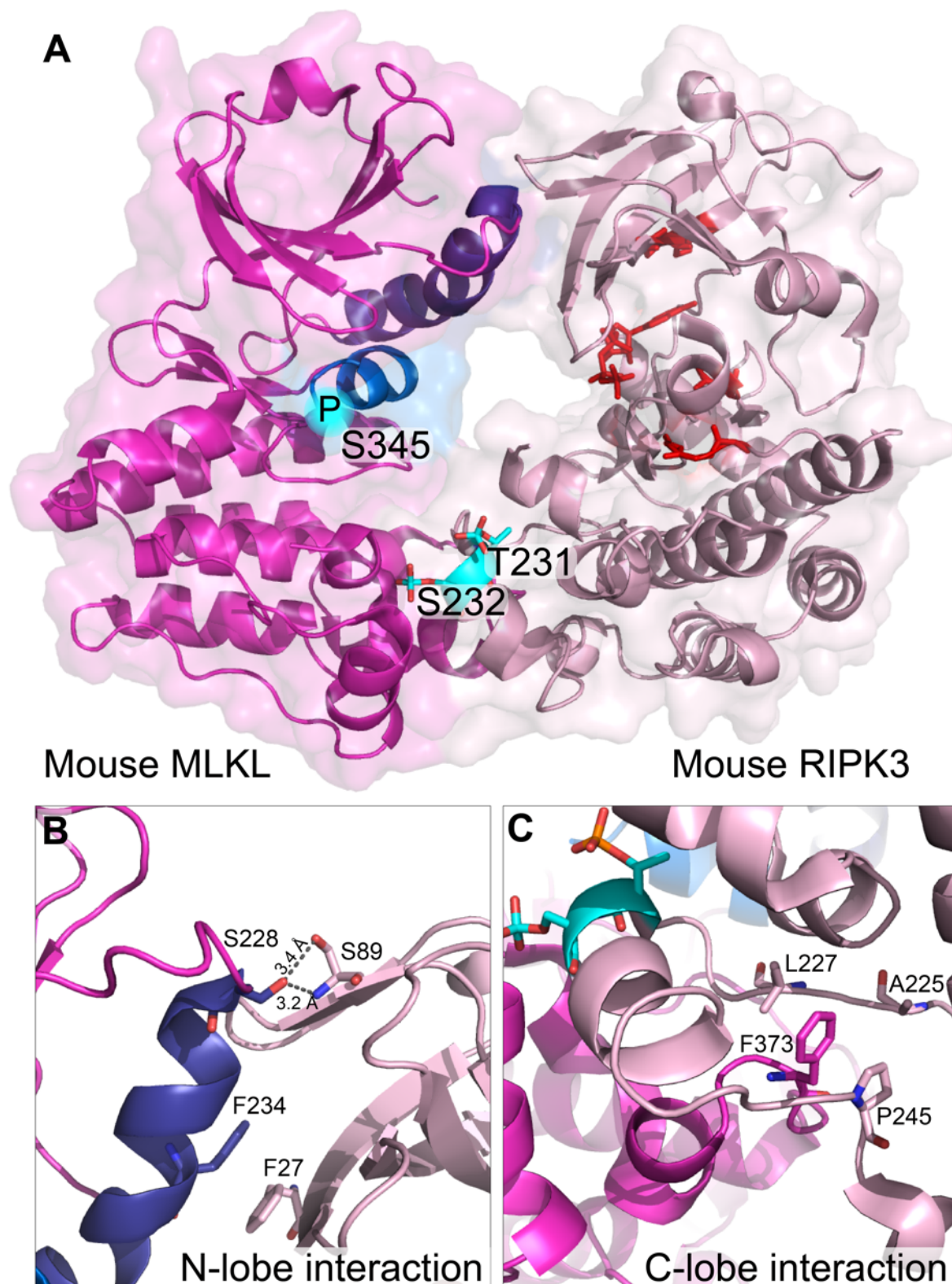
Immunoprecipitation experiments showed that human RIPK3 and human MLKL are associated under basal conditions, and that the interaction is highly enhanced by necroptosis induction (Sun et al., 2012). Multiple groups have shown that RIPK3 must be phosphorylated in order to bind MLKL, on S227 in human RIPK3, and S232 and T231 in mouse (Chen et al., 2013, Sun et al., 2012, Petrie et al., 2019b). Mouse RIPK3 cannot bind human MLKL, and human RIPK3 can only interact with mouse MLKL very weakly, despite the RIPK3s having the same function in cells (Sun et al., 2012, Chen et al., 2013). Chen et al. (2013) were able to mutate mouse RIPK3 to enable binding to human MLKL, by mutating two residues directly adjacent to the RIPK3 phosphosite S232, to the equivalent human sequence. Our understanding of the MLKL RIPK3 binding mode was expanded by a co-crystal structure of the mouse proteins obtained in 2013 (Xie et al., 2013). The structure showed that the pseudokinase domain of MLKL aligns with the kinase domain of RIPK3 N-lobe to N-lobe, C-lobe to C-lobe, and as such there are two main interaction interfaces between the proteins (Figure 1.5). The N-lobe interaction is dominated by H-bonds and  $\pi$ -stacking interactions, whilst the most significant C-lobe interaction is the insertion of F373 from mouse MLKL into a hydrophobic cleft near RIPK3's  $\alpha$ G helix, a residue that is conserved in human MLKL (F386). A recent study has found viral MLKL xenologues have a conserved equivalent aromatic residue that is necessary for their binding to human RIPK3 (Petrie et al., 2019b). Mutation of the equivalent residue in human MLKL (F386A) also attenuated hRIPK3 binding, showing that this binding mode is conserved between human and mouse systems (Petrie et al., 2019b). Of note, in the mouse MLKL:RIPK3 co-crystal structure, the authors found by mass spec that both proteins in the complex are phosphorylated, however compared to an apo RIPK3 structure, the MLKL bound RIPK3 appears to be in an inactive conformation (Xie et al., 2013). Whether this has functional relevance, or is an artefact of crystallisation remains to be determined.

### *MLKL phosphorylation by RIPK3*

Sun et al (2012), identified that human MLKL was phosphorylated by RIPK3 on residues S358 and T357 of the activation loop. By making single alanine mutations they discovered that phosphorylation of either of these residues was sufficient to allow necroptosis, but the double alanine mutant T357A-S358A blocked necroptosis. They suggested that homologous S345 in mouse MLKL would be the RIPK3 phosphorylation site, and this was later confirmed by other groups (Murphy et al., 2013, Rodriguez et al., 2016, Tanzer et al., 2015). Mouse MLKL's activation loop also has two other phosphosites; T249 and S347, with S345 being the most essential and the other two sites playing auxiliary roles (Rodriguez et al., 2016). The mouse MLKL phosphomimetic mutant S345D, is sufficient to cause necroptosis upon expression in mouse cells (Murphy et al., 2013). The story for the human phosphomimetic mutant is more complex with different groups reporting differing activity. Sun et al. (2012) reported that T357E-S358D behaved similarly to wild-type MLKL and could induce cell death with stimulus. Other groups have used human MLKL mutant T57E-S358D as an autoactive mutant in a range of cell lines, including U2Os (Wang et al., 2014a), which do not express RIPK3 (He et al., 2009), and HT29 cells (Yoon et al., 2017, Yoon et al., 2016) a commonly used cell line to study necroptosis. Studies performed by our own group found that human phosphomimetic mutants, T357E-S358D and T357E-S358E, were totally inactive in U937 and HT29 human cells lines, and could not be induced to cause cell death even with necroptotic stimulus (Petrie et al., 2018).

Aside from the residues in MLKL's activation loop, phosphorylation of which are essential for the induction of necroptosis, other phosphosites in MLKL have been identified and shown to tune MLKL activity (Tanzer et al., 2015). Interestingly one of these sites is mouse residue S232, previously identified as an interface site between mouse MLKL and RIPK3 (Xie et al., 2013) (Figure 1.5). The phosphosite was identified in an *in vitro* kinase assay between MLKL and RIPK3, this may suggest that the co-crystal structure represents just one conformational state of the two proteins interaction, and that other binding modes are possible.





**Figure 1.5 Mouse MLKL co-crystal structure with RIPK3**

Mouse MLKL (magenta) in complex with RIPK3 (light pink) (PDB:4M69; (Xie et al., 2013)). **A** The residues of RIPK3's active site and bound ATP analogue ANP are shown in red. S232 and T231 are of RIPK3 are phosphorylated in the structure, and are shown in cyan. The main activating phosphosite of MLKL S345 is not resolved in the structure, its approximate position at the end of the activation loop helix is

highlighted in cyan with P for phosphosite. **B** Some of the N-lobe interactions are highlighted including the H-bonds formed between S228 from mouse MLKL and S89 from RIPK3, and the  $\pi$ -stacking interactions between F234 (MLKL) and F27 (RIPK3). **C** The essential C-lobe interaction; insertion of F373 (MLKL) into a hydrophobic pocket on RIPK3 lined with residues P245, L227 and A225.

#### *The proposed molecular switch mechanism of activation*

RIPK3 binding to MLKL and subsequent phosphorylation of residues within MLKL's activation loop are essential for necroptosis to occur, but how phosphorylation of MLKL's pseudokinase domain leads to release of the killer 4HB domain remains unknown. A possible molecular switch mechanism for MLKL activation was proposed in 2013 (Murphy et al., 2013). The hypothesis states that phosphorylation of activation loop residues in MLKL leads to structural rearrangements in the protein that unleash the killer 4HB domain and allow oligomerisation and subsequent cell death to occur. One suggestion is that the structure of MLKL orthologues such as human or mouse MLKL, could represent different states of activation of MLKL (Murphy et al., 2014a, Czabotar and Murphy, 2015) (Figure 1.4).

In support of this hypothesis, mutations of residues within the pseudokinase domain, distal to the brace region that is responsible for oligomerisation, and the killer 4HB domain, do have an impact on MLKL oligomerisation and cell death. Firstly, in the case of mouse MLKL, phosphomimetic mutation S345D confers MLKL constitutive killing ability (Murphy et al., 2013). Mutations to the residues mediating the hydrogen bond between mouse MLKL's activation loop helix and the  $\beta$ 3 strand VTIK motif (Figure 1.3), Q343A and K219M, also result in autoactive MLKL that can kill upon expression in the absence of necroptotic stimuli (Murphy et al., 2013). It is not clear how these mutations, or indeed phosphorylation of the activation loop could have such a large impact on the activity of the rest of the protein. In the mouse MLKL full-length structure, the 4HB domain does not have any contacts points with the pseudokinase domain, and presumably this conformation may represent activated MLKL, after the 4HB domain has been unleashed (Murphy et al., 2013).

The human phosphomimetic mutant T357E-S358E pseudokinase structure has been solved by crystallography, however by our own group's assays, this mutant is inactive in human cells (Petrie et al., 2018), unlike the mouse counterpart. The mutated residues of the activation loop are not resolved, and the rest of the structure is highly similar to the wild-type human pseudokinase structure (Petrie et al., 2018). This could indicate that mutation or phosphorylation of the pseudokinase domain may not be sufficient to alter the pseudokinase conformation, and one suggestion is that the RIPK3 interaction itself is required to properly activate human MLKL and prepare it to kill cells (Petrie et al., 2018). It is also possible that the residues of the pseudokinase domain may interact with other regions of MLKL directly, so the effect of these mutations and RIPK3 phosphorylation might only be seen in conformation of the full-length protein.

To examine what the inactive human MLKL protein might look like, one paper utilised a mutation of the MLKL pseudokinase domain found in human cancer (Petrie et al., 2018). The mutation, E351K occurs in the highly conserved GFE motif of MLKL (corresponding to DFG in an active kinase; Figure 1.2). Recombinant E351K showed deficits in oligomerisation and liposome membrane permeabilisation, and in cells exhibited slowed kinetics in inducing cell death, making it a suitable representation for inactive MLKL. Using hydrogen-deuterium exchange data from this mutant, paired with distance restraints derived from zero-length crosslinking mass spectrometry data, a model of inactive human MLKL was developed (Petrie et al., 2018) (Figure 1.6), based upon an existing full-length human MLKL open conformation model (Arnež et al., 2016). In the inactive model, the 4HB domain is closely associated with the pseudokinase domain, and phosphorylation of the pseudokinase domain causing unleashing of the 4HB is supported by this model. Such studies have not been replicated for mouse MLKL, and whether the activation mechanism used by the two species is the same remains of outstanding interest.

#### *Other activation requirements post-RIPK3 phosphorylation?*

Phosphorylation of MLKL by RIPK3 was once thought to be sufficient to activate MLKL and induce cell death, however certain metabolites and proteins have been

found to be required for the execution of necroptosis, even after MLKL has been phosphorylated. Three papers independently identified HSP90 as an MLKL interacting partner (Jacobsen et al., 2016, Zhao et al., 2016, Bigenzahn et al., 2016). Zhao et al. (2016) reported that overexpression of HSP90 promoted oligomerisation and membrane translocation of overexpressed human MLKL in HEK239T cells. They also found that both the N-terminal region of MLKL (residues 1-178) and the pseudokinase domain of MLKL interacted with HSP90 using co-immunoprecipitation. Similarly, Jacobsen et al. (2016) showed that inhibition of HSP90 prevented MLKL oligomerisation and membrane translocation in a RIPK3 independent manner, and contributed to the execution of necroptosis in both human and mouse cells. These results implicate HSP90 in the conformational change events following MLKL phosphorylation.

Two recent papers have also implicated the metabolite, inositol phosphate, in human MLKL activation (McNamara et al., 2019, Dovey et al., 2018). Initially, inositol phosphates were investigated by this group as a surrogate for PIP headgroups (Quarato et al., 2016), the lipids that MLKL is thought to interact with at the plasma membrane (Dondelinger et al., 2014). These results are discussed in the membrane section (Section 1.8). Further research showed that inositol phosphates play a role in MLKL activation themselves (Figure 1.6). By knocking down expression of inositol phosphate pathway enzymes IPMK and IPTK1 in human cells, Dovey et al. (2018) found that inositol phosphates were required for necroptosis to occur. The group went on to show using NMR that inositol phosphate 3 (IP<sub>3</sub>), IP<sub>4</sub>, IP<sub>5</sub> and IP<sub>6</sub> can bind to the 4HB domain, and that high concentrations of IP<sub>6</sub> specifically can displace the first brace helix from the 4HB domain (Dovey et al., 2018, McNamara et al., 2019). Recombinant human MLKL constructs that had the first brace helix displaced from the 4HB domain by inositol phosphates had increased liposome permeabilising efficiency. Furthermore, mutants D137A, W133A and M122E that disrupt binding between the first brace helix and the 4HB domain (See Figure 1.6), made a 4HB plus brace human MLKL construct autoactive in cells (McNamara et al., 2019). Based on these results, McNamara et al. (2019) hypothesize that displacement of the first brace helix from the 4HB by IP<sub>6</sub> is required for MLKL activation post RIPK3 mediated

phosphorylation. These studies have so far only been performed in human MLKL systems.

There are other data in the literature which support the idea that interactions between the first brace helix and the 4HB domain are essential for regulating the killing ability of the 4HB domain. Firstly, a mouse MLKL mutation D139V, which is adjacent to the mouse equivalent of human residue D137, has recently been shown to cause auto-activity in full-length mouse MLKL (Hildebrand et al., 2019). Secondly, experiments using chimeras made by swapping different domains of mouse and human MLKL for each other, recently showed that the species identity of the first brace helix was essential for communicating normal MLKL activation signals (Davies et al., 2018) (Chapter 4). Therefore, IP<sub>6</sub> mediated displacement of the brace region from the 4HB may represent an extra step in MLKL's activation mechanism. A potential caveat to this work is that the inositol phosphates are only weak millimolar binders (McNamara et al., 2019, Quarato et al., 2016). Interactions with such affinity do occur in cells, however this weak binding makes recombinant validation of the hypothesis difficult. Indeed, none of the NMR studies could acquire nuclear Overhauser effect (NOE) distant restraints between IP<sub>6</sub> and MLKL to solve a bound structure, due to the weak affinity of interaction.

Another event required for MLKL activation post-RIPK3 phosphorylation has been proposed recently. Genetic knock out of TAM family kinases *Tyro3*, *Mer* and *Axl* made HT29 cells insensitive to TSZ-induced necroptosis (Najafov et al., 2019). Using *in vitro* and in cell kinase assays, the study found that human MLKL was phosphorylated by TAM kinases at the conserved Y376. Drawbacks to the study include use of an 'autoactive' human MLKL mutant Q356A to validate their findings in HEK293T cells, a cell line which does not usually undergo necroptosis due to lack of RIPK3 expression (He et al., 2009). The human Q356A mutant is based on mouse MLKL Q343A (Murphy et al., 2013)), however human MLKL Q356 does not participate in the salt-bridge interaction targeted by this mutation in mouse (Figure 1.4), and there is no evidence in the literature that this human MLKL mutant is indeed autoactive. However, despite these caveats, human MLKL mutant Y376F was unable to

reconstitute necroptosis in HT29s, which could be taken as compelling evidence that this residue, and its potential to be phosphorylated, is important to necroptosis execution. However, this tyrosine residue is not just strictly conserved in MLKL, but is also very highly conserved across most serine/threonine kinases, indicating it might play an important structural role (see alignment (Hanks et al., 1988), equivalent to residue 215). The paper notes that this residue is not solvent exposed in the human pseudokinase structure, so would only be available for phosphorylation with some conformational change, that they postulate would occur post phosphorylation by RIPK3.

The findings from all of these papers point to additional layers of regulation post RIPK3 phosphorylation, presumably to facilitate MLKL oligomerisation or membrane permeabilisation. The factors thought to contribute to MLKL activation are summarised in Figure 1.6. Following MLKL activation, MLKL can form oligomers, evidence for which is examined in the next section.





brace helix and linker region are still associated with the 4HB in this structure. **Inset:** **i** The 4HB+first brace helix human MLKL NMR structure from McNamara et al. (2019) (PDB:6D74). Residues which mediate hydrogen bonding between the brace helix and 4HB are labelled. **ii** A surface structure of the 4HB shows how first brace helix linker residues M122 and W133 insert into pockets on the 4HB and stabilise the linker region. **iii, iv** Mouse MLKL has conserved residues which can mediate the same interactions as human MLKL in **i** and **ii**. These are labelled on the 4HB+brace region taken from the full-length mouse crystal structure. **C** Three studies (McNamara et al., 2019, Dovey et al., 2018, Quarato et al., 2016) suggest that a second activating event post RIPK3 mediated phosphorylation occurs, involving IP<sub>6</sub> removing the inhibitory interaction between the brace and 4HB, by binding to the 4HB. The structure in **C** is for illustrative purposes only, there is no structure of IP<sub>6</sub> bound MLKL currently available.

### *1.7 MLKL oligomerisation*

Oligomerisation of MLKL post phosphorylation by RIPK3, is an attribute that has been observed in multiple studies and is widely agreed upon as an essential step in MLKL executed necroptosis. Artificially dimerised full-length MLKL, and MLKL N-terminal region constructs (4HB + brace) are capable of causing constitutive death cells, without the need for RIPK3 (Tanzer et al., 2016, Quarato et al., 2016). Mutations that disrupt oligomerisation are linked to deficits in, or complete abrogation of, necroptosis in cells (Davies et al., 2018, Cai et al., 2014, Dondelinger et al., 2014).

Interestingly, although MLKL oligomers are associated with activated, phosphorylated MLKL in cells, unphosphorylated purified recombinant MLKL constructs can spontaneously form oligomers *in vitro* (Petrie et al., 2018, Davies et al., 2018, Hildebrand et al., 2014, Wang et al., 2014a, Quarato et al., 2016). This finding suggests that oligomerisation is an intrinsic ability of MLKL and is not dependant on phosphorylation. Instead it is likely that there are regulation mechanisms in cells, that keep MLKL in a conformation which prevents spontaneous oligomerisation, and that the phosphorylation event (or another activating event post phosphorylation) may trigger removal of those mechanisms. One suggested mechanism for human MLKL is constitutive binding of MLKL to RIPK3, and the phosphorylation event being a trigger for MLKL release (Petrie et al., 2018). A further possibility is that the oligomers observed recombinantly actually represent MLKL in



the steady state, and that the oligomers observed in cells, by Blue Native-PAGE (BN-PAGE) for example, are actually much larger assemblies. Mutational analysis shows that MLKL oligomers can form in the cytoplasm (Hildebrand et al., 2014), indicating that oligomerisation is a step that likely precedes membrane association. The importance of understanding MLKL's oligomerisation has been recognised by the MLKL field, with numerous papers on the subject appearing since 2014. The stoichiometry of the oligomer remains of outstanding interest however, due to the many conflicting reports in the literature.

#### *Trimer, tetramer, hexamer or octamer?*

Various groups have reported that MLKL forms trimers (Cai et al., 2014, Davies et al., 2018, Hildebrand et al., 2014), tetramers (Petrie et al., 2018, Chen et al., 2014, Huang et al., 2017, Liu et al., 2017), hexamers (Wang et al., 2014a), octamers (Huang et al., 2017) and large amyloid MLKL polymers (Liu et al., 2017, Johnston and Wang, 2018, Reynoso et al., 2017). Much of the discrepancy in the findings in the field must be due to the varying techniques used to study oligomerisation, and use of mouse and human MLKL orthologues interchangeably. In Chapter 4, I aim to uncover the stoichiometry of the mouse MLKL oligomer, and potential reasons for the discrepancies between reported oligomer stoichiometry are discussed in the introduction to that chapter.

#### *Role of disulfide bonds*

Some studies have found that upon addition of reducing agent MLKL oligomers collapse on SDS-PAGE, which indicates that disulfide bonds may be important for MLKL oligomerisation (Wang et al., 2014a, Chen et al., 2014, Huang et al., 2017). However, these studies rely on cell extracts harvested under non-reducing conditions, so MLKL is being moved from a reducing environment inside the cell to an oxidative one, therefore whether the disulfide bonds are native, or spontaneously form upon cell lysis is an open question.

### *MLKL polymers*

Recently work from one group has suggested that human MLKL forms functional amyloid-like polymers, that are made up of tetramer subunits (Liu et al., 2017, Reynoso et al., 2017, Johnston and Wang, 2018). Liu et al. (2017) showed through semi-denaturing detergent agarose gel electrophoresis (SDD-AGE) that MLKL forms large polymers that cannot migrate through an agarose gel, in MLKL taken from necroptotically stimulated cell extracts. They went on to suggest that the 4HB+brace region of human MLKL is sufficient to form the polymers, however this was achieved by incubating recombinant human MLKL (residues 2-178) at 37°C degrees for 16 hours (Liu et al., 2017). Many proteins can form amyloid structures with perturbation of buffer conditions or high temperature (Stefani and Dobson, 2003), and therefore this does not constitute convincing evidence that MLKL forms functional amyloid in cells. This paper suggested that polymerisation was due to disulphide bonding mediated by C86, because mutation C86S inhibited polymer formation, however this cysteine is not conserved in mouse MLKL (Liu et al., 2017). Another paper from the same group proposed that MLKL is maintained in its disulfide bond reduced monomeric state by thioredoxin-1 (Trx1), a thiol oxidoreductase, and showed that Trx1 inhibition promoted MLKL polymer formation and necroptosis (Reynoso et al., 2017). These studies provide an interesting idea for the steps beyond MLKL oligomerisation in a defined stoichiometry, and indeed there is precedence in the literature for functional amyloid signalling in the necroptotic pathway in the form of RIPK1:RIPK3 functional amyloid signalling (Mompeán et al., 2018, Li et al., 2012). This work remains to be validated by further studies, however, could link back to very large oligomers observed by other groups. For instance, Dondelinger et al. (2014) reported 'high MW complexes' of certain human MLKL constructs expressed in HEK293Ts, that could not enter non-reducing SDS-PAGE gels, and were stuck in the loading well.

Once MLKL oligomerises, it translocates to the plasma membrane. Very little is known about translocation and now we move on to consider MLKL's membrane permeabilisation once it reaches the plasma membrane.

### *1.8 Plasma membrane disruption*

An early report suggested a role for mitochondrial membrane damage in necroptosis, mediated by proteins PGAM 5 and Drp1 (Wang et al., 2012), however, the importance of mitochondrial damage for necroptosis was disputed by many (Moujalled et al., 2014, Tait et al., 2013, Quarato et al., 2016, Tanzer et al., 2016, Murphy et al., 2013), with one study showing that mitochondria are dispensable for the execution of necroptosis (Tait et al., 2013). Although MLKL has shown a preference for membranes containing cardiolipin in some studies (Wang et al., 2014a, Su et al., 2014), this is likely attributable to MLKL's general preference for negatively charged lipids. Three studies have tested MLKL binding to lipid strips, and MLKL showed a strong preference for binding to PIPs, however, could also bind at least weakly with other negatively charged lipids PS, PG cardiolipin and PI (Wang et al., 2014a, Petrie et al., 2018), but not uncharged membrane components PE, PC, cholesterol or sphingomyelin (Wang et al., 2014a, Dondelinger et al., 2014, Petrie et al., 2018). In agreement with these findings the N-terminal region of MLKL was able to permeabilise liposomes with a plasma membrane like-lipid mix more efficiently than those with a mitochondria-like lipid mix (Tanzer et al., 2016). Another study found that MLKL constructs fused with a mitochondrial membrane localisation sequence could not induce necroptosis in cells with activated RIPK3 and caspase inhibition (Quarato et al., 2016). Most recent data suggest that it is MLKL's interaction with the plasma membrane that leads to cell death (Cai et al., 2014, Hildebrand et al., 2014, Chen et al., 2014, Quarato et al., 2016).

Whether MLKL has a preference for any specific domains of the plasma membrane, or requires specific curvature remains an open question. Two papers have reported that MLKL associates with lipid rafts (Fan et al., 2019, Chen et al., 2014), and Su et al. (2014) reported that MLKL cannot interact with nanodiscs, but information to explain these observations has not come to light. Another open question in the literature is the mode of MLKL plasma membrane disruption, with many suggestions having been put forward to date, but no clear model emerging. Multiple papers have focussed on the idea of MLKL as an ion channel, and a second group of papers have

focussed on the biophysical characteristics of the 4HB domain as it interacts with membranes, these themes are discussed below.

#### *Does MLKL form an ion channel?*

Different studies have shown a decrease in necroptosis levels in cells cultured in various ion-deficient media. Cai et al. (2014) showed a smaller percentage of HT29 cells underwent necroptosis in  $\text{Ca}^{2+}$  free media, than with  $\text{Ca}^{2+}$  present, and that there was a significant calcium ion influx into cells after 4 hours of TSZ stimulus, as measured by a  $\text{Ca}^{2+}$  sensitive fluorescent dye FLuo4. They performed a knockdown of the voltage insensitive ion channel TRPM7, based on its previous implication in cell death, and found that knockdown reduced necroptosis levels (but did not completely prevent it) and  $\text{Ca}^{2+}$  influx downstream of TSZ stimulus in HT29 and Jurkat cells (Cai et al., 2014). Chen et al. (2014) similarly showed using a fluorescent  $\text{Na}^+$  probe, that  $\text{Na}^+$  signals increased in L929 cells post TSZ stimulus, and then only decreased once the cell became propidium iodide (PI: a DNA binding dye used to measure cell death) permeable. They used the same  $\text{Ca}^{2+}$  indicator as Cai et al. (2014) in L929 cells and found that there was no significant correlation between  $\text{Ca}^{2+}$  influx and necroptosis. They also found  $\text{K}^+$  levels did not change during necroptosis. They went on to test the effect of  $\text{Na}^+$ ,  $\text{Ca}^{2+}$  and  $\text{K}^+$  deficient media on necroptosis in L929 cells, and found, in contrast to Cai et al. (2014), that  $\text{Na}^+$  deficient media decreased necroptosis, whereas  $\text{Ca}^{2+}$  deficient media had no effect, and  $\text{K}^+$  deficient media accelerated necroptosis (Chen et al., 2014).

A third study also implicated ion influx in the execution of necroptosis, and reported that human MLKL itself acts as a cation channel in the plasma membrane, this time with a preference for  $\text{Mg}^{2+}$  ions (Xia et al., 2016). This paper showed that human MLKL phosphomimetic mutant T357D-S358E behaved like an ion channel using electrophysiology measurements, although to get a channel-like signal they had to titrate MLKL down to very low concentrations, and the 4HB alone (1-125) induced constitutive conductance, consistent with breaking the membrane rather than acting like a channel. The paper suggested that the 4HB domain forms a membrane spanning cation channel, using substituted cysteine accessibility mutagenesis in

combination with MTSET, a bulky positively charged reagent that reacts with exposed cysteines and disrupts channel conductance. They showed that addition of MTSET from either the *cis* or *trans* side of the membrane resulted in differential inhibition of the 'channel'. Considering the amount of solvent facing charged residues on the 4HB domain, it seems unlikely that the 4HB could stably insert into the hydrophobic environment of the bilayer without considerable refolding. The paper did not address this point. The authors also noted that the MLKL 4HB domain has no homology with any known ion channels and would be unique among ion channels in that it has permeability to  $Mg^{2+}$ ,  $Na^+$  and  $K^+$ . Addition of a bulky covalent addition such as MTSET to the 4HB domain could inhibit permeabilisation in many ways other than disrupting conductance through a channel, for instance addition of the eight residue FLAG tag to the N-terminus of mouse MLKL is enough to abrogate necroptosis in cells (Hildebrand et al., 2014). Therefore, the findings of this paper are not entirely convincing.

One other study has dealt with ion influx. Ros et al. (2017) found using Fluo4 that there was calcium influx in all cell lines tested post TSZ stimulus (L292, Nh3, MEF and HT29), however, the timing of the influx varied between cell lines. There was early  $Ca^{2+}$  influx in L929 cells treated with TSZ, pre-PI staining, that was not inhibited by RIPK1 inhibitor Nec1, and was found to be dependent on Smac-mimetic stimulation (Ros et al., 2017). This  $Ca^{2+}$  influx was not important for the execution of necroptosis, and was likely mediated by a specific ion channel, based on its persistence in cells undergoing necroptosis that were incubated with polyethylene glycols (PEGs), which prevent changes in osmotic imbalance mediated by pores but not ion channels (Ros et al., 2017, Tejuca et al., 2001, Sukhorukov et al., 2009). There was a much later  $Ca^{2+}$  influx in HT29 cells, although in only a subset of the cell population, that occurred just before PI staining, that was blocked by inhibitors of MLKL (NSA) and RIPK1 (Nec1), in agreement with the report of Cai et al. (2014). None of the cell lines exhibited reduced necroptosis when cultured in  $Ca^{2+}$  free media, although L929 showed increased cell death. The paper concluded that calcium signalling during necroptosis was cell type specific and did not play a role in execution of cell death, and suggested that the conflicting results in previously published material were due to the different

cell types used. The paper also showed that culturing cells in PEGs, which helps to ameliorate the effects of osmotic imbalance generated by pores (Tejuca et al., 2001, Sukhorukov et al., 2009), delayed PI uptake and cell lysis. This suggests that cell death is mediated by the formation of pores in the plasma membrane. In the presence of PEGs, PI could move through the pores but Dextran 10 could not, indicating that the pores are small in size, likely smaller than roughly 4nm in diameter.

Recently MLKL has also been implicated in the activation of another plasma membrane channel, Pannexin 1, although knockout of Pannexin 1 did not prevent necroptosis from occurring but instead changed the dying cells' permeability to another nuclear dye To-pro-3 (Douanne et al., 2019). The relevance of this finding is still unclear.

The study by Ros et al. (2017) suggested that rather than forming an ion channel, or activating one, MLKL was leading to a change in ion balance in the cell by introducing small holes in the plasma membrane. This is the mode of action presumed in other papers, which take a more biophysical approach to better understand the physical characteristics of the MLKL membrane interaction. NMR has been a key technique used to study this, and features heavily in the papers described below.

#### *Plug-release mechanism*

The first paper to suggest a 'plug-release' mechanism for MLKL membrane permeabilisation was published in 2014 (Su et al., 2014). Su et al. (2014) solved the NMR structure of the human 4HB domain plus brace construct (2-154) in solution, and used NMR to study its interactions with liposomes. They observed peak broadening for human MLKL in the presence of liposomes that indicated binding. They proposed that specific residues became exposed to hydrophobic environments when MLKL was incubated with liposomes based on covalent tagging of the protein with a hydrophobic sensitive dye. The residues that became exposed to the hydrophobic environment were present on all faces of the 4HB domain however, and these results did not suggest a particular interacting face, or direction of insertion into the membrane.

The only residue which did not become exposed to a hydrophobic environment was from the first brace helix, leading the authors to investigate if the brace helix becomes dissociated from the 4HB domain during membrane permeabilisation. A 4HB domain only construct (2-123) was reported to have more potent liposome permeabilising ability than the 4HB+first brace helix construct. Furthermore, two residues of the 4HB domain, K26 and R30 (Figure 1.6) which mediate salt bridges with the first brace helix were mutated to glutamate to disrupt the interaction between the 4HB domain and brace, and this construct also had improved liposome permeabilising ability (Su et al., 2014). They also observed poor packing between two helices ( $\alpha 2$  and  $\alpha 5$ ) of the 4HB, which sit under the brace helix in its interaction with the 4HB (Figure 1.7 A).

Therefore, Su et al. (2014) proposed a 'plug-release' mechanism for MLKL membrane permeabilisation, whereby the first brace helix dissociates from the 4HB removing a stabilising force that helps the  $\alpha 2$  and  $\alpha 5$  helix packing together. They suggested the  $\alpha 2$  and  $\alpha 5$  helices may open up to reveal a hydrophobic membrane binding interface, that would form the outside of an MLKL oligomeric pore (graphically summarised in Figure 1.7). Whilst the opening of the helices in the 4HB domain has not been validated by others to date, other groups have also found the removal of the brace from the 4HB to be an important step for MLKL activity on membranes (McNamara et al., 2019, Quarato et al., 2016).

#### *Mapping PIP binding sites, leads to inositol phosphate discovery*

Quarato et al. (2016) performed another NMR study, that expanded upon the plug release mechanism. Initially the study used inositol phosphates as a surrogate for headgroups to test possible binding sites of human MLKL to PIP lipids (Quarato et al., 2016). These results must now be viewed in a new context, as this study has been expanded into two further studies that examine the role of inositol phosphates in MLKL activation in their own right (Dovey et al., 2018, McNamara et al., 2019) (see Section 1.6, Figure 1.6). However, the information gained by studying inositol phosphate binding is still likely to have relevance for PIP binding and the results are discussed in that light below.

Quarato et al. (2016) first tested binding affinity of IP, IP<sub>2</sub>, IP<sub>3</sub> and IP<sub>4</sub> (adding phosphate groups to inositol phosphate), and found that IP<sub>3</sub> (which would correspond to lipid PI(4,5)P<sub>2</sub>) had the highest binding affinity based on chemical shift perturbations in NMR spectra for the 4HB+first brace helix (hMLKL 2-156). The authors mapped the most substantial chemical shift perturbations to helices 1 and 2 of human MLKL and, through titration, identified residues with the highest affinity for IP<sub>3</sub> including E2, N3 K5 and R30. Many of these residues reside at the very N-terminus of MLKL and the high affinity of these residues for inositol phosphates, and, and by extension to PIP lipids, aligns with other findings in the literature. For instance, in the mouse system, addition of a FLAG tag to the N-terminus of mouse MLKL disrupts necroptosis, potentially by shielding these residues from interaction with PIPs (Hildebrand et al., 2014). Furthermore, the deletion of the first ten residues from the mouse 4HB domain results in a construct that can translocate to the plasma membrane, but cannot mediate death (Chen et al., 2014), potentially because it has lost key PIP binding residues.

After establishing binding affinity for inositol phosphates, this study examined the interaction of human MLKL 1-156 with PIP containing detergent micelles (Quarato et al., 2016). The authors found that high concentrations of PI(4,5)P<sub>2</sub> could induce a disordering of the first brace helix. This was observed through line broadening for most of the peaks within this region, which could be a result of binding to the micelles, and formation of poorly dispersed new peaks between 7.7 and 8.3 ppm (on H axis). This result agrees with the Su et al. (2014) finding that the first brace helix does not become embedded in the hydrophobic environment, and disruption of its interaction with the 4HB domain may precede MLKL embedment in the membrane. Interestingly, Su et al. (2014) reported circular dichroism spectroscopy data showing that the very similar construct human MLKL 2-154 remained mainly  $\alpha$  helical in the presence of liposomes, which does not correlate with the first brace helix becoming disordered. Su et al. (2014) only used phosphatidyl inositol and cardiolipin in their liposomes however, so it is possible that MLKL requires its highest affinity ligand PI(4,5)P<sub>2</sub> to undergo this unfolding event. Indeed, Quarato et al. (2016) found that phosphatidyl inositol did not cause such a large change in the NMR spectra, and only saw new



peaks indicating disordering of the first brace helix at the highest concentration of PI(4,5)P<sub>2</sub> in the micelles. Both papers highlighted the salt bridge interaction between the first brace helix and  $\alpha$ 2 helix (R30, D139), and found that mutation of either residue activated MLKL constructs, inducing death in cells and enhanced activity in liposome permeabilization assays *in vitro*, in agreement with more recent studies (McNamara et al., 2019).

Taken together, the results of Su et al. (2014) and Quarato et al. (2016) both suggest that interactions between the first brace helix and the 4HB domain must be disrupted before or during membrane embedment. The most recent paper on the topic, by McNamara et al. (2019) may suggest, however, that this event happens earlier on in MLKL activation, mediated by cytoplasmic inositol phosphates. Furthermore, it is not clear if the binding sites described by Quarato et al. (2016) are used for both inositol phosphate and PIP binding, before and during membrane permeabilisation.

#### *Mutagenesis to the 4HB domain reveals a complicated picture*

In the previous studies described, and others, various mutations have been made to the MLKL 4HB domain that affect the execution of necroptosis. Assigning a clear role to specific residues of the 4HB domain, and defining a 'membrane interacting face' if there is just one, is complicated by the fact that the mutations appear to affect MLKL in a multitude of ways, with some mutations affecting oligomerisation and or membrane translocation, and others affecting PIP binding etc. Mutations have also been made in either mouse or human MLKL, and as discussed in previous pages, the two orthologues differ in their activity. Therefore, understanding which residues of the 4HB domain are essential for a particular function is complex; a summary of studies in which mutagenesis was performed on the 4HB domain is presented below.

In human MLKL, Dondelinger et al. (2014) first showed that collective mutation of a panel of 9 positively charged residues between residue 15 and 35 on human MLKL's 4HB domain to alanine, prevented MLKL mediated death when MLKL and mutants were expressed in HEK293T cells. The 9A mutations also prevented recombinant construct human MLKL 1-210 from permeabilising liposomes (Dondelinger et al.,

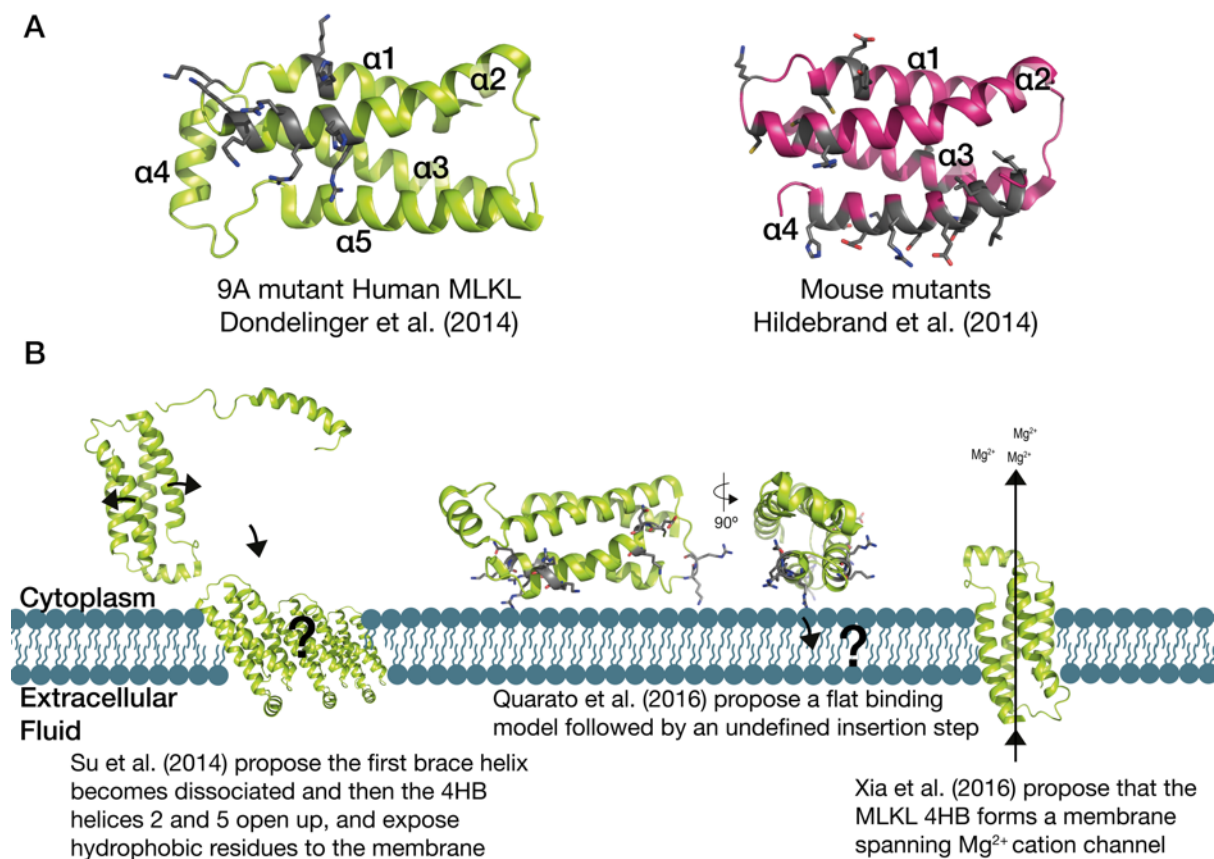
2014). This finding was later replicated by another group (Quarato et al., 2016), this time in mouse embryonic fibroblast cells (MEFs). The conclusion drawn was that these positively charged residues are required for PIP binding. Importantly however, the 9A mutant inhibited oligomerisation and membrane localisation in cells (Dondelinger et al., 2014), therefore the inability to induce death in cells is not necessarily due to a loss of lipid binding, but could be due to poor translocation or oligomerisation. A human MLKL variant in which these 9 residues were mutated to glutamine (9E), showed reduced PIP binding recombinantly (Dondelinger et al., 2014). However, the 9A mutant was not tested so whether the loss of the positively charged residues resulted in a lack of binding, or the addition of 9 negatively charged residues induced repulsion from the negatively charged PIP lipids, is unclear. Only 4 of these positively charged residues are conserved in mouse MLKL, and some of the 9 have charge swaps in the mouse orthologue. Neither paper made finer grain mutations. Therefore, despite the obvious importance of at least some of these residues, it is not clear which in particular are essential for MLKL oligomerisation, membrane translocation or PIP binding.

Using auto-active construct mouse MLKL 1-180 as a base, Hildebrand et al. (2014) identified residues in mouse MLKL 4HB that were essential for cell death and oligomer formation at the membrane, using cell death assays and BN-PAGE. Many of the mutations in this study showed deficits in oligomerisation and membrane translocation, as per the mutants identified in Dondelinger et al. (2014). This suggests that a) the 4HB domain plays a role in stabilising MLKL oligomers and promotes membrane translocation, and b) that these two processes (oligomerisation and membrane translocation) are closely linked. However, some mutants (D106A/R105A and E109A/E110A) from this paper could translocate to the membrane as monomers, showing that oligomerisation is not essential for membrane translocation (Hildebrand et al., 2014).

From these studies and others, many residues of the 4HB domain have been shown to be important for mouse and human MLKL cell death execution. Whether they inhibit death via deficits in oligomerisation, membrane translocation or lipid binding

are not clear. Interestingly, the important residues identified are present over the surface of the 4HB domain, and do not occupy the same face of the 4HB domain in mouse and human MLKL. It remains of outstanding interest as to whether the differences between important residues between mouse and human MLKL reflect differences in mechanism of action or simply that comparable mutations have not been made in each species' MLKL.

A graphical summary of our knowledge of the MLKL membrane binding mode is presented in Figure 1.7.



### Figure 1.7 Summary of potential membrane permeabilisation mechanisms

**A** The mutations made to residues in human and mouse MLKL 4HB that lead to deficits in killing, either via loss of lipid binding, membrane association or oligomerisation (Dondelinger et al., 2014, Hildebrand et al., 2014). Mouse and human MLKL have been mutated independently, and the residues tested between species are not consistent, however some there is overlap of some important residues on the  $\alpha 1$  and  $\alpha 2$  helices. **B** Three of the models for membrane permeabilisation proposed by different groups.

Recently another family of proteins, the ESCRTs, has been implicated as negative regulators of MLKL's execution of necroptosis at the plasma membrane. Three papers found that cells undergoing necroptosis were able to release small 'bubbles' of membrane or 'necroptotic bodies' containing phospho-MLKL into the extracellular milieu (Yoon et al., 2017, Gong et al., 2017, Zargarian et al., 2017). Gong et al. (2017) showed through genetic depletion that it was members of the ESCRT-III family of proteins, that facilitated membrane blebbing, and in fact actively worked to repair membrane damage induced by MLKL to prolong the life of cells undergoing necroptosis. Yoon et al. (2017) found that MLKL's association with ESCRT proteins was not confined to cells undergoing necroptosis. They reported a role for MLKL in endosomal trafficking in the steady state, that was not dependent on, but could be enhanced by RIPK3 mediated phosphorylation of MLKL. Their study found that knockout of MLKL from HT29 cells and HepG2 cells using siRNA, decreased the number of exosomes released by the both cell types (Yoon et al., 2017). This could be restored by wild-type MLKL expression, and the human phosphomimetic T357E-S358D mutant MLKL, but not MLKL 1-180 or MLKL with mutations to key positively charged residues in the 4HB. The exosomes released by cells undergoing necroptosis were found to contain phospho-MLKL and ESCRT-III family members, in agreement with the results of Gong et al. (2017), as well as flotillin proteins.

In 2019, another study found that ESCRT proteins, as well as flotillins can facilitate removal of phospho-MLKL from cells treated with necroptotic stimuli (Fan et al., 2019). Through genetic knockout experiments, they showed that flotillin 1 and 2 are responsible for targeting MLKL for lysosomal degradation, whilst ESCRT protein ALIX contributed to phospho-MLKL release in exosomes (Fan et al., 2019), in agreement with the previous studies. Collectively, these studies suggest that even once phospho-MLKL reaches the plasma membrane, the cell still has mechanisms to stave off necroptotic death, if membrane repair by ESCRTs or phospho-MLKL depletion prevent plasma membrane accumulation of a threshold level of phospho-MLKL.

### *1.9 MLKL function independent of its necroptotic role*

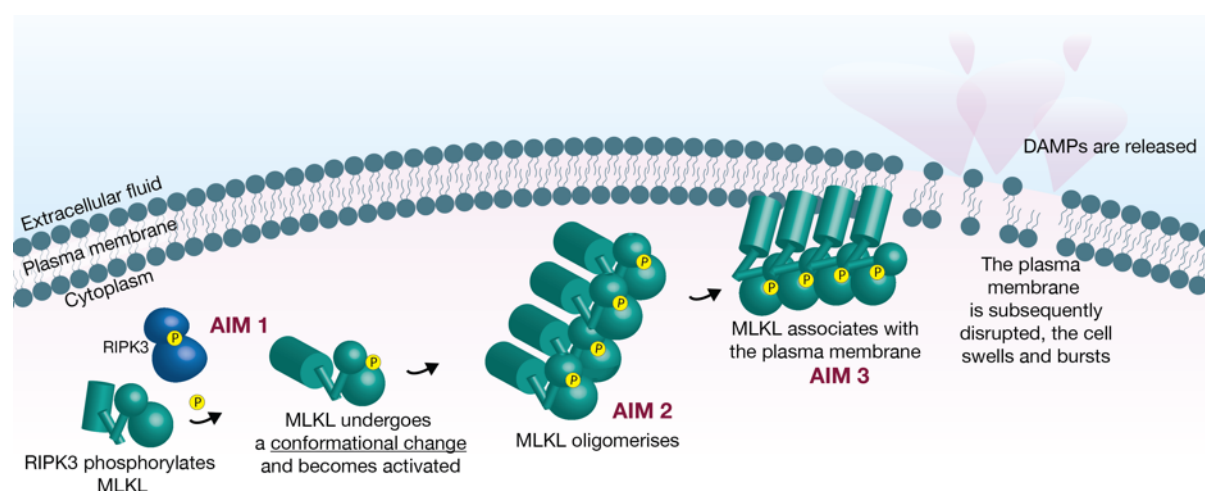
MLKL's proposed function in steady state exosome production (Yoon et al., 2017), is not the only necroptosis independent role reported in the MLKL literature (Ying et al., 2018). Ying et al. (2018) suggest MLKL promotes neuronal regeneration in mouse models of nerve damage. Using western blot and microscopy techniques they found that *Mkl<sup>-/-</sup>* mice showed decreased rates of myelin sheath breakdown post nerve injury, which ultimately lead to a delay in nerve regeneration. Part of their proposed mechanism involves MLKL binding to sulfatide, a neuronal lipid, but only post incubation with damaged nerve extracts. This is not in agreement with other results, where MLKL did not bind strongly to sulfatide on lipid strips in two papers (Dondelinger et al., 2014, Wang et al., 2014a). Mass spectrometry analysis showed that MLKL became phosphorylated on serine 441 after incubation with damage nerve extracts, providing a possible explanation for this discrepancy in results. However, a phosphosite at S441 has never previously been reported, and the kinase that mediates this remains unknown.

Whilst these roles for MLKL outside of necroptosis are poorly understood, and require further validation, it is important to keep in mind that MLKL may have other functions. Keeping abreast of MLKL's other roles may aid development of therapeutics that do not have unexpected side effects.

## 1.10 Summary and aims

This thesis aims to better understand MLKL's molecular mechanism at each step in its role as executioner of necroptosis. The basic hypothesis presented by the literature, that is the foundation for my studies, involves MLKL becoming activated by a molecular switch mechanism, prior to its oligomerisation and translocation to the plasma membrane, where it perturbs the bilayer to cause cell death.

This mechanism of action, and where my own results fit into this picture, is summarised in Figure 1.8.



**Figure 1.8 Schematic overview of MLKL's mechanism of action with aims**

Cytoplasmic monomeric MLKL is activated by RIPK3 phosphorylation. Phosphorylation induces a conformation change event that allows MLKL to oligomerise and translocate to the plasma membrane. MLKL then disrupts the plasma membrane leading to cell death. Aim 1 pertains to RIPK3 compatibility and MLKL activation. Aim 2 examines MLKL oligomerisation, and Aim 3 considers MLKL's action at the membrane.

*AIM 1: To improve our understanding of MLKL activation by RIPK3, and explore issues of species compatibility*

This aim is addressed in Chapter 3, which provides the most comprehensive study of MLKL orthologues to date. This work contributes to better understanding the evolution of MLKL, its RIPK3 compatibility determinants and its activation mechanisms.

*AIM 2: To define the stoichiometry of the mouse MLKL oligomer, examine the oligomer interface and the role of the brace helices*

This aim is addressed in Chapter 4, which provides evidence that mouse MLKL forms a trimer and shows that the brace helices are not only important for oligomerisation, but also communication of activation signals between domains.

*AIM 3: To better understand MLKL's mechanism of action at the plasma membrane*

This broad aim is addressed in Chapter 5. Novel techniques were used in observational studies of MLKL interacting with different membrane systems, to provide new, unbiased insights into its action at the membrane.

## **2.0 Materials and Methods**

Parts of this chapter use text from the Materials and Methods section in Davies et al. (2018), for which I wrote the manuscript. For the full paper see Appendix II.

### *2.1 Cloning*

Whilst I performed some cloning for this thesis, much of the cloning work and construct generation was performed partially or fully by Dr James Murphy and Sam Young. Rui Qin generated the brace mutant chimeric constructs used in Chapter 4. Dr James Murphy and Sam Young also cloned, purified, digested and phosphatase treated all the plasmid vectors used in this work. For a complete list of the vectors, primers, constructs and accession numbers for MLKL sequences used in this thesis see Appendix I.

#### *General cloning protocol*

Insert DNA was amplified from template DNA (see details of templates in Appendix I) by PCR using manually-designed oligonucleotides (synthesized by Integrated DNA Technologies). Specific mutations were generated by overlap PCR. DNA inserts were ligated into a pre-cut and phosphatase treated plasmid vector using *Bam*HI and *Eco*RI digestion (ThermoFisher Scientific, 37°C >1.5 hours) using T4 ligase (ThermoFisher Scientific; room temperature, ~2 hours). 2 µl of the ligation mix was transformed into 50 µl of electro-competent T1-resistant DH10B (Invitrogen) cells via electroporation, and cells plated onto antibiotic (kanamycin (GoldBio) – 50 µg/ml or ampicillin (GoldBio) – 100 µg/ml) containing LG-agar plates (see Table 2.1 for recipe), that were incubated overnight at 37°C. Individual colonies were picked and used to inoculate 3 ml of Super Broth (Table 2.1) with antibiotic, which was grown overnight at 37°C with shaking. Plasmid DNA was isolated using Qiagen Miniprep kits (as per manufacturer's instructions), and eluted in EB buffer (Qiagen; Table 2.1). Presence of insert was verified by a diagnostic digest using *Bam*HI and *Eco*RI upon resolution on TAE-agarose gel. Inserts were Sanger sequenced (AGRF, Parkville, VIC; Micromon, Clayton, VIC). Plasmids were stored at -20 °C.



The resulting purified plasmids were ready to be used for *E. coli* protein expression, bacmid production (for insect cell protein expression), and inoculation into maxi-prep cultures for mammalian cell work (see following sections).

#### *Bacmid production and purification*

Briefly, pFastBac derived vectors (See Appendix I) with insert were transfected into chemically-competent DH10MultiBac *E. coli* cells (ATG Biosynthetics) via heat shock at 42°C for 2 minutes followed by recovery in an ice water bath. Transfected cells were plated onto LG-agar plates supplemented with tetracycline (10 µg/ml; Sigma), gentamycin (7 µg/ml; Sigma), kanamycin (50 µg/ml), IPTG (40 µg/ml; GoldBio) and BluoGal (0.1 mg/ml; Invitrogen) (for antibiotic, and blue and white selection), and incubated at 37°C for 48 hours, to allow colonies sufficient time to metabolise BluoGal and enable blue-white screening. Positive white colonies were streaked onto fresh plates, and after 48 hours single white colonies were used to inoculate 3 ml L-broth (Table 2.1) cultures, grown overnight at 37°C, shaking, in the presence tetracycline (10 µg/ml), gentamycin (7 µg/ml) and kanamycin (50 µg/ml). Bacmid DNA was isolated as previously described (Babon and Murphy, 2013), and resuspended in TE buffer (Invitrogen; Table 2.1). Bacmids were stored at 4°C, freezing bacmids may result in shearing of DNA, due to their large size.

#### *Maxipreps for mammalian cell DNA*

Plasmid DNA containing the desired insert was transformed into electro-competent DH10B cells via electroporation, which were used to inoculate 200 ml of Super broth, and the culture was grown overnight at 37°C with shaking. Maxipreps were performed using Qiagen Maxiprep kits as per the manufacturer's instructions to generate high purity and high yield plasmid preps. DNA concentration was measured by absorbance at 260 nm, and DNA was diluted to ~1 µg/µl in TE buffer (Invitrogen; Table 2.1) if necessary. Plasmids were stored at -20°C.

**Table 2.1 Buffers and media used for cloning and bacterial work**

| Buffer/media                     | Composition                                                                                                                                                                                      |
|----------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| LG-agar (WEHI media kitchen)     | 1 % (w/v) Tryptone,<br>0.5% (w/v) Yeast Extract,<br>0.5% (w/v) NaCl,<br>0.2% (w/v) D-Glucose,<br>10 mM Tris Buffer pH 7.4,<br>1 mM MgCl <sub>2</sub> .6H <sub>2</sub> O,<br>1.5% (w/v) Agar Agar |
| L-Broth (WEHI media kitchen)     | 1% (w/v) Tryptone,<br>0.5% (w/v) Yeast Extract,<br>1% (w/v) NaCl,<br>2.5 mM NaOH pH 7.4                                                                                                          |
| Super Broth (WEHI media kitchen) | 3.5% (w/v) Tryptone,<br>2% (w/v) Yeast Extract,<br>0.5% (w/v) NaCl,<br>5 mM NaOH pH 7.1-7.3                                                                                                      |
| EB buffer (Qiagen)               | 10 mM Tris-HCl pH 8.5                                                                                                                                                                            |
| TE buffer (Invitrogen)           | 10 mM Tris-HCl pH 8.0,<br>0.1 mM EDTA                                                                                                                                                            |

## 2.2 Recombinant protein expression and purification

### *E. coli* expression

Mouse MLKL 1-169 constructs were expressed in electrocompetent BL2-CodonPlus (Agilent) cells as NusA-His<sub>6</sub> fusions, based upon an established expression protocol (Hercus et al., 2013). Fresh transformations were prepared prior to each expression. Multiple colonies were used to inoculate 25 ml of Super Broth containing 50 µg/ml kanamycin, that was incubated at 37°C with shaking. After approximately 3 hours or once the cultures were turbid, 2-10 ml of the starter culture was used to inoculate 1 L Super Broth cultures, containing 50 µg/ml kanamycin, which were incubated at 37°C until optical density at 600 nm reached 0.6-0.8. Cultures were transferred to an 18°C incubator for 15-20 minutes before addition of 500 mM IPTG to induce protein expression overnight (18°C, shaking). Cells were harvested by centrifugation (10,000 *g*, 10 minutes). Pellets were snap frozen in liquid nitrogen and stored at -80°C, or used immediately.

### *E. coli His-tag purification*

Proteins were expressed with a Tobacco Etch Virus (TEV) cleavable His<sub>6</sub>-tagged NusA tag fused to their N-terminus. Cell pellets were resuspended in 1.5 x their weight lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM imidazole, pH 8.0, 2 mM  $\beta$ ME) with 3 mM MnCl<sub>2</sub> and DNase (10 U; Roche). Cells were lysed with an EmulsiflexC5 homogenizer (Avestin). Immediately post-lysis, 1 mM of protease inhibitor phenylmethanesulfonyl fluoride (PMSF; Sigma) was added. The whole lysate was centrifuged (45,000 g, 20 minutes, 4 °C), and the supernatant 0.2  $\mu$ m filtered (Sartorius). The supernatant was passed over a pre-equilibrated (with lysis buffer) Ni<sup>2+</sup> charged 1 ml HiTrap Chelating HP cartridge column (GE Healthcare) twice using a peristaltic pump at room temperature. The column was washed with lysis buffer supplemented with 350 mM NaCl (>10 column volumes; CV), to elute non-specific binding proteins, followed by lysis buffer (>10 CV) to reduce salt concentration. Protein was eluted in lysis buffer supplemented with 100 mM imidazole in 5 ml (5 CV). The eluate was buffer exchanged with TBS (20 mM Tris-HCl pH 8.0, 150 mM NaCl), and concentrated by centrifugation in an Amicon Ultra-15 concentrator (Millipore) to a volume of <2 ml then cleaved overnight (4°C) with 0.5 mg of His<sub>6</sub> tagged TEV protease (produced in-house by Phil Morgan and Dr Cheree Fitzgibbon). Samples of the supernatant, flowthrough from the Ni<sup>2+</sup> column, washes, elution and elution plus TEV were analysed by SDS-PAGE to confirm presence of protein. The TEV protease-cleaved eluate was passed over a 1 ml Ni<sup>2+</sup> cartridge column connected to a 1 ml MonoQ column (Hi Trap Q FF, GE Healthcare), to remove the His-tagged TEV protease, any uncleaved material and cleaved NusA. The MonoQ column binds NusA at pH 8.0 (as in TBS), and is important for removing excess NusA; the main contaminant of this purification. Unbound protein was eluted with up to 3 ml of TBS and collected in 0.5 ml fractions. At this stage, before further purification by size exclusion chromatography, the protein could not be concentrated as this leads to protein precipitation. Therefore, the protein concentration of the 0.5 ml fractions was measured by absorbance at 280 nm, and the fractions with the most protein pooled, up to a maximum 5 ml volume. The pooled flowthrough was loaded onto a HiLoad 16/600 Superdex75 PG size exclusion column (GE Healthcare) that had been pre-equilibrated with TBS at 4°C, and 1.5 ml fractions collected across one CV

(120 ml). Protein purity was assessed by SDS-PAGE with Coomassie Brilliant Blue staining. Pure fractions were pooled, concentrated, and Tris(2-Carboxyethyl) Phosphine (TCEP; Thermofisher) added to 1 mM. Protein concentration was measured using absorbance at 280 nm, and protein snap frozen in liquid nitrogen and stored at -80°C if not to be used immediately.

#### *Baculovirus production and protein expression in Sf21 cells*

All MLKL constructs that incorporate the pseudokinase domain were expressed in insect cells. Sf21 insect cells were cultured in Insect-XPRESS media (Lonza), usually in 1 L schott bottles in a 100-200 ml volume, and incubated at 27°C (120 rpm). To maintain viability, cells were split to  $0.7 \times 10^6$  cells/ml to last 3 days (e.g. Monday to Wednesday) and split to  $0.3 \times 10^6$  cells/ml to last 4 days (e.g. Friday to Monday). Under this splitting regime cells did not exceed density of  $4 \times 10^6$  cells/ml. Cells were maintained in culture for up to 3 months before a new batch was thawed (frozen stocks stored in liquid nitrogen).

#### *Transfection via Cellfectin system*

Transfections were performed as previously described (Babon and Murphy, 2013). Briefly 1 µg of bacmid DNA was diluted into 200 µl of insect cell media and 6 µl of Cellfectin II reagent (Thermofisher), mixed gently, then incubated at room temperature for 30 minutes.  $9 \times 10^5$  Sf21 cells were plated into a 6 well plate in 2 ml of media, and incubated at 27°C for 30 minutes to settle into a monolayer. The DNA:lipid mix was made up to 1 ml with insect cell media. Media was removed from Sf21 cells monolayer and DNA:lipid mix added dropwise. After 3-5 hours, media was removed and replaced with 2 ml of fresh media. After 4 days, the media containing the virus (P1) was harvested from the cells, and stored in the dark at 4°C for up to 1 month if not immediately required

#### *Viral amplification*

To expand virus, P1 was diluted 1/100 into 100-200 ml of Sf21 cells at  $1.5 \times 10^6$  cells/ml, and incubated in a 1 L Schott bottle for 4 days (27°C, 120 rpm). After 4 days, cells were removed via centrifugation (500 g, 5 min) and virus containing

media harvested (P2). P2 virus was used immediately or stored in the dark at 4°C for up to 2 weeks.

#### *MLKL expression*

For expression, 50 ml P2 virus was used to infect 500 ml of  $3-4.0 \times 10^6$  cells/ml in 2.8 L Fernbach flasks. After a 48 hour incubation (27°C, 90 rpm), Sf21 cells containing cytosolically expressed MLKL protein were pelleted by centrifugation (500 g, 5 minutes). Cell pellets were snap frozen in liquid nitrogen, then stored at -80°C, or thawed and used immediately. The snap freezing step is always performed to help break the insect cell membrane, even when pellets are to be used immediately.

#### *His-tagged protein purification (Sf21 cells)*

The human MLKL pseudokinase domain, the rat MLKL constructs, the horse MLKL constructs and all mouse MLKL constructs incorporating the pseudokinase domain, were expressed with a TEV cleavable His<sub>6</sub>-tag, or a Streptavidin Binding Protein (SBP) fused TEV cleavable His<sub>6</sub>-tag fused to their N-terminus (vectors used for each expression construct listed in Appendix I). Cell pellets were resuspended in 50 ml of Lysis Buffer (for buffers see Table 2.2) per 1 L of insect cell culture. Cells were lysed by sonication (Misonix Sonicator 3000) in an ice-water bath, 10 seconds on and 10 seconds off, for a total of 4 minutes at 40% amplitude. The whole lysate was centrifuged (45,000 g, 60 minutes, 4°C) to remove debris; the lysate cannot be filtered due to high glycerol concentration. The supernatant was added to Ni<sup>2+</sup>-NTA agarose resin (cOmplete His-Tag Purification Resin; Roche) (1ml of beads per 1L of insect cell culture), that had been pre-washed with MilliQ (10 CV) then lysis buffer (10 CV), and left to bind, rolling at 4°C for 1-2 hours. The beads were pelleted by centrifugation (300 g, 5 minutes, 4°C), and the unbound fraction decanted. Beads were washed in Lysis Buffer (2 x 20 ml), and 35 mM Imidazole Wash Buffer (2 x 10 ml). Protein was eluted by mixing 5 ml of Elution Buffer with the pooled beads, followed by centrifugation (300 g, 5 minutes, 4°C). The eluted protein was decanted into a fresh tube and the process of elution repeated a further two times to a total of 15 ml. The eluate was mixed with 0.5 mg of His<sub>6</sub>-TEV protease and 1 mM TCEP and incubated (stationary) for 1 hour at room temperature. The eluate and TEV mixture was dialysed

using cellulose dialysis tubing (6,000-8,000 Da molecular weight cut-off, CelluSep, cat. 8030-50) in 1 L of Dialysis Buffer, for 1-3 hours at 4°C. Dialysis buffer was then replaced with fresh buffer (1 L) and left overnight at 4°C, on a magnetic stirrer. The dialysed sample was added to 0.5 ml of Ni<sup>2+</sup> beads and incubated at 4°C for 30-60 minutes to remove the His<sub>6</sub>-TEV, and the His<sub>6</sub> or His<sub>6</sub>-SBP tag and other impurities which bound non-specifically to the Ni<sup>2+</sup> beads in the first purification step. The beads were washed with 3 x 5 ml of Size Exclusion Chromatography Buffer (SEC Buffer), and the washes, which contain the cleaved protein, were collected. For mouse pseudokinase domain constructs, which bound non-specifically to the Ni<sup>2+</sup> beads after the His<sub>6</sub>-tag had been cleaved, the beads were washed with 35 mM Imidazole SEC Buffer, and these washes collected and pooled with the SEC Buffer washes. The washes were filtered through a 0.45 µm filter (Sartorius), then concentrated using an Amicon Ultra-15 concentrator (Millipore) to less than 1 ml volume. The concentrated protein was loaded onto a HiLoad 16/600 Superdex200 PG size exclusion column (GE Healthcare) that had been pre-equilibrated with SEC Buffer at 4°C and 1.5 ml fractions collected. Protein purity was assessed by SDS-PAGE with Coomassie Brilliant Blue staining. Pure fractions were pooled, concentrated, and TCEP (ThermoFisher) added to 1 mM. Protein concentration was measured using absorbance at 280 nm, and protein snap frozen in liquid nitrogen and stored at -80°C if not to be used immediately.

**Table 2.2 Buffers for Sf21 His<sub>6</sub> Purification**

| Buffer                                                     | Components                                                                                                                           |
|------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------|
| Lysis Buffer                                               | 20 mM Tris-HCl, pH 8.0<br>500 mM NaCl<br>10 mM imidazole, pH 8.0<br>20% v/v glycerol<br>1 mM TCEP(Thermofisher)<br>1 mM PMSF (Sigma) |
| 35 mM Imidazole Wash Buffer                                | 20 mM Tris-HCl, pH 8.0<br>500 mM NaCl<br>35 mM imidazole, pH 8.0<br>20% v/v glycerol                                                 |
| Elution buffer                                             | 20 mM Tris-HCl, pH 8.0<br>500 mM NaCl<br>250 mM imidazole, pH 8.0<br>20% v/v glycerol<br>1 mM TCEP                                   |
| Size Exclusion Chromatography Buffer /Dialysis Buffer      | 20 mM HEPES, pH 7.5<br>200 mM NaCl<br>5% v/v glycerol                                                                                |
| 35 mM Imidazole Size Exclusion Chromatography (SEC) Buffer | 20 mM HEPES, pH 7.5<br>200 mM NaCl<br>35 mM imidazole, pH 8.0<br>5% v/v glycerol                                                     |

*GST purification (Sf21 cells)*

The human MLKL full-length constructs were expressed with a TEV protease-cleavable Glutathione S-transferase (GST) tag fused to their N-terminus (vectors used for each expression construct listed in Appendix I). Cell pellets were resuspended in 50 ml GST Lysis Buffer (See Table 2.3) per 1 L of insect cell culture. Cells were lysed by sonication (Misonix Sonicator 3000) in an ice-water bath, 10 seconds on and 10 seconds off, for a total of 4 minutes at 40% amplitude. The whole cell lysate was centrifuged (45,000 *g*, 60 minutes, 4°C) to remove sediment. The supernatant was added to glutathione agarose beads (UBPBio; 1 ml of beads for 1 L of insect cell culture), that had been pre-washed with MilliQ (10 CV) then GST Lysis buffer (10 CV), and left to bind, rolling, at 4°C for 1-2 hours. Beads were pelleted by centrifugation (300 *g*, 5 minutes, 4°C), and the unbound fraction decanted. Beads were washed with GST Wash Buffer (3 x 20 ml). The protein was cleaved off the beads by addition of 0.5 mg of His<sub>6</sub> tagged TEV protease (in 200 µl volume) with TCEP

(1 mM), and left to stand at room temperature for 1 hr, then at 4°C overnight. Cleaved protein was collected by mixing 5 ml of SEC Buffer with the beads, followed by centrifugation (300 g, 5 minutes, 4°C). The 5 ml containing the cleaved protein was decanted to a fresh tube, and the process repeated twice more, so that a total of 15 ml eluate was collected. The eluate was filtered through a 0.45 µm filter (Sartorius) to remove any contaminating beads, then concentrated using an Amicon Ultra-15 concentrator (Millipore) to less than 1 ml volume. The concentrated protein was loaded onto a HiLoad 16/600 Superdex200 PG size exclusion column (GE Healthcare) that had been pre-equilibrated with SEC Buffer at 4°C and 1.5 ml fractions collected. Protein purity was assessed by SDS-PAGE with Coomassie Brilliant Blue staining. Pure fractions were pooled, concentrated, and TCEP (Thermofisher) added to 1 mM. Protein concentration was measured using absorbance at 280 nm, and protein snap frozen in liquid nitrogen and stored at -80°C if not to be used immediately.

**Table 2.3 Buffers for GST purification**

| Buffer                                            | Components                                                                                  |
|---------------------------------------------------|---------------------------------------------------------------------------------------------|
| GST Lysis Buffer                                  | 20 mM Tris-HCL, pH 8.0,<br>200 mM NaCl<br>5% v/v glycerol<br>1 mM TCEP<br>1 mM PMSF (Sigma) |
| GST Wash Buffer                                   | 20 mM Tris-HCL, pH 8.0,<br>200 mM NaCl<br>5% v/v glycerol                                   |
| Size Exclusion Chromatography Buffer (SEC Buffer) | 20 mM HEPES, pH 7.5<br>200 mM NaCl<br>5% v/v glycerol                                       |

### 2.3 Mammalian cell culture

#### *Culturing conditions*

HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies) supplemented with 10% (v/v) Foetal Calf Serum (FCS), penicillin (100 U/mL), streptomycin (100 µg/ml). Mouse dermal fibroblasts (MDFs) are adherent cells, and were cultured in DMEM supplemented with 10% (v/v) FCS, penicillin (100



U/mL), streptomycin (100 µg/ml) and after selection for lines stably transduced with MLKL expression constructs, puromycin (5 µg/ml; Life Technologies, CA). U937 cells (ATCC) are suspension cells and were cultured in human tonicity RPMI medium supplemented with 8–10% (v/v) FCS and, after selection, puromycin (5 µg/ml). Cells were cultured at 37°C in 10% CO<sub>2</sub>. Cell lines were frozen in FCS doped with 10% (v/v) DMSO and stored at -80°C. All cell lines were passaged three times a week to maintain viability, and generally kept in culture for no longer than 2 months.

#### *Making cell lines using lentivirus*

Transductions were performed using the Qiagen Effectene kit. 2 µg of pFTRE3G plasmid DNA encoding a doxycycline-inducible gene of interest, and helper plasmids pCMV-δR8.2 (0.2 µg) and pVSV-G (0.2 µg) that produce the lentiviral capsid and envelope, were diluted in EC buffer (Qiagen) to 366 µl. 32 µl of Enhancer was mixed with the DNA, left for 2 minutes, and then 32 µl of Effectene reagent was mixed in, and this was left for 10 minutes, to allow lipid:DNA complex formation. HEK293T cells at 80% confluency in 10 cm plates were changed into fresh media (DMEM, supplemented with FCS, penicillin and streptomycin), and the lipid:DNA complex added dropwise to the cells. After 48 hours, the virus containing media was collected, filtered (0.45 µm; Sartorius) and polybrene (10 µg/ml for MDFs, 5 µg/ml for U937s and HT29s; Sigma) added. The virus was used to infect up to six biologically independent MDF cell lines (three wild-type and three *Mkl1*<sup>-/-</sup>, derived from different mice), at 60% confluency in 6-well plates. For U937 transductions, the virus was split between two cell lines, one wild-type and one *Mkl1*<sup>-/-</sup>. After 48 hours, puromycin (5 µg/ml with MDFs, or tapered up to 5 µg/ml for U937s) was added to select cells harbouring the lentiviral transposon.

#### *Death assays*

MDFs were seeded at 5 x 10<sup>4</sup> cells/well into 24-well plates, and allowed to adhere overnight. 10 ng/ml doxycycline was added to cells to induce construct expression, and after three hours cells were either left untreated, or treated with TNF (Produced in-house; 100 ng/ml) and Smac-mimetic (Compound A, Tetralogic; 500 nM) (TS), or TS and IDN-6556 (Tetralogic) (5 µM) (TSI) or Q-VD-OPh (Focus Bioscience) (10 µM)

(TSQ). Doxycycline negative controls were also performed. 24 hours after treatment, cells were harvested via trypsinisation and treated with propidium iodide (PI; 100 ng/ml), and PI positive cells counted by BD FACSCalibur flow cytometry. U937 cells were seeded at 50,000 cells per well in 96 well plates, and treated with doxycycline (10 ng/ml) immediately after plating. The rest of the death assay was conducted in the same way as for MDFs, except cells were harvested without trypsin. The death assay protocol has been described in this methods paper (Liu et al., 2018)

#### *Blue Native-PAGE*

MDFs were seeded at  $5 \times 10^5$  cells/well in 6-well plates, and allowed to adhere overnight. Construct expression was induced by addition of 10 ng/ml doxycycline for three hours, before being treated with TSQ or left untreated. After ~6 hours, when cell death had reached ~40% by eye, cells were harvested. Membrane fractionation and Blue Native PAGE (BN-PAGE) was performed as previously described (Hildebrand et al., 2014). Briefly the cells were permeabilised in MELB buffer (Table 2.4) with 0.025% digitonin, and the cytosolic and crude membrane fraction separated by centrifugation. The crude membrane fraction was further solubilised in 1% digitonin and centrifuged to clarify. The cytosolic fraction was made up to 1% digitonin, and samples separated on a 4-16% Bis-Tris Native PAGE gel (Life Technologies). Proteins were transferred onto a PVDF membrane (Osmonics), de-stained and denatured before being probed by western blot (see below).

**Table 2.4 Membrane Fractionation Buffer for BN-PAGE**

| Buffer      | Composition                                                                                                                                                                                              |
|-------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| MELB Buffer | 20 mM HEPES pH 7.5<br>100 mM KCl<br>2.5 mM MgCl <sub>2</sub><br>100 mM sucrose<br>2 µM N-ethyl maleimide<br>5 mM β-glycerophosphate<br>1 mM Na Molybdate<br>1 mM Na Pyrophosphate<br>100 µM Na Fluoride. |

*Western blot*

Following transfer via electrophoresis, PVDF membranes were rinsed in PBS-T (16 mM Na<sub>2</sub>HPO<sub>4</sub>, 4 mM NaH<sub>2</sub>PO<sub>4</sub>.H<sub>2</sub>O, 148.8 mM NaCl, 0.1% (v/v) Tween 20) and blocked with 5% (w/v) skim milk powder in PBS-T for 1 hour (room temperature). They were washed for 3 x 10 minutes in PBS-T, then incubated overnight at 4°C with primary antibody. Primary antibody was removed, the membrane washed for 6 x 10 minutes in PBS-T, and Horse Radish Peroxidase (HRP) conjugated secondary antibody added for 1 hour (room temperature). The membrane was washed (6 x 10 minutes) and bands visualised using Chemiluminescent HRP Substrate (Millipore). Antibodies used are detailed in Table 2.5.

**Table 2.5 Antibodies**

| Antibody                                                                       | Dilution                                 | Source |
|--------------------------------------------------------------------------------|------------------------------------------|--------|
| Anti – MLKL 3H1 (WEHI Monoclonal Lab) available as cat. MABC604 from Millipore | 1/1000                                   | Rat    |
| Anti – actin (Sigma)                                                           | 1/3000                                   | Mouse  |
| Anti-VDAC1 (Voltage Dependent Anion Channel 1; Merck, cat. MABN504)            | 1/500                                    | Mouse  |
| Anti – GAPDH (Cell Signalling Technology)                                      | 1/1000<br>BN-PAGE;<br>1/3000<br>SDS-PAGE | Rabbit |
| Anti-FLAG 9H1 (WEHI Monoclonal Lab)                                            | 1/1000                                   | Rat    |
| Secondary antibodies: anti-rat, anti-mouse, anti-rabbit (Southern Biotech)     | 1/5000                                   | Goat   |

### *Statistical analyses*

Cell death assay results are presented as a mean  $\pm$  SEM of at least three independent experiments (each conducted with a minimum of two biologically independent replicates for MDFs, or one biological replicate for U937s). Pooled, paired T-tests were performed to compare samples, and significance assigned for p values of  $< 0.05$ .

## *2.4 Biophysical methods*

### *Analytical ultracentrifugation*

Sedimentation velocity experiments were performed using an XL-I analytical ultracentrifuge (Beckman Coulter) equipped with UV/Vis scanning optics. Buffer reference (20 mM Tris-HCl, 150 mM NaCl, pH 8.0, 1 mM TCEP) and 380  $\mu$ l of MLKL sample solutions at 40  $\mu$ M were loaded into 12 mm double-sector cells with quartz windows and the cells were mounted in an An-60Ti 4-hole rotor or An-50Ti 8-hole rotor. All experiments were conducted at 50,000 rpm (201,600 g) and 20°C, and radial absorbance data were collected at 280 nm in continuous mode. Data were fitted to a continuous sedimentation coefficient distribution [c(s)] model and converted to standardised sedimentation coefficient (s<sub>20,w</sub>) distributions using SEDFIT (Schuck, 2000). The protein partial specific volumes, buffer density, and buffer viscosity were calculated using SEDNTERP (Laue et al., 1992, Hayes et al., 2006).

### *Thermal shift assay*

Thermal shift assays were performed previously described (Lucet and Murphy, 2017). 0.6  $\mu$ g of protein in SEC Buffer with 1 mM TCEP was assayed in a 25  $\mu$ l volume. The stability of the protein in the conditions listed in Table 2.6 was tested. Thermal denaturation was induced, and measured using a Corbett Real Time PCR machine that detected fluorescence of SYPRO Orange (530 nm; Molecular Probes, CA), a dye which fluoresces upon binding to exposed hydrophobic parts of the protein. A higher thermal stability in the presence of ATP or compound (a positive shift in melting temperature;  $\Delta T_m$ ) signifies binding, and resultant stabilisation of the protein. Two independent assays were performed each in technical duplicate.

**Table 2.6 Conditions tested in thermal shift assays**

| Condition                                                 |
|-----------------------------------------------------------|
| Apo – (SEC Buffer only)                                   |
| 200 $\mu$ M ATP                                           |
| 200 $\mu$ M ATP, 1 mM $Mg^{2+}$                           |
| 1 mM ATP                                                  |
| 1 mM ATP, 5 mM $Mg^{2+}$                                  |
| 40 $\mu$ M MLKL binding control compound in 4% (v/v) DMSO |
| 4% (v/v) DMSO                                             |

### *2.5 Small Angle X-ray Scattering (SAXS)*

SAXS data were collected on the SAXS/WAXS beamline (Australian Synchrotron) using an inline size exclusion chromatography setup (Kirby et al., 2013). 2D scattering data were radially averaged and normalized to sample transmission. 11 x 2sec scatter patterns (mouse MLKL full-length) or 15 x 1sec scatter patterns (rat MKL full-length) from the apex of the elution peak were averaged before background scatter (estimated from the average of 20 scatter patterns from the SEC run prior to protein elution) was subtracted using the ScatterBrain software (written by Stephen Mudie, Australian Synchrotron). The ATSAS suite of software was used for all subsequent SAXS data analyses: Guinier analysis was performed using PRIMUS (Konarev et al., 2003); the pair-wise intra-atomic distance distribution function  $P(r)$  and  $D_{max}$ , the maximum dimension of the particle, were calculated from scattering data using GNOM (Svergun, 1992); rigid body modelling was performed using SASREF (Petoukhov and Svergun, 2005) with P2, P3 or P4 symmetries trialled. All structural models were illustrated using PyMOL. The data collection and processing statistics are presented in Table 4.1 (Chapter 4) for mouse MLKL full-length and Table 3.3 (Chapter 3) for rat MLKL full-length.

## 2.6 Crystallography

### *Rat pseudokinase domain*

Protein was concentrated off SEC to 5 mg/ml, using an Amicon Ultra-15 concentrator (Millipore), snap frozen in liquid nitrogen and stored at -80°C, until required. Crystallisation trials were performed at the C3 facility, Parkville. All trials were set up robotically in 96 well plates, using the vapour diffusion method for crystallisation, with 50 µl reservoir volume and 150 nl protein solution in sitting drops in a 1:1 ratio with reservoir solution. The structure was first solved using crystals that grew within three days in the 0.2 M magnesium chloride, 25% (w/v) polyethylene glycol 3350, 0.1 M bis-tris chloride pH 5.5 condition at 20°C. Crystals were soaked in 0.2 M magnesium chloride, 25% (w/v) polyethylene glycol 3350, 0.1 M bis-tris chloride pH 5.5 supplemented with 20% (v/v) ethylene glycol cryoprotectant before flash freezing in liquid nitrogen, and diffracted to 3.3 Å. Better diffracting crystals grew in 20 mM manganese(II) chloride, 25% (v/v) polyethylene glycol 400, 0.1 M sodium MES pH 6.5. They were flash frozen in mother liquor, utilising the 25% (v/v) polyethylene glycol 400 in the condition as the cryoprotectant, and diffracted to 2.2 Å at the Australian Synchrotron (crystallisation conditions and datasets summarised in Table 2.7).

### *Horse pseudokinase domain*

Protein was concentrated off SEC to 6.7 mg/ml, using an Amicon Ultra-15 concentrator (Millipore), then snap frozen in liquid nitrogen and stored at -80°C until required. Protein was diluted to 5 mg/ml in SEC buffer for crystallisation trials at the C3 facility, Parkville. Horse MLKL pseudokinase domain crystallised within 3 weeks in the 2 M ammonium sulphate, 0.1 M sodium HEPES pH 7.5 condition. The crystal was soaked in 2 M sodium malonate pH 7 as a cryoprotectant, then flash frozen in liquid nitrogen. The crystal diffracted to 2.7 Å.

**Table 2.7 Crystallisation conditions**

| Protein                   | Temperature (°C) | Condition                                                                                    | Dataset resolution |
|---------------------------|------------------|----------------------------------------------------------------------------------------------|--------------------|
| Rat Pseudokinase domain   | 20               | 0.2 M magnesium chloride, 25% (w/v) polyethylene glycol 3350, 0.1 M bis-tris chloride pH 5.5 | 3.3 Å              |
| Rat Pseudokinase domain   | 20               | 20 mM manganese (II) chloride, 25% (v/v) polyethylene glycol 400, 0.1 M sodium MES pH 6.5    | 2.2 Å              |
| Horse pseudokinase domain | 20               | 2 M ammonium sulphate, 0.1 M sodium HEPES pH 7.5                                             | 2.7 Å              |

All crystallisation screens provided by CSIRO's C3 facility.

#### *Data collection and analysis*

Data was collected on the MX2 Beamline at the Australian Synchrotron, using an Eiger detector. Data reduction, integration and scaling was performed with XDS (Kabsch, 2010). The Matthews coefficient for each crystal was estimated using MATTPROB (Weichenberger and Rupp, 2014, Kantardjieff and Rupp, 2003). Both structures were solved by molecular replacement with Phaser.MR (McCoy et al., 2007) within Phenix (Adams et al., 2010) using the human MLKL pseudokinase domain, PDB:4MWI (Murphy et al., 2014a) as the search model. Refinement was performed using Phenix.refine, and model building in Coot (Emsley et al., 2010). Crystal structure figures were created in Pymol.

## *2.7 Liposome experiments*

### *LUV production*

Large Unilamellar Vesicles (LUVs) were prepared using a plasma membrane-like lipid mix (Table 2.8). Lipids were stored as 20 mg/ml (~25 mM for most lipids) stocks in chloroform. 50 µl of plasma membrane-like lipid mix was dried under nitrogen gas, then left to dry further under vacuum for a minimum of 2 hours. Dried lipids were resuspended in either 500 µl of LUV buffer (10 mM HEPES, 135 mM KCl) or LUV buffer with 50 mM 5(6)-carboxyfluorescein dye (Sigma), to form dye filled liposomes. The mixture was then freeze-thawed at least 5 times, by immersion in liquid nitrogen until fully frozen, followed by immersion in a 37°C water bath until contents had

thawed. The lipid mixture was extruded through polycarbonate membranes of 50 nm, 100 nm or 200 nm size cut-off (Avanti Polar Lipids), a minimum of 21 times to form liposomes, using a pre-warmed mini extruder (Avanti Polar Lipids). Liposomes stocks were at approximately 2.5 mM lipid concentration and were stored at 4°C in the dark.

**Table 2.8 Plasma membrane-like lipid mix used to make LUVs**

| Lipid                           | Source              | Proportion of plasma membrane-like mix |
|---------------------------------|---------------------|----------------------------------------|
| Phosphatidylethanolamine (POPE) | Avanti Polar Lipids | 20%                                    |
| Phosphatidylcholine (POPC)      |                     | 40%                                    |
| Phosphatidylinositol (PI)       |                     | 10%                                    |
| Phosphatidylserine (DOPS)       |                     | 20%                                    |
| Phosphatidylglycerol (POPG)     |                     | 10%                                    |

#### *LUV dye release assays*

Protein was diluted to either 2  $\mu$ M, or 500  $\mu$ M (2 x desired final concentration) in LUV buffer, and 50  $\mu$ l aliquoted in triplicate into adjacent wells of a 96 well U-bottom plate (Falcon). 50  $\mu$ l of 2% CHAPS detergent in LUV buffer and 50  $\mu$ l of LUV buffer alone was aliquoted into the 96 well plate in triplicate, as the positive and negative controls respectively. The liposomes (either 50 nm, 100nm or 200 nm diameter filled with 5(6)-carboxyfluorescein dye) were purified from the surrounding dye using a PD-10 desalting column (GE Healthcare), and diluted to 20  $\mu$ M lipid concentration. At the plate reader (Hidex Chameleon Multilabel Microplate Reader (Lab logic)), the protocol was pre-programmed and machine ready to be loaded, before 50  $\mu$ l of liposomes was quickly added to each well of the 96 well plate using a multi-channel pipette. The plate was immediately placed in the plate reader and measurements started. At this stage, speed is essential to capture the beginning of dye release. Fluorescence was measured every 2 minutes for 60 minutes (31 measurements) with excitation wavelength of 485 nm and emission wavelength of 535 nm.

#### *Cryo-electron Microscopy*

Dye-free liposomes and protein were pre-incubated together in LUV buffer for 30 minutes at room temperature prior to snap freezing, with liposomes at 1 mM concentration, and protein at various concentrations. 3  $\mu$ l of the sample was snap frozen onto a copper grid with lacey carbon coating by immersion in liquid ethane



using a Vitrobot under the following conditions; 4°C, 100% humidity, -2 blot force, 3 second blot time. Samples were viewed with a TECNAI F30 transmission electron microscope with a maximum electron dose of 20 e<sup>-</sup>/Å<sup>2</sup>. Dr Wilson Wong assisted with grid freezing and microscope use.

#### *GUV production – plasma membrane mix*

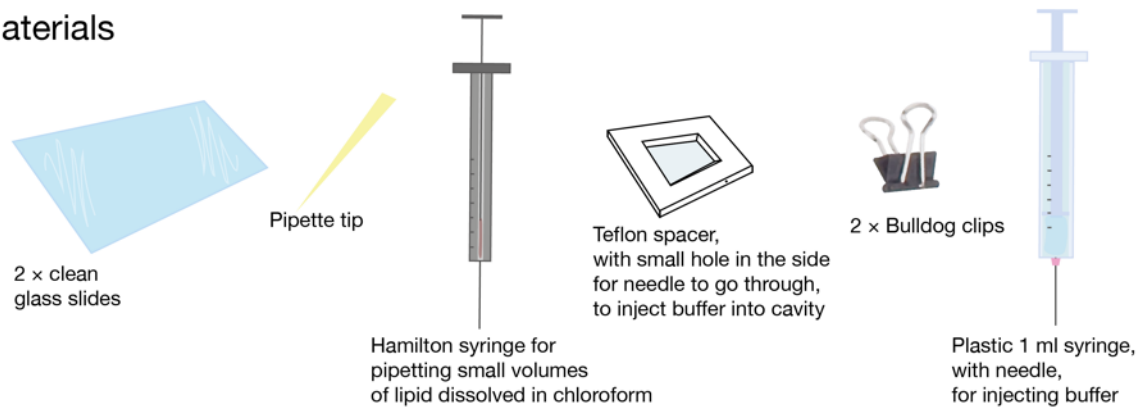
All Giant Unilamellar Vesicle (GUV) experiments were conducted at the Max Planck Institute for Colloids and Interfaces (Potsdam, Germany) with the assistance of Dr Jan Steinkuehler.

GUVs were formed using passive swelling on a Poly Vinyl Alcohol (PVA) hydrogel as previously described (Weinberger et al., 2013). A schematic detailing this method is presented in Figure 2.1, described herein. 5% (w/v) PVA (MW 145000, Merck) solution was prepared by heating PVA and MiliQ H<sub>2</sub>O together, at 90°C with stirring, until PVA fully dissolved. 50 µl of the solution (cooled to room temperature) was spread thinly onto a rectangular glass slide using the side of a pipette tip. The PVA film was dried in an oven at 50°C for 30 minutes. Once dry, a glass Hamilton syringe was used to deposit 2-10 µl of the plasma membrane-like lipid mix (Table 2.8; 4 mM lipid), supplemented with 0.4 % (M) lipophilic carbocyanine DiD dye (ThermoFisher) onto the PVA hydrogel, and lipid was spread into a thin film using the flat of the needle. The glass slide with PVA and lipid film was dried under vacuum for a minimum of 2 hours, to remove any chloroform. To swell GUVs, a rectangular teflon spacer with a small hole in the side wall (See Figure 2.1) was sandwiched between the PVA/lipid coated glass slide and another glass slide (Figure 2.1). Approximately 700 µl of filtered LUV buffer with or without free dye such as rhodamine, was injected into the cavity between the glass slides, through the hole in the teflon spacer. GUVs were left to passively swell for 30 – 60 minutes, and progress monitored under a microscope. GUVs encapsulate buffer components during swelling, so if a dye was included with the buffer, it would be contained inside the GUVs. To harvest GUVs, the bottom glass was gently tapped multiple times to dislodge GUVs from the PVA hydrogel. The top glass was slowly slid off and a P1000 pipette used to harvest the GUV solution

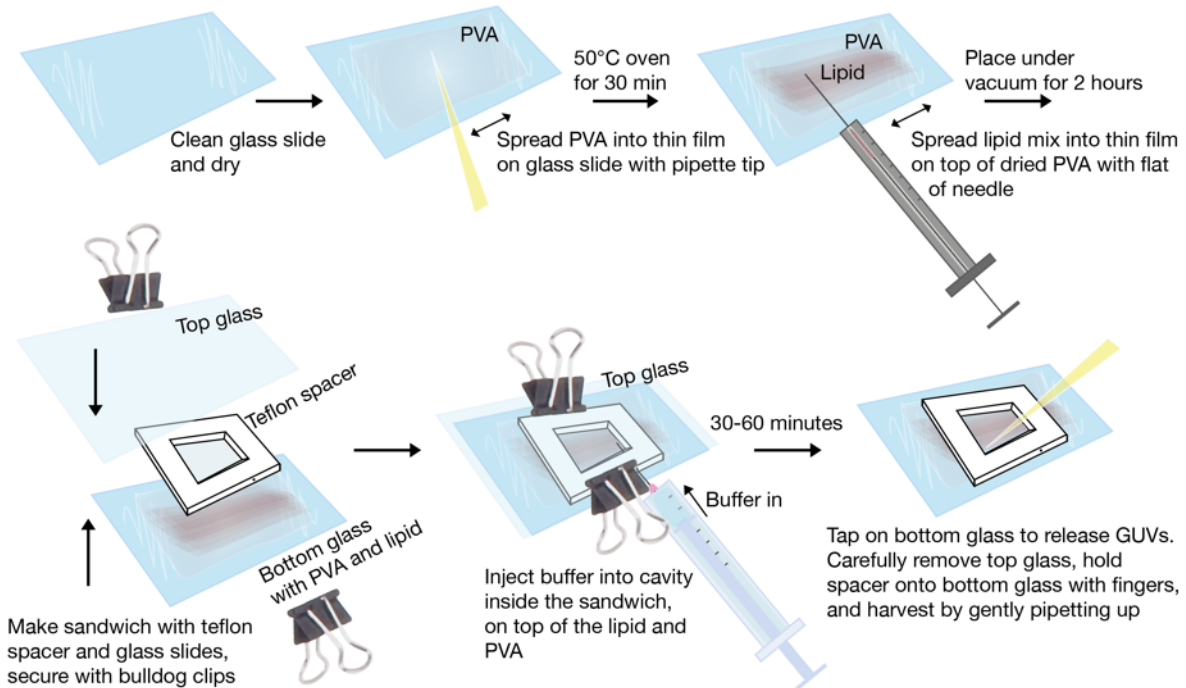
trapped in the cavity between the glass slides. A fresh GUV stock was made every day GUVs were to be used.

To reduce GUV contamination, all tools, glass surfaces, Teflon spacers and anything else used to make the GUVs was rinsed with ethanol followed by MilliQ water and then dried under nitrogen before being used.

## Materials



## Method



**Figure 2.1 Schematic of GUV production via passive swelling on PVA hydrogel**  
**Materials** that are required are pictured. **Method A** Clean glass slide with ethanol, then MilliQ water, then dry under nitrogen stream. **B** Deposit 50  $\mu$ l of PVA solution onto glass slide using a pipette, with the same pipette tip, spread out the PVA on the glass slide to form a film, let dry for 30 minutes. **C** Spread lipid mix on top of PVA hydrogel by swiping needle side to side while depressing Hamilton syringe containing lipid solution, to make a thin film. Let dry under vacuum for 2 hours. **D** Assemble

sandwich as pictured. **E** Inject buffer into cavity using a syringe with needle inserted through the hole in the Teflon spacer, trying to fill the cavity totally without leaving air bubbles. Leave the sandwich on a flat surface for 30 – 60 minutes while the GUVs swell **F** With sandwich still assembled, gently tap on the bottom glass. Unclip the bulldog clips and hold glass slides between your fingers. Carefully slide off top glass from one corner first, insert pipette into cavity and take up GUV solution.

#### *Phase separated GUV production*

Phase separated vesicles were produced by Dr Jan Steinkuehler using a DOPG:eggSM:chol (Avanti Polar Lipids) lipid mix in the ratio 3:5:2 to 10. GUVs were produced as previously described (Pataria et al., 2014).

#### *GUV binding assays*

To monitor protein association with GUVs, GUVs and protein were pre-mixed and incubated at room temp for at least 30 minutes prior to imaging, unless otherwise stated. Binding assays were conducted in LUV buffer (10 mM HEPES pH 7.0, 135 mM KCl), protein concentration was varied between 50 nM and 1  $\mu$ M, while GUV concentration was kept approximately equal between samples, by diluting the GUV stock solution 1/10 into the protein sample.

Glass slides for microscopy were pre-coated in BSA, (10 mg/ml BSA in LUV buffer) incubated for 10 minutes, then excess solution removed with MilliQ water, and glass dried under nitrogen. This prevents the GUVs adsorbing to the glass and bursting. For confocal microscopy, 10  $\mu$ l of GUV:protein sample was spotted onto the pre-coated glass slide, and sandwiched under another glass slide using a plastic spacer.

#### *GUV dye ingress assays*

Protein/GUV samples were prepared as above except with the addition of various dyes to sample solution (See Table 2.9 for dyes, and concentration). Dextran 10 Cascade blue, and Dextran 70 Rhodamine B were mixed with GUVs together, and tested at the same time, due to their different excitation and emission wavelength. Sulphorhodamine B dye was tested separately due to its overlapping excitation and emission with the Dextran 70.

**Table 2.9 Dyes used in GUV ingress assays**

| Dye                                    | Excitation $\lambda$ (nm) | Emission $\lambda$ (nm) | Stokes radius* | Concentration |
|----------------------------------------|---------------------------|-------------------------|----------------|---------------|
| Sulphorhodamine B (Sigma)              | 565                       | 586                     | -              | 0.25 $\mu$ M  |
| Dextran 10 Cascade Blue (Thermofisher) | 400                       | 420                     | ~1.9 nm        | 2.5 $\mu$ M   |
| Dextran 70 Rhodamine B (Thermofisher)  | 570                       | 590                     | ~6.5 nm        | 2.5 $\mu$ M   |

\* From (Armstrong et al., 2004)

### *Confocal microscopy*

The GUVs were formed with a lipophilic carbocyanine DiD conjugated dye molecule (Thermofisher) to allow visualisation of the GUV membrane by confocal fluorescence microscopy, and MLKL constructs expressed with a C-terminal enhanced GFP fusion, to allow their visualisation by fluorescence.

A Leica confocal SP8 microscope was used to collect all GUV binding images, dye ingress images, and videos of ring formation. Phase contrast was used to visualise GUVs with white light. Confocal fluorescence microscopy was used to visualise fluorophores. Excitation wavelengths for fluorophores used and emission wavelengths that were collected are shown in Table 2.10.

**Table 2.10 Excitation and emission  $\lambda$  for each fluorophore**

| Fluorophore             | Excitation $\lambda$ (nm) | Emission $\lambda$ collected (nm) |
|-------------------------|---------------------------|-----------------------------------|
| GFP                     | 488                       | 492-550                           |
| DiD                     | 633                       | 635-698                           |
| Sulphorhodamine B       | 561                       | 566-610                           |
| Dextran 10 Cascade blue | 405                       | 410-450                           |
| Dextran 70 rhodamine B  | 561                       | 566-610                           |

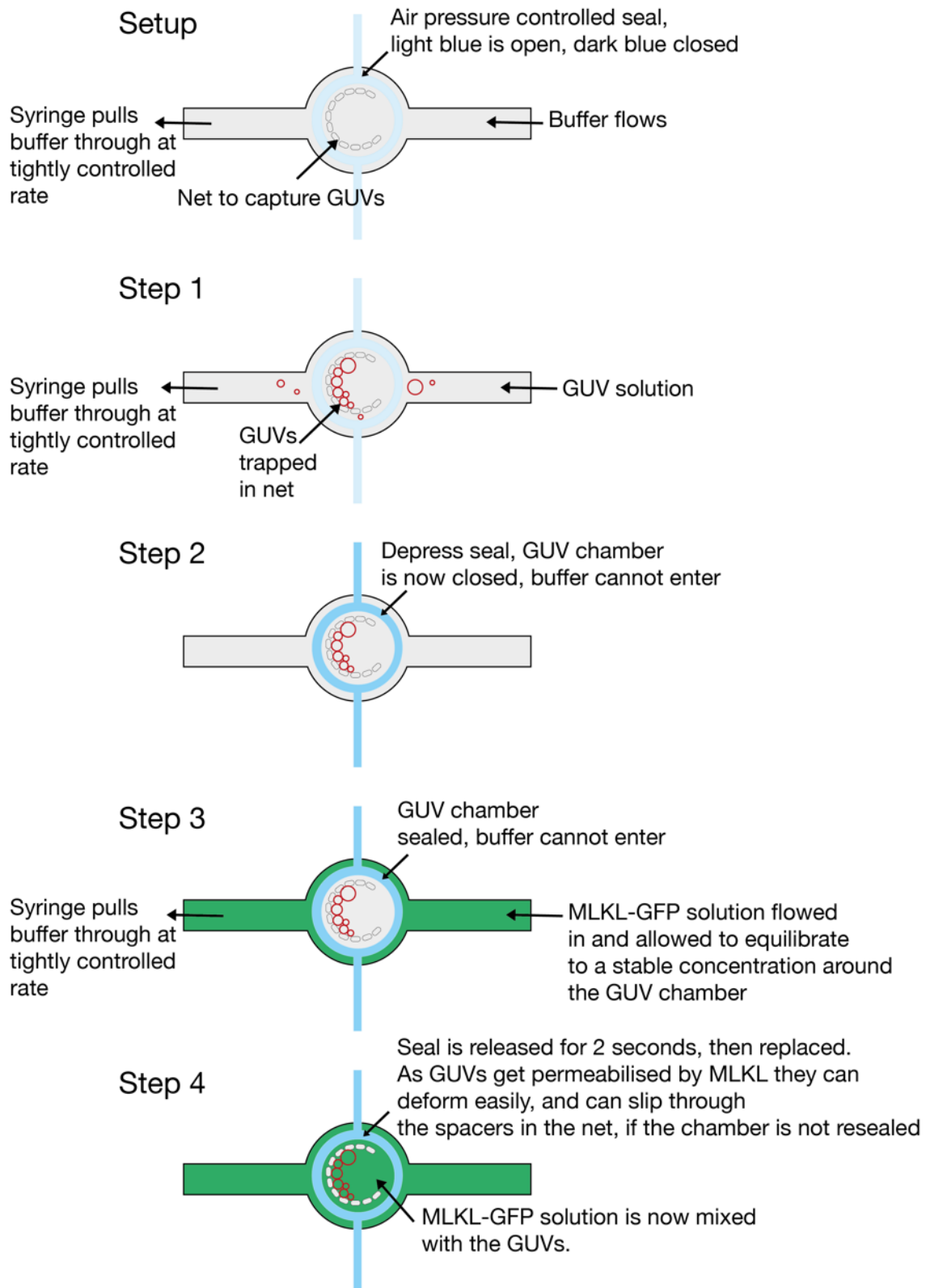
Videos were collected with a frame rate of 1/0.52 seconds, and a 512 x 512 pixel resolution. To capture video from the point where MLKL was first added to GUVs, flow cells were used.

### *Flow cells*

A detailed description of the flow cells used in video experiments is published (Yandrapalli and Robinson, 2019). The cells are designed and manufactured by hand by Dr Tom Robinson's group at the Max Planck Institute for Colloids and Interfaces. Briefly, the flow cells have a reservoir at the flow cell entry, where sample to be flowed through the cell is loaded. The flow is modulated by a neMESYS Syringe Pump (Cetoni), which is connected to the exit of the flow cell. The syringe pump works by accurately pulling small volumes of buffer through the flow cell, fed by the reservoir solution. This allows accurate rate determination (in  $\mu\text{l}/\text{minute}$ ), and provides flow free from pulsation. The flow cells have small chambers inside them with a plastic trap that captures GUVs, as they are flowed through. The flow cells have a seal that can be opened and closed around each GUV holding chamber, using air pressure as a switch. A schematic of what one GUV holding chamber in the flow cell looks like is presented in Figure 2.2.

Flow cells were coated with 10 mg/ml BSA in LUV buffer, to prevent GUV and protein sticking to the walls of the flow cell. The BSA solution was loaded into the reservoir of the flow cells and forced through the channels by centrifugation (8000  $g$ , 10 minutes), then left to bind to the flow cell at room temperature for 1 hour. To change solution in the flow cells (i.e. to add GUVs, then rinse, and add protein), the solution in the reservoir was buffer exchanged with new solution, by pipetting in  $\sim 130\ \mu\text{l}$  of new solution (close to the capacity of the reservoir), mixing with the old reservoir solution, and removal of  $130\ \mu\text{l}$ . This was repeated five times, and after this  $130\ \mu\text{l}$  of the new solution was left in the well. For the GUV ring formation videos, the sequence of buffer addition and seal manipulation was as follows. LUV buffer was flowed through to rinse off excess BSA solution ( $\sim 90\ \mu\text{l}$ ;  $20\ \mu\text{l}/\text{minute}$ ), GUV solution was flowed through until traps were filled with enough GUVs (decided by eye looking through microscope  $\sim 90\ \mu\text{l}$ ,  $5\ \mu\text{l}/\text{minute}$ ). Then, air pressure was supplied to close

seals around GUV chambers, trapping the GUVs inside. Then more LUV buffer was flowed through ( $\sim 60 \mu\text{l}$ ,  $10 \mu\text{l}/\text{minute}$ ), to clear remaining GUVs outside of the trap. MLKL-GFP protein (multiple concentrations trialled, in LUV buffer) was then flowed through ( $\sim 60 \mu\text{l}$ ,  $10 \mu\text{l}/\text{minute}$ ) so that the flow cell was full of the protein solution and equilibrated at the correct concentration. Then the video was started. Under very low flow ( $1 \mu\text{l}/\text{minute}$ ), the seal around the GUV chamber was released for two seconds, then resealed. This allowed mixing of the MLKL-GFP solution with the GUVs in the chamber, and resealed the GUVs inside. Figure 2.2 shows a schematic of this process.



**Figure 2.2 Schematic of flow cell usage in GUV ring formation videos**

A simplified representation of one of the chambers in the flow cell is presented. The steps taken to flow buffer through as described above are illustrated.

### *Image analysis*

All data analysis for GUVs was performed in ImageJ. Scripts were used to manage image processing for the production of montages to show MLKL GUV binding, and to compare dye fluorescence inside GUVs compared to the outside solution, in order to measure dye ingress. Scripts were written by myself in collaboration with Dr Jan Steinkuehler, and can be found in Appendix III. Video slices were formed by averaging 10 consecutive frames per image, beginning at each time point indicated. This was done because individual frames only have low contrast, and averaging multiple frames improves contrast, and improves visual clarity. In some cases, the field of view was moved within the 10 selected frames, so the frames were hand aligned so that the GUV was aligned in each frame before averaging.

### *2.8 Neutron reflectometry*

All neutron reflectometry experiments were conducted at the Australian Centre for Neutron Scattering (ACNS; Lucas Heights, Australia) with the assistance of Dr Anton Le Brun.

#### *Preparation of SAM monolayer for neutron reflectometry*

Silicon wafers, coated with a ~80 Å thick chromium layer and ~280 Å thick gold layer, that were used for neutron reflectometry experiments were purchased from the Melbourne Nano Fabrication Facility (Figure 2.4). Wafers were cleaned in a Jelight UV-ozone cleaner for 15 minutes and then washed thoroughly using 0.5% (v/v) Hellmanex and MilliQ water, then dried with nitrogen stream. Care was taken at all steps to avoid touching the surface of the wafer. Formation of the self-assembled monolayer followed a previously described protocol (Hughes et al., 2014). A solution of 1-oleoyl-2-(16-thiopalmityl)-*Sn*-glycero-3-phosphocholine (herein called thiolipid) at concentration of 0.25 mg/mL in 1 % (w/v) octyl-β-D-glucopyranoside (OG), 10 mM HEPES pH 7.6 was pipetted onto the surface of the wafer until the entire wafer was covered. The wafer was incubated in a 50°C oven for 1-2 hours. During this time, the thiolated lipid tails react covalently with the gold to form a self-assembled monolayer (SAM) (see Figure 2.3). After 1-2 hours, excess lipid solution was poured off and the wafer washed again with 0.5% (v/v) Hellmanex, MilliQ water and dried under nitrogen



stream. This process was repeated twice to ensure best coverage of the thiolipid SAM.

*Preparation of floating supported bilayers for neutron reflectometry*

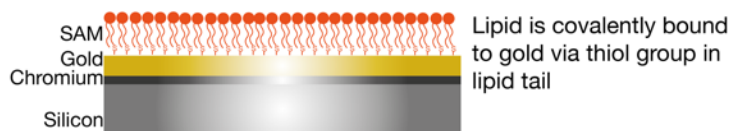
Floating bilayers were prepared as described in (Hughes et al., 2014) using a Langmuir-Blodgett/Langmuir-Schaefer dipping trough (NIMA Ltd., Coventry, UK) at ANSTO (See Figure 2.3). Briefly, the wafer with SAM was cleaned with 0.5% (v/v) Hellmanex and MilliQ, then dried under nitrogen stream. Without touching the surface of the wafer, the wafer was attached to a holder, and positioned on the dipping arm perpendicular to the surface of the trough solution, and then lowered under the surface of the trough solution. The trough solution (10 mM  $\text{CaCl}_2$ ), was cleaned extensively, by aspirating dust and other contaminants off the surface of the solution until surface tension was  $< 0.5 \text{ mN m}$  (milliNewton metres). Lipid to form the inner leaflet (DPPC; 10 mg/ml in chloroform) was added dropwise to the surface of the trough solution and the chloroform allowed to evaporate. The monolayer was then compressed until surface tension reached  $35 \text{ mN m}$ , corresponding to the assembly of a monolayer on the surface of the solution that is in the gel phase (Figure 2.3). The Langmuir-Blodgett dip was then conducted by the wafer being slowly raised vertically out of the trough at  $5 \text{ mm/minute}$ . Barrier arms maintained the surface tension of the trough solution as the wafer was raised, so that monolayer packing was maintained. As the wafer was being raised the SAM headgroups came into contact with the DPPC headgroups that were assembled at the air:trough solution interface, and through polar interactions pulled the monolayer up from the trough, onto the SAM on the wafer (See Figure 2.3, First Dip). Once the wafer was fully raised from the trough, it was re-positioned horizontally above the trough, with the surface of the wafer being parallel to the surface of the solution.

For the second Langmuir-Schaefer dip, the trough was cleaned extensively to remove any lipid remaining from the first dip, until surface tension reached  $< 0.5 \text{ mN m}$ . Lipid for the outer leaflet (DPPC/PI(4,5) $\text{P}_2$  or POPC/PI(4,5) $\text{P}_2$  mix; 1 mg/ml in chloroform) was added dropwise to the trough surface until surface tension reached  $35 \text{ mN m}$ , corresponding to assembly of a monolayer at the air:trough solution interface. Then

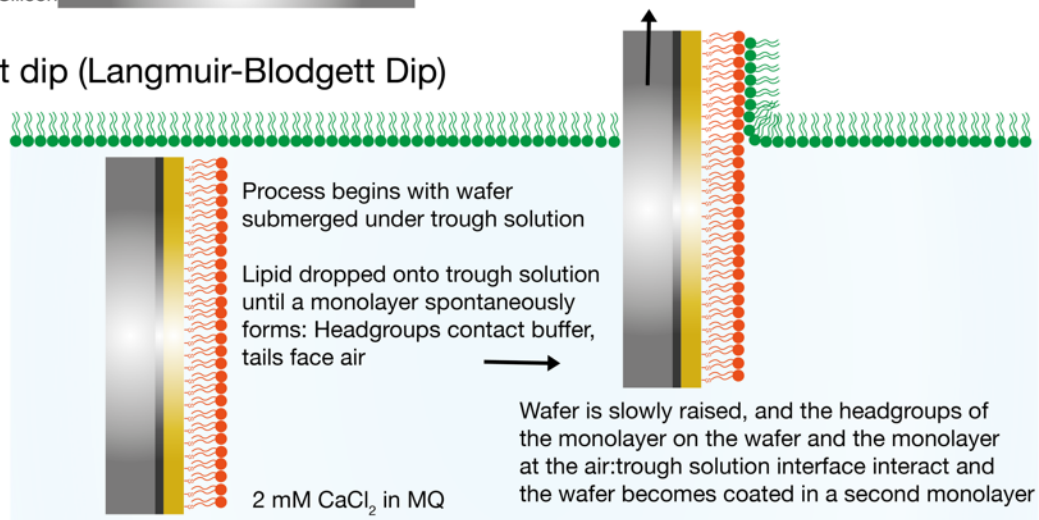
the wafer was aligned to be exactly parallel to the surface of the solution using a laser scanner, which simultaneously scans the trough solution and wafer surfaces and adjusts the wafer position until parallel with the solution surface. Once aligned, the wafer was slowly dipped (6 mm/minute) onto the monolayer at the air:trough solution interface. The hydrophobic tails on the wafer and at the trough surface meet in this dip, and the floating bilayer is formed (Figure 2.3; Second Dip).

The full chip was then assembled under the surface of the trough, (Figure 2.3). Lipids used for each bilayer, in the different experiments are detailed in Table 2.11.

## Initial state of wafer

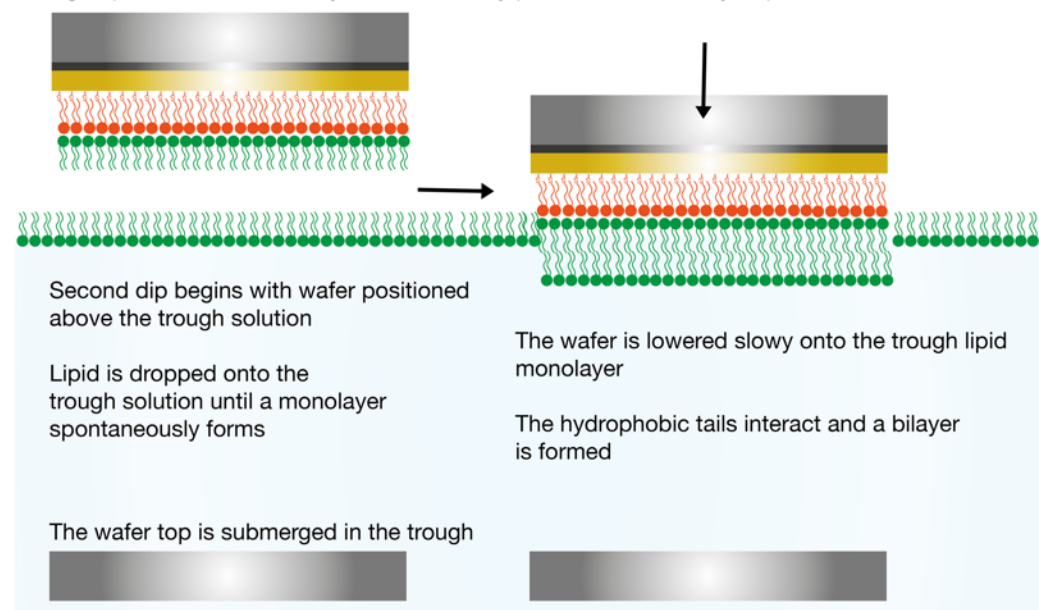


## First dip (Langmuir-Blodgett Dip)

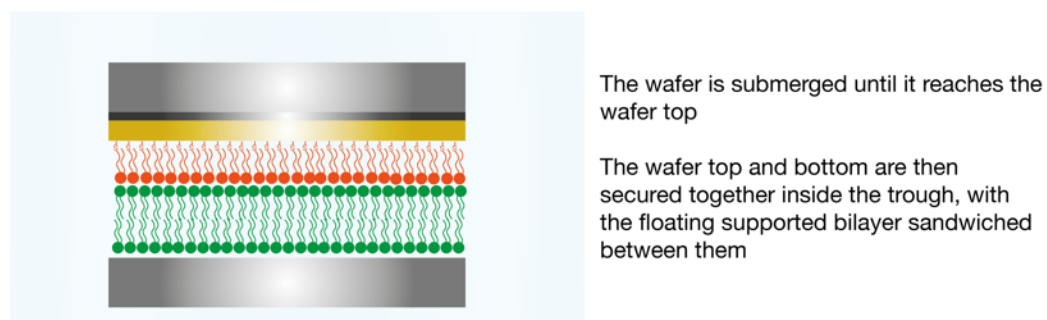


## Second dip (Langmuir-Schaefer Dip)

Wafer now has thiolated monolayer (orange), and second monolayer (green)  
Headgroups of the two monolayers associate by polar interactions, hydrophobic tails are stable in air



## Wafer with floating supported bilayer is assembled



### Figure 2.3 Formation of Floating Supported Bilayer (FSB) on silicon wafer for neutron reflectometry experiments

The schematic graphically depicts the process described in the preceding paragraphs. In the initial state of the wafer, the main silicon wafer, the chromium layer and the gold layer are labelled. The orange lipids form a self-assembled monolayer (SAM) that is covalently bound to the gold. The process of each dip is described briefly in the schematic.

#### *Experimental design*

Two experiments were performed using neutron reflectometry, the lipid mix for each is in Table 2.11. Deuterated mouse MLKL construct 1-169 was expressed in *E. coli* by Karyn Wilde at the National Deuteration facility as described previously (Duff et al., 2015), and purified as described in Section 2.2 for these experiments. For each experiment protein was diluted to 0.5 mg/ml in D<sub>2</sub>O LUV buffer (10 mM HEPES, pH 7.1 (equivalent to pH 7.5 in H<sub>2</sub>O), 135 mM KCl made up in D<sub>2</sub>O), in a volume of at least 2 ml, to inject onto the wafer. Neutron data was collected in three different buffer contrasts, either in D<sub>2</sub>O LUV buffer, H<sub>2</sub>O LUV buffer (10 mM HEPES, pH 7.5, 135 mM KCl, made up in H<sub>2</sub>O), or a mixture of these two buffers (38% D<sub>2</sub>O LUV buffer, 62% H<sub>2</sub>O LUV buffer), called protein matched water buffer (PMW).

**Table 2.11 Lipids used in each neutron reflectometry experiment**

|                     | Inner leaflet*                                                    | Outer leaflet                  |
|---------------------|-------------------------------------------------------------------|--------------------------------|
| <b>Experiment 1</b> | DPPC                                                              | 9:1 POPC:PI(4,5)P <sub>2</sub> |
| <b>Experiment 2</b> | D62DPPC<br>(deuterated lipid; Avanti<br>Polar Lipids cat. 860355) | 9:1 DPPC:PI(4,5)P <sub>2</sub> |

\*The inner leaflet is the leaflet of the bilayer closer to the wafer

#### *Neutron reflectometry data collection*

Neutron reflectometry experiments were performed on the Platypus time-of-flight neutron reflectometer at the Australian Centre for Neutron Scattering (James et al., 2011). The neutron source is the 20 MW OPAL Research Reactor and the instrument uses cold-neutrons that are moderated by a liquid deuterium cold-neutron source. As Platypus is a time-of-flight neutron reflectometer, neutrons are released in pulses by a double disc chopper system, and the 2D <sup>3</sup>He detector records their time-of-flight

(see Figure 2.4). The time-of-flight of the neutron is proportional to its wavelength through the following equation:

$$\lambda = \frac{ht}{m_n d}$$

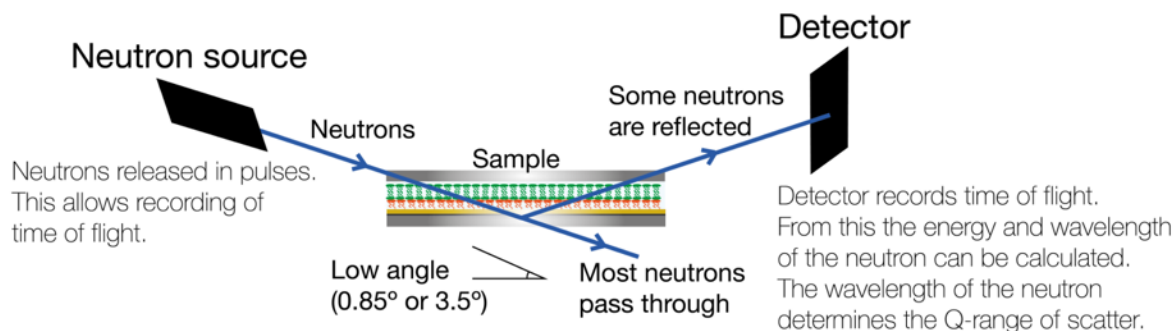
Where  $h$  is Planck's constant,  $t$  is time-of-flight,  $m_n$  is the mass of a neutron, and  $d$  is the distance from the first chopper to the detector. The wavelength of the neutrons that are reflected from the sample is used to determine the momentum transfer ( $Q$ ) range of the neutron scatter, as determined by the following equation:

$$Q = \frac{4\pi \sin\theta}{\lambda}$$

where  $\theta$  is the angle of reflection (either  $0.85^\circ$  or  $3.5^\circ$ ), and  $\lambda$  is the wavelength of the neutron.

Data were collected at two angles,  $0.85^\circ$  and  $3.5^\circ$  using 3.17 mm/0.68 mm and 13.06mm/2.81 mm collimation slits respectively. Reflectivity at angle 1 ( $0.85^\circ$ ) was measured for 10 minutes and angle 2 ( $3.5^\circ$ ) was measured for 60 minutes. As more neutrons are reflected at the lower angle that data is collected more quickly. At the higher angle, more neutrons pass through the sample and fewer are reflected, and thus a longer data collection time is required. The beam was aligned for each wafer to ensure the angle of reflection was accurate (See Figure 2.4 for schematic of data collection).

A minimum of 2 ml of protein sample was injected over prepared wafers for protein experiments. A High Performance Liquid Chromatography (HPLC) pump was used to flush over a minimum of 5 ml of each buffer, either D<sub>2</sub>O LUV buffer, PMW LUV buffer or H<sub>2</sub>O LUV buffer, before each buffer contrast was collected. Data is collected in multiple buffers to allow contrast matching (explained in Chapter 5, Figure 5.13), and to provide extra restraints for the data fitting and analysis steps.



### Figure 2.4 Neutron reflectometry data collection set up

Neutrons are released in timed pulses. The neutrons interact with the sample, and a small proportion are reflected from the sample and hit the detector, where their time-of-flight is recorded, while most neutrons pass through the sample.

#### *Data reduction and analysis*

Data collected from each angle was stitched together at the appropriate Q-range using Fizzy (ANSTO in-house software, (Nelson, 2010)). The lower angle ( $\theta$ ) gives data on the lower Q-range, and the higher angle gives data at higher Q range with some overlap in the middle. The data reduction software stitches the two datasets together at the overlap region, subtracts the background, re-bins the data to the appropriate instrument resolution, and corrects for detector efficiency. The final data are then presented as Reflectivity (reflected intensity/incident intensity) vs. Q. Reduced data was analysed using Motofit software (version 1.8) (Nelson and Prescott, 2019). The system is divided in a series of layers and each layer is fitted according to its three properties of thickness, scattering length density, and roughness. Scattering Length Density (SLD,  $\rho$ ) is analogous to a neutron's refractive index and is determined by the chemical and isotopic composition of the material according to:

$$\rho = \frac{\sum_{i=1}^n b_i}{V_m}$$

Where  $b_i$  is the coherent scattering length of  $i$ th of  $n$  atoms in a molecule and  $V_m$  is the molecular volume. Once an initial model is determined a theoretical reflectivity profile is calculated using the Abele's matrix method (Nelson, 2006) and compared to the collected data. The parameters of the model are then adjusted until the calculated reflectivity best matches the collected data through a least squares regression. The best fit to the data is determined by the model with the lowest  $\chi^2$

value. To achieve the minimum  $\chi^2$  a genetic algorithm is used so that realistic limits on each parameter can be imposed and to avoid trapping in local minima.

### **3.0 Studying MLKL Orthologues**

#### *3.1 Study rationale*

Published studies have shown differences in the basic biology of mouse MLKL and human MLKL and their role in necroptosis. The two orthologues form oligomers of different stoichiometry (Davies et al., 2018, Petrie et al., 2018), and human MLKL cannot reconstitute necroptosis in a mouse cell line (Tanzer et al., 2016), likely due to the fact that mouse RIPK3 cannot bind to human MLKL (Sun et al., 2012, Chen et al., 2013). Phosphomimetic mutations to the residues targeted by RIPK3 also show different phenotypes; S345D in mouse MLKL bypasses the need for RIPK3, and is autoactive in mouse cells (Murphy et al., 2013). The equivalent human phosphomimetic T357E-S358E, cannot kill human cells (Petrie et al., 2018). Furthermore, there are conformational differences between human and mouse MLKL in structures of the pseudokinase domain (Murphy et al., 2013, Murphy et al., 2014a) and the 4HB (Murphy et al., 2013, Su et al., 2014) (Figure 1.4, Figure 1.7).

Whilst there has been comparison of human and mouse MLKL in the literature, currently only one paper (Tanzer et al., 2016), and some reviews (Dondelinger et al., 2016, Shan et al., 2018), consider other MLKL orthologues. Studies of additional MLKL orthologues will potentially provide a better understanding of MLKL's basic mechanism of action. The differences between human and mouse pseudokinase structures have been suggested to represent alternate conformations of MLKL's molecular switch mechanism (Murphy et al., 2014a, Czabotar and Murphy, 2015). Other orthologue structures could yield further information on the conformational flexibility possible for MLKL proteins. The divergent sequence of an MLKL orthologue could affect the most stable conformation, enhancing the chance to capture an alternate conformation through techniques such as crystallography. The size of the MLKL protein, and even its individual domains such as the pseudokinase, are too large for use in more conventional techniques such as NMR to study protein dynamics. Whilst an orthologue structure with a new conformation would not constitute direct evidence that human or mouse MLKL could take on a similar conformation, it could serve as a starting point for other experiments to test MLKL's molecular switch mechanism.



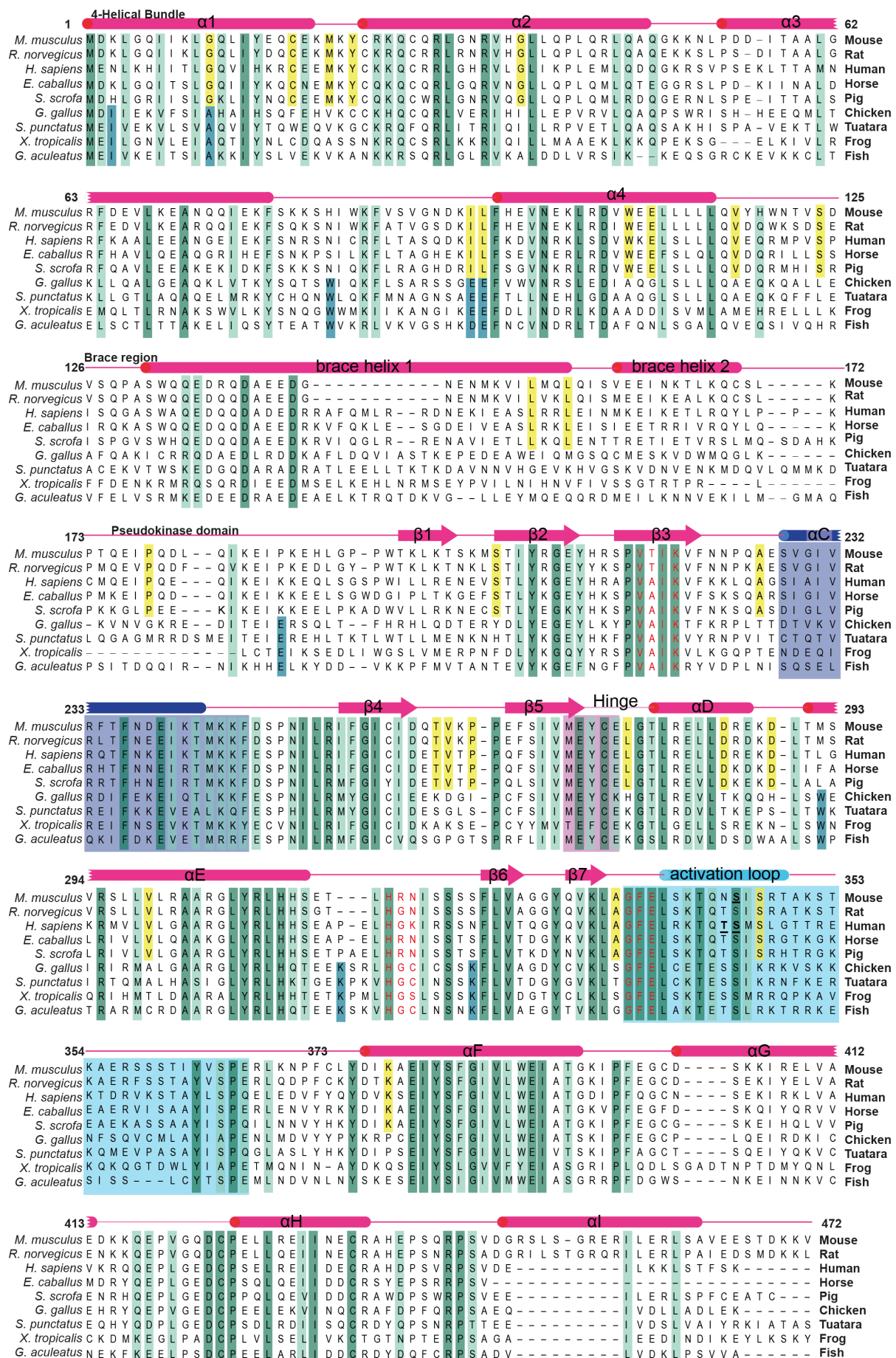
Though MLKL seems to have evolved to combat viral infection and enhance innate immune defences, it is surprisingly poorly conserved across animal classes (Dondelinger et al., 2016). The MLKL gene first appears in invertebrate animals, along with RIPK3-like genes. Through evolution, both genes have been lost in some animals in independent events. Birds for instance, retain the MLKL gene, however have lost RIPK3, and therefore have presumably evolved another activation mechanism for MLKL. The class *Carnivora* has lost MLKL but not RIPK3, this class includes animals such as cats and dogs. Marsupials have lost both RIPK3 and MLKL (Dondelinger et al., 2016). Why MLKL persists in some species and not in others is unknown. One study found a correlation between litter size and retention of the MLKL gene (Shan et al., 2018). They found that animals with large litter sizes and small time intervals between litters were more likely to have lost MLKL, with mouse and rat being conspicuous outliers. This group hypothesized that necroptosis may play a role in aborting unfit embryos, based on a few examples of *Mkl* or *Ripk3* genetic knockout rescuing the embryonic lethality associated with other mutations (Shan et al., 2018). Whilst this may be true for a few specific mutations, this hypothesis is yet to be borne out with more general examples. There may be other reasons why MLKL is important to animals with relatively few precious offspring, for instance having improved defence against viral infection may improve survival past juvenile stages, and allow genes to be passed on. Understanding the differences in activity, if there are any, between MLKL orthologues might help answer this question. For example, if some species' MLKLs are more active, perhaps this would correlate with living conditions that required a more alert innate immune system.

Finally, an important reason to better understand MLKL orthologues, and particularly their similarity to human MLKL, is that human MLKL is currently the target of drug discovery efforts. The necroptosis pathway in mouse appears to be divergent from human, and therefore an appropriate animal model will need to be found to investigate the safety and efficacy of lead compounds, for therapeutic programs to progress.

So far one paper has dealt with the activity of MLKL orthologues in cells (Tanzer et al., 2016). The study tested the cross compatibility of human and mouse MLKL in human and mouse cell lines, and found that the human N-terminal region (residues 1-180) could not kill in mouse cells whilst the equivalent mouse MLKL construct could. They went on to test if N-terminal region constructs from horse, chicken, fish (stickleback species) and frog MLKL could also kill in mouse cells. Horse and frog could, while chicken and fish could not. Some orthologue MLKLs were expressed recombinantly and tested in a liposome dye release assay. Chicken and frog MLKL could both permeabilise liposomes, despite their differing activity in cells. However, whether the orthologues could not kill in cells due to species incompatibility in the 4HB, or lack of activation stimuli, was not determined in that study.

To expand on the work from this previous study, and to better understand orthologue signalling and activation, nine full-length MLKL orthologues were chosen for further study. The orthologues included those from Tanzer et al. (2016): mouse, human, horse, chicken, frog and fish, with the addition of rat, pig, and tuatara MLKL. This selection of orthologues provides five mammalian MLKLs for comparison pig, horse, mouse, human and rat, plus example species from other vertebrate animal classes *Osteichthyes* (fish), *Reptilia* (tuatara), *Amphibia* (frog) and *Aves* (chicken). Rats (Barber, 2013), frogs (Blum and Ott, 2018, Lee-Liu et al., 2017), fish (Witten et al., 2017, Barber, 2013) and pigs (Mair et al., 2014) are all used as model organisms, and could be useful for drug discovery programs, should they behave similarly to human MLKL.

To compare the orthologues, a sequence alignment of the full-length MLKL proteins was performed (Figure 3.1).



### Figure 3.1 Sequence alignment of assayed orthologues

Sequence alignment of orthologues, scientific 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. Residues that are conserved with identity in mammalian MLKL are highlighted in yellow, and residues conserved with identity in non-mammalian MLKL are highlighted in teal. Regions equivalent to catalytic motifs on canonical kinases are coloured red, and residues of the  $\alpha$ C helix (dark blue) and activation loop (light blue) are highlighted. The domain architecture of mouse MLKL (PDB:4BTF) is illustrated above the alignment, lines are loops, cylinders are helices and arrows  $\beta$ -sheets. Residue numbering is for mouse MLKL.

From the sequence alignment, the best conserved region between the nine MLKL orthologues is the pseudokinase domain, followed by the 4HB. The brace region which links the 4HB to the pseudokinase domain is the most divergent (Figure 3.1). This could relate to the strict requirements for the 4HB to maintain membrane permeabilising ability, and for the pseudokinase domain to retain its molecular switch mechanism. The brace region has been shown to play important roles in oligomerisation and communication between the two domains. The sequence alignment suggests there is less stringency in the residues needed to perform this function, and multiple modes of oligomerisation and interdomain communication may exist between orthologues. The alignment also reveals many residues that are well conserved in mammals but differ in other orthologues (highlighted in yellow in Figure 3.1). This reflects mammals sharing a more recent common ancestor and could indicate that MLKL from these species will behave more similarly than those from other classes. All orthologues show a high degree of conservation of pseudocatalytic motifs; the VAIK motif, GFE (equivalent to DFG in a kinase) and HRN (equivalent to HRD in a kinase), although the non-mammalian pseudokinases have a cysteine or serine in place of the asparagine in the HRN motif (Figure 3.1). Even though birds lack a RIPK3 gene, chicken MLKL is not obviously more divergent compared to other orthologues. Its brace region does have two motifs (IQMG and VNVG) similar to the consensus sequence of the RIPK1 and RIPK3 RHIM domains (IQIG/VQVG) (Sun et al., 2002b, Mompeán et al., 2018) that the eight other MLKLs do not have (Figure 3.1). However, these motifs are not conserved in all birds (conserved in turkey, but not flycatcher or rifleman, from Uniprot, data not shown).

**Table 3.1 Sequence identity of orthologues over different MLKL domains**

| Species        | Mouse<br>Identity (Similarity) % |             |             | Human<br>Identity (Similarity) % |             |             |
|----------------|----------------------------------|-------------|-------------|----------------------------------|-------------|-------------|
|                | Full-length                      | 4HB+brace   | PsK         | Full-length                      | 4HB+brace   | PsK         |
| <b>Mouse</b>   | -                                | -           | -           | 61.8 (84.0)                      | 51.7 (82.6) | 69.3 (86.6) |
| <b>Rat</b>     | 85.8 (96.3)                      | 82.7 (97.1) | 87.9 (96.2) | 62.2 (84.2)                      | 53.9 (80.3) | 68.1 (87.9) |
| <b>Pig</b>     | 60.2 (83.2)                      | 52.4 (77.5) | 66.1 (87.3) | 63.0 (88.6)                      | 57.8 (87.2) | 68.3 (91.8) |
| <b>Horse</b>   | 63.0 (83.3)                      | 56.1 (78.1) | 67.6 (87.1) | 65.3 (88.3)                      | 57.9 (87.4) | 70.2 (89.0) |
| <b>Chicken</b> | 39.4 (66.6)                      | 31.7 (66.5) | 50.8 (74.8) | 40.2 (70.4)                      | 32.4 (69.0) | 50.5 (76.0) |
| <b>Tuatara</b> | 39.8 (70.4)                      | 29.1 (69.8) | 49.1 (76.2) | 41.0 (73.6)                      | 30.5 (70.2) | 52.9 (79.7) |
| <b>Frog</b>    | 36.0 (71.1)                      | 23.5 (68.7) | 45.7 (74.3) | 35.9 (65.8)                      | 23.8 (64.5) | 45.2 (70.3) |
| <b>Fish</b>    | 35.1 (65.9)                      | 28.1 (63.7) | 41.8 (71.1) | 35.8 (67.8)                      | 24.2 (62.9) | 44.4 (71.2) |

Alignments performed with lalign (<https://www.ebi.ac.uk/Tools/psa/lalign/>) (Madeira et al., 2019). Sequence accession codes can be found in Appendix I.

Comparison of percent identity between orthologues in Table 3.1 demonstrates the higher similarity between mammalian orthologues as compared to other vertebrate classes. Rat and mouse shared very high similarity and identity, and apart from rat, each mammalian orthologue showed a comparable level of sequence conservation between the human and mouse MLKL. Fish MLKL shared the least homology with mouse and human MLKL at only ~35% identity.

To test the ability of the MLKL orthologues to reconstitute necroptosis, MLKL orthologues were introduced into either *Mkl<sup>-/-</sup>* mouse (mouse dermal fibroblast; MDF) or *MLKL<sup>-/-</sup>* human (U937) cell lines. MDFs and U937s are used frequently to study necroptosis and are sensitive to necroptosis induction through the TNF pathway (Davies et al., 2018, Hildebrand et al., 2014, Murphy et al., 2013). Use of these cell lines permits direct comparison of results with previous studies, as cell type differences have been noted to influence cell death assay results (Tanzer et al., 2016). The MDF cell lines are derived from *Mkl<sup>-/-</sup>* mice (Murphy et al., 2013), so biological replicate cell lines are used for cell experiments (called #391, #392 or #393). U937s are an established human monocytic cell line (Chanput et al., 2015) and therefore comprise only one cell line for biological replicates.

### 3.2 Introducing MLKL orthologues constructs into mouse and human cell lines

*Mkl1*<sup>-/-</sup> MDFs and *MLKL*<sup>-/-</sup> U937 cell lines were infected with lentivirus, encoding a puromycin resistance gene to allow for selection, and doxycycline (dox)-inducible MLKL orthologue constructs. Orthologues with divergent sequence at the 3H1 MLKL antibody (Murphy et al., 2013) epitope were expressed as C-terminal FLAG tag fusions (Figure 3.2).

|                | 3H1 peptide: C Q P A S W Q Q E D R Q D A E E D |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
|----------------|------------------------------------------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| <b>Mouse</b>   | W                                              | N | T | V | S | D | V | S | Q | P | A | S | W | Q | Q | E | D | R | Q | D |
| <b>Rat</b>     | W                                              | K | S | D | S | E | V | S | Q | P | A | S | W | Q | Q | E | D | Q | Q | D |
| <b>Human</b>   | R                                              | M | P | V | S | P | I | S | Q | G | A | S | W | A | Q | E | D | Q | Q | D |
| <b>Horse</b>   | R                                              | I | L | L | S | S | I | R | Q | K | A | S | W | Q | Q | E | D | Q | Q | D |
| <b>Pig</b>     | R                                              | M | H | I | S | R | I | S | P | G | V | S | W | H | Q | E | D | Q | Q | D |
| <b>Chicken</b> | K                                              | Q | A | L | L | E | A | F | Q | A | K | I | C | R | R | Q | D | A | E | D |
| <b>Tuatara</b> | K                                              | Q | F | F | L | E | A | C | E | K | V | T | W | S | K | E | D | G | Q | D |
| <b>Frog</b>    | R                                              | E | L | L | L | K | F | F | D | E | N | K | R | M | R | Q | S | Q | R | D |
| <b>Fish</b>    | S                                              | I | V | Q | H | R | V | F | E | L | V | S | R | M | K | E | D | E | E | D |

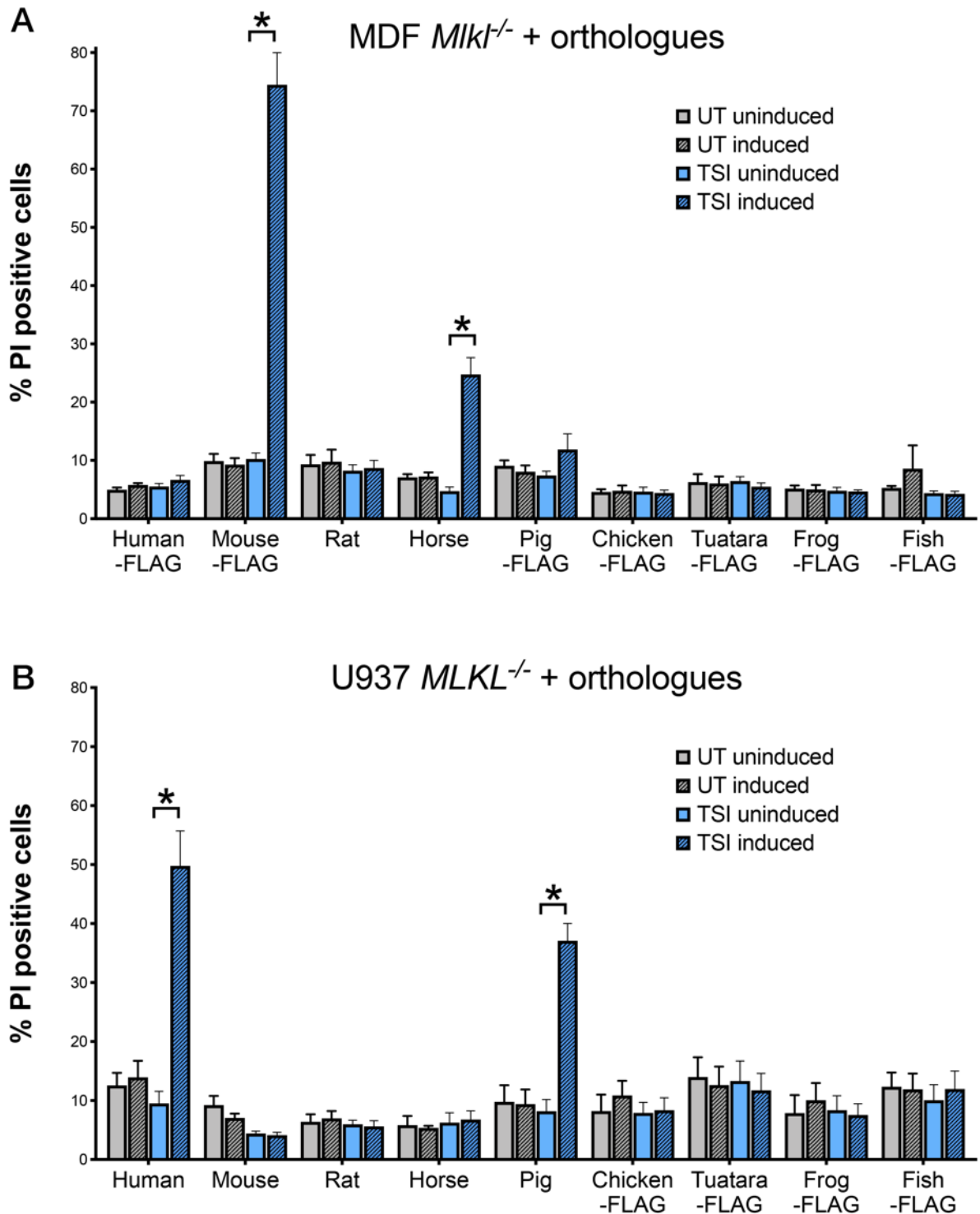
**Figure 3.2 Epitope compatibility with MLKL antibody**

The peptide that antibody 3H1 was raised against is compared to the equivalent brace region sequence of MLKL orthologues to determine which constructs to express as C-terminal FLAG fusions. Residues conserved across all species are highlighted in green.

Chicken, tuatara, frog and fish MLKL were made as FLAG fusions based on their divergent sequence at the 3H1 epitope (Figure 3.2). Horse MLKL has been shown to be detected by 3H1 (Tanzer et al., 2016). Pig MLKL was initially made without a FLAG tag but was not detectable by western blot in multiple independent experiments (data not shown), despite high sequence similarity to the peptide (Figure 3.2). Experiments were repeated with a FLAG tagged pig MLKL in MDFs, but efforts to generate a FLAG tagged pig MLKL construct in U937s failed, so the results for the untagged pig MLKL protein are shown. To ensure the FLAG tag did not perturb MLKL function, mouse MLKL-FLAG and human MLKL-FLAG control constructs were introduced into MDFs alongside the orthologue constructs, and their death assay results compared to published data on the untagged proteins (Murphy et al., 2013, Tanzer et al., 2016).

### *3.3 Testing necroptosis reconstitution by MLKL orthologues in human and mouse cells*

Cells harbouring orthologue constructs were treated with or without dox to induce orthologue expression, then treated with or without necroptotic stimuli (TNF, to stimulate the TNF pathway, Smac-mimetic to block cIAPs and induce formation of Complex IIa, and IDN a caspase-8 inhibitor to block signalling through Complex IIa and induce necroptosis; TSI, see Figure 1.1). This experimental setup can determine if orthologue MLKL is auto-active (kills without TSI stimulus) or if they can reconstitute necroptosis with TSI stimulation (Figure 3.3). A control group of cells were also treated with T and S to induce apoptosis as a control for normal TNF pathway signalling in all death assays. Cell lines were only used if TS death induced after 24 hours was greater than 30% (data not shown). Cell death was measured 24 hours after TSI treatment, by uptake of a DNA binding dye, propidium iodide (PI), using flow cytometry.

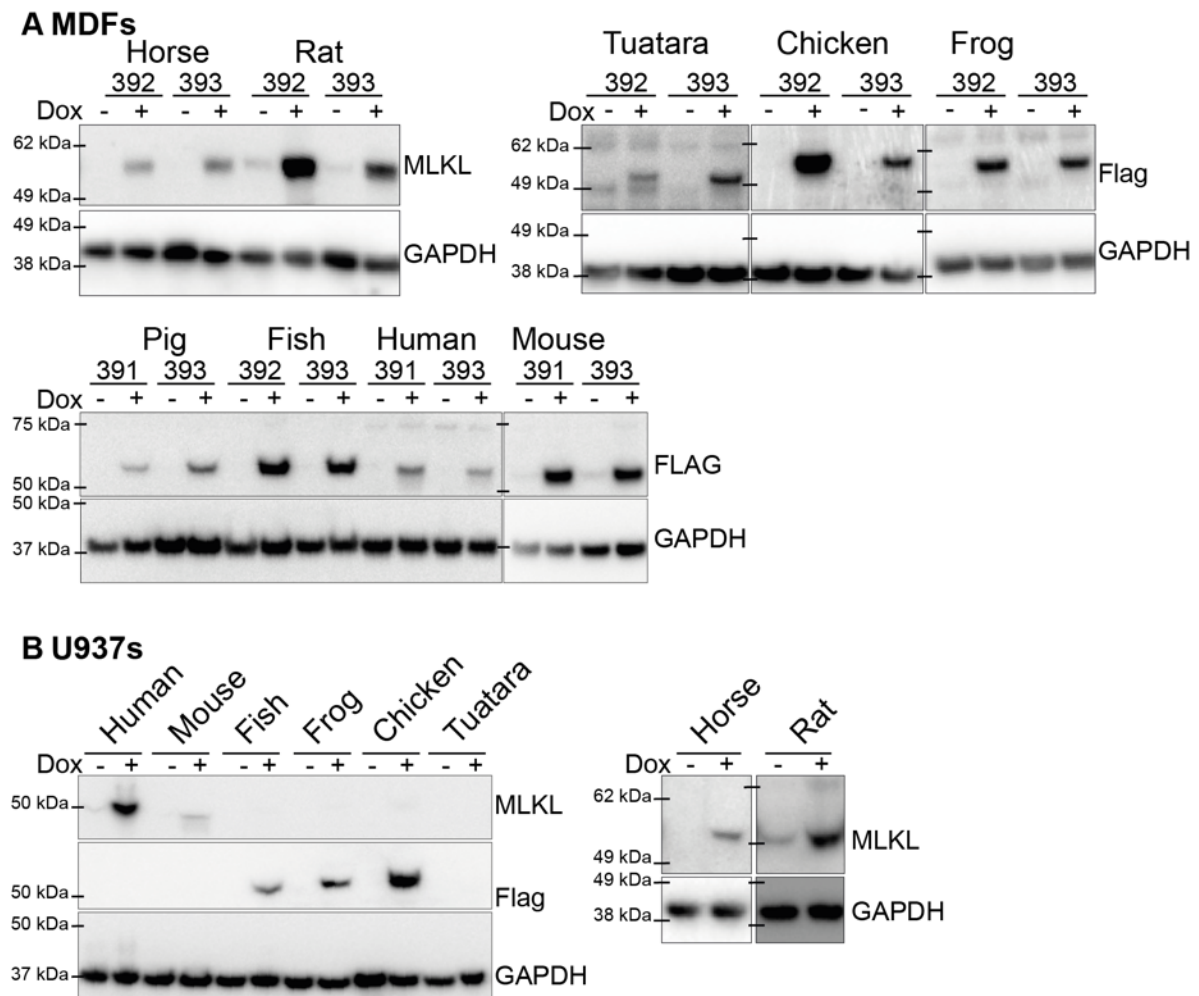


**Figure 3.3 Certain orthologues can reconstitute necroptosis in either mouse or human cell lines, but not both**

Doxycycline was used to induce expression of orthologue MLKL constructs, and TSI was used as necroptotic stimulus. Death was measured by propidium iodide (PI) uptake by flow cytometry after 24 hours. **A** MDF assays were conducted in biological duplicate (i.e. 2 MDF cell lines) in at least 3 independent experiments (n=6-10). **B** U937 was assayed with one biological replicate in at least 3 independent experiments (n=3-5). All error bars are  $\pm$  SEM. Significant differences ( $p < 0.05$ ) calculated by T-test between uninduced and induced samples are indicated with an asterisk.



Most orthologues could not reconstitute necroptosis in either mouse or human cells (Figure 3.3 A, B). Pig MLKL reconstituted necroptosis in U937 cells treated with dox and TSI ( $37.1 \pm 2.97\%$  cell death), but not MDF cells. In MDFs, the pig MLKL-FLAG (in Figure 3.3) and pig MLKL (data not shown) constructs behaved comparably, and did not significantly induce cell death upon TSI stimulation. Horse MLKL reconstituted death in MDFs (dox, TSI  $24.7 \pm 2.90\%$ ) but not U937 cells. In TSI treated cells in the absence of dox, there was no increase in cell death above baseline, so necroptosis was due to the presence of the orthologue constructs. In cells treated with dox but not TSI, there was no significant increase in cell death compared to uninduced cells, showing that none of the orthologue constructs are auto-active. Mouse MLKL-FLAG and human MLKL-FLAG behaved in agreement with published data (Tanzer et al., 2016, Murphy et al., 2013); mouse MLKL induced necroptosis robustly, but human MLKL was unable to reconstitute necroptosis in mouse cells. This demonstrated the FLAG tag did not affect MLKL function. To ensure deficits in necroptosis reconstitution were not attributable to defects in protein expression, protein expression was verified by western blot. (Figure 3.4). All orthologues, except for tuatara in U937s, were expressed, although no western blot could be performed for pig MLKL in U937s due to a lack of antibody compatibility (Figure 3.4). The western blot showed different intensity bands for many of the orthologues, which could be due to species differences affecting protein stability or folding. The differences in expression between MDF replicate cell lines could be due to differing lentiviral infection efficiencies for each replicate, resulting in copy number variation of orthologue genes incorporated into each cell line. Despite this variation, orthologues that showed relatively low expression levels could still reconstitute signalling (compare horse MLKL expression to rat, in Figure 3.4 A), and those with some of the highest expression levels such as chicken MLKL, could not reconstitute necroptosis.



**Figure 3.4 Most orthologues can be expressed in mouse and human cells**

Cells were treated with dox for three hours, cell extracts were run on SDS-PAGE and western blotting performed to detect MLKL levels. **A** MDFs *MLKL*<sup>-/-</sup> cell lines reconstituted with orthologues of MLKL. 391 392 and 393 are biological replicate cell lines. **B** U937 *MLKL*<sup>-/-</sup> cell line reconstituted with orthologues of MLKL. Representative blot shown of two independent experiments.

Fish, frog and chicken MLKL could not reconstitute necroptosis in human or mouse cell lines, and tuatara could not reconstitute necroptosis in mouse cell lines in which it was expressed (Figure 3.3 A). Pig and horse MLKL are both mammalian orthologues with similar percentage sequence identity both to human and mouse MLKL (Table 3.1), however pig could only reconstitute necroptosis in human cells, and horse could only do so in mouse cells. Rat MLKL, which has nearly identical sequence to mouse in important structural motifs such as the  $\alpha$ C helix and the activation loop (Figure 3.1), could not reconstitute death in mouse cells. Comparing sequences and percentage identity could explain why some distantly related orthologues cannot reconstitute

necroptosis in mouse and human cell lines. For closely related orthologues, however, merely comparing percentage similarity does not provide an explanation for the death assay results. Therefore, a structural biology approach was used to compare and contrast the orthologues further.

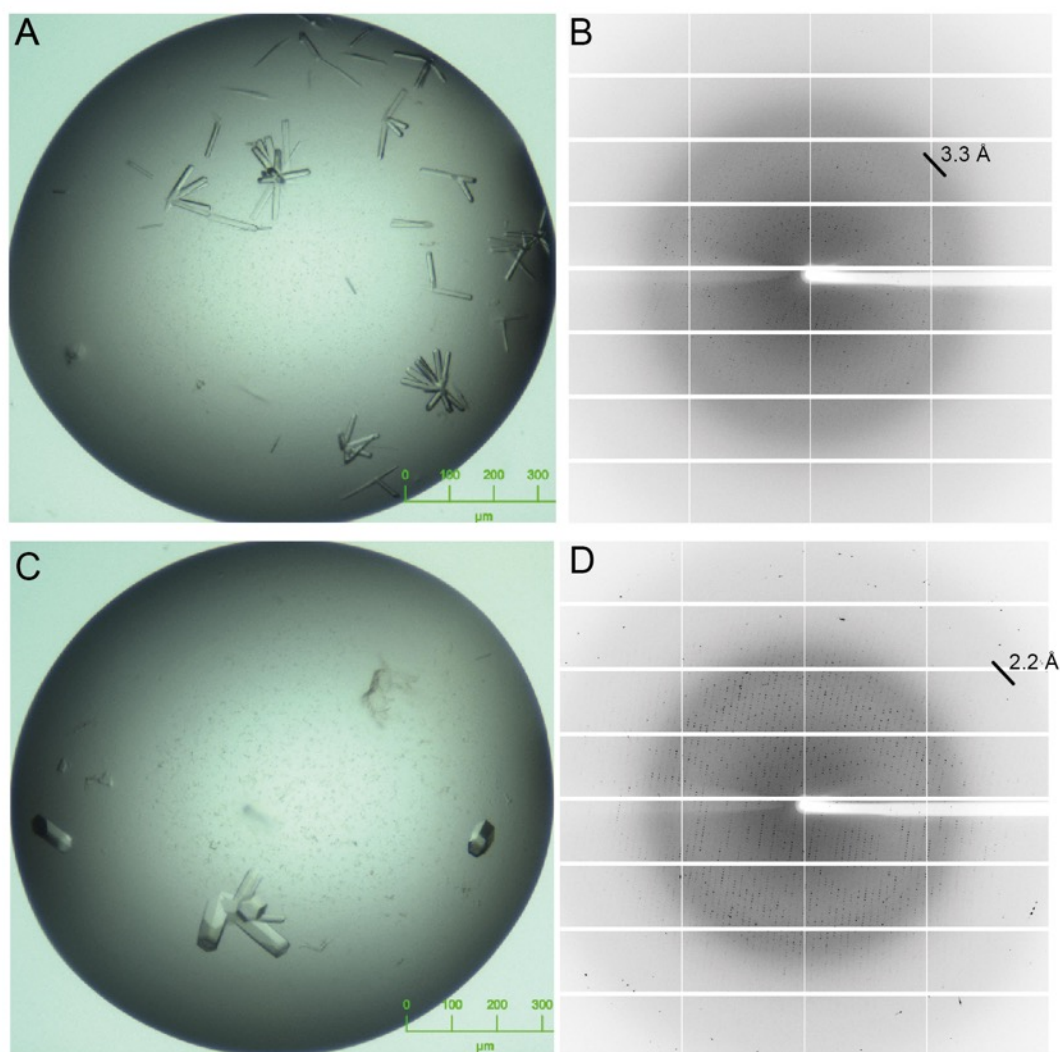
### *3.4 Determining structures of MLKL orthologues*

The structures of full-length mouse MLKL (Murphy et al., 2013) and the human MLKL pseudokinase domain (Murphy et al., 2014a, Xie et al., 2013) have been solved, and display differences in conformation in key structural motifs (See Figure 1.4). For instance, the mouse MLKL activation loop takes on an atypical helical conformation, while the human MLKL activation loop is a flexible loop. By solving the structures of other MLKL orthologues we hoped to better understand their varying ability to reconstitute necroptosis in mouse and human cells.

Pig, horse, and rat MLKL were chosen as the focus of structural determination efforts, as examples of orthologues that either kill in human cells (pig), kill in mouse (horse) or kill in neither despite high sequence homology (rat). Full-length and pseudokinase domain-only constructs were cloned and expression trialled in Sf21 insect cells. Soluble protein was produced for rat MLKL full-length, and pseudokinase domain and for the horse MLKL pseudokinase domain, and crystallisation trials were undertaken at the CSIRO Collaborative Crystallisation Centre ([www.csiro.au/C3](http://www.csiro.au/C3)), for both of these constructs.

#### *The rat pseudokinase crystal structure*

The rat pseudokinase protein crystallised in multiple conditions (Figure 3.5). The best crystals were flash frozen in cryo-protectant and taken to the Australian Synchrotron MX2 beamline for data collection.



**Figure 3.5 Crystals and diffraction of rat pseudokinase domain**

**A** The initial crystals for the rat pseudokinase grew in 0.2 M magnesium chloride, 25% (w/v) polyethylene glycol 3350, 0.1 M bis-tris chloride pH 5.5, they diffracted to 3.3 Å at the Australian Synchrotron (**B**). Optimisation yielded the crystals in **C**, (20 mM manganese(II) chloride, 25% (v/v) polyethylene glycol 400, 0.1 M sodium MES pH 6.5), that diffracted to 2.2 Å resolution (**D**).

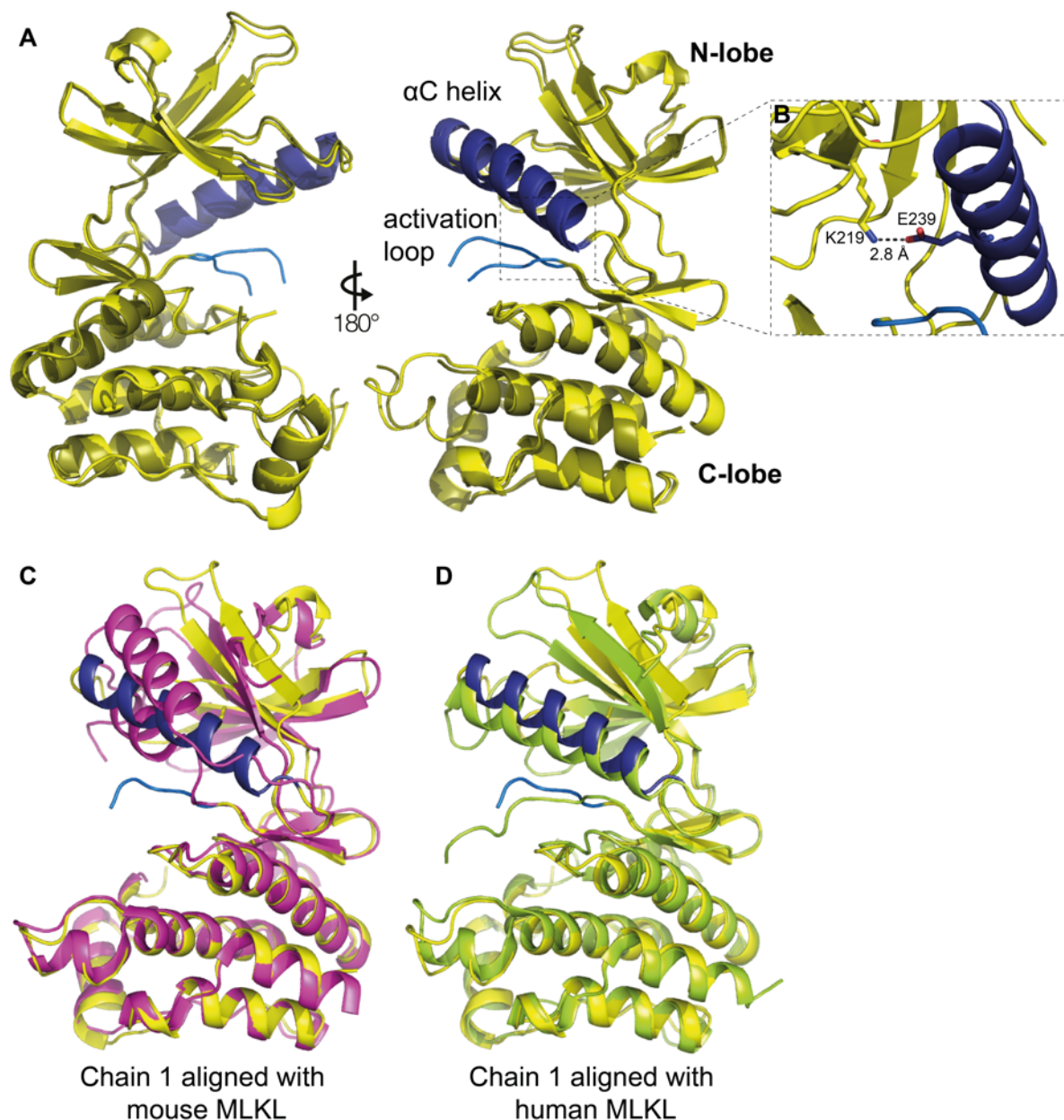
The rat pseudokinase structure was solved using data collected from the crystals in Figure 3.5 A, XDS was used for data reduction, integration and scaling. Both the human and mouse pseudokinase structures were trialled as models for molecular replacement using Phenix Phaser-MR; the human model provided the best solution. The higher resolution data from Figure 3.5 D was subsequently collected and merged with the original data set, and the R-free set extended to the higher resolution shells.

**Table 3.2. Rat pseudokinase domain structure statistics**

(Statistics for the highest-resolution shell are shown in parentheses)

|                                                          |                             |
|----------------------------------------------------------|-----------------------------|
| Resolution range (Å)                                     | 47.51 - 2.192 (2.27 - 2.20) |
| Space group                                              | P 6 <sub>1</sub> 2 2        |
| Unit cell a, b, c (Å), $\alpha$ , $\beta$ , $\gamma$ (°) | 190.0 190.0 77.76 90 90 120 |
| Total reflections                                        | 862802 (83143)              |
| Unique reflections                                       | 42689 (4093)                |
| Multiplicity                                             | 20.2 (20.3)                 |
| Completeness (%)                                         | 100 (98)                    |
| Mean I/sigma(I)                                          | 24.83 (3.04)                |
| Wilson B-factor                                          | 40.3                        |
| R-merge                                                  | 0.1024 (1.24)               |
| R-meas                                                   | 0.105 (1.271)               |
| R-pim                                                    | 0.02322 (0.2786)            |
| CC1/2                                                    | 1 (0.89)                    |
| CC*                                                      | 1 (0.97)                    |
| Reflections used in refinement                           | 42667 (4080)                |
| Reflections used for R-free                              | 4264 (406)                  |
| R-work                                                   | 0.1852 (0.2348)             |
| R-free                                                   | 0.2161 (0.2913)             |
| CC(work)                                                 | 0.960 (0.902)               |
| CC(free)                                                 | 0.938 (0.828)               |
| Number of non-hydrogen atoms                             | 4304                        |
| macromolecules                                           | 4112                        |
| ligands                                                  | 6                           |
| Protein residues                                         | 512                         |
| RMS(bonds) (Å)                                           | 0.009                       |
| RMS(angles) (°)                                          | 1.0                         |
| Ramachandran favored (%)                                 | 96.6                        |
| Ramachandran allowed (%)                                 | 3.4                         |
| Ramachandran outliers (%)                                | 0                           |
| Rotamer outliers (%)                                     | 1.1                         |
| Clashscore                                               | 5.92                        |
| Average B-factor                                         | 51.33                       |
| macromolecules                                           | 51.21                       |
| ligands                                                  | 91.83                       |
| solvent                                                  | 52.73                       |

The crystals were in space group P 6<sub>1</sub> 2 2 with two chains in the asymmetric unit. Model building in Coot and refinement in Phenix.refine led to a final model with an R-work and R-free of 18.52% and 21.61% respectively (Table 3.2).



**Figure 3.6 Crystal structure of rat MLKL pseudokinase domain**

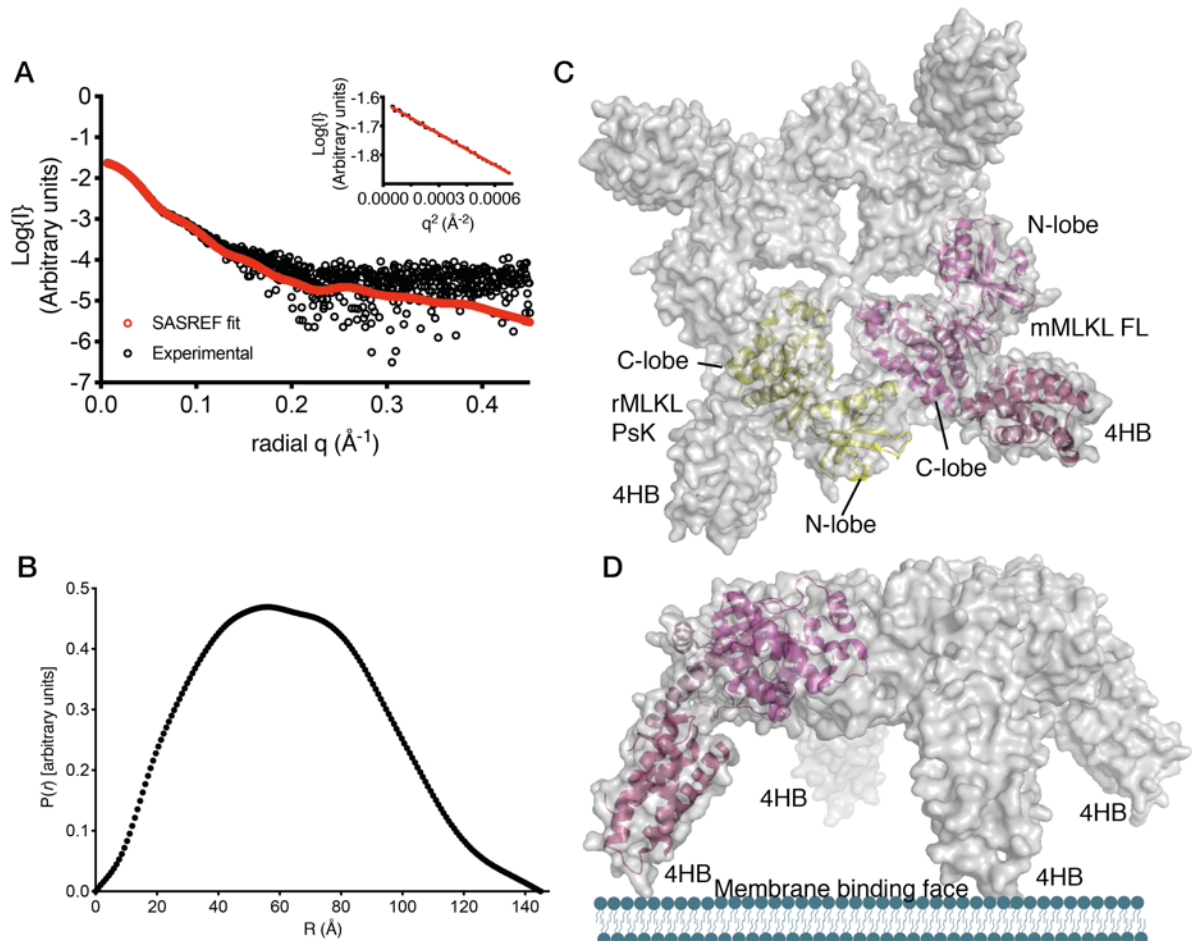
**A** The structure has two molecules in the asymmetric unit shown here aligned in yellow and olive. The N-lobe, C-lobe,  $\alpha$ C helix (deep blue) and activation loop (light blue) are labelled. **B** Shows the salt bridge interaction between E239 of the  $\alpha$ C helix and K219 of the VAIK motif. **C** Rat MLKL pseudokinase (Chain 1) aligned with the mouse MLKL pseudokinase domain from the full-length mouse MLKL crystal structure (PDB:4BTF). **D** Rat MLKL pseudokinase (Chain 1) aligned with the human MLKL pseudokinase domain crystal structure (PDB:4MWI).

Both chains in the asymmetric unit had very similar folds, and aligned with an RMSD of 0.539 Å. The rat structure had a largely unstructured activation loop helix (Figure 3.6 A), similar to the human MLKL structure (Figure 3.6 D). E239 of the  $\alpha$ C helix held a salt-bridge interaction with K219 (Figure 3.6 B) which is also seen in the human



structure between equivalent residues, but not in the mouse structure, where Q343 of the activation loop helix takes the place of the glutamate in the  $\alpha$ C helix (Murphy et al., 2013) (Figure 1.4). The rat pseudokinase domain structure aligned to the human pseudokinase with an RMSD of 0.962 Å, and to the mouse with an RMSD of 1.027 Å. This is due to the common position of the  $\alpha$ C helix seen in both the human and rat structure, and the rat structure's lack of an activation loop helix, that is seen in the mouse structure (Figure 3.6 C, D).

Diffraction crystals of full-length rat MLKL were not obtained. The protein eluted off gel filtration in two species, a monomer and an oligomer. The oligomer was taken for SAXS analysis at the Australian Synchrotron, to further compare with mouse and human MLKL.



**Figure 3.7 SAXS analysis of rat MLKL full-length oligomer in solution**

**A** Scattering profile of averaged and background subtracted data from apex of inline size exclusion peak of rat MLKL full-length oligomer (black circles). Theoretical scatter values calculated from the SASREF rigid body fit with P4 symmetry are shown

in red circles. The P4 symmetry fit has a  $\chi$  value of 0.39. Inset: Guinier analysis of experimental SAXS data for  $q$ . The linearity of the plot indicates the protein is not aggregated. **B** The  $P(r)$  real space interatomic distribution for rat MLKL full-length, calculated with GNOM, with maximum particle dimension ( $D_{\max}$ ) of 140 Å. **C** Rigid body modelling of the full-length rat MLKL tetramer calculated from the monomeric mouse MLKL crystal structure coordinates (PDB:4BTF) using SASREF. The surface of the tetramer model calculated by SASREF is shown in grey. A cartoon representation of the mouse MLKL full-length crystal structure (mMLKL FL, pink), that was used as the envelope to calculate the model is overlaid with one protomer, and the 4HB, brace helices and N-lobe are labelled. The structure of the rat MLKL pseudokinase domain is aligned to the model in yellow (rMLKL PsK), the C-lobe and N-lobe are indicated. **D** The tetramer model oriented with the 4HB on one face, which would presumably form the membrane binding face.

**Table 3.3 Rat MLKL full-length SAXS data collection and analysis statistics**

| Data-collection parameters                |                                                                                                                                  |
|-------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------|
| Instrument                                | Australian Synchrotron SAXS/WAXS beamline                                                                                        |
| Beam geometry                             | 120 $\mu\text{m}$ x 400 $\mu\text{m}$ source                                                                                     |
| Wavelength (Å)                            | 1.033                                                                                                                            |
| Exposure time                             | 1 second exposures                                                                                                               |
| Temperature (K)                           | 285                                                                                                                              |
| $q$ range (Å <sup>-1</sup> ) <sup>a</sup> | 0.0069 to 0.45                                                                                                                   |
| Protein concentration                     | 60 $\mu\text{l}$ of 3.85 mg/ml protein via inline gel filtration chromatography<br>0.2M NaCl, 20mM HEPES pH 7.5, 5% v/v glycerol |
| Structural parameters                     |                                                                                                                                  |
| $I(0)$ (cm <sup>-1</sup> ) [from $P(r)$ ] | $0.02380 \pm 0.0001$                                                                                                             |
| $R_g$ (Å) [from $P(r)$ ]                  | $49.06 \pm 0.244$                                                                                                                |
| $D_{\max}$ (Å)                            | 145                                                                                                                              |
| $I(0)$ (cm <sup>-1</sup> ) (from Guinier) | $0.02398 \pm 0.0002$                                                                                                             |
| $R_g$ (Å) (from Guinier)                  | $49.7 \pm 0.624$                                                                                                                 |
| Software employed                         |                                                                                                                                  |
| Primary data reduction                    | Scatterbrain (Australian Synchrotron)                                                                                            |
| Data processing                           | PRIMUS, GNOM                                                                                                                     |
| Computation of model intensities          | CRYSOL                                                                                                                           |
| Rigid body fitting                        | SASREF                                                                                                                           |

<sup>a</sup>  $q$  is the magnitude of the scattering vector, which is related to the scattering angle ( $2\theta$ ) and the wavelength ( $\lambda$ ) as follows:  $q = (4\pi/\lambda)\sin\theta$



SAXS analysis was performed using concentrated protein from an oligomeric peak of rat MLKL full-length off size exclusion chromatography (SEC), which was loaded onto an inline SEC column and SAXS data was collected on the peak that eluted. The scatter displayed a linear Guinier region which indicated that protein aggregates did not contribute to the scatter (Figure 3.7). The radius of gyration ( $R_g$ ) predicted by the  $P(r)$  distribution and Guinier analysis were in good agreement at 49.7 Å and 49.1 Å respectively (Table 3.3). The  $D_{max}$  predicted from the  $P(r)$  curve was 145 Å. SASREF was used to calculate a rigid body model for the scattering data using the full-length mouse structure as a model (Figure 3.7 C, D). Multiple symmetries were trialled (see Table 3.4) and the tetramer model, which fit the data best is depicted in Figure 3.7. In the SASREF model the pseudokinase domain of rat MLKL forms the tetramer contact points (Figure 3.7 C). In the model the 4HB domains all face one direction, which could correspond to a membrane binding face of the oligomer (Figure 3.7 D). Importantly the SASREF model is restricted by the starting model, in this case the mouse full-length monomer crystal structure, that is used for fitting. The mouse full-length monomer model may not represent the conformation of the rat full-length protein in the context of a tetramer.

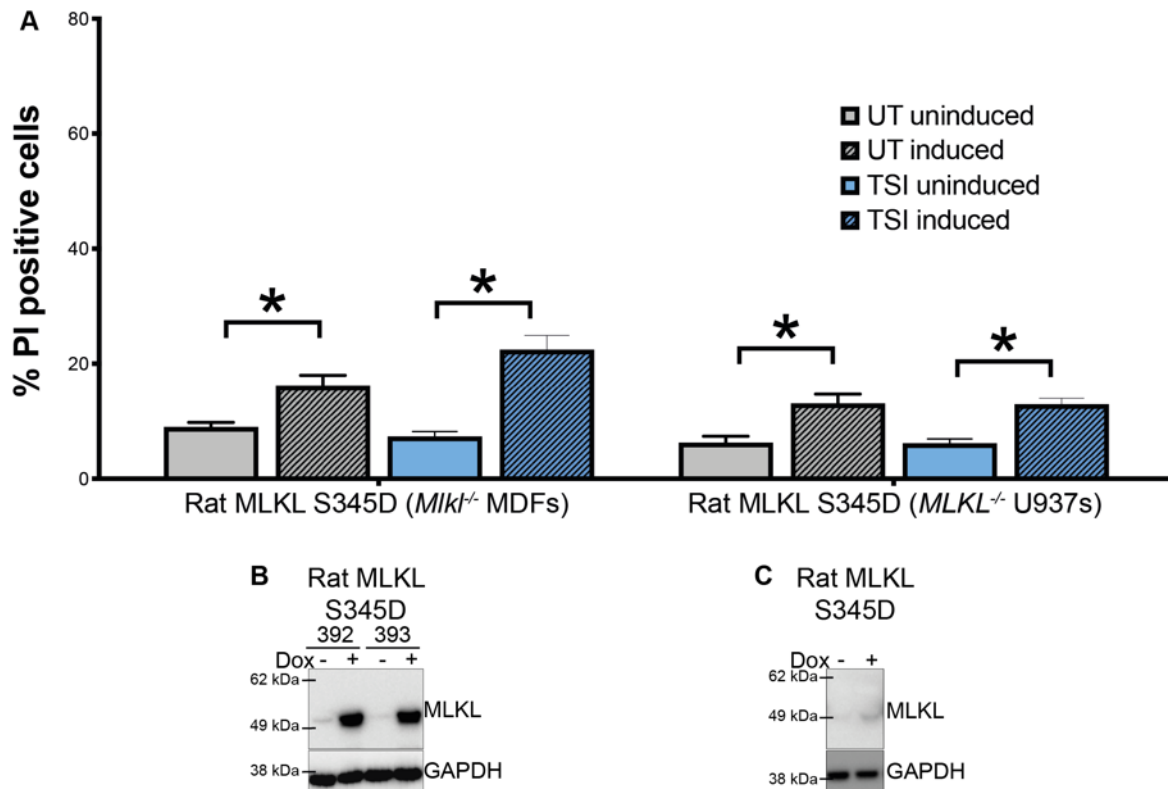
The rat MLKL full-length protein elutes from size exclusion chromatography at a more similar elution volume to human MLKL than to mouse MLKL full-length (data not shown), and our group suspect that the human MLKL forms a tetramer and mouse MLKL forms a trimer in solution. Therefore, multiple possible symmetries for rigid body modelling were trialled with SASREF to see which sized oligomer fitted the data better.

**Table 3.4 Chi values for rat MLKL with different symmetry**

| Symmetry          | $\chi$ value of best fit |
|-------------------|--------------------------|
| Monomer (CRY SOL) | 5.56                     |
| P2 (dimer)        | 2.29                     |
| P3 (trimer)       | 0.49                     |
| P4 (tetramer)     | 0.39                     |

The tetramer model fitted the scattering data very well ( $\chi=0.39$ ), coming close to an optimal  $\chi$  value of 0.3 (Table 3.4). The trimer model also exhibited a very good fit with  $\chi$  value of 0.49. The tetramer model is depicted above because it has the best fit, but more experiments are needed other than SAXS to distinguish between the trimer and tetramer models, similar to the case with mouse MLKL (see Chapter 4).

Overall the rat structural data indicates that its conformation is more similar to human than to mouse, and even may form a tetramer like human MLKL (Petrie et al., 2018). Rat MLKL shares a high sequence similarity with mouse in the activation loop, the site of RIPK3 phosphorylation. The human phosphomimetic mutant T357E-S358E, is inactive in human cells, whereas the mouse phosphomimetic mutant (S345D) is autoactive. The phosphomimetic construct rat MLKL S345D was introduced into *Mlkl*<sup>-/-</sup> MDFs and *MLKL*<sup>-/-</sup> U937s using the same lentiviral system as in Figure 3.3, and tested in death assays to determine if it would act more like the human or the mouse phosphomimetic in cells (Figure 3.8)



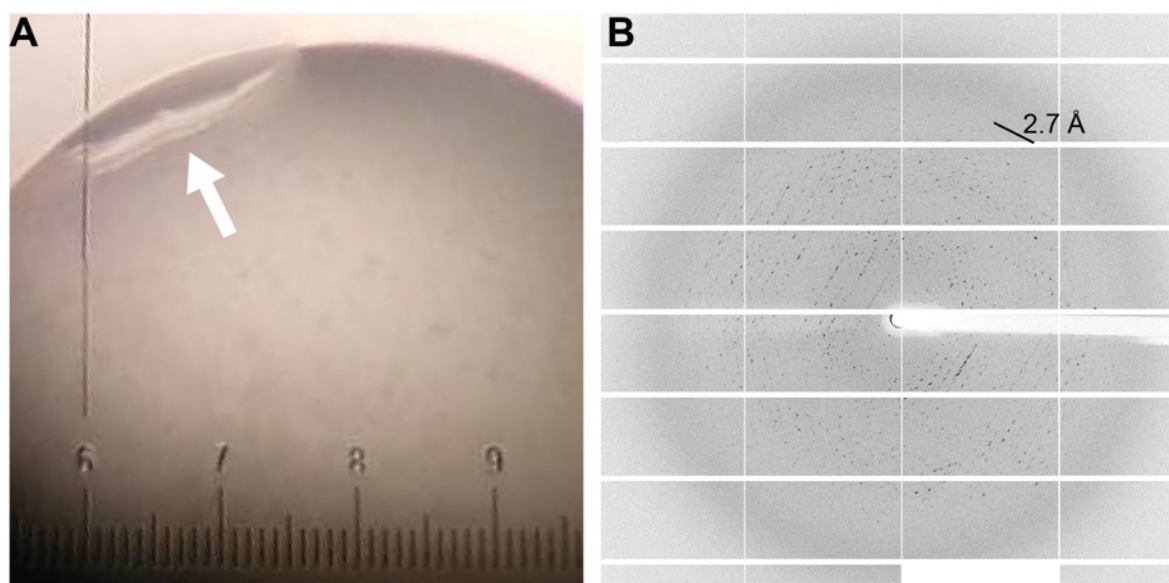
**Figure 3.8 Rat MLKL phosphomimetic mutant S345D is autoactive in mouse and human cells**

**A** Doxycycline was used to induce expression of rat MLKL S345D, and cells were then treated with a necroptotic death stimulus (TSI). Death was measured by propidium iodide (PI) uptake by flow cytometry after 24 hours. MDF assays were conducted in biological duplicate in 3 independent experiments (n=6). One U937 cell line was assayed in 3 independent experiments (n=3). All error bars are  $\pm$  SEM. Significant differences ( $p < 0.05$ ) calculated by T-test between uninduced and induced samples are indicated with an asterisk. **B** Western blot analysis of *Mdk1*<sup>-/-</sup> MDFs reconstituted with rat MLKL S345D, 392 and 393 are biological replicate cell lines. **C** Western blot analysis of U937 *MLKL*<sup>-/-</sup> cell line reconstituted with rat MLKL S345D. For both MDFs and UP37s cells were treated with dox for three hours. Cell extracts were run on SDS-PAGE and western blotting performed to detect MLKL levels. Representative blots shown of two independent experiments.

Rat MLKL S345D was able to induce low levels of necroptosis upon expression in both human and mouse cells, showing that it is somewhat autoactive, and similar to mouse MLKL S345D in that regard. The protein was expressed in both MDF cell lines, but only to low levels in U937 cells, following addition of dox (Figure 3.8).

### *The horse pseudokinase crystal structure*

The horse pseudokinase domain protein also crystallised in trials at the C3 crystallisation facility. The best diffracting crystal took three weeks to grow.



**Figure 3.9 Horse pseudokinase domain crystal and diffraction pattern**

**A** Crystal of horse MLKL pseudokinase domain in 2 M ammonium sulphate, 0.1 M sodium HEPES pH 7.5. The crystal grew on the edge of the drop, indicated with white arrow. Large ticks on scale bar are 100 μm apart. **B** Diffraction of the crystal in **A**, reflections reached 2.7 Å resolution.

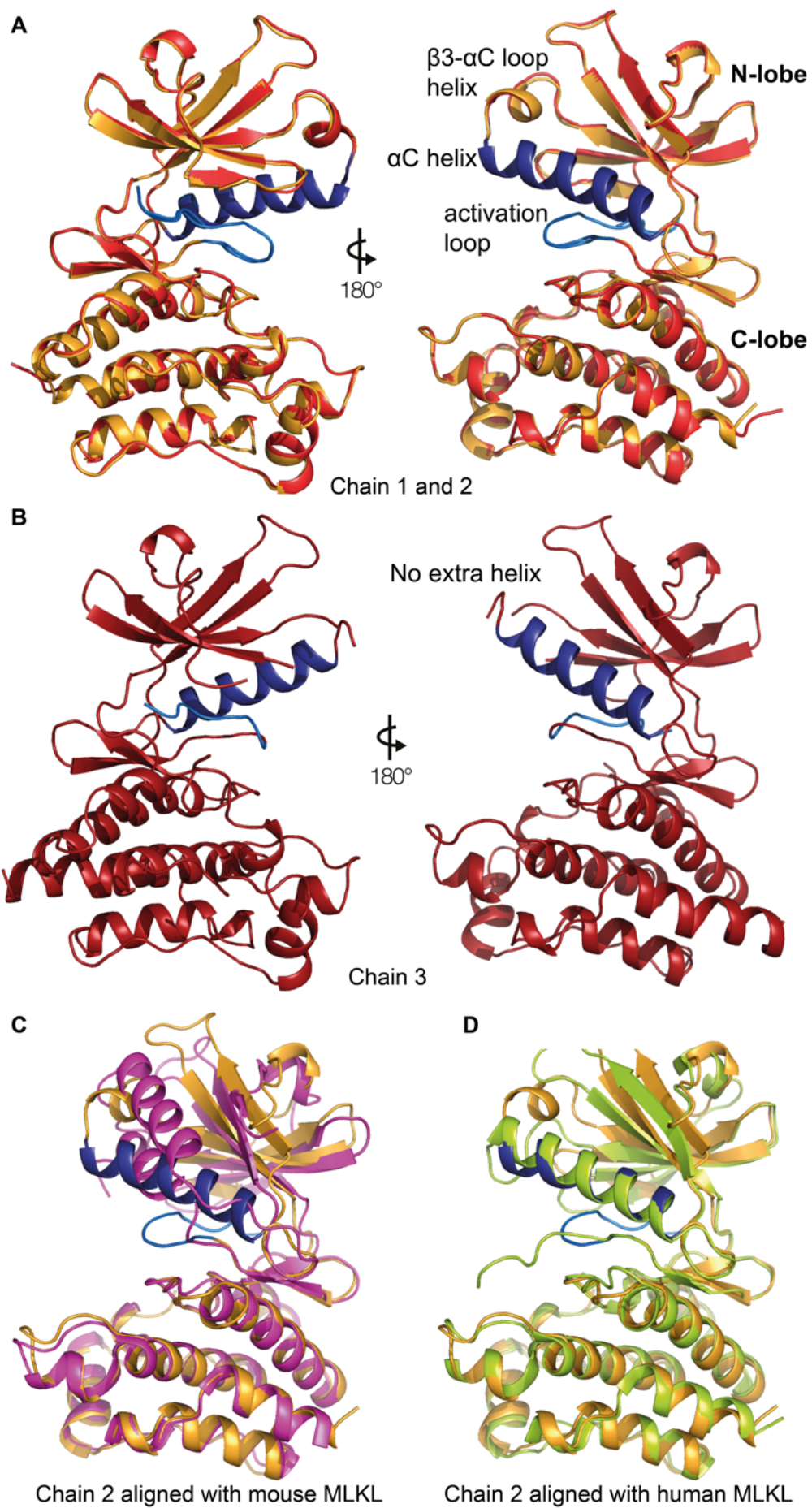
The crystal diffracted to 2.7 Å (Figure 3.9). XDS was used for data reduction, integration and scaling, and the structure was solved by molecular replacement with Phenix Phaser-MR using the human pseudokinase domain as a search model. There were three chains in the asymmetric unit.

**Table 3.5 Horse pseudokinase domain structure statistics**

(Statistics for the highest-resolution shell are shown in parentheses)

|                                                          |                              |
|----------------------------------------------------------|------------------------------|
| Resolution range (Å)                                     | 40.10 - 2.75 (2.85 - 2.75)   |
| Space group                                              | C 1 2 1                      |
| Unit cell a, b, c (Å), $\alpha$ , $\beta$ , $\gamma$ (°) | 155.5 124.8 81.1 90 112.7 90 |
| Total reflections                                        | 256589 (22583)               |
| Unique reflections                                       | 37126 (3346)                 |
| Multiplicity                                             | 6.9 (6.2)                    |
| Completeness (%)                                         | 99 (90)                      |
| Mean I/sigma(I)                                          | 8.79 (0.99)                  |
| Wilson B-factor                                          | 53.52                        |
| R-merge                                                  | 0.1914 (1.548)               |
| R-meas                                                   | 0.2072 (1.687)               |
| R-pim                                                    | 0.07878 (0.6624)             |
| CC1/2                                                    | 0.992 (0.443)                |
| CC*                                                      | 0.998 (0.783)                |
| Reflections used in refinement                           | 36830 (3346)                 |
| Reflections used for R-free                              | 2000 (182)                   |
| R-work                                                   | 0.1834 (0.3138)              |
| R-free                                                   | 0.2379 (0.3601)              |
| CC(work)                                                 | 0.958 (0.687)                |
| CC(free)                                                 | 0.924 (0.446)                |
| Number of non-hydrogen atoms                             | 6645                         |
| macromolecules                                           | 6585                         |
| ligands                                                  | 12                           |
| Protein residues                                         | 813                          |
| RMS(bonds) (Å)                                           | 0.01                         |
| RMS(angles) (°)                                          | 1.38                         |
| Ramachandran favored (%)                                 | 96.2                         |
| Ramachandran allowed (%)                                 | 3.8                          |
| Ramachandran outliers (%)                                | 0                            |
| Rotamer outliers (%)                                     | 5.3                          |
| Clashscore                                               | 10.64                        |
| Average B-factor                                         | 59.35                        |
| macromolecules                                           | 59.44                        |
| ligands                                                  | 65.59                        |
| solvent                                                  | 45.13                        |

The structure was iteratively built with Coot and refined using Phenix.refine, to an R-work and R-free of 18.34% and 23.79% respectively (Table 3.5).

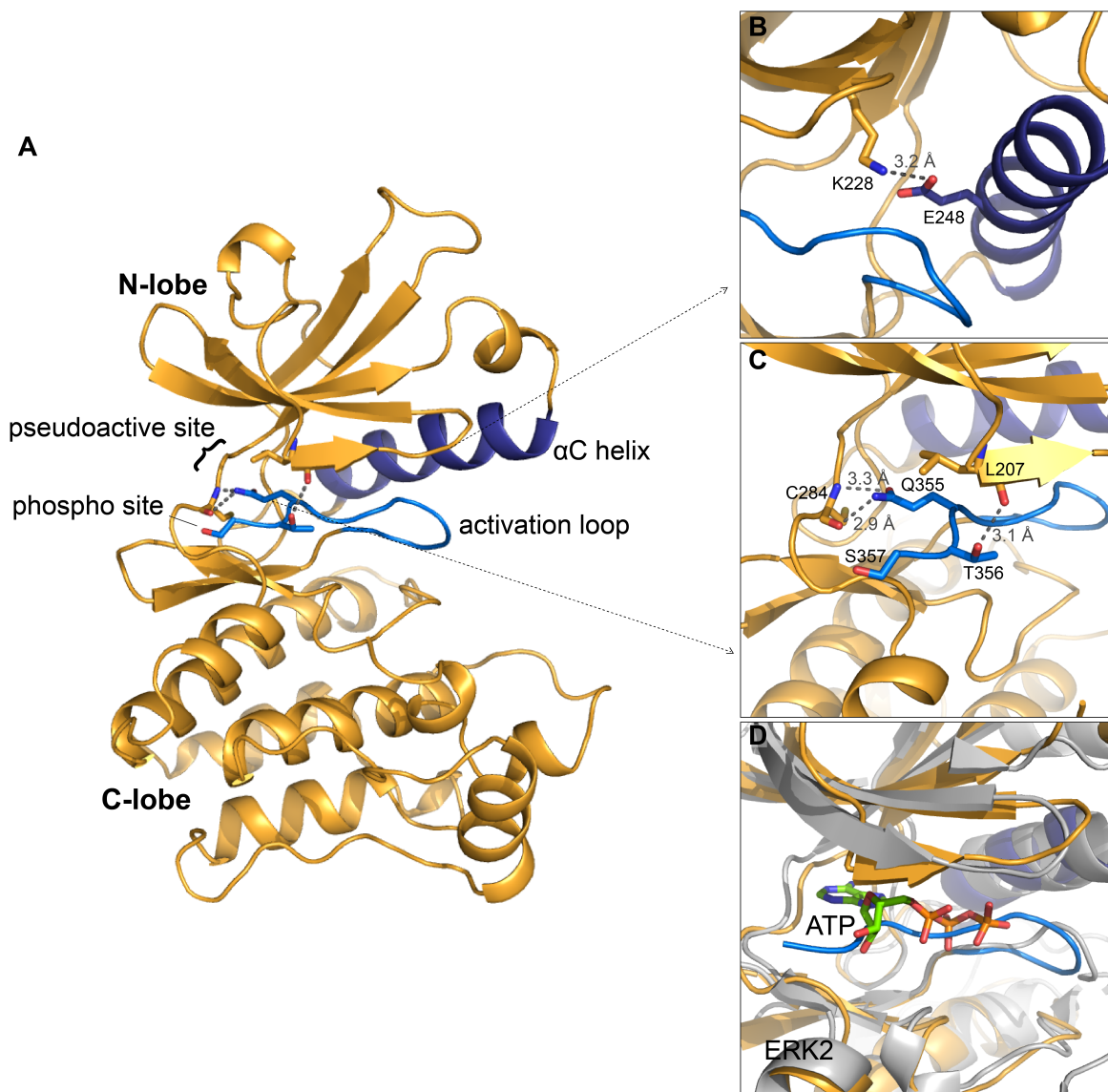


### Figure 3.10 Structure of horse MLKL pseudokinase domain

The structure had three molecules in the asymmetric unit, two of which were similar, and had an extra helix in the N-lobe, termed the  $\beta 3$ - $\alpha C$  loop helix (**A**), and a third (**B**) that did not form the extra helix due to crystal packing. The N-lobe, C-lobe,  $\alpha C$  helix (deep blue), activation loop (light blue) are labelled. **C** Horse MLKL pseudokinase (Chain 2) aligned with the mouse MLKL pseudokinase domain from the full-length mouse MLKL crystal structure (PDB:4BTF). **D** Horse MLKL pseudokinase (Chain 2) aligned with the human MLKL pseudokinase domain crystal structure (PDB:4MWI).

The horse MLKL pseudokinase domain more closely resembled both the human and rat structures than the mouse, in terms of position of the  $\alpha C$  helix (Figure 3.10). It aligned with the human pseudokinase structure with an RMSD of 0.712 Å (Figure 3.10 D) and with mouse, 1.099 Å (Figure 3.10 C). The structure revealed an extra helix in the  $\beta 3$ - $\alpha C$  loop, positioned directly above the  $\alpha C$  in two (Figure 3.10 A) of the three molecules in the asymmetric unit. The third chain (Figure 3.10 B) had crystal contacts at the point where the extra helix was located in the other in the other chains. The horse structure differed from the human and rat MLKL pseudokinase domain structures in regards to its activation loop, which was more fully resolved, and the equivalent residues to those that are phosphorylated by RIPK3 in human MLKL (T356 and S357 in horse) were visible. The activation loop is a key motif in regulation of kinase activity (Huse and Kuriyan, 2002, Möbitz, 2015) so this part of the structure was examined more closely (Figure 3.11).





**Figure 3.11 The horse MLKL activation loop adopts a novel conformation**

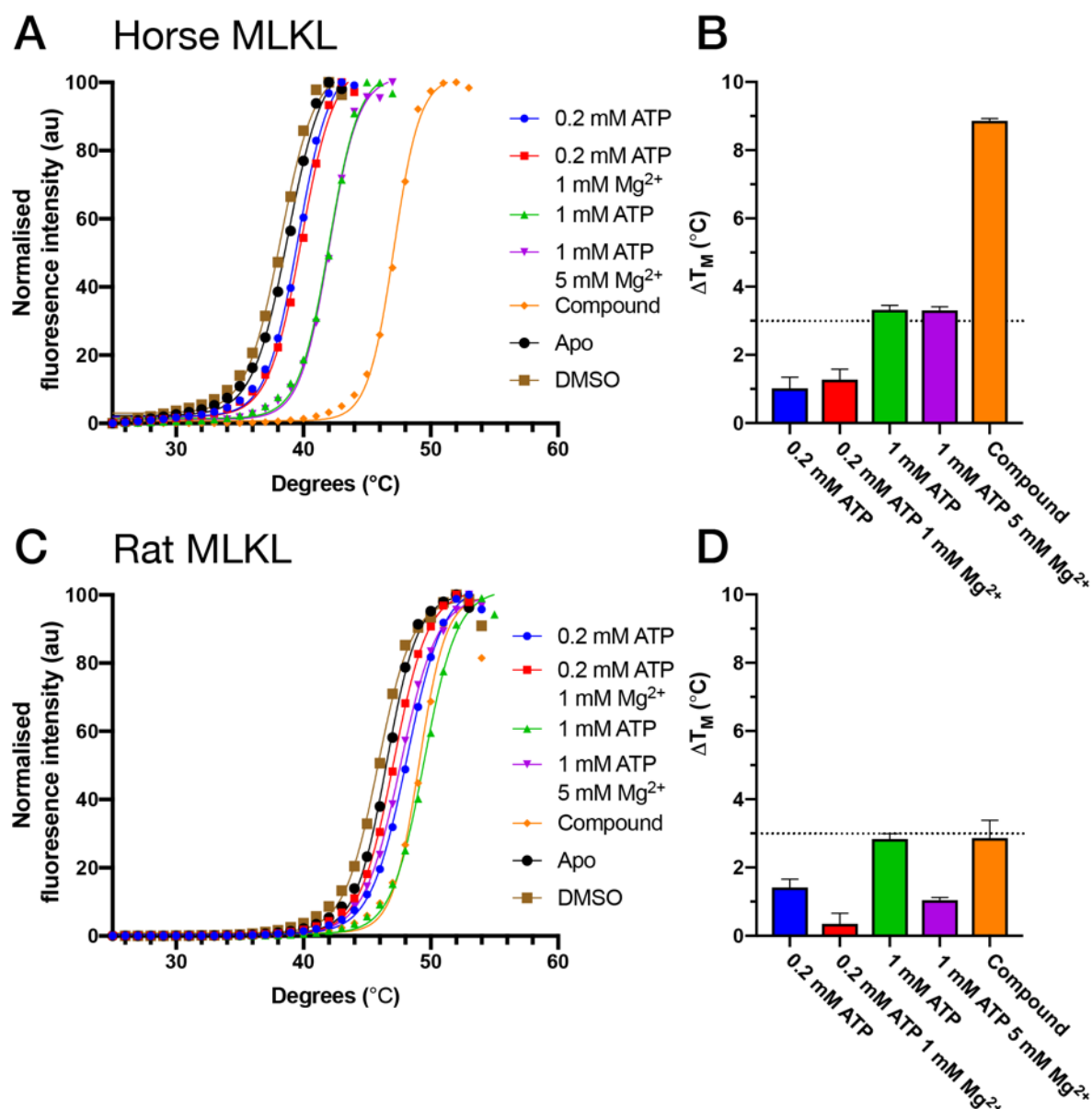
**A** Annotation of chain 2 of the horse pseudokinase structure, highlighting the pseudoactive site and the activation loop. **B** Horse MLKL makes a salt bridge interaction between E248 of the  $\alpha$ C and K228 of the VAIK motif. **C** Activation loop residues Q355 and T356 make hydrogen bonds with the backbone carbonyl of C284 and of L207. T356 and S357 are the residues equivalent to the site of RIPK3 phosphorylation in human MLKL. **D** The horse MLKL pseudokinase aligned with ATP binding kinase ERK2 (grey, PDB:4gt3), ATP is shown in green.

The activation loop was better resolved in this structure, likely because it was stabilised by a series of hydrogen bonds that position the loop in between the two lobes of the pseudokinase domain in the pseudoactive site (Figure 3.11). The hydrogen bonds involved multiple residues along the length of the activation loop including T356, which is equivalent to T357 in human MLKL and is one of the residues



phosphorylated by RIPK3. Adjacent residue Q355 formed a hydrogen bond with the backbone carbonyl group of C284 (Figure 3.11C). E248 retained the conserved salt-bridge with K228 of the VAIK motif (Figure 3.11B), seen in the human and rat MLKL pseudokinase domain structures. Alignment with active kinases such as ERK2 (pictured in Figure 3.11 D) showed that the activation loop occupied the ATP binding site of a typical kinase. The same hydrogen bonding network was present in all three chains in the asymmetric unit.

Given that the activation loop was binding in a site that would be occupied by ATP in a canonical kinase, the ATP binding ability of horse MLKL was tested using a thermal shift assay (Lucet and Murphy, 2017). The rat pseudokinase domain was also tested for comparison (Figure 3.12).



**Figure 3.12 The horse and rat pseudokinase domains can bind ATP**

**A, C** Horse and rat pseudokinase domain binding to ATP was measured using a thermal shift assay with ATP at two concentrations, with and without Mg<sup>2+</sup>. An MLKL binding control compound was also tested. The curves are representative of two independent experiments. **B, D** Shift in melt temperature ( $T_m$ ) relative to apo (ATP; Mg<sup>2+</sup>) and DMSO (compound), 3°C shift on the graph is indicated with a line. Bars are the mean of two independent experiments performed in technical duplicate, error bars are SD.

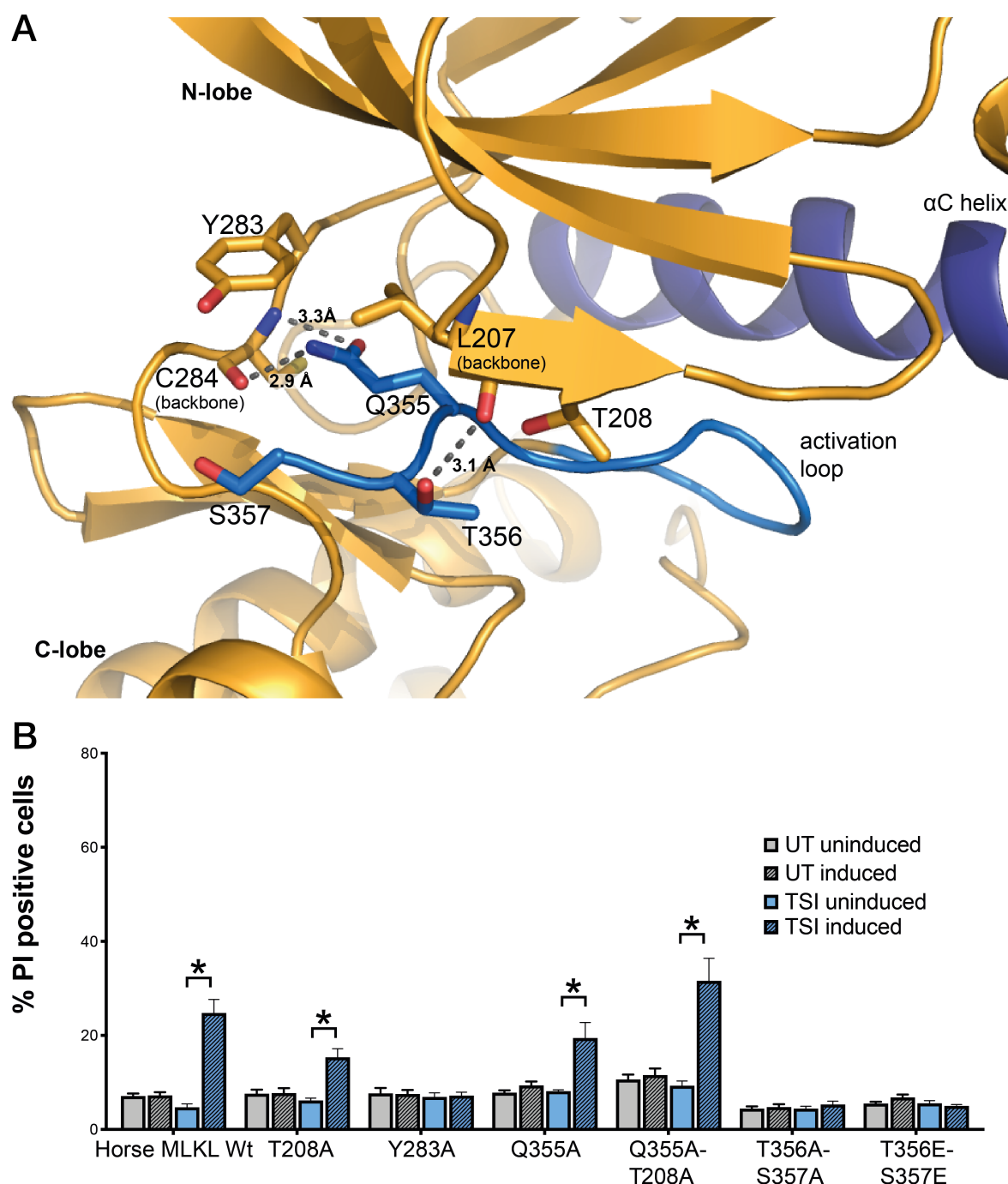
The thermal shift assay has been established as a means of measuring ligand binding of pseudokinases such as MLKL in the literature (Lucet and Murphy, 2017, Murphy et al., 2014a, Murphy et al., 2014b). When ligand binds, it stabilises the protein and therefore leads to an increase in the melting temperature of the protein. In the literature, a threshold of positive 3°C  $T_m$  shift (Murphy et al., 2014b) has been used to

classify pseudokinases as ATP binders, in the presence of 0.2 mM ATP. The apo horse MLKL pseudokinase had a lower thermal stability ( $T_m$ :  $38.6 \pm 0.145^\circ\text{C}$ ) than the rat MLKL pseudokinase ( $46.6 \pm 0.065^\circ\text{C}$ ) (Figure 3.12). Both rat and horse showed modest increases in  $T_m$  with the addition of 0.2 mM ATP, but only reached close to a  $3^\circ\text{C}$  shift in  $T_m$  upon inclusion of 1 mM ATP (horse:  $3.32 \pm 0.127^\circ\text{C}$ ; rat:  $2.84 \pm 0.156^\circ\text{C}$ ) (Figure 3.12 B, D). The horse pseudokinase domain showed an equivalent thermal shift in the presence of ATP regardless of the presence of  $\text{Mg}^{2+}$ , whereas the thermal shift in rat was reduced in the presence of  $\text{Mg}^{2+}$ . Based on these results neither horse nor rat MLKL would be classed as ATP binding. Horse MLKL showed the largest thermal shift when incubated with an MLKL binding compound, sourced from a collaboration, that has been shown to bind to the mouse and human MLKL pseudoactive site in other binding assays (not published).

The pseudokinase domain structures of two MLKL orthologues, rat, which could not kill in either human or mouse cells; and horse, which reconstituted necroptosis in mouse cells, were determined. The next step was to use these structures to try and understand interspecies compatibility. Horse MLKL mediated necroptosis in MDFs was used as a test system.

### 3.5 Structure guided mutagenesis of horse MLKL

The phosphorylation state and position of the activation loop modulates the activity of canonical kinases (Huse and Kuriyan, 2002, Möbitz, 2015) and mutation to residues of the activation loop can modulate MLKL's activity and oligomerisation state (Petrie et al., 2018, Murphy et al., 2013). To investigate the role of the novel horse MLKL activation loop conformation, alanine mutations were made to disrupt the hydrogen bonding network that presumably holds the activation loop in place. Q355A was designed to prevent hydrogen bonding to the C284 backbone. Depending on the rotamer of T356, which was not possible to assign with confidence at the 2.7 Å resolution, T356 appeared to form a H-bond with the backbone carbonyl of L207 in the N-lobe (as depicted in Figure 3.11 C), or with the hydroxyl group in the side chain of adjacent residue T208. Because the backbone hydrogen bond cannot be targeted by mutation, a T208A mutant was made on its own, and paired with the Q355A mutant to form double mutant Q355A:T208A (Figure 3.11, 3.13). The activation loop is positioned against the hinge region of MLKL, the location of the highly conserved MEYCE motif (Figure 3.1). Y283 from this motif was mutated to alanine, to see if removal of bulk in this area would affect necroptosis. T356 and S357, the equivalent residues to those which are phosphorylated by RIPK3 in human MLKL were mutated to alanine to prevent phosphorylation by RIPK3, or to glutamate to act as phosphomimetics. Full-length horse MLKL constructs harbouring these mutations were stably introduced into MDF cells as dox-inducible expression constructs via lentiviral transduction, and cells were challenged with necroptotic stimuli (Figure 3.13), as previously described (Figure 3.3).

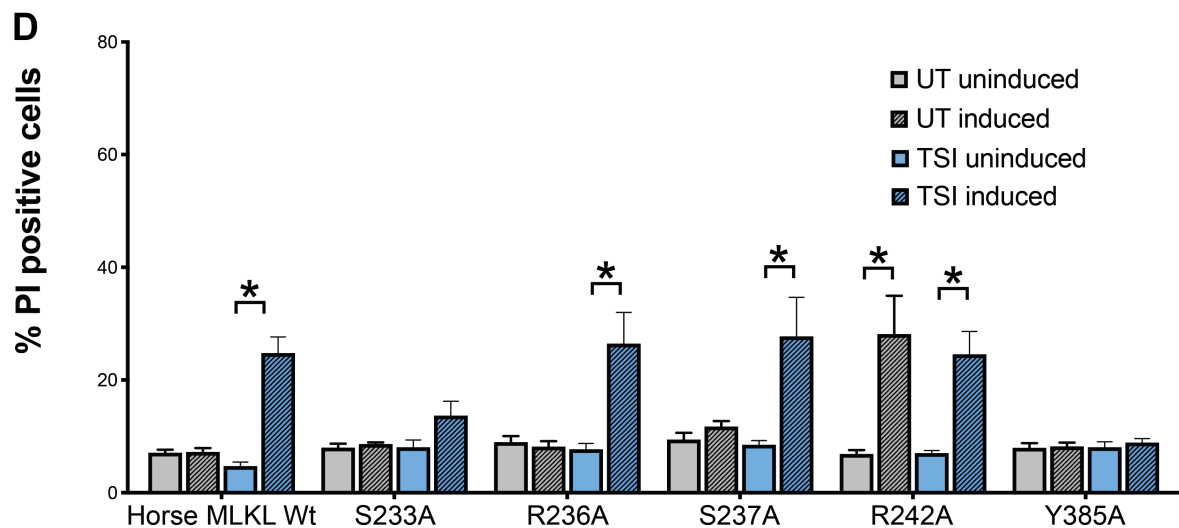
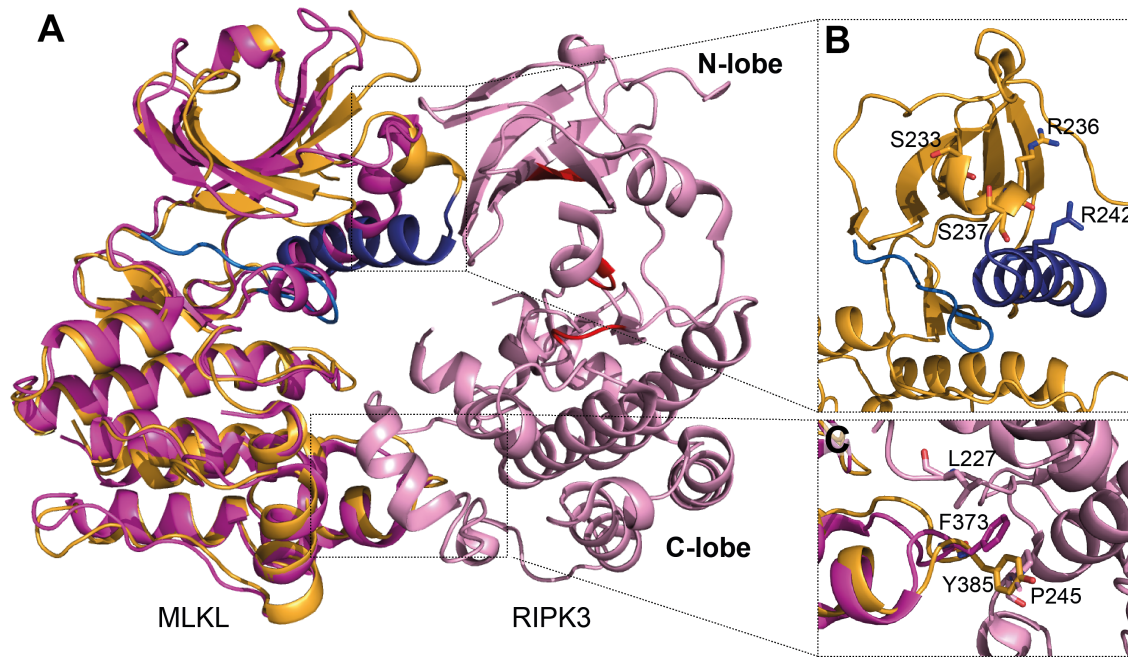


**Figure 3.13 Mutations made to horse activation loop can disrupt necroptosis**

**A** The activation loop viewed to display the full hydrogen bonding network, and residues subject to mutation. **B** Expression of horse MLKL constructs with alanine mutations were induced in MDFs with doxycycline, and their ability to reconstitute necroptosis tested with TSI. Horse MLKL wild-type data is the same as in Figure 3.3 and included for comparison. Assays were conducted in biological duplicate in at least 3 independent experiments ( $n=6-10$ ). Error bars are  $\pm$  SEM. T-tests were performed between induced and uninduced samples, significant differences ( $p<0.05$ ) are indicated with an asterisk.

Mutations that targeted residues involved in the hydrogen bonding network illustrated in Figure 3.13 A; T208A, Q355A and double mutant Q355-T208A did not prevent cells from undergoing necroptosis (Figure 3.13 B). This may indicate that T356 hydrogen bonds with the backbone of L207, or that the N-lobe:activation loop H-bonds that stabilise the activation loop in this conformation (from Q355 and T356) are not essential for horse MLKL function. Mutation to the highly conserved Y283 of the hinge region abrogated necroptosis. Because this residue is so highly conserved (Figure 3.1) this could be due to structural perturbation to MLKL. In order to test this, the Y283A mutant was incorporated into a horse pseudokinase construct to be expressed in insect cells, with a view to perform a thermal shift assay on the recombinant protein. The Y283A recombinant protein expressed was insoluble however, indicating that it is less stable than the wild-type horse protein. Both the alanine mutations to prevent phosphorylation of T356 and S357, and the phosphomimetic mutation T356E-S357E abrogated necroptosis in this system. This result indicates that T356 and S357 are likely to be the target of mouse RIPK3 phosphorylation in MDF cells. Interestingly, the horse MLKL phosphomimetic mutation shares the human phosphomimetic T357E-S358E phenotype, in that is totally inactive, not auto-active like the mouse and rat S345D mutants.

To test if horse MLKL was interacting with mouse RIPK3 by the same interface as the mouse protein does, the horse MLKL pseudokinase structure was aligned with the mouse MLKL pseudokinase domain from the co-crystal structure of mouse RIPK3 with mouse MLKL from Xie et al. (2013) (Described in Section 1.6, Figure 1.5). Horse MLKL appears to be able to share the two main contact points between mouse MLKL and RIPK3, through its N-lobe and C-lobe (Figure 3.14). Based on the alignment, residues that might mediate the horse MLKL mouse RIPK3 interaction were mutated to alanine in the horse MLKL protein (S233A, R236A, S237A, R242A and Y385A), and the resulting constructs tested in death assays in MDFs (Figure 3.14).

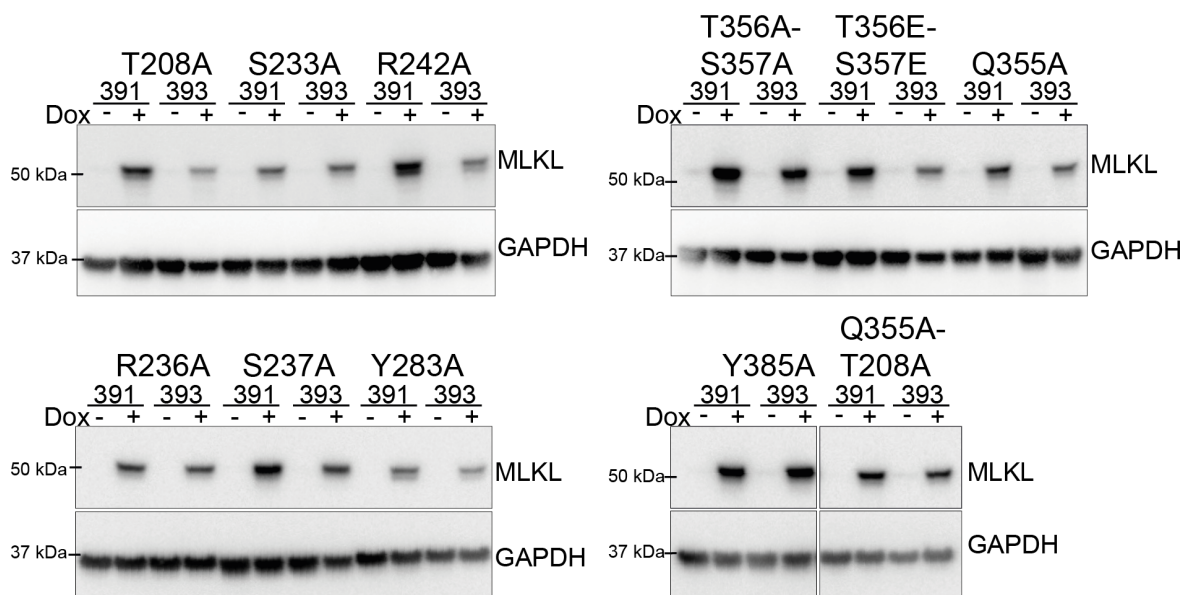


**Figure 3.14 Mutations to potential RIPK3 interaction sites prevent necroptosis**

**A** Alignment of horse MLKL pseudokinase domain (orange) with mouse MLKL pseudokinase (magenta) in the mouse MLKL-mouse RIPK3 (light pink) co-crystal structure, PDB: 4M69. Catalytic residues of RIPK3 are highlighted in red. **B** Residues of horse MLKL that may interact with the RIPK3 N-lobe that were targeted by alanine mutation are labelled. **C** F373 is purported to form a key hydrophobic interaction between the C-lobes of mouse MLKL and mouse RIPK3 (Xie et al., 2013). Orthologous Y385 in horse was mutated to alanine. **D** Expression of horse MLKL constructs with alanine mutations were induced in MDFs with doxycycline, and their ability to reconstitute necroptosis tested with TSI. Wild-type horse MLKL data is the same as in Figure 3.3 and included for comparison. Assays were conducted in biological duplicate in at least 3 independent experiments (n=6-10). Error bars are  $\pm$  SEM. T-tests were performed between induced and uninduced samples, significant differences ( $p < 0.05$ ) are indicated with an asterisk.

Two mutations made to the residues potentially involved in an N-lobe interaction, S233A and R242A, affected necroptosis, whilst the other two (R236A and S237A) did not measurably impact horse MLKL's capacity to reconstitute necroptosis signalling in *Mkl<sup>-/-</sup>* MDF cells (Figure 3.14). S233A horse MLKL appears to allow a modest degree of necroptosis to occur upon TSI stimulation, but the increase in death relative to dox induction alone was not statistically significant. On the other hand, R242A horse MLKL induced necroptosis upon expression regardless of presence of necroptotic stimulus. Mutation of Y385 to alanine in horse MLKL completely abrogated necroptosis, indicating that this residue is important for the interaction between horse MLKL and mouse RIPK3.

To ensure that the alanine mutations made to horse MLKL in Figures 3.13, and 3.14 did not affect protein expression levels, western blots were performed (Figure 3.15).



**Figure 3.15 Expression blots of horse MLKL mutants**

MDFs *Mkl<sup>-/-</sup>* cell lines reconstituted with horse MLKL mutants. 391, 392 and 393 are biological replicate cell lines. Cell extracts were run on SDS-PAGE and western blotting performed to detect MLKL levels. Representative blots shown of two independent experiments.

The blots in Figure 3.15 showed that all the mutants were expressed to similar levels, including mutant Y283A, that it is located in a highly-conserved motif at the hinge region of MLKL (Figure 3.1).



### 3.6 Discussion: comparing and contrasting MLKL orthologues

The ability of MLKL orthologues to reconstitute necroptosis was tested in mouse and human cell lines to determine which, if any, species shared compatibility in necroptotic signalling. Since 2012, when MLKL's role in necroptosis was discovered (Sun et al., 2012, Zhao et al., 2012), many studies have produced conflicting conclusions about MLKL's oligomerisation (Cai et al., 2014, Chen et al., 2014, Wang et al., 2014a), and killing ability (Tanzer et al., 2016). Over time it has become clear that mouse and human MLKL differ in many respects (Petrie et al., 2019a), including in structure (Murphy et al., 2014a), oligomerisation stoichiometry (Davies et al., 2018, Petrie et al., 2018), and cell death activity (Tanzer et al., 2016), and interspecies differences may account for some of the conflicting results in the literature. Therefore we sought to better understand what factors contribute to the differences between mouse and human MLKL, by looking further afield at other MLKL orthologues.

#### *Death assay results*

Nine orthologues; human, mouse, rat, horse, pig, chicken, tuatara, frog and fish, were tested in the initial screen for necroptosis reconstitution in mouse and human *MLKL*<sup>-/-</sup> cell lines. Of these, only two orthologues could reconstitute necroptosis: horse MLKL reconstituted necroptosis in mouse cells, and pig MLKL reconstituted in human cells (Figure 3.3, A, B). Neither were able to reconstitute necroptosis to the same levels as the native MLKL; the difference was especially stark between mouse MLKL ( $74.5 \pm 5\%$  necroptotic death in MDFs) and horse MLKL ( $24.8 \pm 2.9\%$  necroptotic death) (Figure 3.3). This could be due to poor communication with host machineries involved in steps following activation, such as oligomerisation, translocation or even membrane permeabilisation. An example of this could be poor compatibility with mouse or human HSP90, which been shown to be important for necroptosis execution post MLKL activation (Jacobsen et al., 2016, Zhao et al., 2016). The difference could also be due to low binding affinity with RIPK3 and subsequent low efficiency in MLKL phosphorylation and activation.

### *RIPK3 compatibility is key*

None of the MLKL orthologues were able to kill in mouse or human cells without TSI stimulation. This suggests that all MLKL orthologues have regulatory mechanisms that keep them inactive under steady state, and must be activated before they can induce cell death, like human and mouse MLKL. All orthologue species tested have a RIPK3 protein except for chicken (Dondelinger et al., 2016), so a likely activator candidate is RIPK3. In the previous orthologue study (Tanzer et al., 2016), the 4HB+brace of frog, horse, chicken and fish MLKL were expressed in MDFs and cell death measured. The 4HB+brace of chicken and fish MLKL did not cause cell death upon expression, like the 4HB+brace of human MLKL, even though the recombinant 4HB+brace constructs of human and chicken MLKL were capable of permeabilising liposomes. The 4HB+brace of horse MLKL and frog MLKL could kill upon expression in cells, suggesting their regulation is more similar to mouse MLKL, which does induce cell death upon expression of the 4Hb+brace in *Mlkl*<sup>-/-</sup> and *Mlkl*<sup>-/-</sup> *Ripk3*<sup>-/-</sup> mouse cells (Tanzer et al., 2016). In Figure 3.3, full-length horse MLKL, but not frog MLKL could induce killing upon TSI stimulus, suggesting that horse MLKL can be activated by mouse RIPK3 whereas frog MLKL cannot, and that RIPK3 compatibility is likely to be the deciding factor for whether these orthologues can kill in mouse or human cells.

### *What does sequence say?*

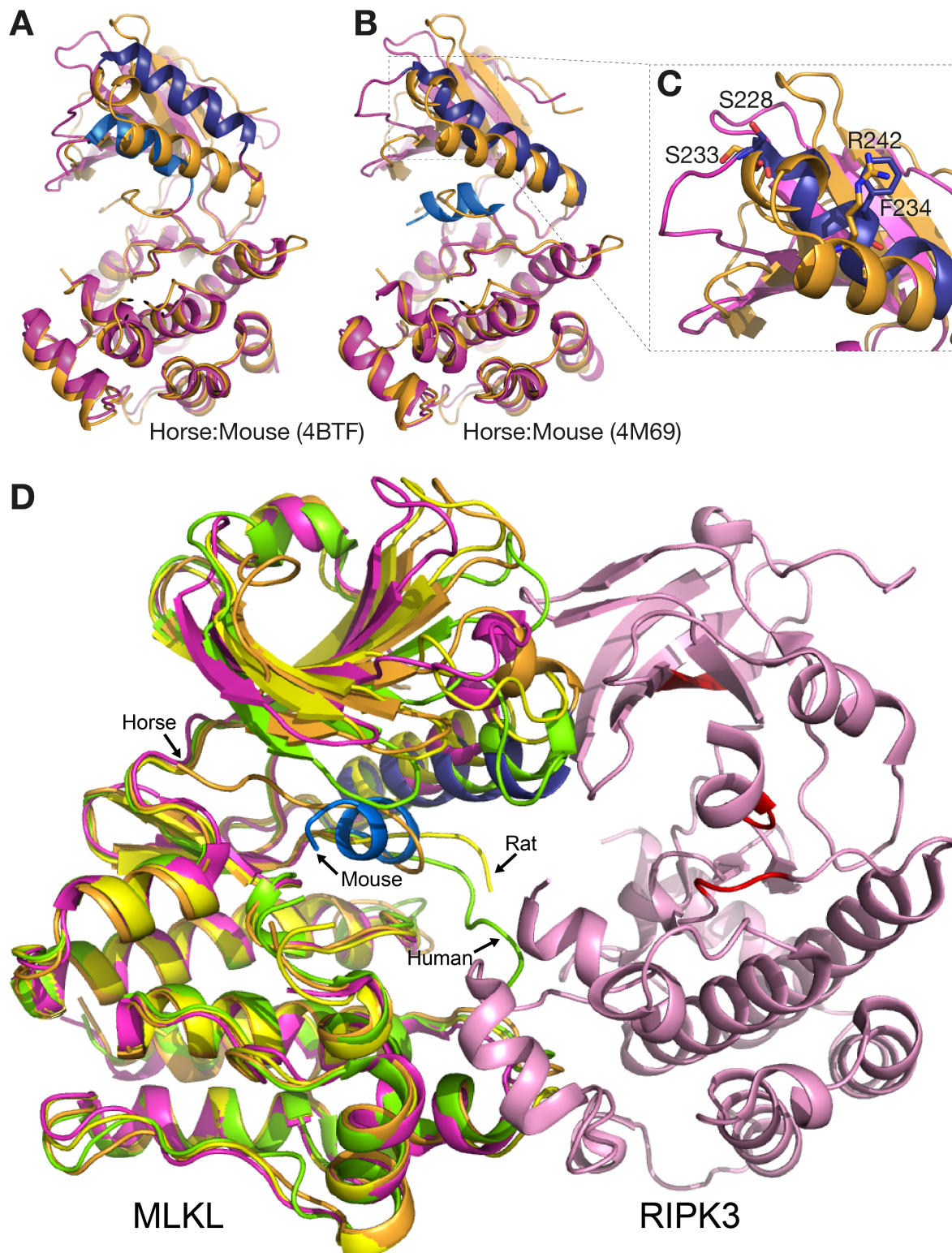
Reptile, amphibian, fish and avian MLKL's were the least closely related orthologues to mouse and human MLKL tested (Figure 3.1, Table 3.1). None were able to reconstitute in the mammalian system (although tuatara did not express in U937s, Figure 3.4 B). This could be due to the protein sequence being too varied compared to the native MLKL, and these species are the most divergent in the regions considered to be important to MLKL function, such as the activation loop, (Figure 3.1). For the orthologues that could reconstitute necroptosis, it is not clear from sequence comparisons why either should be more mouse-like or human-like. Rat MLKL has the highest sequence identity to mouse (85.8%), however it cannot reconstitute necroptosis in MDFs, while horse MLKL (identity 63.0%) can (Table 3.1). Pig MLKL has overall less sequence identity with human MLKL (63.0%) than horse

does (65.3%), however it can kill U937 cells. Pig MLKL has the highest sequence similarity to human MLKL in the pseudokinase domain (91.8%) (Table 3.1), which could be important for communication with human RIPK3. Sequence similarity does not fully explain why some orthologues share RIPK3 compatibility however, because rat MLKL (96.2% similarity to mouse in the pseudokinase domain; Table 3.1) could not communicate with mouse RIPK3. Xie et al. (2013) who solved the co-crystal structure of pseudokinase domain of mouse MLKL bound to mouse RIPK3, suggested that other portions of the full-length MLKL protein probably contributed to the binding face. This has been recently supported by a finding that the brace region of human MLKL was important for human RIPK3 binding (Petrie et al., 2019b). The exact determinants in the brace region for this interaction remain to be determined however.

The best validated RIPK3 binding determinant in the literature is the highly conserved aromatic residue in the C-lobe of MLKL, F373 in mouse. In studies in mouse (unpublished), human MLKL (F386A) and viral MLKLs, mutation of this residue to alanine has abrogated RIPK3 binding (Petrie et al., 2019b). In horse MLKL mutation of the equivalent residue Y385 to alanine abrogated necroptosis (Figure 3.14), showing that it is likely binding to RIPK3 through the *bona fide* MLKL:RIPK3 interacting interface. This residue is a conserved aromatic in most orthologues (Figure 3.1), but in fish and frog it is an asparagine, the polarity of which might not be well accommodated in the predominantly hydrophobic pocket, which could be a reason that fish and frog failed to reconstitute necroptosis in mouse and human cells. The residue is conserved in all the other orthologues tested however, so while it is probably necessary for RIPK3 binding, it's presence is not sufficient to confer RIPK3 compatibility. Therefore, there must be other commonalities between horse and mouse MLKL.

#### *What does structure say?*

Of the available structures, the mouse and horse MLKL pseudokinase domains are the only that have demonstrated shared compatibility with mouse RIPK3, so the structures of those are closely compared in Figure 3.16.



**Figure 3.16 The horse, rat and human MLKL pseudokinase domain structures aligned with the mouse MLKL:mouse RIPK3 co-crystal structure**

**A** Horse MLKL (orange) is aligned with the mouse MLKL pseudokinase domain (magenta) from the full-length mouse MLKL crystal structure (PDB:4BTF), and **B** the mouse MLKL pseudokinase from the RIPK3 kinase domain co-crystal structure (PDB:4M69). **C** Residues that affected the RIPK3 interaction in the N-lobe are labelled for horse and mouse. **D** Orthologues human (green; PDB:4MWI), rat (yellow) and

horse (orange) MLKL are aligned with mouse MLKL (magenta) in complex with mouse RIPK3 (light pink). In all panels the activation loop of mouse MLKL is coloured in light blue, and its  $\alpha$ C is in dark blue. The activation loop belonging to each species is labelled with an arrow. The residues of RIPK3's active site are coloured in red.

The horse MLKL pseudokinase domain aligns better overall with the human counterpart than the mouse, (RMSD of 0.712 Å with human MLKL and 1.099 Å with mouse). However, the extra  $\beta$ 3- $\alpha$ C loop helix present on horse MLKL confers the protein with some similarities in conformation to the mouse structure. When aligned with the apo mouse pseudokinase (Figure 3.16 A), the  $\beta$ 3- $\alpha$ C loop helix is positioned where the end of the mouse  $\alpha$ C is, and the horse  $\alpha$ C is pushed down to the position of the activation loop helix – this confers horse MLKL some structural similarity to mouse MLKL, and may contribute to recognition by mouse RIPK3. In the RIPK3 bound form (Figure 3.16 B), the mouse MLKL activation loop helix shifts, aligning now with the horse activation loop, and the mouse MLKL  $\alpha$ C helix changes position and becomes kinked, aligning with the horse MLKL  $\alpha$ C and  $\beta$ 3- $\alpha$ C loop helices. This  $\beta$ 3- $\alpha$ C loop helix positions horse MLKL residue, S233, that was necessary for the horse MLKL to induce cell death in mouse cells (Figure 3.14), in the same place as mouse residue S228. Mouse residue S228 mediates H-bonding to mouse RIPK3 in the co-crystal structure solved by Xie et al. (2013) (see Figure 1.5, Introduction). The same residue was also discovered as a site of RIPK3 mediated MLKL phosphorylation (Tanzer et al., 2015), and mutation of this residue to alanine or glutamate in mouse MLKL was found to decouple MLKL activation from RIPK3, yielding auto-active constructs. These results suggest that this serine residue is a RIPK3 interaction modulator, and the common positioning of this serine between mouse and horse may be a reason for their compatibility.

Another residue that was tested as a potential RIPK3 interactor was R242 of horse MLKL. Alignment of horse MLKL to mouse MLKL in the MLKL:RIPK3 co-crystal structure, shows that R242 is positioned in a similar position to F234 of mouse MLKL (Figure 3.16 C). F234 forms a  $\pi$ -stacking interaction with F27 of mouse RIPK3 (Figure 1.5, Introduction) (Xie et al., 2013), and the guanidinium group of horse MLKL's R242 may participate similarly in a cation- $\pi$  interaction (Flocco and Mowbray, 1994). Mutant R242A made horse MLKL auto-active in mouse cells, meaning that R242A horse

MLKL is either constitutively phosphorylated by RIPK3 or it has been released from requiring RIPK3 phosphorylation to induce cell death. I was not able to experimentally test the two explanations, because neither the mouse nor human phospho-MLKL antibody was able to detect phospho-horse MLKL (data not shown). Interestingly, although R242 has spatial conservation with F234 of mouse MLKL, in the sequence alignment in Figure 3.1, the arginine residue is highly conserved in MLKL's  $\alpha$ C helix sequence amongst all orthologues except fish. This suggests that the arginine may be important for MLKL function, and correct execution of the molecular switch mechanism. Therefore, the R242A mutant's autoactivity may reflect dysregulation of the switch and not an enhanced RIPK3 interaction.

In the alignment of horse MLKL with RIPK3 bound mouse MLKL, the activation loops of both orthologues aligned to the same position (Figure 3.16 B), which was not observed in the alignment of the horse with the apo mouse pseudokinase structure (Figure 3.16 A). Therefore the other orthologue structures were aligned with mouse MLKL in the mouse:RIPK3 structure to see how their activation loops, the target site of RIPK3 phosphorylation, were positioned (Figure 3.16 D).

One feature that horse MLKL has in common with the mouse structure, and not human or rat, is that the activation loop is well ordered. In the mouse, it is ordered in an  $\alpha$ -helix, in the horse structure the loop located in the ATP binding site where it is ordered by a series of hydrogen bonds. In both the horse and mouse MLKL crystal structures the activation loops are resolved further past the GFE motif (+8 residues in chain 1 of horse MLKL, and +11 residues in mouse) than in the rat and human structures (+2 and +5 residues respectively), where the loops are solvent facing and likely highly flexible, and therefore poorly resolved. Using the mouse RIPK3 mouse MLKL co-crystal structure as a template for understanding, one can speculate that the human and rat MLKL activation loops appear to be easily accessible to the RIPK3 active site (Figure 3.16 D), whereas for both mouse and horse, some disordering event of activation loop from its crystallised conformation must occur before the activation loop is available for phosphorylation by RIPK3. This could affect the binding energy of the interaction between MLKL and RIPK3, and perhaps this similarity

between mouse and horse MLKL makes their RIPK3 binding mode compatible. Evidence against this idea is that mutations made to disrupt the activation loop conformation did not strongly affect horse MLKL activity in mouse cells (Figure 3.13). The only mutations to the horse MLKL activation loop that affected activity were the mutations to the residues that are likely targeted by RIPK3 phosphorylation, equivalents of which have been made in other species, discussed below.

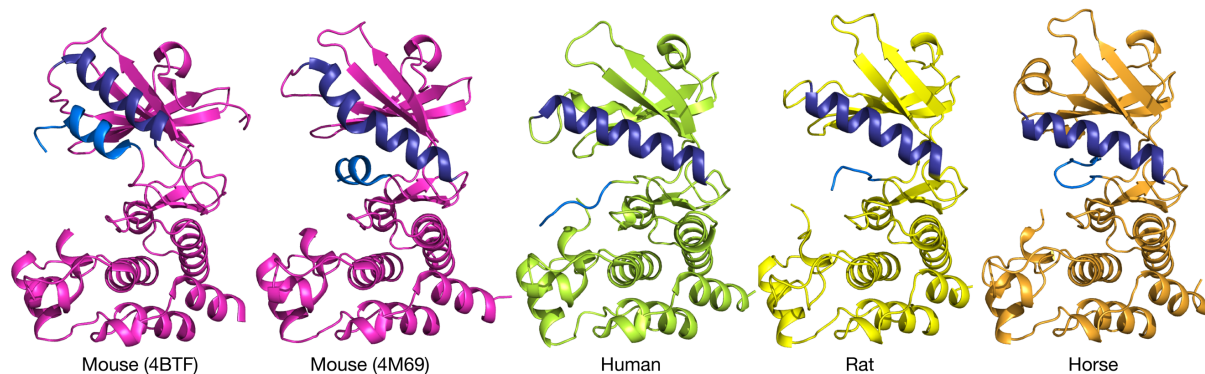
#### *Phosphomimetic mutations and ATP binding*

The mouse and human MLKL phosphomimetic mutants, mouse S345D and human T357E-S358E, have opposite activity in cells. The mouse S345D mutation turns MLKL into a constitutive killer (Murphy et al., 2013), whereas the human T357E-S358E becomes incapable of executing necroptosis (Petrie et al., 2018). The two phosphomimetic mutations that were tested here, rat MLKL S345D (Figure 3.8 A) and horse MLKL T356E-S357E (3.13), also had opposing functions. Rat MLKL S345D was able to induce a low, but statistically significant, level of cell death upon expression in both mouse and human cells, even though rat MLKL could not kill in either mouse or human cells with TSI stimulation. This shows that when the requirement for RIPK3 activation is removed, rat MLKL can be active in mouse and human cells, albeit to a low level. The phosphomimetic horse MLKL mutant was rendered totally inactive, and in this regard behaved more like human MLKL, even though it is active in mouse, but not human cell lines. Both human and mouse MLKL can bind ATP although not in the presence of magnesium (Murphy et al., 2014a, Murphy et al., 2013). In thermal shift assays rat MLKL showed very weak binding to ATP, that was sensitive to the addition of magnesium (Figure 3.12). Horse MLKL domain showed a slightly larger thermal shift in the presence of ATP that was insensitive to addition of magnesium (Figure 3.12). The horse MLKL activation loop is positioned within the interlobe cleft in which ATP is expected to bind (Figure 3.11 D). This provides the possibility that the horse MLKL activation loop conformation may be disrupted by ATP binding in cells. ATP has previously been shown to modulate the permeabilising activity of human MLKL on liposomes (Petrie et al., 2018), and this activation loop conformation may be a means of MLKL sensing ATP binding. However, horse MLKL appears to be less able to bind ATP than human MLKL, based on published data (Murphy et al., 2014a), and

another interpretation could be that the pseudoactive site/ATP binding site of horse MLKL allows the activation loop to bind because it is structurally divergent from human MLKL, and no longer favours ATP binding.

*What more can the different structures tell us?*

All of the available MLKL pseudokinase orthologue structures are compared in Figure 3.17.



**Figure 3.17 Comparison of horse, rat, mouse human MLKL pseudokinase domain structures.**

All orthologues have the  $\alpha$ C helix in dark blue, and activation loop in light blue. Mouse (PDB 4BTF; 4M69) (Murphy et al., 2013, Xie et al., 2013) is in magenta, human (PDB 4MWI) (Murphy et al., 2014a) in green, rat in yellow and horse in orange.

Of the orthologues in Figure 3.17, mouse MLKL is the only one that displays the activation loop helix conformation, which is maintained in the apo and RIPK3 bound structures. This is surprising given the high sequence similarity between rat and mouse MLKL (96.2%, Table 3.1). This result can be interpreted in two ways. It may be an explanation for why rat MLKL cannot kill in mouse cells, they have a different structures, and therefore rat MLKL may have lost mouse RIPK3 compatibility. However, rat MLKL also does not kill human cells and it has a very similar conformation to human MLKL. Moreover, crystal structures only show a snapshot of protein dynamics. An alternative interpretation is that the different conformations of mouse and rat MLKL, viewed in light of their high sequence similarity, represent two conformations that the pseudokinase domain of each MLKL can adopt. This interpretation could give insight into MLKL's proposed molecular switch mechanism; the idea that RIPK3 phosphorylation in the pseudokinase induces structural changes that are translated through the entire protein leading to its activation (Murphy et al.,



2013). It is possible that the conformations in these structures represent different states of activation, and potentially even the novel activation loop conformation present in horse could be a part of this mechanism because other MLKL orthologues have the necessary residues to form the same hydrogen bonding network seen in the horse structure. A combination of these two interpretations may also be true, in that different MLKL proteins adopt multiple conformations, but the conformer that is most stable for each orthologue could differ, thereby altering their RIPK3 compatibilities and activation thresholds.

In conclusion, at first glance the new orthologue structures showed surprising similarity to the human MLKL pseudokinase domain, which did not appear to correlate with either sequence conservation (i.e. rat and mouse) or activity in cells (horse MLKL kills mouse cells). Upon closer inspection however, both rat and horse MLKL do have their own similarities with mouse. Rat MLKL cannot communicate with mouse RIPK3, however, its phosphomimetic mutant form was autoactive in MDFs suggesting that it does have similar regulation to mouse MLKL. The horse structure had an extra  $\beta 3$ - $\alpha C$  loop helix, positioned above the  $\alpha C$  helix, rather than an activation loop helix positioned below the  $\alpha C$  as in mouse MLKL, however, this extra helix actually positioned some key residues in the equivalent location to their counterparts in mouse MLKL in its RIPK3 bound form (Figure 3.16, 3.17). Pig MLKL was able to reconstitute necroptosis in human U937 cells, which could be useful to the therapeutic programs currently trying to find MLKL inhibitors. It is of great interest to solve the pig pseudokinase structure, however so far, no expression constructs have resulted in soluble protein in insect cells. Taken together, the orthologue work has made some inroads into better understanding MLKL and RIPK3 compatibility, and in the future when more is known about the basic mechanism of the MLKL RIPK3 interaction, reanalysis of the results may yield more insights.

## ***4.0 Brace Region Mediated Oligomerisation and Interdomain Communication***

Much of the work in this chapter has been published in a co-first paper Davies and Tanzer et al. (2018) (see Appendix II), for which I wrote the manuscript and prepared figures during my PhD. The data presented in this chapter focus on the work that I performed during my PhD. Contributions by other persons, and work performed by myself before the commencement of my PhD are indicated in figure legends.

MLKL oligomerisation is a key step in MLKL's mechanism of action. Numerous studies have shown that abrogation of MLKL's ability to oligomerise, either totally or partially prevents necroptosis from occurring in cells (Davies et al., 2018, Cai et al., 2014, Hildebrand et al., 2014, Dondelinger et al., 2014). Conversely, forced dimerization/oligomerisation is sufficient to induce spontaneous necroptosis (Quarato et al., 2016, Tanzer et al., 2016, Chen et al., 2014). Therefore the protein:protein contacts that mediate MLKL oligomerisation could be targeted by novel necroptosis inhibitors. Furthermore, understanding the MLKL oligomer may help us to understand how it can translocate to and/or disrupt membranes. Some discussion of what is known about MLKL oligomerisation is presented in the Introduction (Section 1.7). This chapter begins with a discussion of the techniques used to examine the stoichiometry of the MLKL oligomer, and previously published work (Hildebrand et al., 2014, Davies et al., 2018) that forms the foundation of the research presented in this chapter.

As mentioned in Chapter 1 (Section 1.7), the MLKL oligomer has been reported to comprise varying numbers of subunits in different studies, ranging from trimers (Cai et al., 2014, Davies et al., 2018, Hildebrand et al., 2014), tetramers (Petrie et al., 2018, Chen et al., 2014, Huang et al., 2017, Liu et al., 2017), hexamers (Wang et al., 2014a) and octamers (Huang et al., 2017) to large amyloid MLKL polymers (Liu et al., 2017, Johnston and Wang, 2018, Reynoso et al., 2017). It is likely that much of the discrepancy between these findings arises from the techniques used to estimate the molecular weight of the oligomer, and a reliance on non-reducing SDS-PAGE gels in many of these studies.

To generate MLKL oligomer samples, most groups stimulate various cell lines with necroptotic stimuli (e.g. TSZ), and then run cell extracts on non-reducing gels (Chen et al., 2014, Cai et al., 2014, Wang et al., 2014a). The first possible source of inconsistency in results is that each group prepares their cell lysates differently prior to running on the gel. Most use gentle detergent lysis on the cells (Cai et al., 2014, Cai and Liu, 2018, Wang et al., 2014a), however Wang et al. (2014) who detected the hexamer, incubated their cell lysates at 30°C under non-reducing conditions prior to analysis by non-reducing SDS-PAGE. This heat treatment could lead to non-specific aggregation of the protein (Delahaije et al., 2015, Creamer et al., 2004, Schon et al., 2015, Kirley and Norman, 2018), and might contribute to the high molecular weight species they observed.

The second source of inconsistency comes from drawing conclusions about molecular weight of the oligomer based on its migration through a non-reducing SDS-PAGE gel. Some proteins, especially membrane associated proteins like the MLKL oligomer, migrate anomalously on SDS-PAGE compared to protein molecular weight markers (Rath and Deber, 2013, Rath et al., 2009). This is due to the detergent SDS not binding membrane associated proteins in a standard manner (Rath et al., 2009), meaning that the charge of the protein:SDS complex may not reflect its length, and the protein may not be linearized fully. Both of these factors are requirements for accurate size determination by SDS-PAGE (Reynolds and Tanford, 1970b, Reynolds and Tanford, 1970a). The use of non-reducing SDS-PAGE (lacking a reducing agent in order to preserve disulfide bonds between protein subunits) may compound the difficulty in estimating molecular weight. The presence of disulfide bonds between protein subunits, ensures that the entire complex cannot be totally linearized by SDS, and the shape of the protein chain will affect migration through the gel (Pitt-Rivers and Impiombato, 1968, Dunker and Kenyon, 1976). One study illustrated this by running linearized 3X and 4X MLKL constructs alongside their oligomer sample on non-reducing SDS-PAGE and found that the 'oligomer' ran in between these two constructs (Huang et al., 2017), showing that molecular weights determined from a gel are not definitive.

Some studies have also employed chemical cross-linkers to study the MLKL oligomer (Huang et al., 2017, Wang et al., 2014a, Petrie et al., 2018). Huang et al. (2017) used addition of a cross-linker upon cell lysis to find an MLKL octamer, which they suggested was composed of two tetramers. Chemical cross-linkers can be useful in determining oligomeric states of proteins (Arora et al., 2017), however though Huang et al. (2017) showed that RIPK1 and RIPK3 were not part of the octamer, they did not perform mass spectrometry on the octamer band and rule out MLKL crosslinking to another protein in the cell lysate causing the large shift in oligomer weight. Wang et al. (2014) and Petrie et al. (2018) both made use of cross-linkers on recombinant protein. Like Huang et al. (2017), Wang et al. (2014) then ran these crosslinked species on SDS-PAGE, which may not be accurate in determining molecular weight as discussed above. Petrie et al. (2018) took another approach, to use the cross-linked recombinant protein, not to prove oligomer stoichiometry, but to try and understand the arrangement of subunits in the oligomer, the mass of which was by determined by other means, discussed below.

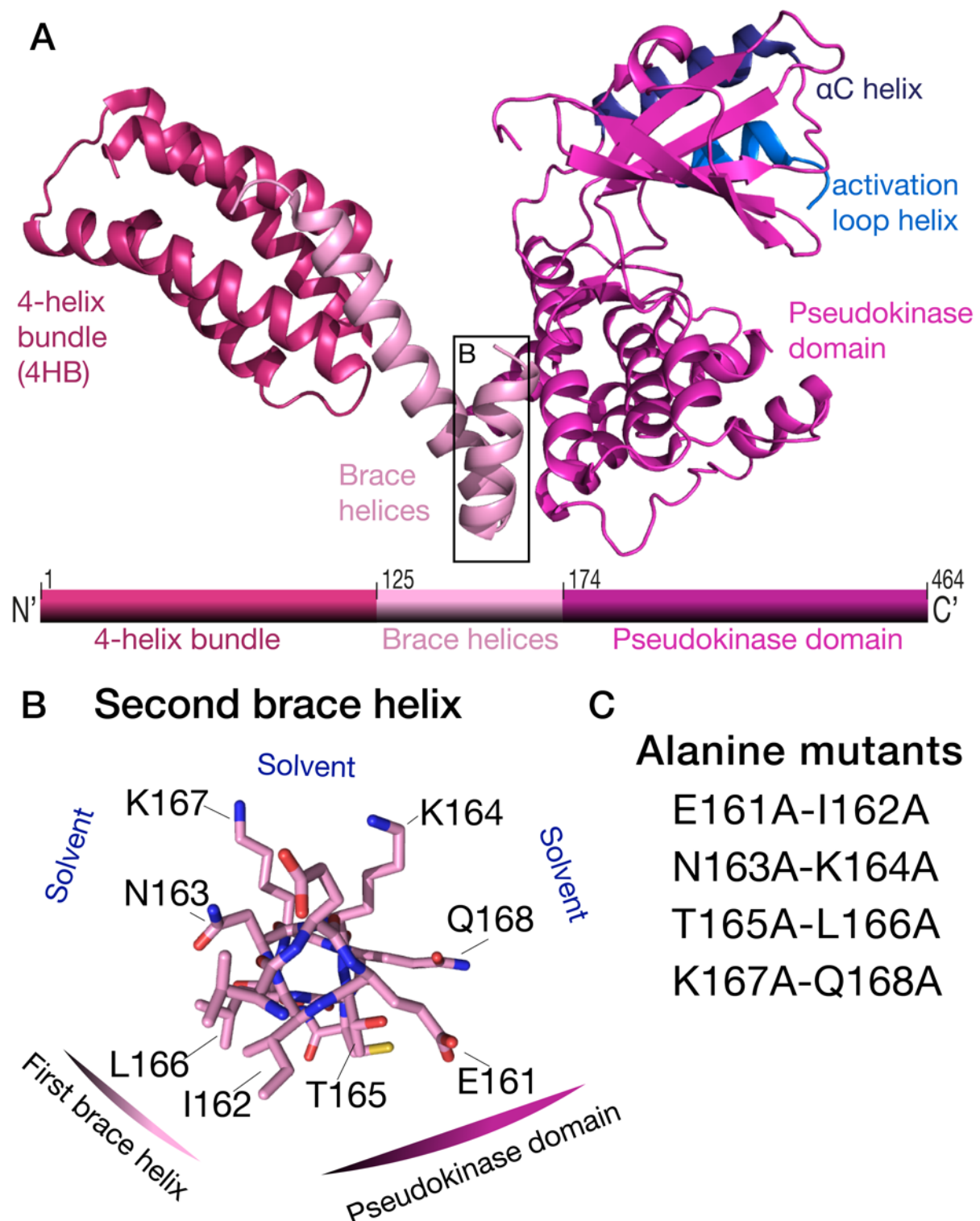
Moving away from the use of gels to determine the molecular weight of the MLKL oligomer proved useful for Petrie et al. (2018). Native mass spectrometry of purified recombinant human MLKL protein, showed unambiguously an oligomeric species of 216,190 Da, corresponding to an MLKL tetramer (Petrie et al., 2018). Furthermore, scattering data from SAXS analysis were most consistent with a tetramer model, and distance restraints gained from mass spectrometry on cross-linked MLKL tetramer were used to make a model of the human MLKL tetramer. This study provided strong evidence that human MLKL does form a tetramer, as suggested in some other studies (Huang et al., 2017, Liu et al., 2017).

Another study used analytical ultracentrifugation (AUC) to show that recombinant truncated mouse MLKL construct mMLKL 1-169 formed a trimer in solution (Hildebrand et al., 2014). This construct has a cysteine to serine mutation at residue 169 (C169S), which does not affect its ability to oligomerise. This construct, mMLKL 1-169 C169S (herein referred to as mMLKL 1-169), forms the basis for my study of mouse MLKL oligomerisation.

#### *4.1 The second brace helix of mMLKL 1-169 mediates oligomerisation*

The mMLKL 1-169 construct comprises the 4HB of mouse MLKL plus the two brace helices, as defined by the mouse MLKL full-length crystal structure (Murphy et al., 2013) (Figure 4.1). Studies of human MLKL indicate that constructs incorporating the 4HB and both brace helices could form oligomers (Quarato et al., 2016, Su et al., 2014), like mMLKL 1-169, however removal of the second brace helix inhibited oligomerisation (Quarato et al., 2016). Therefore, to examine whether the same principle was true for mouse MLKL, a new mouse MLKL construct, mMLKL 1-158, which lacks the second brace helix was expressed and purified. Size exclusion chromatography (SEC) is a technique used to differentiate between proteins on the basis of Stokes' radius or rotational radius in solution, and decreased elution volume can correspond to increases in molecular weight or more extended conformations of a protein. On SEC, mMLKL 1-169 eluted at a higher volume than expected for its molecular weight, indicating that it forms an oligomer. mMLKL 1-158 eluted at the expected volume for a ~20 kDa protein, indicating that loss of the second brace helix also inhibits oligomer formation in mouse MLKL (see Figure 1, Appendix II) (Davies et al., 2018).

Based on this result, it appeared that the second brace helix of mouse MLKL confers oligomerisation ability to the mMLKL 1-169 construct. To define which residues of the second brace helix were important for this, a series of alanine mutants to the second brace helix were designed (Figure 4.1).



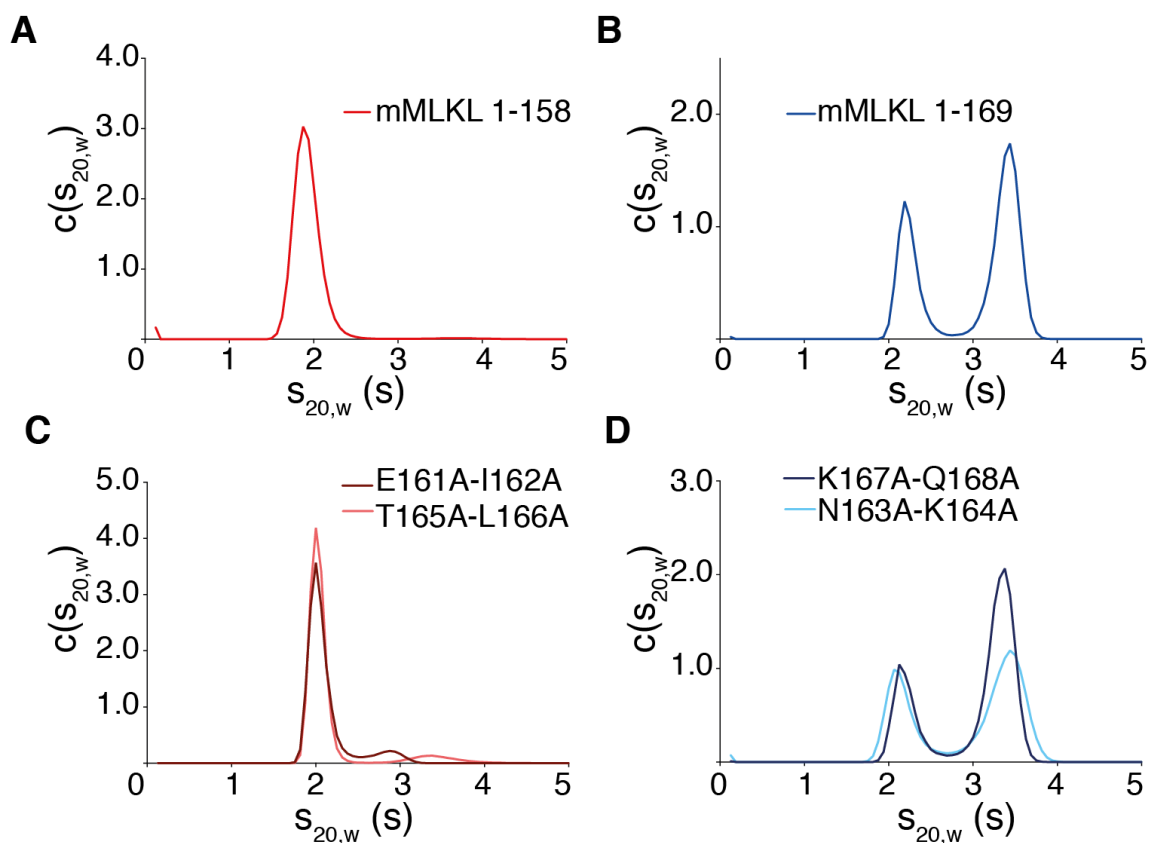
**Figure 4.1 Double alanine mutations to the second brace helix designed to test oligomerisation requirements**

**A** The structure of full-length mouse MLKL is depicted with residue boundaries of reach domain annotated. The brace region is shown in light pink (PDB:4BTF). **B** Looking down the helical axis of the second brace helix, the side chains of the residues targeted by alanine scanning mutagenesis are labelled. The relative position of the first brace helix, pseudokinase domain, and solvent in the full-length MLKL

crystal structure are indicated. **C** A list of the double alanine mutations that were made to the mMLKL 1-169 construct.

Double alanine mutants were chosen to reduce the number of mutant constructs which needed to be purified (Figure 4.1 C). All mutant constructs were cloned, expressed in *E. coli*, and purified using nickel-affinity chromatography during my Honours year. Size exclusion chromatography showed that double alanine mutants N163A-K164A and K167A-Q168A eluted similarly to trimeric construct mMLKL 1-169. The two other mutants E161A-I162A and T165A-L166A eluted similarly to monomer mMLKL 1-158 (see Davies et al. (2018); Appendix II, Figure 1).

To confirm the results from size exclusion chromatography, during my PhD AUC was performed on the two control constructs mMLKL 1-158 and mMLKL 1-169, and all the double alanine mutants (Figure 4.2).



**Figure 4.2 AUC analysis for mMLKL 1-158, mMLKL 1-169 and double alanine mutants**

**A** mMLKL 1-158 sedimented as a single species, in AUC. **B** mMLKL 1-169 sedimented in two distinct species. **C** E161A-I162A, and T165A-L166A sedimented as single species. **D** K167A-Q168A and N163A-K164A sedimented as two species.

mMLKL 1-158 sedimented as a single species consistent with a monomer of ~20 kDa (Figure 4.2 A). Alanine mutants E161A-I162A, and T165A-L166A, showed very similar sedimentation coefficients to mMLKL 1-158, both with only a minor species that corresponded to a higher molecular weight (Figure 4.2 C). This indicated that as observed by size exclusion chromatography, the constructs existed predominantly as monomers in solution. mMLKL 1-169 on the other hand sedimented in two species, consistent with a mixture of monomer and oligomer in solution. K167A-Q168A and N163A-K164A both showed similar curves and sedimentation coefficients to mMLKL 1-169, showing that they retain the ability to oligomerise. These results indicated that some or all of residues E161A, I162A, and T165A or L166A play an important role in mMLKL 1-169 oligomerisation.

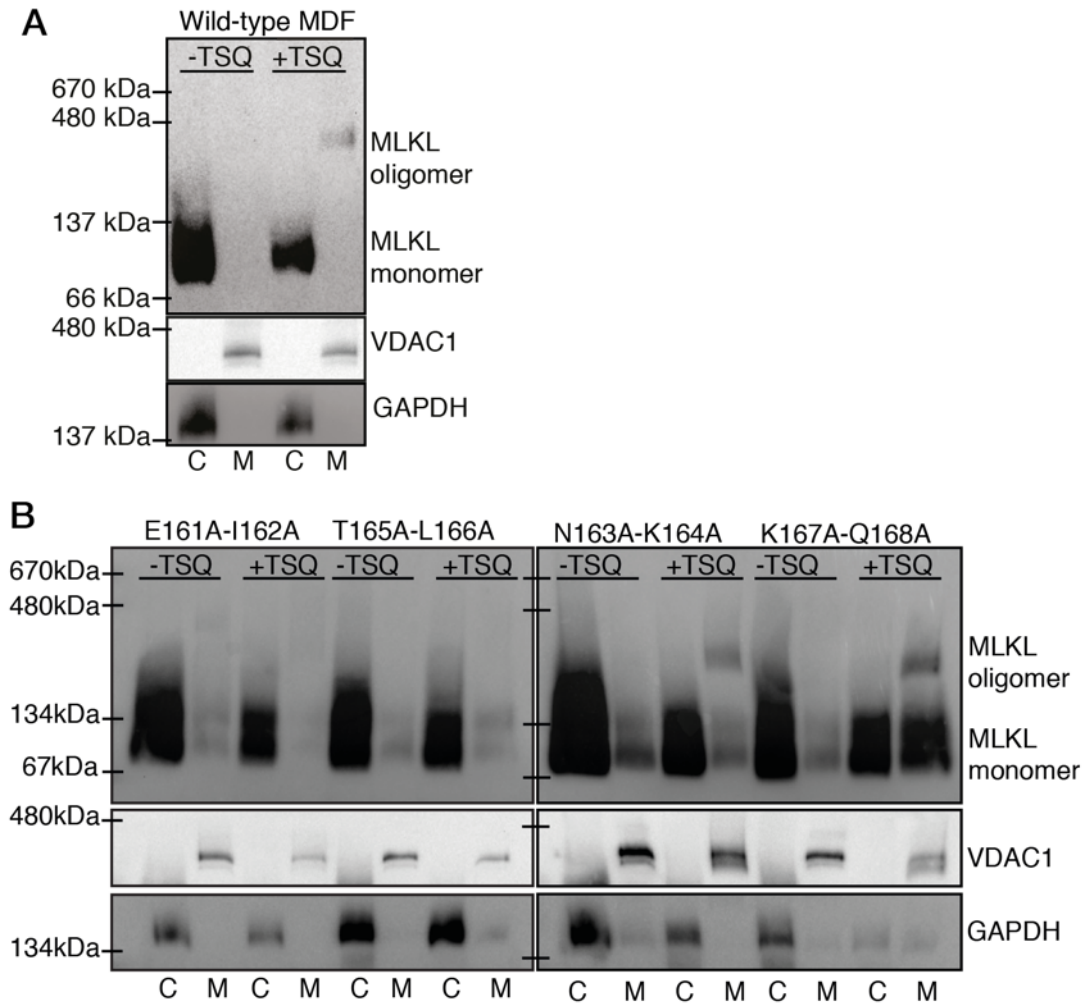
#### *4.2 Introducing double alanine mutants into full-length MLKL*

The truncated construct mMLKL 1-169 was useful for studying the oligomerisation properties of the second brace helix recombinantly, because it can be readily expressed in *E. coli*, and forms oligomers spontaneously in solution (Hildebrand et al., 2014). The oligomer of most importance however, is the full-length MLKL oligomer which forms in cells and is essential to MLKL mediating cell death. To confirm that the mMLKL 1-169 trimer reflected the full-length MLKL oligomer, the same alanine mutations were introduced into full-length mouse MLKL. The double alanine mutant constructs, as well as a wild-type mouse MLKL were stably transduced into *Mlkl*<sup>-/-</sup> mouse dermal fibroblasts (MDFs) using lentivirus to introduce puromycin selectable, doxycycline (dox) inducible MLKL constructs into cell lines.

To test the oligomerisation potential of the mouse full-length constructs harbouring alanine mutations in cells, doxycycline was added to MDF cell lines to induce expression of MLKL constructs. The cells were then treated with necroptotic stimulus TSQ; TNF and Smac-mimetic, and QVD a pan-caspase inhibitor, (Introduction, Figure 1.1), to cause MLKL activation and translocation of MLKL oligomers to the plasma



membrane (Hildebrand et al., 2014). After 7 hours of TSQ treatment, cells were harvested on ice, and subjected to gentle detergent lysis and subcellular fractionation, and a blue native-PAGE (BN-PAGE) was performed (Figure 4.3). BN-PAGE allows detection of the MLKL oligomer and can map its subcellular localisation either to the cytoplasm, or to a crude membrane fraction (Hildebrand et al., 2014), which includes the plasma membrane.

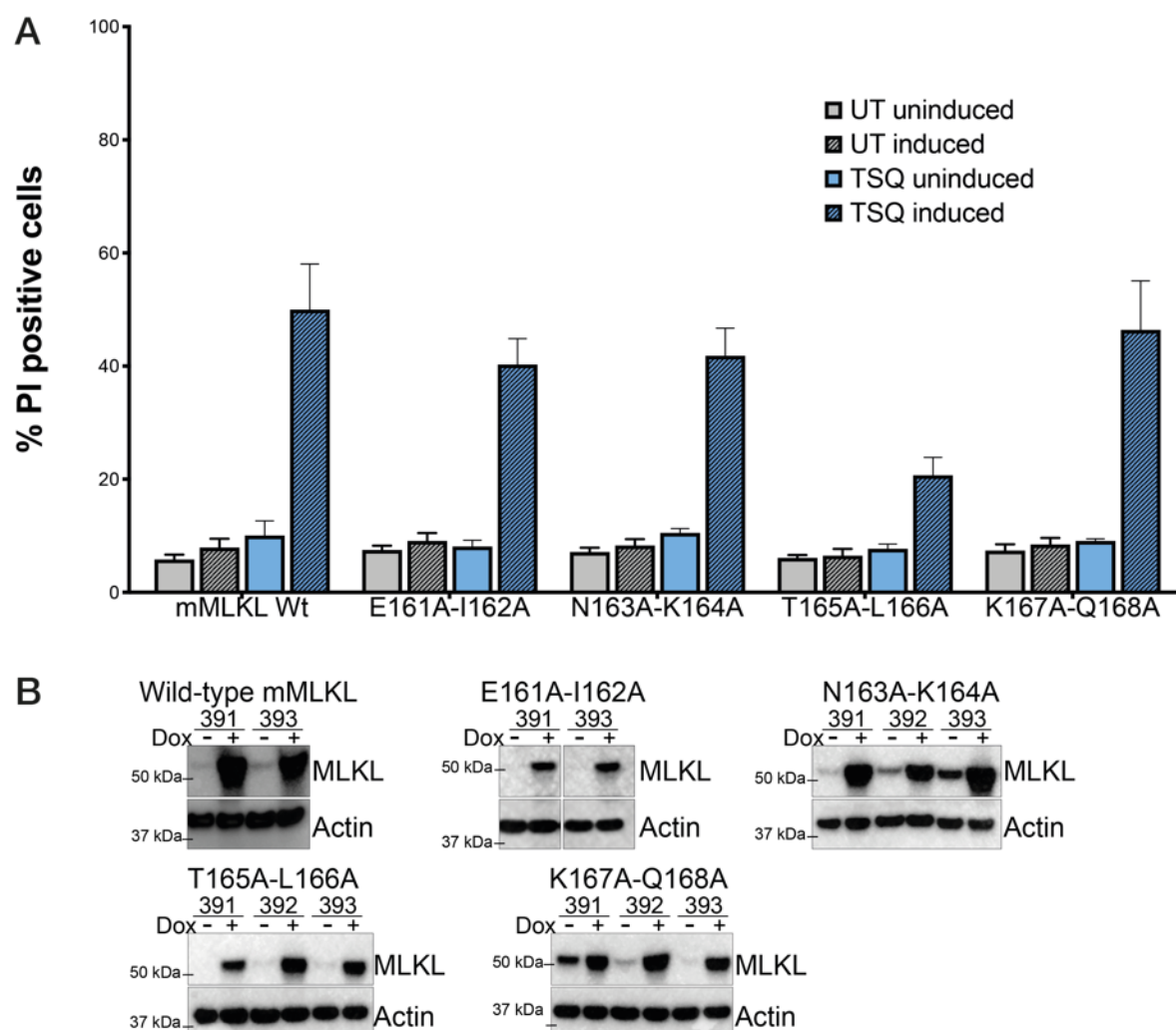


**Figure 4.3 E161A-I162A, T165A-L166A show deficits in oligomerisation in cells**

All MDF samples were treated with doxycycline for 3 hours, then half were treated TSQ for an additional 7 hours. Cells were then harvested, and fractionated using digitonin. Cytoplasmic and crude membrane fractions were run on BN-PAGE, then transferred onto a PVDF membrane and blotted. VDAC1 is a loading control for the membrane fraction, and GAPDH is a loading control for cytoplasmic fraction. The blots are representative of two independent experiments. **A** BN-PAGE western blot of wild-type MDFs expressing endogenous levels of MLKL. **B** BN-PAGE western blot of *MLKL*<sup>-/-</sup> MDFs expressing mMLKL double alanine mutants via treatment with dox.

E161A-I162A and T165A-L166A did not form oligomers after 7 hours of TSQ treatment, by which time endogenous wild-type MLKL and mutant constructs N163A-K164A and K167A-Q168A showed strong oligomer bands in the membrane fraction of the TSQ treated samples (Figure 4.3 A, B). This indicated that the mMLKL 1-169 trimer indeed corresponds to the full-length mouse functional oligomer, and that the residues E161A-I162A and T165A-L166A were required for oligomerisation.

Cells were then tested in death assays, to determine if this defect in oligomerisation reduced cell death by necroptosis (Figure 4.4). Doxycycline was used to induce mutant MLKL expression, then MDFs were challenged with apoptotic stimulus TS (as a control for TNF pathway signalling) and necroptosis stimulus (TSQ). After 24 hours, the cells were harvested and death measured by PI uptake with flow cytometry quantitation to determine percentage cell death. Only the untreated (UT) and TSQ samples with and without dox are shown for clarity. The TS stimulation is a control to ensure that the cell lines remain sensitive to TNF pathway stimulation and cell death, and only data from cell lines that respond to TS are used to test necroptosis. Cell lines were also tested with TSI stimulus (using the IDN-6556 caspase inhibitor instead of QVD-OPh) during my Honours year; these data were published in Davies et al. (2018) (Appendix II).



**Figure 4.4 Only alanine mutant T165A-L166A causes deficit in necroptosis**

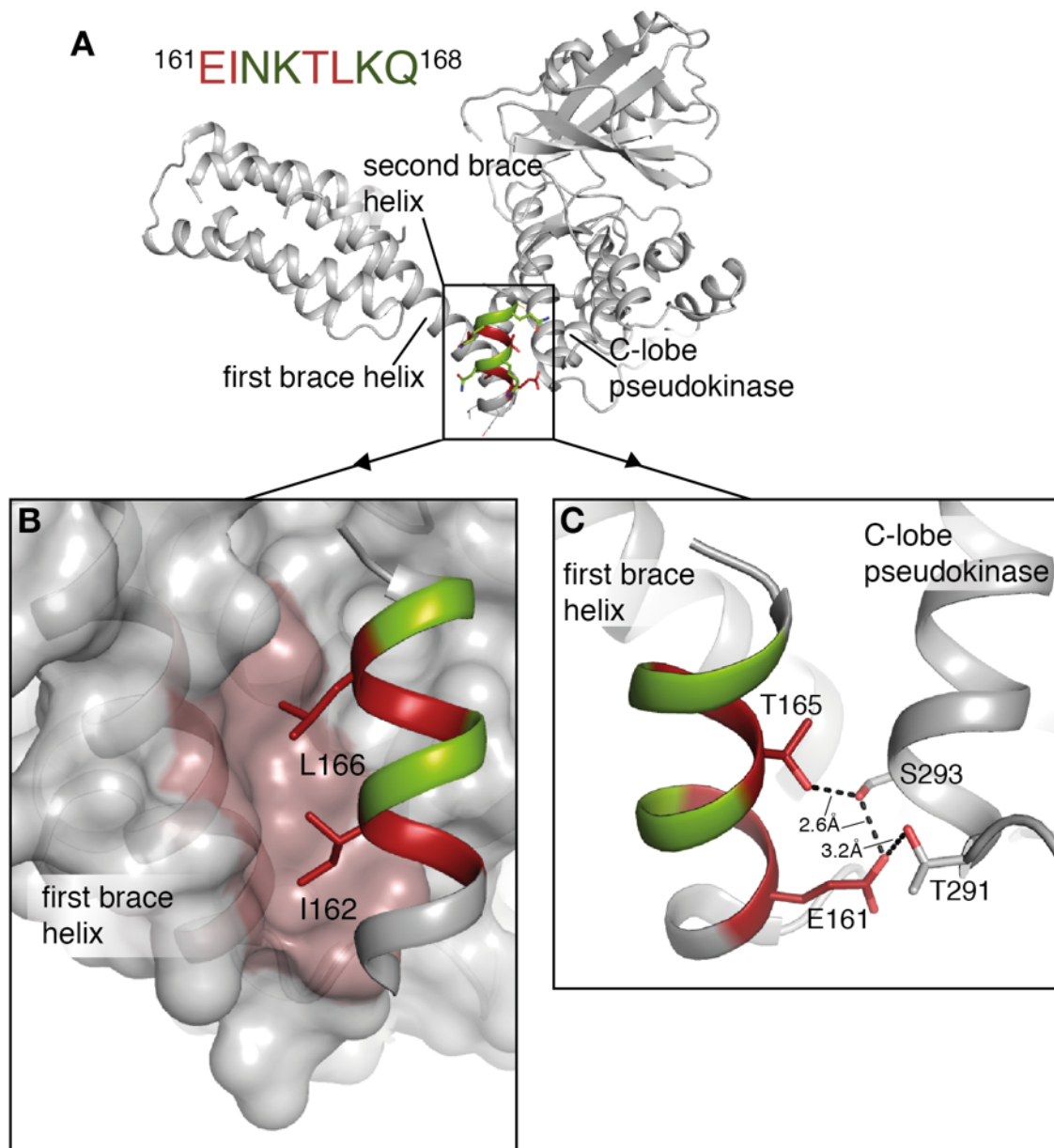
**A** Doxycycline was used to induce expression of double alanine mutant MLKL constructs, and TSQ was used as necroptotic stimulus. Death was measured by propidium iodide (PI) uptake by flow cytometry after 24 hours. MDF assays were conducted in biological duplicate or triplicate in at least 3 independent experiments (n=6-9). **B** *Mlkl*<sup>-/-</sup> MDF cell lines reconstituted with wild-type mouse MLKL and double alanine mutants. 391 392 and 393 are biological replicate *Mlkl*<sup>-/-</sup> MDF lines. Cells were treated with dox for three hours, cell extracts were run on SDS-PAGE and western blotting performed to detect MLKL levels. I made cell lines N163A-K164A, T165A-L166A and K167A-Q168A during my Honours year, and performed western blots on these cell lines in my Honours year. The remaining cell lines and western blots were prepared during my PhD and all death assay data was collected during my PhD.

One of the mutants, T165A-L166A mMLKL, showed reduced killing compared to the wild-type counterpart (Figure 4.4 A), (proportion of cells that underwent TSQ induced death:  $20.71 \pm 3.18\%$  vs  $50.01 \pm 8.04\%$ ), whilst the other mutants, E161A-I162A, N163A-K164A and K167A-Q168A, had levels of activity comparable to wild-type

mouse MLKL. Interestingly, this included construct E161A-I162A (TSQ induced death:  $40.29 \pm 4.56\%$ ), which showed a lack of oligomerisation via BN-PAGE (Figure 4.3) and in the context of the truncated construct mMLKL 1-169 (Figure 4.2). Therefore it was likely that some oligomerisation could still occur in E161A-I162A MLKL full-length and to a lesser extent in the T165A-L166A construct. This oligomer likely formed with either very low efficiency and was not detectable by BN-PAGE, or oligomerisation suffered a kinetic defect due to the mutations made.

#### *4.3 Refining the alanine mutations to better define interfaces in MLKL necessary for oligomerisation*

Alanine mutants E161A-I162A and T165A-L166A, prevented oligomerisation in the context of the truncated construct mMLKL 1-169 (Figure 4.2). However, the failure of these same alanine mutants to totally abrogate cell death/oligomerisation in the full-length MLKL system (Figure 4.4) suggested that the mutations may not completely disable the full-length MLKL oligomerisation interface. Therefore, the positioning of these mutations was re-evaluated in the context of the mouse MLKL full-length crystal structure, in order to see if other regions of MLKL might compensate for deficits in oligomerisation induced by the brace region alanine mutations (Figure 4.5).



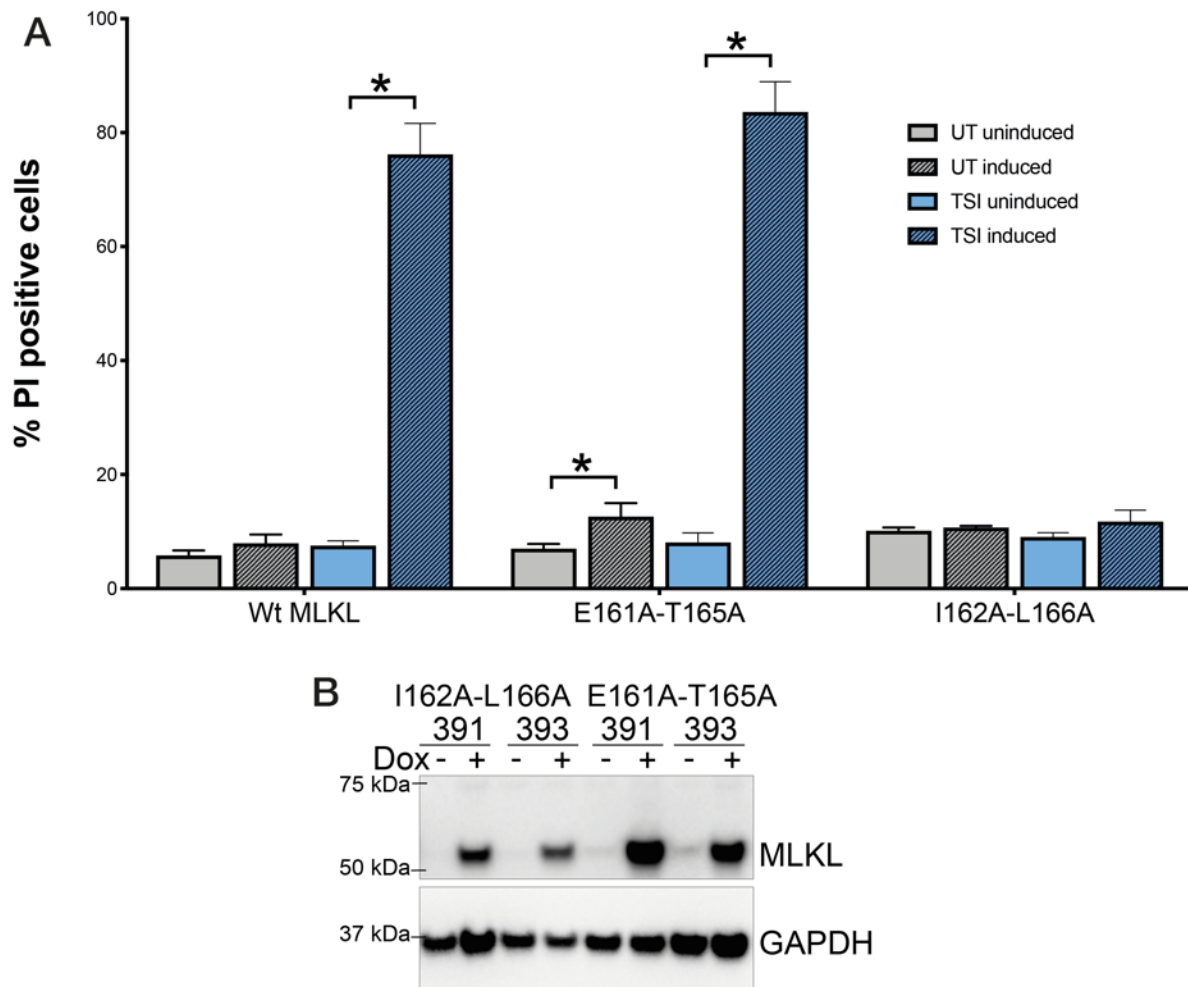
**Figure 4.5 Two possible interfaces affected by alanine mutations**

The residues mutated in the double alanine scanning mutagenesis are highlighted in this figure. Residues which did not affect oligomerisation when mutated are shown in green; N163, K164, K167 and Q168. Residues to which mutation did affect oligomerisation are shown in red sticks; E161, I162, T165 and L166. **A** Shows the mutations in the context of the full-length mouse MLKL structure (PDB:4BTF). **B** Shows one face of the second brace helix involving I162 and L166 that form hydrophobic packing interactions with the first brace helix (the surface of the interaction is shown in pink). **C** shows another face of the second brace helix, where E161 and T165 form hydrogen bonds to the C-lobe of MLKL.

Residues N163, K164, K167 and Q168 were all solvent facing in the mouse MLKL crystal structure and mutation to these residues did not affect oligomerisation (Figure 4.5). I162 and L166 were involved in hydrophobic packing interactions with

hydrophobic side chains of the first brace helix. T165 and E161 on the other hand were both involved in hydrogen bonds with the C-lobe the MLKL pseudokinase domain. Therefore both mutants E161A-I162A and T165A-L166A were targeting two different protein packing interactions through each alanine mutation (Figure 4.5). Given that one residue from each pair contributed to a different interface it was not possible to tell which interaction was key for MLKL oligomerisation.

If one of the interfaces out of Figure 4.5 B and C, was essential for oligomerisation and the other was not, then only half disrupting the important interface could explain the residual levels of cell death, in cells expressing each of the E161A-I162A and T165A-L166A constructs (Figure 4.4). To address this, each interface was targeted with a new double alanine mutant, E161A-T165A for the C-lobe interaction and I162A-L166A for the first brace helix interaction. These two mutant constructs were made in the context of full-length mouse MLKL, introduced into *Mlkl*<sup>-/-</sup> MDFs and their ability to reconstitute necroptosis tested (Figure 4.6), as described for Figure 4.4, this time using a different caspase inhibitor, IDN-6556.



#### Figure 4.6 I162A-L166A and not E161A-T165A precludes necroptosis in MDFs

**A** MLKL mutant expression was induced with doxycycline, and cells then treated with death stimulus (TSI). Death was measured by propidium iodide uptake (PI) by flow cytometry after 24 hours. MDF assays were conducted in biological duplicate in at least 3 independent experiments (n=6-10). **B** *Mkl1*<sup>-/-</sup> MDF cell lines reconstituted with wild-type or mutant MLKL, 391 and 393 are biological replicate cell lines. Cells were treated with dox for three hours, cell extracts were run on SDS-PAGE and western blotting performed to detect MLKL levels.

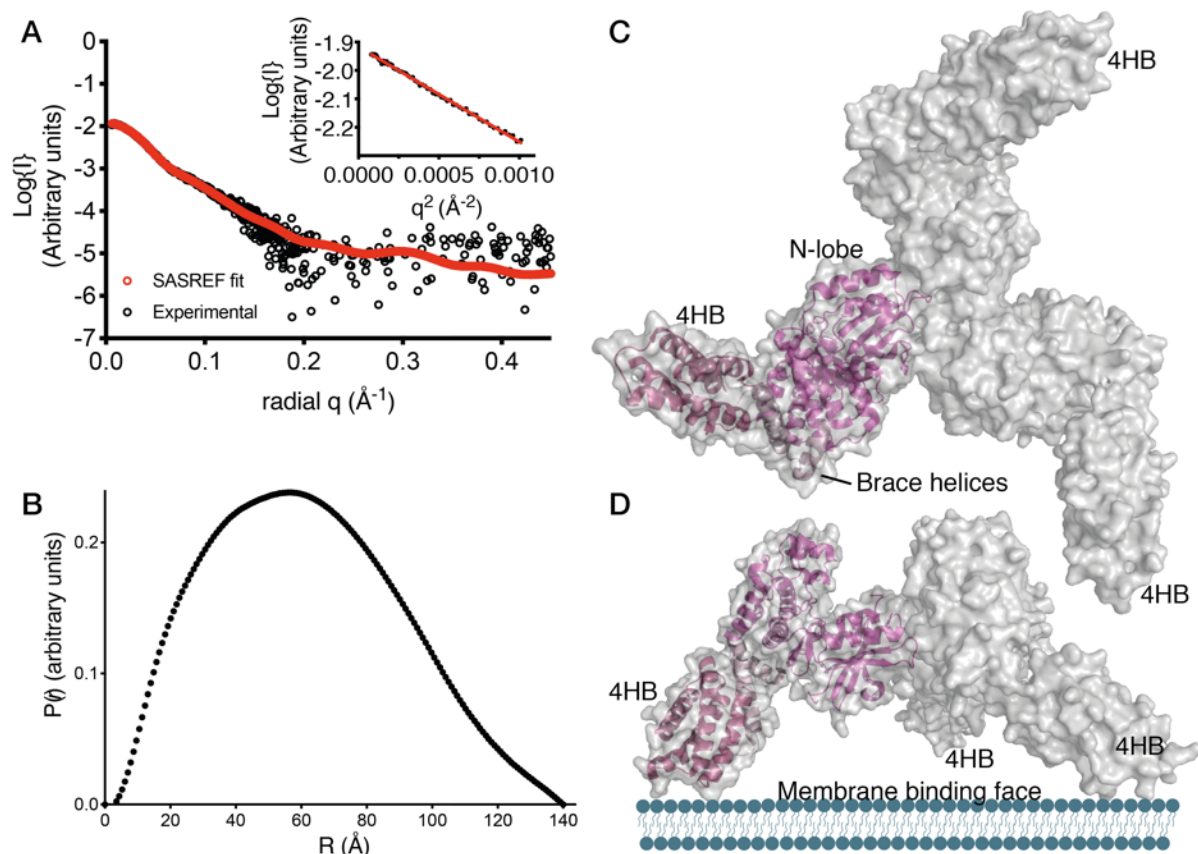
The amount of necroptosis observed for wild-type MLKL was higher with TSI than it was with TSQ ( $76.19 \pm 5.45$  vs  $50.01 \pm 8.04\%$ ), likely because IDN-6556 is a more potent caspase-8 inhibitor (Brumatti et al., 2016), which strongly drives cells to undergo necroptosis (Figure 4.6). The data for the original double alanine mutants (Figure 4.4) assayed with TSI can be found in Davies et al. (2018) (Appendix II). Mutant E161A-T165A reconstituted death signalling to levels comparable to the wild-type counterpart (TSI treated induced death  $83.63 \pm 5.305\%$ ), whilst mMLKL I162A-L166A was not able to induce cell death above background levels (TSI:  $11.74\% \pm 2.01\%$ ).

E161A-T165A exhibited a small, but statistically significant, ability to induce constitutive cell death upon its expression (Figure 4.6). Compared to the original double alanine mutants E161A-I162A and T165A-L166A, which were still capable of inducing cell death, albeit to slightly lower levels than wild-type MLKL (Figure 4.4), the new alanine mutations E161A-T165A and I162A-L166A results were clearer and showed that residues I162 and L166 were essential to death, and residues E161 and T165 were not. The data in Figure 4.6 and Figure 4.4 also indicated that mutating only one of the hydrophobic residues in the context of full-length MLKL was not enough to completely prevent oligomerisation.

#### *4.4 SAXS analysis is consistent with full-length mouse MLKL forming trimers in solution*

Published AUC data (Hildebrand et al., 2014) and SAXS analysis (Davies et al., 2018) on mMLKL 1-169 indicate that it forms a trimer, and the results presented previously in this chapter indicate that full-length MLKL oligomer is related to the mMLKL 1-169 trimer. However, there are currently no published data studying the oligomerisation of the recombinant full-length mouse MLKL. Improvements made to the expression of full-length mouse MLKL during my PhD made it possible to purify enough of the recombinant MLKL oligomer to perform SAXS analysis (Figure 4.7).





**Figure 4.7 SAXS analysis supports trimeric model for mouse MLKL in solution**

**A** Scattering profile of averaged and background subtracted data from apex of inline size exclusion peak of mMLKL full-length oligomer (black circles). Theoretical scatter values calculated from the SASREF rigid body fit are shown in red circles. The fit has a  $\chi$  value of 0.47. Inset: Guinier analysis of experimental SAXS data for  $q$ . The linearity of the plot indicates the protein is not aggregated. **B** The  $P(r)$  real space interatomic distribution for mMLKL full-length, calculated with GNOM, with maximum particle dimension ( $D_{\text{max}}$ ) of 140  $\text{\AA}$ . **C** Rigid body modelling of the full-length mMLKL trimer calculated from the monomeric crystal structure coordinates (PDB:4BTf) using SASREF. The surface of the trimer model calculated by SASREF is shown in grey. A cartoon representation of the mouse MLKL full-length crystal structure (magenta), that was used as the envelope to calculate the model is overlaid with one protomer, and the 4HB, brace helices, and N-lobe of the pseudokinase domain is labelled. **D** The trimer model oriented with the 4HB on one face, which would presumably form the membrane binding face.

**Table 4.1 Mouse MLKL full-length SAXS data collection and analysis statistics**

| Data-collection parameters                 |                                                                                                                                 |
|--------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------|
| Instrument                                 | Australian Synchrotron SAXS/WAXS beamline                                                                                       |
| Beam geometry                              | 120 $\mu\text{m}$ x 400 $\mu\text{m}$ source                                                                                    |
| Wavelength ( $\text{\AA}$ )                | 1.033                                                                                                                           |
| Exposure time                              | 2 second exposures                                                                                                              |
| Temperature (K)                            | 285                                                                                                                             |
| q range ( $\text{\AA}^{-1}$ ) <sup>a</sup> | 0.0106 to 0.45                                                                                                                  |
| Protein concentration                      | 45 $\mu\text{L}$ of 6.7 mg/ml protein via inline gel filtration chromatography<br>0.2M NaCl, 20mM HEPES pH 7.5, 5% v/v glycerol |
| Structural parameters                      |                                                                                                                                 |
| $I(0)$ ( $\text{cm}^{-1}$ ) [from $P(r)$ ] | $0.01198\text{E} \pm 0.00007$                                                                                                   |
| $R_g$ ( $\text{\AA}$ ) [from $P(r)$ ]      | $47.70 \pm 0.223$                                                                                                               |
| $D_{\text{max}}$ ( $\text{\AA}$ )          | 140                                                                                                                             |
| $I(0)$ ( $\text{cm}^{-1}$ ) (from Guinier) | $0.01217 \pm 0.0001$                                                                                                            |
| $R_g$ ( $\text{\AA}$ ) (from Guinier)      | $48.6 \pm 0.815$                                                                                                                |
| Software employed                          |                                                                                                                                 |
| Primary data reduction                     | Scatterbrain (Australian Synchrotron)                                                                                           |
| Data processing                            | PRIMUS, GNOM                                                                                                                    |
| Computation of model intensities           | CRY SOL                                                                                                                         |
| Rigid body fitting                         | SASREF                                                                                                                          |

<sup>a</sup> q is the magnitude of the scattering vector, which is related to the scattering angle ( $2\theta$ ) and the wavelength ( $\lambda$ ) as follows:  $q = (4\pi/\lambda)\sin\theta$

The mouse MLKL full-length protein elutes from size exclusion chromatography in a monomer and oligomer peak (data not shown). 45  $\mu\text{l}$  of the concentrated oligomer peak was loaded onto an inline SEC column, and SAXS data collected was on the peak that was eluted. The data displayed a linear Guinier region, indicating that protein aggregates did not contribute to the scattering profile (Figure 4.7A). A  $P(r)$  curve, which shows the distribution of interatomic distances within the protein (reflecting size and shape), was calculated from the scattering profile, that showed a  $D_{\text{max}}$  of 140  $\text{\AA}$ , and a radius of gyration of 47.7  $\text{\AA}$ . This was in good agreement with the values of  $D_{\text{max}}$  and  $R_g$  calculated from the Guinier plot (Table 4.1). SASREF was

used to generate a rigid body model to fit the experimental data, with imposed P3 symmetry but no imposed contacts, based on the previous SAXS and AUC performed on mMLKL 1-169. The mouse MLKL full-length crystal structure (PDB:4BTF) was used to generate the envelope used in fitting. The model which best fit the data, with a  $\chi$  value of 0.47 is shown in Figure 4.7 C. The 4HB faces outward in the model and the oligomeric contact points are within the N-lobe of the pseudokinase domain based on the model.

The SAXS data was fitted as a trimer based on the previous results for mMLKL 1-169 (Davies et al., 2018). To validate that a trimer was the correct choice of symmetry for the rigid body modelling, SASREF was used to model the mouse MLKL full-length with P2 (corresponding to dimer) and P4 (corresponding to tetramer) symmetry as well (Table 4.2).

**Table 4.2 SASREF fitting of rigid body models with P2, P3 and P4 symmetry**

| <b>Symmetry</b>   | <b><math>\chi</math> value of best fit</b> |
|-------------------|--------------------------------------------|
| Monomer (CRY SOL) | 3.60                                       |
| P2 (dimer)        | 1.06                                       |
| P3 (trimer)       | 0.47                                       |
| P4 (tetramer)     | 0.64                                       |

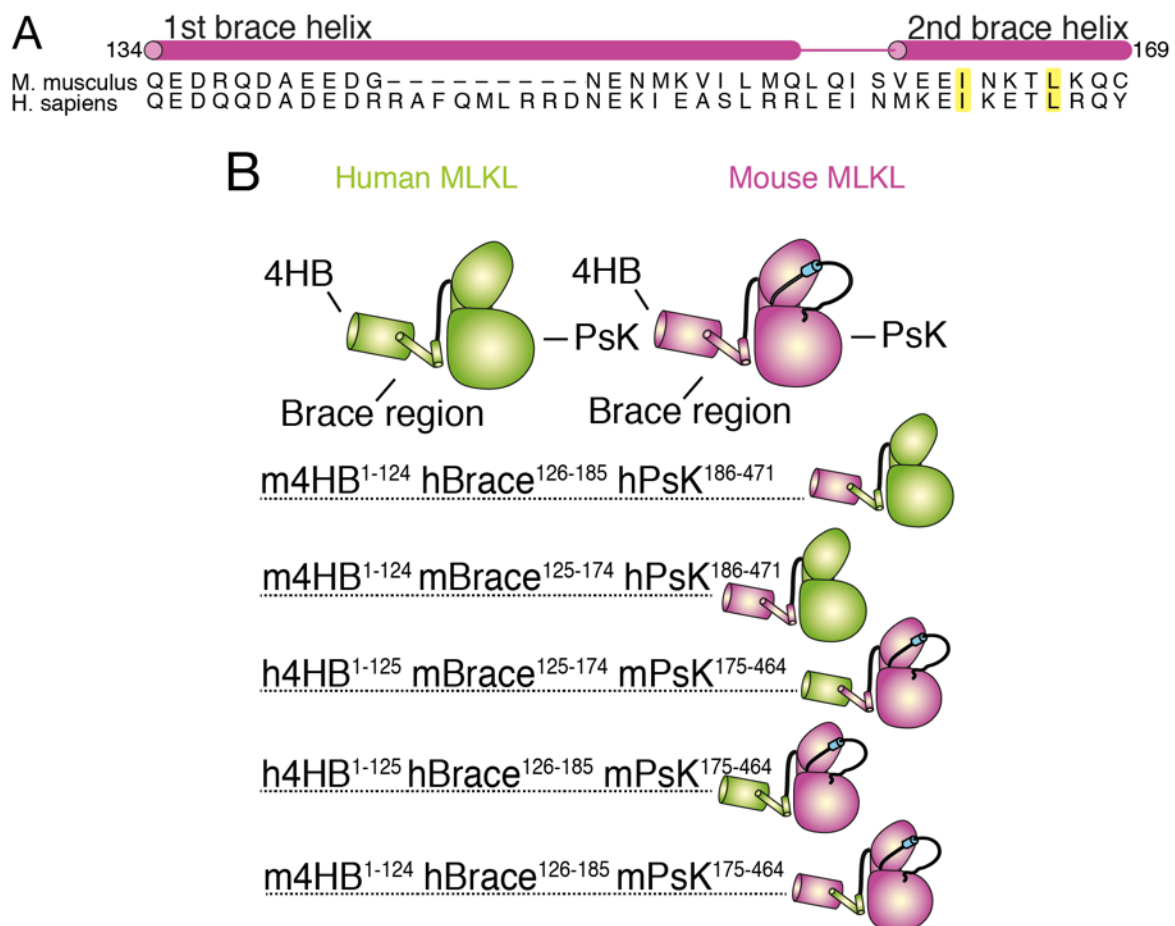
Table 4.2 showed that the trimeric model was the best fit for the SAXS data, but that the tetramer model had a plausible  $\chi$  value too (0.64).  $\chi$  values under 1 are considered plausible, and the dimeric and monomeric fits had  $\chi$  values of greater than 1, showing that mouse MLKL full-length is not a monomer or dimer in solution.

#### *4.5 Examining differences between mouse and human MLKL brace regions*

The first half of this chapter illustrates the importance of the brace region to MLKL's oligomerisation and killing ability. However, despite this importance, there is much sequence variation in the brace between MLKL orthologues (Chapter 3, Figure 3.1), including between mouse and human MLKL. The residues, I162 and L166, that are required for oligomerisation in mouse MLKL are conserved in human MLKL; however, human MLKL has nine additional residues in the brace region relative to the mouse sequence (Figure 4.8 A). Mouse and human constructs comprising the 4HB+brace

have different killing ability in mouse and human cell lines respectively. The mouse 4HB+brace construct is constitutively active in mouse cells, but the human 4HB+brace construct is not active in human cells (Tanzer et al., 2016). The reasons for this difference are unclear.

To test how the differences in the brace region sequence may affect communication between domains, and potentially enforce species-specificity or the particular behaviour of each species' MLKL, chimeric MLKL constructs were designed. These chimeras swap elements of the human and mouse sequences at domain boundaries identified in the mouse MLKL full-length crystal structure (Figure 4.8). The sequence boundaries for human were made based on sequence alignment with the mouse protein. Details of chimeric constructs are described in Figure 4.8 B.



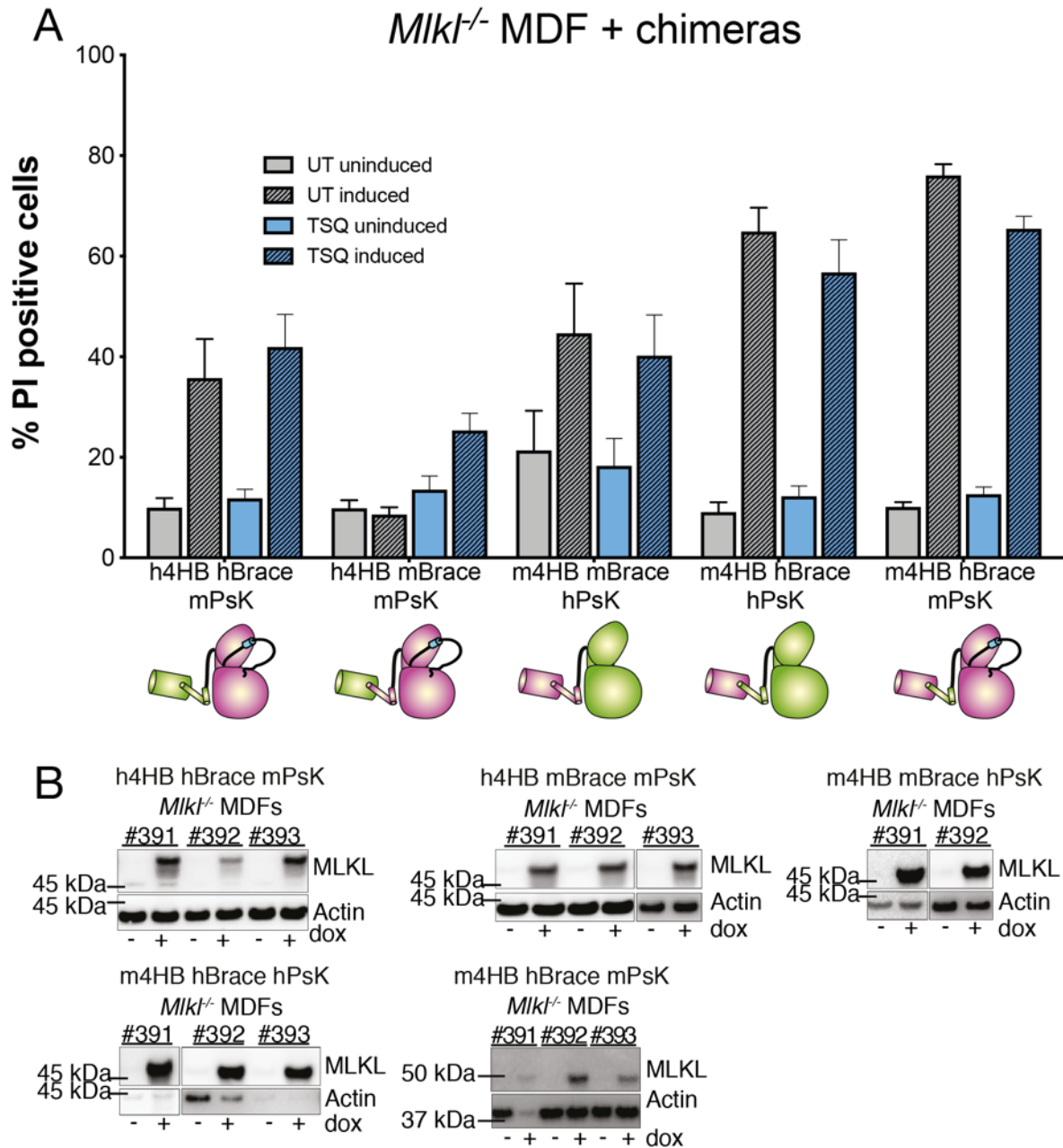
**Figure 4.8 Schematic of chimeric mouse and human MLKL constructs**

**A** An alignment of the mouse and human brace region. I162 and L166 are conserved in human MLKL (highlighted in yellow), but human MLKL has a nine residue insertion

in the brace region. **B** Details of the five chimeric constructs made. Human MLKL components are coloured green, and mouse MLKL is coloured pink. The chimera names, and domain boundaries are labelled.

The chimeras were tested in both human (HT29, and U937) and mouse (MDF) cell lines. Only the data from the mouse cell lines are presented here, because I performed death assays on the mouse cells during the course of my PhD, but not human cells. The human data were published in Davies et al. (2018) (See Appendix II).

The chimeras were expressed in two or three biologically-independent *Mlkl*<sup>-/-</sup> MDF cells lines, using a lentiviral system that generates stably transduced dox inducible constructs. Cells were then either left untreated or treated with necroptotic stimuli (TSQ) in the presence or absence of doxycycline to determine how the chimeric constructs could contribute to necroptotic cell death (Figure 4.9)

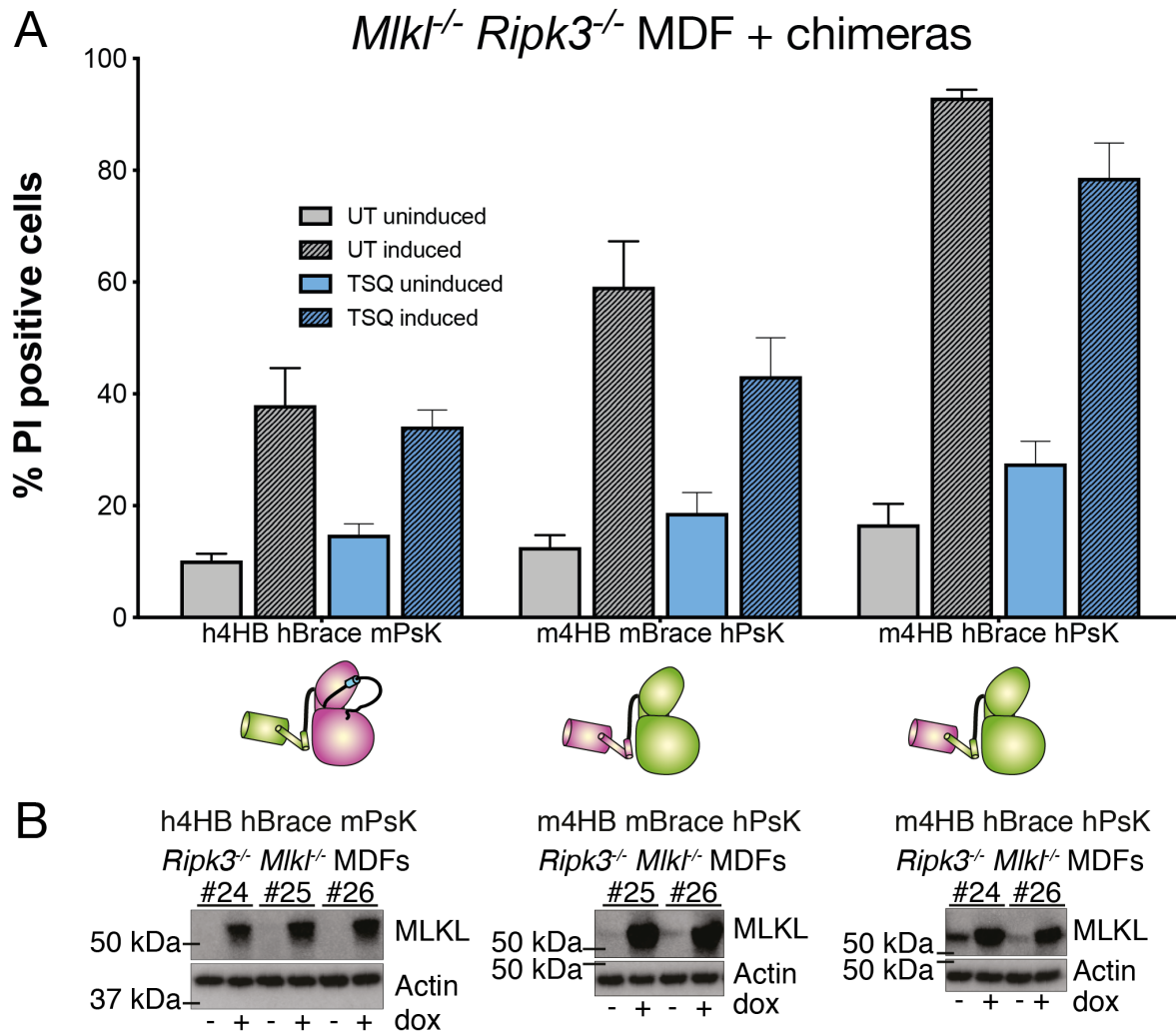


**Figure 4.9 Most chimeric MLKL constructs are constitutively active in MDFs**

**A** Chimeric MLKL expression was induced in *Mlkl*<sup>-/-</sup> MDF cells using doxycycline, and then cells were treated with death stimulus (TSQ). Death was measured by flow cytometry after 24 hours. MDF assays were conducted in biological duplicate or triplicate in at least three independent experiments (n=6-10). **B** MDFs *Mlkl*<sup>-/-</sup> cell lines reconstituted with MLKL chimeras. 391, 392 and 393 are biological replicate cell lines. Cells were treated with dox for three hours, cell extracts were run on SDS-PAGE and western blotting performed to detect MLKL levels. Maria Tanzer prepared all the cell lines in this figure, and performed western blots on them, except for the m4HB-hBrace-mPsK cell line which I prepared, and performed western blots on. Death assays repeats were shared, Maria Tanzer performed 27/42 repeats for this figure, and I performed 15/42.

Four of the five chimeric constructs (h4HB-hBrace-mPsK, m4HB-mBrace-hPsK, m4HB-hBrace-hPsK and m4HB-hBrace-mPsK), were able to induce necroptosis in MDFs upon expression by addition of dox alone (Figure 4.9). Only h4HB-mBrace-mPsK was not auto-active, and furthermore only showed weak induction of necroptosis ( $25.36 \pm 3.41\%$ ) even upon addition of TSQ stimulus. Addition of TSQ in the MDFs expressing auto-active constructs, did not increase levels of necroptosis. All chimeras were expressed to similar levels in MDFs (4.9 B), including h4HB-mBrace-mPsK. This indicated that the deficit in necroptotic activity was due to the make-up of the chimera, not a lack of expression in cells.

To test if RIPK3 was contributing to necroptosis mediated by the chimeras, by constitutive recruitment for instance, three of the auto-active chimeras were expressed in *Mkl1<sup>-/-</sup> Ripk3<sup>-/-</sup>* MDFs, and their activity tested in death assays (Figure 4.10).



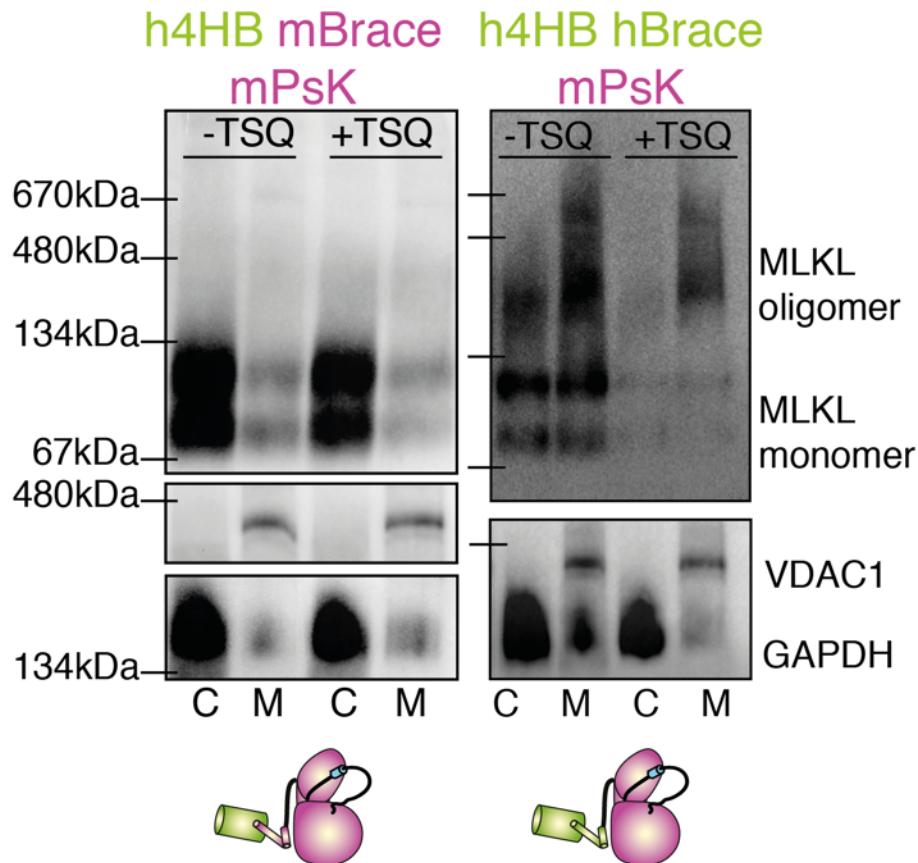
**Figure 4.10 The constitutive activity of chimeras is RIPK3 independent**

**A** Doxycycline was added to *Mlkl<sup>-/-</sup> Ripk3<sup>-/-</sup>* MDF cells to induce expression of chimeric MLKL constructs, and then cells were treated with necroptotic stimulus (TSQ). Death was measured by propidium iodide (PI) uptake by flow cytometry after 24 hours. MDF assays were conducted in biological duplicate or triplicate in at least 3 independent experiments (n=6-10). **B** *Mlkl<sup>-/-</sup> Ripk3<sup>-/-</sup>* MDF cell lines reconstituted with MLKL chimeras, 24, 25 and 26 are biological replicate cell lines, derived from different mice. Cells were treated with dox for three hours, cell extracts were run on SDS-PAGE and western blotting performed to detect MLKL levels.

h4HB-hBrace-mPsK, m4HB-mBrace-hPsK and m4HB-hBrace-hPsK were all constitutively active in *Mlkl<sup>-/-</sup> Ripk3<sup>-/-</sup>* MDF cells, and were expressed to similar levels (Figure 4.10). The results of the death assays in the double knockouts (Figure 4.10) were very similar to the *Mlkl<sup>-/-</sup>* MDFs (Figure 4.9), indicating that RIPK3 was not required for necroptosis caused by the chimeras h4HB-hBrace-mPsK, m4HB-mBrace-hPsK and m4HB-hBrace-hPsK. Therefore, constitutive RIPK3 association could not be the mode for auto-activity; these data suggest an activation



mode that is MLKL-intrinsic. BN-PAGE was performed to test for the presence of MLKL oligomers in the membrane fraction of unstimulated cells, that could account for the constitutive death. Auto-active construct h4HB-hBrace-mPsK and inactive construct h4HB-mBrace-mPsK were tested (Figure 4.11).



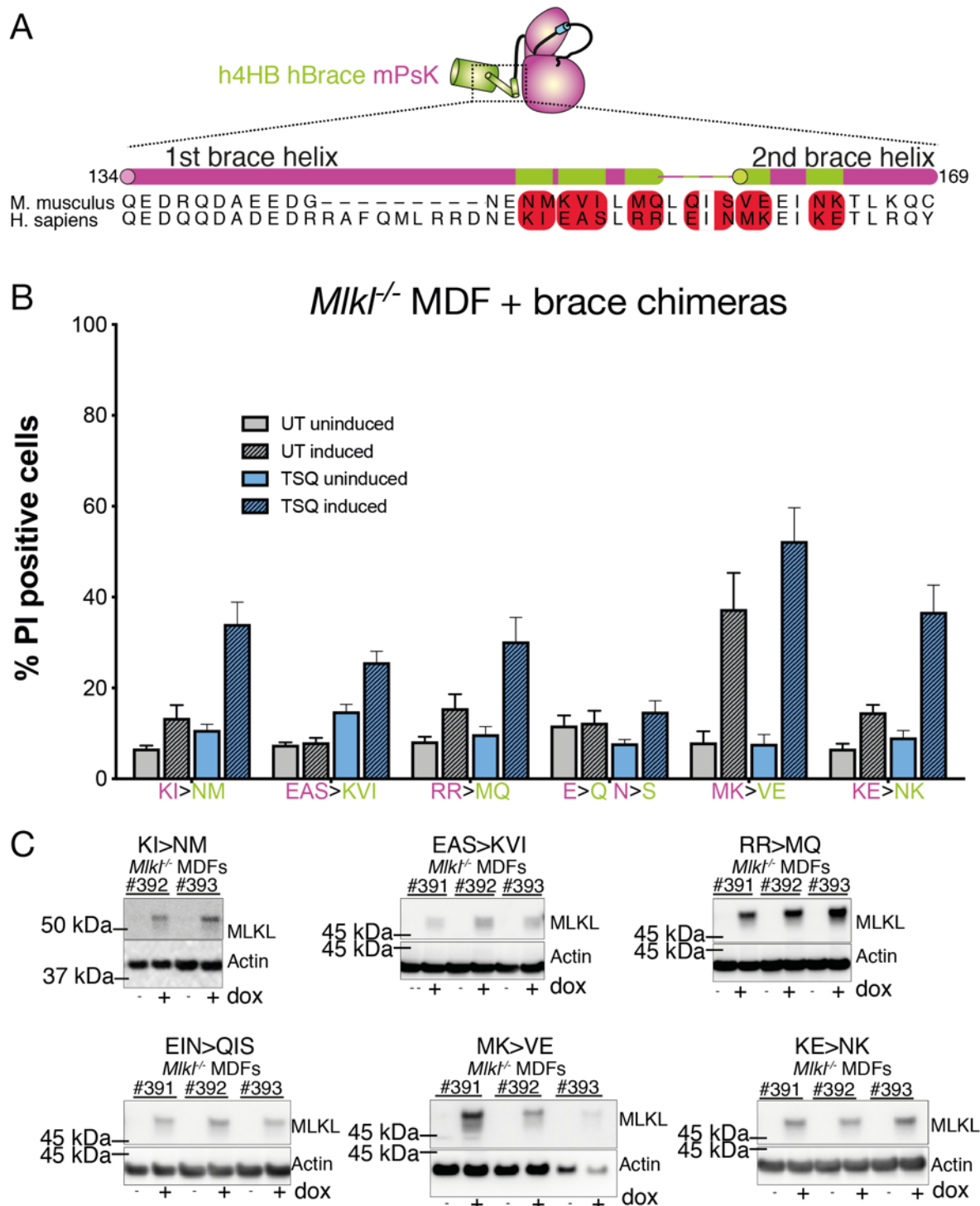
**Figure 4.11 h4HB-hBrace-mPsK forms oligomers constitutively but h4HB-mBrace-mPsK cannot oligomerise**

All MDF samples were treated with doxycycline for 3 hours, then half were treated with TSQ. h4HB-mBrace-mPsK harbouring cells were harvested 7 hours after TSQ treatment, h4HB-hBrace-mPsK expressing cells had to be harvested after 18 hours to achieve sufficient MLKL signal to be detected by BN-PAGE western blot. Cells were fractionated using digitonin. Cytoplasmic and crude membrane fractions were run on BN-PAGE, then transferred onto a PVDF membrane and blotted. VDAC1 is a loading control for membrane, and GAPDH is a loading control for cytoplasm. The blots are representative of two independent experiments.

Chimera h4HB-mBrace-mPsK was unable to form oligomers, even in TSQ treated cells (Figure 4.11), which accounted for the low levels of cell death upon TSQ stimulus observed in Figure 4.9. h4HB-hBrace-mPsK formed oligomers upon expression even in the absence of TSQ stimulation. Interestingly for the h4HB-hBrace-mPsK, the TSQ

stimulated sample showed less MLKL monomer remaining in the cytoplasm than in the dox only treated sample, suggesting that the construct may interact with RIPK3. This was not manifested in the death assay results however (Comparing Figure 4.11 with and without TSQ, and Figure 4.9 A), where death was not enhanced substantially by TSQ addition.

The inactive h4HB-mBrace-mPsK chimera, and the auto-active h4HB-hBrace-mPsK chimera, differ only in the sequence of the brace region, yet one chimera is totally inactive and incapable of forming membrane associated oligomers, while the other is a constitutive oligomeric killer (Figure 4.11). In order to better understand which specific residues of either the mouse or human brace region could lead to such differences in activity, a series of mutations were made to the brace region in the auto-active h4HB-hBrace-mPsK construct (Figure 4.12). The mutations were targeted to residues which differs between the mouse and human brace region, outside of the nine-residue insertion in human MLKL. On the background of the human brace in the h4HB-hBrace-mPsK chimera, these specific residues were mutated to the mouse sequence in groups of two or three. Six brace mutant constructs were made (Figure 4.12 A) and tested in death assays, as previously described in this chapter (Figure 4.12 B).



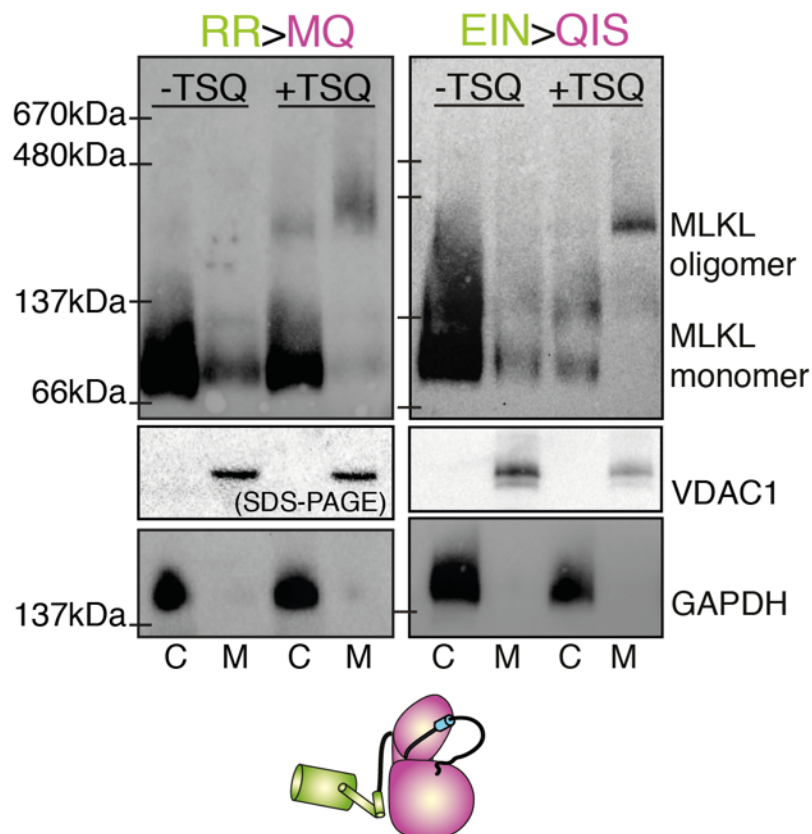
**Figure 4.12 Fine mutations made to the brace region of h4HB-hBrace-mPsK**

**A** Alignment of the mouse and human brace region, with residues that differ between the two species, that were targeted by mutation, highlighted in red. Each red bubble signifies one construct that has residues in that bubble mutated from the human sequence to the mouse sequence in the background of the h4HB-hBrace-mPsK chimera. **B** The brace mutant chimeras were challenged in death assays. Doxycycline was used to induce expression of chimeric brace mutant MLKL constructs *Mlkl*<sup>-/-</sup> MDF cells, and then treated with death stimulus (TSQ). Death was measured by propidium iodide (PI) uptake by flow cytometry after 24 hours. MDF assays were conducted in biological duplicate or triplicate in at least three independent experiments (n=6-10).

Maria Tanzer prepared all cell lines for this figure, and performed all western blots except for KI>NM, which I performed the western blot for. Maria Tanzer contributed 7/55 death assay repeats, Emma Petrie contributed 20/55 death assay repeats and I contributed 28/55 repeats.

All the brace mutant chimeras, except MK>VE, lost ability to cause high levels of constitutive necroptosis upon expression (Figure 4.12 A, B). MK>VE appeared to behave most similarly to the base construct h4HB-hBrace-mPsK (Figure 4.9). In particular KI>NM, RR>MQ and KE>NK all displayed more 'wild-type-like' behaviour, than the base construct, with reduced auto-activity, and necroptosis being induced by conventional necroptotic stimulus TSQ.

To test if the new brace mutant chimeras really did resemble wild-type MLKL in their necroptotic signalling, BN-PAGE was performed to monitor for the appearance of oligomers in response to TSQ stimulation (Figure 4.13).



**Figure 4.13 BN-PAGE of fine mutation chimeras**

MDF samples were treated with doxycycline for 3hours, then half were treated with TSQ. Cells were harvested 7 hours after TSQ treatment and then fractionated using

digitonin. Cytoplasmic (C) and crude membrane (M) fractions were run on BN-PAGE, then transferred onto a PVDF membrane and blotted. VDAC1 is a loading control for membrane, and GAPDH is a loading control for cytoplasm. The blots are representative of two independent experiments.

Both RR>MQ and EIN>QIS were not able to form MLKL oligomers in the membrane fraction without TSQ stimulus, though a small amount of MLKL monomer was present in the membrane fraction of each chimera (Figure 4.13). Both mutants were able to form oligomers at the membrane in response to TSQ stimulus, in the fashion of wild-type MLKL. It must be noted however, that both mutants, especially EIN>QIS, showed deficits in levels of necroptosis in cells compared to wild-type MLKL (compare with wild-type mouse MLKL in MDFs treated with TSQ Figure 4.4).

#### *4.6 Discussion*

Since MLKL was first identified as the final executioner of necroptosis, the stoichiometry of its oligomer has been a point of contention in the literature. Furthermore, no high-resolution structure of the oligomer is currently available, and the residues which form the oligomeric interface are unknown. Data from our group has suggested that human and mouse MLKL exhibit different characteristics in their cell killing ability (Tanzer et al., 2016), their structure (Murphy et al., 2014a), and also their oligomerisation stoichiometry (Davies et al., 2018, Petrie et al., 2018, Hildebrand et al., 2014). The study presented in this chapter tried to understand which residues of the mouse MLKL brace region contribute to mouse MLKL oligomerisation, and understand how differences in the sequence of the brace region between mouse and human MLKL may contribute to their differences in cell killing ability and regulation (Davies et al., 2018).

#### *The functional MLKL oligomer in cells*

In 2014, the recombinant construct mMLKL 1-169 comprising the mouse 4HB plus the two brace helices was reported to spontaneously form a trimer in solution (Hildebrand et al., 2014). The oligomerisation of constructs comprising of the 4HB+brace region but not the 4HB alone had been observed by multiple groups in studies of human MLKL (Quarato et al., 2016, Su et al., 2014). We identified that it

was the second brace helix of mouse MLKL, which conferred oligomerisation ability to mMLKL 1-169 through the generation of the mMLKL 1-158 construct (Davies et al., 2018). Based on this, alanine scanning mutagenesis of the second brace helix was performed and two mutations which disrupted oligomerisation in the recombinant system were identified; E161A-I162A, and T165A-L166A (Figure 4.1, 4.2). The same mutations introduced into full-length mouse MLKL prevented the formation of oligomers in the membrane fraction of cells after 7 hours of TSQ treatment via BN-PAGE (Figure 4.3). However, these mutations did not totally abrogate cell death (Figure 4.4), suggesting that some oligomers may still form in the presence of these mutations, albeit with slower kinetics, or in reduced levels.

The persistent necroptosis that could occur through E161A-I162A and T165A-L166A MLKL constructs (Figure 4.4) hinted that the precise determinants of the second brace helix required for oligomerisation had not been completely targeted. Examining the positions of E161-I162 and T165-L166 in the crystal structure of full-length mouse MLKL revealed that alanine substitutions of these pairs targeted two possible interfaces (Figure 4.5). One interface was a hydrophobic packing interaction between the second and first brace helix, and the other was hydrogen bonds between the second brace helix and the pseudokinase domain of MLKL. Mutant constructs E161A-T165A and I162-L166A were designed to disrupt either the hydrophobic or hydrophilic interface, and it was the hydrophobic residues, I162 and L166, that were essential for MLKL mediated cell death (Figure 4.6). Based on the BN-PAGE data on related constructs, E161A-I162A and T165A-L166A (Figure 4.3) it is likely that residues I162 and L166 are crucial for oligomerisation.

#### *Possible mode of oligomerisation?*

The residues found to be responsible for disrupting oligomerisation by alanine scanning, leucine and isoleucine, both have hydrophobic sidechains. Mutation of these residues to alanine, which also has a hydrophobic sidechain, is not a dramatic change, but a loss of bulk. This subtle change resulted in complete abrogation of killing function. A well-known class of oligomers that rely on hydrophobic interactions are coiled coil domains (Woolfson, 2017). Coiled coil oligomers are formed by

hydrophobic packing interactions between helices from different protomer subunits, relying predominantly on hydrophobic residues such as I, L, V and M, which fall on the 'a' and 'd' positions of a heptad repeat motif characteristic of coiled coils i.e. a(I,L,V,M)-b(X)-c(X)-d(I,L,V,M)-e(X)-f(X)-g(X), where X is any amino acid (Parry et al., 2008). Proteins which do not have perfect heptad repeats can still form coiled coils, and identity of amino acids within the heptad repeat, either at the 'a' and 'd' positions, or in surrounding positions, and the spacing between hydrophobic residues, can influence the stoichiometry of the oligomer formed (Nie et al., 2016, Woolfson, 2017, Bjelic et al., 2013, Harbury et al., 1993, Ciani et al., 2010, Parry et al., 2008). Alanine can also be found in heptad repeat motifs at the 'a' or 'd' position, however mutation of two or more bulkier hydrophobic residues to alanine has been shown to disrupt oligomer formation via coiled coils (Luo et al., 1999), so the alanine mutation I162A-L166A may disrupt a coiled coil oligomer.

One study has previously explored the idea that human MLKL forms oligomers via a coiled coil motif (Cai et al., 2014). They used Multicoil, a bioinformatics program that predicts the likelihood of both dimeric and trimeric coiled coil motifs in proteins based on their sequence (Wolf et al., 1997). Multicoil identified two sections of human MLKL that had some likelihood of forming a coiled coil, a region between residues 50-78 in the 4HB, and the region comprising the first and second brace helix of MLKL (Cai et al., 2014). Cai et al. (2014) showed that in the first predicted coiled coil motif, substitution of specific residues of the heptad repeat with glycine could not disrupt oligomerisation in cells. They did find however, that mutation of the equivalent residues to I162 and L166 in human MLKL to glycine, in the second predicted coiled coil region, disrupted oligomerisation of human MLKL in cells.

Our two studies reached agreement on the residues essential for oligomerisation using two different starting points, and in different MLKL species. Our study identified I162 and L166 in an unbiased way from the two initial double alanine mutations E161A-I162A and T165A-L166A. Cai et al. (2014) made mutations based on a hydrophobic heptad repeat predicted by Multicoil, with I162 falling on a 'd' position and L166 on an 'a' position. The heptad repeat identified in Cai et al. (2014) is

reproduced in Figure 4.14. The repeat contains mainly hydrophobic residues at the ‘a’ and ‘d’ positions however does have some residues less commonly found in coiled coils at those positions such as asparagine (Figure 4.14). Cai et al. (2014) did not publish results for mutation to any of the other residues which fell on the ‘a’ and ‘d’ position of the predicted heptad repeat. In order to validate if the proposed heptad repeat is important, mutation to other components of it would need to be made. My own study only examined the second brace helix, and found that mutation to residues not situated at the ‘a’ and ‘d’ positions of the heptad repeat (E161, N163, K164, T165, K167 and Q168) did not affect oligomerisation. This could constitute evidence that the heptad repeat is important, however better proof would be targeting other residues in the ‘a’ and ‘d’ position, by removing their hydrophobic side chains.



**Figure 4.14 The heptad repeat identified by the Multicoil Program (reproduced from Cai et al. (2014))**

The sequence of mouse and human MLKL brace region is aligned with heptad repeat predicted by Multicoil (Wolf et al., 1997). Residues at the ‘a’ and ‘d’ position are highlighted in yellow.

Another interesting implication of the coiled coil hypothesis is that it might help to explain why the human and the mouse protein form a tetramer and trimer respectively (Petrie et al., 2018, Davies et al., 2018). Slight changes to the amino acid sequences of coiled coils can change their oligomerisation stoichiometry, and the human MLKL predicted coiled coil extends into its nine-residue insertion, giving it a substantial difference to mouse.

The best way to examine whether MLKL forms a coiled coil oligomer would be to generate a high-resolution structure, which was an aim of my PhD. However, despite optimisation of crystals of mMLKL 1-169 and similar constructs, no crystals diffracting better than 7 Å were obtained. In the absence of a high-resolution structure, SAXS was used to gain low resolution structural information about the trimer.



#### *Mouse MLKL full-length protein likely forms trimers in solution*

The SAXS results show that a trimeric model of mouse MLKL full-length is favoured compared to the dimer or tetramer models (Figure 4.7, Table 4.2). This is in agreement with the AUC and SAXS data collected for mMLKL 1-169 and together these results suggest that mouse MLKL is a trimer. However, because we suggest the mouse oligomer has a different stoichiometry than the human tetramer, and because the tetramer model provided a plausible fit for the mouse MLKL SAXS data, it is necessary to provide further evidence for this assertion. In future, to confirm unambiguously the stoichiometry of the mouse MLKL oligomer, native mass spectrometry should be performed on the mouse full-length oligomer, as Petrie et al. (2018) did for human MLKL.

Unlike the trimer model generated for mMLKL 1-169 (Davies et al., 2018) in which the brace helices are modelled as the contact point between trimer subunits, in the full-length MLKL model (Figure 4.7) the N-lobe of the pseudokinase domain appears to form the contact points. Because this model is derived from the coordinates of the monomeric mouse MLKL crystal structure (Murphy et al., 2013), we cannot exclude the possibility that the domains adopt a different conformation in the context of an oligomeric assembly. The general organisation of the model appears to be plausible, because the 4HB domains are all oriented on one face of the oligomer, which could constitute a membrane binding face (Figure 4.7 D).

#### *Chimeras illustrate the role of the brace region in interdomain communication*

The domain swapped chimeras between mouse and human MLKL demonstrated the essential role that the brace region plays in interdomain communication, and regulation of MLKL's killing ability (Figure 4.8, 4.9). All the chimera constructs that included the mouse 4HB domain were auto-active. m4HB or m4HB+brace constructs have been shown to be auto-active in mouse cells when expressed alone (Hildebrand et al., 2014). Therefore, the domain swapped chimeras appear to have lost MLKL's mechanism of regulating the mouse 4HB, allowing it to act independently of the rest of the protein. Importantly, the species specific pseudokinase domain is not enough

to regulate activity of the 4HB domain; the correct species of the brace helices is also essential, as the m4HB-hBrace-mPsK chimera was auto-active.

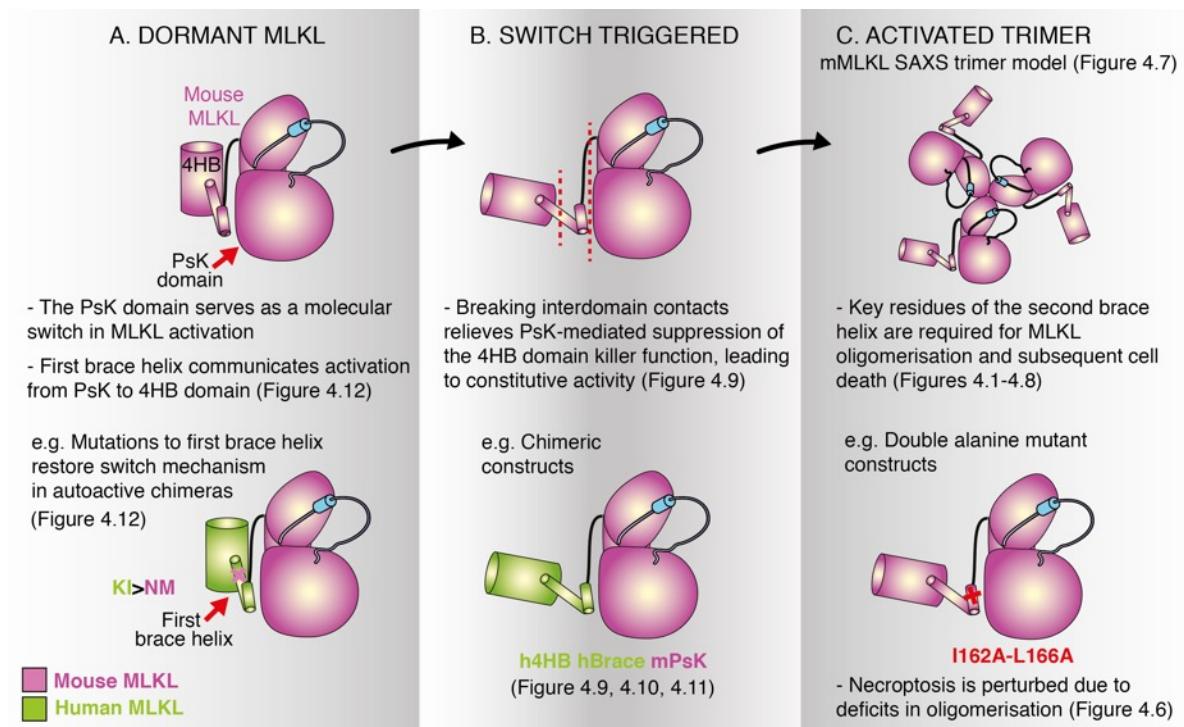
The results of the chimeras that contained the human 4HB domain were more surprising. The h4HB or h4HB+brace have previously been shown to be inactive in *MLKL*<sup>-/-</sup> MDFs, even with artificial dimerization (Tanzer et al., 2016). h4HB-hBrace-mPsK was auto-active in cells, showing that the human 4HB itself is capable of permeabilising the plasma membrane of mouse cells. Instead the h4HB or h4HB+brace alone must be incapable of permeabilising MDFs for other reasons. These could include lack of membrane association, lack of oligomerisation or in the case of the artificially dimerised 4HB, a difference in form of the artificial oligomer to the functional oligomer. A h4HB-hBrace construct (hMLKL 1-182) has been shown to form oligomers when purified as a recombinant protein (Quarato et al., 2016), but a very similar construct hMLKL 1-180 was not able to form membrane associated oligomers in human cells (U937) when examined by BN-PAGE (Tanzer et al., 2016). The h4HB-hBrace-mPsK chimera was capable of constitutive oligomerisation and membrane translocation in MDFs (Figure 4.11). This suggests that inclusion of the mouse pseudokinase domain relieved some suppression of oligomerisation that the h4HB-brace has in cells. Or, inclusion of the mouse pseudokinase domain could confer compatibility with partner proteins that may promote oligomerisation, an example could be mouse HSP90 (Introduction, Section 1.6). The related chimera m4HB-hBrace-mPsK was also auto-active, so potentially the combination of mouse pseudokinase with human brace favours the formation of oligomers.

Conversely, the h4HB-mBrace-mPsK chimera, was incapable of oligomerising and therefore causing cell death. This implies that the presence of the human 4HB domain inhibits the formation of oligomers via the mouse brace region. Recent findings suggesting that an inhibitory interaction between the first brace helix and the 4HB domain must be released for human MLKL to become properly activated (McNamara et al., 2019) might provide an alternative explanation for the lack of activity. The mouse brace region may be a potent inhibitor of the human 4HB domain and perhaps

in this chimera, the mechanisms for removing this inhibitory interaction are compromised.

The chimeras, h4HB-hBrace-mPsK and h4HB-mBrace-mPsK, show that the species-specific sequence of the brace region has a potent effect on killing ability. The cell death assays on the brace mutation chimeras (Figure 4.12) suggest that specific residues of the brace region are essential for normal MLKL regulation. The residues of the first brace helix seem to be more involved, as mutation to some residues of the second brace helix did not abolish auto-activity (Figure 4.12, also more clearly seen in TSI data, published in Davies et al. (2018), Appendix II). Interestingly, the specific residues that were mutated to the mouse sequence in the context of the h4HB-hbrace-mPsK chimera did not induce the inhibition of oligomerisation that was observed in the h4HB-mBrace-mPsK chimera. Therefore, it is likely that more substantial changes from the human brace to the mouse brace are mediating the inhibition of oligomerisation; for example, the deletion of nine residues in the mouse brace compared to the human brace may contribute.

Figure 4.15 provides a graphical summary of what has been learnt from studying the MLKL brace region in this chapter. The data suggest that the brace region plays an essential role in much of MLKL's mechanism of action. This includes communication of regulatory and activation signals from the pseudokinase domain to the 4HB (Figure 4.15 A, B), and playing an integral role in the formation of the functional MLKL oligomer, which kills cells (Figure 4.15 C).



**Figure 4.15 Summary of the roles of the brace region**

The data presented in this chapter show that the brace region plays an essential role in the suppression of MLKL mediated killing (**B**), normal communication of activation signals (**A**), and formation of the active MLKL oligomer (**C**).

## **5.0 MLKL at the Membrane**

As discussed in the Introduction (Section 1.8), only a handful of studies have examined MLKL's membrane permeabilisation ability from a biophysical standpoint, and those that have, have focussed on NMR studies of MLKL associating with liposomes (Su et al., 2014) or detergent micelles (Quarato et al., 2016). The data emerging from those and other studies indicated that: (1) MLKL has a preference for binding phosphatidyl inositol phosphate lipids; (2) the site of MLKL membrane permeabilisation is the plasma membrane; and (3) that MLKL requires dissociation of the brace region from the four helical bundle (4HB) to be fully active on membranes (Dondelinger et al., 2014, Quarato et al., 2016, Wang et al., 2014a, Su et al., 2014).

In regards to models of plasma membrane permeabilisation, however, there is a lack of evidence to strongly support many of the proposed models in the literature. Su et al. (2014) suggested that the MLKL 4HB undergoes an unfolding event which reveals hydrophobic portions of the protein to the lipid tails within the bilayer. Through this mechanism, the 4HB would form a transmembrane segment that is part of an oligomeric MLKL pore (Su et al., 2014). Xia et al. (2016) proposed that the 4HB acts as a transmembrane ion channel. Quarato et al. (2016) found evidence for a PIP binding face on hMLKL's 4HB. They proposed a model where MLKL first associates with the bilayer surface, based on the PIP headgroup binding sites, then goes onto insert into the membrane in an undefined second step (Quarato et al., 2016). Recent studies from that group have also implicated the PIP binding sites as inositol phosphate bindings site, important for MLKL activation (Introduction, Section 1.6) (Dovey et al., 2018, McNamara et al., 2019). Therefore, the specific PIP binding sites, and the role they play in membrane permeabilisation, have to be deconvoluted from the inositol phosphate binding sites and their role in activation. These proposed models for MLKL mediated membrane permeabilisation are graphically described in Figure 1.7 of the Introduction.

In this chapter I describe work examining MLKL membrane permeabilisation using a range of techniques not previously used to study MLKL. My aim was to use different techniques to provide new information on MLKL's membrane association to support

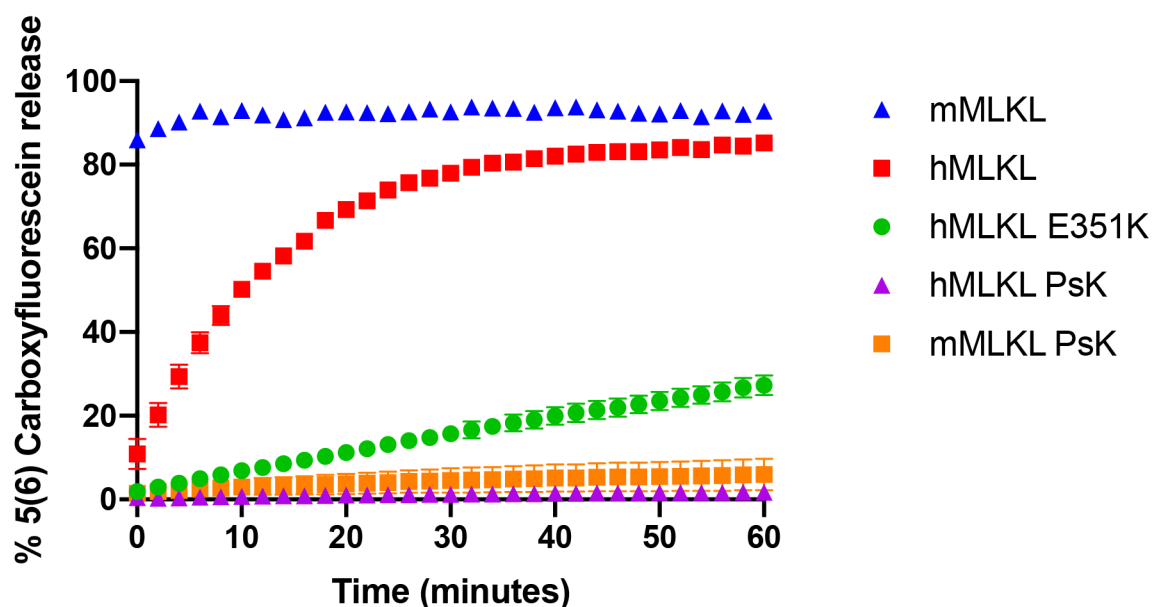
or dispute the existing contending models for membrane association and permeabilisation.

### *5.1 Defining the activity of constructs using conventional liposome assays; addressing questions of curvature*

To date most membrane studies performed with MLKL use liposomes as the model membrane system. This system has been useful in identifying domains of MLKL with liposome permeabilising ability (Tanzer et al., 2016, Su et al., 2014, Dondelinger et al., 2014), mutants that disrupt function (Petrie et al., 2018, Quarato et al., 2016), and identifying lipid mixes that MLKL has preference for (Tanzer et al., 2016, Wang et al., 2014a). Our own group has used 100 nm diameter liposomes with a plasma membrane-like lipid mix (10% PI, 20% POPE, 40% POPC, 20% DOPS and 10% POPG) to show that MLKL has robust permeabilising ability (Petrie et al., 2018, Tanzer et al., 2016). However, there are a number of reasons why liposomes may not be a representative model for the cell membrane, the most obvious being the high membrane curvature of liposomes compared to the plasma membrane of a cell. Furthermore, in liposome dye release assays, MLKL approaches the membrane from the outside rather than from within, as it would in a cell. Two studies attempted to use nanodiscs as a model membrane system, however MLKL was reported not to interact with them (Su et al., 2014, Wang et al., 2014a). The authors suggested that due to the constrained nature of the lipids in a nanodisc, the lipids could not be displaced to allow MLKL insertion in this system. However, another possibility was that the low curvature of the nanodisc membrane could prevent MLKL interaction.

We were interested in the question of membrane curvature, and how this might affect MLKL's function. First, we established the membrane permeabilising activity of five constructs (mMLKL full-length, mMLKL Pseudokinase (PsK), hMLKL full-length, hMLKL E351K full-length and hMLKL PsK) on plasma membrane-like lipid mix liposomes of 100 nm in diameter, as a point of comparison to published liposome dye release assays from our own group (Petrie et al., 2018, Tanzer et al., 2016) and others (Figure 5.1). Previously the pseudokinase domain of MLKL has been shown to not permeabilise liposomes (Tanzer et al., 2016). The activation loop mutant hMLKL

E351K, which shows deficits in oligomerisation ability and is a monomer in solution is less able to permeabilise liposomes than wild-type hMLKL, which exists in a monomer:tetramer equilibrium (Petrie et al., 2018). In dye release assays, MLKL constructs are incubated with liposomes filled with a high concentration of the self-quenching dye, 5(6)-carboxyfluorescein. As the membrane is permeabilised the dye is released and fluorescence read on a plate reader (Figure 5.1).



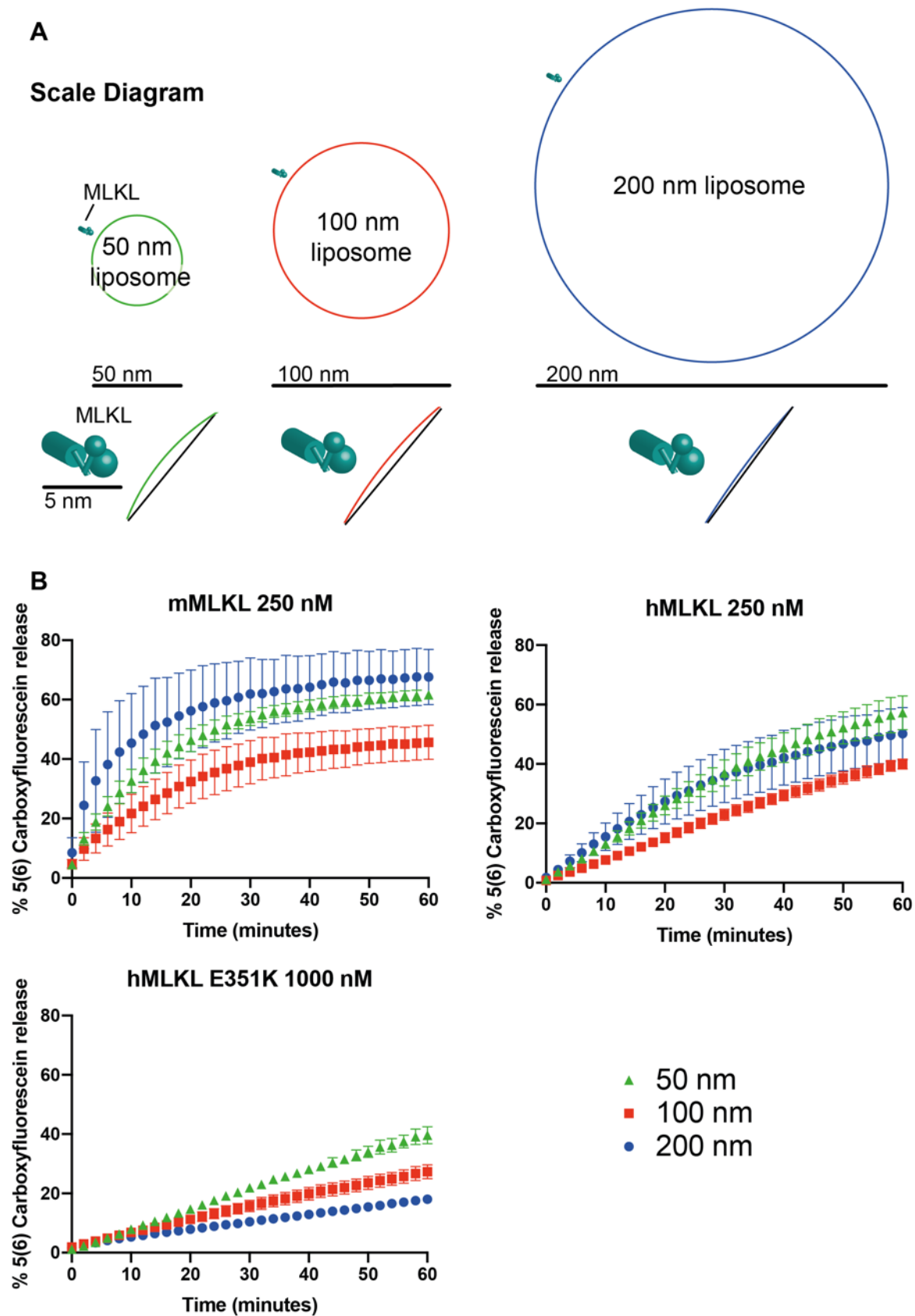
**Figure 5.1 Relative membrane permeabilising activity of different MLKL constructs**

100 nm plasma membrane-like lipid mix liposomes were formed via extrusion in the presence of high concentrations of the self-quenching dye 5(6)-carboxyfluorescein, as described in Chapter 2, Section 2.7. MLKL constructs were added to the liposomes in a 10:1 lipid to protein ratio, and membrane permeabilisation detected by an increase in fluorescence as 5(6)-carboxyfluorescein was released. Dye release was normalised using a buffer plus liposome control to set the baseline fluorescence, and 1% (v/v) CHAPS plus liposomes as 100% dye release fluorescence. Assays were conducted in technical triplicate. mMLKL permeabilisation was performed once (n=1, no error bars shown), hMLKL has two repeats (n=2) and the remaining constructs hMLKL E351K, mMLKL pseudokinase (PsK) and hMLKL PsK have three (n=3). Error bars are  $\pm$  SEM of independent experiments.

Mouse MLKL full-length permeabilised the liposomes most effectively, followed by hMLKL full-length and then hMLKL E351K (Figure 5.1), in agreement with published data (Petrie et al., 2018). The human MLKL pseudokinase and mouse MLKL pseudokinase showed negligible liposome permeabilising ability, again in agreement with published data (Tanzer et al., 2016).

The active proteins (mMLKL, hMLKL, hMLKL E351K) were then tested on liposomes of 50 nm, 100 nm and 200 nm diameter respectively to see if dye release could be enhanced or repressed by changes in membrane curvature (Figure 5.2).





**Figure 5.2 Testing the effect of membrane curvature on MLKL membrane permeabilisation**

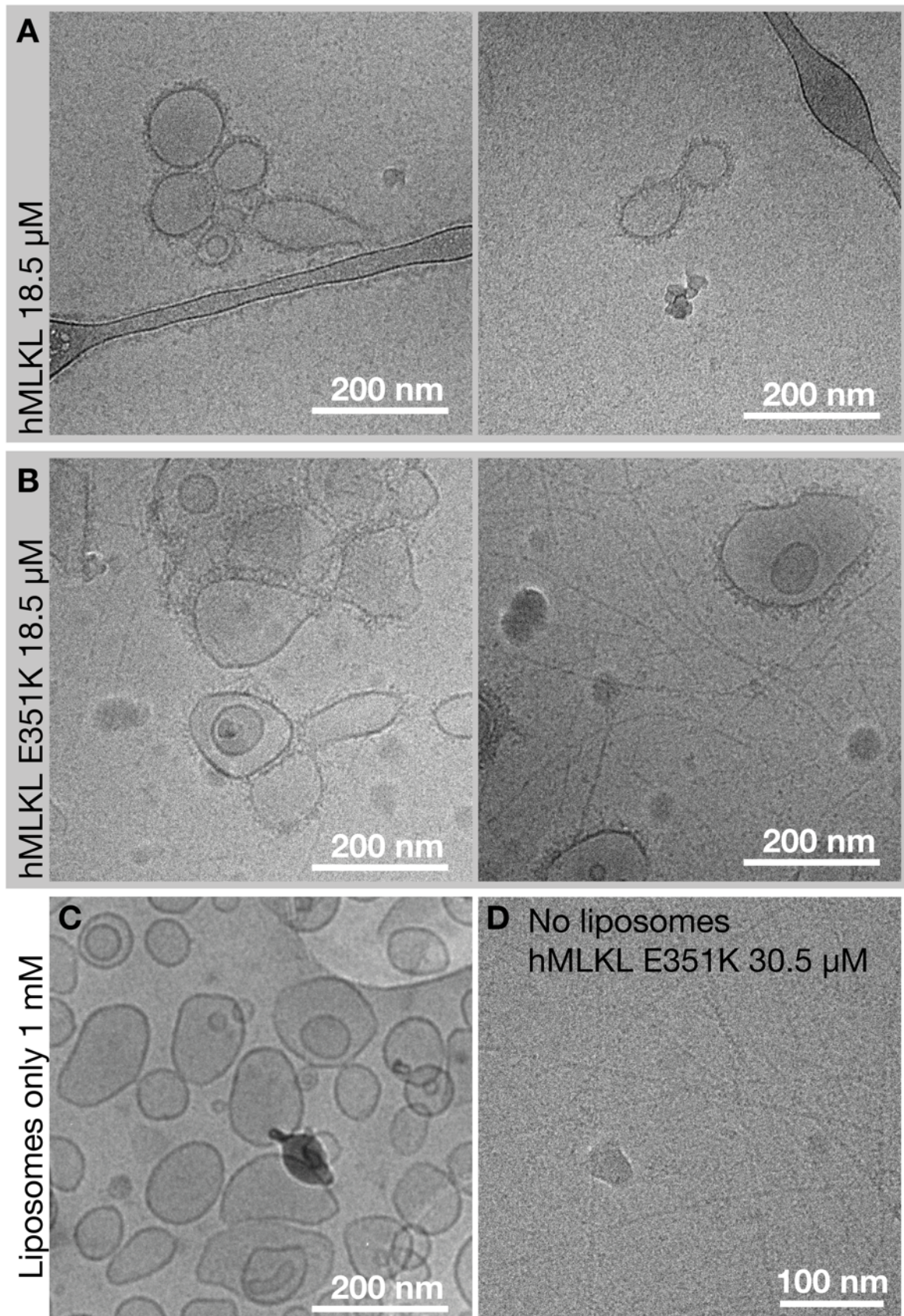
**A** Scale diagram that illustrates the relative size of the MLKL protein (based on the full-length mouse crystal structure (PDB:4BTF)), compared to the liposomes of

varying diameter, and how the curvature of the membrane varies with size. The width of the bilayer is not to scale. **B** Mouse MLKL, human MLKL and mutant hMLKL E351K were tested in liposome dye release assays. Assays were conducted in technical triplicate in three independent experiments (n=3) except for E351K 50 nm where n=2. Error bars are  $\pm$  SEM, some are too small to be seen over the symbol.

The relative size of the MLKL full-length protein and the curvature of the membrane for the 50 nm, 100 nm and 200 nm liposomes is illustrated in Figure 5.2 A. For these assays, mouse and human MLKL were diluted to a lower concentration (250 nM) than the less active mutant E351K (1000 nM) (Figure 5.2 B), so that subtle effects in liposome permeabilising ability on membranes of different curvature could be observed. Only hMLKL E351K showed a clear trend with higher membrane pitch leading to greater permeabilisation activity (dye release%: 50 nm > 100 nm > 200 nm). Whilst the 50 nm liposomes appeared to be permeabilised more efficiently than the 100 nm liposomes for both mouse and human MLKL full-length, the largest liposomes of 200 nm diameter, were permeabilised with similar efficiency to the 50 nm liposomes by these two constructs. The 200 nm diameter liposomes also showed the greatest variation between repeat experiments with mMLKL and hMLKL. Overall testing the activity of MLKL on liposomes did not suggest a strong preference for higher or lower membrane curvature, within the range of curvatures tested in these experiments.

## *5.2 CryoEM on MLKL binding to 100 nm liposomes*

Another disadvantage of the liposome assay as a means for testing MLKL's permeabilisation activity is that the technique offers only a population view, and the liposome membrane is not monitored whilst MLKL is permeabilising it. CryoEM was used to visualise 100 nm liposomes after incubation with MLKL constructs for 30 minutes at room temperature (Figure 5.3). The molar lipid to protein ratio used in the CryoEM experiments was higher (~50 lipid : 1 protein) than the ratio used in liposome assays (10 lipid : 1 protein; with 1000 nM protein), based upon optimisation performed in my Honours year.



**Figure 5.3 CryoEM shows MLKL binding to liposome membranes**

**A, B** Examples of liposomes incubated with MLKL for 30 minutes at room temperature, prior to being snap frozen onto lacey carbon/copper grids in a vitreous

ice layer using liquid ethane. **C** Liposome only control (from my Honours year), prepared in the same way as A and B except without protein. **D** hMLKL E351K incubated at room temperature for 30 minutes before being snap frozen onto lacey grids as in **A**, **B** and **C**.

Liposomes incubated with MLKL exhibited a rough coating that protruded approximately 10 nm outward from the liposome membrane (Figure 5.3). Human MLKL seemed to destroy most of the liposomes, because it was very difficult to find liposomes to image on the grid after incubation with full-length hMLKL. From three independent experiments, liposomes with hMLKL bound were only captured once (pictured in Figure 5.3 A). hMLKL E351K on the other hand showed many more coated liposomes on the grid and the spread of liposomes was more comparable to the liposome only control (Figure 5.3 B, C). The protein coating appeared to clump liposomes together. Both wild-type hMLKL and E351K exhibited similar morphologies of binding to the liposomes, which is intriguing given that E351K showed markedly reduced liposome permeabilisation ability. This suggests that E351K hMLKL can still bind liposomes, but does not destroy them as potently as wild-type hMLKL. Along with the E351K hMLKL coated liposomes, long thin fibrils were also observed. Adding E351K hMLKL to grids alone (Figure 5.3 D) showed that the fibrils were formed by the protein and this was observable over multiple protein preparations. The liposome only control showed that whilst many liposomes are approximately 100 nm in diameter there is some variation in size of the liposomes.

These data serve as a good starting point for future studies, and expanding the study by performing CryoEM tomography on these samples has the possibility of revealing what ordered structures, if any, MLKL forms on the plasma membrane. Furthermore, studying the fibril of E351K hMLKL, even if it is an artefact of the mutation, as it was not observable in the wild-type hMLKL protein, could provide an avenue to solving the structure of full-length human MLKL. These experiments fell beyond the scope of my PhD but are exciting avenues for further study.

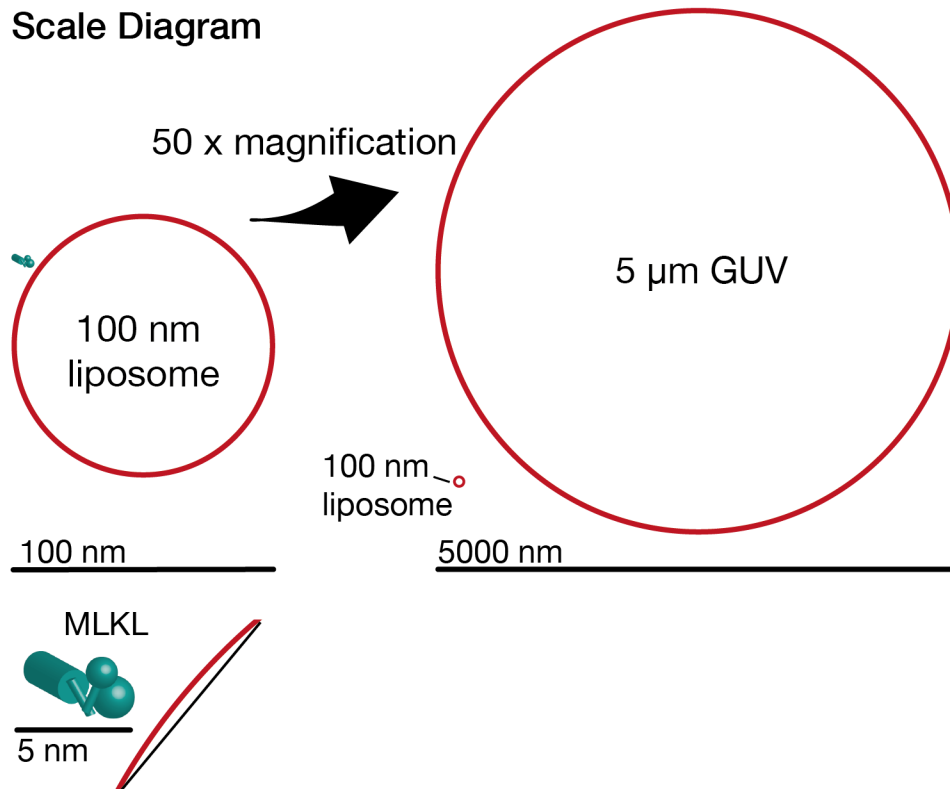
One issue with examining MLKL membrane association via CryoEM, is that the images are slices in time. Furthermore, as mentioned above, 100 nm liposomes are



very small compared to the size of a cell and thus have higher curvature. The data from Figure 5.3 shows that MLKL can bind stably to intact liposomes, which had not been previously observed. The assay in Figure 5.2 showed that even though some constructs have reduced activity on membranes of lower pitch (i.e. E351K hMLKL), all MLKL constructs tested retained permeabilisation. Taken together, these data were an indication that MLKL's membrane permeabilisation could be tested using another membrane system, Giant Unilamellar Vesicles (GUVs).

### *5.3 Making GUVs to test MLKL binding*

Giant Unilamellar Vesicles (GUVs) are substantially larger than vesicles that can be formed by membrane extrusion or sonication, and typically have a diameter of 5-35  $\mu\text{m}$ , a similar size range to mammalian cells. Their large size means that for proteins the size of MLKL, the membrane surface is relatively flat (Figure 5.4). GUVs are also large enough that they can be observed by confocal microscopy. This technique has the advantage that, unlike for CryoEM, MLKL membrane association with GUVs can be monitored in real time.



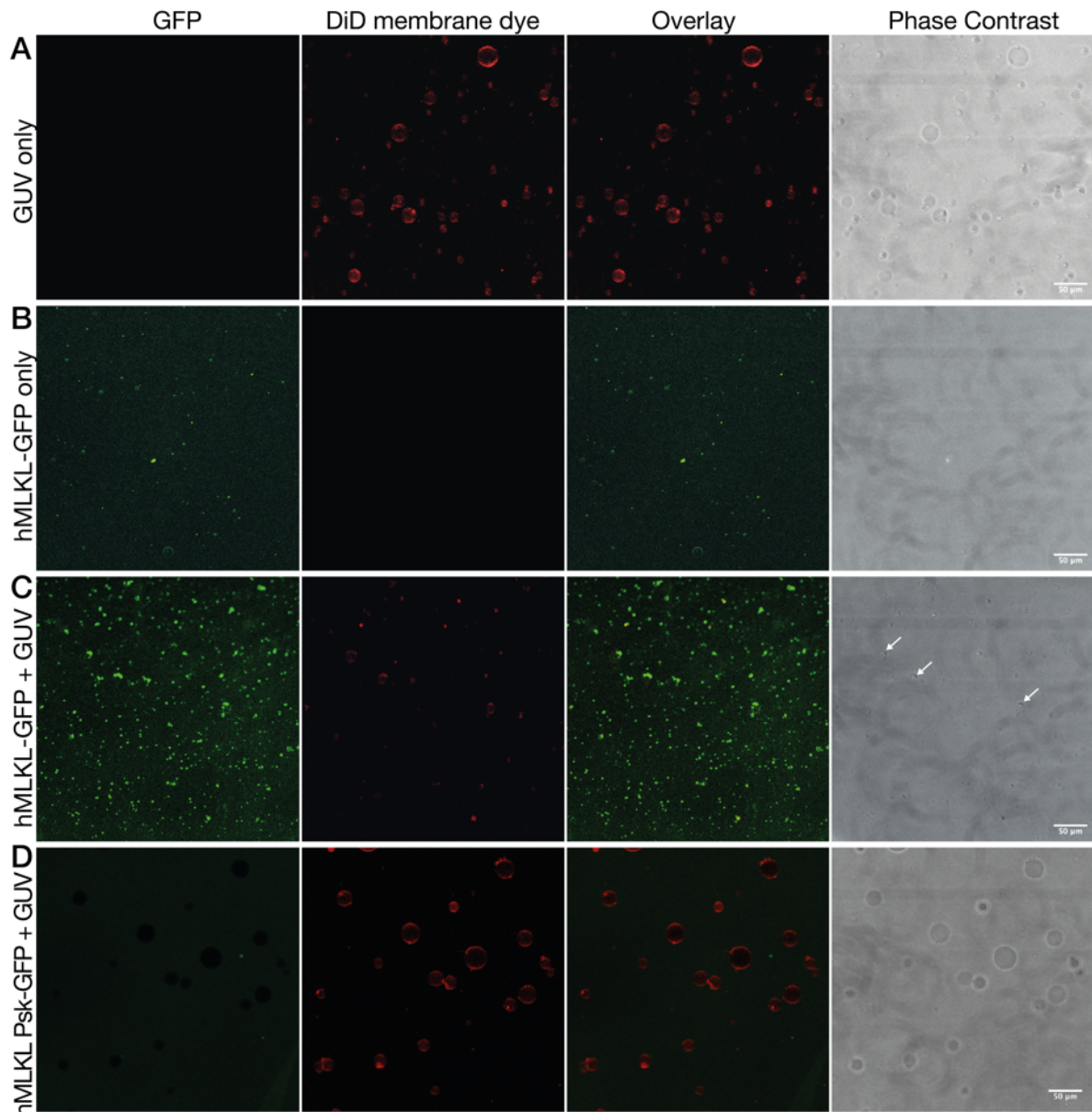
**Figure 5.4 GUVs are much larger than 100 nm liposomes, and have lower membrane curvature**

The relative size of MLKL compared to a 100 nm diameter liposome is depicted, as well as the curvature of the membrane. The same 100 nm liposome is shown next to a small GUV of 5 µm in diameter.

To maintain consistency with our previous studies, we formed GUVs in the same buffer used in liposome experiments (10 mM HEPES pH 7.5, 135 mM KCl) and with the same plasma membrane-like lipid mix that had been previously used by our group. To form GUVs under these relatively high salt concentrations, and with a complex lipid mix, passive swelling from a PVA hydrogel was used (Weinberger et al., 2013). To allow visualisation by confocal fluorescence microscopy GUVs were formed with a small amount of fluorescent membrane dye DiD, which has a long hydrocarbon tail that incorporates into bilayers. This conferred red fluorescence to the GUV membrane. We generated recombinant constructs of enhanced GFP (herein referred to as GFP) fused to the C-terminus of MLKL (hMLKL-GFP, hMLKL PsK-GFP, hMLKL E351K-GFP, mMLKL-GFP and mMLKL PsK-GFP) to enable monitoring of MLKL binding to GUVs by fluorescence microscopy. As the N-terminal 4HB domain is considered to be predominantly responsible for membrane binding and disruption (Hildebrand et al., 2014, Dondelinger et al., 2014, Su et al., 2014, Tanzer et al., 2015),

we reasoned that GFP fusion to the C-terminus of MLKL would not affect membrane binding and permeabilisation.

Fluorescently-tagged MLKL constructs were added to GUVs, left to bind for >30 minutes, before being observed by confocal microscopy using phase contrast and fluorescence (Figure 5.5). GUVs grown by passive swelling on hydrogel incorporate the large molecular weight PVA molecules on their inside as they swell. This results in the inside of the GUV having different optical properties to the outside buffer, which does not contain PVA. This is observable as a white halo around the GUV, and a darker grey colour inside the GUV when viewed by phase contrast microscopy, as shown in the GUV only and hMLKL PsK-GFP panels in Figure 5.5 (A, D). If the GUV is permeabilised, its contents equilibrate with the outside buffer and as a result when viewed with phase contrast the inside of the GUV will be the same colour as the outside of the GUV and the halo will be lost.



**Figure 5.5 Full-length hMLKL dissolves most GUVs and exposure to membranes induces protein aggregation**

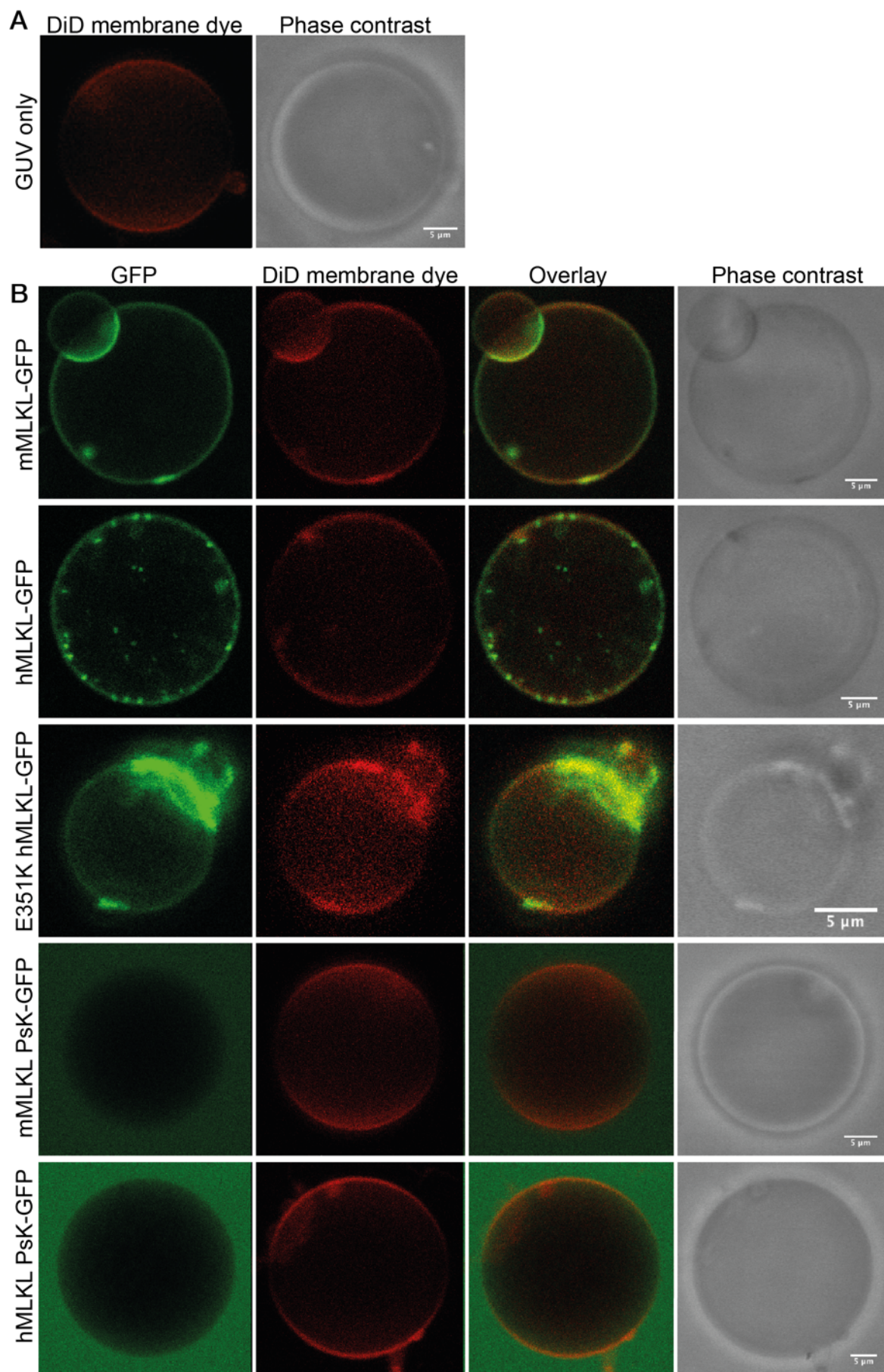
**A** GUVs alone are predominantly unilamellar and defect free, and have phase contrast. **B** hMLKL-GFP at 500 nM without GUVs. **C** hMLKL-GFP at 500 nM incubated with the GUVs for 30 min. Most GUVs have disappeared, the protein forms aggregated clumps and also binds to the remaining membrane in clumps. Remaining GUVs are the same colour as the background solution because they have lost phase contrast, these are highlighted with white arrows. **D** hMLKL Psk-GFP at 1000 nM incubated with GUVs for 30 minutes. GUVs are visible in the DiD membrane dye channel, MLKL-GFP constructs through the GFP channel and the overlay channel combines the GFP and DiD signals. Scale bar is 50  $\mu$ m.

Compared to GUV only controls, addition of active protein, such as hMLKL-GFP, resulted in loss of most GUVs, leaving a much smaller population (hMLKL-GFP 500 nM used as an example, also true for mMLKL-GFP observed in other



experiments; Figure 5.5). The remaining GUVs also lost phase contrast, and as a result are much harder to see in the phase contrast images (highlighted with white arrows in the Figure 5.5 C). GUVs are sensitive to changes in osmolarity, however, their disappearance was likely not due changes in osmolarity because all experiments were carried out in the same buffer as the GUVs were grown in. Incubation with hMLKL PsK-GFP protein, did not cause a reduction in the number of GUVs compared to the GUV protein alone (Figure 5.5 D). Incubation of hMLKL-GFP with GUVs also induced protein aggregation compared to an hMLKL-GFP only control (Figure 5.5 B), which could be due to hMLKL-GFP protein binding to broken GUV fragments.

These findings are in agreement with the CryoEM results in which hMLKL full-length appeared to destroy most of the liposomes. Based on this, for equilibrium experiments in which GUVs and protein were incubated >30 minutes before confocal microscopy, the population of remaining GUVs were those that were examined. On the remaining GUVs, we looked for protein binding which would be seen as accumulation of GFP signal at the membrane.

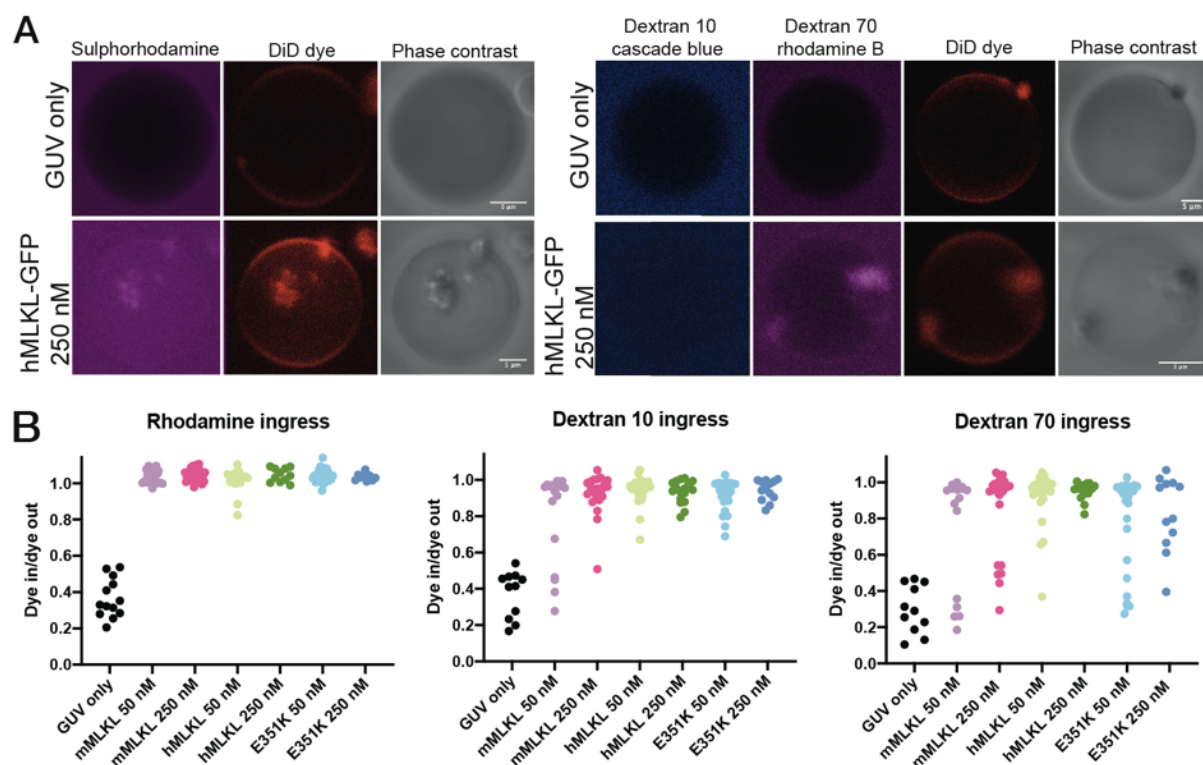


**Figure 5.6 Full-length MLKL binds to GUV membrane, and causes loss of phase contrast through permeabilisation**

**A** A representative image of a GUV without protein added. The membrane was visualised by addition of 0.4% (M) membrane dye DiD. The GUVs were also observed using phase contrast microscopy to monitor permeabilisation. GUVs alone are unilamellar and unpermeabilised. **B** mMLKL-GFP, hMLKL-GFP and the mutant construct hMLKL E351K-GFP all bound to the GUV membrane at 250 nM and showed a loss of phase contrast indicative of permeabilization. The pseudokinase only constructs did not bind, even at 1  $\mu$ M, and did not induce loss of phase contrast. Images are representative of at least 10 GUVs. GUVs are visible in the DiD membrane dye channel, MLKL-GFP constructs through the GFP channel and the overlay channel combines the GFP and DiD signals.

Mouse MLKL-GFP, hMLKL-GFP and hMLKL E351K-GFP all exhibited binding to GUVs, as shown by co-localisation between the GFP signal and the DiD membrane dye signal (Figure 5.6). mMLKL PsK-GFP and hMLKL PsK-GFP were unable to bind, even at higher concentrations, with the GFP signal remaining diffuse and in solution, rather than localising to the membrane. All active constructs showed increased GFP signal intensity at sites of GUV membrane deformities, or points where two GUVs touched, as can be seen in the DiD and phase contrast panels. The phase contrast effect was lost on GUVs incubated with mMLKL-GFP and hMLKL-GFP; the inside of the GUV became the same colour as the outside buffer (Figure 5.6 B), which indicates that the GUVs were permeabilised. For the PsK-GFP constructs, the majority of GUVs retained phase contrast, showing that they were not permeabilised.

As a more direct test of membrane permeabilisation, dye ingress assays were performed (Figure 5.7). Dyes of varying size were added to the solution outside of the GUVs, and incubated with mMLKL-GFP, hMLKL-GFP and hMLKL E351K-GFP for one hour before imaging by confocal microscopy (Figure 5.7).



**Figure 5.7 Full-length MLKL constructs can permeabilise GUVs**

**A** Representative image of GUV only and GUV plus protein incubated with different dyes: sulphorhodamine, dextran 10 cascade blue, and dextran 70 rhodamine. The sulphorhodamine experiment was carried out separately because of fluorescence overlap with the dextran 70 rhodamine. **B** The ratio of mean fluorescence intensity in a set area inside vs outside the GUV is plotted to show degree of permeabilisation. GUV only is plotted in black to show the baseline fluorescence ratio when there is no protein. Dye ingress for each dye with either mMLKL-GFP, hMLKL-GFP or E351K-GFP are plotted separately with comparison of 50 and 250 nM protein concentrations. Each data point represents measurement for individual GUVs taken over two independent experiments ( $n=2$ ).

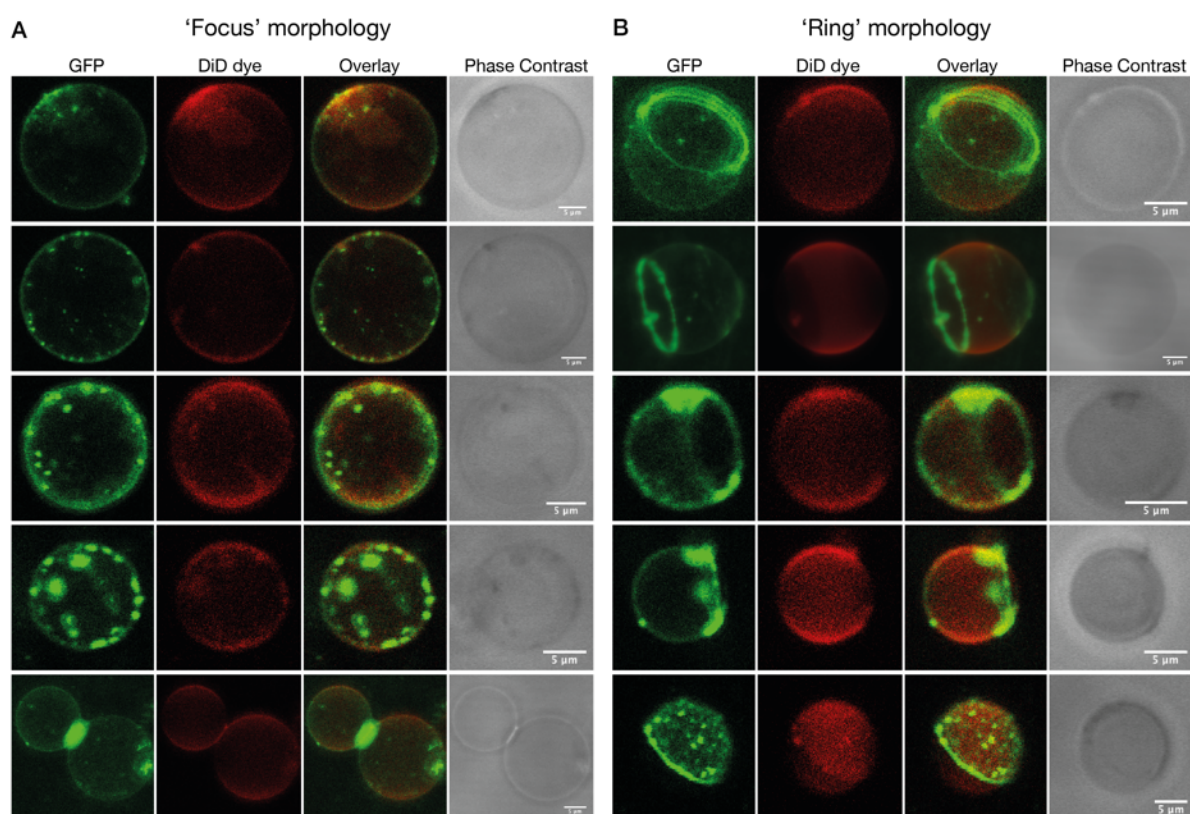
To measure dye ingress, the mean fluorescence intensity inside a specified area of the GUV was divided by the mean fluorescence intensity in a specified area outside the GUV (where the dye is added). There was a low level of background fluorescence inside the GUVs even in GUV only controls (Figure 5.7A) likely due to dye above or below the GUV in solution. Therefore, dye in/ dye out ratios of GUV controls are shown as an indication of background (black dots). A ratio of 1 indicated that the inside of the GUV had completely equilibrated with the dye outside, and the GUV had fully permeabilised.

Mouse MLKL-GFP, hMLKL-GFP and E351K-GFP (at both 50 nM and 250 nM) allowed the small dye rhodamine into the GUVs, with a dye ingress ratio of close to 1 (Figure 5.7 B). Most constructs tested also made GUVs permeable to the Dextran 10 dye. However, for mMLKL-GFP at 50 nM there was a subset of GUVs that were impermeable to dextran 10, with a similar fluorescence ratio to the GUV only control. Of all the constructs tested, hMLKL at 250 nM was most active at the population level at enabling transit of dextran 70 across the GUV membrane. A subset of GUVs treated with mMLKL (50 nM and 250 nM), hMLKL (50 nM) and hMLKL-E351K (50 nM and 250 nM) were impermeable to the largest dye dextran 70. Some degree of concentration dependent increase in permeability was observable. A greater proportion of GUVs appeared to be impermeable to dextran 10 and dextran 70 at the 50 nM protein concentration compared to the 250 nM concentration. While more measurements, e.g. using higher throughput methods, would be required to test for statistical significance, these data allow us to observe qualitative differences for permeabilisation activity of the different constructs.

MLKL was therefore able to bind to and permeabilise GUVs, which have a very low membrane curvature compared to the previously characterised 100 nm liposomes. During experimentation, we found that GUVs were not suitable for high throughput experiments, or generation of high quality quantitative measurements, due to the large variation in GUV yield between preparations, and the lack of an accurate way to measure GUV concentration in each experiment. We therefore sought to use GUVs as an observation system.

We noted that hMLKL-GFP displayed different membrane binding behaviour to mMLKL-GFP on GUVs. The mouse protein bound uniformly over the GUV surface (as per Figure 5.5). However, the human protein showed a variety of morphologies on GUVs membranes, involving the MLKL forming diffraction limited foci (Figure 5.5, 5.8).





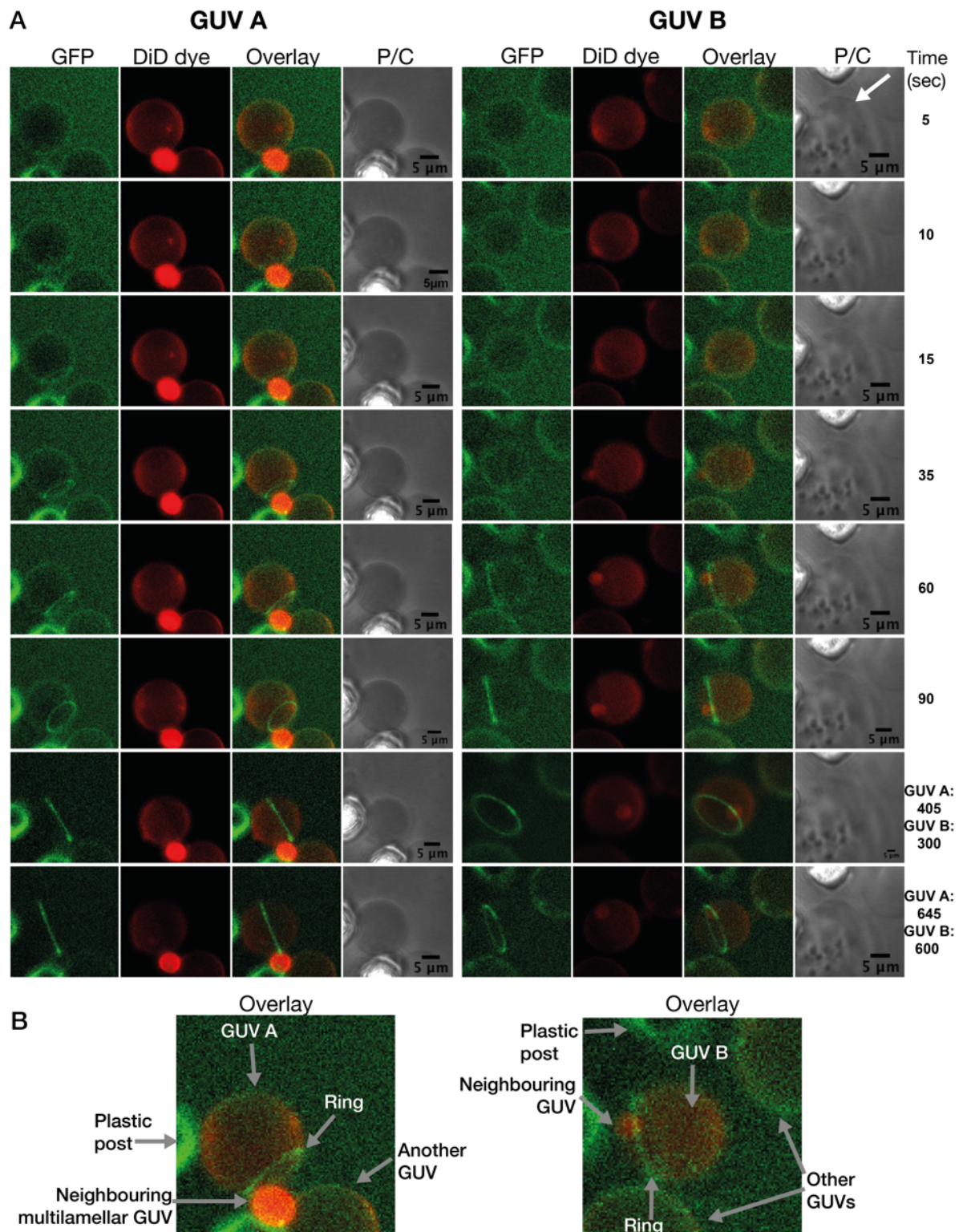
**Figure 5.8 hMLKL-GFP binds GUVs in foci and ring like structures**

**A** In most cases foci of varying size and number were observed, often associated with contact points between GUVs or membrane deformities evident in the phase contrast images **B** In some cases, hMLKL-GFP ring like structures were also observed. Scale bars are all 5 μm.

The 'focus' morphology shown in Figure 5.8 occurred more frequently on GUVs than the 'ring' morphology, but the ring was consistently observed on some GUVs in all independent experiments with hMLKL-GFP. The observed 'ring' morphology was not a large pore; GUVs with the ring were still intact and often retained phase contrast. Moreover, a pore of this size would cause the GUV to collapse. In all cases where a ring was formed, hMLKL-GFP binding was predominantly localised to one side of the ring. MLKL has not previously been reported to form large organised structures on membranes, such as the rings we observed, so we sought to better understand how the ring might form.

To provide more information on ring formation we used flow cells to enable GUVs to be videoed from the time of protein addition. The details of the flow cells used in these experiments have been described previously (Yandrapalli and Robinson, 2019). A description of the experimental process with flow cells is provided in Chapter 2,

Figure 2.3. Videos were recorded from the point of MLKL addition to GUVs, and still images of ring growth from the resulting videos are shown in Figure 5.9.



**Figure 5.9 Dynamics of hMLKL-GFP ring growth on GUVs**

**A** Snapshots from video for hMLKL-GFP at 250 nM mixed with GUVs in flow cells. Ring growth was captured on two GUVs, GUV A and GUV B in the same video. Time

is indicated on the right in seconds. The bright white cube-like structures in the phase contrast channel are plastic posts used to trap GUVs inside the flow cell. hMLKL-GFP protein binds to these posts non-specifically so they are also visible in the GFP channel. GUV B is difficult to see through the phase contrast channel due to debris partially obscuring it, and is indicated with a white arrow. **B** Enlarged images from the 60 second time point of GUV A and GUV B with labels to aid explanation. P/C stands for phase contrast. Scale bars are 5  $\mu$ M.

Ring formation was only observed on a small number of GUVs and therefore we had limited opportunities to record their growth in the flow cells. Ring growth was only captured on two GUVs from start to finish at this concentration (250 nM) and two more videos were collected which did not capture ring growth from the very start of protein addition, and at a different hMLKL-GFP concentration (500 nM, data not shown).

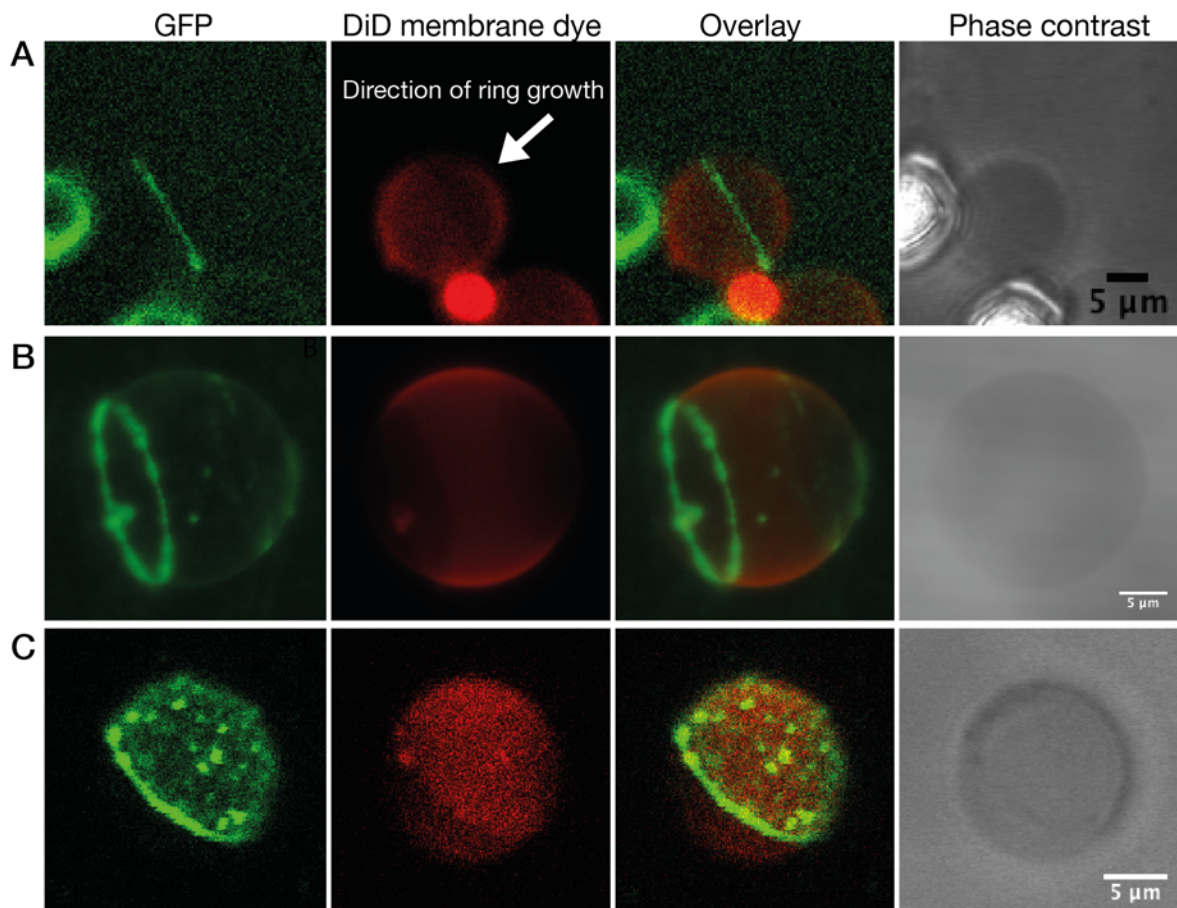
For GUV A, the ring grew from an initial nucleation of concentrated hMLKL-GFP that formed at a contact point with a small neighbouring GUV (Figure 5.9 GUV A 5, 10 seconds). The neighbouring GUV for GUV A was the bright red circle, likely a multilamellar GUV (Figure 5.9 B). The nucleus of growth was most visible in the GFP channel of GUV A at the 5 and 10 second time points. GUV B also had a small neighbouring GUV, however, it was positioned behind GUV B at the start of the video, migrating to the foreground in later time points (Figure 5.9 A, B). For GUV B, a small faint ring first appeared at the 15 and 35 second time points, close to where the small neighbouring GUV was positioned. Potentially a nucleation of concentrated hMLKL-GFP may have formed on GUV B, next to the neighbouring GUV, as with GUV A, but was difficult to observe due to the position of the neighbouring GUV. The initial small ring on both GUVs appeared to enlarge around the circumference of the vesicle. A ring was first clearly visible by 15 seconds for GUV A and 35 seconds for GUV B. Within ~5 minutes the rings had grown to their maximum size, almost equatorially bisecting the GUVs.

The videos showed that rings grew rapidly from a small ring to a large one. A commonality between GUV A and B was that they were both adjacent to small neighbouring GUVs contacting their membrane (Figure 5.9 B). However, it should be noted that in the same experiments rings were not observed on other GUVs that also



had small adjacent GUVs touching their membrane. Therefore, while the small neighbouring GUV might promote ring formation, it does not necessarily enforce it.

In many GUVs with rings, the uniform distribution of DiD membrane dye was impacted (Figure 5.8, 5.9), as exemplified in Figure 5.10.

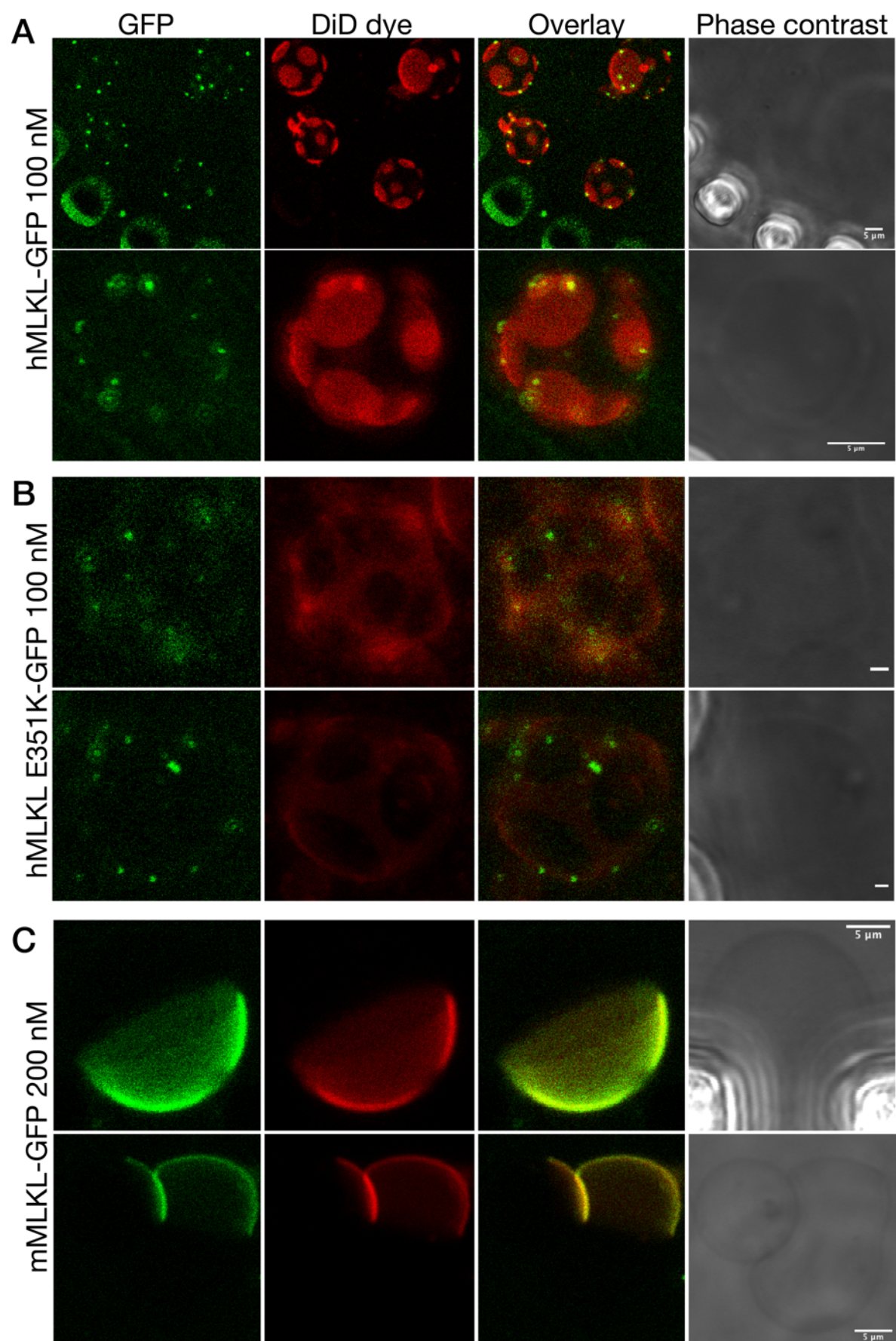


**Figure 5.10 Ring growth induces a redistribution of DiD membrane dye and hMLKL-GFP binding**

These GUVs show examples of the change in membrane dye distribution in the GUV membrane which occurs concurrent with the presence of the ring. **A** is GUV A from Figure 5.9, the direction of ring growth is indicated. **B, C** Still images of human MLKL 250 nM incubated with GUVs for >30 min.

In Figure 5.10 are examples of GUVs in which the dye was enriched in one hemisphere of the GUV as demarcated by the ring, with one side having brighter red fluorescence than the other. Human MLKL-GFP also preferentially bound to the same hemisphere that the membrane dye was enriched in (see especially Figure 5.10 C). The redistribution of membrane dye on these GUVs is reminiscent of the non-uniform distribution that occurs on phase separated GUVs (Patarraia et al., 2014). In those

cases, phase separated GUVs have bright patches of dye due to the hydrocarbon tail of the DiD predominantly localising to the liquid-disordered phase of the membrane. Under normal conditions, the plasma membrane lipid mix used in my experiments does not undergo phase separation. As the redistribution in membrane dye occurred concurrent with ring growth, we wanted to test if phase separation was important to MLKL ring formation. Furthermore, because the ring was positioned at the boundary between the dye-enriched and dye-depleted parts of the membrane, we speculated that ring formation might occur at phase separation boundaries. To test this hypothesis we performed experiments with the MLKL-GFP constructs on phase separated SM:chol:POPG GUVs (Figure 5.11).



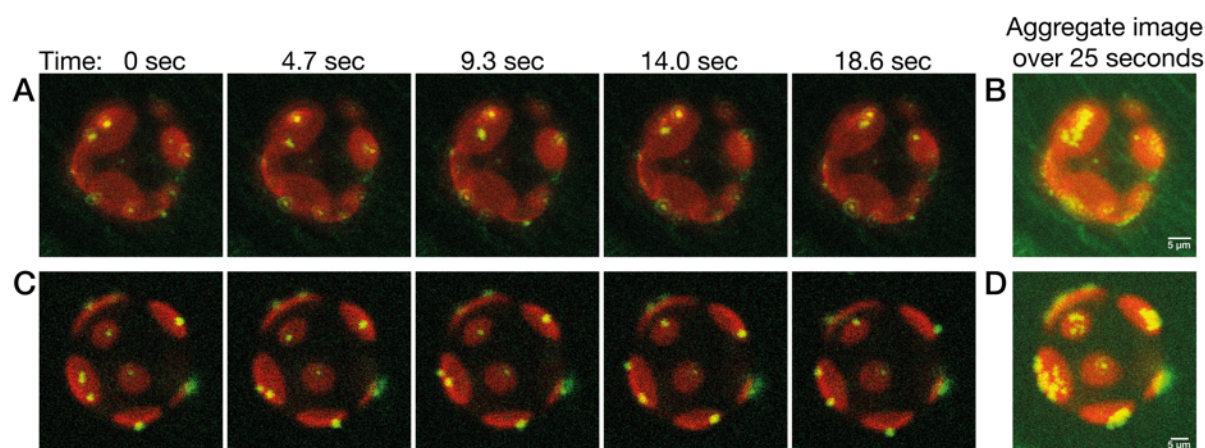
### **Figure 5.11 MLKL preferentially binds to the liquid-disordered phase in the GUV membrane**

POPG:Sphingomyelin:Chol GUVs formed phase separated vesicles, as expected, as demonstrated by localised patches of the membrane dye DiD which favours the liquid-disordered phase. MLKL bound to these GUVs, however did not form rings. hMLKL-GFP, E351K-GFP and mMLKL-GFP all preferentially bound to the liquid-disordered phase of the GUV bilayer. Mouse MLKL-GFP bound uniformly to the disordered patches, whilst hMLKL-GFP and E351K-hMLKL formed foci that were mobile within the patches (observed by video, see Figure 5.12). **A, B** Human MLKL-GFP and E351K-GFP at 250 nM were introduced to GUVs in a flow cell and imaged immediately. **C** Mouse MLKL-GFP at 200 nM added to GUVs in a flow cell and imaged immediately. All scale bars are 5  $\mu\text{m}$ .

Based on the ring demarcating what could be two different phases on the GUVs in Figure 5.10, we wondered if MLKL would localise to the boundary between the liquid-disordered and liquid-ordered phases and therefore form rings. Instead, MLKL bound entirely to the liquid-disordered phase in these GUVs. Human MLKL showed a focus morphology, and mouse MLKL showed a uniform binding modality, in agreement with observations on the plasma membrane mix GUVs (Figure 5.5). This illustrated that the difference in binding mode between species was not particular to the lipid mix used in the original GUVs. POPG has double bonds in its hydrocarbon tails and therefore would preferentially localise to the disordered phase of the GUV. However, POPG is also negatively charged. Attempts were made to make phase separated GUVs in which negatively charged lipid would either be equally present in both phases or would be confined to the liquid-ordered phase, but these were unsuccessful. Therefore, it was not possible to conclude whether MLKL preferred the liquid-disordered phase, or whether it had a preference for negatively charged phospholipids, as already proposed in the literature (Dondelinger et al., 2014, Wang et al., 2014a, Quarato et al., 2016, Petrie et al., 2018).

The phase separated vesicles did not enforce ring formation, and were not sufficient to determine if MLKL had a preference for a certain lipid phase. However, the patches of liquid-disordered phase allowed clear observation of diffusion of the hMLKL-GFP foci laterally through the membrane. Frames from two videos which capture the diffusion are shown in Figure 5.12.





**Figure 5.12 hMLKL foci can diffuse on the GUV membrane**

Examples of hMLKL-GFP (100 nM, videoed in flow cell) foci diffusing in the liquid-disordered patches of POPG:Spingomyelin:Chol phase separated vesicles. **A, C** The first five panels are individual frames from a video, each 4.65 seconds apart. **B, D** A maximum intensity aggregate image of 11 frames from the videos in A and C, illustrating the area of the liquid-disordered patch that the foci occupied over that time frame. Scale bars are 5  $\mu\text{m}$ .

The foci were able to diffuse through the membrane, and each focus remained localised to a specific liquid-disordered patch on the GUV (Figure 5.12). Contrary to our prediction that MLKL would prefer to bind at a phase boundary, the foci did not favour the boundary between the liquid-ordered and liquid-disordered phase, but spent most of the time within the liquid-disordered patch, and not at the edges (Figure 5.12, B, D).

Overall the GUV work was an interesting observational study, which illustrated that human MLKL and mouse MLKL exhibit different binding modes to GUVs, and that human MLKL is capable of forming oligomeric foci with flexible stoichiometries of protein grouping. We were also able to observe human MLKL diffusing laterally on the membrane, during ring formation, and also as foci. In subsequent experiments, we employed neutron reflectometry to provide a different view of MLKL activity at the membrane.

#### 5.4 Neutron reflectometry

Confocal microscopy gives a view of the surface of a membrane, allowing observation of lateral movement of proteins across a membrane, but does not allow

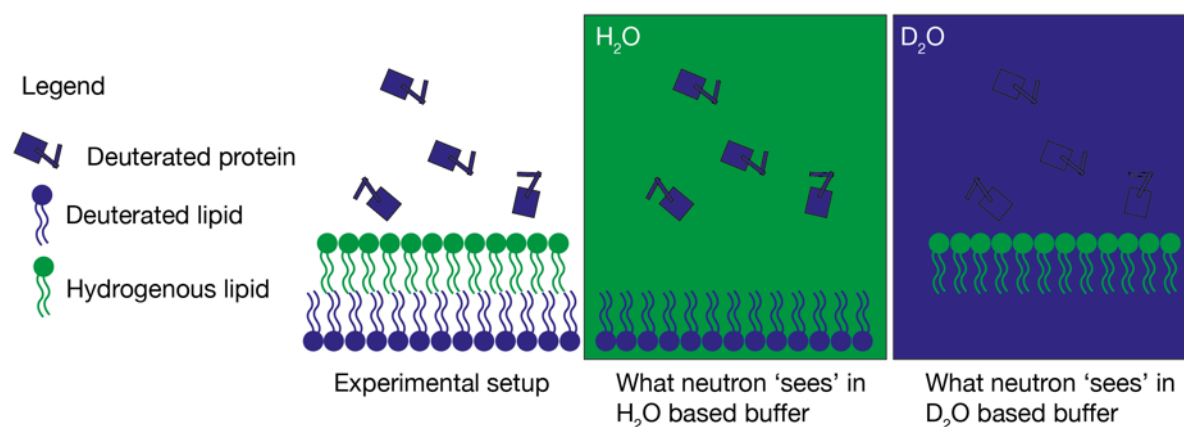
investigation through the depth of the membrane. To complement the confocal microscopy studies, we used neutron reflectometry, a technique that gives information on the changes to the cross section of the membrane.

Neutron reflectometry is useful for investigating properties of very thin films (typically in the size range of 1 to 500 nm), in our case a floating supported bilayer (FSB; See Methods, Figure 2.3). Neutrons are projected through the sample at a very low angle ( $<5^\circ$ ), and some neutrons reflect off different components of the sample which are then measured with a detector (Figure 2.4). The Platypus reflectometer at the Australian Centre for Neutron Scattering is a time-of-flight reflectometer that uses the time taken for neutrons to hit the detector to determine their wavelength, and uses this information to construct the Q-range of the resultant scatter (Methods: Section 2.7). The resultant scatter gives the intensity of the neutron interaction with the sample vs the Q-range (determined by the wavelength of the neutron and the angle of reflection), and the scatter profile contains information about relative distances between contrasting layers within a sample, in a manner analogous to Small Angle X-ray Scattering (SAXS), as well as the Scattering Length Density (SLD; discussed below) of different components in the sample.

Neutron reflectometry is an information poor technique, as information on the phase of amplitude the neutron is lost. Therefore, neutron reflectometry data is analysed by preparing a theoretical model for the system being analysed, and then comparing its back-calculated scatter to the observed data. Neutron reflectometry is similar to SAXS in this regard, much of the theory for data analysis of neutron reflectometry and SAXS is the same. The starting theoretical model is modified and refitted to the experimental data. Thus, by an iterative process the model is improved until it fits the experimental data.

Neutrons interact with nuclei based on their specific mix of protons and neutrons and as such have characteristic scattering interactions with nuclei of each elemental isotope, referred to as the Scattering Length Density (SLD;  $\text{\AA}^{-2}$ ). The scattering lengths for hydrogen (one proton) and deuterium (proton and neutron) are very different

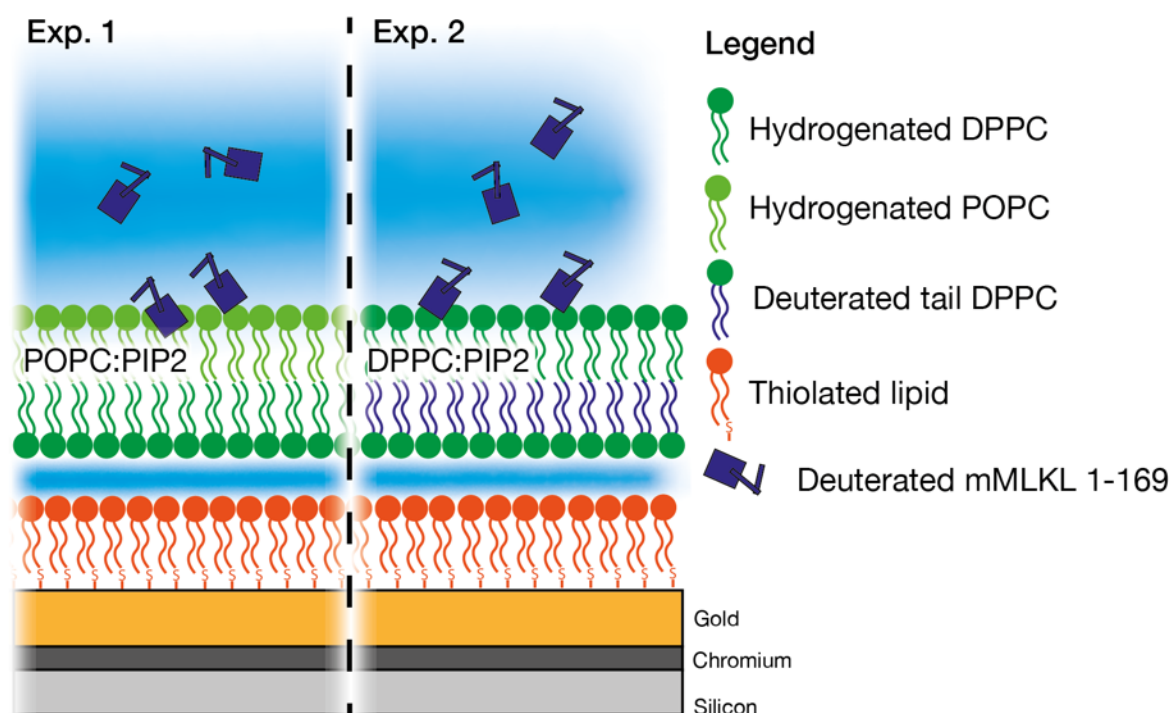
at  $-3.739 \times 10^{-5} \text{ \AA}$  and  $6.671 \times 10^{-5} \text{ \AA}$  respectively. This is useful because it means the SLD of  $\text{H}_2\text{O}$  ( $-0.56 \times 10^{-6} \text{ \AA}^{-2}$ ) and  $\text{D}_2\text{O}$  ( $6.34 \times 10^{-6} \text{ \AA}^{-2}$ ) are very different. This is exploited in neutron contrast matching experiments. For example, to a neutron, the tail region in a hydrogenous bilayer in  $\text{H}_2\text{O}$  will be almost invisible, but a deuterated bilayer in  $\text{H}_2\text{O}$  will stand out, and vice versa (Figure 5.13).



**Figure 5.13 Contrast matching in neutron reflectometry**

To a neutron, hydrogenous lipid in a solution of  $\text{H}_2\text{O}$  is nearly invisible, because they have similar SLD values. This property allows specific parts of an experimental set up to be highlighted by using buffers of different SLDs such as  $\text{H}_2\text{O}$  and  $\text{D}_2\text{O}$  in a process called contrast matching.

We planned to perform experiments using neutron reflectometry that would allow monitoring of the depth of MLKL entry into the bilayer, as in the literature some articles have suggested that MLKL forms a membrane spanning pore (Xia et al., 2016), whilst others have only provided evidence for models of peripheral membrane association (Quarato et al., 2016). We also performed an experiment using an asymmetrically deuterated bilayer to determine if MLKL would induce lipid mixing, and experiments on both liquid-ordered and liquid-disordered phase bilayers to investigate the open question of membrane phase association raised in our phase separated GUV experiments described above. A schematic of the experiments performed is illustrated in Figure 5.14.



**Figure 5.14 Summary of neutron reflectometry experiments**

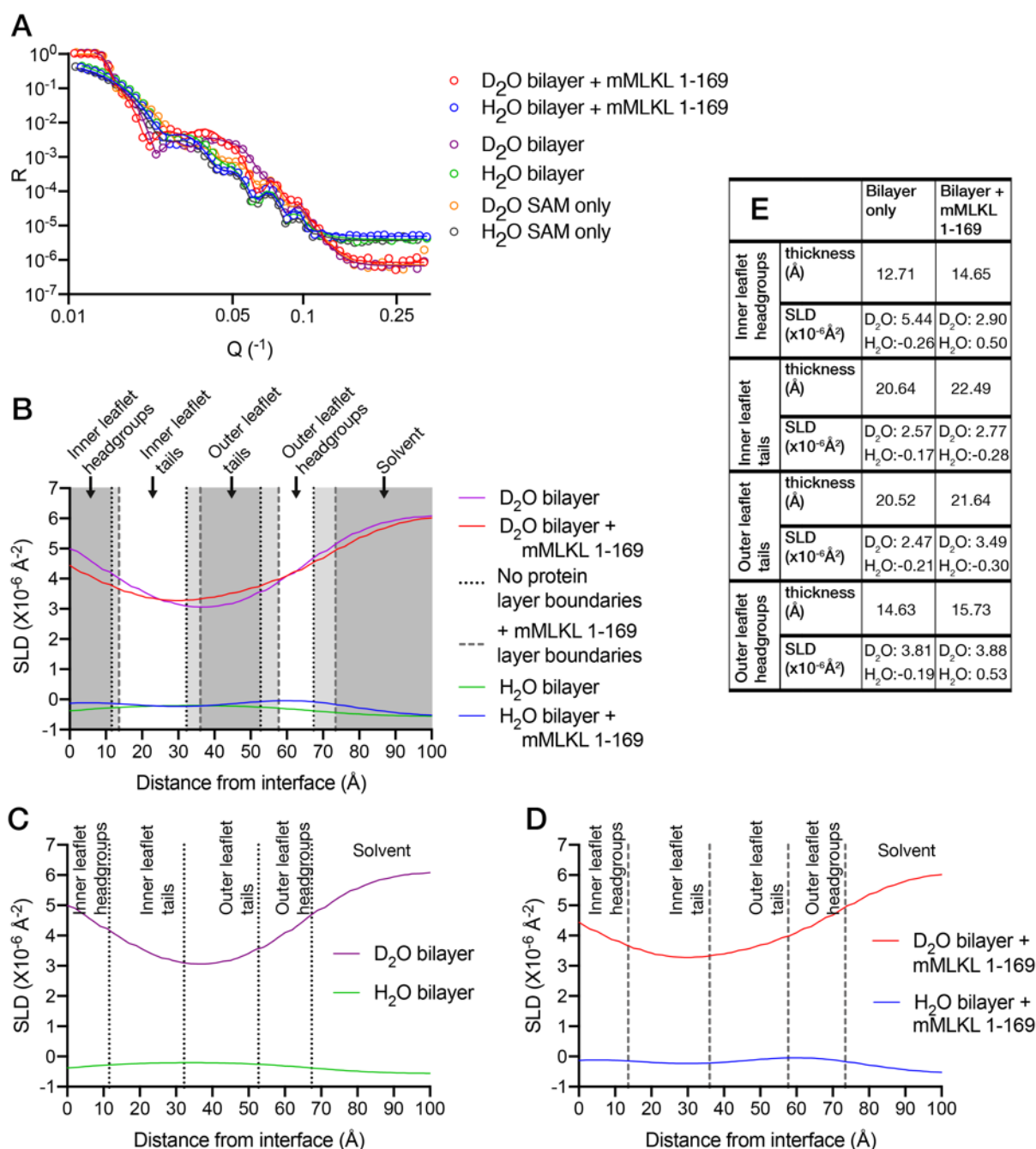
This figure summarizes the neutron reflectometry experiments successfully completed. Experiment 1 uses a hydrogenous DPPC lower leaflet, hydrogenous 9:1 POPC|PIP<sub>2</sub> upper leaflet, and deuterated MLKL 1-169. Experiment 2 uses a tail deuterated DPPC lipid (d62DPPC) in the lower leaflet, and hydrogenous 9:1 DPPC|PIP<sub>2</sub> upper leaflet, and deuterated MLKL 1-169.

Experiment 1 was performed with deuterated protein on a hydrogenous bilayer. The depth of penetration of the deuterated protein into the bilayer should be easily detectable by an increase in SLD in the layers of the membrane i.e. the outer headgroups, tails or inner head groups where the protein is present. Experiment 1 uses POPC as the lipid to form the outer leaflet that MLKL will first interact with. The oleic acid tail of this lipid has a double bond, which induces a kink in the tails and prevents close packing, and this lipid forms a liquid-disordered phase at room temperature. Experiment 2 uses DPPC in the outer leaflet. DPPC hydrocarbon palmitic acid chains are fully saturated, they can pack together closely and form a liquid-ordered phase bilayer at room temperature (Nagle and Tristram-Nagle, 2000). By using a comparison between the two experiments we wanted to investigate MLKL's activity on liquid-ordered and liquid-disordered membranes, to follow up on our GUV data. Experiment 2 also uses an asymmetrically deuterated bilayer where the tails of the inner leaflet are deuterated. If the bilayer is intact, the inner leaflet tail



layer will exhibit a higher SLD than surrounding layers. If there is a redistribution of high SLD across both leaflets once protein is added, it will indicate that MLKL membrane association induces lipid mixing between leaflets, which has been suggested by some recent studies (Zargarian et al., 2017, Yoon et al., 2017, Gong et al., 2017).

Experiment 1 was analysed first, as it is a more simple system without the deuterated lipid, to determine how deeply deuterated mMLKL 1-169 inserts into the bilayer (Figure 5.15).



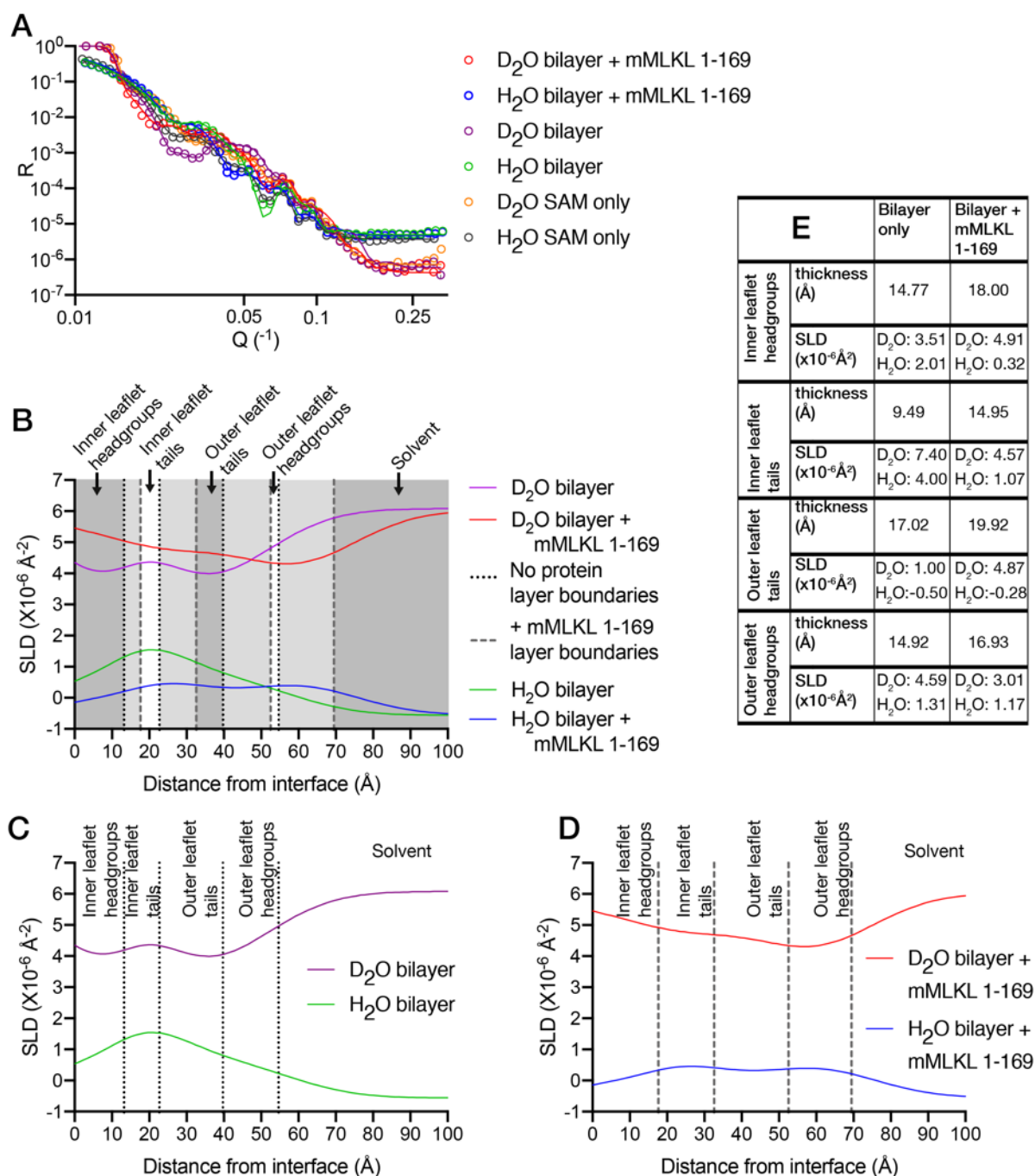
**Figure 5.15 Experiment 1 analysis and results**

**A** Raw scattering curves for Experiment 1, showing the self-assembled monolayer alone (SAM), the SAM with the bilayer formed on top, and the SAM with the bilayer and protein, at two buffer contrasts (D<sub>2</sub>O and H<sub>2</sub>O). Raw data is plotted in circles and the fits are solid lines. **B** The SLD vs “distance from interface” plot derived from the model fitted in A, for the bilayer alone and bilayer plus protein. For simplicity, the data presented is only for the inner leaflet layer and above, as the self-assembled monolayer is the same for each experiment. Black and grey lines delineate layer boundaries of the bilayer that the fitted SLD corresponds to. Because the thickness of the bilayer changes with addition of protein, the layers with and without protein do not perfectly align, regions where the layers align are indicated with black arrows and a label. **C, D** the individual graphs used to make B, shown separately for clarity. **E** A

table showing the changes to the bilayer thickness, and changes to the SLD of each layer in the D<sub>2</sub>O and H<sub>2</sub>O buffer before and after addition of protein.

Differences to the bilayer were observed after addition of protein, which were apparent in the raw reflectivity curves (Figure 5.15 A), comparing the D<sub>2</sub>O contrast before mMLKL 1-169 addition, in purple, to after protein addition in red. The protein did not totally destroy the bilayer however, as the reflectivity curves post protein addition did not resemble the underlying self-assembled monolayer (SAM) curves, that were collected before the bilayer was formed upon the SAM (Figure 5.15 A, compare the red curve to the orange curve). The experiment was best fitted with a model in which there was no additional layer for protein on top of the bilayer, which suggested that mMLKL 1-169 may insert entirely into the bilayer. Importantly this construct only includes the 4HB and brace region of MLKL. This construct was used for this experiment because it can be expressed in *E. coli* and therefore can be deuterated, unlike the full-length MLKL construct, which can only be expressed in insect cells. Despite the mMLKL 1-169 being deuterated (will have an SLD of  $7.04 \times 10^{-6} \text{ \AA}^{-2}$  in D<sub>2</sub>O and  $6.00 \times 10^{-6} \text{ \AA}^{-2}$  in H<sub>2</sub>O), and presumably incorporating fully into the membrane, there was only a small increase in the SLD of the outer leaflet tails after addition of protein (Figure 5.15 B, E). The thickness of the bilayer as a whole increased slightly with addition of the protein (Figure 5.15 E).

In the next experiment, to better understand what affect MLKL might have on the membrane, the inner leaflet of the bilayer was formed using a lipid with deuterated tails. In this experiment, the mixing of lipid between leaflets could be monitored (Figure 5.16).



**Figure 5.16 Experiment 2 analysis and results**

**A** Raw scattering curves for Experiment 2, showing the self-assembled monolayer alone (SAM), the SAM with the bilayer formed on top, and the SAM with the bilayer and protein, at two buffer contrasts (D<sub>2</sub>O and H<sub>2</sub>O). Raw data is plotted in circles and the fits are solid lines. **B** The SLD vs “distance from interface” plot derived from the model fitted in A, for the bilayer alone and bilayer plus protein. For simplicity, the data presented is only for the inner leaflet layer and above, as the self-assembled monolayer is the same for each experiment. Black and grey lines delineate layer boundaries of the bilayer that the fitted SLD corresponds to. Because the thickness of the bilayer changes with addition of protein, the layers with and without protein do not perfectly align, regions where the layers align are indicated with black arrows and a label. **C**, **D** the individual graphs used to make B, shown separately for clarity. **E** A

table showing the changes to the bilayer thickness, and changes to the SLD of each layer in the D<sub>2</sub>O and H<sub>2</sub>O buffer before and after addition of protein.

Large changes in thickness of the bilayer were induced with addition of mMLKL 1-169 (Figure 5.16 E). All layers increased in thickness, with the most marked increase in the inner leaflet tail region from a thickness of 9.49 Å before protein, to 14.95 Å with protein. These large changes make direct comparison of curves for before and after protein addition on the same axis difficult (see Figure 5.16 B). Therefore, the results are best observed in the separated graphs in 5.16 C and D. Before addition of protein in the H<sub>2</sub>O contrast (Figure 5.16 C; green line), there was a large spike in SLD in the inner leaflet of the tails ( $4.00 \times 10^{-6} \text{ Å}^{-2}$  in H<sub>2</sub>O), compared to the tails of the outer leaflet ( $-0.50 \times 10^{-6} \text{ Å}^{-2}$  in H<sub>2</sub>O), indicating that an asymmetric bilayer had been formed. After addition of protein (Figure 5.16 D; yellow line) the SLD became more evenly distributed between the two leaflets (inner tails:  $1.07 \times 10^{-6} \text{ Å}^{-2}$ , outer tails  $-0.28 \times 10^{-6} \text{ Å}^{-2}$  in H<sub>2</sub>O) indicating that the deuterated lipids had been mixed with the non-deuterated lipids. The experiment was best fitted when no extra layer for MLKL was included after addition of protein, in agreement with Experiment 1. The depth of penetration of the deuterated MLKL protein into the leaflet is not as easily monitored in this experiment due to the presence of the deuterated lipid, so any increase in SLD in the membrane layers, particularly in the tail regions cannot be solely attributed to the presence of the MLKL protein.

## 5.5 Discussion

This chapter describes reductionist approaches using model membrane systems to better understand MLKL's mechanism of membrane disruption. Many of the results described in this chapter are at this stage observational, and understanding the detailed biophysics underlying these phenomena will likely require further in-depth studies. This discussion will bring together the various observations on MLKL membrane association from different techniques, and discuss some of the caveats for each technique used.

### *Membrane curvature*

The question of whether MLKL can interact with membranes of low curvature is a simple one, yet it has not been addressed so far in the literature. Su et al. (2014) reported that MLKL could not interact with nanodiscs. The two most obvious reasons for this, are that either MLKL cannot interact with membranes of such low curvature, or, that there is some kind of steric hindrance for insertion of MLKL due to the constrained nature of the bilayer in nanodiscs.

MLKL mediated dye release from liposomes of varying diameter (Figure 5.2) illustrated that MLKL does not appear to have a strong preference for permeabilising membranes of a particular curvature. The effect of curvature on permeabilisation ability was most pronounced for the E351K hMLKL mutant, which has weak membrane permeabilising ability (Figure 5.1) (Petrie et al., 2018), that was slightly enhanced on liposomes of higher membrane curvature (Figure 5.2). The more active constructs, full-length mMLKL and full-length hMLKL, showed a small increase in permeabilisation activity against both the 50 nm and 200 nm diameter liposomes compared to 100 nm. For these constructs, the slight differences in activity on different sized liposomes were only apparent once protein concentration was decreased (250 nM compared to 1000 nM for E351K). This indicates that with high enough concentration, MLKL can efficiently permeabilise membranes despite their curvature.

The liposome assay results showed only subtle differences, and one reason for this could be that although the liposomes were formed by extrusion with a membrane that limited the liposome size to either 50, 100 or 200 nm, smaller liposomes may have also been present in the samples, as was observed in CryoEM images of 100 nm liposomes (Figure 5.3 C). The fact MLKL can interact with GUVs that have a very low curvature compared to liposomes (Figure 5.4) and floating supported bilayers, which are formed on a flat surface, shows that MLKL can interact with membranes of varying curvature. Therefore, the reason that MLKL was found to not interact with nanodiscs (Su et al., 2014, Wang et al., 2014a), is likely due to steric hindrance stopping insertion into those constrained bilayers.

### *Stable interaction with membranes*

My CryoEM experiments suggest that wild-type human MLKL might destroy liposomes, leaving very few to be observed as compared to the liposome only controls (Figure 5.3 A, C). The same was true in the GUV experiments, where hMLKL-GFP destroyed most of the GUVs (Figure 5.5), but hMLKL PsK-GFP did not (Figure 5.5 D). The remaining vesicles in both the CryoEM and GUV experiments represent an unknown subset of the total population; it is not clear why they persist when others are lost. In the CryoEM images, the remaining liposomes appeared to be coated in a layer of MLKL protein, and this coating looked very similar between wild-type hMLKL and the less active mutant hMLKL E351K. On the surviving subset of GUVs, all MLKL constructs harbouring the 4HB were able to bind to and permeabilise GUV membranes (Figure 5.6). Interestingly in the CryoEM images the 100 nm liposomes appear to be evenly coated, whilst on the GUVs hMLKL appeared to form foci at the membrane. However, the foci were quite dispersed and regions between foci appeared to have uniform GFP signal (Figure 5.6). Therefore, it is possible that the foci do not form with sufficient frequency to be present on the smaller surface area of the 100 nm liposomes. Furthermore, the exact size of the foci is unknown, due to the resolution limit of the microscope used, therefore it is possible that the foci may cover larger areas than that of the 100 nm liposome. As most liposomes incubated with hMLKL were lost in CryoEM experiments, the dye release seen in liposome assays is likely from the destroyed liposomes. In the GUV experiments, whilst many vesicles were lost with protein addition, the remaining MLKL bound GUVs were permeabilised. This illustrates that MLKL can induce permeabilisation while it is coating the membrane, prior to total destruction of the vesicle.

### *Permeabilisation*

To investigate the size of membrane disruption MLKL caused to GUV membranes, GUVs were incubated with protein and either a small molecular weight dye (sulphorhodamine) or dyes conjugated to 10 or 70 kilodalton dextrans (Figure 5.7), with hydrodynamic radii of ~1.9 nm and 6.5 nm respectively (Armstrong et al., 2004). Previously, Ros et al. (2017) suggested that MLKL can form pores smaller than 4 nm

in diameter in cells, based on the observation that cells incubated with PEGs, that prevent osmotic damage to the cell membrane, were permeable to propidium iodide when undergoing necroptosis, but impermeable to dextran 10. In contrast, in the GUV experiments hMLKL, hMLKL E351K and mMLKL were all capable of allowing transit of dextran 70 across the membrane for at least some GUVs (Figure 5.7). It is possible that the size of molecules that can transfer through the membrane is not reflective of a certain sized pore formed by MLKL, but rather that small pores formed by MLKL could be enlarged by small osmotic imbalances between the GUV and the buffer, allowing larger molecules to transfer. To mitigate against this affect, the GUVs were formed in the same buffer as the experiments were conducted in. However, the GUV membrane is less stable than a cell membrane, and one cannot rule out small osmotic differences having some effect.

Mouse MLKL, and the low activity construct hMLKL E351K, appeared to be better able to allow diffusion of high molecular weight dextrans across the GUV membrane at 250 nM protein concentration than at 50 nM (Figure 5.7). Wang et al. (2014) previously showed a similar result: with addition of increasing concentration of a full-length hMLKL phosphomimetic mutant (TSED) to liposomes, larger dextrans could egress. They reported that dextran 40 could cross the liposome membrane at the highest concentration of protein tested (Wang et al., 2014a), at odds with the results reported in Ros et al. (2017) in cells. Another possible explanation for different sized openings in cells versus GUVs is that the amount of MLKL present at cell membranes is lower than the amount of MLKL added to GUVs. Unfortunately, this is difficult to compare or calculate. In the GUV experiments, although the amount of lipid used to make GUVs is known, the process of GUV growth varies in efficiency and it is difficult to quantify how many GUVs are formed per preparation, therefore it is difficult to determine a lipid to protein ratio. There are also other possible factors at play in cells, for example, it has been proposed that the ESCRT proteins repair membrane damage by MLKL (Gong et al., 2017), and a recent paper showed that ESCRT proteins affected how quickly DAMP protein HMGB1 is released from necroptotic cells (Murai et al., 2018), indicating that in cells there is active modulation of the level of membrane disruption.



### *Different binding modes between human and mouse MLKL*

Another observation from my GUV studies was in regards to the different binding modes exhibited by human and mouse MLKL. As previous chapters, and MLKL literature have highlighted, mouse and human MLKL differ in their mode of action in cells. The different morphologies of binding mode by the recombinant proteins on this model membrane system illustrates again that the proteins are intrinsically poised to perform differently.

### *What is the significance of the ring morphology?*

The ring morphology that human MLKL exhibits on the GUV membrane (Figure 5.8), is highly unusual and has not been observed before with other proteins binding to GUVs to our knowledge. The ring is a large, seemingly organised oligomeric structure. It does not resemble a small circular pore that other pore forming proteins may form in the membrane, where protein:protein contacts between adjacent subunits enforce a certain pitch and 'n' number for pore formation (Iacovache et al., 2010). The ring can grow rapidly, and during growth from a small ring to a large one, as observed in Figure 5.9, the curvature of the ring changes. This suggests that the protein:protein interactions which form the ring are flexible, do not have a defined pitch of interaction, and likely have a fast on- and off-rate to allow incorporation of new subunits into the ring. The ring, although unusual, constitutes reasonable evidence that MLKL can form higher order oligomeric structures on the membrane. It is possible that the foci formed by hMLKL-GFP could be formed by a similar protein:protein interaction as formed by the ring, but that conditions are not optimal for ring growth. It is also tempting to link the ring structure to the filaments formed by hMLKL E351K observed by CryoEM microscopy (Figure 5.3). However, wild-type hMLKL did not form filaments when incubated under the same conditions as E351K, and it was wild-type hMLKL that predominantly formed rings on GUVs.

### *Phase separation, lipid sorting or both?*

Redistribution of membrane dye was seen concurrent with ring growth (most apparent in GUV B, by 60-90 second time point, Figure 5.9). This could indicate that some lipids are more likely to end up on one side of the oligomer as the ring grows,

or potentially that the protein:protein interface that forms the rings allows diffusion of lipids across it, and by favouring diffusion of lipids of a certain shape or charge, could induce some kind of lipid sorting. It was interesting to note that rings did not form around the patches of liquid-disordered phase on phase separated GUVs (Figure 5.11), meaning that the ring was not triggered to form at phase boundaries. The foci that formed in the liquid-disordered phase patches were mobile, showing that both higher order MLKL oligomers observed, the foci and the ring, can diffuse laterally through the cell membrane (Figure 5.9, 5.12). The phase separated GUV experiments (Figure 5.11, 5.12) raised the question of how membrane phase can affect MLKL activity. Some papers have suggested that MLKL binds to lipid rafts (Fan et al., 2019, Chen et al., 2014), which are enriched in cholesterol and are liquid-ordered phase (Sonnino and Prinetti, 2013). Our GUV results indicated, that MLKL might prefer the liquid-disordered phase, with the caveat that we do not know the distribution of POPG between the liquid-ordered and disordered phases on the phase separated GUV membrane.

To address the question of phase by another technique, we formed floating supported bilayers of either liquid-ordered or liquid-disordered phases to test by neutron reflectometry. The two experiments presented in this chapter used either POPC or DPPC as the main lipid of the outer leaflet (Figure 5.14), which form liquid-disordered phase or liquid-ordered phase membranes at room temperature respectively (Figure 5.15, 5.16). MLKL was able to interact with both membranes, illustrating that it is likely that MLKL does not have such a strong preference for a particular phase as suggested by the GUV experiments (Figure 5.11, 5.12). Rather, MLKL's localisation to one side of the ring, may indicate that negatively-charged lipids were enriched in one hemisphere of the ring, rather than the formation of a different lipid phase. Likewise, MLKL's preferential binding to the liquid-disordered patches on the phase separated GUVs is likely due to the predominant localisation of negatively charged POPG to those patches.

The identity of the main lipid in the outer leaflet of the bilayer in Experiment 1 and Experiment 2 was not the only variable to change between these two experiments. In

Experiment 2, a deuterated tail lipid was used in the inner leaflet, making direct comparison of the models generated to describe Experiment 1 and Experiment 2 difficult. For a more direct comparison of differences in MLKL's interaction with the two types of outer leaflet, we performed an experiment identical to Experiment 1 except using DPPC/PIP2 in the outer leaflet, the analysis of which is still ongoing (data not shown). This experiment can be more directly compared to Experiment 1 (Figure 5.15), and the results from this comparison should more clearly show any differences that MLKL may exhibit between interaction with a liquid-ordered phase or liquid-disordered phase membrane.

#### *Depth of insertion into bilayer*

The neutron reflectometry results indicate that most of the 4HB + brace construct (mMLKL 1-169) incorporated into the bilayer rather than sitting on top, as the model that best represented the data for both experiments did not include a protein layer above the bilayer. Interestingly there was not much of an increase in the SLD of the bilayer with addition of deuterated protein (Figure 5.15), even though the protein has a much higher SLD than the lipids (SLD of  $7.04 \times 10^{-6} \text{ \AA}^{-2}$  in  $\text{D}_2\text{O}$  for protein, compared to  $\sim 1.80 \times 10^{-6} \text{ \AA}^{-2}$  for lipid headgroups and  $\sim -0.30 \times 10^{-6} \text{ \AA}^{-2}$  for lipid tails). A possible explanation for this is that, over the entire area of the membrane perhaps only a small amount of protein inserted into the bilayer, and as such the SLD of the entire layer did not increase by much. Another possible explanation is that the protein disrupted the bilayer, allowing more solvent into the bilayer, which would counteract the increase in SLD from protein inserting, as the solvent (both the  $\text{D}_2\text{O}$  buffer and  $\text{H}_2\text{O}$  buffer) have a lower SLD than the deuterated protein. Experiments were also performed with full-length mMLKL, but unfortunately the floating supported bilayers used in those experiments were of poor quality and no useable data could be obtained.

#### *Lipid mixing between leaflets*

The data from Experiment 2 indicates that MLKL insertion into the membrane increased lipid mixing between leaflets. The induction of lipid mixing between leaflets of the bilayer is in support of recently published studies that MLKL causes PS

exposure on cells undergoing necroptosis (Zargarian et al., 2017, Yoon et al., 2017, Gong et al., 2017, Chen et al., 2019). Our result suggests that rather than activating a specific scramblase at the membrane, the mixing between leaflets is part of MLKL's mode of membrane permeabilisation, which could occur by the formation of a toroidal pore, in which headgroups from the two leaflets join at the pore lumen, allowing mixing between the two leaflets (Gilbert et al., 2014). The other important result from neutron reflectometry is that the 4HB and most of the brace become embedded in the membrane. If, as suggested by the lipid mixing between leaflets, MLKL induces toroidal pore formation in the membrane, MLKL may remain in a predominantly hydrophilic environment in the pore lumen, by associating with the headgroups of the bilayer in a toroidal pore, and this may satisfy the requirements of the positively charged amino acids that coat the 4HB. Importantly multiple models of toroidal pore formation by membrane interacting proteins exist, some of which require the protein to interact with the hydrophobic tails of the bilayer as well, (see carpet model, vs toroidal model; (Brogden, 2005). How MLKL may satisfy hydrophobic binding requirements in the bilayer remains unknown, and further experiments would need to be performed to confirm the exact nature of the membrane disruption formed by MLKL.

### *Conclusions*

The mechanism of MLKL membrane association and permeabilisation is a contentious area in the MLKL literature. In this study, I employed biophysical techniques that are novel to the study of MLKL membrane association. Using this approach, I collected a range of observations that bring new information to the MLKL membrane interaction field. It appears that MLKL can bind to the membrane of liposomes and GUVs stably in at least a subset of vesicles, MLKL treated GUVs were permeable to large dextran conjugate dyes, and at the membrane human MLKL is capable of assembling into higher order oligomers such as rings. Formation of structures such as this may be a mechanism for MLKL to increase its local concentration at the membrane, in order to induce membrane stress. Ring growth resulted in lipid sorting, as shown by redistribution of membrane dye, and MLKL's preferential association with one hemisphere of the GUV as demarcated by the ring,

suggesting that the lipids MLKL has affinity for were predominantly localised to one hemisphere. The neutron reflectometry results from membranes of different phase suggest that MLKL may not be a phase sensing protein, but that MLKL association with the bilayer can induce lipid mixing between leaflets. This result is useful for ruling a model suggested in the literature. If MLKL formed a cation channel as suggested by Xia et al. (2016), this would not promote lipid mixing between leaflets, and therefore this model is not supported by the neutron reflectometry experiment. As the study of the MLKL membrane interaction continues, other observations from this study may contribute to understanding subsequent data, and bring the MLKL field closer to an accurate model of MLKL membrane association and permeabilisation.

## **6.0 Conclusions and Future Directions**

The scope of the data presented in this thesis covers MLKL's entire proposed mechanism of action in the execution of necroptosis (represented in Figure 1.8), with the possible exception of MLKL's translocation to the plasma membrane. I employed a broad range of methodologies to better understand its function; including established techniques such as cell death assays and X-ray crystallography, and techniques applied to the study of MLKL for the first time, such as neutron reflectometry and confocal microscopy on Giant Unilamellar Vesicles. My aims set out to answer specific questions on different stages of MLKL function, and as such three distinct bodies of work emerged, which are all discussed in their respective chapters. The relationship between the data emerging from my three aims will be discussed in this chapter, as well as experiments which are yet to be performed that will further illuminate MLKL's mode of action.

While only one of my aims initially sought to understand the differences between MLKL orthologues, studying the disparity between the mouse and human MLKL protein became an overarching theme of my research, which permeated into my other studies on MLKL oligomerisation and membrane association. Often mouse and human orthologue proteins have similar molecular mechanisms. Mice are routinely utilised as preclinical model organisms for human drug discovery. The differences in mode of action between mouse and human MLKL, and differential killing ability in cells, were therefore somewhat unexpected. These differences have led to some discontinuity in the field, for instance with use of mouse specific mutations in the human MLKL context (Najafov et al., 2019), or disparity in the size of the reported MLKL oligomer (Hildebrand et al., 2014, Huang et al., 2017), and killing ability of the 4HB+brace in cells (Tanzer et al., 2016). Investing in the idea that necroptosis has evolved to combat pathogen infection (Silke and Hartland, 2013, Nailwal and Chan, 2019, Pearson and Murphy, 2017), the stark differences may have arisen due to different selection pressures from the varying pathogen repertoire that humans and mice are exposed to, which call for a necroptosis pathway more, or less, prone to activation.

The first results chapter deals with the question of MLKL orthology. Most orthologues tested were not capable of reconstituting necroptosis in mouse or human *MLKL*<sup>-/-</sup> cell lines. Pig MLKL was capable of reconstituting necroptosis in *MLKL*<sup>-/-</sup> human cell lines to near wild-type human MLKL levels. Unfortunately, soluble recombinant pig MLKL protein could not be expressed in insect cells. It is of outstanding interest to design a pig MLKL construct that can be expressed and purified in order to perform structural studies, which may help illuminate why pig MLKL has compatibility with human RIPK3. Horse MLKL was able to reconstitute cell death in *Mkl1*<sup>-/-</sup> MDFs. The structural similarities between mouse and horse MLKL, particularly in horse MLKL's potential RIPK3 binding interface, suggest that it is RIPK3 compatibility that first and foremost decides whether an MLKL orthologue has the capacity to reconstitute necroptosis in a foreign cell system. This assertion is reinforced by other data. While full-length frog MLKL cannot reconstitute necroptosis in MDFs, the frog 4HB+brace induced constitutive necroptosis in mouse cells (Tanzer et al., 2016). This shows that when the requirement for RIPK3 phosphorylation of the pseudokinase is removed, the remainder of the frog protein is compatible with the mouse system. Furthermore, the killing activity of the chimeric h4HB-mBrace-mPsk construct in MDFs showed that given the correct RIPK3 interacting domain, the human 4HB was able to kill mouse cells, even though it alone, or in the context of full-length human is inactive in MDFs (Davies et al., 2018, Tanzer et al., 2016).

I was intrigued to find that rat MLKL was not active in mouse cells, despite the high sequence similarity between the rat and mouse proteins. The mouse phosphomimetic mutant, mMLKL S345D is autoactive in mouse cells. When the equivalent mutation was made to rat MLKL, this construct induced low level autoactivity in both mouse and human cells, showing that when the requirement for activation by RIPK3 is removed, rat MLKL can kill in mouse cells. Though the mouse MLKL and rat MLKL sequence conservation is very high (85.8% identity, 96% similarity), the mouse RIPK3 and rat RIPK3 sequence similarity is lower (76.4% identity, 88% similarity). Potentially the differences between rat and mouse MLKL, though they are few, relate to how each MLKL can bind their species specific RIPK3. This finding emphasises how finely tuned MLKL:RIPK3 pairings must be for each species. The lack of shared

compatibility between mouse and rat MLKL, makes the observed differences between human and mouse MLKL more plausible, given the relative evolutionary distances between these species.

A follow up experiment to further test if it is RIPK3 that determines species specificity, would be to co-express RIPK3 and MLKL orthologue pairs from a particular species in human or mouse *Ripk3*<sup>-/-</sup> *Mlkl*<sup>-/-</sup> cell lines. Assuming the orthologue RIPK3 could communicate with upstream signalling partners, it would be possible to test if MLKL orthologues can function in foreign cell systems downstream of RIPK3 mediated activation. One group has found a way to circumvent the requirement for orthologue RIPK3 compatibility with upstream partners, by artificially oligomerising hRIPK3, using a dimerization fusion protein cassette (Quarato et al., 2016). Quarato et al. (2016) reconstituted *Ripk3*<sup>-/-</sup> *Mlkl*<sup>-/-</sup> MEFs with an oligomerisable hRIPK3 construct and hMLKL, and found that with addition of dimerising agent, and a pan caspase inhibitor, necroptosis was induced via hMLKL in MEFs. It would be interesting to perform the reciprocal experiment in human cells, or in cells of other orthologues.

Another sought after, but elusive, piece of evidence to better understand MLKL activation by RIPK3 is a high-resolution structure of either hRIPK3 alone, or a co-crystal structure of hMLKL and hRIPK3. Understanding how the hMLKL:hRIPK3 interaction works, and how it differs from the mMLKL:mRIPK3 interaction, will help put into context the structures of the other orthologues solved in Chapter 3. Mutational analysis has gone some way to describing the different requirements for mouse and human MLKL RIPK3 binding (Chen et al., 2013, Petrie et al., 2019b, Petrie et al., 2018), but without a structure these results are difficult to interpret. Another potential avenue to improving our understanding of the MLKL:RIPK3 relationship, would be molecular dynamics simulations. These experiments might be able to describe the RIPK3:MLKL interaction pre- and post-MLKL phosphorylation, and to depict transient states difficult to capture with crystallography. One theory put forward suggests that phosphorylation of hMLKL may lead to its dissociation from hRIPK3, supported by the fact that phosphomimetic mutant hMLKL T357E/S358E shows decreased binding to hRIPK3 (Petrie et al., 2018, Petrie et al., 2019b).



Understanding how phosphorylation affects the interaction between the two proteins may be important in understanding if RIPK3 plays any other role in MLKL's mechanism of action, such as templating oligomerisation, or influencing subcellular localisation.

RIPK3 phosphorylation therefore may be the gatekeeper of species compatibility, but downstream of this event other factors could play a role. Our group has suggested that the mouse and human MLKL oligomers may differ in stoichiometry, with the mouse being a trimer (Davies et al., 2018, Hildebrand et al., 2014), and the human forming a tetramer (Petrie et al., 2018). These suggestions are based upon biophysical techniques such as analytical ultracentrifugation (Hildebrand et al., 2014) and native mass spectrometry (Petrie et al., 2018). During my PhD, I performed SAXS analysis on both the mouse full-length and rat full-length oligomer. Using the mouse full-length crystal structure as a rigid body model, the rat MLKL scattering data favoured a tetramer model whilst the mouse favoured a trimeric model. Based upon the sequence similarity between these two constructs, it seemed unlikely that they would form an oligomer of a different size. However, they do have some sequence variation at the brace region, in and around the residues which form the putative heptad repeat (Cai et al., 2014), and variations in the residues of the heptad repeat can affect the stoichiometry of the coiled coil that forms (Harbury et al., 1993). Furthermore, the human MLKL tetramer model proposes that human MLKL takes on a daisy chain conformation with the brace regions at contact points (Petrie et al., 2018). Given the tetramer is not a dimer of dimers, it seems plausible that one protein subunit could be left out of the daisy chain to form a trimer, with similar protein contacts still mediating oligomerisation.

To unambiguously determine the stoichiometry of the mouse MLKL oligomer, as well as the rat, for comparison with human MLKL, we plan to perform native mass spectrometry on the oligomers from these proteins in the same manner as Petrie et al. (2018). This has previously not been possible due to difficulty in generating enough material of full-length mouse MLKL oligomer. Confirming the stoichiometry of the mouse oligomer is important, because our assumptions that it forms a trimer are

based upon AUC on a truncated construct mMLKL 1-169, and SAXS analysis which shows that whilst the mouse scattering data is best supported by a trimeric model, a tetramer could make a plausible model as well. In support of the conclusion that mouse MLKL full-length forms a trimer, I have linked the truncated construct mMLKL 1-169 to the full-length oligomer in cells by functional analysis in Chapter 4. Therefore, based on the existing data I would predict that native mass spectrometry will reveal the mouse oligomer to be a trimer.

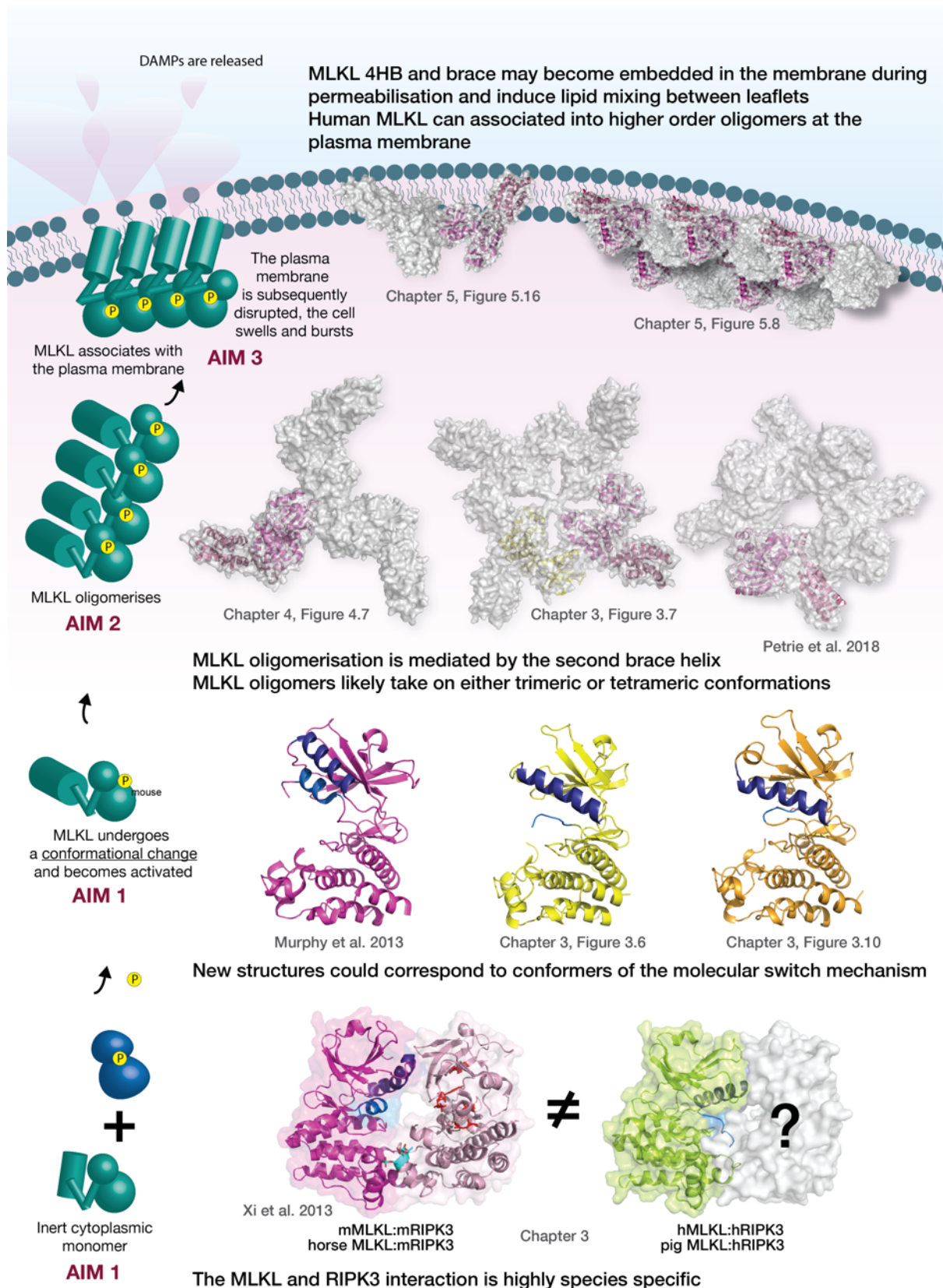
If native mass spectrometry shows that mouse MLKL in fact forms a tetramer like human MLKL, it will raise some interesting questions. In that case, the mouse tetramer would appear to behave quite differently to the human tetramer. For instance, the mouse full-length oligomer has a larger elution volume on gel filtration than human MLKL, and the mouse MLKL SAXS data favours a trimer. This would suggest that the overall conformation or envelope of the two species' oligomers are quite different, even if they are made up of the same number of subunits. This could still represent a key difference between species, leading to functional differences in cells.

Downstream of the oligomerisation step, my studies found further differences in human and mouse MLKL at the membrane. Chapter 5 examined the activity of human and mouse MLKL on Giant Unilamellar Vesicles (GUVs) for the first time. Mouse and human MLKL displayed varying modes of membrane association, with human MLKL appearing to be intrinsically poised to form large oligomers at the membrane, whilst mouse MLKL maintained a uniform binding modality to the membrane. This may suggest that either human and mouse MLKL disrupt membranes in different ways, or it might suggest that mouse MLKL requires other interacting partners to form higher order oligomers at the membrane. In any case, oligomers such as the ring, or the foci, or even the filaments formed by the hMLKL E351K mutant under CryoEM, all represent oligomers of a much larger size than just the tetramer or trimer oligomer that we have so far investigated, and may have similarities to the MLKL polymer observed by others (Liu et al., 2017, Johnston and Wang, 2018). These observations point to the fact that MLKL may undergo higher order assembly at membranes. This

may constitute a previously unexplored new step in MLKL's mechanism of action (Figure 1.8), and the protein:protein interaction interfaces or other factors that lead to higher order assemblies remain to be understood.

As mentioned in the Chapter 5, it would be of great interest to perform CryoEM tomography on the liposome membranes coated in MLKL, as MLKL's membrane association mode remains of outstanding interest. The data in this thesis suggest that MLKL does not form a transmembrane ion channel as suggested by Xia et al. (2016). Instead, my interpretation of the data is that MLKL can accumulate at the membrane and form large oligomers, and induce formation of a toroidal, not proteinaceous pore, which suggests that MLKL binding induces stress upon the plasma membrane until it breaks. This interpretation is based upon a collection of observations from different techniques, and relies upon some inference and conjecture. To prove or disprove this model will require further experiments, perhaps including super high resolution microscopy of MLKL interacting with either GUVs or the cell membrane, and better characterisation of the higher order oligomers that MLKL may form at membranes.

My contributions to our understanding of MLKL's mechanism of action as relating to the proposed molecular mechanism in Figure 1.8 and each of my aims, is summarised in Figure 6.1. The contributions of other studies which I have compared and contrasted my data with are indicated with citations in the figure.



**Figure 6.1 Contributions from this thesis to better understanding MLKL's molecular mechanism of action**

Results from Aim 1 indicated that each species' MLKL and RIPK3 pair show stringent requirements for binding, and that MLKL orthologues are rarely compatible with mouse or human RIPK3. The two new structures of the MLKL pseudokinase domain,

from the rat and horse orthologues, could represent different conformations of the MLKL molecular switch mechanism, and in particular the different structures of mouse and rat suggest that each protein could take on the other's conformation. In the course of examining Aim 1 and 2, I performed SAXS on the mouse and rat MLKL full-length oligomers, which revealed that mouse and rat may have different stoichiometries of oligomerisation, both models are shown next to the human MLKL SAXS model from Petrie et al. (2018). I showed conclusively that some residues of the second brace helix are required for mouse MLKL oligomerisation. The results from Aim 3 provided evidence of human MLKL forming higher order assemblies at the plasma membrane, and found that MLKL induces toroidal pore formation on membranes.

Viewing MLKL activity through the lens of mouse and human MLKL differences is an unusual perspective to take, and sometimes a frustrating one. Given the differences between the two proteins, discoveries made about the human or mouse proteins may need to be confirmed in the reciprocal system. However, it is an interesting conundrum, and a greater understanding of the differences between the species will in turn help us better understand how each MLKL works, and be important for ensuring that drugs developed against human MLKL have an appropriate animal model that can be used to test their efficacy. Furthermore, these interspecies differences may eventually highlight the different selection pressures the necroptosis effectors MLKL and RIPK3 have been subjected to, and may help us to understand why necroptosis has evolved and what role it plays in the context of human health and disease.

## Appendix I

Each vector used imparts a different tag or expression system compatibility (Table 7.1). Some vectors were commercially sourced, and others are commercial vectors with modifications. Vector cloning was performed by Dr James Murphy and Sam Young.

**Table 7.1 Vectors used**

| Vector                   | Purpose                                                                                                       | Antibiotic resistance | Source                                            | References                                                                               |
|--------------------------|---------------------------------------------------------------------------------------------------------------|-----------------------|---------------------------------------------------|------------------------------------------------------------------------------------------|
| pF TRE3G PGK puro        | Mammalian cell expression with puromycin selection                                                            | ampicillin            | Modified from pF 5x UAS puro (Vince et al., 2007) | (Murphy et al., 2013, Hildebrand et al., 2014, Tanzer et al., 2016)                      |
| p FastBac HTB            | Insect cell expression (via bacmid production), in-frame N-terminal TEV cleavable His tag                     | ampicillin            | Life technologies                                 | (Murphy et al., 2013)                                                                    |
| pF FastBac HTB SBP       | Insect cell expression (via bacmid production), in-frame N-terminal TEV cleavable His tag followed by SBP tag | ampicillin            | Modified from pF FastBac HTB (Life Technologies)  | Unpublished                                                                              |
| p FastBac1 GST           | Insect cell expression (via bacmid production), in-frame N-terminal TEV cleavable GST tag                     | ampicillin            | Modified from pF FastBac1 (Life Technologies)     | (Murphy et al., 2013, Petrie et al., 2018)                                               |
| pETNusH HTB              | E. coli expression, in-frame N-terminal TEV cleavable NusA protein tag                                        | kanamycin             | Modified from pETM60 (De Marco et al., 2004)      | (Murphy et al., 2010, Murphy et al., 2013, Davies et al., 2018, Hildebrand et al., 2014) |
| pF TRE3G PGK C-FLAG puro | Mammalian cell expression with C-terminal FLAG tag and puromycin selection                                    | ampicillin            | Modified from pF TRE3G PGK puro                   | (Murphy et al., 2013, Hildebrand et al., 2014, Tanzer et al., 2016)                      |
| pF TRE3G PGK GFP puro    | Mammalian cell expression with C-terminal GFP tag and puromycin selection                                     | ampicillin            | Modified from pF TRE3G PGK puro                   | (Murphy et al., 2013, Hildebrand et al., 2014, Tanzer et al., 2016)                      |

The full-length or truncation constructs of MLKL that were used for recombinant protein expression or mammalian expression (Table 7.2). The source of the DNA sequence for the template DNA is listed, as well as the primers used to amplify the insert from the template. The vector that the constructs were inserted into is listed, showing the expression system for the construct and any tags put on it. These constructs were also used for other point mutations made (Table 7.5).

**Table 7.2 Full-length, and truncation constructs of orthologue MLKLs**

| Species, residue                         | Accession, source                          | Forward                                                 | Reverse                                          | Vector                                   |
|------------------------------------------|--------------------------------------------|---------------------------------------------------------|--------------------------------------------------|------------------------------------------|
| Mouse MLKL full-length (1-464)           | NM_029005.3; DNA2.0, CA, USA               | cgcggtatccgcgcca<br>ccatggataaattggga<br>cagatcatcaag   | cgcggtatccttacac<br>cttctgtccgtggatt<br>c        | p FastBac HTB SBP,<br>pF TRE3G PGK puro, |
| Mouse MLKL pseudokinase domain (179-464) | NM_029005.3; DNA2.0, CA, USA               | cgcggtatccgcgcca<br>ccatgcaagatctcca<br>aatcaaggagattcc | cgcggtatccttacac<br>cttctgtccgtggatt<br>c        | pF TRE3G PGK puro,<br>p FastBac HTB SBP  |
| Mouse MLKL (1-158)                       | NM_029005.3; DNA2.0, CA, USA               | cgcggtatccgcgcca<br>ccatggataaattggga<br>cagatcatcaag   | cgcggaattcagcta<br>ttgcaactgcatcagg<br>ataac     | pETNusH HTB                              |
| Human MLKL full-length (2-471)           | NP_001135969.1; DNA2.0, CA, USA            | cgcggtatccgcgcca<br>ccatggaaaattgga<br>catattatcaccc    | cgcggtatcctacttag<br>aaaagggtggagag              | pF TRE3G PGK puro,<br>p FastBac1 GST,    |
| Human MLKL pseudokinase domain (190-471) | NP_001135969.1; DNA2.0, CA, USA            | cgcggtatcccaagag<br>caaatcaaggagatca<br>ag              | cgcggtatcctacttag<br>aaaagggtggagag              | p FastBac HTB                            |
| Rat MLKL full-length (1-464)             | XP_003753159; ATUM, CA, USA                | cgcggtatccgataaatt<br>gggacagatcatcaag<br>ttag          | cgcggaattcacagctt<br>ttgtccatagag                | pF TRE3G PGK puro,<br>p FastBac HTB-SBP  |
| Rat MLKL pseudokinase domain (179-464)   | XP_003753159; ATUM, CA, USA                | cgcggtatcccaagatt<br>ccaagtcaaggagatt<br>cc             | cgcggaattcacagctt<br>ttgtccatagag                | p FastBac HTB                            |
| Horse MLKL full-length (1-475)           | XP_005608486; Bioneer, South Korea         | cgcggtatccgcgcca<br>ccatggataaac                        | tcgctgtgctagcgaa<br>ttcagttgaatacaca<br>gcttg    | pF TRE3G PGK puro                        |
| Horse MLKL pseudokinase (188-475)        | XP_005608486; Bioneer, South Korea         | cgcggtatcccaagatc<br>aaatcaaggagattaa<br>gaaag          | tcgctgtgctagcgaa<br>ttcagttgaatacaca<br>gcttg    | p FastBac HTB                            |
| Pig MLKL full-length (1-477)             | XP_003481839; ATUM, CA, USA                | cgcggtatccgcgcca<br>ccatggatc                           | cgcggaattcaacacg<br>tggttcacaaaag                | pF TRE3G PGK puro                        |
| Chicken MLKL full-length* (1-486)        | XP_015134716; Bioneer, South Korea         | n/a                                                     | n/a                                              | pF TRE3G PGK puro                        |
| Tuatara MLKL full-length (1-486)         | ENSSPUT00000023207.1; Bioneer, South Korea | cgcggtatccgaaattgt<br>ggagaagggtgctgagt<br>g            | gcggaattcagcttgc<br>tgttgctatcttc                | pF TRE3G PGK puro                        |
| Stickleback MLKL full-length (1-470)     | ENSGACT00000011307.1; GeneArt, Germany     | cgcggtatccagccac<br>catggaaattgtgaaag<br>agattac        | ttcgctgtgctagcgg<br>ccaccacgctgggc<br>agttatc    | pF TRE3G PGK puro                        |
| Frog MLKL full-length (1-498)            | XP_002931711; Bioneer, South Korea         | cgcggtatccagccac<br>catggagatcctggg                     | tttcgctgtgctagcgt<br>gttttttagtgagacc<br>atattgc | pF TRE3G PGK puro                        |

\*The chicken full-length gene was ordered from ATUM, and cut out from a plasmid with *Bam*HI/*Eco*RI digest and then cloned into its respective vectors

Mouse MLKL 1-169 point mutation constructs (Chapter 4) were generated by PCR with mismatch in reverse primer (Table 7.3). The forward primer is the same as for mouse MLKL full-length Table 7.2.

**Table 7.3 mMLKL 1-169 and mutant constructs**

| Construct            | Reverse primer sequence (5'-3')                        | Vector         |
|----------------------|--------------------------------------------------------|----------------|
| mMLKL 1-169<br>C169S | cgcggaattcagcttgcttcagggtttgttgatttctccac              | PetNusH<br>HTB |
| V159A-E160A          | cgcggaattcagcttgcttcagggtttgttgatttctgccgcgctaattgcaac | PetNusH<br>HTB |
| E161A-I162A          | cgcggaattcagcttgcttcagggtttgttggtgcttccacgctaattg      | PetNusH<br>HTB |
| N163A-K164A          | cgcggaattcagcttgcttcagggttgcggcgatttctccacgc           | PetNusH<br>HTB |
| T165A-L166A          | cgcggaattcagcttgcttcagggtttgttgatttctccacgctaattg      | PetNusH<br>HTB |
| K167A-Q168A          | cgcggaattcagcttgcggccagggtttgttgatttctccac             | PetNusH<br>HTB |

The chimeric domain swapped constructs were synthesized by Bioneer, South Korea. Chimeric construct h4HB-hBrace-mPsk was used as a template for point mutations that were generated by Rui Quin (primers Table 7.4), via overlap PCR.

Mouse MLKL-GFP and mouse MLKL Psk-GFP (Chapter 5) were generated by Rupoj Sarbaswa, by cloning the mouse MLKL full-length gene, and the mouse pseudokinase domain gene into a pF TRE3G PGK GFP puro vector (Table 7.1). The mMLKL-GFP fusions were amplified by PCR then cloned into p FastBac HTB vector (Table 7.1). The human MLKL-GFP fusions were synthesised by ATUM, USA, then cloned into p FastBac derived vectors (Table 7.1) via *EcoRI* and *BamHI* digestion, followed by T4 ligase ligation, as described in Chapter 2.

Mutant constructs were made and often tested in the context of mammalian cell expression to look for deficits in killing ability. The mutations were made by overlap PCR using the templates listed in Table 7.2 with the same forward and reverse primers as the base construct in Table 7.2, the mutagenesis primer is listed in Table 7.4.



**Table 7.4 Mutant constructs and mutagenesis primers**

| Mutant construct                                    | Mutagenesis forward primer (5'-3')    | Vector                               |
|-----------------------------------------------------|---------------------------------------|--------------------------------------|
| Mouse MLKL full-length E161A-I162A                  | caaattagcgtggaagcagccaacaaaaccctg     | pF TRE3G PGK puro                    |
| Mouse MLKL full-length N163A-K164A                  | cgtggaagaaatcgccgcaaccctgaagc         | pF TRE3G PGK puro                    |
| Mouse MLKL full-length T165A-L166A                  | gaagaaatcaacaaagccgcgaagcaatgctc      | pF TRE3G PGK puro                    |
| Mouse MLKL full-length K167A-Q168A                  | caacaaaaccctggcggcatgctcactaaaac      | pF TRE3G PGK puro                    |
| Mouse MLKL full-length E161A-T165A                  | gcgtggaagcaatcaacaaagccctgaagc        | pF TRE3G PGK puro                    |
| Mouse MLKL full-length I162A-L166A                  | gcgtggaagaagccaacaaaaccgcgaagcaatgctc | pF TRE3G PGK puro                    |
| Horse MLKL full-length T208A                        | gatggaatcccactagcgaaaggtagttc         | pF TRE3G PGK puro                    |
| Horse MLKL full-length Y283A                        | cgtcatggaggcctgtgagctcgg              | pF TRE3G PGK puro,<br>pF FastBac HTB |
| Horse MLKL full-length Q355A                        | gagttgagcaaaacagcgactccatcagtc        | pF TRE3G PGK puro                    |
| Horse MLKL full-length Q355A-T208A                  | gagttgagcaaaacagcggtccatcagtcg        | pF TRE3G PGK puro                    |
| Horse MLKL full-length TSAA                         | gttgagcaaaacacaggctgccatcagtcgagg     | pF TRE3G PGK puro                    |
| Horse MLKL full-length TSEE                         | gttgagcaaaacacaggaggaaatcagtcgaggaac  | pF TRE3G PGK puro                    |
| Horse MLKL full-length S233A                        | gtattcagcaaagcacaggccagaagc           | pF TRE3G PGK puro                    |
| Horse MLKL full-length R236A                        | gcaaatcacaggccgcaagcattggaatag        | pF TRE3G PGK puro                    |
| Horse MLKL full-length S237A                        | cacaggccagagccattggaatagtg            | pF TRE3G PGK puro                    |
| Horse MLKL full-length R242A                        | gcattggaatagtggcgcatacttc             | pF TRE3G PGK puro                    |
| Horse MLKL full-length Y385A                        | catttctgcagcagccatctcacctg            | pF TRE3G PGK puro                    |
| Rat MLKL full-length S345D                          | gcaaaacacagactgatataagccgggc          | pF TRE3G PGK puro                    |
| h4HB-hBrace-mPsk KI <sup>h</sup> >NM <sup>m</sup>   | gacggaaatgaaaagatcaaagttatcctgatg     | pF TRE3G PGK puro                    |
| h4HB-hBrace-mPsk EAS <sup>h</sup> >KVI <sup>m</sup> | ggaaatgaaaatatggaagctagcctgatgcagttg  | pF TRE3G PGK puro                    |
| h4HB-hBrace-mPsk RR <sup>h</sup> >MQ <sup>m</sup>   | gaaagttatcctgaggcgggtgcaaattagcg      | pF TRE3G PGK puro                    |
| h4HB-hBrace-mPsk EIN <sup>h</sup> >QIS <sup>m</sup> | cactgagactacaaatcagcatgaaagaaatcaag   | pF TRE3G PGK puro                    |
| h4HB-hBrace-mPsk MK <sup>h</sup> >VE <sup>m</sup>   | cgattagaaatcaacgtggaagaaatcaaggaaac   | pF TRE3G PGK puro                    |
| h4HB-hBrace-mPsk KE <sup>h</sup> >NK <sup>m</sup>   | catgaaagaaatcaataaaactttgaggcag       | pF TRE3G PGK puro                    |
| Human MLKL E351K                                    | gcttgcaggatttaagttgaggaaaac           | pF FastBac1 GST                      |



## The brace helices of MLKL mediate interdomain communication and oligomerisation to regulate cell death by necroptosis

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### Abstract

The programmed cell death pathway, necroptosis, relies on the pseudokinase, Mixed Lineage Kinase domain-Like (MLKL), for cellular execution downstream of death receptor or Toll-like receptor ligation. Receptor-interacting protein kinase-3 (RIPK3)-mediated phosphorylation of MLKL's pseudokinase domain leads to MLKL switching from an inert to activated state, where exposure of the N-terminal four-helix bundle (4HB) 'executioner' domain leads to cell death. The precise molecular details of MLKL activation, including the stoichiometry of oligomer assemblies, mechanisms of membrane translocation and permeabilisation, remain a matter of debate. Here, we dissect the function of the two 'brace' helices that connect the 4HB to the pseudokinase domain of MLKL. In addition to establishing that the integrity of the second brace helix is crucial for the assembly of mouse MLKL homotrimers and cell death, we implicate the brace helices as a device to communicate pseudokinase domain phosphorylation event(s) to the N-terminal executioner 4HB domain. Using mouse: human MLKL chimeras, we defined the first brace helix and adjacent loop as key elements of the molecular switch mechanism that relay pseudokinase domain phosphorylation to the activation of the 4HB domain killing activity. In addition, our chimera data revealed the importance of the pseudokinase domain in conferring host specificity on MLKL killing function, where fusion of the mouse pseudokinase domain converted the human 4HB + brace from inactive to a constitutive killer of mouse fibroblasts. These findings illustrate that the brace helices play an active role in MLKL regulation, rather than simply acting as a tether between the 4HB and pseudokinase domains.

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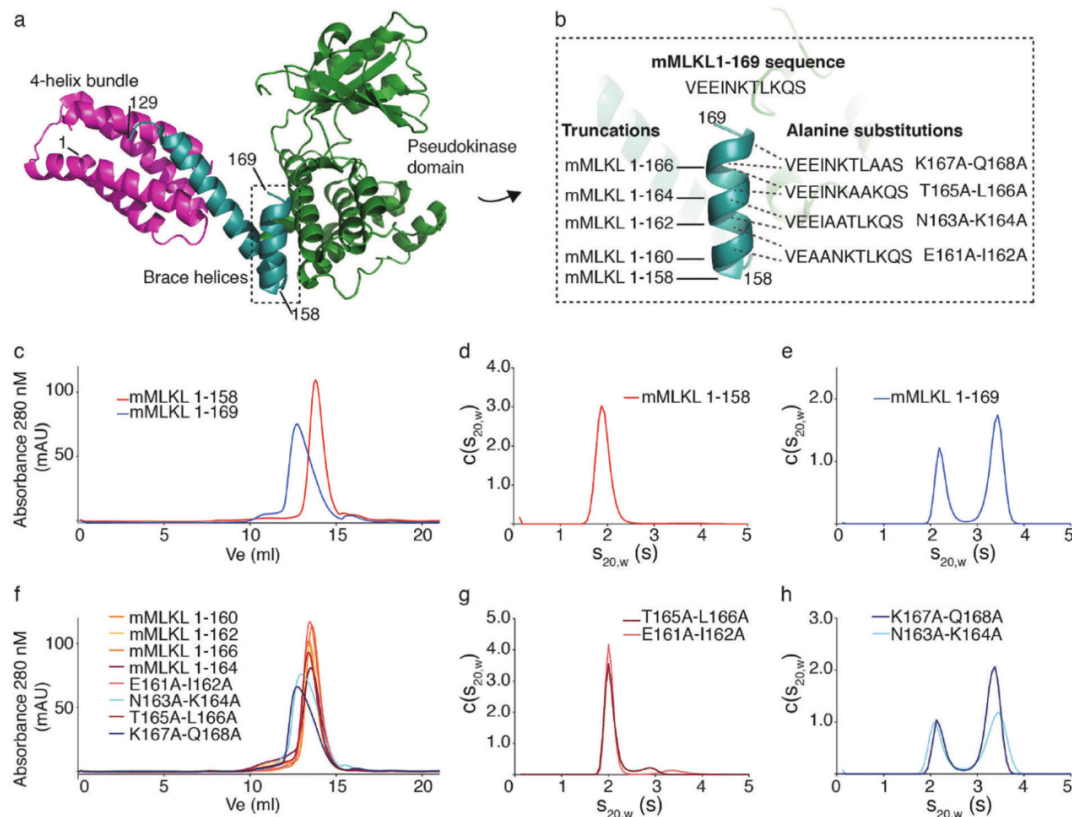
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### Introduction

Mixed Lineage Kinase domain-Like protein (MLKL) is the most terminal known obligate effector of the programmed cell death pathway necroptosis [1, 2, 3, 4]. Necroptosis is an inflammatory form of cell death, in which the plasma membrane becomes permeabilised, releasing damage-associated molecular patterns (DAMPs) to alert the innate immune system [5, 6, 7, 8]. It has been implicated in tissue damage associated with a range of human pathologies, including ischaemic reperfusion injuries [9, 10, 11, 12], and diseases of the liver [13, 14, 15]. Death receptor and Toll-like receptor (TLR)–3 and –4 ligation are known to trigger necroptosis, however the best studied pathway is death induction via TNF signalling. In certain cellular contexts, TNF signalling can activate a kinase cascade in which (auto)phosphorylated RIPK1 forms a functional amyloid oligomer with RIPK3 [16, 17], which can then activate MLKL by phosphorylation [1, 2, 4, 18, 19, 20]. This leads to a conversion of MLKL from an inert cytoplasmic

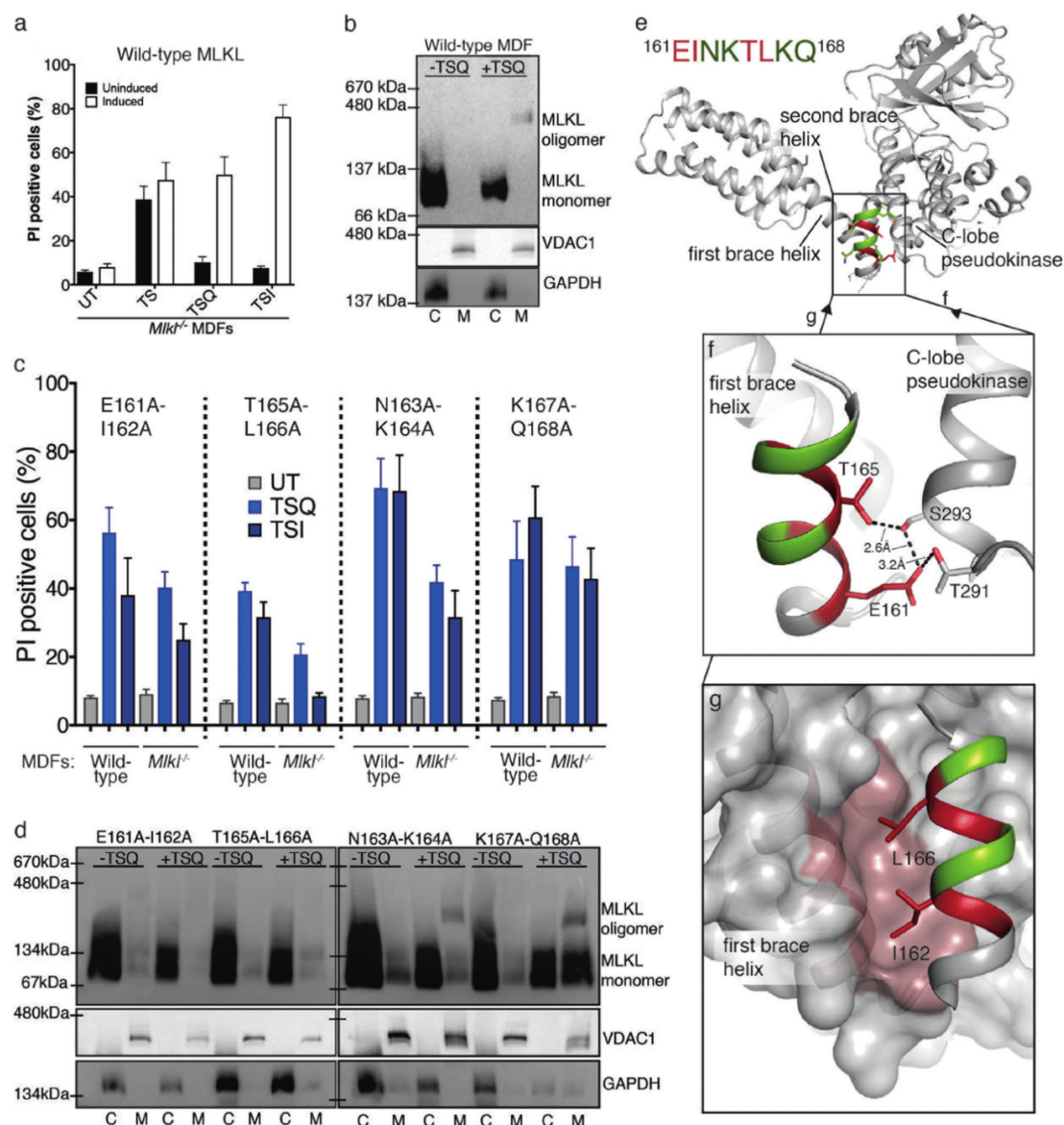


**Fig. 1** Residues 159–169 of the second brace helix are responsible for trimerisation of mMLKL 1–169 in vitro. **a** The structure of full-length mouse MLKL (PDB:4BTF) [1]. The 4HB is coloured magenta, the PsK domain green and the two-helix brace in teal. **b** Brace truncations and mutations are highlighted on the second brace helix. **c** Overlay of analytical gel filtration of mMLKL 1–158 and 1–169. **d**, **e** Standardised continuous sedimentation coefficient  $c(s_{20,w})$  distributions for mMLKL 1–158 and mMLKL 1–169. **f** Gel filtration of truncations, mMLKL 1–160, 162, 164 and 166, and mMLKL 1–169 harbouring

the E161A-I162A, T165A-L166A, N163A-K164A, or K167A-Q168A alanine mutations. Constructs that behave similarly to mMLKL 1–169 are coloured in shades of blue, while those that behave similarly to mMLKL 1–158 are coloured in shades of red. **g** Standardised continuous sedimentation coefficient  $c(s_{20,w})$  distributions for E161A-I162A and T165A-L166A. **h** Standardised continuous sedimentation coefficient  $c(s_{20,w})$  distributions for N163A-K164A and K167A-Q168A

monomer, to an oligomeric, membrane-associated effector of necroptosis [21, 22, 23, 24]. The key trigger in this conversion is RIPK3-mediated phosphorylation of the activation loop in MLKL's C-terminal pseudokinase domain to induce exposure of the N-terminal four-helix bundle (4HB) domain [23], which is presumed to induce necroptotic cell death via its membrane permeabilisation activity [15, 25, 26]. The mechanism by which a phosphorylation event in the C-terminal pseudokinase domain leads to exposure of the killer N-terminal 4HB domain is currently unclear. Here, we explore the hypothesis that the two interdomain helices, termed the 'brace' helices, are key to communicating a phosphorylation event in the pseudokinase domain to exposure of the 4HB domain to enable its participation in membrane localisation, permeabilisation and cell death.

MLKL oligomerisation is an essential step in necroptosis [23], however there is no consensus on the stoichiometry of the complex. Trimers [22, 23, 24], tetramers [21, 22], hexamers [15], and octamers [27] have been reported, as well as data showing forced dimerisation of MLKL is sufficient to cause auto-activation and cell death [24, 26]. The oligomerisation interface is unknown, and there is also contention as to whether disulphide bonds are required for oligomer formation, or merely a consequence of higher-order assembly [15, 21, 22, 25, 28]. Previous studies have shown that recombinant mouse and human MLKL constructs comprising the 4HB domain and the two 'brace' helices can form trimers [23, 24], whereas the pseudokinase (PsK) domain alone does not [29]. Notably, truncated forms of recombinant human MLKL (residues 2–154 or 1–140) lacking portions of the brace region do not oligomerise



[24, 30], implicating the brace region as a possible mediator of oligomerization. Similar studies are yet to be reported for MLKL orthologues, however, and the precise molecular details remain to be determined.

Involvement of the brace region in the molecular switch mechanism and oligomerisation has so far been inferred. Here, we directly interrogated the function of the MLKL 'brace' helices. In addition to revealing an important role for the second brace helix in mediating oligomerisation and cell death through truncation and mutational analyses, our studies of mouse–human MLKL chimeras—in which the 4HB,

brace or pseudokinase domain were swapped between mouse and human MLKL (and vice versa)—indicate that the integrity of the brace helices is crucial to interdomain communication. We observed that disrupting communication between the N and C-terminal domains of MLKL is capable of flipping the molecular switch to generate constitutively active constructs. A working switch mechanism could be restored by substitution of specific residues in the predicted first brace helix and adjacent loop, such that necroptotic stimuli was required for cell death to ensue. These data highlight the importance of the brace region to



**Fig. 2** Cellular activity of MLKL bearing second brace helix mutations parallels behaviour of recombinant proteins. **a** *Mkl<sup>+/+</sup>* MDFs reconstituted with wild-type MLKL were stimulated with TNF (T), Smac-mimetic (S) and Q-VD-OPh (Q) or emricasan/IDN-6556 (I), and cell death was quantified using propidium iodide (PI) staining and flow cytometry. **b** Western blot of Blue-Native PAGE of wild-type MDFs treated  $\pm$  TSQ to measure localisation to cytoplasmic (C) or membrane (M) fractions and oligomerisation state. VDAC1 and GAPDH western blot reprobes were used to assess quality of fractionation. **c** Full-length MLKL constructs containing indicated alanine mutations were stably incorporated into MDFs via lentiviral infection, and protein expression induced by doxycycline (dox) treatment. Cells were either left untreated (UT), or treated with TSQ or TSI to induce necroptosis 3 h post dox induction. Cell death was measured using PI staining and flow cytometry 24 h after death stimulation. Responses to an apoptotic stimulus (TS), and death in the presence or absence of dox-induction, are shown in entirety in Supplementary Figure 2. **d** Western blot of Blue-Native PAGE on cell fractions was used to monitor the formation of MLKL higher-order species and determine cellular localisation in untreated or TSQ stimulated *Mkl<sup>+/+</sup>* MDFs, expressing alanine mutant constructs. C; cytoplasmic fraction, M; membrane fraction. Western blots for VDAC1 (membrane associated) and GAPDH (cytoplasmic protein) were used to assess the quality of fractionation. BN-PAGE blots are representative of 2 independent experiments. Cell death data are the mean  $\pm$  s.e.m. of three independent experiments with 2–3 biologically independent replicates per experiment ( $n = 6$ –9). **e** Residues subjected to alanine mutagenesis were mapped on to the full-length mouse MLKL structure (PDB: 4BTF) [1]. Mutation of residues in green (N163, K164 and K167, Q168) does not preclude oligomerisation, while those in red (E161, I162 and T165, L166) block oligomerisation. **f** Zoomed depiction of second brace helix structure in **e**. Hydrogen bonds between the second brace helix (E161 and T165) and C-lobe of the pseudokinase domain (T291 and S293) are indicated in black dashed lines with interatomic distances annotated. **g** Atoms from the first brace helix that form hydrophobic packing interactions with I162 and L166 from the second brace helix are coloured pink and shown in a surface representation

two related facets of MLKL activation: as an interface that contributes to MLKL oligomerisation, and in the relay of the proposed molecular switch mechanism to connect pseudokinase domain phosphorylation to the release or activation of the 4HB domain executioner function.

## Results

### Residues in the second brace helix mediate trimerisation of mouse MLKL N-terminal domain

Our previous analytical ultracentrifugation (AUC) analyses showed that the mouse MLKL (mMLKL) 4HB + brace (residues 1–169) exists in solution in vitro in a monomer-trimer equilibrium with a  $K_D$  for self-association of  $7.2 \mu\text{M}^2$  (ref. 23). Subsequent biophysical studies of human MLKL (hMLKL) constructs indicated that while a construct incorporating the 4HB and two brace helices (1–182) oligomerised, truncation of one (1–154) or both (1–140) brace helices compromised oligomerisation [24, 30]. Here we

sought to validate whether the brace region of mMLKL mediates oligomerisation, and to perform detailed mutational analyses to more clearly map the intersubunit interface.

Initially, we generated an mMLKL construct encompassing the 4HB domain and first brace helix (residues 1–158), as defined from our earlier crystal structure of mMLKL [1]. Compared to the homotrimeric mMLKL 1–169, which encompasses the 4HB + two brace helices (Fig. 1a, b), mMLKL 1–158 was monomeric. First, mMLKL 1–158 eluted at a higher retention time from analytical size exclusion chromatography than mMLKL 1–169 (Fig. 1c). Second, by sedimentation velocity analytical ultracentrifugation (SV-AUC), we observed a single peak with sedimentation coefficient of 1.8 for mMLKL 1–158 (Fig. 1d), while two species with sedimentation coefficients of 2.1 and 3.3 were observed for mMLKL 1–169 (Fig. 1e), consistent with its propensity for self-association into higher-order species. Sequential deletion of terminal residues in the second brace helix led to behaviour comparable to mMLKL 1–158 in analytical size exclusion chromatography (Fig. 1b,f; Supplementary Figure 1a), indicating that integrity of the second brace helix is crucial to higher-order assembly in solution.

To further define which residues mediate mMLKL trimerisation, we substituted pairs of residues in the second brace helix of mMLKL 1–169 with alanine to generate E161A-I162A, N163A-K164A, T165A-L166A and K167A-Q168A mutants (Fig. 1b, Supplementary Figure 1a). Analytical size exclusion chromatography and SV-AUC experiments identified two mutant constructs, N163A-K164A and K167A-Q168A, which retained the ability to form trimers (Fig. 1h), and two constructs, E161A-I162A and T165A-L166A, which were primarily monomeric (Fig. 1g). These results indicate that residues E161, I162, T165 and L166, which are involved in the packing of the second brace helix against the first brace helix and the pseudokinase domain in the mMLKL structure, contribute to mMLKL oligomerisation.

### Trimerisation via the second brace helix is necessary for necroptosis

To test whether residues that were important for mMLKL 1–169 oligomerisation, E161, I162, T165, and L166, play an important role in necroptosis, we introduced alanine substitutions into full-length mouse MLKL and examined their capacity to induce death in response to necroptotic stimuli. Doxycycline (Dox)-inducible wild-type or mutant mMLKL lentiviral constructs were stably transduced into wild-type and *Mkl<sup>+/+</sup>* mouse dermal fibroblasts (MDFs), and their effects on cell viability measured by propidium iodide (PI) staining using flow cytometry following no stimulation

(untreated, UT), an apoptotic (TNF, T; Smac mimetic, S), or necroptotic (TSQ; Q-VD-OPh, Q; or emricasan/IDN-6556, I) stimulus (Fig. 2; Supp. Figure 2). Many experimenters induce necroptosis with the pan-caspase inhibitor, Q-VD-OPh (Q) [1, 19, 23, 26, 31], however emricasan (IDN-6556, I) has recently been shown to inhibit caspase-8 hetero- and homodimers [32] more potently and might therefore be a better tool than Q-VD-OPh. Comparing stimulation of necroptosis with TSQ and TSI side-by-side, revealed TSI to be a more potent driver of necroptosis in *Mkl<sup>-/-</sup>* mouse fibroblasts reconstituted with inducibly expressed wild-type mMLKL (Fig. 2a). Our previous work has established that under basal conditions, mMLKL is cytoplasmic and exists as a presumed monomer [19, 23, 31]. Upon stimulation with necroptotic stimuli, such as TSQ, mMLKL translocates to membranes and assembles into a higher molecular weight species whose assembly can be monitored by Blue-Native (BN) PAGE (Fig. 2b).

The ability of each mutant to reconstitute the necroptosis pathway was assessed upon induction of protein expression in both wild-type and *Mkl<sup>-/-</sup>* MDFs (Fig. 2c). All cell lines expressed MLKL constructs comparably (Supplementary Figure 3). Full-length mMLKL constructs harbouring the alanine mutations that prevented oligomerisation of mMLKL 1–169 in analytical size exclusion chromatography and AUC, E161A-I162A and T165A-L166A, showed reduced ability to kill in both wild-type and *Mkl<sup>-/-</sup>* MDFs (Fig. 2c) compared to wild-type mMLKL (Fig. 2a). The capacity of mMLKL to induce necroptosis was almost entirely abrogated by the T165A-L166A mutation, while E161A-I162A mMLKL exhibited attenuated, but measurable, cell death upon TSQ or TSI stimulation (Fig. 2c). In BN-PAGE experiments, in contrast to wild-type mMLKL (Fig. 2b), neither mutant formed detectable oligomers 6 h post TSQ treatment (Fig. 2d), consistent with deficits in the capacity of these mutants to induce cell death.

Expression of full-length mMLKL constructs harbouring mutations that were capable of forming oligomers in the context of recombinant mMLKL 1–169, N163A-K164A and K167A-Q168A, in wild-type and *Mkl<sup>-/-</sup>* MDFs led to levels of death similar to that observed of wild-type MLKL upon TSQ stimulation (~45% death), although to a lesser extent when treated with TSI (Fig. 2c). Like wild-type mMLKL, both mutants translocated to the membrane fraction and assembled into oligomers in BN-PAGE experiments after 6 h of treatment with TSQ (Fig. 2d).

In the context of the full-length mouse crystal structure, residues which do not influence oligomerisation (N163, K164, K167 and Q168) are solvent facing (green in Fig. 2e), whereas those that disrupt trimerisation when mutated to alanine face their two flanking regions: the first brace helix and pseudokinase domain (red, Fig. 2e). E161 and T165 form hydrogen bonds with T291 and S293

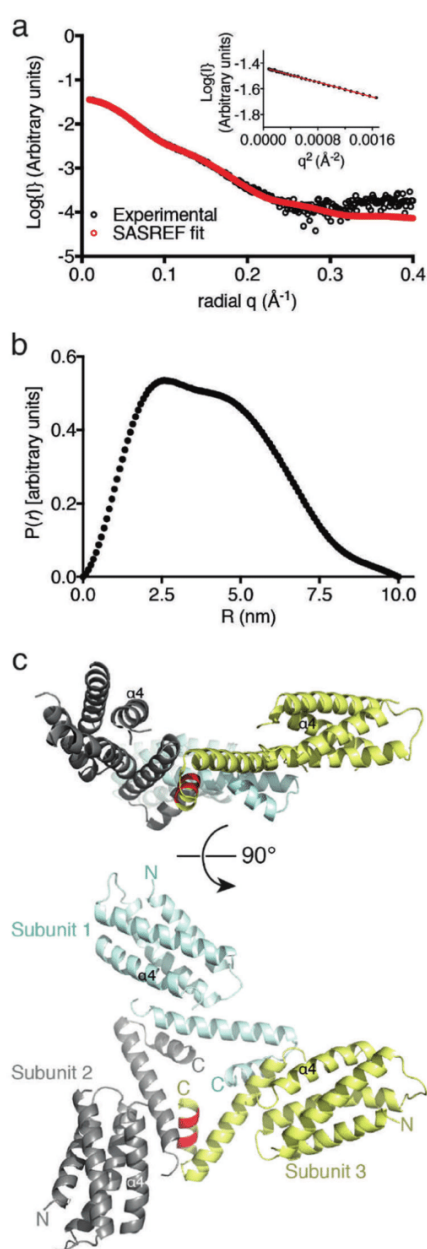
located on the C-lobe of the pseudokinase (PsK) domain (Fig. 2f), while I162 and L166 interact with a shallow hydrophobic pocket on the first brace helix (Fig. 2g). These data implicate the second brace helix as a key determinant of mMLKL trimerisation and assert the functional importance of mMLKL trimerisation in necroptotic signalling.

### The mouse MLKL 4HB + brace adopts a radial trimer conformation in solution

To gain a structural insight into the topology of the mMLKL N-terminal region in solution, we analysed recombinant mMLKL 1–169 using Small Angle X-ray Scattering (SAXS; Supplementary Table 1). To ensure the mMLKL (1–169) monomer-oligomer equilibrium favoured the oligomer form, protein at 7 mg/mL (>340  $\mu$ M), a markedly higher concentration than the  $K_D$  for trimerisation (7.2  $\mu$ M<sup>2</sup>) previously reported for this protein [23], was resolved by inline size exclusion chromatography eluting protein into the path of the X-ray beam at the Australian Synchrotron SAXS/WAXS beamline. The resulting data were of high quality, with no indications of polydispersity evident from Guinier analysis (Fig. 3a; inset), with the interatomic distance distribution indicating a maximum dimension of 100 Å (Fig. 3b). Rigid body fitting of the mMLKL 1–169 coordinates (from our full-length mMLKL crystal structure [1]) using SASREF with P3 symmetry led to a trimeric model with excellent agreement between experimental data and theoretical scattering calculated for the trimeric model, as signified by  $\chi = 0.60$ . This model is consistent with our mutational analyses: the second brace helix serves an important function as an inter-protomer contact point (Fig. 3c; face of helix with E161, I162, T165, L166 shown in red); and key residues on the  $\alpha$ 4-helix of the 4HB domain required for MLKL to execute necroptosis identified in our previous work [23] are presented on one face of the trimer model. Furthermore, these data were only consistent with the assembly of the mMLKL 4HB + Brace region into homotrimers, as monomeric and other oligomerisation regimes led to poor fits, with  $\chi$  values >3.

### The MLKL brace region functions as a communication lever between pseudokinase and 4HB domains in a species-specific manner

Previously, we noted that the species of origin of both MLKL and the host cell line governs the capacity of MLKL to induce cell death. For example, *Mkl<sup>-/-</sup>* MDFs cannot be reconstituted by expression of hMLKL [26]; and mouse, but not human, MLKL 4HB constitutively kill cells in the absence of stimuli [26] (summarised in Fig. 4c). Furthermore, cell death was not induced by expression of full-length hMLKL, h4HB + Brace and m4HB + Brace in the



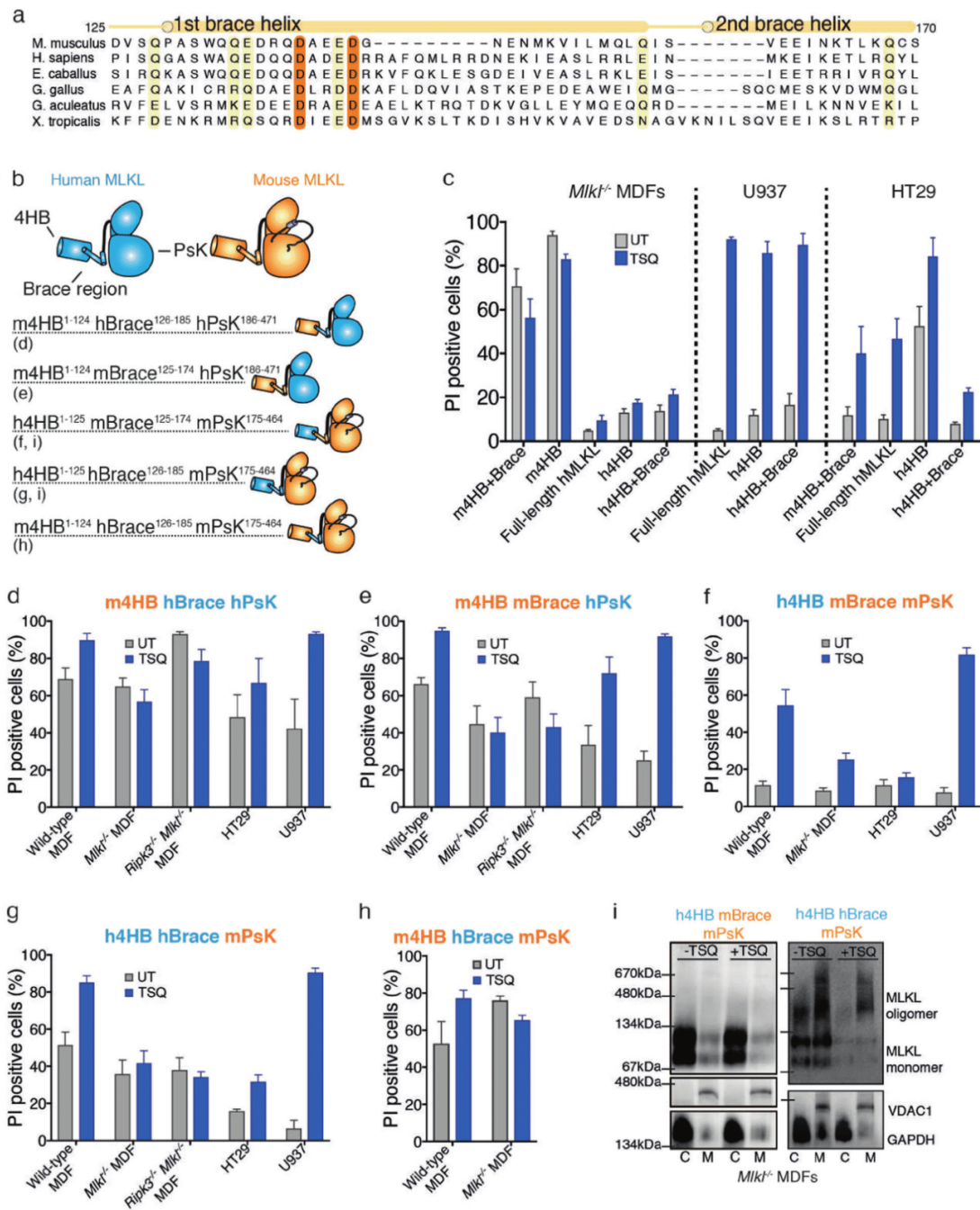
**Fig. 3** mMLKL 4HB + Brace forms a radial homotrimer in solution. **a** Small angle scatter profile of averaged, and background subtracted, data from apex of inline size exclusion peak (black circles). Agreement between the experimental and theoretical scatter (red circles) calculated from a rigid body, trimeric model in SASREF is reflected in the  $\chi$ -value of 0.60. Inset: Guinier plot of experimental SAXS data for  $q$ .  $R_g < 1.3$ . Linearity of the plot indicates that polydisperse (aggregated species) do not contribute measurably to the scatter. **b** Interatomic distance distribution plot,  $P(r)$ , profile calculated from scattering data with GNOM. Maximum particle dimension,  $D_{max}$ , was estimated as 100 Å. **c** Orthogonal views of the trimeric model of mMLKL 4HB + brace. The three units are shown in grey, cyan and yellow, and the residues important for trimerisation are shown in red

region of MLKL from multiple species, including a nine-residue insertion in the human brace region compared to the mouse counterpart (Fig. 4a). To define the extent to which variation in the brace helices would impact MLKL activation, oligomerisation and host cell species-specificity, we prepared a series of mouse:human chimeras. The chimeras were fused between mouse and human sequence at key functional domain boundaries selected for mMLKL based on the full-length crystal structure [1]: 4HB, residues 1–124; brace, 125–174; and pseudokinase domain (PsK), 175–464. Corresponding residues were selected for human MLKL by sequence alignment as 1–125 (4HB), 126–185 (brace), and 186–471 (PsK). Initially, five chimeric constructs were generated; m4HB<sup>1–124</sup>-hBrace<sup>126–185</sup>-hPsK<sup>186–471</sup>, h4HB<sup>1–125</sup>-mBrace<sup>125–174</sup>-mPsK<sup>175–464</sup>, m4HB<sup>1–124</sup>-mBrace<sup>125–174</sup>-hPsK<sup>186–471</sup>, h4HB<sup>1–125</sup>-hBrace<sup>126–185</sup>-mPsK<sup>175–464</sup>, and m4HB<sup>1–124</sup>-hBrace<sup>126–185</sup>-mPsK<sup>175–464</sup> (Fig. 4b). With these chimeric constructs, we aimed to determine the role of the brace helices in communication between domains through species-specific compatibilities. Chimeras were expressed upon dox-induction in MDFs and two human cell lines, HT29 and U937, and death of untreated (UT), and cells subjected to necroptotic (TSQ) (Fig. 4d–h) or apoptotic (TS) (Supp. Figure 4) stimuli was assessed. Chimeric proteins were expressed to comparable levels, as assessed by western blot (Supp. Figure 5), indicating that folding or expression defects do not contribute to signalling deficits.

Expression of mouse 4HB kills MDFs [23] (Fig. 4c), and it retained this ability when fused to the human brace and pseudokinase (m4HB-hBrace-hPsK) (Fig. 4d). Furthermore, expression of m4HB-hBrace-hPsK also killed the human cell lines, HT29 and U937 (Fig. 4d). This suggests that the brace region and pseudokinase domain from hMLKL cannot restrain the innate ability of the m4HB to kill cells, in contrast to the mouse brace and pseudokinase domain in the context of unstimulated wild-type mMLKL. The m4HB-mBrace-hPsK MLKL chimera also constitutively killed MDFs and, to a lesser extent, human cell lines (Fig. 4e), and m4HB-hBrace-mPsK, showed strong constitutive killing of MDFs (Fig. 4h). These data illustrate that mouse

human cell lines, HT29 and U937, in the absence of stimulation of the endogenous necroptotic machinery with TSQ [26] (summarised in Fig. 4c). Interestingly, the h4HB could induce cell death of HT29, but not U937, in the absence of stimuli [26] (Fig. 4c), consistent with differences in endogenous host cell factors contributing to necroptotic death. There is substantial sequence divergence in the brace





pseudokinase and the mouse brace are required to work together to suppress the m4HB.

To further define species-specificity, we made reciprocal constructs comprising the N-terminus of hMLKL and the

C-terminus of mMLKL. In contrast to m4HB-hBrace-hPsK, but consistent with our published observations on the h4HB domain [26] (Fig. 4c), h4HB-mBrace-mPsK, did not constitutively kill when expressed in either human or mouse



**Fig. 4** Chimeric mouse:human MLKL constructs reveal key determinants for constitutive killing of human and mouse cell lines. **a** Sequence alignment of MLKL brace regions from multiple species. Residue number for mouse and a schematic of the position of brace helices in the mouse structure are presented above the alignment. **b** Schematics of mouse (orange) and human (blue) MLKL chimeras. **c** Mouse m4HB + Brace (residues 1–180), mouse 4HB (1–125), and (undimerised) gyrase fusions of full-length hMLKL (1–471), h4HB + Brace (1–180) and h4HB (1–125) were inducibly expressed in *Mlkl*<sup>-/-</sup> MDF, human HT29 and U937 cells, and death of untreated or TSQ-stimulated cells measured by PI-staining and flow cytometry. These data were reported previously [23, 26], and are included for comparison with the chimera and mutant data reported herein. **d–h** Wild-type, *Mlkl*<sup>-/-</sup> and *Mlkl*<sup>-/-</sup> *Ripk3*<sup>-/-</sup> MDFs, HT29, and U937 cells were stably transfected with doxycycline inducible chimeric constructs. Following induction of protein expression with dox, cells were either left untreated (UT) or stimulated with TSQ/TSI. MDF cell death was quantified by PI uptake after 24 h; data are the mean  $\pm$  s.e.m. of three independent experiments with 2–3 biologically independent replicates per experiment ( $n = 6–9$ ). Death assays on the human cell lines, HT29 and U937, were performed similarly to MDFs, except that cell death was measured at 48 h post-stimulation. Human cell line data are the mean  $\pm$  s.e.m. of three independent experiments ( $n = 3$ ), except for those in **g**, where  $n = 2$ . Responses to an apoptotic stimulus (TS), and death in the presence or absence of dox-induction, are shown in entirety in Supplementary Figure 4. **i** Western blot of Blue-Native PAGE on fractionated *Mlkl*<sup>-/-</sup> MDFs expressing inactive and constitutively-activated chimeras treated  $\pm$  TSQ. h4HB-hBrace-mPsK cells were harvested 18 h after dox treatment; h4HB-mBrace-mPsK were harvested at 6 h. C cytoplasmic fraction, M membrane fraction. VDAC1 (membrane associated) and GAPDH (cytoplasmic protein) blots were used as fractionation controls. All BN-PAGE blots are representative of two independent experiments

cells, and this construct did not reconstitute necroptotic signalling in *Mlkl*<sup>-/-</sup> MDFs (Fig. 4f). Furthermore, higher-order oligomers were not observed in *Mlkl*<sup>-/-</sup> MDFs expressing h4HB-mBrace-mPsK following TSQ stimulation and MLKL remained localised to the cytoplasm in BN-PAGE analysis (Fig. 4i), indicating that this chimera is unable to oligomerise and translocate to the plasma membrane. In addition to the MLKL monomer, we detected a second band that migrated near the 134 kDa marker on BN-PAGE, possibly representing a dimer (Figs. 4i, 5d). This is distinct from the higher-order species associated with cell death that migrates just below the 480 kDa marker on BN-PAGE. In contrast to the h4HB-mBrace-mPsK chimera, inclusion of the hMLKL brace region within the h4HB-hBrace-mPsK construct conferred the ability to constitutively induce cell death when expressed in wild-type, *Mlkl*<sup>-/-</sup> and *Mlkl*<sup>-/-</sup> *Ripk3*<sup>-/-</sup> MDFs (Fig. 4g). Induction of this construct for 18 h in *Mlkl*<sup>-/-</sup> MDFs led to formation of oligomers by BN-PAGE in the absence of stimuli (Fig. 4i). The same construct did not induce constitutive cell death in the human lines, U937 and HT29 (Fig. 4g). These data implicate the pseudokinase domain as a key adaptor domain that dictates species-selectivity of MLKL and whether an MLKL orthologue can interact with the host machinery.

### In concert with the PsK domain, the brace helices act as a latch to restrain the executioner function of the MLKL 4HB domain

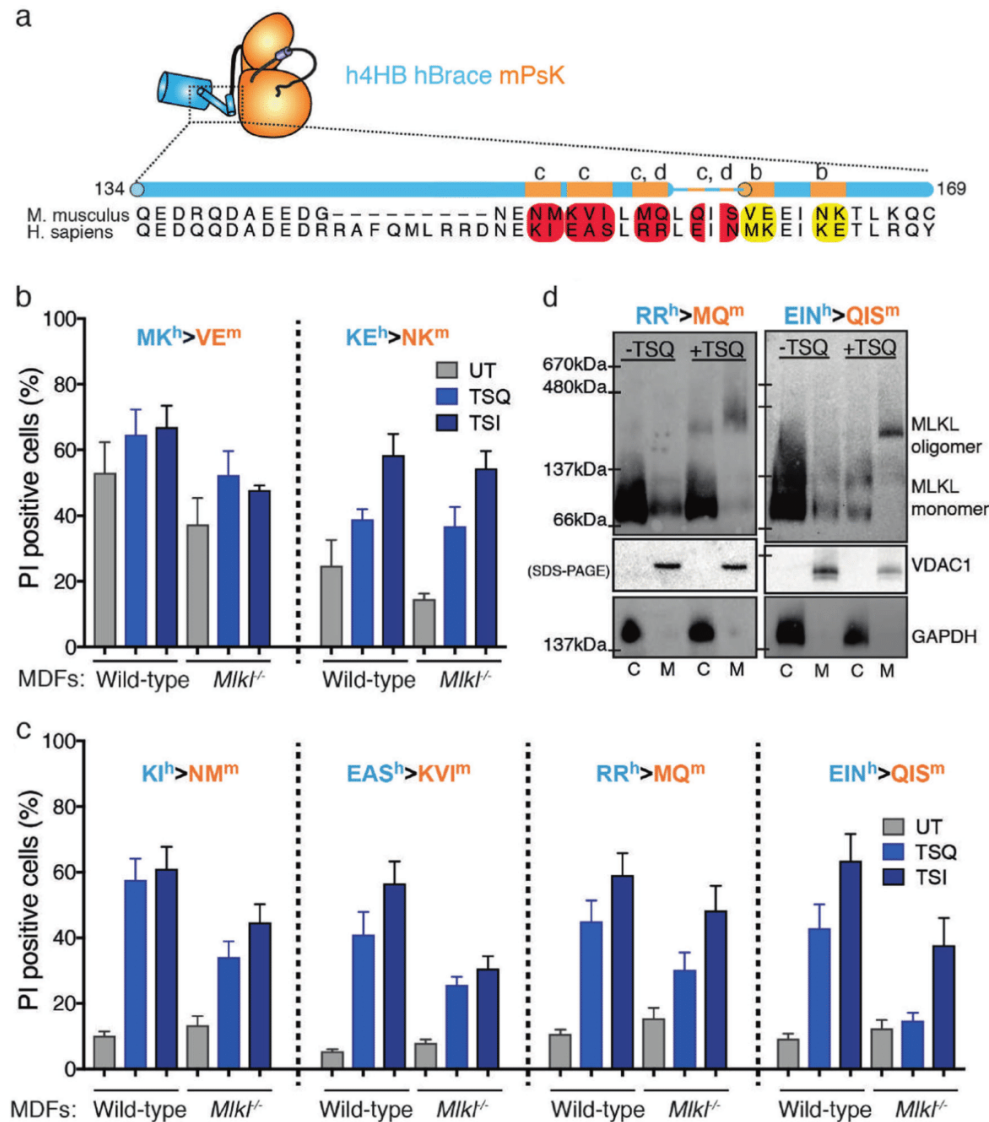
The h4HB-hBrace-mPsK construct constitutively killed MDFs in the absence of stimulus (Fig. 4g), yet only differs from the inactive h4HB-mBrace-mPsK (Fig. 4f) in the brace region. Considering that a necroptotic stimulus (TSQ) does not relieve the repressive interaction within the h4HB-mBrace-mPsK chimera or induce oligomerisation, our data favour the existence of an inhibitory interaction between the mouse brace region and the human 4HB that blocks oligomerisation and therefore MLKL-driven cell death.

To pinpoint the differences that lead to such different killing abilities in MDFs, we made pairwise mutations along the brace helices in the h4HB-hBrace-mPsK construct, substituting human brace residues with counterparts from the mouse sequence (Fig. 5a). These constructs were inducibly expressed in wild-type and *Mlkl*<sup>-/-</sup> MDFs (Supp. Figure 7), and their capacity to induce cell death in the absence of necroptotic stimuli, or to reconstitute necroptosis upon TSQ or TSI stimulation, were evaluated. Chimeras containing the human to mouse substitutions, MK<sup>h</sup> > VE<sup>m</sup> and KE<sup>h</sup> > NK<sup>m</sup>, that lie in the second helix retained the capacity to induce stimulus-independent death of MDFs (Fig. 5b). This suggests that the second brace helix, whilst being important for oligomerisation, does not play a substantive role in tipping the balance between activated and inactive MLKL. The insensitivity of these residues from the second brace helix to mutation is not altogether surprising, given that the in the mouse full-length structure, residues at equivalent points in the helix are all solvent facing.

In contrast, four constructs, harbouring KI<sup>h</sup> > NM<sup>m</sup>, EAS<sup>h</sup> > KVI<sup>m</sup>, RR<sup>h</sup> > MQ<sup>m</sup> or EIN<sup>h</sup> > QIS<sup>m</sup> substitutions, no longer exhibited constitutive killing of MDFs, but were able to reconstitute the necroptosis pathway in *Mlkl*<sup>-/-</sup> MDFs, where cell death (Fig. 5c) and membrane translocation/oligomerisation (Fig. 5d) ensued only following treatment with necroptotic stimuli. Despite comprising the human MLKL 4HB + brace and the mouse pseudokinase domain, it was possible to confer the properties of wild-type mMLKL on the construct through subtle changes in the predicted first brace helix and subsequent loop. Substitution of hBrace residues that preclude an inhibitory interaction with the mouse PsK domain within h4HB-hBrace-mPsK enabled restoration of the switch mechanism (summarised in Fig. 6).

## Discussion

The precise molecular details underlying MLKL's activation and cell killing mechanism remain incompletely

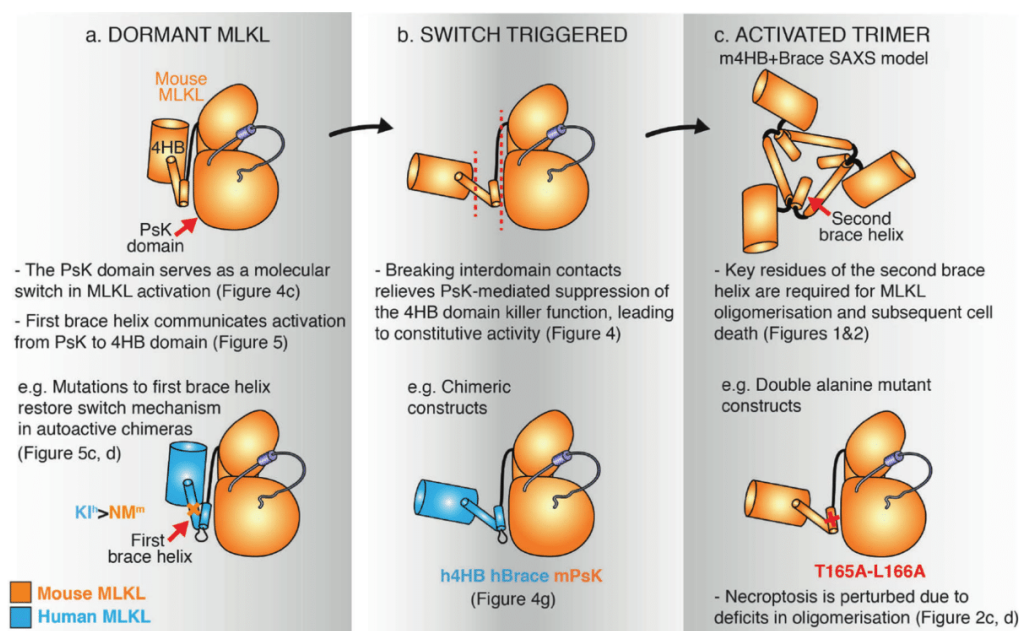


**Fig. 5** Residue swaps between human and mouse brace region suggest distinct roles for the first and second brace helix. **a** Schematic showing the base construct, h4HB-hBrace-mPsK, and the sequence of both mouse and human MLKL at the brace region. Residues mutated from the human sequence to the equivalent mouse residues are highlighted in red (first brace helix and proceeding loop) or yellow (second brace helix). Location of brace helices (based on mMLKL structure [1]), and letters corresponding to figure panels are indicated. **b**, **c** Expression of chimeric constructs harbouring the indicated mutations in wild-type and *Mlkl*<sup>-/-</sup> MDFs was induced by dox, and cell death in untreated or

necroptotic (TSQ/TSI) conditions measured by PI uptake and flow cytometry. Death assays represent mean  $\pm$  s.e.m. of three independent experiments with 2-3 biologically independent replicates per experiment ( $n = 6-9$ ). Responses to an apoptotic stimulus (TS), and death in the presence or absence of dox-induction, are shown in entirety in Supplementary Figure 6. **d** Western blots of BN-PAGE performed on fractionated cell lysates from h4HB-hBrace EIN<sup>h</sup>>QIS<sup>m</sup> mPsK and h4HB-hBrace RR<sup>h</sup>>MQ<sup>m</sup> mPsK, with and without TSQ stimulation. Blots are representative of two independent experiments

understood. The consensus among published studies is that MLKL activity relies on higher-order assemblies [21, 22, 23, 26, 28], however, the stoichiometry, and the importance of intersubunit disulphide crosslinks and post-translational

modifications [33] within the MLKL oligomer remain unclear. Additionally, there have been conflicting reports regarding the capacity of exogenously expressed MLKL 4HB domains to kill cells. Noting that sequences of



**Fig. 6** Summary of the roles of the brace helices in orchestrating the transition of MLKL from dormant to killer oligomeric form. **a** The pseudokinase domain suppresses the executioner activity of the 4HB domain in the dormant form of MLKL. Introduction of the KI<sup>h</sup> > NM<sup>m</sup>, EAS<sup>h</sup> > KVI<sup>m</sup>, RR<sup>h</sup> > MQ<sup>m</sup> or EIN<sup>h</sup> > QIS<sup>m</sup> mutations (Fig. 5c, d) into the first brace helix and adjacent loop of the constitutively active h4HB-hBrace-mPsK chimera abrogated constitutive activity, and restored responsiveness to necroptotic stimuli. The data implicated the first brace helix and adjacent loop in maintenance of the dormant MLKL species and communication of PsK domain activation to the

4HB domain. **b** Four of the five chimeric constructs generated exhibited constitutive killing activity, highlighting that the native interface between each subdomain (the 4HB, the brace and the PsK) conferred by either mouse or human sequence, is required for maintenance of the molecular switch. Breaking these interfaces triggers the switch for MLKL, resulting in a species primed to kill (Fig. 4). **c** Key residues of the second brace helix mediate oligomerisation of the mouse 4HB + Brace (Fig. 1), which forms trimers in solution (Fig. 3). These residues are required for oligomerisation of full-length MLKL and consequent necroptosis in MDF cells (Fig. 2)

the brace helices vary in length and composition among MLKL orthologues, we sought to establish the extent to which the brace helices govern MLKL activation via oligomerisation and communication between the PsK and 4HB domains.

Previously, we reported that mMLKL 4HB + brace (residues 1–169) assembled into homotrimers in vitro [23]. Subsequently, an NMR study of hMLKL 4HB + first brace helix (2–154) [30] and a SAXS study of the hMLKL PsK [29] demonstrated that both of these domains exist as stable monomers. From these data, we predicted that the second brace helix was a key determinant in MLKL oligomerisation. From our current analyses, we deduced that any truncations to the second brace helix of mMLKL (residues 159–169) compromise homotrimer assembly, with alanine scanning mutagenesis pinpointing E161/I162 and T165/L166 as key determinants. Full-length mMLKL with these mutations failed to assemble into higher molecular weight complexes and were compromised in their ability to execute necroptosis. The T165A/L166A mutation almost completely abrogated mMLKL-mediated death, while the

E161A/I162A mMLKL retained the capacity to induce necroptotic cell death after 24 h. This incomplete abrogation of cell death suggests that the full-length MLKL E161A-I162A mutant can still oligomerise, albeit with delayed kinetics, as no oligomer is observed after 6 h in Blue-Native PAGE. This in turn suggests that in the context of the full-length protein, there are additional interfaces/interactions apart from those targeted by the alanine substitutions, that stabilise the trimer.

To further dissect the role of the brace helices in MLKL activation, and whether the brace helices contribute to the species-specificity previously noted in MLKL-mediated death of human and mouse cell lines [26], we generated chimeric constructs containing combinations of mouse and human MLKL subdomains. These studies implicated the host species brace + PsK as serving a modulatory role in restraining or promoting the 4HB domain-mediated cell death, depending on the cellular context. In mouse cells, we showed that it is the species-specific mBrace and mPsK together that are essential for repressing the m4HB, as any chimera containing the m4HB caused constitutive cell



death. On the other hand, the h4HB-hBrace-mPsK construct, which caused constitutive cell death in MDFs independent of mRIPK3, did not cause constitutive death in HT29 or U937 cells, implicating the hPsK as an important determinant of MLKL-driven necroptosis in human cells. Intriguingly, whilst the h4HB-hBrace-mPsK chimera exhibited stimulus-independent killing of mouse fibroblasts, the h4HB fused to the mouse brace + PsK was completely inactive. Based on this finding, we exploited the contrasting killing activities to better understand determinants that suppress the 4HB domain executioner function. Mutational analyses revealed residues at the end of the predicted first brace helix and in the subsequent loop as key elements of the molecular switch mechanism, which communicates activating phosphorylation events in the PsK domain to the executioner 4HB domain. Our data are most consistent with a role for the first brace helix mutants disfavouring spontaneous assembly of MLKL oligomers, or enabling inhibitory interactions with the pseudokinase domain to be restored. Furthermore, our chimera data reveal an underappreciated role of the MLKL pseudokinase domain in conferring host specificity. h4HB or h4HB + brace constructs do not induce death of mouse fibroblasts [26], and full-length human MLKL does not reconstitute necroptotic signalling in *Mlkl*<sup>-/-</sup> fibroblasts [26, 34], even with forced dimerisation [26]. Here we observed that fusion to the mouse PsK domain was sufficient to enable the h4HB + brace, but not the h4HB alone, to kill MDFs. These findings indicate that the mMLKL PsK domain may function as a protein interaction domain to recruit host factors that contribute to necroptosis signalling, akin to the role reported for the co-chaperone system, HSP90:Cdc37, in MLKL activation [31, 35, 36]. The h4HB-hBrace-mPsK construct constitutively killed *Ripk3*<sup>-/-</sup> *Mlkl*<sup>-/-</sup> fibroblasts, therefore the constitutive oligomerisation observed by BN-PAGE, and killing activity of this construct cannot be explained by constitutive activation by RIPK3.

In the present work, we defined a crucial role for the second brace helix of mouse MLKL in facilitating trimer formation and necroptotic cell death (Fig. 6c), and the first brace helix and adjacent interhelix loop as important regulatory elements that convey the activation signal from the pseudokinase domain to the executioner 4HB domain (Fig. 6a). Collectively, these data illustrate that the brace helices are important regulatory elements in MLKL activation, and not simply an interdomain linker, whose regulatory functions appear to have co-evolved with its neighbouring domains and diverged among species. Owing to their central role in MLKL activation, future studies should consider the contribution of the brace helices to the molecular switch mechanism and in efforts to pharmacologically target MLKL to modulate necroptotic activity in human pathologies.

## Materials and methods

### Expression constructs

Insert DNA was amplified from a mouse MLKL template (synthesised by DNA2.0, CA) by PCR using manually-designed oligonucleotides (Integrated DNA Technologies). For recombinant protein constructs, DNA inserts were cloned into vector (Kan<sup>r</sup>) pETNusH HTB (derived from pETM60) [37, 38], using *Bam*HI and *Eco*RI restriction sites as an inframe fusion C-terminal to a TEV-protease cleavable NusA-His<sub>6</sub> tag. The mouse MLKL (1–169) construct is as described previously [23], and harbours a C169S substitution. Inserts were Sanger sequenced (AGRF, Parkville, VIC; or Micromon, Clayton, VIC). For mammalian cells, mutations were encoded by oligonucleotides (IDT) and introduced into full-length mMLKL or h4HB-hBrace-mPsK templates by overlap PCR with terminal *Bam*HI and *Eco*RI restriction sites used to clone into the (Amp<sup>r</sup>) pFTRE3G PGK puro vector [1]. Chimeric DNA inserts were synthesised by Bioneer (South Korea) and subcloned analogously into pFTRE3G PGK puro. HEK293T cells at 80% confluency in 10 cm plates were transfected with 1 µg of pFTRE3G plasmid, helper plasmids pCMV-δR8.2 (2.0 µg) and pVSV-G (0.8 µg) to produce the lentiviral capsid and envelope, and a pGFP (0.1 µg) reporter plasmid. After 48 h, the virus containing media was filtered and polybrene (10 µg/ml; Sigma) added. The virus was used to infect six biologically independent Mouse Dermal Fibroblast (MDF) cell lines (three wild-type and three *Mlkl*<sup>-/-</sup>, derived from different mice), at 60% confluency in a six-well plate. After 48 h puromycin (5 µg/mL) media was added to select cells harbouring the pFTRE3G plasmid.

### Recombinant protein expression and purification

mMLKL NTD constructs were expressed in BL21-CodonPlus cells. One liter Super broth cultures containing 50 µg/mL kanamycin were incubated at 37 °C until optical density at 600 nm reached 0.6–0.8, cultures were transferred to an 18 °C incubator for 15–20 min before adding 500 mM IPTG (Gold Biotechnology) to induce protein expression. Cultures were incubated overnight (18 °C) and cells harvested by centrifugation (10,000 × g, 10 min).

Proteins were expressed with a Tobacco Etch Virus (TEV) cleavable hexa-histidine tagged NusA tag fused to their N-terminus. Cell pellets were resuspended in lysis buffer (20 mM Tris-HCL, pH 8.0, 150 mM NaCl, 10 mM imidazole, pH 8.0, 2 mM βME) with 3 mM MnCl<sub>2</sub> and DNase (10 U; Roche). Cells were lysed with an EmulsiflexC5 homogeniser (Avestin). Immediately post-lysis 1 mM of protease inhibitor phenylmethanesulfonyl fluoride (PMSF; Sigma) was added. The whole lysate was

centrifuged ( $45,000 \times g$ , 20 min, 4 °C), and the supernatant 0.2  $\mu$ M filtered. The supernatant was passed over a Ni<sup>2+</sup> charged 1 mL HiTrap Chelating HP cartridge column (GE Healthcare) twice. The column was washed extensively with lysis buffer supplemented with 350 mM NaCl, followed by lysis buffer. Protein was eluted in lysis buffer with 100 mM imidazole. The eluate was buffer exchanged to reduce imidazole concentration, and concentrated, then cleaved overnight (4 °C) with 0.5 mg of hexa-histidine tagged TEV protease. The next day, eluate was passed over a nickel cartridge to remove TEV protease, any uncleaved material and cleaved NusA. The flowthrough containing the NTD construct was loaded onto a HiLoad 16/600 Superdex75 PG size exclusion column (GE Healthcare) and 1.5 mL fractions collected. Protein purity was assessed by SDS-PAGE with Coomassie Brilliant Blue staining. Pure fractions were pooled, concentrated, and TCEP (ThermoFisher) added to 1 mM.

### Qualitative size exclusion chromatography

All NTD constructs were run on the same Superdex75 10/300 GL column (GE Healthcare), at 4 °C, in TBS (20 mM Tris pH 7.5, 150 mM NaCl), on the same day. Three hundred microliter at 1 mg/mL was loaded for each protein, and 0.5 mL fractions collected.

### Analytical ultracentrifugation

Sedimentation velocity experiments were performed using an XL-I analytical ultracentrifuge (Beckman Coulter) equipped with UV/Vis scanning optics. Buffer reference (20 mM Tris-HCl, 150 mM NaCl, pH 8.0, 1 mM TCEP) and 380  $\mu$ L of mMLKL sample solutions at 40  $\mu$ M were loaded into 12 mm double-sector cells with quartz windows and the cells were mounted in an An-60Ti 4-hole rotor or An-50Ti 8-hole rotor. All experiments were conducted at 50,000 r.p.m. ( $201,600 \times g$ ) and 20 °C, and radial absorbance data were collected at 280 nm in continuous mode. Data were fitted to a continuous sedimentation coefficient distribution [c(s)] model and converted to standardised sedimentation coefficient ( $s_{20,w}$ ) distributions using SEDFIT [39]. The protein partial specific volumes, buffer density, and buffer viscosity were calculated using SEDNTERP [40].

### Small angle X-ray scattering (SAXS)

SAXS data were collected on the SAXS/WAXS beamline (Australian Synchrotron) using an inline size-exclusion chromatography setup [41]. Two-dimensional scattering data were radially averaged, normalised to sample transmission, and  $11 \times 2$  s scatter patterns from the apex of the

elution peak were averaged before background scatter (estimated from the average of 20 scatter patterns from the SEC run prior to protein elution) was subtracted using the ScatterBrain software (written by Stephen Mudie, Australian Synchrotron). The ATSAS suite of software was used for all subsequent SAXS data analyses: Guinier analysis was performed using PRIMUS [42]; the pairwise intratomic distance distribution function  $P(r)$  and  $D_{max}$ , the maximum dimension of the particle, were calculated from scattering data using GNOM [43]; rigid body modelling of the mMLKL(1–169) homotrimer was performed using SASREF with P3 symmetry [44]. All structural models were illustrated using PyMOL. The data collection and processing statistics are presented in Supplementary Table 1.

### Cell lines

MDFs were cultured in Dulbecco's Modified Eagle Medium (Life Technologies) supplemented with 10% (v/v) Foetal Calf Serum, penicillin (100 U/mL), streptomycin (100  $\mu$ g/mL) and, after selection, puromycin (5  $\mu$ g/mL). U937 and HT29 cells were cultured in human tonicity RPMI medium supplemented with 8–10% v/v fetal calf serum (FCS) and puromycin (5  $\mu$ g/mL) for lines stably transduced with MLKL expression constructs.

### Death assays

MDFs were seeded at  $5 \times 10^4$  cells/well into 24-well plates, and allowed to adhere overnight. Cells were treated with 10 ng/ml doxycycline, and after 3 h either left untreated, or treated with TNF (Produced in-house; 100 ng/mL) and Smac-mimetic (Compound A, Tetralogic; 500 nM) (TS), or TS and IDN-6556 (Idun Pharmaceuticals) (5  $\mu$ M) (TSI) or Q-VD-OPh (10  $\mu$ M) (TSQ). Doxycycline negative controls were also performed. 24 h after treatment, cells were harvested and treated with propidium iodide (PI; 100 ng/mL), and PI positive cells counted by BD FACSCalibur flow cytometry. Human cell death assays were conducted analogously, except doxycycline treatment was for 4 h. Cell viability was quantitated by PI staining and flow cytometry 48 h post-stimulation for human cell lines.

### Blue-Native PAGE

MDFs were seeded at  $5 \times 10^5$  cells/well in six-well plates, and allowed to adhere overnight. Cells were treated with 10 ng/ml dox for 3 h, before being treated with TSQ or left untreated. After ~6 h, when cell death had reached ~40% by eye, cells were harvested. Membrane fractionation and Blue-Native PAGE (BN-PAGE) was performed as previously described [23]. Briefly the cells were permeabilised

in MELB buffer with 0.025% digitonin, and the cytosolic and crude membrane fraction separated by centrifugation. The crude membrane fraction was further solubilised in 1% digitonin and centrifuged to clarify. The cytosolic fraction was made up to 1% digitonin, and samples separated on a 4–16% Bis-Tris Native PAGE gel (Life Technologies). The gel was transferred onto a PVDF membrane (Osmonics), de-stained and denatured before being probed by western blot.

### Western blot

Following transfer via electrophoresis, PVDF membranes were rinsed in PBS-T and blocked with 5% w/v milk powder in PBS-T for 1 h (RT). They were washed for  $3 \times 10$  min in PBS-T, then incubated overnight (4 °C) with primary antibody. Primary antibody was removed, the membrane washed for  $6 \times 10$  min, and Horse Radish Peroxidase (HRP) conjugated secondary antibody added for 1 h (RT). The membrane was washed ( $6 \times 10$  min) and bands visualised using Chemiluminescent HRP Substrate (Millipore). Antibodies used were rat anti-MLKL [1] (1/1000; clone 3H1, available as cat. MABC604 from Millipore), rabbit anti-GAPDH (1/1000; glyceraldehyde-3-phosphate dehydrogenase; Cell Signalling Technology), mouse anti-VDAC1 (1/500; Voltage Dependent Anion Channel 1; Merck, cat. MABN504) and mouse anti-actin (1/3000; Sigma).

### Statistical analyses

Cell death assay results are presented as a mean  $\pm$  s.e.m. of at least three independent experiments (each conducted with a minimum of two biologically independent replicates for MDFs).

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### Compliance with ethical standards

**Conflict of interest** J.M.M., J.S., P.E.C., E.J.P. and S.N.Y. contribute to a project funded by Anaxis Pharma to develop necroptosis inhibitors. The remaining authors declare that they have no conflict of interest.

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## SUPPLEMENTARY TABLE

**Supplementary Table 1. SAXS data collection and analysis statistics**

| mMLKL 1-169; C169S                      |                                                                                                            |
|-----------------------------------------|------------------------------------------------------------------------------------------------------------|
| <b>Data-collection parameters</b>       |                                                                                                            |
| Instrument                              | Australian Synchrotron<br>SAXS/WAXS beamline                                                               |
| Beam geometry                           | 120 micron point source                                                                                    |
| Wavelength (Å)                          | 1.033                                                                                                      |
| Exposure time                           | 2 sec exposures                                                                                            |
| Temperature (K)                         | 285                                                                                                        |
| q range (Å <sup>-1</sup> ) <sup>a</sup> | 0.00912 to 0.400                                                                                           |
| Protein concentration                   | 50 µL of 7 mg/ml protein<br>via inline gel filtration<br>chromatography<br>0.2M NaCl, 20mM<br>HEPES pH 7.5 |
| <b>Structural parameters</b>            |                                                                                                            |
| I(0) (cm <sup>-1</sup> ) [from P(r)]    | 0.03664 ± 0.0002                                                                                           |
| R <sub>g</sub> (Å) [from P(r)]          | 31.55 ± 0.14                                                                                               |
| D <sub>max</sub> (Å)                    | 100                                                                                                        |
| I(0) (cm <sup>-1</sup> ) (from Guinier) | 0.03600 ± 0.0002                                                                                           |
| R <sub>g</sub> (Å) (from Guinier)       | 31.30 ± 0.32                                                                                               |
| <b>Software employed</b>                |                                                                                                            |
| Primary data reduction                  | Scatterbrain (Australian<br>Synchrotron)                                                                   |
| Data processing                         | PRIMUS, GNOM                                                                                               |
| Computation of model<br>intensities     | CRY SOL                                                                                                    |
| Rigid body fitting                      | SASREF                                                                                                     |

<sup>a</sup> q is the magnitude of the scattering vector, which is related to the scattering angle (2θ) and the wavelength (λ) as follows:  $q = (4\pi/\lambda)\sin\theta$



## SUPPLEMENTARY FIGURE LEGENDS

**Supplementary figure 1. SDS-PAGE of all mMLKL NTD purified constructs, CD spectroscopy, and supplementary AUC spectra.** (a) Each purified protein construct was resolved by reducing SDS-PAGE to assess purity. (b) Circular dichroism spectroscopy showing mMLKL NTD constructs retain  $\alpha$ -helical secondary structure. All constructs show double-minima at 208 nm and 222 nm respectively. Each point on the curve is a mean of 4 second averaging at each wavelength. (c-h) Sedimentation velocity analytical ultracentrifugation data. Raw radial absorbance data (circles) are shown overlaid with the best fit to the continuous sedimentation coefficient [c(s)] distribution model (solid lines). Radial absorbance scans collected at 18 minute intervals during sedimentation are shown.

**Supplementary figure 2. Cellular activity of MLKL bearing second brace helix mutations parallels behaviour of recombinant proteins.** Complete dataset, including uninduced vs dox-induced and TS only controls, for experiments presented in Figure 2. TNF (T); Smac-mimetic (S); emricasan (IDN-6556; I); Q-VD-OPh (Q). (a-d) Wild-type and *Mlkl*<sup>-/-</sup> MDFs had full-length mMLKL harbouring the indicated alanine substitutions stably incorporated under the control of a doxycycline (dox) inducible promoter using lentiviral infection. Cells were either left untreated (UT), treated with TS (apoptosis) or treated with TSI or TSQ (necroptosis) 3 hrs post dox induction. Cell death was measured using PI staining and flow cytometry 24 hours after death stimulation. Cell death data are the mean  $\pm$  SEM of three independent experiments with 2-3 biologically independent replicates per experiment (n=6-9). The dox-induced (white bars), untreated (UT) and TSQ-stimulated data are shown in Figure 2.

**Supplementary figure 3. Western blots showing inducible expression of alanine scanning mutagenesis MLKL constructs with addition of doxycycline.** (a) *Mlkl*<sup>-/-</sup> MDFs reconstituted with wild-type full length mMLKL. (b-e) Wild-type and *Mlkl*<sup>-/-</sup> MDFs expressing full length MLKL with indicated alanine mutations. Each line has either two or three biological replicates (indicated by #1 and #2 for wild-type; #391, #392 and #393 for *Mlkl*<sup>-/-</sup>). Actin was used as a loading control.

**Supplementary figure 4. Chimeric mouse:human MLKL constructs reveal key determinants for constitutive killing of human and mouse cell lines.** Complete dataset, including uninduced vs dox-induced and TS only controls, for experiments presented in Figure 4. (a, c, e, g, i) Wild-type, *Mlkl*<sup>-/-</sup> and *Mlkl*<sup>-/-</sup> *Ripk3*<sup>-/-</sup> MDFs were stably transfected with doxycycline inducible chimeric constructs. In the presence or absence of dox, cells were either left untreated (UT) or stimulated with TS, or TSQ/TSI. Cell death was analysed by PI uptake 24 hours after death stimuli. Data are the mean  $\pm$  SEM of three independent experiments with 2-3 biologically independent replicates per experiment (n=6-9).

(b, d, f, h) Human cell lines, HT29 and U937, stably transduced with doxycycline inducible chimeric constructs. Cell death assays were performed similarly to MDFs, except that cell death was measured 48 hrs after death stimulus for human cell lines. Data are the mean  $\pm$  SEM of 3 independent experiments (n=3), except for (h) where n=2 for U937 cells. The dox-induced (white bars), untreated (UT) and TSQ-stimulated data are shown in Figure 4.

**Supplementary figure 5. Western blots showing inducible expression of chimeric MLKL constructs with addition of doxycycline in MDFs, U937 and HT29 cells.** (a) HT29 cells reconstituted chimeric MLKL (one biological replicate per chimera). (b) U937 cells reconstituted chimeric MLKL (one biological replicate per chimera). (c-g) Either wild-type and *Mlkl*<sup>-/-</sup> MDFs, or wild-type and *Mlkl*<sup>-/-</sup> and *Mlkl*<sup>-/-</sup> *Ripk3*<sup>-/-</sup> MDFs expressing chimeric

constructs. Each MDF line has either two or three biological replicates (indicated by #1, #2 and #3 for wild-type; #391, #392 and #393 for *Mlkl*<sup>-/-</sup>; #24, #25 and #26 for *Mlkl*<sup>-/-</sup> *Ripk3*<sup>-/-</sup>). Actin was used as a loading control.

**Supplementary figure 6. Residue swaps between human and mouse brace region suggest distinct roles for the first and second brace helix.** Complete dataset, including uninduced vs dox-induced and TS only controls, for experiments presented in Figure 5.

(a-f) Wild-type and *Mlkl*<sup>-/-</sup> MDFs were stably transfected with doxycycline inducible chimeric constructs harbouring the indicated mutations. Cells were stimulated with TS, TSQ or TSI and cell death was measured by PI uptake and flow cytometry. Death assays represent mean ± SEM of three independent experiments with 2-3 biologically independent replicates per experiment (n=6-9). The dox-induced (white bars), untreated (UT) and TSQ/TSI-stimulated data are shown in Figure 5.

**Supplementary figure 7. Western blots showing inducible expression of residue swapped chimeric MLKL constructs with addition of doxycycline in MDFs.** (a-f) Wild-type and *Mlkl*<sup>-/-</sup> MDFs expressing residue swapped chimeric constructs. Each MDF line has either two or three biological replicates (indicated by #1, #2 and #3 for wild-type; #391, #392 and #393 for *Mlkl*<sup>-/-</sup>). Actin was used as a loading control.

## SUPPLEMENTARY METHODS

### Circular dichroism spectroscopy

Circular dichroism (CD) spectroscopy experiments were performed on the Aviv Circular Dichroism Spectrometer, Model 430. Proteins between 0.15-0.5 mg/ml were analysed in TBS (150 mM NaCl, 20 mM Tris-HCl, pH 7.5) with 1mM TCEP. Sample was placed in a 0.1 cm pathlength quartz cuvette, and readings taken between 190–260 nm, with 4 second averaging at each wavelength. Buffer blank adjusted readings were converted from machine units (millidegrees) to mean residue ellipticity (MRE) by the following formulae:

$$MRE = \frac{Machine\ units \times MRW}{10 \times pathlength \times protein\ concentration} \quad (deg.cm^2.dmol^{-1}.residue^{-1})$$

Where MRW = Mean residue weight, pathlength is cm, concentration is mg/ml.

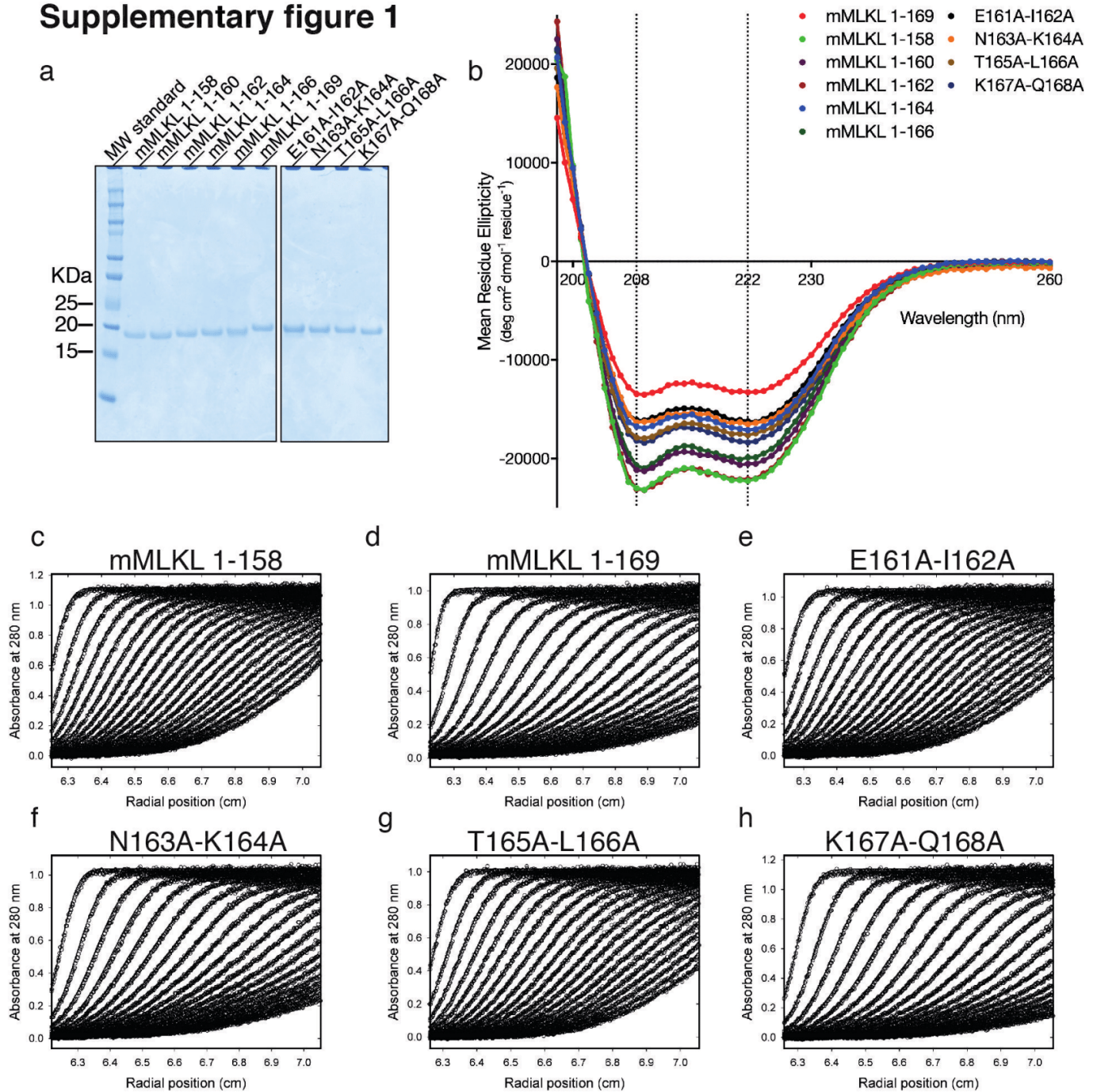
$$MRW = \frac{Molecular\ weight\ (Da)}{Number\ of\ residues} \quad (Da.residue^{-1})$$

(<http://dichroweb.cryst.bbk.ac.uk/html/home.shtml><sup>1</sup>)

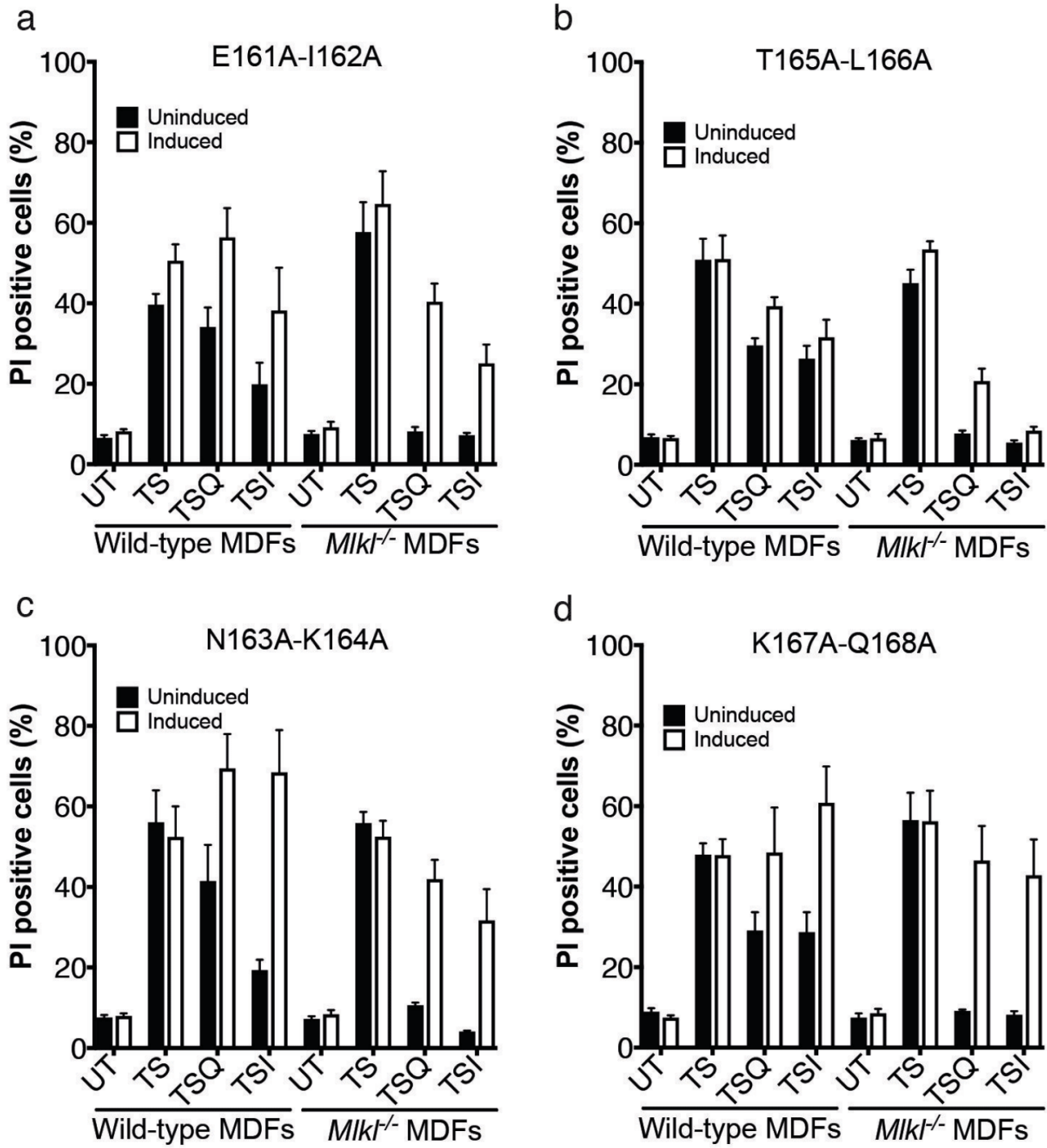
## SUPPLEMENTARY REFERENCE

1. Lobley A, Whitmore L, Wallace BA. DICHROWEB: an interactive website for the analysis of protein secondary structure from circular dichroism spectra. *Bioinformatics* 2002, **18**(1): 211-212.

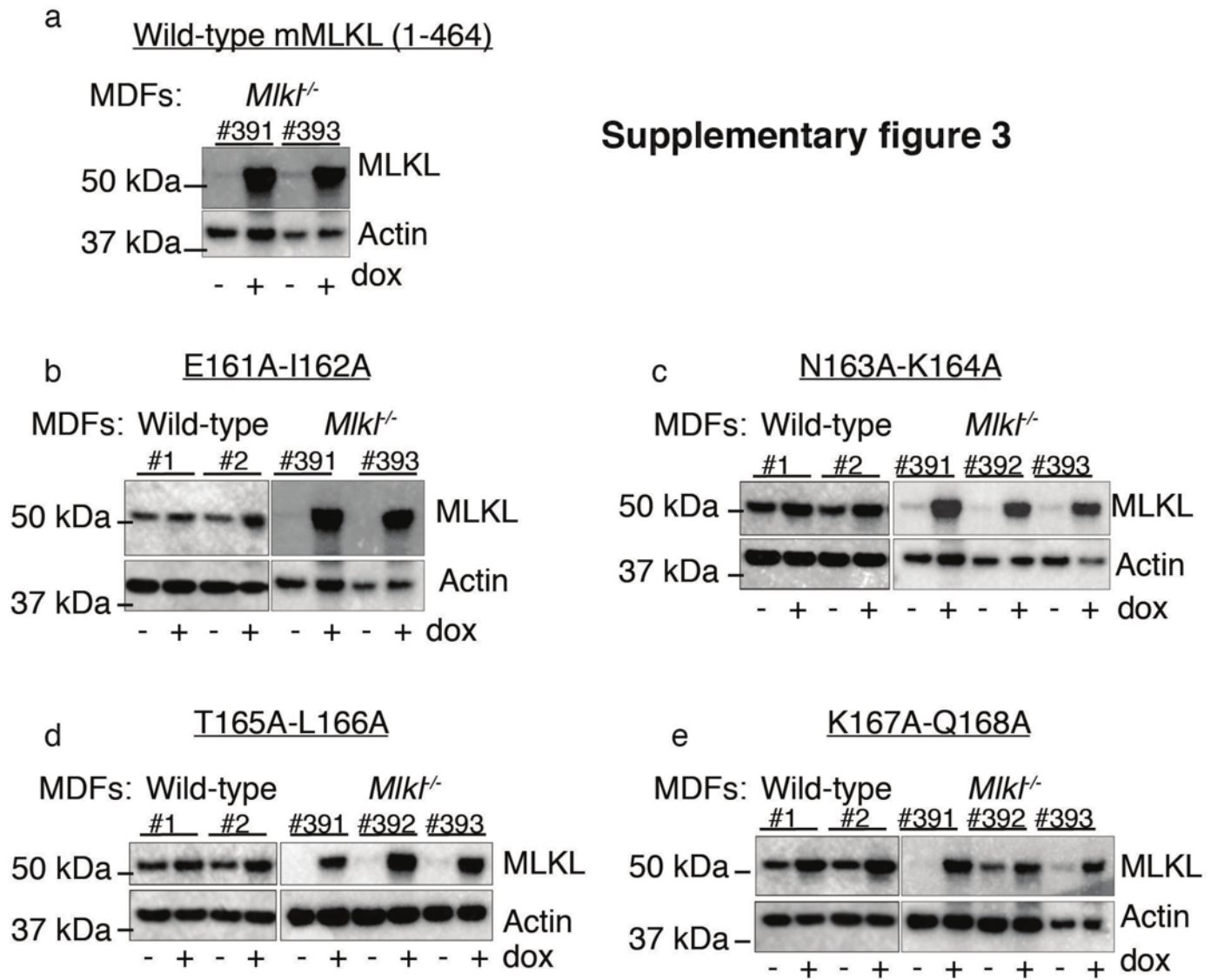
# Supplementary figure 1



Supplementary figure 2

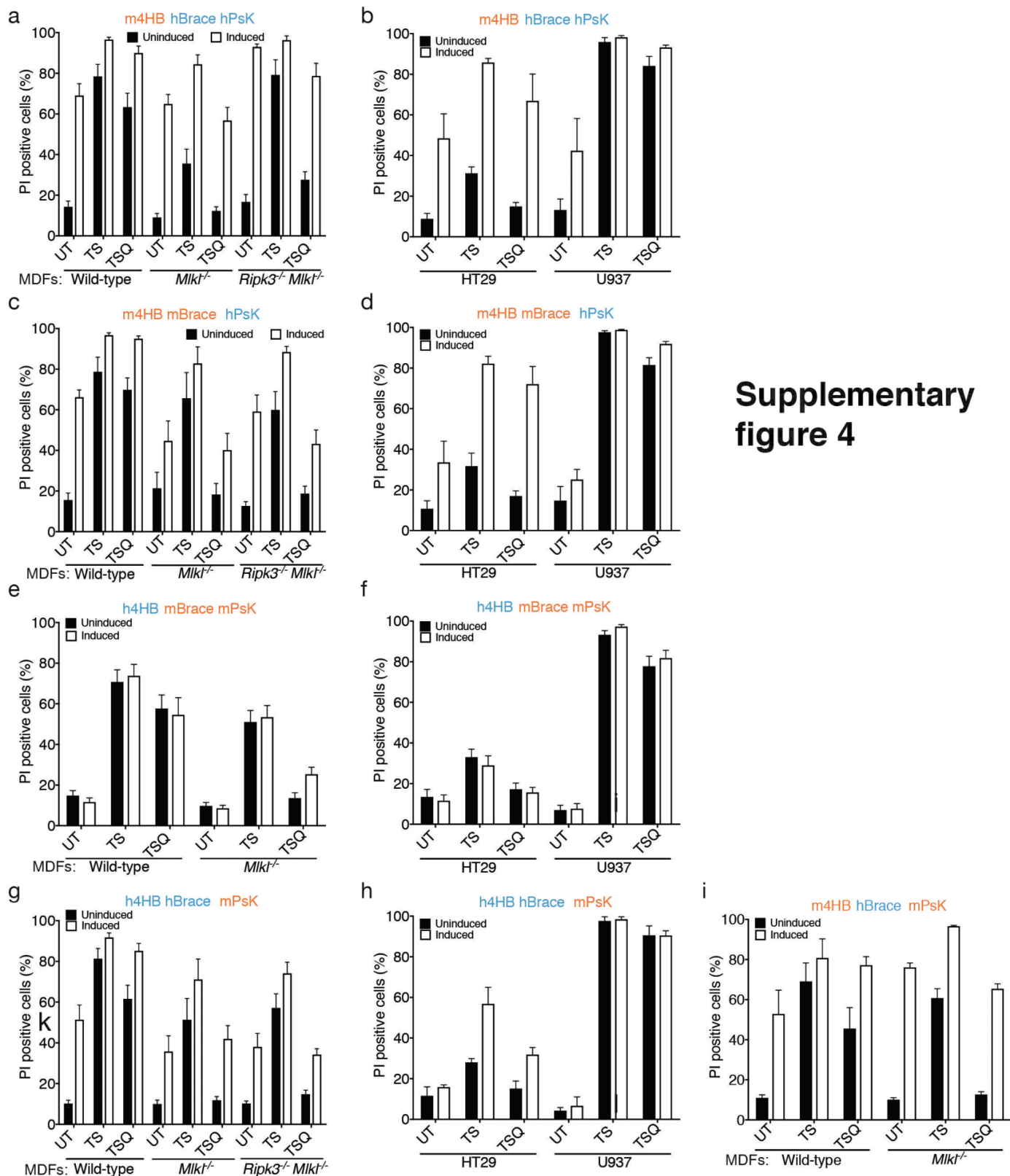


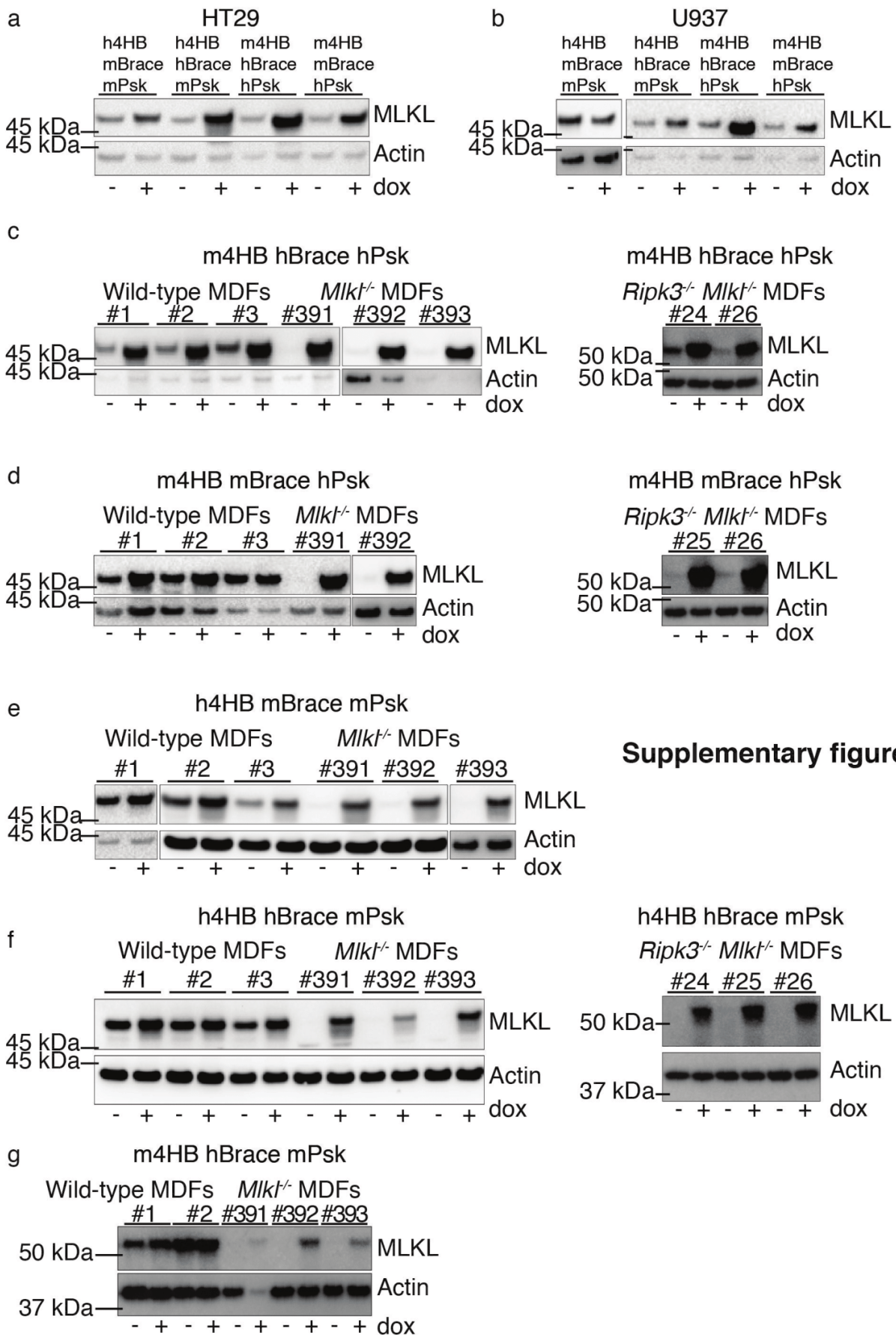
## Supplementary figure 3





# Supplementary figure 4

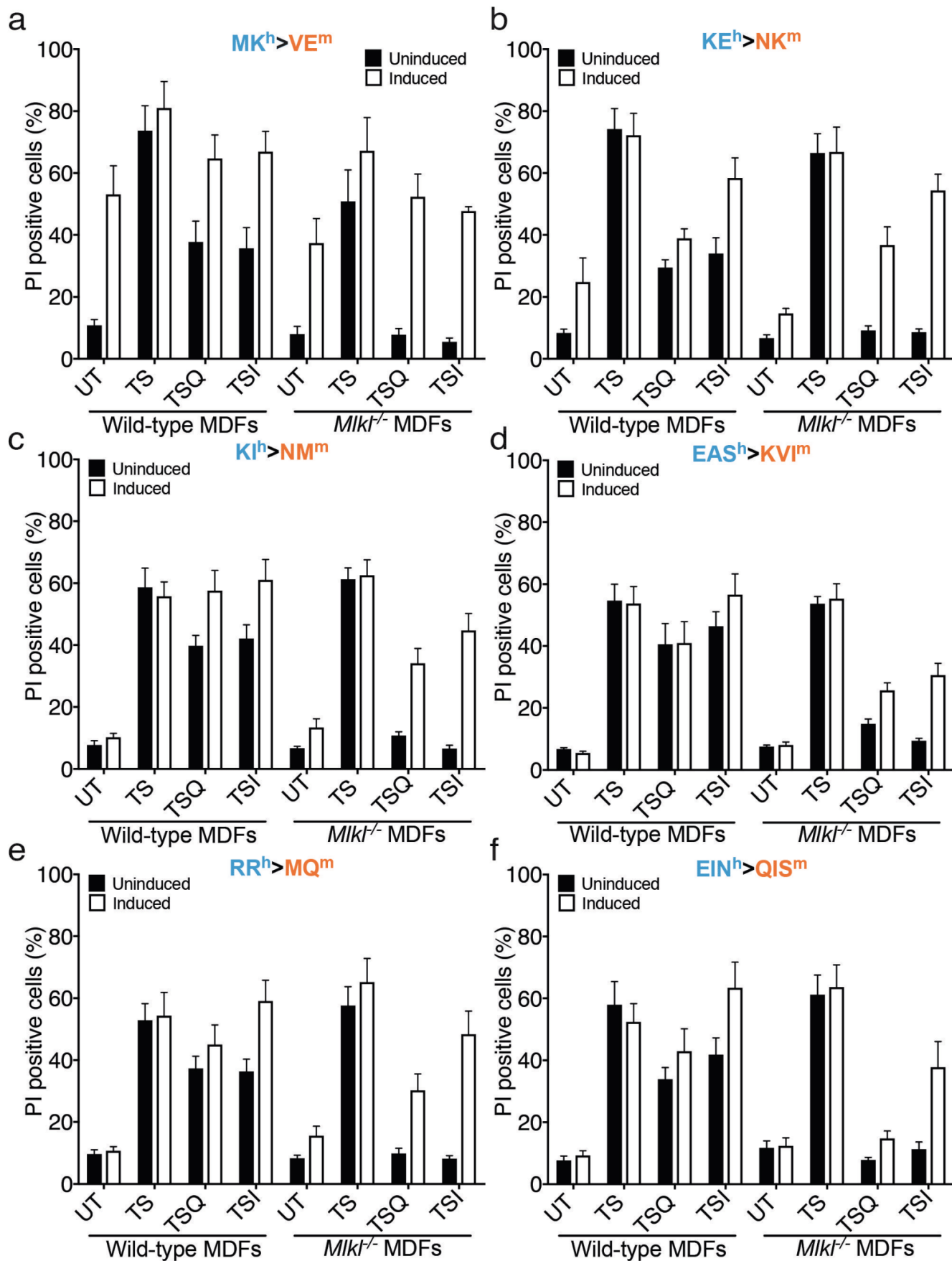




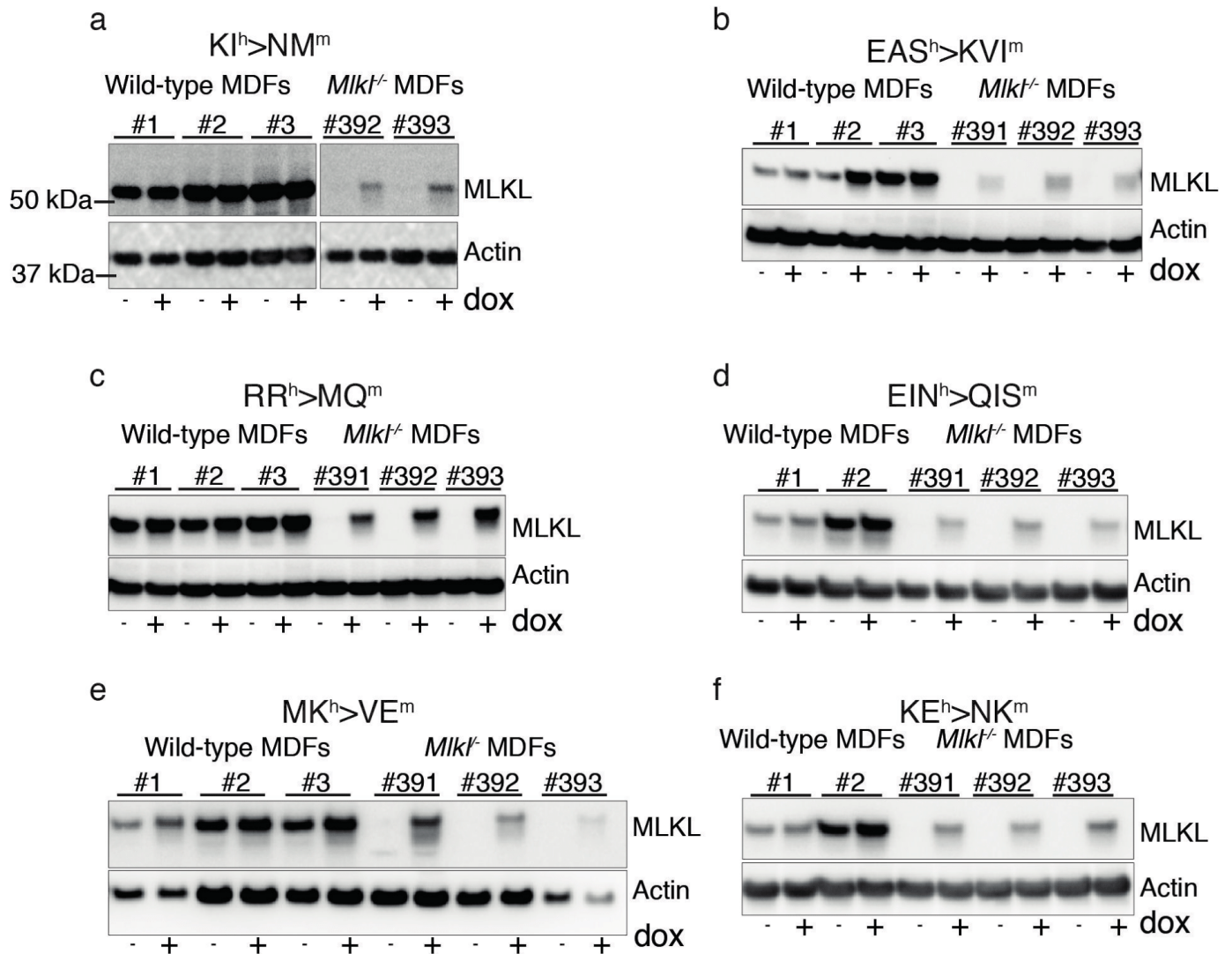
**Supplementary figure 5**



## Supplementary figure 6



## Supplementary figure 7



## Appendix III

Macros were written in ImageJ to manage image processing for GUV experiments. Two example macros are included below.

### *Macro for making montages*

This macro works on stack image files with the naming convention c:x/y - X, where c is channel, 'x' is current the current channel of 'y' numbers of channels, and X is extra information. The macro assigns the desired look-up-table (LUT) to the correct channel, makes an overlay between two specified channels, arranges the channels into a montage with a scale bar, then saves the montage to a specified location, increasing efficiency of image processing.

Annotation describing the purpose of different parts of the macro is in green text.

```
1 myImageID = getTitle;
2 run("Duplicate...", "duplicate channels=1-3");
3 makeRectangle(140, 140, 206, 206);
4 waitForUser("move ROI")
5 run("Crop");
6 //above steps make region of interest (ROI) around GUV, and crop to correct size
7 label=getInfo("slice.label");
8 variable=split(label,"/");
9 run("Stack to Images");
10 selectWindow("c:1/"+variable[1]);
11 rename("1");
12 run("Green");
13 //set the look-up-table (LUT) for GFP channel to green
14 selectWindow("c:2/"+variable[1]);
15 rename("2");
16 run("Red");
17 //sets the LUT for membrane dye to red
18 run("Merge Channels...", "c1=[2] c2=[1] create keep");
19 selectWindow("Composite");
20 run("Stack to RGB");
21 rename("3");
22 //makes composite of red and green
23 selectWindow("c:3/"+variable[1]);
24 rename("4");
25 run("Grays");
26 //sets phase contrast image to grey
27 run("Images to Stack", "name=Stack title=[] use");
28 setSlice(3);
29 run("Copy");
30 run("Delete Slice");
31 setSlice(3);
32 run("Add Slice");
33 run("Paste");
34 run("Scale Bar...", "width=5 height=4 font=14 color=White background=None location=[Lower Right] bold");
35 run("Make Montage...", "columns=4 rows=1 scale=1");
36 //makes montage with 5 um scale bar
37 name=myImageID+"Montage"
38 waitForUser("Check");
39 //lets user check montage is correct before saving to a specified location
40 rename(label+"Montage");
41 saveAs("PNG", "/Users/davies.k/Desktop/GUV/MLKL-7-9-8-8/hMLKL 500 nM Montage/"+name+".png");
42 close():close("Stack"):close("Composite"):close(myImageID):
```

### *Macro for measuring dye ingress*

This macro generates two equal sized regions of interest (ROI), and places one inside and one outside the GUV. Then it records the mean fluorescence intensity within both ROIs for each fluorescent channel of interest, and stores the data in a table. The table can then be exported to excel and used to calculate the ratio of fluorescence intensity inside versus outside the GUV.

Annotation describing the purpose of different parts of the macro is in green text.

```
1 run("Set Measurements...", "mean display redirect=None decimal=3");
2 //instructs fiji to measure mean fluorescence intensity to 3 decimal places
3 myImageID = getTitle;
4 run("Duplicate...", "duplicate channels=1-4");
5 setSlice(4);
6 //sets slice on phase contrast image so that regions of interest (ROI) can be positioned inside and outside GUV
7 makeOval(55, 72, 94, 94);
8 //generates region of interest of defined size (94 pixels by 94 pixels)
9 waitForUser("move ROI");
10 roiManager("Add");
11 makeOval(218, 243, 94, 94);
12 waitForUser("move ROI");
13 roiManager("Add");
14 roiManager("Show All");
15 //following steps measure fluorescence intensity inside and outside the GUV (using ROIs) for each fluorescent channel
16 setSlice(1);
17 run("Measure");
18 setResult("Label", nResults-2, myImageID+"dex10");
19 setSlice(3);
20 run("Measure");
21 setResult("Label", nResults-2, "dex70");
22 roiManager("Select", 0);
23 roiManager("Delete");
24 roiManager("Select", 0);
25 roiManager("Delete");
26 close();
27 close(myImageID);
28 //measurements are stored in a table, labelled with image number and dye identity to be processed in excel
```

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