

The role of Receptor Interacting Protein Kinase 3 in inflammatory signalling

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St Cross College
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*A thesis submitted for the degree of
Doctor of Philosophy*

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Abstract

Inflammatory signalling and programmed cell death are fundamental processes employed by higher order organisms as defence mechanisms against foreign substances. Necroptosis, a proinflammatory type of programmed cell death mediated by the kinase RIPK3, has emerged as an important innate immune defence mechanism against viruses. The necroptosis process is accompanied by *de novo* synthesis of cytokines and chemokines within the dying cells. However, how inflammatory signalling is induced in the necroptotic cell remains poorly understood.

In this study, I describe that RIPK3, independently of its cell death-inducing activity, stimulates inflammatory signalling pathways mediated by the kinases TAK1 and IKKs. I provide evidence that RIPK3 can stimulate inflammatory signalling through two distinct mechanisms, the scaffolding-dependent pathway and the RIPK3 kinase activity-dependent pathway. In the scaffolding-dependent pathway, RIPK1 connects RIPK3 to the ubiquitin ligases, cIAPs and LUBAC, for the activation of TAK1 and IKKs through K63- and M1-linked polyubiquitin, independent of RIPK3's kinase activity. In contrast, the RIPK3 kinase activity-dependent pathway does not require cIAPs or LUBAC, but is mediated by RIPK3's kinase activity and its substrate, MLKL, and is suppressed by the RIPK1-recruited protease caspase-8.

Taken together, this study indicates that RIPK3, like its family members RIPK1 and RIPK2, is a scaffolding kinase able to promote inflammatory signalling. RIPK3 promotes inflammatory signalling through kinase-dependent and -independent mechanisms. Given the role of necroptosis in the host defence against viruses that often encode caspase inhibitors, RIPK3 kinase activity-dependent signalling may serve not only as an additional mechanism to ensure the activation of inflammatory signalling, but also as a regulatory checkpoint between RIPK3-mediated inflammatory signalling and cell death.

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Abstract

Inflammatory signalling and programmed cell death are fundamental processes employed by higher order organisms as defence mechanisms against foreign substances. Necroptosis, a proinflammatory type of programmed cell death mediated by the kinase RIPK3, has emerged as an important innate immune defence mechanism against viruses. The necroptosis process is accompanied by *de novo* synthesis of cytokines and chemokines within the dying cells. However, how inflammatory signalling is induced in the necroptotic cell remains poorly understood.

In this study, I describe that RIPK3, independently of its cell death-inducing activity, stimulates inflammatory signalling pathways mediated by the kinases TAK1 and IKKs. I provide evidence that RIPK3 can stimulate inflammatory signalling through two distinct mechanisms, the scaffolding-dependent pathway and the RIPK3 kinase activity-dependent pathway. In the scaffolding-dependent pathway, RIPK1 connects RIPK3 to the ubiquitin ligases, cIAPs and LUBAC, for the activation of TAK1 and IKKs through K63- and M1-linked polyubiquitin, independent of RIPK3's kinase activity. In contrast, the RIPK3 kinase activity-dependent pathway does not require cIAPs or LUBAC, but is mediated by RIPK3's kinase activity and its substrate, MLKL, and is suppressed by the RIPK1-recruited protease caspase-8.

Taken together, this study indicates that RIPK3, like its family members RIPK1 and RIPK2, is a scaffolding kinase able to promote inflammatory signalling. RIPK3 promotes inflammatory signalling through kinase-dependent and -independent mechanisms. Given the role of necroptosis in the host defence against viruses that often encode caspase inhibitors, RIPK3 kinase activity-dependent signalling may serve not only as an additional mechanism to ensure the activation of inflammatory signalling, but also as a regulatory checkpoint between RIPK3-mediated inflammatory signalling and cell death.

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List of Abbreviations

4HB	Four-helix bundle, the oligomerisation domain in MLKL
aa	Amino acid residue
ATP	Adenosine triphosphate
AIM2	Absent in melanoma 2, a cytosolic DNA receptor
AP-1	Activator protein-1, an inflammatory transcription factor
ATF	Activating transcription factor, a family of inflammatory transcription factors
BMDM	Bone-marrow-derived macrophage
BSA	Bovine serum albumin
CARD	Caspase recruitment domain
c/EBP	CCAAT-enhancer-binding protein
cIAP	Cellular inhibitor of apoptosis protein
CpA	Compound A, a Smac mimetic
CREB	cAMP response element-binding protein
CrmA	Cytokine response modifier A, a virus-encoded caspase inhibitor
CXCL	C-X-C motif ligand
CXCR	C-X-C chemokine receptor
CYLD	Cylindromatosis, a deubiquitinase
DAMP	Danger-associated molecular pattern
DD	Death domain
DED	Death effector domain
Dox	Doxycycline
DR	Death receptor
dsRNA	Double-strand RNA
DTT	Dithiothreitol

DUB	Deubiquitinase
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinases, a type of MAPK
EPEC	Enteropathogenic <i>Escherichia coli</i>
FADD	Fas associated via death domain, an adaptor protein
FBS	Foetal bovine serum
FLIP	FLICE (Caspase-8)-like inhibitory protein
FKBP12	12kDa FK506-binding protein
FV	FKBP12-F36V, dimerisation domain
GSDMD	Gasdermin D
HCMV	Human cytomegalovirus
HMGB1	High-mobility group box 1, a DAMP protein
HOIL-1	Haem-oxidised iron-responsive element-binding protein 2 ubiquitin ligase 1, a subunit of LUBAC
HOIP	HOIL-1-interacting protein, the catalytic subunit of LUBAC
HSV-1	Herpes simplex virus type 1
IAV	Influenza A virus
IE1	Immediate early gene 1, a protein encoded by HCMV
IFI16	IFN γ inducible protein 16, a cytosolic DNA sensor
IFN	Interferon
IκB	Inhibitor of NF- κ B
IKK	I κ B kinase
IL	Interleukin
IL-1R	IL-1 receptor
IRAK	IL-1R-associated kinase
IRF	Interferon regulatory factor
ISG	Interferon-stimulated gene
JNK	c-Jun NH ₂ -terminal kinase, a type of MAPK
LPS	Lipopolysaccharide
LSB	Laemmli sample buffer

LUBAC	Linear ubiquitin chain assembly complex
KO	Knockout
MAL	Myelin and lymphocyte protein, an adaptor protein for TLR4 located on cell surface
MAPK	Mitogen-activated protein kinase
MAP2K	MAPK kinases
MAP3K	MAP2K kinase
MCMV	Murine cytomegalovirus
MDP	Muramyl dipeptide
MEK	= MAP2K
MLK	Mixed lineage kinase
MLKL	Mixed lineage kinase domain-like
MK2	MAPK-activated protein kinase 2
MKK	= MAP2K
MyD88	Myeloid differentiation primary response 88, an adaptor protein for TLR signalling pathways
Nec1(s)	Necrostatin-1(s), RIPK1 kinase activity inhibitors
NEM	N-Ethylmaleimide, a DUB inhibitor
NEMO (IKKγ)	NF- κ B essential modulator
NES	Nuclear export signal
NF-κB	Nuclear factor κ -light-chain-enhancer of activated B cells, an inflammatory transcription factor
NIK	NF- κ B-inducing kinase, a component of the noncanonical NF- κ B pathway
NLR	NOD-like receptor
NLRC	NLR family CARD-containing
NLRP	NLR family PYD-containing protein
NOD	Nucleotide oligomerisation domain
NZF	Nuclear protein localization 4 zinc finger domain, a K63-linked polyubiquitin binding domain
OTULIN	Ovarian tumor domain deubiquitinase with linear linkage specificity
PAMP	Pathogen-associated molecular pattern

PBS	Phosphate buffered saline
PBST	PBS-Tween
PCR	Polymerase chain reaction
PDG	Peptidoglycan
PIP	Phosphatidylinositol phosphate
PRR	Pattern recognition receptor
PtdSer	Phosphatidylserine
PYD	Pyrin domain
QVD	Quinolyl-valyl-O-methylaspartyl-[-2, 6-difluorophenoxy]-methyl ketone (Q-VD-Oph), a pan-caspase inhibitor
RHIM	RIP homotypic interaction motif
RIPK (RIP) . . .	Receptor interacting protein kinase
SDS	Sodium dodecyl sulphate
SHARPIN	Src homology domain 3 and multiple ankyrin repeat domains protein-associated regulator of G-protein signaling homology domain interactor, a subunit of LUBAC
SM	SMAC mimetic
SMAC	Second mitochondria-derived activator of caspases
SUB	Linkage specific ubiquitin binder
TAB	TAK1-binding protein
TAK1	TGF β -activated kinase 1, a MAP3K
TANK	TRAF family member-associated NF- κ B activator, a protein in the TBK1-IRF pathway
TBK1	TANK-binding protein 1
TBS	Tris-buffered saline
TEMED	Tetramethylethylenediamine
TGF-β	Transforming growth factor beta
TIR	Toll/IL-1 receptor signalling domain
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFSF	TNF superfamily

TNFR		Tumour necrosis factor receptor
TPL2		Tumor progression locus 2, a MAP3K
TRADD		TNFR1-associated death domain protein
TRAF		TNFR-associated factor, a group of proteins in multiple inflammatory signalling pathways, some of which possess ubiquitin ligase activity
TRAIL		TNF-related apoptosis-inducing ligand
TRAIL-R		TRAIL receptor
TRAM		Translocating chain-associated membrane protein, an adaptor protein for endosome-located TLR4
TRIF		TIR-containing adapter-inducing IFN- β , an adaptor protein in TLR3 and TLR4 pathways
UBA		Ubiquitin-associated domain
UBAN		Ubiquitin-binding domain in A20 binding and inhibitor of NF- κ B (ABIN) proteins and NEMO
UBD		Ubiquitin-binding domain
UIM		Ubiquitin-interacting motif
vFLIP		Viral FLICE (caspase-8) inhibitory protein
vIAP		Viral inhibitor of apoptosis protein
vICA		Viral inhibitor of caspase activation
vIRA		Viral inhibitor of RIP activation (vIRA)
WNV		West Nile Virus
WT		Wild-type
XIAP		X-linked inhibitor of apoptosis protein
ZBD		Z-nucleic acid binding domain
ZBP1		Z-nucleic acid binding protein 1
ZIKV		Zika virus
zVAD		Carbobenzoxy-valyl-alanyl-aspartyl-fluoromethylketone (z-VAD-fmk), a pan-caspase inhibitor

1

Introduction

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1.1 Inflammatory signalling

Inflammation is widely used among simple and complex organisms to respond to undesired substances (Hotamisligil 2017). In this section, I give an overview of the inflammatory process and ways to activate it.

1.1.1 An overview of inflammatory signalling

The inflammatory process

"To set on fire" - as the Latin word it derives from means, inflammation features symptoms that have been described in 4 words since 2000 years ago: redness and swelling with heat and pain (Majno 1991). Physiologically, the described symptoms arise from the dilation of vascular walls, plasma leakage and leukocyte migration from the circulatory system into sites of inflammation (Medzhitov 2010). The ultimate goal of an inflammatory response is the clearance of undesired substances. This includes expelling non-self from self, for example during pathogen infection, and repairing damaged tissues, as in the case of injury (Hotamisligil 2017).

Inflammatory signalling is the underlying molecular mechanisms in the inflammatory process. During an inflammatory response, signalling takes place in 3 steps. At the initiation stage, an inflammatory stimulus is sensed by cells, which results in outputs

of proinflammatory molecules like cytokines and chemokines. Next, surrounding cells and leukocytes detect the proinflammatory molecules and amplify the inflammatory signal by producing more inflammatory molecules. Third, inflammatory molecules are sensed by functional cells, which are activated to eliminate the inflammatory inducer (Medzhitov 2010).

Consistent with their role in combating pathogen and injury, inflammatory inducers mainly fall into 2 categories, pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). PAMPs are pathogen-specific molecules, for example, bacteria outer membrane components lipopolysaccharide (LPS) and peptidoglycan (PDG), and nucleic acids with bacteria and virus features like double strand RNA (dsRNA) and unmethylated CpG. DAMPs are host-derived intracellular molecules that are not released to the environment except in times of tissue injury or cell death, for example, the adenosine triphosphate (ATP) molecule and high-mobility group box 1 (HMGB1) protein. Both PAMPs and DAMPs are recognised by a group of structurally and functionally diverse receptors, collectively referred to as pattern recognition receptors (PRRs). PRRs can localise on the cell surface or in the cytosol. Their activation marks the initiation of an inflammatory response (Newton and Dixit 2012).

Activation of PRRs results in the production of early-response cytokines like tumour necrosis factor (TNF), interleukin (IL)-6 and IL-1 β . They are the key molecules for the amplification of the inflammatory signal in the second step. Cytokines are recognised by their corresponding receptors that are widely expressed by tissues and tissue-resident immune cells. The activation of receptors for TNF, IL-6 and IL-1 β results in amplified production of the same cytokine as well as the production of other cytokines, chemokines and proinflammatory molecules (Turner et al. 2014).

Chemokines attract leukocytes to sites of inflammation (Turner et al. 2014). For example, the chemokine C-X-C motif ligand (CXCL) 8 (also called IL-8) is expressed by a wide variety of cells in response to inflammatory stimuli. Its receptor C-X-C chemokine receptor (CXCR) 1 and CXCR2 (also known as IL-8RA and IL-8RB) are widely expressed on leukocytes like neutrophils and monocytes (Russo et al. 2014). During the inflammatory process, CXCL8 activation of CXCR1 on neutrophils and

monocytes induces their chemotaxis towards sites of inflammation (Turner et al. 2014). At the site, TNF, IL-1 β and CXCL8 promote the extravasation of the immune cells by stimulating endothelial cells to enhance the levels of leukocyte adhesion molecules on their surface (Newton and Dixit 2012). At sites of inflammation, immune cells like neutrophils and monocyte-derived macrophages resolve inflammation by attacking pathogen or engulfing tissue debris. They can also initiate adaptive immune responses or support tissue repair when necessary (Sugimoto et al. 2016).

In summary, by distinguishing self from non-self and injured from healthy, inflammatory signalling plays an important role in maintaining tissue homeostasis.

Pathological inflammation

The self-amplifying feature of inflammatory signalling means that unresolved inflammation could develop into a chronic pathological condition. While acute inflammatory signalling can be resolved within hours or days, chronic inflammation can last weeks, months or even years. Chronic inflammation could result from persistent pathogen infection, recurring tissue damage, or dysregulated inflammatory response (Nathan and Ding 2010; Nasef et al. 2017).

Local chronic inflammation could result in severe disturbance of tissue function. Unresolved inflammation leads to excessive plasma leakage and constitutive infiltration of tissues by short-lived immune cells, as is the case in chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease. Further, unresolved tissue damage from chronic inflammation could induce active fibroblasts in the plasma to deposit collagen on the damaged site, and the expansion of collagen could lead to fibrosis that further weakens the physiological function of the tissue. For example, fibrosis as has been seen in arteries, the liver, the lung or the gut undergoing chronic inflammation (Nathan and Ding 2010).

Aside from detectable chronic inflammation, low-level chronic inflammatory signalling underlies many chronic diseases (Grivennikov et al. 2010). For example, chronic inflammatory signals from adipose tissue macrophages contribute to insulin resistance in

obesity (Reilly and Saltiel 2017). The chronic inflammatory condition is also associated with generally increased risk of cancer (Grivennikov et al. 2010).

In conclusion, inflammatory signalling is a double-edged sword, being able to both safeguard and disrupt tissue homeostasis. Therefore, targeting inflammatory signalling has clinical values in both directions.

Therapeutic targeting of inflammatory signalling

Therapeutic lowering of inflammation levels dates back centuries ago with records of treatment with salicylate-containing plant extracts (Dinarello 2010). Today, aspirin, the acetylated form of salicylic acid, is still the most commonly used drug to prevent cardiovascular disease. It works by blocking the synthesis of the inflammatory mediators prostaglandins and thromboxanes (Dinarello 2010; Patrignani and Patrono 2016). Today, many more small molecule inhibitors are being developed to block inflammatory signalling at different stages. Thus, much effort has been put into characterising the molecular mechanisms of inflammatory signalling pathways to develop novel ways to target them.

While treating chronic inflammatory conditions require inhibitory drugs, fighting cancer may require the opposite. In a mature tumour microenvironment, inflammatory signals from tumour cells shape the recruited immune cells to establish an immune-suppressive environment (Nakamura and Smyth 2017). Efforts to block immunosuppressive signals from tumour cells have resulted in the anti-programmed death-1 (PD1) therapy, which blocks the effect of the programmed death ligand-1 (PD-L1) molecule abnormally expressed on tumour cells to suppress T cell activation (Chen and Han 2015). Another way to revoke antitumour immune responses is to awake the immune cells' potential to kill tumour cells. This can be achieved by inducing immunogenic cell death of tumour cells or priming the immune cells with type I interferons (IFNs). Both have been shown to improve the effect of the anti-PD1 therapy (Nakamura and Smyth 2017).

To conclude, targeting the inflammatory signalling pathway has therapeutic value in many disease scenarios. The discovery of improved ways to target the inflammatory signalling pathway relies on better understanding of the underlying molecular mechanisms.

1.1.2 The inflammatory signalling pathways

The production of cytokines in response to PAMPs and DAMPs is a crucial event in the inflammatory process. In humans, around 100 PRRs sense various PAMPs and DAMPs, to trigger the expression of cytokines through a limited number of transcription factors, mainly nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B), activator protein-1 (AP-1), interferon regulatory factor (IRF), cAMP response element-binding protein (CREB) and CCAAT-enhancer-binding protein (c/EBP) (Newton and Dixit 2012). In this section, I will introduce the NF- κ B, AP-1 and IRF transcription factors and ways to activate them.

The NF- κ B pathway

The NF- κ B family of transcription factors consists of 5 members: NF- κ B1 (also named p105, precursor of the p50 subunit), NF- κ B2 (also named p100, precursor of the p52 subunit), RelA (also named p65), RelB and c-Rel (Liu et al. 2017b). After activation, they form homo- or heterodimers and translocate from the cytoplasm into the nucleus. In the nucleus, the NF- κ B transcription factors bind 9-10 base-pair (bp) DNA sequence called κ B sites to promote the transcription of cytokines, chemokines, growth factors, cell adhesion molecules and proinflammatory enzymes (Gilmore 2006; Taniguchi and Karin 2018).

Depending on the involved subunits, the NF- κ B pathway takes place in 2 forms, the canonical pathway and the noncanonical (or alternative) pathway (Figure 1.1). The canonical NF- κ B pathway predominantly relies on p50-p65 and p50-c-Rel heterodimers, and is activated downstream of PRRs and the vast majority of cytokine receptors (Liu et al. 2017b). In contrast, the noncanonical NF- κ B pathway is mediated by the RelB-p52 heterodimer, and is activated by a limited number of TNF superfamily (TNFSF) ligands (Lawrence 2009).

The activation of the canonical NF- κ B pathway relies on the function of the ubiquitin system. Ubiquitin is a 76-amino acid (aa) post-translational modification moiety. It is typically attached to the ϵ -amino group of a lysine residue on the substrate via its C-terminal carboxyl group. This process is referred to as ubiquitylation (or ubiquitination). After being conjugated onto the substrate, the ubiquitin moiety can be further ubiquitylated

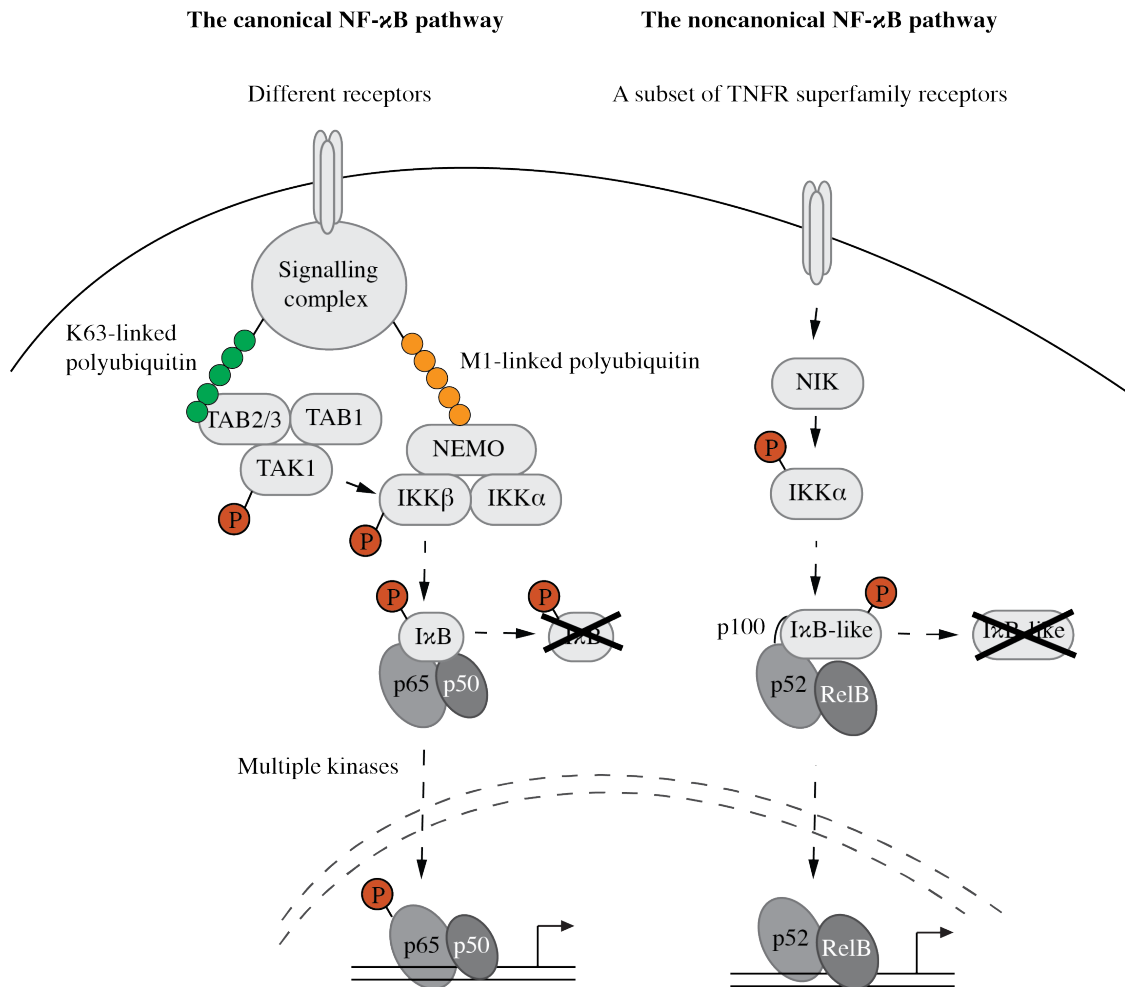


Figure 1.1: The NF-κB pathways. In the canonical NF-κB pathway, TAK1 is recruited through the ubiquitin-binding activity of its interaction partner, TAB2 or TAB3, and the IKK complex is recruited via the ubiquitin-binding activity of NEMO. TAK1 is activated after its recruitment and further phosphorylates IKKβ to activate it. Activated IKKβ phosphorylates IκB for its proteasomal degradation. IκB constantly holds the transcription factor subunits p65 and p50 in the cytosol. With its degradation, the p65-p50 heterodimer is released for nuclear translocation. During the process, multiple kinases target p65 for phosphorylation to modulate its activity. In the noncanonical NF-κB pathway, NIK is stabilised after receptor activation, which phosphorylates IKKα and subsequently p100. Phosphorylation of p100 promotes its proteasomal processing into the p52 subunit and degradation of its IκB-like subunit. The released p52 dimerises with RelB for nuclear translocation.

on one of its 7 lysine residues (K6, K11, K27, K29, K33, K48 and K63) or the N-terminal amino group of M1, forming a polyubiquitin chain (Kulathu and Komander 2012). Polyubiquitin chains assembled in different linkages have distinct conformation and recruit specific binding proteins for signal transduction (Husnjak and Dikic 2012). The most studied ubiquitin chain type is K48-linked polyubiquitin, which targets the

substrate for proteasomal degradation. Next to it is K63- and M1-linked polyubiquitin, which play scaffolding roles in inflammatory pathways (Kwon and Ciechanover 2017; Zinngrebe et al. 2014). Little is known about the function of other types of linkages (Kwon and Ciechanover 2017).

In the canonical NF- κ B pathway, the integration point of different stimuli is the ubiquitin-dependent recruitment and activation of transforming growth factor beta (TGF β)-activated kinase 1 (TAK1). TAK1 exists in a complex with its regulatory partners TAK1-binding protein (TAB) 1 and TAB2 or TAB3 (Shibuya et al. 1996; Takaesu et al. 2000; Ishitani et al. 2003; Cheung et al. 2004). When K63- and M1-linked polyubiquitin are assembled in the activated inflammatory receptor complex, TAB2 or TAB3 binds K63-linked polyubiquitin assembled in the upstream signalling complex via their ubiquitin-binding domains, leading to the recruitment of TAK1 (Kanayama et al. 2004). After recruitment, the TAK1-TAB complex undergoes phosphorylation, ubiquitylation and other modifications to activate TAK1 (Hirata et al. 2017).

After its activation, TAK1 activates the inhibitor of NF- κ B (I κ B) kinase (IKK) complex. The IKK complex consists of 3 subunits, IKK α , IKK β and NF- κ B essential modulator (NEMO, also called IKK γ). NEMO mediates the recruitment of the IKK complex through binding M1-linked polyubiquitin assembled in the inflammatory complex (Rahighi et al. 2009). In the canonical NF- κ B pathway, p50 and p65 are constantly held in the cytosol by the I κ B protein. Activated TAK1 phosphorylates IKK β , which in turn phosphorylates I κ B, leading to its ubiquitylation and proteasome-mediated degradation. This allows the p50-p65 heterodimer to translocate into the nucleus (Figure 1.1) (Newton and Dixit 2012). During this process, several kinases target the p65 subunit for phosphorylation to modulate the activity and specificity of the heterodimer (Jeong et al. 2005; Sabatel et al. 2012).

The central protein in the non-canonical NF- κ B pathway is NF- κ B-inducing kinase (NIK). With no signal input, NIK is constantly targeted for proteasome-mediated degradation (Liao et al. 2004). With the activation of the non-canonical NF- κ B pathway, NIK is stabilised and activates the IKK α subunit of the IKK complex. NIK and IKK α phosphorylates the p100 protein, which consists of the N-terminal p52 subunit and the

C-terminal I κ B-like inhibitory subunit. Under steady conditions, p100 couples with RelB and stays in the cytosol. When phosphorylated, p100 is processed by the proteasome and converted to p52, which subsequently couples with RelB to be translocated (Figure 1.1) (Sun 2012).

The MAPK pathway

Mitogen-activated protein kinases (MAPKs) are a group of serine-threonine protein kinases (Schaeffer and Weber 1999). The majority of MAPKs mediate responses to stress and inflammatory stimuli. Activated MAPKs phosphorylate a vast range of transcription factors and other kinases, resulting in diverse outcomes, including cell proliferation, differentiation, inflammation, autophagy, etc (Kyriakis and Avruch 2012).

There are 3 types of MAPKs: extracellular signal-regulated kinases (ERKs), p38 MAPKs and c-Jun NH₂-terminal kinases (JNKs). All MAPKs can be activated in response to signals from cytokine receptors and PRRs, and induce proinflammatory transcription by activating the transcription factor AP-1 when activated (Kyriakis and Avruch 2012).

MAPKs are activated in a kinase cascade. Signal inputs first activate MAPK kinase kinases (MAP3Ks), which activate MAPK kinases (MAP2Ks, also abbreviated as MKKs or MEKs), which in turn activate MAPKs (Kyriakis and Avruch 2012).

In inflammatory signalling, the MAP3K TAK1 is activated after recruitment to the assembled K63-linked polyubiquitin (Newton and Dixit 2012). TAK1 promotes MAPK signalling in 2 ways. First, TAK1 phosphorylates MAP2Ks MKK6, MKK3 and MKK4, which in turn phosphorylate-activate MAPKs p38 and JNK (Figure 1.2) (Wang et al. 2001; Moriguchi et al. 1996; Kyriakis and Avruch 2012). Second, TAK1 phosphorylates IKK β , which in turn phosphorylates p105, an inhibitory protein for another MAP3K, tumor progression locus 2 (TPL2) (Beinke et al. 2004). Phosphorylation of p105 leads to the release of TPL2 which then phosphorylate-activates MEK1 and MKK3/6, the MAP2Ks for ERK and p38 (Figure 1.2) (Beinke et al. 2004; Senger et al. 2017).

Activated p38, JNK and ERK phosphorylate and stabilise the subunits of the transcription factor AP-1, including the Jun family transcription factors like c-Jun and JunD, the Fos family transcription factors like c-Fos, and the activating transcription factor (ATF)

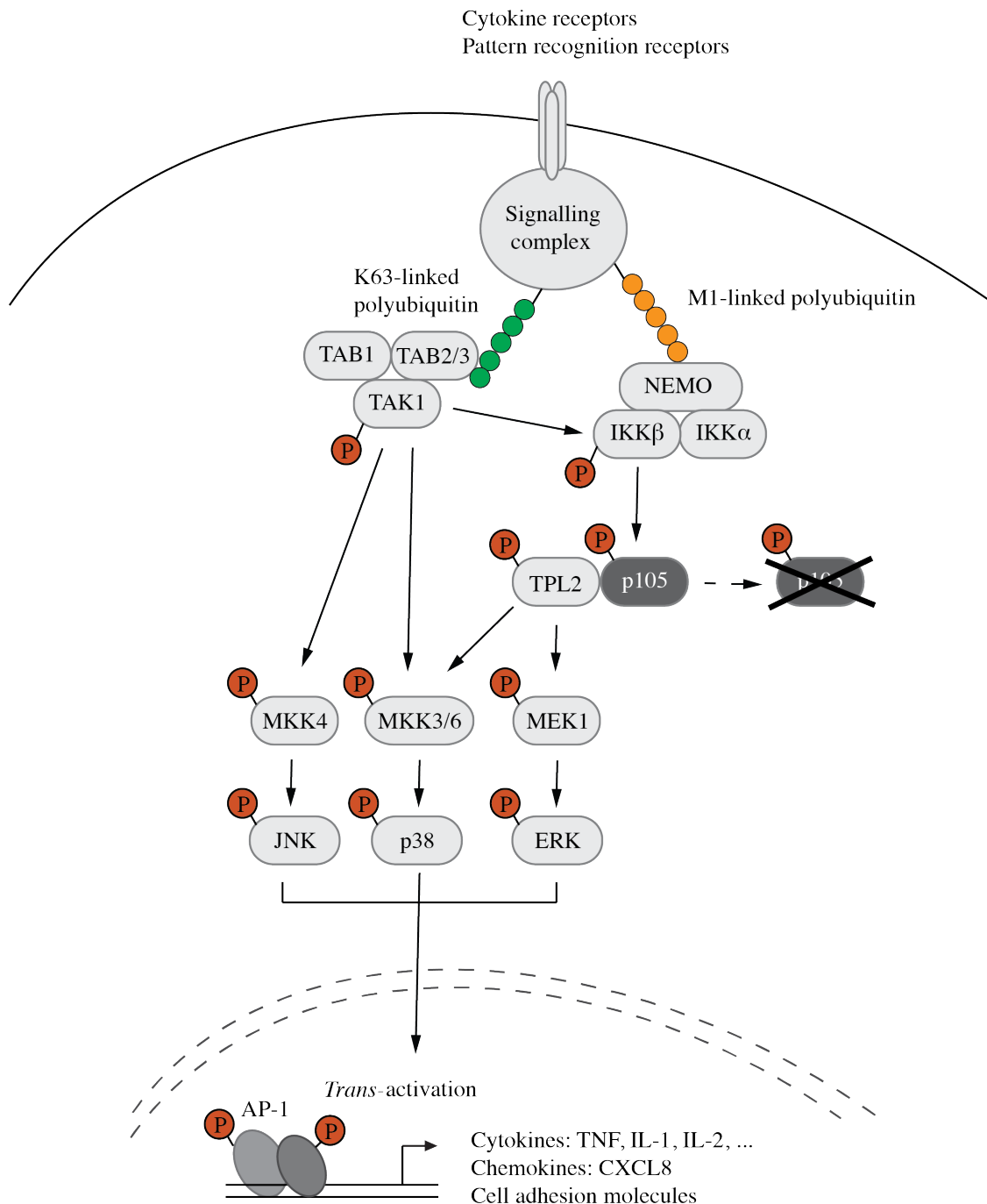


Figure 1.2: The MAPK pathway. In the inflammatory complex, TAK1 is a MAP3K that phosphorylate-activates MAP2Ks MKK4 and MKK3/6, which in turn phosphorylate-activate MAPKs JNK and p38. TAK1 also phosphorylates IKK β , which in turn phosphorylates p105 to release another MAP3K, TPL2. TPL2 activates MAP2Ks MKK3/6 and MEK1, which activate MAPKs p38 and ERK. Activated MAPKs phosphorylate subunits of the AP-1 transcription factor for their activation and stabilisation.

family transcription factor like ATF2 (Shaulian and Karin 2002). Activated AP-1 is a

homo- or heterodimer that contains at least 1 subunit from the Jun family. The AP-1 transcription factor regulates many processes, including cell growth, differentiation and survival. Among its many functions, AP-1 is a *trans*-activator of proinflammatory genes like cytokines, chemokines and cell adhesion molecules (Kyriakis and Avruch 2012).

The IRF pathway

The interferon regulatory factor (IRF) transcription factor family has 9 members, IRF1 to IRF9 (Mamane et al. 1999). All of them are involved in the regulation of inflammatory signalling pathways (Savitsky et al. 2010). Of them, IRF3 is the best characterised. It can be activated by a group of PRRs like Toll-like receptor (TLR) 3, TLR4 and cytosolic nucleic acid sensors (Yamamoto et al. 2003; Stetson and Medzhitov 2006; Ishii et al. 2006; Takaoka et al. 2007).

IRF3 is directly phosphorylate-activated by IKK-related kinases TNFR-associated factor (TRAF) family member-associated NF- κ B activator (TANK)-binding protein (TBK) 1 and IKK ϵ (Figure 1.3) (Pomerantz and Baltimore 1999; Fitzgerald et al. 2003; Mori et al. 2004). In addition to IRF3, IRF5 and IRF7 are also involved in responses to TLR and cytosolic DNA receptors (Honda and Taniguchi 2006). Activated IRFs dimerise to promote the transcription of type I interferons (IFNs) and chemokines (Honda and Taniguchi 2006).

1.2 Cell death responses in the inflammatory process

In addition to inducing the transcription of proinflammatory molecules, many inflammatory signals also induce cell death (Chan et al. 2014; Paludan et al. 2019). Programmed cell death is an integral part of an inflammatory response. Programmed death of the infected cell is thought to be a host-beneficial process that both eliminates the replication niche of the intracellular pathogen and signals danger to immune cells and/or surrounding tissue (Weinlich et al. 2017; Yatim et al. 2017). In this section, I will introduce the 3 main forms of programmed cell death carried out by infected cells in response to proinflammatory signals, apoptosis, necroptosis and pyroptosis.

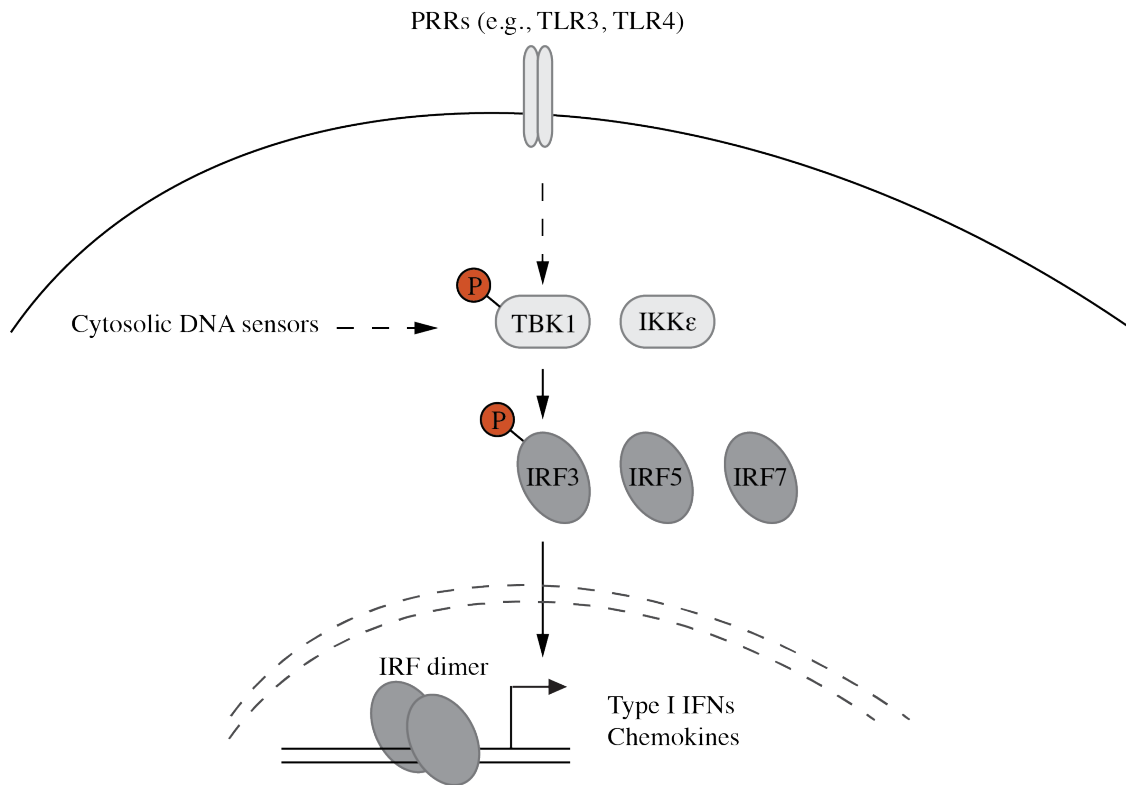


Figure 1.3: The IRF pathway. The IRF pathway is activated by PRRs like TLR3, TLR4 and cytosolic DNA sensors through the IKK-related kinases TBK1 and IKKε. TBK1 and IKKε phosphorylate IRF3 and possibly also other IRFs, leading to the formation of IRF dimers to promote the expression of type I IFNs and chemokines.

1.2.1 Apoptosis

Overview

Apoptosis takes its name from the Greek "falling off" of leaves. It is an immune-suppressive form of cell death (Nagata 2018). During apoptosis, cells shrink and bleb with condensed nuclei and chromatin (Kerr et al. 1972). The apoptotic cells display "eat-me" signals on their surface to recruit macrophages and dendritic cells to phagocytose them, and thereby avoid the exposure of their cellular contents to the surroundings (Nagata 2018).

The importance of apoptosis in immune defence is implicated by virus-encoded proteins targeting the pathway, e.g., viral inhibitor of caspase activation (vICA) encoded by cytomegaloviruses, cytokine response modifier A (CrmA) encoded by Cowpox virus, viral FLICE (caspase-8) inhibitory protein (vFLIP) encoded by γ -herpesvirus and viral

inhibitor of apoptosis proteins (vIAPs) encoded by Baculoviruses (Skaletskaya et al. 2001; Zhou et al. 1997; Bertin et al. 1997; Clem and Miller 1994; Best 2008).

Caspases

Apoptosis is carried out by a cascade of cysteine aspartyl proteases named caspases (Alnemri et al. 1996). The human genome encodes 12 caspases (Nagata 2018). They all contain two catalytic subunits at their C-terminus, one ~20kDa large subunit that contains the catalytic active site and one ~10kDa small subunit (Salvesen and Riedl 2008). Some caspases contain death-fold superfamily domains like death effector domains (DEDs) or caspase recruitment domains (CARDs) at their N-termini to mediate homotypic interaction (Lahm et al. 2003).

Caspases can be categorised based on their structure and function (Salvesen and Riedl 2008). Caspases-8 and -10 contain DEDs, and caspase-9 contains CARD, allowing them to be recruited for activation during apoptosis. They are categorised as apoptosis initiator caspases. In contrast, caspases-3, -6 and -7 do not have death-fold superfamily domains. During apoptosis, they are activated by initiator caspases and cleave over 1500 substrates once activated, giving rise to the apoptotic phenotype. They are referred to as apoptosis executioner caspases (Salvesen and Ashkenazi 2011; Julien and Wells 2017).

The extrinsic apoptosis pathway

Apoptosis can be induced by both intrinsic and extrinsic signals (Taylor et al. 2008). The former are signals from inside the cell such as DNA damage and endoplasmic reticulum (ER) stress, and the latter include PAMPs and DAMPs (Jorgensen et al. 2017).

Extrinsic apoptosis can be activated by PRRs as well as a subgroup of TNFR superfamily receptors named death receptors (DRs), including TNFR1, Fas, TNF-related apoptosis-inducing ligand (TRAIL)-receptor (TRAIL-R) 1 and TRAIL-R2 (Weber et al. 2010; Thapa et al. 2016; Walczak 2013).

Death domain (DD)- and DED-containing proteins bridge activated receptors with initiator caspases. Both Fas and TRAIL-Rs contain DDs, while PRRs and TNFR1 recruit a DD-containing protein, receptor-interacting protein kinase (RIPK) 1, after activation (Itoh

and Nagata 1993; Pan et al. 1997; Walczak 2013; Micheau and Tschopp 2003). During apoptosis, DDs from Fas, TRAIL-Rs and RIPK1 recruit the adaptor protein Fas associated via death domain (FADD) (Figure 1.4) (Wang et al. 2010; Micheau and Tschopp 2003). When FADD is recruited to DDs, a conformation change is induced, exposing its DED to recruit DED-containing initiator caspases, i.e., caspase-8 in mice, and caspase-8 and caspase-10 in human. A DED-containing regulatory protein, FLICE-like inhibitory protein (FLIP), is also recruited (Wang et al. 2010). As the function of caspase-10 is not clear, I will focus on caspase-8 for the description of the extrinsic apoptosis pathway.

Caspase-8 is synthesised and recruited in its zymogen form procaspase-8 (Muzio et al. 1998). It has 2 main isoforms, p55 and p53 (Scaffidi et al. 1997). After its recruitment, procaspase-8 forms homodimers or heterodimers with FLIP (Irmeler et al. 1997).

When forming homodimers, the protease activity of caspase-8 is activated. The large subunit of caspase-8, p18, first catalyses the release of the small subunit, p10, and then catalyses the release of itself from the N-terminus. Two p18 subunits and two p10 subunits then assemble to form mature caspase-8 (Figure 1.5) (Chang et al. 2003). Mature caspase-8 cleaves zymogens of executioner caspases caspase-3 and caspase-7, leading to their activation (Figure 1.4) (Stennicke et al. 1998; Zhou and Salvesen 1997). Active executioner caspases cleave over 1500 substrates with diverse cellular functions at different rates, giving rise to the apoptosis phenotype in a few hours (Julien and Wells 2017). Among the cleaved proteins are phospholipid translocases that control the distribution of a phospholipid molecule, phosphatidylserine (PtdSer), on extracellular and intracellular sides of the cell membrane. The cleavage of phospholipid translocases leads to the rapid and irreversible accumulation of PtdSer on the cell surface to be recognised by macrophages to induce the engulfment of the apoptotic cell (Nagata et al. 2016; Nagata 2018) (Figure 1.4).

The caspase-8-FLIP heterodimer

FLIP is a caspase-8-like protein and an NF- κ B-target gene (Kreuz et al. 2001; Micheau et al. 2001). It is expressed in 2 major isoforms, the long isoform FLIP_L and the short isoform FLIP_S. FLIP_L is a 55kDa protein that has exactly the same domain

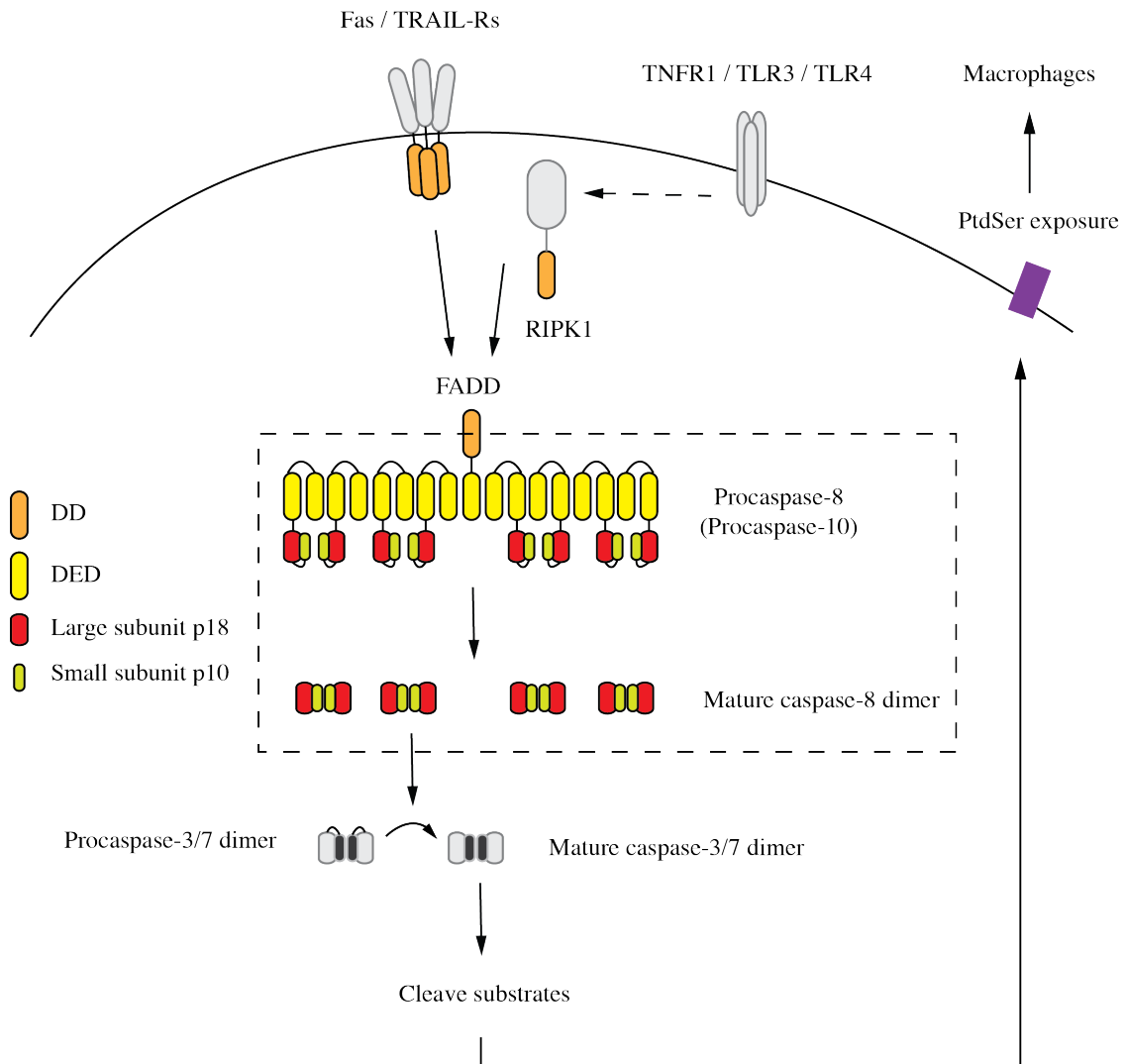


Figure 1.4: The extrinsic apoptosis pathway. Activation of the extrinsic apoptosis pathway depends on DD-mediated recruitment of FADD. FADD recruits procaspase-8 dimers via DEDs. In human cells, procaspase-10 is also recruited. Procaspase-8 dimers catalyse the maturation of caspase-8 dimers in a process illustrated in Figure 1.5. Mature caspase-8 dimers catalyse the activation of caspase-3 and caspase-7. Active executioner caspases cleave thousands of substrates in cells, leading to the apoptotic phenotype and the exposure of PtdSer on cell surface to recruit macrophages.

organisation as procaspase-8 but lacks the catalytic cysteine (Irmeler et al. 1997). FLIP_S is a 26kDa protein that consists of 2 DEDs, resembling the N-terminus of procaspase-8 (Figure 1.6) (Irmeler et al. 1997).

FLIP_S inhibits apoptosis by competitive binding of caspase-8 (Micheau et al. 2002; Hughes et al. 2016) (Figure 1.6). In contrast, FLIP_L-binding activates caspase-8 to process the release of the small subunits (Micheau et al. 2002) (Figure 1.6). The resultant p43/41-

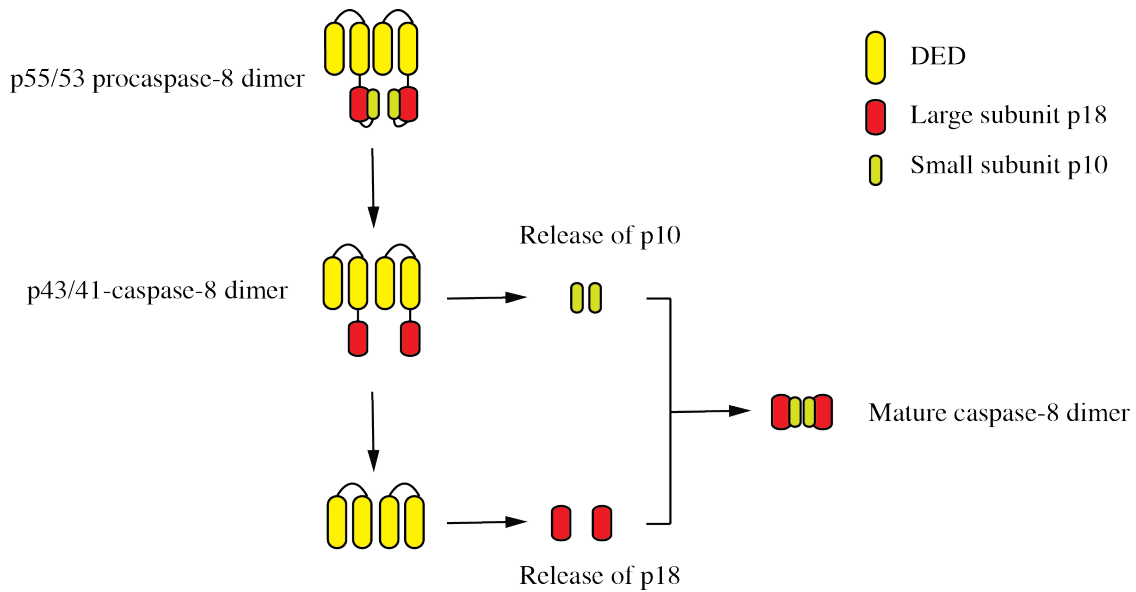


Figure 1.5: The processing of caspase-8. Caspase-8 is synthesised and recruited in its zymogen form p55/53. The formation of a homodimer activates the catalytic p18 subunit (shown in red) to catalyse the cleavage and release of the small subunit, which subsequently matures into p10, leaving behind the p43/41-caspase-8 dimer. In the p43/41-caspase-8 dimer, p18 catalyses the release of itself. The released p18 and p10 subunits assemble into the mature caspase-8 dimer with two catalytic active sites.

caspase-8-p43-FLIP heterodimer has partial catalytic activity. It can catalyse the activation of caspase-8 homodimers, but has much reduced activity towards caspase-3 zymogen (Pop et al. 2011). Therefore, it is proposed that low levels of FLIP_L activates caspase-8 homodimers to drive apoptosis, whereas high levels of FLIP_L competitively binds caspase-8 to inhibit the process (Hughes et al. 2016). In addition to regulating apoptosis, the caspase-8-FLIP_L heterodimer regulates the necroptosis pathway by cleaving the essential proteins in it, for example, RIPK1, RIPK3 and cylindromatosis (CYLD) (Micheau et al. 2002; Feng et al. 2007; O'Donnell et al. 2011; Oberst et al. 2011). As FLIP is an NF- κ B target gene, the regulation of caspase-8 activity by FLIP proteins ensures that the cell death pathways are suppressed in the presence of inflammatory signalling (Figure 1.6).

1.2.2 Necroptosis

Overview

Contrary to apoptosis which minimizes the exposure of cellular contents to the environment, necroptosis is a lytic form of programmed cell death. It features cell swelling,

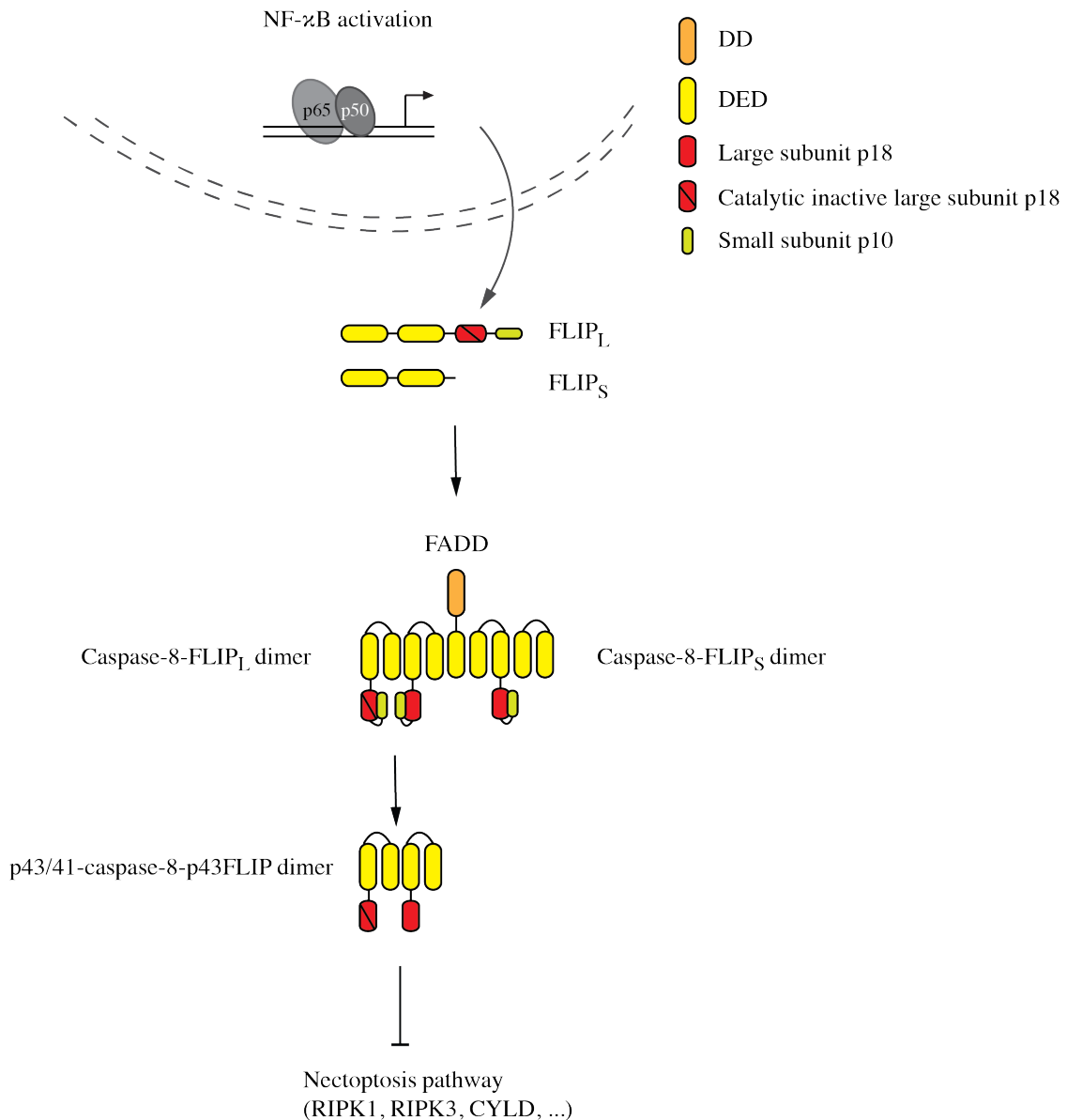


Figure 1.6: FLIP regulates the activity of caspase-8. NF-κB promotes the expression of FLIP in 2 isoforms, FLIP_L and FLIP_S. They form heterodimers with procaspase-8. While FLIP_S-binding blocks the processing of caspase-8, FLIP_L-binding promotes the processing of caspase-8 and itself to release the p10 subunits from both proteins, forming a p43/41-caspase-8-p43-FLIP heterodimer. The p43/41-caspase-8-p43-FLIP heterodimer cannot cleave-activate executioner caspases, but is able to cleave-inactivate RIPK1, RIPK3 and CYLD, which are the key proteins in the necroptosis pathway.

membrane leakage and DAMP release, and is thus thought to be highly proinflammatory (Kaczmarek et al. 2013).

Like apoptosis, necroptosis can be triggered by PAMPs and DAMPs. Receptors that activate the necroptosis pathway are the same sets that activate extrinsic apoptosis, e.g.,

PRRs like TLR3, TLR4 and DRs like TNFR1, Fas and TRAIL-Rs (Kaiser et al. 2013; He et al. 2011; Zhou and Yuan 2014). Due to the activity of the caspase-8-FLIP_L heterodimer, necroptosis is only induced when the apoptosis pathway is compromised. Therefore, necroptosis is considered as a reserved way that the host cell fights pathogens that encode apoptosis inhibitors. By necroptosis, infected cells release DAMPs to stimulate anti-microbial responses in the surroundings as well as eliminate the replication niche of the intracellular pathogen (Weinlich et al. 2017).

The importance of necroptosis in eliminating infection is supported by the identification of pathogen-encoded necroptosis inhibitor proteins. Examples include EspL, encoded by enteropathogenic *Escherichia coli* (EPEC), viral inhibitor of RIP activation (vIRA), encoded by murine cytomegalovirus (MCMV), immediate early gene 1 (IE1), encoded by human cytomegalovirus (HCMV), and ribonucleotide reductase large subunit ICP6, encoded by herpes simplex virus type 1 (HSV-1) (Figure 1.7) (Pearson et al. 2017; Upton et al. 2012; Omoto et al. 2015; Guo et al. 2015).

The RIP homotypic interaction motif

Different necroptosis pathways converge at the activation of receptor-interacting protein kinase 3 (RIPK3). RIPK3 consists of an N-terminal kinase domain, a so-far unique intermediate region with no specific domain prediction, and a C-terminal RIP homotypic interaction motif (RHIM) (Sun et al. 2002). RHIM is a short motif of ~15-20 amino acids with the core 4 amino acids being XQXG, where the first X can be either valine or isoleucine, and the second X can be one of valine, isoleucine, leucine or cysteine (Nanson et al. 2019). The activation of RIPK3 relies on its recruitment to other RHIM-containing proteins via homotypic interaction.

So far, 4 RHIM-containing proteins have been identified in the mammalian genome, RIPK1, RIPK3, Toll/IL-1 receptor (IL-1R) signalling domain (TIR)-containing adapter-inducing IFN- β (TRIF), and a cytosolic nucleic acid sensor Z-nucleic acid-binding protein 1 (ZBP1) (Nanson et al. 2019; Kuriakose and Kanneganti 2018). Among the receptors that activate necroptosis, ZBP1 is able to directly interact with RIPK3, TLR3 and TLR4

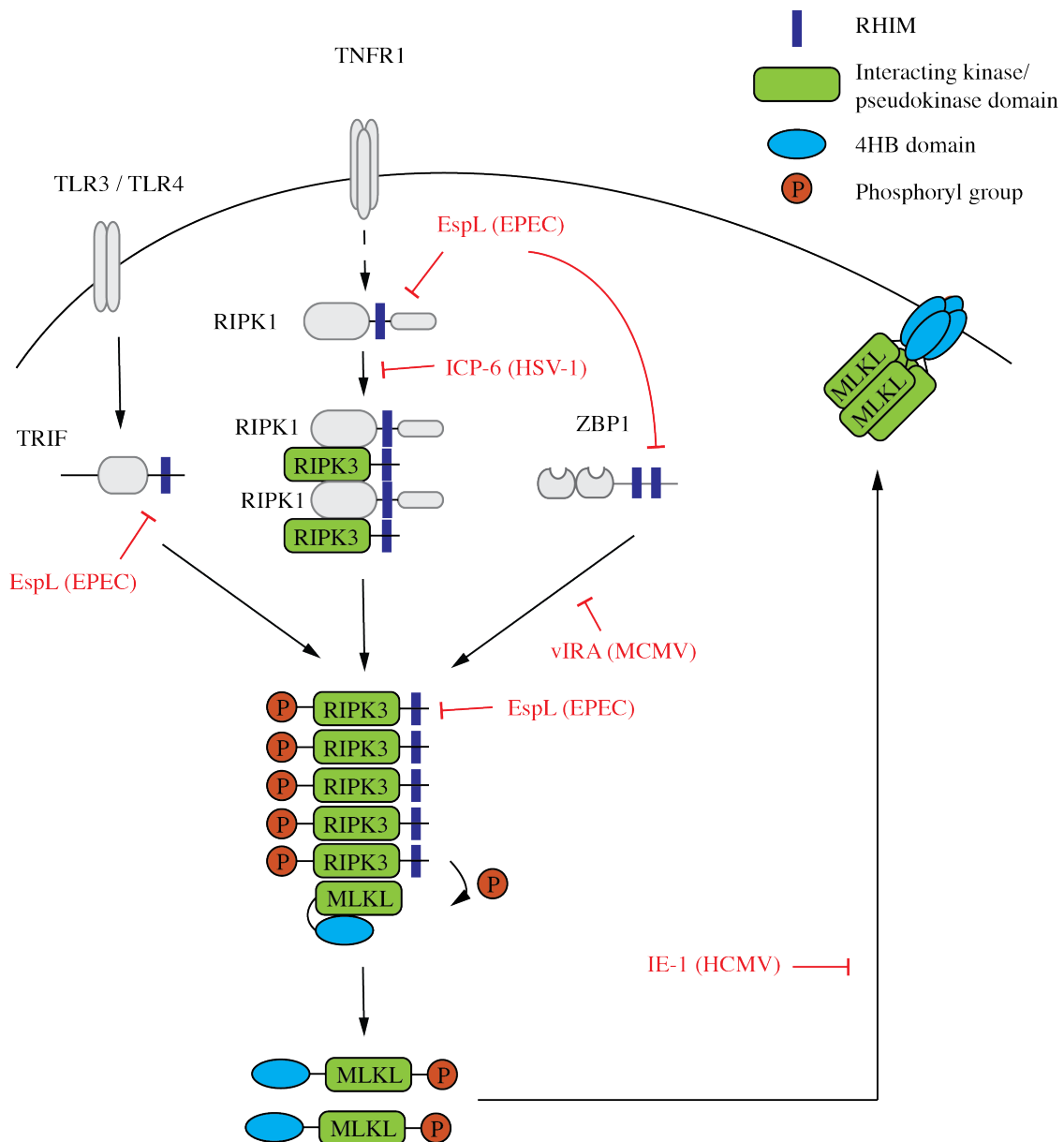


Figure 1.7: The necroptosis pathway. RIPK3 is recruited through interaction with RHIM-domain-containing proteins, including ZBP1, TRIF downstream of TLR3 and TLR4, and RIPK1 downstream of TNFR1. The recruitment of RIPK3 seeds the formation of a RIPK3 amyloid, where RIPK3 undergoes autophosphorylation or cross-phosphorylation for its activation. Phosphorylated RIPK3 recruits MLKL through the interaction between the kinase domain and pseudokinase domain. RIPK3 phosphorylates MLKL on its pseudokinase domain, leading to a change in its conformation and the exposure of the N-terminal 4HB domain for its oligomerisation. The MLKL oligomer translocates onto the plasma membrane and disrupts its integrity. The necroptosis pathway is targeted by a number of pathogen-encoded effector proteins. EPEC-encoded EspL cleaves RHIM-containing proteins. MCMV-encoded vIRA is a RHIM-containing protein that disturbs ZBP1-RIPK3 interaction. ICP-6 encoded by HSV-1 disturbs RIPK1-RIPK3 interaction, and IE-1 encoded by HCMV inhibits MLKL-mediated membrane rupture after its activation.

recruit TRIF which in turn recruits RIPK3, while DRs recruit RIPK3 through RIPK1 (Upton et al. 2012; Zhou and Yuan 2014).

Execution of the necroptosis pathway

How RIPK3 is activated after its recruitment is characterised in TNF-induced RIPK3 activation. During TNF-induced necroptosis, RIPK1 recruits RIPK3 to form heteroamyloids, followed by the recruitment of more RIPK3 molecules to form RIPK3 homoamyloids (Mompeán et al. 2018). In the RIPK1-RIPK3 heteroamyloid, RIPK3 undergoes autophosphorylation or cross-phosphorylation at multiple sites (Cho et al. 2009; He et al. 2009; Sun et al. 2012; McQuade et al. 2013). RIPK3 is also shown to phosphorylate RIPK1 in the heteroamyloid, though its function is not clear (Cho et al. 2009).

Among the phosphorylation sites on RIPK3 is serine 227 (S227) on its kinase domain. S227 phosphorylation stabilises the interaction between RIPK3 and its substrate, mixed lineage kinase domain-like (MLKL). MLKL is a pseudokinase that is structurally closest to the mixed lineage kinase (MLK) family (Sun et al. 2012). It consists of an N-terminal four-helix bundle (4HB) domain and a C-terminal pseudokinase domain tethered by a 2 helix linker (Murphy et al. 2013). MLKL interacts with RIPK3 via its pseudokinase domain (Sun et al. 2012). Upon binding, RIPK3 phosphorylates MLKL on multiple sites on the activation loop of the pseudokinase domain (Xie et al. 2013; Rodriguez et al. 2016). Phosphorylation of MLKL in the pseudokinase domain leads to conformational changes that cause the exposure of the 4HB domain (Hildebrand et al. 2014). The exposed 4HB domain mediates the oligomerisation of MLKL molecules, forming trimers in mouse and tetramers in human (Cai et al. 2014b; Chen et al. 2014). Human MLKL also contains a cysteine residue on its 4HB domain that mediates the formation of disulfide bonds among MLKL oligomers, leading to the formation of amyloid-like oligomers (Liu et al. 2017a).

MLKL oligomers translocate to the plasma membrane, where they disrupt membrane integrity by interacting with phosphatidylinositol phosphates (PIPs) on the membrane and depolarises the cell by forming cation channels, leading to the necrotic phenotype (Figure 1.7) (Wang et al. 2014; Dondelinger et al. 2014; Xia et al. 2016).

Necroptosis and inflammatory signalling

Several lines of evidence suggest that necroptosis is accompanied by the activation of inflammatory signalling pathways in a cell-autonomous manner.

RIPK3 has been reported to mediate inflammatory signalling during LPS- and TNF-induced necroptosis (Najjar et al. 2016; Zhu et al. 2018). In addition, oligomerisation of mouse RIPK3 was shown to activate NF- κ B mediated inflammatory signalling prior to cell death, which is required for necroptotic cells to prime an adaptive immune response (Yatim et al. 2015). At later stages of necroptosis, cation flux as a result of MLKL activation activates the inflammasome for the maturation of IL-1 β (Conos et al. 2017).

Production of proinflammatory molecules within the dying cell enhances the inflammatory response in the surrounding cells. The discoveries that the necroptosis process is accompanied by active production of proinflammatory molecules gave rise to the concept of inducible DAMPs (iDAMPs). As opposed to constitutive DAMPs (cDAMPs) like ATP and HMGB1 that are constantly expressed in the cells, iDAMPs are produced during cell death via mechanisms that are dependent on the underlying cell death pathway. It is proposed that iDAMPs could be a marker for the immune system to distinguish programmed cell death from accidental ones for specific responses (Yatim et al. 2017).

1.2.3 Pyroptosis

Overview

Pyroptosis was coined from the Greek "pyro" meaning "fire" for its strong ability to induce inflammation (Cookson and Brennan 2001). Phenotypically, pyroptosis shares the features of apoptosis and necroptosis by having a cell swelling lytic phenotype as well as DNA fragmentation and chromosome condensation (Cookson and Brennan 2001). Molecularly, pyroptosis is defined by the involvement of the gasdermin family proteins to disrupt membrane integrity and the maturation and release of the IL-1 family cytokines IL-1 β and IL-18 (Shi et al. 2017). Pyroptosis can be triggered by various PAMPs and other signals (Shi et al. 2017). It is important in immunity against pathogens of all categories, including bacteria, viruses, fungi and protozoan (Man et al. 2017).

The inflammatory caspases are central to the execution of pyroptosis, including caspases-1 and -11 in mice, and caspases-1, -4 and -5 in human (Martinon and Tschopp 2004). They all contain a CARD at the N-terminus which is important for their activation (Martinon and Tschopp 2004).

The canonical pyroptosis pathway

Pyroptosis can be activated through canonical and noncanonical pathways (Shi et al. 2017). Canonical pyroptosis is activated by a group of PRRs, including nucleotide oligomerisation domain (NOD)-like receptor (NLR) family receptors NLR family PYD-containing (NLRP) 3, NLRP1, NLR family CARD-containing 4 (NLRC4), and DNA sensors absent in melanoma 2 (AIM2) and IFN γ inducible protein 16 (IFI16). Noncanonical pyroptosis is proposed to be activated by LPS sensing of caspases-4 and -5 in human and caspase-11 in mice (Platnich and Muruve 2019).

The canonical pyroptosis pathway relies on the death-fold domains pyrin domain (PYD) and CARD for signal transduction (Lu et al. 2014). The convergent point for the canonical pyroptosis pathway is the recruitment of the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) (Jorgensen et al. 2017). Similar to the domain organisation of FADD, ASC consists of an N-terminal PYD and a C-terminal CARD (Martinon et al. 2002). It is recruited to PYD-containing PRRs after their activation, and in turn recruits CARD-containing procaspase-1 (Cai et al. 2014a). The PYD-containing PRR, ASC and procaspase-1 form a multi-protein platform named the inflammasome via interaction between PYDs and CARDS for the activation of caspase-1 (Figure 1.8) (Lu et al. 2014; Cai et al. 2014a).

IL-1 β and IL-18 are expressed in their inactive form with the activation of the NF- κ B pathway (Bauernfeind et al. 2009). Cleavage of pro-IL-1 β and pro-IL-18 by caspase-1 releases their inhibitory N-terminus, leading to their maturation (Hailey et al. 2009). In addition to cytokines, caspase-1 cleaves gasdermin D (GSDMD) to release its C-terminal inhibitory domain (Shi et al. 2015). Like that of MLKL, the N-terminus of GSDMD oligomerises and binds lipids on the plasma membrane to form pores to

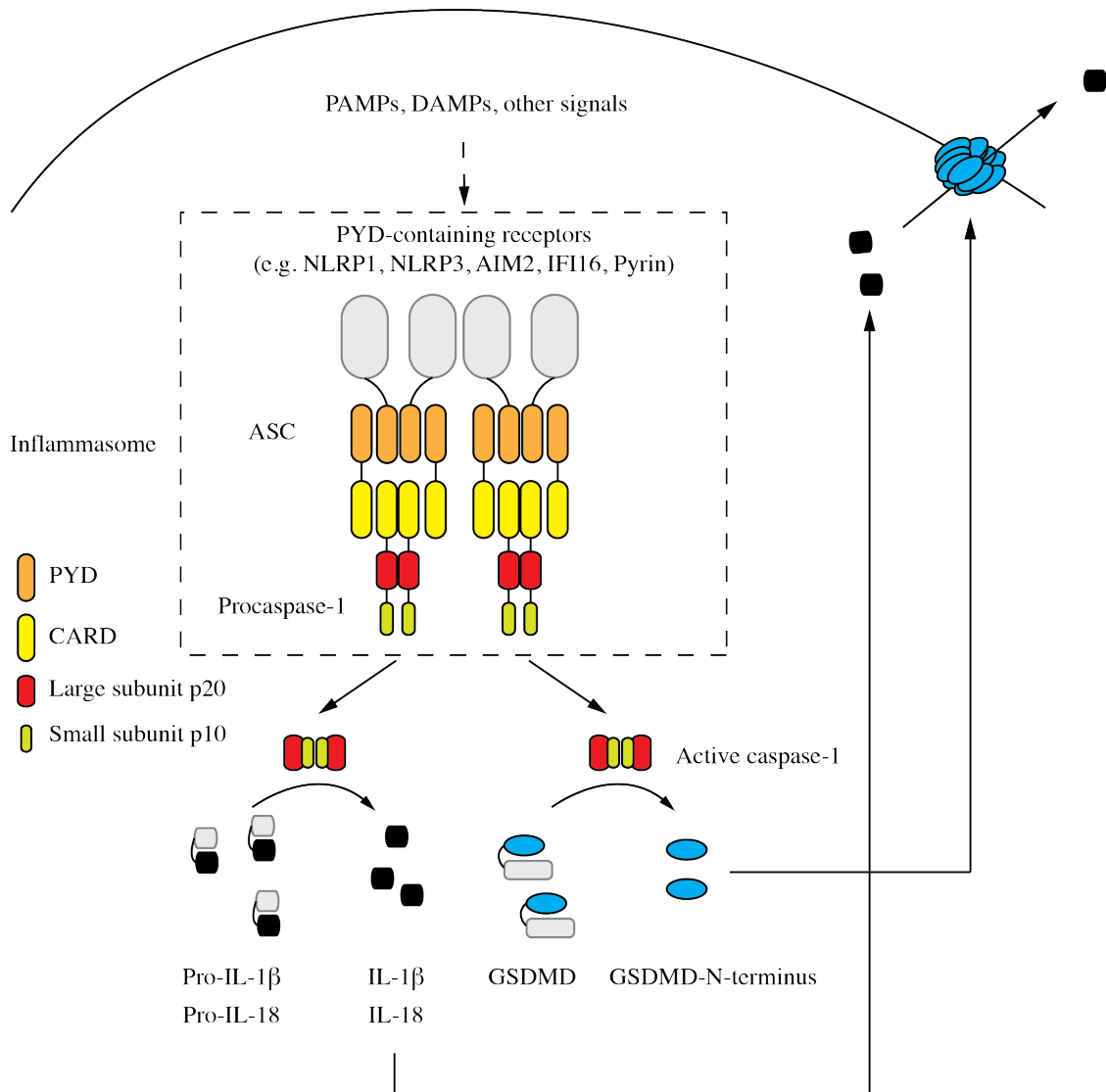


Figure 1.8: The canonical pyroptosis pathway. Various signals lead to the oligomerisation of PYD-containing proteins, including NLRP1, NLRP3, AIM2, IFI16 and Pyrin. Oligomerisation of PYD-containing receptors leads to the recruitment of ASC via interaction between PYD. ASC in turn recruits procaspase-1 via CARD. Dimerisation of procaspase-1 results in its cleavage and maturation. Active caspase-1 cleaves pro-IL-1 β , pro-IL-18 and GSDMD to their active form. Active GSDMD forms oligomer pores on the membrane, and active IL-1 β and IL-18 are released through it.

release mature IL-1 β and IL-18 as well as DAMP-containing cytosolic contents (Figure 1.8) (Ding et al. 2016).

1.3 Crosstalk between cell death and inflammation

As can be seen from the above discussion, many PAMPs and DAMPs are able to induce both inflammatory signalling and cell death, like LPS and TNF. After the activation of their receptors, intricate mechanisms control the crosstalk between inflammatory signalling and different modes of cell death. In this section, I will introduce the crosstalk between cell death and inflammation after the activation of TNFR1, TLR3/TLR4 and ZBP1.

1.3.1 The TNFR1 signalling pathway

TNF is a key cytokine in the inflammation process. There are 2 TNF receptors in mammalian cells, TNFR1 and TNFR2. TNFR2 is mainly expressed on immune cells and endothelial cells, the activation of which results in inflammatory responses (Wajant and Siegmund 2019). In contrast, TNFR1 is expressed in almost every type of mammalian cells (Wajant and Siegmund 2019). TNFR1 has a DD in its intracellular region, which enables it to activate cell death in addition to MAPK- and NF- κ B-mediated inflammatory signalling (Tartaglia et al. 1993).

TNFR1-mediated inflammatory signalling

Within minutes of TNFR1 activation, a proinflammatory complex Complex I is assembled (Wajant and Siegmund 2019). TNFR1 oligomerisation leads to changes in the conformation of its intracellular region to recruit the adaptor proteins TNFR1-associated death domain protein (TRADD) and RIPK1 via their common DD (Hsu et al. 1995). TRADD recruits TNFR associated factor (TRAF) 2 with its N-terminal region (Park et al. 2000). TRAF2 exists in a complex with cellular inhibitor of apoptosis protein (cIAP) 1 and cIAP2 via its cIAP interaction motif (Zheng et al. 2010; Dondelinger et al. 2016).

TRAF2, cIAP1 and cIAP2 are ubiquitin ligases that assemble K63-linked polyubiquitin in the complex, though the function of cIAPs is more prominent (Vince et al. 2009). RIPK1 is the major K63-linked ubiquitylation target in the TNFR1 complex, but K63-linked polyubiquitin can also be built on TNFR1 or free of substrates to mediate inflammatory signalling (Bertrand et al. 2008; Xia et al. 2009).

As discussed in Chapter 1.1.2, K63-linked polyubiquitin recruits TAK1 via TAB2 or TAB3 to activate the MAPK pathway (Kanayama et al. 2004). In addition, K63-linked polyubiquitin recruits the M1-linked polyubiquitin ligase complex linear ubiquitin chain assembly complex (LUBAC) (Haas et al. 2009). LUBAC consists of 3 components, haem-oxidised iron-responsive element-binding protein 2 ubiquitin ligase 1 (HOIL-1), HOIL-1-interacting protein (HOIP) and Src homology domain 3 and multiple ankyrin repeat domains protein-associated regulator of G-protein signaling homology domain interactor (SHARPIN) (Kirisako et al. 2006; Tokunaga et al. 2011). Both HOIP and HOIL-1 contain NZF domains that bind K63-linked polyubiquitin, allowing the recruitment of LUBAC to K63-linked polyubiquitin (Haas et al. 2009). LUBAC assembles M1-linked polyubiquitin on TNFR1 and its associated complex, which recruits the IKK complex via NEMO, leading to the activation of the NF- κ B pathway (Ea et al. 2006; Draber et al. 2015) (Figure 1.9).

TNFR1-mediated apoptosis

Hours after TNFR1 activation, a cytoplasmic complex Complex II forms with TNFR1 internalisation that could mediate apoptosis. Complex II contains TRADD, RIPK1, FADD, caspase-8, caspase-10 and FLIP but not TNFR1 (Micheau et al. 2002).

The stability and activity of Complex II are regulated by components in Complex I (Annibaldi and Meier 2018). For example, Complex I-associated inflammatory kinases IKKs, MAPK-activated protein kinase 2 (MK2) and TBK1 phosphorylate RIPK1 to limit its association with components in Complex II and suppress cell death (Dondelinger et al. 2015; Dondelinger et al. 2019; Dondelinger et al. 2017; Menon et al. 2017; Jaco et al. 2017; Geng et al. 2017; Dondelinger et al. 2018; Xu et al. 2018; Lafont et al. 2018). Ubiquitylation on RIPK1 also helps to keep RIPK1 stabilised in Complex I (Jaco et al. 2017). Additionally, NF- κ B target proteins FLIP and A20 restrict cell death. FLIP restricts apoptosis by regulating caspase-8 activity, and A20 suppresses cell death partly by modifying RIPK1 with K48-linked polyubiquitin to promote its proteasomal degradation (Wertz et al. 2004).

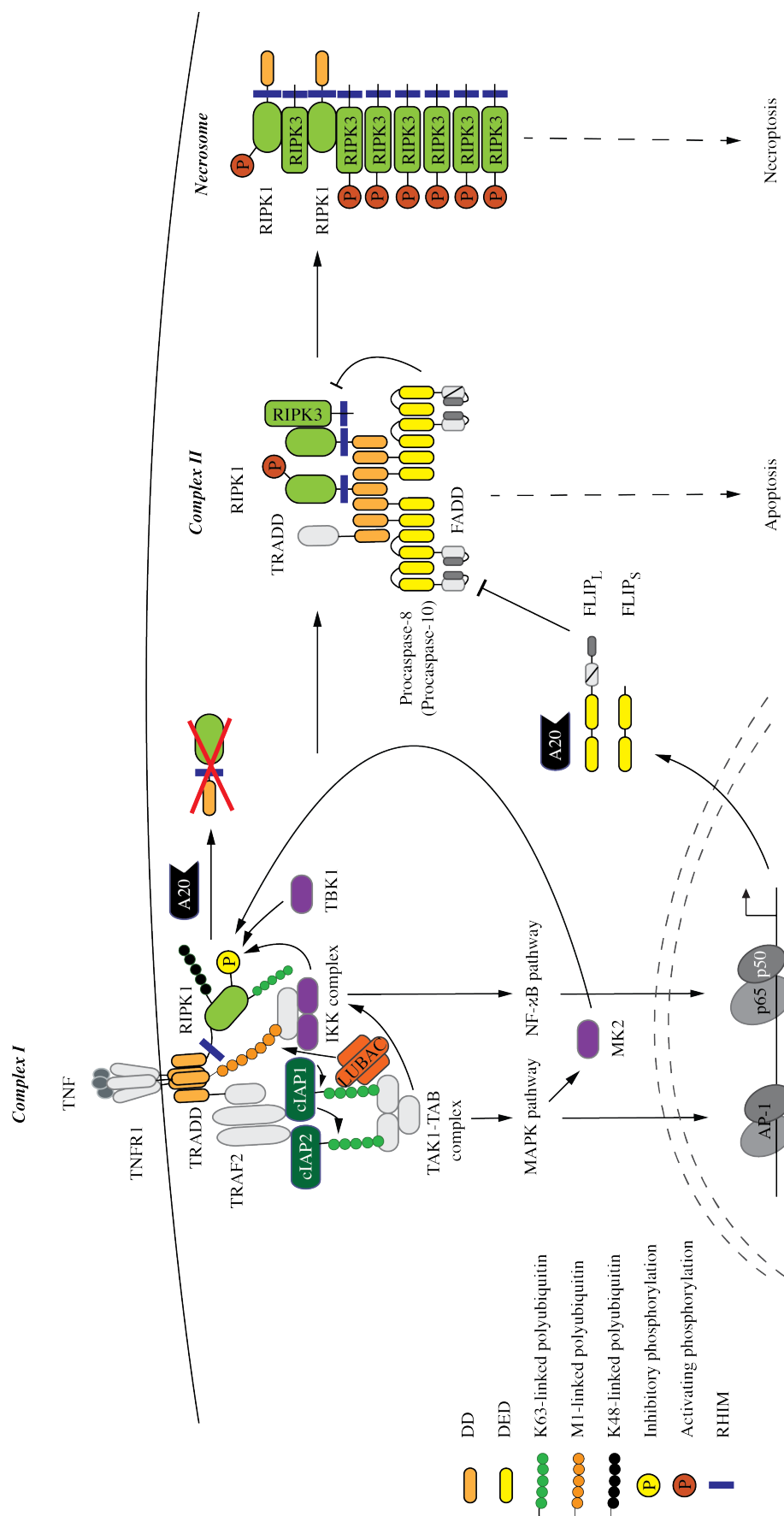


Figure 1.9: The TNFR1 signalling pathway. Activated TNFR1 recruits TRADD and RIPK1 through its DD. TRADD recruits TRAF2 in a complex with ubiquitin ligases cIAP1 and cIAP2. cIAPs assemble K63-linked polyubiquitin on RIPK1, TNFR1 and other components. K63-linked polyubiquitin recruits the TAK1-TAB complex to activate the MAPK pathway and the LUBAC complex to assemble M1-linked polyubiquitin on components of the complex, which in turn recruits the IKK complex to activate the NF- κ B pathway. Under apoptosis conditions, RIPK1 undergoes autophosphorylation and promote apoptosis in a cytosolic complex Complex II through interaction among DDs and DEDs. Several layers of restraints prevent TNFR1-induced cell death. Kinases in the inflammatory pathway, including MK2, IKKs and TBK1, put on inhibitory phosphorylation on RIPK1 to prevent its autophosphorylation. A20, a product of NF- κ B pathway, put on K48-linked polyubiquitin on RIPK1, leading to its degradation. Another target of NF- κ B, RIPK3, counteracts the activation of caspase-8 by forming heterodimers with it. Defects in the inflammatory pathway license the initiation of apoptosis. RIPK1 and RIPK3 enucleate the necrosome, mediating TNFR1-induced necroptosis. If caspase-8 is blocked, RIPK1 and RIPK3 enucleate the necrosome, mediating TNFR1-induced necroptosis.

When Complex I or the inflammatory pathway is defective, Complex II drives apoptosis. This could happen physiologically with the infection of pathogens that encode inhibitory proteins of the inflammatory pathway, for example, *Shigella* and *Yersinia*, or by experimental depletion of essential elements in the inflammatory pathway (Ashida et al. 2010; Ashida et al. 2014). Under these conditions, failure to control RIPK1's activity induces its autophosphorylation at S166, dissociation from Complex I and attachment to components of Complex II (Newton et al. 2016; Xu et al. 2018). Caspases-8 and -10 are activated in Complex II, followed by caspases-3 and -7 in a process described in Chapter 1.2.1 (Micheau and Tschopp 2003).

TNFR1-mediated necroptosis

The necroptosis-activating protein RIPK3 is recruited to the Complex II by RIPK1 through interaction between their RHIMs (Dondelinger et al. 2013). However, the recruitment of RIPK3 does not license the start of necroptosis. As discussed in Chapter 1.2.1, the p43/41-caspase-8-p43-FLIP heterodimer cleaves RIPK1, RIPK3 and other components in the necroptosis pathway to inactivate it (Oberst et al. 2011). In the absence of caspase-8 or its activity, such as with the infection of viruses that encode caspase-8 inhibitors, excessive RIPK1 and RIPK3 form heteroamyloids that seed the assembly of the necrosome to activate necroptosis through the process described in Chapter 1.2.2 (Cho et al. 2009).

In summary, TNFR1 by default induces inflammatory signalling, which inhibits cell death through inflammatory kinases, scaffolding ubiquitin chains and the production of prosurvival proteins. When the inflammatory pathway is inhibited, autophosphorylated RIPK1 activates apoptosis in a complex with FADD and caspase-8. If caspase-8 is also inhibited, autophosphorylated RIPK1 activates RIPK3 to induce necroptosis (Figure 1.9).

1.3.2 The TLR3/TLR4 signalling pathways

TLR3 and TLR4 belong to the Toll-like receptor family of PRRs. TLR3 recognises dsRNA in endosomes following phagocytosis, and TLR4 is a membrane-resident receptor that recognises LPS (Matsumoto et al. 2003; Poltorak et al. 1998). The activation of TLR3 and TLR4 induces various responses under different conditions, including inflammatory

signalling, apoptosis, necroptosis and pyroptosis (Figure 1.10) (Ramnath et al. 2017). TLR4 is able to recruit two adaptor proteins via the TIR domain after its activation, TIR-containing adapter-inducing IFN- β (TRIF) and myeloid differentiation primary response 88 (MyD88), while TLR3 recruits TRIF but not MyD88 (Yamamoto et al. 2002). Whereas MyD88 only mediates inflammatory signalling, TRIF is the central adaptor coordinating inflammation and cell death pathways (Ramnath et al. 2017).

MyD88-mediated inflammatory signalling

Activated TLR4 on the plasma membrane recruits MyD88 and myelin and lymphocyte protein (MAL) to induce inflammatory signalling via TIR domain-mediated interaction (Kagan and Medzhitov 2006). Besides a C-terminal TIR domain, MyD88 also contains an N-terminal DD that recruits another DD-containing protein IL-1R-associated kinase (IRAK) 4, which in turn recruits DD-containing IRAK2 through DD homotypic interaction (Lin et al. 2010). DDs of MyD88, IRAK4 and IRAK2 stack, forming the Myddosome that brings together the kinase domains of IRAKs for phosphorylation and activation (Lin et al. 2010). The Myddosome further recruits and activates DD-containing IRAK1 (Wang et al. 2017). Despite IRAKs are activated in the Myddosome, the kinase activity of IRAK4 and IRAK1 is not always required for MyD88-mediated inflammatory signalling, similar to that of RIPK1 in TNFR1-mediated inflammatory signalling (Rhyasen and Starczynowski 2015).

Both IRAK1 and IRAK2 contain a TRAF6-binding domain to recruit TRAF6, which assembles K63-linked polyubiquitin (Ye et al. 2002; Deng et al. 2000). In addition, IRAK1 and IRAK4 phosphorylate another ubiquitin ligase Pellino 1, enhancing its activity to assemble K63-linked polyubiquitin (Ordureau et al. 2008). As discussed before, K63-linked polyubiquitin recruits the TAK1-TAB complex and LUBAC to generate M1-linked polyubiquitin to recruit the NEMO-IKK complex, leading to the activation of MAPK and NF- κ B pathways (Kanayama et al. 2004; Haas et al. 2009; Rahighi et al. 2009).

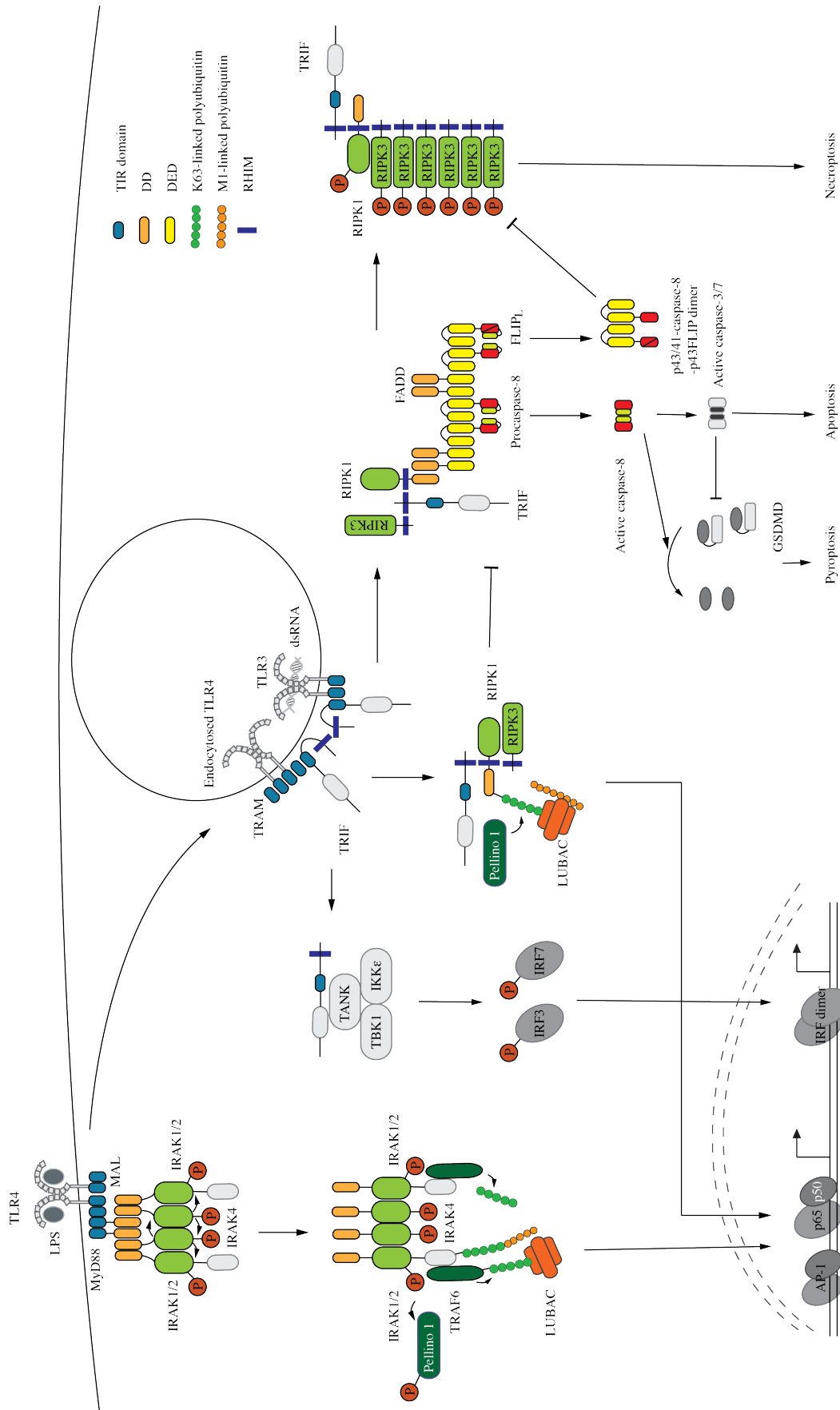


Figure 1.10: The TLR3/TLR4 signalling pathways. TLR4 is activated on the cell surface by LPS, and recruits MyD88 and MAL via their TIR domains. MyD88 recruits IRAK4 and IRAK2 via DDs, resulting in the formation of the Myddosome that further recruits IRAK1. The IRAKs are activated by crossphosphorylation to recruit TRAF6 and phosphorylate Pellino 1, which assembles K63-linked polyubiquitin to recruit LUBAC and the TAK1-TAB complex to for the activation of MAPK- and NF- κ B pathways. After its internalisation, TLR4 recruits another 2 adaptors, TRIF and TRAM via its TIR domain. TLR3, localised on endosomes, is activated by engulfed dsRNA and directly recruits TRIF through TIR domains. TRIF can interact with TANK to activate TBK1 and IKK ϵ for the IRF pathway, resulting in the production of Type I IFNs. TRIF also recruits RIPK1 and RIPK3 via RHIMs. RIPK1 is modified with K63-linked polyubiquitin by Pellino 1, which recruits LUBAC for the activation of MAPK and NF- κ B pathway. Compromission of the inflammatory signalling pathway sensitises cells to TRIF-mediated cell death, where a complex consisting of RIPK1, FADD, procaspase-8 and FLIP to regulate caspase-8 activation. Depending on the cell type, activated caspase-8 cleaves caspases-3 and -7 to activate apoptosis or GSDMD for pyroptosis. Activation of executioner caspases inhibits pyroptosis. The p43/41-caspase-8-p43-FLIP heterodimer antagonises necroptosis. When caspase-8 is inhibited, TRIF mediates necroptosis through RIPK3 activation.

TRIF-mediated inflammatory signalling

TRIF is recruited to TLR3 directly after its activation and to TLR4 with help from another adaptor protein, translocating chain-associated membrane protein (TRAM) after its endocytosis and translocation to the endosome (Oshiumi et al. 2003; Kagan et al. 2008).

TRIF can promote the production of type I IFNs by binding TRAF family member-associated NF- κ B activator (TANK), which in turn interacts with TBK1 and IKK ϵ for the activation of the IRF pathway using IRF3 and IRF7 (Guo and Cheng 2007).

In addition, TRIF promotes the activation of the NF- κ B pathway by recruiting RIPK1 via RHIM-mediated homotypic interaction (Meylan et al. 2004; Cusson-Hermance et al. 2005). The recruited RIPK1 is ubiquitylated with K63-linked polyubiquitin by Pellino 1, which activates the MAPK and NF- κ B pathways by recruiting the TAK1-TAB complex and LUBAC that in turn recruits the IKK complex (Chang et al. 2009; Kanayama et al. 2004; Haas et al. 2009; Rahighi et al. 2009).

RIPK3 is also recruited to TRIF, but its role in TRIF-mediated inflammatory signalling is not clear. In cortical neuron culture, RIPK3 is involved in LPS-induced production of chemokines (Daniels et al. 2017). Whether this holds true in other tissues and species remains to be verified.

TRIF-mediated cell death

Besides inflammatory signalling, the TRIF-RIPK1 axis mediates cell death pathways. Similar to the situation in the TNF signalling pathway, compromisation of the inflammatory signalling pathways, such as with the protein synthesis inhibitor cycloheximide (CHX) or cIAP antagonists, sensitises cells to TRIF-mediated apoptosis, which is mediated by RIPK1-mediated recruitment of FADD and caspase-8 but not dependent on RIPK3 (Weber et al. 2010; Feoktistova et al. 2011; Estornes et al. 2012).

As in the TNFR1-mediated situation, the p43/41-caspase-8-p43-FLIP heterodimer antagonises necroptosis. In the absence of caspase activity, the direct interaction between TRIF and RIPK3 results in the activation of the latter for necroptosis (Feoktistova et al. 2011; He et al. 2011; Kaiser et al. 2013). The contribution of RIPK1 in TRIF-mediated necroptosis is not clear. Although the RIPK1 kinase inhibitor Necrostatin-1

(Nec1) inhibits the process, loss of RIPK1 does not affect TRIF-mediated necroptosis (Feoktistova et al. 2011; He et al. 2011; Kaiser et al. 2013).

Recently, the activation of TLR4 is reported to induce pyroptosis in murine macrophages through caspase-8-mediated cleavage of GSDMD, when the inflammatory pathway is compromised (Gram et al. 2019; Orning et al. 2018; Sarhan et al. 2018). Meanwhile, caspase-3 and caspase-7 are reported to cleave GSDMD at a different site to prevent pyroptosis (Taabazuing et al. 2017). Taken together, TRIF-mediated pyroptosis is likely mediated by the same machinery as TRIF-mediated apoptosis until the point of caspase-8 activation, when the activation of apoptosis or pyroptosis depends on the activity of executioner caspases and the availability of substrates (Figure 1.10).

1.3.3 The ZBP1 signalling pathway

The ZBP1 receptor

Z-nucleic-acid-binding protein 1 (ZBP1) is a cytosolic nucleic acid sensor. It is comprised of two Z-nucleic-acid-binding domains (ZBDs) at its N-terminus, denoted as $Z\alpha$ and $Z\beta$ (or $Z\alpha1$ and $Z\alpha2$), followed by two RHIMs that mediate its interaction with RIPK1 and RIPK3 and a C-terminal region that could activate TBK1 (Ha et al. 2008; Takaoka et al. 2007; Kaiser et al. 2008; Rebsamen et al. 2009).

ZBP1 is an IFN-stimulated gene (ISG) that is induced to express by type I and type II IFNs (Fu et al. 1999; Takaoka et al. 2007). It is required for controlling the proliferation of vIRA-mutant MCMV (MCMV-M45mutRHIM) and the influenza A virus (IAV) in mice (Upton et al. 2012; Kuriakose et al. 2016; Thapa et al. 2016). Activation of the ZBP1 receptor could result in many different outcomes, including inflammatory signalling, IFN production, apoptosis, necroptosis and pyroptosis (Kuriakose et al. 2016).

The *in vivo* ligand for ZBP1 is unresolved. Like many other PRRs, overexpression of ZBP1 leads to its activation in cells (Kaiser et al. 2008; Rebsamen et al. 2009). Structural studies found that the ZBDs of ZBP1 are specialised for binding the Z-form DNA (Schwartz et al. 2001). In addition, a region close to the $Z\beta$ domain of ZBP1 is found to have B-DNA binding ability (Takaoka et al. 2007). As an evidence, the double-strand B-DNA ligand poly(dA:dT) is able to activate ZBP1 (Takaoka et al. 2007).

As the physiological existence and function of Z-DNA in mammalian cells are not clear, it is suggested that ZBDs may bind B-DNA and convert it to Z-DNA (Ravichandran et al. 2019). Recently, ZBP1 is proposed to sense Z-RNA during MCMV-M45mutRHIM infection, as transcriptional activities after MCMV infection is required for activation of ZBP1 (Sridharan et al. 2017; Maelfait et al. 2017). As the physiological appearance of Z-RNA is not clear either, it is possible that ZBP1 binds A-form RNA and converts it to Z-form (Maelfait et al. 2017).

ZBP1-induced inflammatory signalling

ZBP1 dimerises on binding its ligand (Wang et al. 2008; Ha et al. 2008). B-DNA-activated ZBP1 recruits TBK1 through its C-terminus, leading to the phosphorylation of IRF3 to amplify the production of IFN β (Figure 1.11) (Takaoka et al. 2007). ZBP1-induced IFN β and ISG expression was found to be dependent on RIPK1 and RIPK3, while how they are involved is not clear (Kaiser et al. 2008; Daniels et al. 2018).

In HEK293T cells, ZBP1 expression activates NF- κ B signalling. ZBP1-induced NF- κ B activation is dependent on the RHIM-mediated recruitment of RIPK1 and RIPK3 (Kaiser et al. 2008; Rebsamen et al. 2009). How RIPK1 and RIPK3 contribute to inflammatory signalling after ZBP1 recruitment is not clear.

ZBP1-induced cell death responses

ZBP1 activation by MCMV-M45mutRHIM drives necroptosis that is dependent on RIPK3 but not the kinase activity of RIPK1 (Upton et al. 2012). On the contrary, RIPK1 was found to counteract ZBP1-RIPK3-induced necroptosis, possibly by the competitive binding of RHIM (Figure 1.11) (Newton et al. 2016; Lin et al. 2016).

While MCMV infection induces ZBP1-mediated necroptosis, IAV infection induces a mixture of ZBP1-mediated apoptosis and necroptosis in murine fibroblasts, as demonstrated by the involvement of both FADD-caspase-8 and RIPK3-MLKL axes in the process (Thapa et al. 2016). RIPK1 is required to recruit FADD and caspase-8 for ZBP1-induced apoptosis (Figure 1.11) (Thapa et al. 2016).

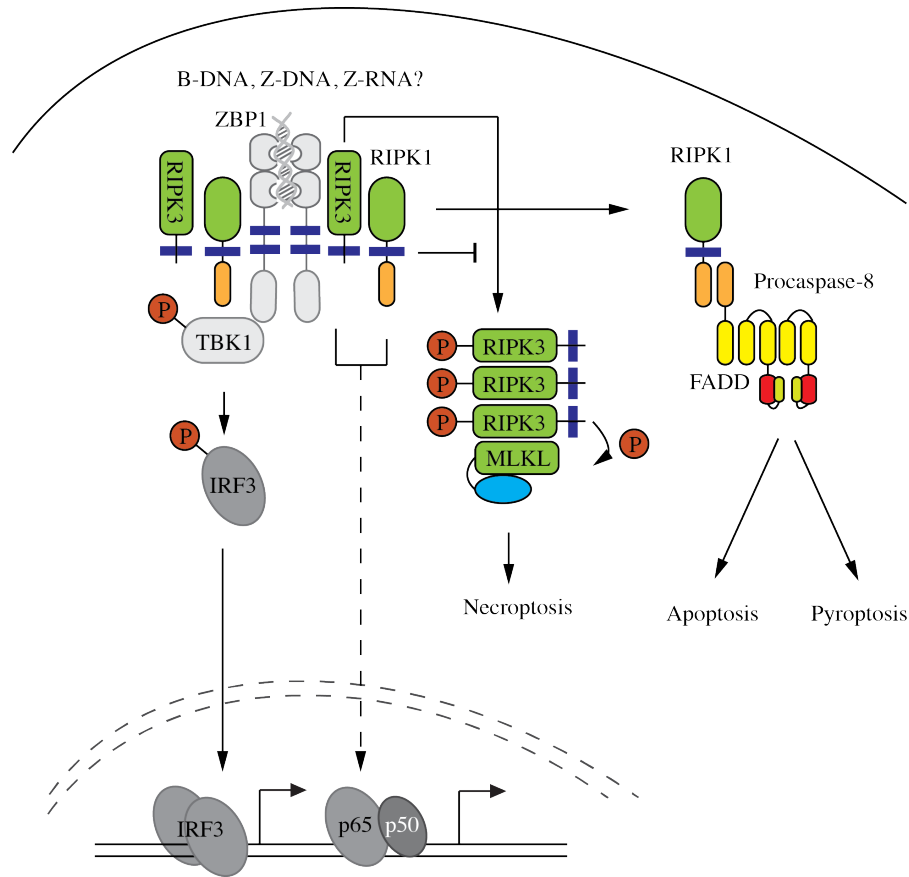


Figure 1.11: The ZBP1 signalling pathway. Activation of ZBP1 can activate the TBK1-IRF pathway in a process that is so far not clear. Activated ZBP1 recruits RIPK1 and RIPK3 to mediate inflammatory signalling and cell death responses. ZBP1 overexpression-induced NF- κ B activation requires both RIPK1 and RIPK3. ZBP1-induced necroptosis in mice is mediated by its interaction with RIPK3 and antagonised by the RHIM of RIPK1. ZBP1-induced apoptosis depends on the RIPK1-FADD-caspase-8 complex. The mechanism of ZBP1-induced pyroptosis is not clear but likely similar to TLR-induced pyroptosis.

In contrast to fibroblasts, IAV-activated ZBP1 in bone marrow derived macrophages (BMDMs) induces a mixture of apoptosis and pyroptosis, demonstrated by both the activation of caspase-3 and the NLRP3-dependent activation of caspase-1 and production of IL-1 β (Kuriakose et al. 2016). It is likely that pyroptosis is initiated with caspase-8 cleavage of GSDMD, and that the inflammasome is formed after membrane rupture, in the same way as TRIF-mediated pyroptosis.

In summary, the outcome and mechanisms of the ZBP1 signalling pathway are context-dependent. While ZBP1 expression leads to RIPK1- and RIPK3-dependent NF- κ B activation in HEK293T cells, ZBP1 activation in murine cells leads to necroptosis, apoptosis and pyroptosis depending on the cell type.

1.4 Receptor interacting protein kinase 3

1.4.1 Scaffolding kinases in inflammatory signalling

In the above examples of inflammatory signalling pathways, scaffolding protein kinases RIPK1, RIPK3 and IRAKs play a central regulatory role. With both kinase activity and scaffolding function, scaffolding kinases could contribute to inflammatory signalling in three ways.

First, scaffolding kinases can act as adaptor proteins by connecting the upstream and downstream components of the inflammatory signalling pathway with their scaffolding domains, and by serving as substrates for ubiquitylation.

Second, scaffolding kinases could activate components of the inflammatory signalling pathway using their kinase activity, e.g., IRAKs phosphorylate the ubiquitin ligase Pellino 1 (Ordureau et al. 2008).

Third, scaffolding kinases could regulate the switch between different responses through their scaffolding or kinase activity. For example, RIPK1 functions as an adaptor protein for ubiquitylation in TNFR1-mediated inflammatory responses independently of its kinase activity, and the activation of its kinase activity promotes cell death (He and Wang 2018).

Due to their key roles in inflammatory signalling, scaffolding kinases are potential targets for drug development for chronic inflammatory diseases (Rhyasen and Starczynowski 2015; Degterev et al. 2019).

There are 2 major families of scaffolding kinases, the IRAKs and the RIPKs. IRAKs (IRAK1 to IRAK4) are core mediators of TLR and IL-1R signalling pathways, while the function of RIPKs is more diverse (Rhyasen and Starczynowski 2015).

1.4.2 The receptor interacting protein kinase family

The RIPK family consists of 7 members, RIPK1 to RIPK7. Of them, RIPK1, RIPK2 and RIPK3 are characterised in the regulation of innate immune pathways. They all contain an N-terminal kinase domain followed by scaffolding domains that mediate their recruitment to immune receptors.

The role of RIPK1 has been extensively described in Chapter 1.3. RIPK2 is a central adaptor for NOD2-mediated inflammatory signalling, a NLR-family intracellular PRR that detects a bacteria cell wall component, muramyl dipeptide (MDP). RIPK2 is recruited to NOD2 via homotypic interaction between their CARDs and mediates NOD2-induced MAPK- and NF- κ B activation by serving as the ubiquitylation substrate. Although RIPK2 undergoes autophosphorylation after its recruitment to NOD2, its kinase activity is not required for NOD2-mediated inflammatory signalling (Hrdinka et al. 2018).

While RIPK3 was initially characterised in the necroptosis pathway, the discovery that it is directly recruited to ZBP1 indicates its potential function as a scaffolding kinase in ZBP1-induced inflammatory signalling and cell death responses.

1.4.3 The structure of RIPK3

Full-length human RIPK3 has 518 amino acid residues (aa). It contains an N-terminal kinase domain (aa 21-287) that is homologous among the RIPK family and a C-terminal RHIM (aa 440-451), the core residues of which are VQVG (aa 458-461) (He and Wang 2018).

The kinase domain of RIPK3 has a canonical kinase fold of an N-lobe, a C-lobe and an intervening activation loop (Xie et al. 2013). Studies on murine RIPK3 proposed that RIPK3 can exist in active and inactive conformations, the former is required for both its kinase activity and its RHIM-mediated interaction with RIPK1. Dimerisation of its kinase domain induces RIPK3 to adopt an active conformation, while interaction with the pseudokinase domain of its substrate, MLKL, induces RIPK3 to adopt an inactive conformation, when further activation signal is required for necroptosis to take place (Raju et al. 2018; Xie et al. 2013).

1.4.4 The function of RIPK3

As detailed in Chapters 1.2.2 and 1.3, one major function of RIPK3 is mediating necroptosis. In addition, RIPK3 has been proposed to activate apoptosis and NF- κ B-mediated inflammatory signalling.

RIPK3-mediated apoptosis was described with inhibitor-bound or D161N-mutant murine RIPK3 (mRIPK3) (Newton et al. 2014; Mandal et al. 2014). Although mutations of the ATP-binding site K50 (K51 on human RIPK3), magnesium-binding site D161 (D160 on human RIPK3) or the catalytic aspartate D143 (D142 on human RIPK3) all result in the loss of kinase activity, only the D161N-mutant or kinase inhibitor GSK'872-bound murine RIPK3 promotes apoptosis through engaging a complex containing RIPK1, FADD, caspase-8 and FLIP (Mandal et al. 2014). The gain-of-function D161N mutant or GSK'872-bound RIPK3 was proposed to adopt an active conformation, which facilitates its interaction with RIPK1 and engagement with the apoptosis-inducing complex, while K50- or D143-mutant RIPK3 was proposed to adopt an inactive conformation (Raju et al. 2018). However, D160N or GSK'872-bound human RIPK3 does not induce apoptosis, indicating a difference between the kinase domain structures of human and murine RIPK3 (Mandal et al. 2014; Geserick et al. 2015).

When it was cloned, RIPK3 expression was found to induce the expression of NF- κ B luciferase reporter (Yu et al. 1999). With the identification of RIPK3 as a necroptosis executioner, the role of RIPK3 in promoting NF- κ B signalling was set aside. Recently, it was rediscovered that RIPK3 could mediate inflammatory signalling independently of necroptosis in LPS- and TNF-induced RIPK3 activation systems, where the kinase activity of RIPK1 and RIPK3 is required (Najjar et al. 2016; Zhu et al. 2018). In addition, induced oligomerisation of murine RIPK3 was shown to activate NF- κ B signalling through RIPK1, independently of its necroptosis-inducing function (Yatim et al. 2015). How RIPK3 promotes inflammatory signalling remains elusive.

1.5 Aims of the study

Necroptosis and the activation of RIPK3 have been implicated in many pathological conditions, including ischemia reperfusion, sepsis, neurodegenerative diseases and chronic inflammatory diseases, rendering the necroptosis pathway a therapeutic target (Linkermann et al. 2013; Duprez et al. 2011; Zhang et al. 2017; Welz et al. 2011; Günther et al. 2011). In addition, introducing cells undergoing RIPK3-mediated cell death and inflammatory signalling into the tumour microenvironment has been shown

to boost antitumour immune responses, providing a way to augment the existing anti-PD1 therapy (Snyder et al. 2019).

To elucidate how RIPK3 mediates inflammatory signalling could help in understanding existing anti-necroptosis therapies as well as guiding the development of new ways to target RIPK3-mediated processes. Therefore, the aim of my DPhil study is to investigate the molecular mechanisms that control RIPK3-mediated inflammatory signalling.

2

Materials and methods

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2.1 Materials

2.1.1 Antibodies

The following antibodies were used in this study (Tables 2.1 and 2.2).

Table 2.1: Primary antibodies

Antibodies	Supplier	Catalogue number
Anti-2A peptide	Millipore	ABS31
Anti-Actin, clone C4	Millipore	MAB1501
Anti-Caspase-8, clone C15	Adipogen	AG-20B-0057
Anti-Caspase-8 (1C12)	Cell Signaling	9246
Anti-Caspase-8 (12F5)	Enzo	ALX-804-242-C100
Anti-Caspase-8 (D35G2)	Cell Signaling	4790
Anti-cIAP1	Enzo	ALX-803-335
Anti-FADD	Cell Signaling	2782S
Anti-Flag	Sigma	F1804
Anti-FLIP	Cell Signaling	3210
Anti-HOIP	R&D	AF8039
Anti-I κ B α	Cell Signaling	9242
Anti-JNK	Cell signaling	9258
Anti-MLKL	Cell Signaling	14993
Anti-p38 MAPK	Cell Signaling	9212
Anti-p65	Cell Signaling	8242
Anti-p44/42 MAPK (ERK1/2)	Cell Signaling	4695
Anti-Phospho-I κ B α	Cell Signaling	2859
Anti-Phospho JNK (pT183/pY185)	BD	612540
Anti-Phospho-MLKL (Ser358)	Cell Signaling	91689
Anti-Phospho-p38 MAPK	Cell Signaling	4511
Anti-Phospho-p44/42 MAPK (ERK1/2)	Cell Signaling	4370
Anti-Phospho-p65	Cell Signaling	3033
Anti-Phospho-RIPK1 (S166)	Cell Signaling	65746
Anti-Phospho-RIPK3 (S227)	Abcam	ab209384
Anti-RIPK1	Cell signaling	3493s
Anti-RIPK3	Cell Signaling	13526

Continuation of Table 2.1

Antibody	Supplier	Catalogue number
Anti-RIPK3	Santa Cruz	sc-47368
Anti-Tet repressor	Novus	NB600-234
Anti-TRIF	Cell Signaling	4596
Anti-Tubulin	Santa Cruz	sc-17787
Anti-ZBP1 (Zippy-1)	Adipogen	AG-20B-0010

Table 2.2: Secondary antibodies

Antibodies	Supplier	Catalogue number
Anti-Goat IgG-HRP	Santa Cruz	sc-2354
Anti-Mouse immunoglobulins-HRP	Dako	P0447
Anti-Rabbit IgG (H+L) -HRP	Bio-Rad	1706515
Anti-Rat IgG (H+L)-HRP	ThermoFisher	31470
Anti-Sheep IgG-HRP	R&D Systems	HAF016

2.1.2 Plasmids

The following plasmids were used in this study (Table 2.3).

Table 2.3: Plasmids used in this study

Plasmid	Insert	Backbone	Source
LZRS-zeo-RIPK3-2xFV-2A-mCherry	RIPK3-2xFV-2A-mCherry	LZRS-zeo	A. Oberst (UW, Seattle)
LZRS-zeo-RIPK3dC-2xFV-2A-mCherry	RIPK3 (aa 1-451)-2xFV-2A-mCherry	LZRS-zeo	A. Oberst (UW, Seattle)
NF- κ B reporter plasmid	Luciferase	pBIIX	Saksela and Baltimore 1993
RIP1 CRISPR/Cas9 KO plasmids gRNAa	RIPK1 gRNAa	-	Santa Cruz
RIP1 CRISPR/Cas9 KO plasmids gRNAb	RIPK1 gRNAb	-	Santa Cruz
RIP1 CRISPR/Cas9 KO plasmids gRNAc	RIPK1 gRNAc	-	Santa Cruz
pBabe-puro	NA	pBabe	M. Jaattela (Copenhagen)
pBabe-puro-RIPK3-Flag	RIPK3-Flag	pBabe	This study
pBabe-puro-RIPK3-2xFV-Flag	RIPK3-2xFV-Flag	pBabe	This study
pBabe-puro-RIPK3dC-2xFV-Flag	RIPK3dC-2xFV-Flag	pBabe	This study

Continuation of Table 2.3

Plasmid	Insert	Backbone	Source
pDestination-Tet-GFP-NES	GFP-NES	pDestination Tet	Gyrd-Hansen lab
pDestination-Tet-GFP-K63-SUB-NES	GFP-K63-SUB-NES	pDestination Tet	Gyrd-Hansen lab
pDestination-Tet-GFP-M1-SUB-NES	GFP-NES-M1-SUB-NES	pDestination Tet	Gyrd-Hansen lab
pLenti-CMV-TetR	Tet Repressor	pLenti-CMV	Gyrd-Hansen lab
pLenti6.3-ZBP1-wt-V5	ZBP1-V5	pLenti6.3	J. Rehwinkel (Oxford)
pLenti6.3-ZBP1-ZBDMut-V5	ZBP1-ZBDMut-V5	pLenti6.3	J. Rehwinkel (Oxford)
pLKO.1-puro/shCaspase-8-1	Caspase-8 shRNA-A	pLKO.1	Sigma TRCN0000377309
pLKO.1-puro/shCaspase-8-5	Caspase-8 shRNA-B	pLKO.1	Sigma TRCN0000376481
pLKO.1-puro/shMLKL-1	MLKL shRNA-1	pLKO.1	Sigma TRCN0000196317
pLKO.1-puro/shMLKL-2	MLKL shRNA-2	pLKO.1	Sigma TRCN0000196741
pLKO.1-puro/shMLKL-3	MLKL-shRNA-3	pLKO.1	Sigma TRCN0000194846
pLKO.1-puro/shMM	Control shRNA	pLKO.1	Sigma SHC002
pMD.G	VSVG	pMD.G	B. Jordan (Einstein, NY)
psPAX2	HIV-pol, HIV-gag	psPAX2	B. Jordan (Einstein, NY)
SV40-Renilla plasmid	Renilla luciferase	pRL	Gyrd-Hansen lab

2.1.3 Cell lines

The following cell lines were used in this study (Table 2.4).

Table 2.4: Cell lines

Cell line	Source
HEK293FT	Gyrd-Hansen lab
HEK293FT/mCherry	This study
HEK293FT/RIPK3-2xFV	This study
HEK293FT/RIPK3dC-2xFV	This study
HCT116	Gyrd-Hansen lab
HCT116/HOIP-KO	Gyrd-Hansen lab
HCT116/HOIP-KO/RIPK3-2xFV	This study
HCT116/RIPK1-KO clone 8c	This study
HCT116/RIPK1-KO clone 8c/RIPK3-2xFV	This study
HCT116/RIPK1-KO clone 12c	This study

Continuation of Table 2.4

Cell line	Source
HCT116/RIPK1-KO clone 12c/RIPK3-2xFV	This study
HCT116/RIPK1-KO clone 12c/RIPK3dC-2xFV	This study
HCT116/RIPK1-KO clone 15c/RIPK3-2xFV	This study
HCT116/RIPK3-2xFV	This study
HCT116/RIPK3-2xFV/shCasp8-A	This study
HCT116/RIPK3-2xFV/shCasp8-B	This study
HCT116/RIPK3-2xFV/shMLKL-1	This study
HCT116/RIPK3-2xFV/shMLKL-2	This study
HCT116/RIPK3-2xFV/shMLKL-3	This study
HCT116/RIPK3-2xFV/shMM	This study
HCT116/RIPK3-D142N-2xFV	This study
HCT116/RIPK3-K50A-2xFV	This study
HCT116/RIPK3-RHIM ^{AAA} -2xFV	This study
HCT116/shCtrl	Gyrd-Hansen lab
HCT116/shCtrl/RIPK3-2xFV	This study
HCT116/shCtrl/RIPK3-D328A-2xFV	This study
HCT116/shCYLD	Gyrd-Hansen lab
HCT116/shCYLD/RIPK3-2xFV	This study
HCT116/shCYLD/RIPK3-D328A-2xFV	This study
HCT116/Tet-On-GFP	This study
HCT116/Tet-On-GFP-K63-SUB	This study
HCT116/Tet-On-GFP-M1-SUB	This study
HCT116/Tet-On-GFP/RIPK3-2xFV	This study
HCT116/Tet-On-GFP-K63-SUB/RIPK3-2xFV	This study
HCT116/Tet-On-GFP-M1-SUB/RIPK3-2xFV	This study
HCT116/XIAP-KO	B. Vogelstein (Baltimore)
HCT116/XIAP-KO/RIPK3-2xFV	This study
HT29	Gyrd-Hansen lab
HT29/Tet-On-ZBP1	J. Maelfait (Ghent)
HT29/Tet-On-ZBP1-ZBDmut	J. Maelfait (Ghent)
HT29/RIPK1-KO clone aA3	This study
HT29/RIPK1-KO clone aA3/RIPK3-2xFV	This study
HT29/RIPK3-2xFV	This study
HT29/RIPK3-2xFV/shCasp8-A	This study
NIH3T3/mRIPK3-2xFV	A. Oberst (UW, Seattle)
NIH3T3/mRIPK3dC-2xFV	A. Oberst (UW, Seattle)
Phoenix-Ampho	Gyrd-Hansen lab
U2OS/NOD2	Gyrd-Hansen lab
U2OS/NOD2/RIPK3	This study
U2OS/NOD2/RIPK3-2xFV	This study
U2OS/NOD2/RIPK3dC-2xFV	This study
U2OS FlpIn	Gyrd-Hansen lab
U2OS/RIPK1-KO clone a	This study

Continuation of Table 2.4

Cell line	Source
U2OS/RIPK1-KO clone a/RIPK3-2xFV	This study
U2OS/RIPK1-KO clone b	This study
U2OS/RIPK1-KO clone b/RIPK3-2xFV	This study
U2OS/RIPK1-KO clone c	This study
U2OS/RIPK1-KO clone c/RIPK3-2xFV	This study
U2OS/RIPK3-2xFV	This study

2.1.4 Reagents

The sources of reagents used in this study are listed in Table 2.5.

Table 2.5: Reagents

Reagent	Supplier	Code
0.45 μ m cellulose acetate syringe filter	Sartorius stedim	16555-K
13.2ml polyallomer Beckman centrifuge tubes	Beckman Coulter	331372
30% w/v Acrylamide Bis-Acrylamide solution	Severn Biotech	20-2100-10
(5Z)-7-Oxozeaenol (TAK1 inhibitor)	Tocris	3604
Acetic acid	Fisher	A/0360/PB17
Anchored oligo(dT) (T18VN)	Sigma	Custom synthesised
Agarose	Web Scientific	AGR-500
Ammonium persulphate	Sigma-Aldrich	A3678
Ampicillin	Sigma-Aldrich	A9518
B/B Homodimerizer (AP20187, Dimeriser)	Clontech	635059
Benchmark pre-stained protein ladder	Novex	10748010
Bovine serum albumin (BSA)	Sigma	A9647
Protein assay dye reagent concentrate	Bio-Rad	500-0006
Brij-35 (30% w/v)	Acros	329581000
Bromophenol blue	Fisher	BP510-500
CellTiter-Glo 2.0 Cell Viability Assay	Promega	G9242
Cloning discs	Sigma	Z374431-100EA
cOmplete protease inhibitor cocktail tablets	Roche	04 693 124 001
Cutsmart buffer	NEB	B7204S
Culturplate-96 white opaque 96-well microplate	PerkinElmer	6005680
Dimethyl sulphoxide (DMSO)	Sigma	D2650
Dithiothreitol (DTT)	Sigma	43819
DMEM, high glucose, GlutaMAX, pyruvate	Thermo Fisher	31966-021
dNTPs	NEB	N0446S
Doxycycline	Sigma-Aldrich	D3072
Dried skimmed milk	Marvel	3023034
Dual-Luciferase Reporter Assay System	Promega	E1960
DuoSet ELISA Ancillary Reagent Kit 2	R&D Systems	DY008

Continuation of Table 2.5

Reagent	Supplier	Code
DuoSet human CXCL1/GRO α ELISA kit	R&D Systems	DY275
DuoSet human IL-8/CXCL8 ELISA kit	R&D Systems	DY208
Dulbecco's phosphate-buffered saline (DPBS)	ThermoFisher	14190
ECL western blotting detection reagent	GE healthcare	RPN2209
ECL Select western blotting detection reagent (enhancer)	GE healthcare	RPN2235
EcoRI-HF	NEB	R3101
Ethylenediaminetetraacetic acid (EDTA)	Fluorochem	239931
Ethanol	Merck	32221
Foetal bovine serum (FBS)	Labtech	FCS-SA
FuGENE 6	Promega	E2692
FuGENE HD	Promega	E2312
Gel Filtration Markers Kit	Sigma	MWGF1000
Gentamycin	Lonza	BE02-12E
Glycerol	Honeywell	G7757
Glycine	Fisher	G/0800/60
GSK'872	Merk Millipore	5.30389.001
Hexadimethrine bromide (polybrene)	Sigma	H9268
Hoechst 33342	Thermo Fisher	62249
Human TNF- α neutralising antibody	R&D	MAB210
Hydrochloric acid (HCl)	Fisher	H/1200/PB15
IKK inhibitor VII	Millipore	401486
IKK inhibitor XII	Millipore	401491
IPEGAL CA-630 (NP-40)	Sigma-Aldrich	I3021
IPTG	Sigma	I6758
Lambda phosphatase	NEB	P0753S
LB medium	Ludwig Institute	-
LB-Agar	Ludwig Institute	-
Lipofectamine RNAiMAX	Invitrogen	100014472
McCoy's 5A (modified), GlutaMAX medium	Thermo Fisher	36600
Micro BioSpin columns	BioRad	7326204
MluI	NEB	R0198S
MluI-HF	NEB	R3198S
MOPS running buffer	Invitrogen	NP0001-02
Mouse IgG Agarose	Sigma	A0919
Mouse IgG (for IP)	Santa Cruz	sc-2025
Mouse IgG1 isotype control (for stimulation)	R&D	MAB002
MycoAlert mycoplasma detection kit	Lonza	LT07-318
N-Ethylmaleimide (NEM)	Sigma	E1271
NEBuffer 3.1	NEB	B7203S
Necrostatin-1s (Nec1s)	Enzo	BV-2535-1
Necrosulfonamide (NSA)	R&D Systems	5025/10
Nitrocellulose membrane	GE Healthcare	10600002

Continuation of Table 2.5

Reagent	Supplier	Code
Nucleospin gel and PCR clean-up kit	Machery-Nagel	740609.250
Nucleospin plasmid miniprep kit	Machery-Nagel	740588.250
NuPAGE 4-12% Bis-Tris midi gels	Invitrogen	WG1403BX10
OptiMEM	Gibco	31985
pGEM-T-Easy vector systems	Promega	A1360
Polyethylene glycol (PEG)-8000	Aldrich	P2139
Phosphate buffered saline (PBS) (10x)	Fisher	BP399-20
Penicillin/Streptomycin (5000 U/mL)	Thermo Fisher	15070
Pentadecamers	IDT	169190224
peqGREEN DNA dye	Peqlab	37-5010
PhosSTOP phosphatase inhibitor cocktail tablets	Roche	04 906 837 001
Phusion polymerase	NEB	M0530L
Phusion polymerase reaction buffer (5x)	NEB	B0518S
Pierce Silver Stain for Mass Spectrometry	Pierce	24600
PMP buffer (10x)	NEB	B0761S
Propidium Iodide (PI) Solution	BioLegend	421301
Protein A/G PLUS agarose beads	Santa Cruz	sc-2003
Purelink HiPure Midiprep kit	Invitrogen	K210014
Puromycin	Invivogen	ant-pr
PVDF membrane	GE healthcare	10600023
QIAprep spin miniprep kit	Qiagen	27106
Quick-gDNA Blood prep Microprep kit	Zymo research	D3071
Q-VD-Oph (QVD)	R&D	OPH001
ReBlot Plus Strong Antibody Stripping Solution (10x)	Millipore	2504
RevertAid reverse transcriptase with 5x Reaction buffer	Thermo	EP0441
RiboLock	Thermo	EO0381
RIP CRISPR/Cas9 KO plasmids	Santa Cruz	sc-400377
RNase-free DNase set	Qiagen	79254
RNeasy Mini kit	Qiagen	74106
SalI	NEB	R0138S
SalI-HF	NEB	S3138S
SeeBlue prestained protein ladder	Invitrogen	LC5925
Shrimp Alkaline Phosphatase (rSAP)	NEB	M0371S
Sodium azide	Sigma	S2002
Sodium chloride	SLS	CHE3316
Sodium deoxycholate	Sigma-Aldrich	D6750
Sodium dodecyl sulphate (SDS)	Fisher	1281-1680
Sucrose	Amresco	0335-500
SYBR Select master mix	Applied Biosystems	4472908
Sytox Green	Thermo Fisher	S7020

Continuation of Table 2.5

Reagent	Supplier	Code
T4 DNA ligase	NEB	M0202L
T4 ligation buffer (10x)	NEB	B0202S
Taq DNA polymerase PCR buffer (10x)	Invitrogen	18067017
Taq polymerase	Invitrogen	18038
Tetramethylethylenediamine (TEMED)	Amresco	0761
TNF (recombinant human)	Peprotech	300-01A
TOP10 competent cells	Gyrd-Hansen lab	-
Trichloroacetic acid (TCA)	Acros	421451000
Tris base	Fisher	BP152-1
Trypsin-EDTA (0.05%), phenol red	Gibco	25300
Tween-20	Fisher	BP337-500
Urea	Prolabo	28877.292
X-gal	Invitrogen	15520-018
Zeocin	Invitrogen	R250
z-VAD(OH)-fmk (zVAD)	Santa Cruz	sc-311560

The sequences and sources of small-interfering RNAs (siRNAs) used in this study are listed below (Table 2.6).

Table 2.6: siRNAs

siRNA	Source	Sequence
siCtrl (for caspase-8)	Sigma SIC001	mismatch
siCtrl (for TRIF)	Invitrogen 12935-300	mismatch
siCasp8-a	Sigma SASI_Hs02_00314221	CAUCUCAGUUCACUGGUUU[dT][dT]
siCasp8-b	Sigma SASI_Hs02_00314222	GAUUUAUCAUACCUCAAA[dT][dT]
siTRIF-A	Invitrogen TICAM1HSS152364	CCAUGAUGAGCAACCUCACGCGACA
siTRIF-B	Invitrogen TICAM1HSS152365	CCCAUUGACGGUGUUUCGGACUGGA
siTRIF-C	Invitrogen TICAM1HSS175528	UCCAUCACUCCUAGCGCCUUCGACA

2.1.5 Buffer composition

The following buffers were prepared with the indicated composition (Table 2.7).

Table 2.7: Homemade buffer composition

Buffer	Composition
20% sucrose buffer	20% (w/v) sucrose, 50mM Tris (pH 7.4), 150mM NaCl, 5mM EDTA, 1% Triton X-100

Continuation of Table 2.7

Buffer	Composition
60% sucrose buffer	60% (w/v) sucrose, 50mM Tris (pH 7.4), 150mM NaCl, 5mM EDTA, 1% Triton X-100
Caspase-8 IP lysis buffer	20mM Tris-HCl (pH 7.5), 150mM NaCl, 1mM EDTA, 1% Triton X-100
Laemmli sample buffer (LSB)	50mM Tris (pH 6.8), 10% Glycerol, 100mM DTT, 2% SDS, 0.1% Bromophenol blue
PBS-Tween (PBST)	1.47 mM KH ₂ PO ₄ , 2.68 mM KCl, 136.9 mM NaCl, 7.97 mM Na ₂ HPO ₄ , 0.1% Tween-20
Resolving gel	10% or 12% Acrylamide-Bis-Acrylamide, 375mM Tris (pH 8.8), 0.1% SDS, 0.1% Ammonium persulfate, 0.004% TEMED
RIPA lysis buffer	50mM Tris (pH 7.5), 150mM NaCl, 1.0% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS
SDS running buffer	192 mM Glycine, 25 mM Tris base, 0.1 % SDS
Stacking gel	4% Acrylamide-Bis-Acrylamide, 125mM Tris (pH 6.8), 0.1% SDS, 0.1% Ammonium persulfate, 0.01% TEMED
Sucrose gradient lysis buffer	50mM Tris (pH 7.4), 150mM NaCl, 5mM EDTA, 1% Triton X-100
TAE buffer	40mM Tris (pH 8.5), 1mM EDTA, 20mM acetic acid
Tris-buffered saline (TBS)	20mM Tris-HCl (pH 7.4), 150mM NaCl

2.1.6 Instruments

The following instruments were used in this study (Table 2.8).

Table 2.8: Instruments used in this study

Instrument	Supplier	Code
CLARIOstar Plus microplate reader	BMG LABTECH	-
FACSCanto	BD	-
LightCycler 480 Instrument II (qPCR machine)	Roche	05015243001
Mini-PROTEAN Tetra Vertical Electrophoresis Cell	Bio-Rad	1658004
Gradient master 108	Biocomp	-
Optima L-100 XP Ultracentrifuge	Beckman Coulter	-
Owl Semidry Electroblotting Systems	Thermo Fisher	HEP-1
PowerPac Basic Power Supply	Bio-Rad	1645050
PowerPac HC High-Current Power Supply	Bio-Rad	1645052
Refridgerated microcentrifuge	Eppendorf	5424R
SW41 Ti Swinging-Bucket Rotor	Beckman Coulter	331362
Veriti 96-Well Thermal Cycler (PCR)	Applied Biosystems	4375786
Zeiss LSM 710 Confocal microscope	Zeiss	-

2.2 Methods

2.2.1 Cell culture and stimulation

Cell culture

All cell lines were cultured at 37°C and 5% CO₂ in growth medium supplemented with 10% v/v foetal bovine serum (FBS), 60µg/ml penicillin and 100µg/ml streptomycin (PS). The growth medium for HT29 and HCT116 cells was McCoy's 5A (Modified) (Gibco), and for HEK293FT, Phoenix-Ampho and U2OS cells was DMEM (Gibco). Cells were subcultured every 2-3 days in T75 or T175 flasks (Corning) and tested for mycoplasma every month.

When subculturing, media was aspirated for removal before cells were washed once with Dulbecco's phosphate-buffered saline (Gibco). Cells were then dissociated with 0.02ml/cm² 0.05% Trypsin-EDTA (Gibco) at 37°C until they detached. The detached cells were then resuspended in complete growth media and replated at 10-20% confluency.

1-2 million cells per vial were cryopreserved for each cell line at early passages. HEK293FT- and U2OS-based cell lines were cryopreserved in 90% FBS+10% DMSO in a freezing container buffered with isopropanol overnight, and HCT116- and HT29-based cell lines were cryopreserved in 95% complete growth media+5% DMSO.

Cell stimulation

Unless otherwise stated, cells were pretreated with inhibitors or their solvent control for 1 hour (h) where applicable before stimulation. Treatment time refers to the time length after the stimulation. Compounds were used at the following concentrations unless otherwise stated (Table 2.9).

Table 2.9: Concentrations for cell stimulation

Compound	Description	Working concentration
(5Z)-7-oxozeaenol Compound A (CpA)	TAK1 inhibitor (TAK1i) SMAC mimetic	1µM 100nM for cell death assays and ELISAs, 2µM for qPCR
Doxycycline (Dox)	Induce protein expression	50ng/ml for ZBP1, 100ng/ml for GFP-SUBs
GSK'872	RIPK3 kinase inhibitor	10µM

Continuation of Table 2.9

Compound	Description	Concentration
IgG1 isotype control	Control for TNF neutralising antibody	0.5 μ g/ml
IKK Inhibitor VII	Used in combination with IKK inhibitor XII as IKKi	1 μ M
IKK Inhibitor XII	Used in combination with IKK inhibitor VII as IKKi	5 μ M
Nec1s	RIPK1 kinase inhibitor	10 μ M
NSA	MLKL oligomerisation inhibitor	1 μ M
Q-VD-Oph (QVD)	Pan-caspase inhibitor	20 μ M
TNF	TNFR1 ligand	2ng/ml
TNF-Ab	TNF neutralising antibody	0.5 μ g/ml
zVAD(OH)-fmk (zVAD)	Pan-caspase inhibitor	20 μ M

When stimulating, all compounds were diluted in 5 μ l OptiMEM for each well. Conditions in the same experiment were topped up with solvent so that each condition received the same amount of solvent per well.

2.2.2 Plasmid transfection

FuGENE HD (Promega) was used for the transfection of HEK293FT and Phoenix-Ampho cells. For transfection, OptiMEM (Gibco), FuGENE HD and plasmids were mixed at a ratio of 20 μ l : 1 μ l : 250ng. Mastermixes were made where possible. The mixture was incubated for 15-30 minutes (min) at room temperature before added dropwise to wells.

FuGENE 6 (Promega) was used for the transfection of HCT116 cells following the same protocol as FuGENE HD.

2.2.3 siRNA-mediated gene knockdown

For siRNA-mediated knockdown of protein expression, cells were plated at 40% confluency in 6-well plates. The next day, siRNA was transfected using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol at an amount of 600 μ l OptiMEM, 6.4 μ l Lipofectamine RNAiMAX and 0.16nmol siRNA per well. 24h after transfection, cells were split equally into 3 wells and cultured in complete growth medium

for 48h. 72h after transfection, one of the three wells in each condition was washed 2x with PBS and frozen at -80°C for western blot, while the other two were stimulated as indicated for qPCR.

2.2.4 Generation of cell lines

CRISPR/Cas9-mediated gene knockout

RIPK1-knockout (KO) clones were generated in multiple cell lines. For the generation of RIPK1-KO clones, RIPK1 CRISPR/Cas KO plasmid mix (Santa Cruz) was purchased and separated by transforming into TOP10 competent cells followed by picking single clones for miniprep and sequencing. The three different RIPK1 CRISPR/Cas KO plasmids numbered a, b and c were transfected separately into indicated cell lines.

For transfection, low-passage parental cells were seeded at 50% confluency in 6-well plates. The next day, each well was transfected with 2µg CRISPR/Cas9 knockout plasmid a, b or c. Media were replaced for all wells 24h after transfection. 36h after transfection, cells were resuspended in complete growth media with 50 µg/ml Gentamycin (Lonza) and sorted to collect the top 10% GFP-positive cells (Jenner Institute, Oxford). The collected cells were plated at 500-1000 cells per 15cm dish in complete growth media supplemented with 50 µg/ml Gentamycin and replaced with complete growth media one week later. Following this, media were replaced every week until colonies could be seen by naked eyes. Then, 48 colonies were transferred to 24-well plates using cloning disks to be expanded for western blotting and cryopreservation.

Selected knockout clones on western blots were genotyped for confirmation. Genome DNA were extracted with Quick-gDNA Blood prep Microprep kit (Zymo research) following the manufacturer's protocol. DNA sequence around the knockout locus was amplified by polymerase chain reaction (PCR) with Phusion-HF (NEB) using primers 5'-CCTGGGCGACATTTTACAAA-3' / 5'-GGCTAAGTCCTCACAAGCA-3' for guide RNA (gRNA)a- and gRNAb-transfected clones and 5'-TGTGAGGAAGTGAGAAACCG-3' / 5'-TGATCCTCCTGCCAAAAGTG-3' for gRNAc-targeted clones. The PCR products were excised from 1.2% agarose gels, purified with on-column purification system (Machery-Nagel), tailed with poly-adenosine using Taq DNA polymerase (Invitrogen)

and inserted into pGEM-T-Easy vector (Promega) by TA-cloning. After ligation, the products were transformed into TOP10 chemically competent cells and plated on LB Agar plates containing 100 μ g/ml Ampicilin (Sigma), 1mM IPTG (Signal) and 50 μ g/ml X-gal (Invitrogen) for selection. Multiple inserted plasmids were sequenced (Eurofins Genomics) for each knockout clone until the likelihood of the appearance of a new sequence was less than 0.05. Only knockout clones carrying frameshifting indels on all alleles were used in the study.

Retrovirus-based construction of stable cell lines

Retroviral particles were used for the stable expression of RIPK3-2xFV and its derivatives in multiple cell lines. For the production of viral particles, 3.5 million Phoenix-Ampho cells were plated in 10cm dishes in 15ml complete growth media. The next day, they were transfected with a mixture of 1.5ml OptiMEM, 36 μ l FuGENE HD, 1.2 μ g pMD.G (VSVG) and 10.8 μ g retroviral vector (see Table 2.10 for vectors used for each cell line). 24h after transfection, the transfection reagent-containing media were replaced with 8 ml complete growth media. 48h after transfection, virus-containing media were collected while cells were given another 8ml complete growth media to collect at 72h after transfection. Virus-containing media were filtered through a 0.45 μ m cellulose acetate syringe filter. Media were directly used for transduction whenever possible. The remaining media were topped up to 8.5 ml with complete growth media and mixed with 0.5 ml 3 M NaCl and 1 ml 50% PEG-8000 solution. Viral particles were precipitated by rotating overnight at 4°C followed by spinning down at 3500xg for 15 min at 4°C. The resulting pellet was resuspended in 150 μ l DPBS, aliquoted into 25 μ l per tube and stored at -80°C for future use.

For transduction, parental cells were plated at 100,000 or 300,000/well in 6-well plates. The next day, their media were replaced with 2ml complete growth media containing 1ml virus-containing supernatant or 1 aliquot of precipitated virus and 5 μ g/ml polybrene. One no-virus control well was set up for every transduction experiment. 24h after transduction, control or virus-containing media were replaced with complete growth media for every well. 48h after transduction, all wells were selected with corresponding antibiotic selection markers (see Table 2.10). Antibiotics-containing complete growth

media were replaced every 2-3 days until cells in the control well were completely killed. From then, antibiotics were kept for another two passages before cells were cryopreserved and used as stable cell lines.

Specially, HEK293FT/RIPK3-2xFV cells consist of one low mCherry fluorescence population and one high mCherry fluorescence population after selection. The mixed population was FACS-sorted to separate the two populations (Jenner Institute, Oxford). Western blotting found the high mCherry population to be RIPK3-free, which was then kept as HEK293FT/mCherry as control. The low-fluorescence population was used as HEK293FT/RIPK3-2xFV cells.

For the construction of HCT116/Tet-On cell lines, the transduced population were plated in 15cm dishes at 500 cells/dish after one week of selection. 24 clones were selected for western blot analysis. One clone with high Tet repressor expression level was selected for experiments.

Lentivirus-based construction of stable cell lines

Lentivirus vectors were used to construct shRNA-mediated stable knockdown cell lines and expression of Dox-inducible GFP-SUBs.

For the production of lentiviral particles, HEK293FT cells were plated in the same way as for retroviral particles. The next day, they were transfected with a mixture of 1.5ml OptiMEM, 36 μ l FuGENE HD, 6 μ g psPAX2 vector, 1.5 μ g pMD.G (VSVG) and 4.5 μ g pLKO.1-based lentiviral vector encoding the shRNA sequence (Sigma). 24h after transfection, transfection reagent-containing media were replaced with 10ml complete growth media. Three days after replacing media, virus-containing supernatant was filtered through a 0.45 μ m cellulose acetate syringe filter. The virus-containing supernatant was directly used for transduction whenever possible, and the remaining virus-containing supernatant was precipitated and preserved in the same way as described for retroviral particles.

For the transduction of lentiviral particles, parental cells were plated at 50,000/well in 12-well plates. The next day, their media were replaced with 1ml complete growth media containing 5 μ g/ml polybrene and 20 μ l virus-containing supernatant or 5 μ l precipitated

virus. One well of no-virus control was set up for every transduction experiment. 24h after transduction, control or virus-containing media were replaced with complete growth media for every well. 72h after transduction, all wells were selected with corresponding antibiotic selection markers (see Table 2.10). Antibiotics-containing complete growth media were replaced every 2-3 days until cells in the control well were completely killed. From then, antibiotics were kept on for another 2 passages before cells were sampled for western blotting and cryopreserved as stable cell lines.

Table 2.10: Construction of stable cell lines

Cell line	Vector	Parental	Selection
HEK293FT/RIPK3-2xFV	LZRS-zeo-RIPK3-2xFV-2A-mCherry	HEK293FT	Zeocin 0.25mg/ml
HEK293FT/RIPK3dC-2xFV	LZRS-zeo-RIPK3dC-2xFV-2A-mCherry	HEK293FT	Zeocin 0.25mg/ml
HCT116/HOIP-KO/RIPK3-2xFV	LZRS-zeo-RIPK3dC-2xFV-2A-mCherry	HCT116/HOIP-KO	Zeocin 0.25mg/ml
HCT116/RIPK1-KO clone 8c/RIPK3-2xFV	LZRS-zeo-RIPK3-2xFV-2A-mCherry	HCT116/RIPK1-KO clone 8c	Zeocin 0.25mg/ml
HCT116/RIPK1-KO clone 12c/RIPK3-2xFV	LZRS-zeo-RIPK3-2xFV-2A-mCherry	HCT116/RIPK1-KO clone 12c	Zeocin 0.25mg/ml
HCT116/RIPK1-KO clone 12c/RIPK3dC-2xFV	LZRS-zeo-RIPK3dC-2xFV-2A-mCherry	HCT116/RIPK1-KO clone 12c	Zeocin 0.25mg/ml
HCT116/RIPK1-KO clone 15c/RIPK3-2xFV	LZRS-zeo-RIPK3-2xFV-2A-mCherry	HCT116/RIPK1-KO clone 15c	Zeocin 0.25mg/ml
HCT116/RIPK3-2xFV	LZRS-zeo-RIPK3-2xFV-2A-mCherry	HCT116	Zeocin 0.25mg/ml
HCT116/RIPK3-2xFV/shCasp8-A	pLKO.1-puro/shCaspase-8-1	HCT116/RIPK3-2xFV	Zeocin 0.25mg/ml, puromycin 1µg/ml
HCT116/RIPK3-2xFV/shCasp8-B	pLKO.1-puro/shCaspase-8-5	HCT116/RIPK3-2xFV	Zeocin 0.25mg/ml, puromycin 1µg/ml
HCT116/RIPK3-2xFV/shMM	pLKO.1-puro/shMM	HCT116/RIPK3-2xFV	Zeocin 0.25mg/ml, puromycin 1µg/ml

Continuation of Table 2.10

Cell line	Vector	Parental	Selection
HCT116/RIPK3-2xFV/shMLKL-1	pLKO.1-puro/shMLKL-1	HCT116/RIPK3-2xFV	Zeocin 0.25mg/ml, puromycin 1 μ g/ml
HCT116/RIPK3-2xFV/shMLKL-2	pLKO.1-puro/shMLKL-2	HCT116/RIPK3-2xFV	Zeocin 0.25mg/ml, puromycin 1 μ g/ml
HCT116/RIPK3-2xFV/shMLKL-3	pLKO.1-puro/shMLKL-3	HCT116/RIPK3-2xFV	Zeocin 0.25mg/ml, puromycin 1 μ g/ml
HCT116/RIPK3-RHIM ^{AAAA} -2xFV	LZRS-zeo-RIPK3-RHIM ^{AAAA} -2xFV-2A-mCherry	HCT116	Zeocin 0.25mg/ml
HCT116/RIPK3-D142N-2xFV	LZRS-zeo-RIPK3-D142N-2xFV-2A-mCherry	HCT116	Zeocin 0.25mg/ml
HCT116/RIPK3-K50A-2xFV	LZRS-zeo-RIPK3-K50A-2xFV-2A-mCherry	HCT116	Zeocin 0.25mg/ml
HCT116/shCtrl/RIPK3-2xFV	LZRS-zeo-RIPK3-2xFV-2A-mCherry	HCT116/shCtrl	Zeocin 0.25mg/ml, puromycin 1 μ g/ml
HCT116/shCYLD/RIPK3-2xFV	LZRS-zeo-RIPK3-2xFV-2A-mCherry	HCT116/shCYLD	Zeocin 0.25mg/ml, puromycin 1 μ g/ml
HCT116/shCtrl/RIPK3-D328A-2xFV	LZRS-zeo-RIPK3-D328A-2xFV-2A-mCherry	HCT116/shCtrl	Zeocin 0.25mg/ml, puromycin 1 μ g/ml
HCT116/shCYLD/RIPK3-D328A-2xFV	LZRS-zeo-RIPK3-2xFV-D328A-2A-mCherry	HCT116/shCYLD	Zeocin 0.25mg/ml, puromycin 1 μ g/ml
HCT116/Tet-On clone C4	pLenti CMV TetR Blast	HCT116	Blasticidin 5 μ g/ml
HCT116/Tet-On-GFP	pDestination-Tet-GFP-NES	HCT116/Tet-On clone C4	Blasticidin 5 μ g/ml, puromycin 1 μ g/ml

Continuation of Table 2.10

Cell line	Vector	Parental	Selection
HCT116/Tet-On-GFP-K63-SUB	pDestination-Tet-GFP-K63-SUB-NES	HCT116/Tet-On clone C4	Blasticidin 5 μ g/ml, puromycin 1 μ g/ml
HCT116/Tet-On-GFP-M1-SUB	pDestination-Tet-GFP-M1-SUB-NES	HCT116/Tet-On clone C4	Blasticidin 5 μ g/ml, puromycin 1 μ g/ml
HCT116/Tet-On-GFP/RIPK3-2xFV	LZRS-zeo-RIPK3-2xFV-2A-mCherry	HCT116/Tet-On-GFP	Blasticidin 5 μ g/ml, puromycin 1 μ g/ml, zeocin 0.25mg/ml
HCT116/Tet-On-GFP-K63-SUB/RIPK3-2xFV	LZRS-zeo-RIPK3-2xFV-2A-mCherry	HCT116/Tet-On-GFP-K63-SUB	Blasticidin 5 μ g/ml, puromycin 1 μ g/ml, zeocin 0.25mg/ml
HCT116/Tet-On-GFP-M1-SUB/RIPK3-2xFV	LZRS-zeo-RIPK3-2xFV-2A-mCherry	HCT116/Tet-On-GFP-M1-SUB	Blasticidin 5 μ g/ml, puromycin 1 μ g/ml, zeocin 0.25mg/ml
HCT116/XIAP-KO/RIPK3-2xFV	LZRS-zeo-RIPK3-2xFV-2A-mCherry	HCT116/XIAP-KO	Zeocin 0.25mg/ml
HT29/RIPK3-2xFV	LZRS-zeo-RIPK3-2xFV-2A-mCherry	HT29	Zeocin 0.25mg/ml
HT29/RIPK3-2xFV/shCasp8-A	pLKO.1-puro/shCaspase-8-1	HT29/RIPK3-2xFV	Zeocin 0.25mg/ml, puromycin 1 μ g/ml
U2OS/NOD2/RIPK3	pBabe-puro-RIPK3-Flag	U2OS/NOD2	Puromycin 1 μ g/ml
U2OS/NOD2/RIPK3-2xFV	pBabe-puro-RIPK3-2xFV-Flag	U2OS/NOD2	Puromycin 1 μ g/ml
U2OS/NOD2/RIPK3dC-2xFV	pBabe-puro-RIPK3dC-2xFV-Flag	U2OS/NOD2	Puromycin 1 μ g/ml
U2OS/RIPK1-KO clone a/RIPK3-2xFV	pBabe-puro-RIPK3-2xFV-Flag	U2OS/RIPK1-KO clone a	Puromycin 1 μ g/ml
U2OS/RIPK1-KO clone b/RIPK3-2xFV	pBabe-puro-RIPK3-2xFV-Flag	U2OS/RIPK1-KO clone b	Puromycin 1 μ g/ml

Continuation of Table 2.10

Cell line	Vector	Parental	Selection
U2OS/RIPK1-KO clone c/RIPK3-2xFV	pBabe-puro-RIPK3-2xFV-Flag	U2OS/RIPK1-KO clone c	Puromycin 1 μ g/ml
U2OS/RIPK3-2xFV	pBabe-puro-RIPK3-2xFV-Flag	U2OS FlpIn	Puromycin 1 μ g/ml

2.2.5 Plasmid construction

Subcloning

For the subcloning of RIPK3-2xFV and its derivative sequences into pBabe-puro plasmids, sequences were amplified from the corresponding template with the indicated primers in Table 2.11. The forward primer carries a MluI restrictive digestion site, and the reverse primer carries a C-terminal Flag-tag sequence followed by a SalI restrictive digestion site. PCR reactions were set up with Phusion-HF DNA polymerase (NEB) following the manufacturer's protocol. PCR products were resolved on a 0.8% agarose gel and excised for purification.

Table 2.11: Templates and primers used for subcloning

Vector	Template	Primers
pBabe-puro-RIPK3-Flag	LZRS-zeo-RIPK3-2xFV-2A-mCherry	5'-acgcgtATGTCGTGCGTCAAGTTATG-3' 5'-gtcgacttactatcgtcgatcatccttgtaatc TTCCCGCTATGATTATACCAAC-3'
pBabe-puro-RIPK3-2xFV-Flag	LZRS-zeo-RIPK3-2xFV-2A-mCherry	5'-acgcgtATGTCGTGCGTCAAGTTATG-3' 5'-gtcgacttactatcgtcgatcatccttgtaatc TTCCAGTTTTAGAAAGCTCCAC-3'
pBabe-puro-RIPK3dC-2xFV-Flag	LZRS-zeo-RIPK3dC-2xFV-2A-mCherry	5'-acgcgtATGTCGTGCGTCAAGTTATG-3' 5'-gtcgacttactatcgtcgatcatccttgtaatc TTCCAGTTTTAGAAAGCTCCAC-3'

Purified PCR product and 5 μ g pBabe-puro plasmid were digested with MluI-HF (NEB) and SalI-HF (NEB) in CutSmart buffer (NEB) overnight at 37 °C. Shrimp alkaline phosphatase (rSAP) (NEB) was added into the system for the last 1h of restrictive enzyme digestion. Linearised plasmids were resolved on 0.8% agarose gel and excised

for purification. Digested PCR product was purified with the Gel and PCR cleanup kit (Machery-Nagel).

100ng linearised vector was used to set up the ligation reaction with a 7:1 molar ratio of PCR product to vector. Ligation was performed overnight with T4 DNA ligase in a total volume of 20 μ l, from which 5 μ l was used for transformation into TOP10 competent cells.

Around a dozen colonies were cultured in LB+Ampicillin media overnight before plasmids were extracted from them. Around 1 μ g extracted plasmids were digested with 3 units MluI (NEB) and SalI (NEB) for over 2h in 1xNEBuffer3.1 in 10 μ l. Digested vectors were resolved on 0.8% agarose gel to identify inserted clones, which were subsequently sequenced for verification.

Mutagenesis

Mutagenesis of RIPK3 was performed with Q5 site-directed mutagenesis kit (NEB) according to the manufacturer's protocol. The following primers were used for mutagenesis:

Table 2.12: Mutagenesis primers for RIPK3

Mutant	Primers
D142N	5' - CCTGCACCGG _a ACCTCAAGCC-3' 5'-AGCACCGGGTTCTGGTCG-3'
D328A	5' -ACAGAAATGG _c TGGCTTTAGGAGAAC-3' 5'-CCCTCCTTGGCCTGACTC-3'
K50A	5' -TGTGGCGGTC _g cGATCGTAAACTCG-3' 5' -TCGTAGCCCCACTTCCTA-3'
RHIM ^{AAAA}	5' -gctgcaGGAGACAACA _A ACTACTTG-3' 5' -tgccgcCCCAGAGCAGTTGTATATG-3'

Full-length of the coding region of the plasmid was sequenced to make sure the plasmids carry the desired mutation.

2.2.6 Cell lysis, immunoprecipitation and western blot

Cell lysis

For western blots, cells were lysed in RIPA buffer unless otherwise stated. Lysis buffer was supplemented with proteasome inhibitors. Phosphatase inhibitors were supplemented when blotting for phosphorylated proteins. The collected cells were washed 2x with

PBS and kept at -80°C prior to use. $10\mu\text{l}/\text{cm}^2$ lysis buffer was added to vessels unless otherwise stated. Cells were scraped in lysis buffer, and the lysates were transferred to eppendorf tubes and incubated on ice for at least 15min before spun down at over $16000\times g$ for 10min at 4°C . If used for western blots, 4x LSB was added to the cleared lysates, and the mixtures were heated at 95°C for over 5min.

Lambda-phosphatase treatment

Under indicated conditions, lambda-phosphatase (λPP) was used to confirm the phosphorylation modification on indicated proteins. Samples to be treated with λ -phosphatase were lysed in TBS+0.5% NP40 buffer supplemented with protease inhibitors. 10x PMP buffer (NEB), 1mM MnCl_2 (NEB) and 100x λ -phosphatase (NEB) were added to the lysates. The mixtures were incubated at 30°C for 30 min before adding LSB and heating at 95°C to make western blotting samples.

Immunoprecipitation

For caspase-8 immunoprecipitation, cells were plated in 10cm dishes per condition at 8.6 million/dish. The next day, cells were stimulated as needed, washed twice with 5ml PBS and lysed immediately or stored at -80°C until use.

0.5ml caspase-8 IP lysis buffer (see Table 2.7 for composition) supplemented with protease inhibitors and phosphatase inhibitors were used per dish for cell lysis. Cells were scraped in lysis buffer, transferred into 1.5ml EP tubes and incubated on ice for 30 min. The lysates were then centrifuged at 14000rpm , 4°C for 10 min. $30\mu\text{l}$ supernatant was mixed with 4x LSB and heated at over 95°C for 5min as input samples.

To reduce nonspecific binding, the remaining supernatant was incubated with $5\mu\text{l}$ mouse IgG-Agarose beads (Sigma) per sample for at least 30min at 4°C . After incubation, beads were discarded, while $1\mu\text{l}$ caspase-8 antibody (1F12) was added to each sample. The lysates and antibody were incubated rotating at 4°C overnight. Following incubation, $5\mu\text{l}$ protein A/G-coupled agarose beads (Santa Cruz) were added to each sample and incubated rotating at 4°C for at least 1h. After incubation, beads were collected in micro-spin columns while supernatants were removed. The beads were washed on column with

0.5ml lysis buffer each time for 4 times. After the last wash, the beads were centrifuged at 900xg, 2min to drain the remaining lysis buffer. Beads were then incubated in 30 μ l 2x LSB for 30min under room temperature before centrifuged again at 900xg, 2min to collect eluates. Eluates were heated at 95°C for 5min as pull-down samples.

For western blots, 10-15 μ l pull-down samples or 5 μ l input samples were loaded onto each lane.

Sucrose gradient assay

The sucrose gradient assay was performed under the supervision and instructions from Dr Emma Fenech in the Christianson lab (University of Oxford).

For sucrose gradient assays, cells were plated in 6cm dishes at 2 million/dish and stimulated as needed the next day. After stimulation, cells were lysed directly or snap-frozen as pellets and stored under -80°C until use.

Cells from each sample were lysed in 0.4ml sucrose gradient lysis buffer (see Table 2.7 for composition) supplemented with protease inhibitors and phosphatase inhibitors. Cells were incubated in lysis buffer on ice for 30min before centrifuged at 14000rpm, 10min, 4°C. 45 μ l cleared lysates were mixed with 4x LSB and heated at 95°C for 5min as input.

Bradford assays were used to measure protein concentrations in the lysates. The appropriate volume for 1mg protein was taken from samples, and topped up to 0.3ml with lysis buffer.

The continuous 20-60% (w/v) sucrose gradient was prepared in 13.2ml thin-wall polyallomer Beckman centrifuge tubes. 60% sucrose buffer (see Table 2.7 for composition) supplemented with protease inhibitors and phosphatase inhibitors was loaded until half the height of the tube. Then, 20% sucrose buffer (see Table 2.7 for composition) supplemented with protease inhibitors and phosphatase inhibitors was loaded until full tube capacity. The tube was then sealed and transferred onto the platform of Gradient Master 108. The 20-60% (w/v) program was selected for gradient formation.

After the formation of the gradient, 0.3ml buffer was removed from the top and replaced with 1mg lysate in 300 μ l lysis buffer. The loaded sucrose gradient tubes were fitted into SW41 rotor swing buckets (Beckman). Opposite swing buckets were balanced

with lysis buffer until the difference was not more than 0.02g. The cell lysates were centrifuged at 39000rpm, 4°C for 16h in Optima L-100 XP ultracentrifuge.

After centrifugation, buffer from each tube was separated into 12 sections by collecting 940 μ l from the top every time. To precipitate protein, each section was mixed thoroughly with 190 μ l 50% trichloroacetic acid and incubated on ice for 60 min. The precipitated protein was pelleted by centrifugation at 17000xg for 5 min at 4°C. The resulting pellet was washed twice with 1ml ice-cold acetone and airdried for 10min before resuspended by vortexing in 2x LSB. 1M Tris (pH 9.5) was used to adjust the pH of samples until Bromophenol-Blue in the LSB turned blue in every tube. The samples were then heated at 56°C for 10min for western blots.

For western blots, 20 μ l sample from each section and the equivalent volume of 20 μ g protein of input samples were resolved on 12% SDS gels.

As a reference for the size of protein complexes, standard proteins from the Gel Filtration Markers Kit (Sigma) were resolved on a 20-60% sucrose gradient following the same protocol (Table 2.13). Samples from fractions of the reference tube were resolved on a 6% handcast SDS gel and detected by silver stain using the Pierce Silver Stain for Mass Spectrometry kit following the manufacturer's protocol.

Table 2.13: Markers used for sucrose gradient

Protein	Molecular weight	Amount
Albumin	66kDa	15 μ g
Alcohol dehydrogenase (ADH)	150kDa	10 μ g
Beta-amylase	200kDa	15 μ g
Apo ferritin	443kDa	30 μ g
Thyroglobin	669kDa	30 μ g

Western blots

Samples were resolved on homecast 10% or 12% Bis-Tris gels or precast 4-12% gradient gels (Invitrogen). Sample made from approximately 1×10^5 cells was loaded onto each lane unless otherwise stated.

Samples on homecast gels were resolved in SDS running buffer at 120V until Bromophenol blue reached the stacking gel. Then they were resolved at 160V for

another 1h. Benchmark prestained protein ladder (Novex) was used to mark the size of proteins. Samples on precast gels were resolved in MOPS running buffer at 190V for 70min with SeeBlue prestained protein ladder (Invitrogen).

The resolved gels were transferred onto nitrocellulose membrane (GE) unless otherwise stated. Transfer was conducted at 100 mA/gel for homecast gels and 200mA/gel for precast gels, maximum voltage 24V, for 70min. The membrane was blocked in 5% w/v milk in PBST with 0.2% sodium azide for over 30min at room temperature (RT) or 4°C overnight. Primary antibodies were diluted in PBST with BSA and 0.2% sodium azide. Membranes were incubated in primary antibody overnight at 4°C, washed 3 times for 10min/time with PBST, incubated with secondary antibody diluted 1:10000 in PBST for 1h at room temperature and washed another 3 times for 10min/time with PBST. Membranes were then developed with 0.025ml/cm² ECL solution using Fuji films. For weak signals, 10-20% enhancer solution was added to the ECL solution.

Membranes were selected for reblotting when necessary. When reblotting with antibody of the same host species, membranes were incubated in stripping buffer (Millipore) for 15min, washed 3x10min with PBST, blocked for at least 30min before incubating with a different primary antibody. When reblotting with antibody of a different host species, membranes were directly washed 3x10min with PBST and incubated with the primary antibody.

2.2.7 Cell death assays

Cell viability assay

Cell viability was determined with CellTitre-Glo reagent (Promega) according to the manufacturer's protocol. Specifically, cells were plated in the central wells of opaque 96-well plates at 3300 cells/well for 72h time courses, and 13000 cells/well for 24h time courses in 100µl complete growth medium. Wells surrounding the used ones were filled with 100 µl DPBS to avoid media evaporation. The next day, cells were treated with indicated drugs diluted in 5µl OptiMEM per well. The plate was incubated under normal culturing conditions until measurement. When measuring, the plate and the CellTitre-Glo reagent were incubated at room temperature for 30min. The CellTitre-Glo

reagent was added at 100 μ l/well, shaken at 200rpm for 2min, incubated again for 10min before luminescence values were read at 1s/well on the microplate reader. Relative viability is calculated by dividing the average of technical replicates for each treatment condition by that of untreated wells.

Sytox Green permeability assay

Sytox Green permeability assays were performed to count the percentage of dead cells as measured by their membrane permeability to Sytox Green. HCT116-based cells were plated in transparent 96-well plates at 2000 cells/well, and 3 wells were plated for each stimulation condition as technical replicates. Cells were stimulated as planned. At the endpoint, 7.32 μ l 20mM Hoechst 33342 (Thermo Fisher) and 0.12 μ l 5mM Sytox Green (Invitrogen) were added to each well and incubated for 5min. Following incubation, the 96-well plate was imaged in 2 channels, Hoechst 33342 and Sytox Green, under the Zeiss LSM 710 confocal microscope. Fiji (ImageJ) was used to count the number of Hoechst 33342 positive nuclei and Sytox Green positive nuclei. A macro was compiled to automatically count all images. For each channel, the threshold was set to (550,65535) under the "Percentile dark no-reset" function, choosing "Dark background". Following this, the script ran "Convert to Mask" followed by "Watershed" to separate close nuclei. Finally, the number of nuclei was counted using the "Analyze Particles" function, setting the size to 20-500. The Sytox Green positive percentage was calculated by dividing the number of nuclei in the Sytox Green channel by that in the Hoechst 33342 channel.

2.2.8 Quantitative real-time PCR (qRT-PCR)

For qRT-PCR, cells were stimulated for 3h unless otherwise specified. RNA extraction from stimulated cells was performed with RNeasy Mini kit (Qiagen) and on-column DNase digestion was performed with the RNase-free DNase Set (Qiagen) according to the manufacturer's protocol. When not used immediately, stimulated cells were lysed in lysis buffer RLT from the kit, and the lysate was stored under -80°C until use. Following extraction, RNA concentrations were measured on Nanodrop and adjusted to 100ng/ μ l with RNase-free water from the kit.

1 μ g RNA was used for reverse transcription. In the first step, 1 μ l 10 μ M random pentadecamers (IDT), 0.5 μ l 100 μ M anchored oligo dT primers (Sigma) and 2 μ l 10mM dNTPs (NEB) were mixed with 10 μ l 100ng/ μ l RNA. The mixture was incubated at 65 °C for 5 min in a thermo cycler. After incubation, 4 μ l 5x RevertAid reverse transcriptase buffer (Thermo Fisher), 0.5 μ l RevertAid reverse transcriptase (Thermo Fisher), 0.5 μ l RiboLock RNase inhibitor (Thermo Fisher) and 1.5 μ l RNase-free H₂O (Qiagen) were added to each tube. Mastermixes were made wherever possible. Samples were then incubated in a thermo cycler at 25°C for 10min, 42°C for 60min, and 70°C for 10min. Synthesised cDNA was diluted with 80 μ l RNase-free H₂O and stored at -20°C until use.

Quantitative PCR from the cDNA was performed using the SYBR Select qPCR master mix (Applied Biosystems). For each reaction, 2 μ l cDNA, 2 μ l RNase-free H₂O and 1 μ l primer mixture containing 10 μ M forward primer and 10 μ M reverse primer were added to 5 μ l 2x mastermix (see Table 2.14 for primer used for each gene). cDNA from the same condition was loaded twice on 384-well qPCR plates as technical replicates.

Table 2.14: qRT-PCR primers used in this study

Name	Sequence
CXCL1	5'-TCCTGCATCCCCCATAGTTA-3' 5'-CTTCAGGAACAGCCACCAGT-3'
CXCL8	5'-TCTGGCAACCCTAGTCTGCT-3' 5'-AAACCAAGGCACAGTGGAAC-3'
HPRT	5'-AGCCAGACTTTGTTGGATTTG-3' 5'-TTTACTGGCGATGTCAATAGG-3'
TNF	5'-TGCTGCAGGACTTGAGAAGA-3' 5'-GAGGAAGGCCTAAGGTCCAC-3'

qPCR was run on the Lightcycler 480 II qPCR machine (Roche) with the following program:

Table 2.15: qPCR program

Name	Temp.	Duration	Ramp rate	Cycles	Acquisition
Warming	50 °C	2 min	4.4 °C/sec	1	None
Melting	95 °C	2 min	4.4 °C/sec	1	None
Amplification	95 °C 60 °C	15 sec 1 min	4.4 °C/sec 2.2 °C/sec	40	- Quant
Melting curve	95 °C	1 min	4.4 °C/sec	1	-

Continuation of Table 2.15

Name	Temp.	Duration	Ramp rate	Cycles	Acquisition
	55 °C	30 sec	2.2 °C/sec		-
	95 °C	-	0.11 °C/sec		Continuous (5/1 °C)

Only wells with a good melting curve were used for data analysis. Interpolation of CT values was performed with the inbuilt software of the qPCR machine. Relative mRNA expression levels were calculated with the comparative CT method based on the housekeeping gene *HPRT* (Schmittgen and Livak 2008). Data from biological replicates were analysed using Graphpad Prism 7.

2.2.9 Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed to measure cytokine levels in cell culture supernatant. Samples were centrifuged at 300xg for 5min to remove floating cell debris. The supernatant was aliquoted into 1.5ml eppendorf tubes and kept under -80°C until measurement. ELISA was performed with kits from R&D Systems (see Table 2.5) as described in the manufacturer's protocol. Absorbance values were read for each well at 450nm and 540nm wavelengths on a microplate reader. For data analysis, the read at 540nm was subtracted from that at 450nm. The resultant values for standard wells were simulated with the built-in hyperbola simulation function in GraphPad Prism 8, from which the values from sample wells were interpolated using the built-in function. Average concentrations of technical replicates were calculated after interpolation as one biological replicate.

2.2.10 Dual luciferase assay

Dual luciferase assays were performed with the Dual-Luciferase Reporter Assay System from Promega (E1960). For ZBP1 expression induced NF- κ B activation experiments, HEK293FT cells were plated in 24-well plates at 400,000 cells/well. The next day, they were transfected with 125ng NF- κ B reporter plasmid, 25ng SV40-Renilla reporter plasmid and an appropriate amount of the gene of interest (GOI) in a ratio of 20 μ l OptiMEM : 1 μ l Fugene HD : 250ng DNA. Compounds were added immediately after transfection where applicable. 24h after transfection, wells were lysed by shaking at

200rpm in 75 μ l passive lysis buffer (Promega) for 20min. 10 μ l lysates were loaded twice on a white opaque 96-well plate for measurement of luminescence on CLARIOstar. 50 μ l of each luciferase assay reagent was injected into each well at a time interval of 12 seconds (s), and luminescence was read every 0.5s. The average of read values from 2s to 10.5s was used as the level of the NF- κ B luciferase activity, and the average of read values from 14.5s to 23s was used as the level of the control Renilla luciferase activity. The average of 2 technical replicates was recorded as one biological replicate value.

For data analysis, the NF- κ B induction level was calculated by dividing the value of NF- κ B luciferase activity by the value of control Renilla luciferase activity. Where necessary, relative NF- κ B induction levels were calculated by dividing all NF- κ B/Renilla values by that of the control condition.

Leftover lysates from the measurement were mixed with 4x LSB, heated at 95°C for 5min and kept at -20°C for western blotting.

2.2.11 Flow cytometry

GFP intensity in cells were measured on the FITC channel on the FACSCanto flow cytometer. Cells were trypsinised, collected and resuspended in PBS for flow cytometry. Data were collected with the built-in software and analysed using FlowJo 10.

2.2.12 Statistical analysis

All statistical analyses were performed with GraphPad Prism 7 or GraphPad Prism 8. Data is plotted as mean $\pm$ standard error of the mean (SEM) for each figure. Unless otherwise stated, ordinary one-way ANOVA and Sidak's multiple comparison test were used to test for statistical differences as indicated. ns, not significant, *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, ****, $p<0.0001$.

3

RIPK3 activation drives inflammatory signalling

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In this Chapter, several lines of evidence show that RIPK3 activation induces MAPK- and NF- κ B-mediated inflammatory signalling. Except in the TNFR1-induced condition, RIPK3 activation induces inflammatory signalling without detectable impact on cell viability.

3.1 ZBP1 expression induces RIPK3-mediated inflammatory signalling

Aberrant ZBP1 expression in RIPK1-deficient mouse keratinocytes leads to RIPK3-mediated cell death and inflammation (Newton et al. 2016; Lin et al. 2016). In HEK293 cells, ectopic expression of ZBP1 induces the activation of NF- κ B reporters (Kaiser et al. 2008; Rebsamen et al. 2009). However, as HEK293T cells do not express RIPK3 or MLKL under normal conditions, it is not clear whether ZBP1 expression induces cell death in human cells in the presence of endogenous RIPK3 and MLKL (Morgan and Kim 2015; Zhao et al. 2012).

3.1.1 ZBP1 expression induces inflammatory signalling independently of cell death

HT29 is a RIPK3-expressing human colorectal carcinoma cell line widely used in necroptosis research (He et al. 2009). To investigate whether ZBP1 expression induces inflammatory signalling independently of cell death, I obtained HT29 cells engineered to express ZBP1 in response to doxycycline (Dox) in mimic of IFN-stimulated expression from Dr Jonathan Maelfait (Ghent, Belgium). First, I investigated the cell death and inflammatory responses of HT29 cells to different induction levels of ZBP1 expression (Figures 3.1A and 3.1B). Induced expression of ZBP1 at low levels (50ng/ml or 100ng/ml Dox) did not compromise the viability of HT29 cells, but at high levels (500ng/ml Dox) did (Figure 3.1C). In contrast, induced expression of a ZBD-mutant version of ZBP1 which does not have nucleic acid-binding ability did not compromise viability at any expression level, indicating that the loss of viability is specific to the substrate recognition ability of ZBP1 (Figures 3.1B and 3.1C).

To investigate if ZBP1 activation induces inflammatory signalling independently of cell death, I measured the levels of two NF- κ B target chemokines, CXCL8 and CXCL1, after treatment with 50ng/ml Dox for 24h. The treatment induced the production of the two chemokines without detectable loss of viability (Figure 3.2). A panel of inhibitors were used to study the dependency of ZBP1-induced chemokine production.

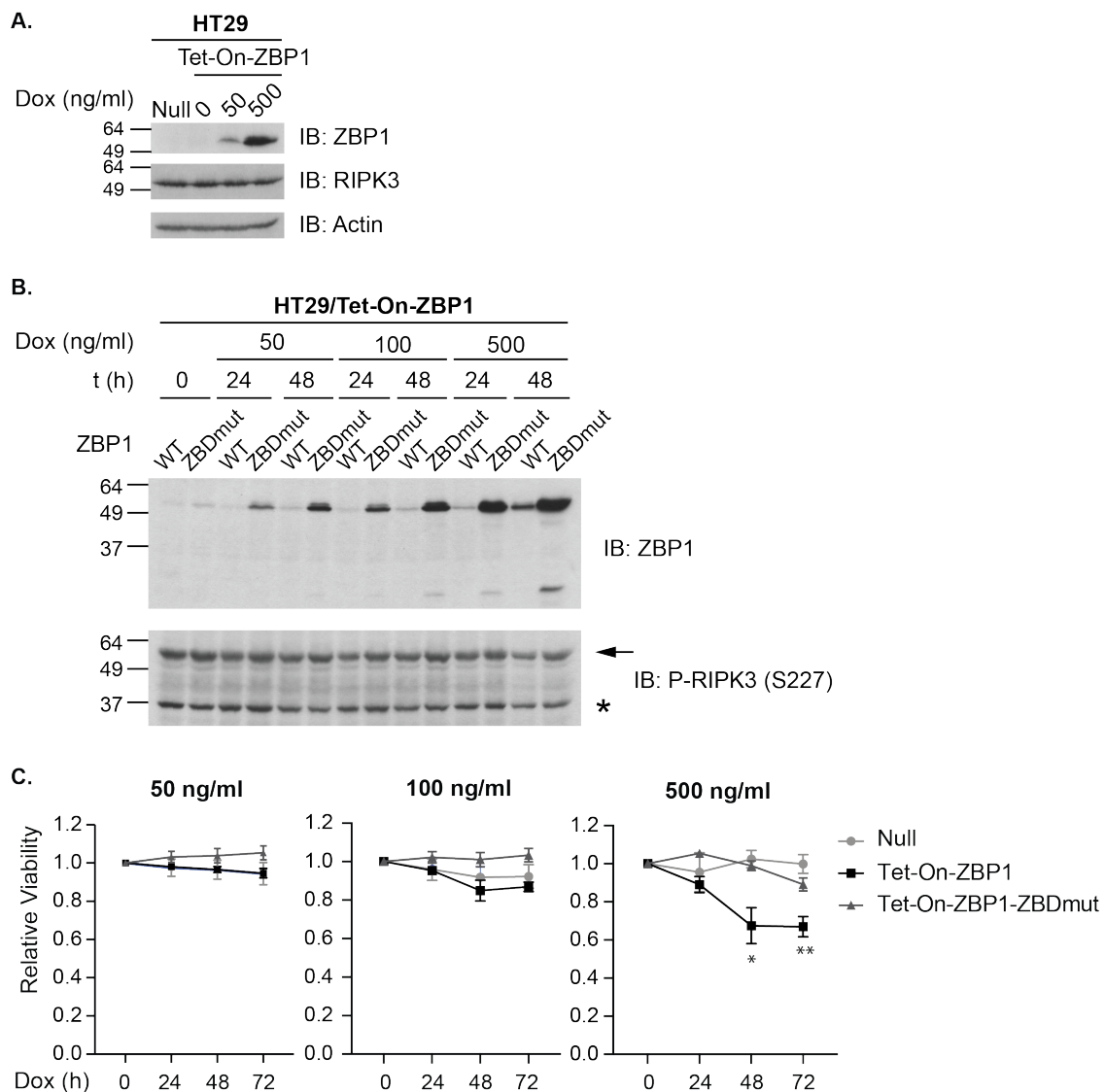


Figure 3.1: High levels of ZBP1 expression in HT29 cells reduce cell viability. (A) HT29/Tet-On-ZBP1 cells were treated with indicated concentrations of Dox for 24h. HT29 cells were plated and collected at the same time without treatment as control. (B, C) HT29, HT29/Tet-On-ZBP1 and HT29/Tet-On-ZBP1-ZBDmut cells were treated with indicated concentrations of Dox for the indicated length of time. (B) Arrow indicates the P-RIPK3 signal band. Asterisk indicates the antibody background band. (C) Cell viability was measured at the end of treatment with ATP-based CellTitre-Glo Assay (n=3). Ordinary 2-way ANOVA and Tukey's multiple comparison test were used to test the differences between HT29/Tet-On-ZBP1 and HT29 (Null) cells at 48h and 72h. *, p<0.05, **, p<0.01. *HT29/Tet-On-ZBP1 and HT29/Tet-On-ZBP1-ZBDmut cells were generated by Dr Jonathan Maelfait in Ghent University, Belgium.*

ZBP1 expression-induced chemokine production was completely inhibited by (5Z)-7-oxozeaenol, an inhibitor of MAP3K TAK1, and the combination of two IKK inhibitors,

IKK VII and IKK XII (denoted as IKKi) (Figure 3.2A) (Wu et al. 2013; Waelchli et al. 2006). Meanwhile, inhibitors of RIPK1 kinase activity (Nec1s), RIPK3 kinase activity (GSK'872) or MLKL oligomerisation (Necrosulfonamide, NSA) did not have an effect on ZBP1-induced chemokine production (Figure 3.2A) (Takahashi et al. 2012; Kaiser et al. 2013; Sun et al. 2012). Thus, the production of CXCL8 and CXCL1 is dependent on the kinase activity of TAK1 and IKK, but not on that of RIPK1, RIPK3 or the oligomerisation of MLKL, which necroptosis depends on. This indicates that ZBP1-induced inflammatory signalling and necroptosis are independent processes with different mechanisms.

3.1.2 ZBP1-induced inflammatory signalling is RIPK3-dependent

I next investigated whether ZBP1-induced inflammatory signalling is dependent on RIPK3, using NF- κ B reporter assays in HEK293FT cells stably transduced with full-length (FL) or a C-terminal truncated (dC) version of human RIPK3 that lacks RHIM. For the convenience of future studies, the RIPK3 transduced was fused with two 12kDa FK506-binding protein (FKBP12)-F36V (abbreviated as FV) domains for inducible oligomerisation, hence referred to as RIPK3-2xFV and RIPK3dC-2xFV (Rodriguez et al. 2016).

I first titrated the amount of ZBP1 to determine the optimal condition of the system (Figure 3.3A). Transfection of increasing amounts of ZBP1 induced an increase followed by a decrease in the NF- κ B reporter activity and a gradual decrease in the control SV40-Renilla reporter activity (Figure 3.3A). 0.5ng ZBP1-expressing plasmids was selected for further experiments as a balance between optimal induction of NF- κ B reporter activity and maintenance of control Renilla activity.

Transfection of wild-type (WT) or ZBD-mutant ZBP1 into HEK293FT expressing FL or dC RIPK3 showed that both the ZBD of ZBP1 and the RHIM of RIPK3 are needed to induce the activation of NF- κ B (Figure 3.3B). Similar to the HT29/Tet-On-ZBP1 system, ZBP1 expression induced NF- κ B activation independent of the kinase activity of RIPK1, RIPK3 or the oligomerisation of MLKL, although the RIPK3 kinase inhibitor GSK'872 reduced NF- κ B reporter and SV40-Renilla activities by the same fold in both empty vector (EV)- and ZBP1-transfected cells (Figure 3.3C). Unlike the HT29/Tet-On-ZBP1

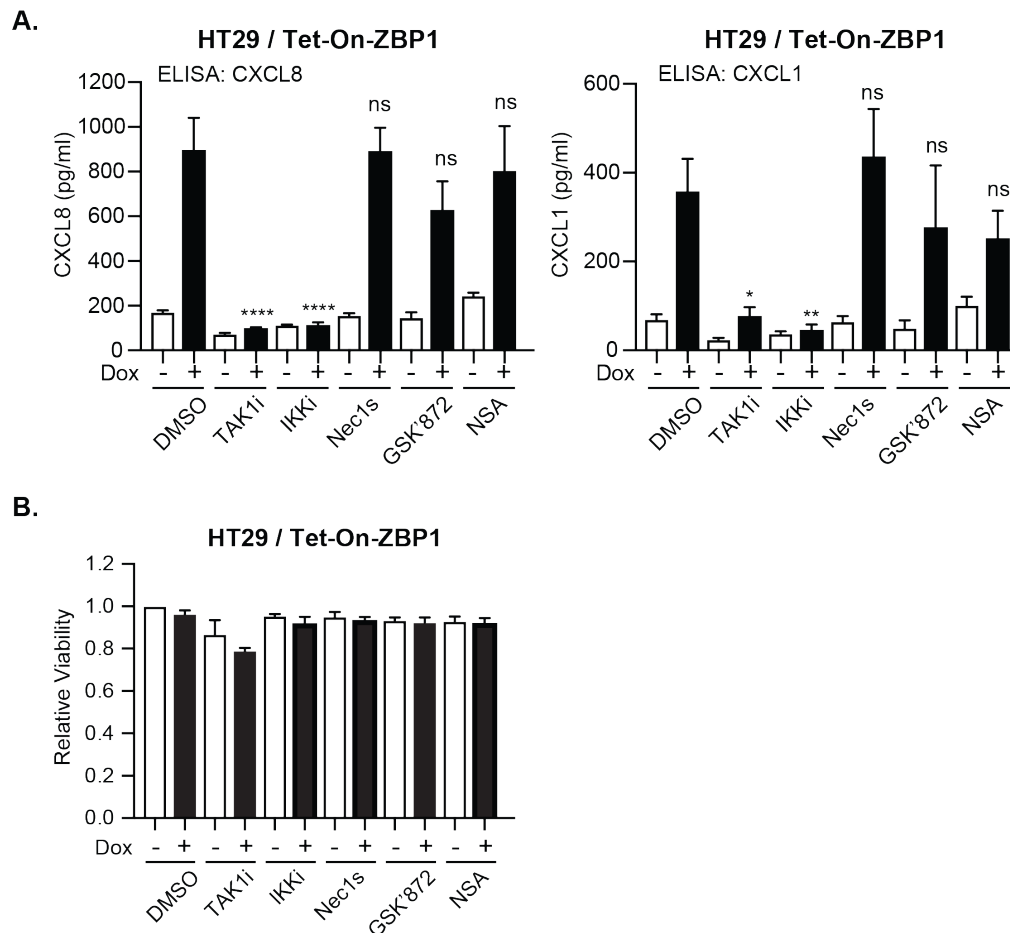


Figure 3.2: Induced ZBP1 expression leads to TAK1- and IKK-dependent chemokine production independently of cell death. HT29/Tet-On-ZBP1 cells were treated with indicated inhibitors in combination with 0 or 50 ng/ml Dox for 24h (n=3). Nec1s, RIPK1 kinase activity inhibitor, GSK'872, RIPK3 kinase activity inhibitor, NSA, MLKL oligomerisation inhibitor. See Table 2.9 for compound concentrations. (A) ELISA measurements of chemokine levels in the supernatant. Ordinary 1-way ANOVA and Sidak's multiple comparison tests were used to test the difference between Dox-induced samples treated with corresponding inhibitors and treated with DMSO. ns, not significant, *, $p < 0.05$, **, $p < 0.01$, ****, $p < 0.0001$. (B) Cell viability assay performed under the same conditions in (A).

system, inhibition of TAK1 did not affect ZBP1-induced NF- κ B activation, and the inhibitory effect of IKK inhibitors was incomplete. This could result from the difference between the activation of the NF- κ B reporter plasmid and that of genomic κ B sites (Figure 3.3C). In all, consistent with previous reports, I confirmed that RIPK3 is required for ZBP1-induced NF- κ B activation (Kaiser et al. 2008; Rebsamen et al. 2009).

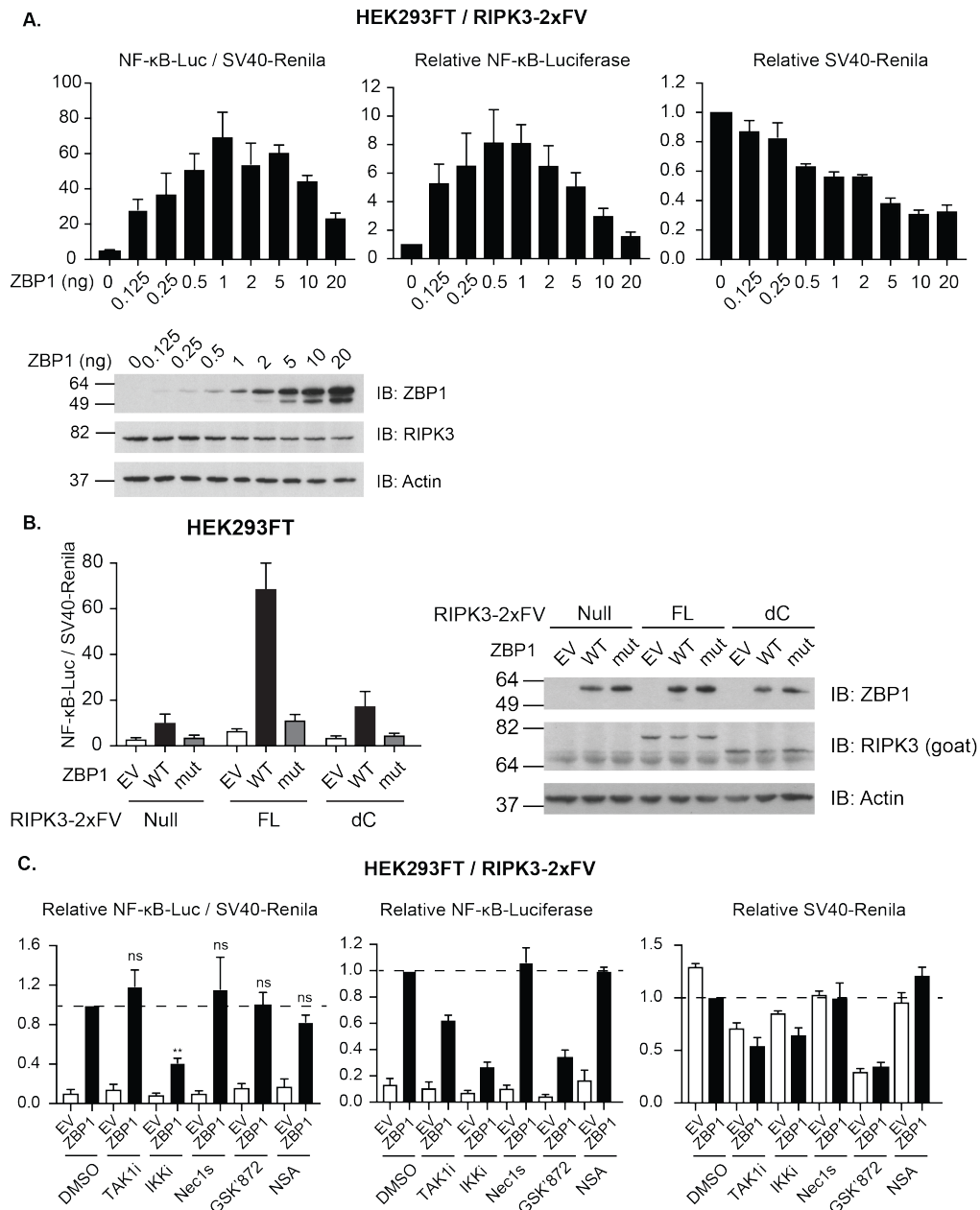


Figure 3.3: RIPK3 mediates ZBP1-induced activation of NF- κ B reporters. (A) Cells were transfected with titrated amounts of ZBP1-expressing plasmids along with the NF- κ B luciferase reporter and control SV40-Renilla reporter plasmids. The total DNA amount in each condition is topped up to the same with empty vector ($n=3$). 24h later, lysates were measured for luciferase activity and blotted for indicated proteins. (B) HEK293FT, HEK293FT/RIPK3-2xFV and HEK293FT/RIPK3dC-2xFV cells were transfected with 0.5ng empty vector, WT or ZBDmut ZBP1-expressing plasmids along with the NF- κ B luciferase reporter and control SV40-Renilla reporter plasmids ($n=3$). 24h later, lysates were measured for luciferase activity and blotted for indicated proteins. (C) Cells were transfected with 0.5ng empty vector or ZBP1-expressing plasmids along with the NF- κ B luciferase reporter and control SV40-Renilla reporter plasmids and treated with indicated compounds ($n=3$). 24h later, lysates were measured for luciferase activity. Ordinary 1-way ANOVA and Sidak's multiple comparison test were used to test the difference between ZBP1-transfected conditions treated with DMSO and with inhibitors. **, $p<0.01$, ns, not significant.

3.2 **TNF-induced RIPK3 activation leads to inflammatory signalling**

The activation of RIPK3 has been extensively characterised in TNFR1-induced necroptosis (Pasparakis and Vandenabeele 2015). In order to investigate whether TNF-induced RIPK3 activation also mediates inflammatory signalling, I stably expressed RIPK3-2xFV in HCT116 cells that do not express endogenous RIPK3 to compare the inflammatory responses under TNF-induced necroptosis conditions in the presence or absence of RIPK3 (Morgan and Kim 2015).

To achieve the TNF-induced necroptosis condition, I used the SMAC mimetic Compound A (CpA) to deplete cIAPs to compromise TNFR1-induced Complex I, in combination with the pan-caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-fluoromethylketone (z-VAD-fmk, zVAD) to inhibit the anti-necroptotic activity of apoptosis initiator caspases. As a validation of the method, TNF+CpA+zVAD induced prominent MLKL phosphorylation and ~80% loss of viability in HT29 cells, consistent with previous reports (Figures 3.4A and 3.4B) (He et al. 2009). However, HCT116/RIPK3-2xFV cells responded mildly in MLKL phosphorylation and cell death percentage when treated with the same condition, the reason for which remains unclear (Figures 3.4A, 3.4C and 3.4D).

Strikingly, TNF+CpA+zVAD treatment induced an increase in the CXCL8 mRNA level in HCT116/RIPK3-2xFV cells, which is dependent on the existence of both RIPK1 and exogenous RIPK3 (Figure 3.5A). Consistent with the role of RHIM in the recruitment of RIPK3, RHIM-mutant RIPK3 did not support inflammatory responses with TNF+CpA+zVAD treatment (Figures 3.5B and 3.5C). Different from ZBP1-induced RIPK3 activation, RIPK1 kinase activity, RIPK3 kinase activity and MLKL oligomerisation are required in TNF+CpA+zVAD-induced inflammatory signalling (Figures 3.5A, 3.5B, 3.5D and 3.5E). The same process was described in HT29 cells in response to TNF+SMAC mimetic+zVAD (Zhu et al. 2018).

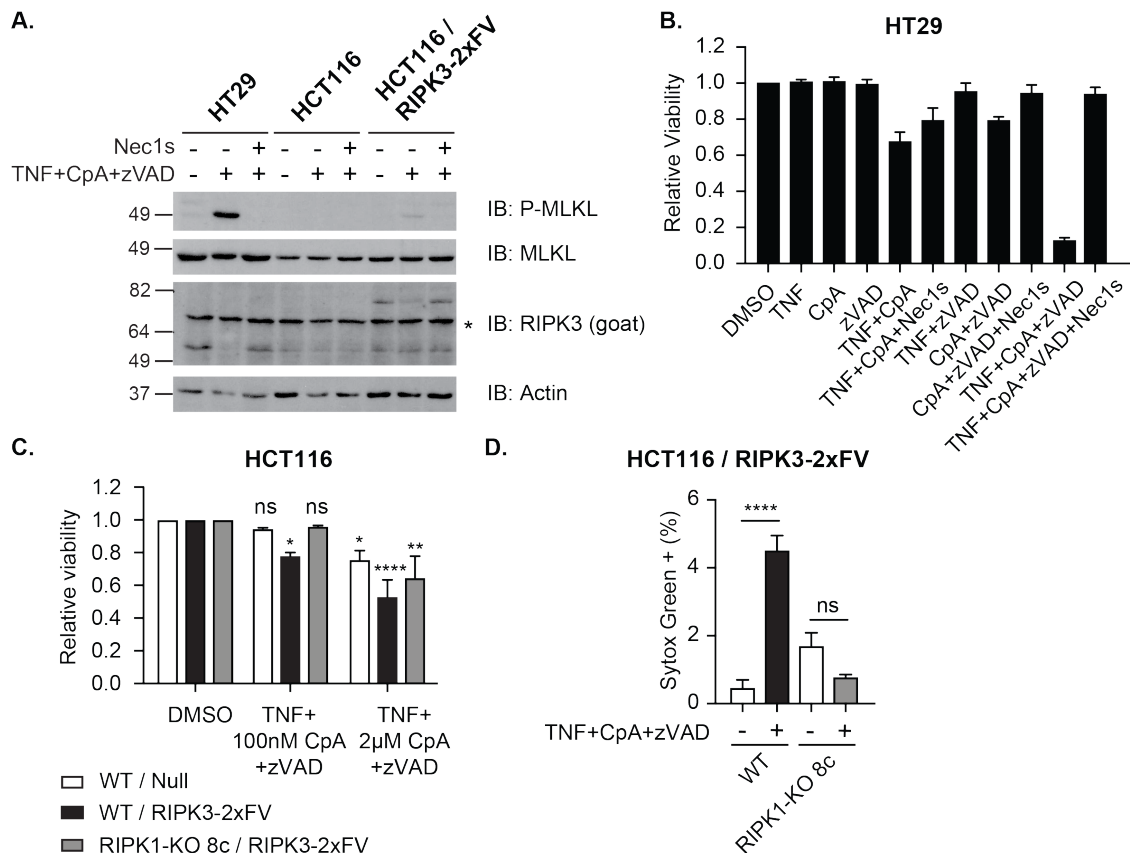


Figure 3.4: TNF+CpA+zVAD induces compromised necroptosis response in HCT116/RIPK3-2xFV cells. (A) Cells were treated as labelled for 6h. CpA, 100nM, TNF, 20ng/ml. (B) Cells were treated as indicated for 24h. TNF, 2ng/ml, CpA, 100nM. (C) HCT116, HCT116/RIPK3-2xFV or HCT116/RIPK1-KO clone 8c/RIPK3-2xFV cells were treated as indicated for 24h. TNF, 1ng/ml. 2-way ANOVA and Tukey's multiple comparison test were used to test the difference between indicated conditions and DMSO-treated condition of the same cell line. (D) HCT116/RIPK3-2xFV and HCT116/RIPK1-KO clone 8c/RIPK3-2xFV cells were treated for 24h and stained with Sytox Green and Hoechst 33342. Ordinary one-way ANOVA and Sidak's multiple comparison test was used to test for the statistical difference between indicated conditions. TNF, 1ng/ml, CpA, 100nM. ns, not significant, *, $p < 0.05$, **, $p < 0.01$, ****, $p < 0.0001$.

3.3 Chemical-induced RIPK3 oligomerisation drives inflammatory signalling

3.3.1 The chemical-induced RIPK3 oligomerisation system

Chemical-induced RIPK3 oligomerisation was reported to induce MAPK- and NF- κ B-mediated inflammatory signalling and necroptosis in NIH3T3 mouse fibroblasts (Yatim et al. 2015). When fused with two FV domains, RIPK3 can be induced to oligomerise with a cell-permeable bivalent ligand AP20187 (hence referred to as dimeriser), which

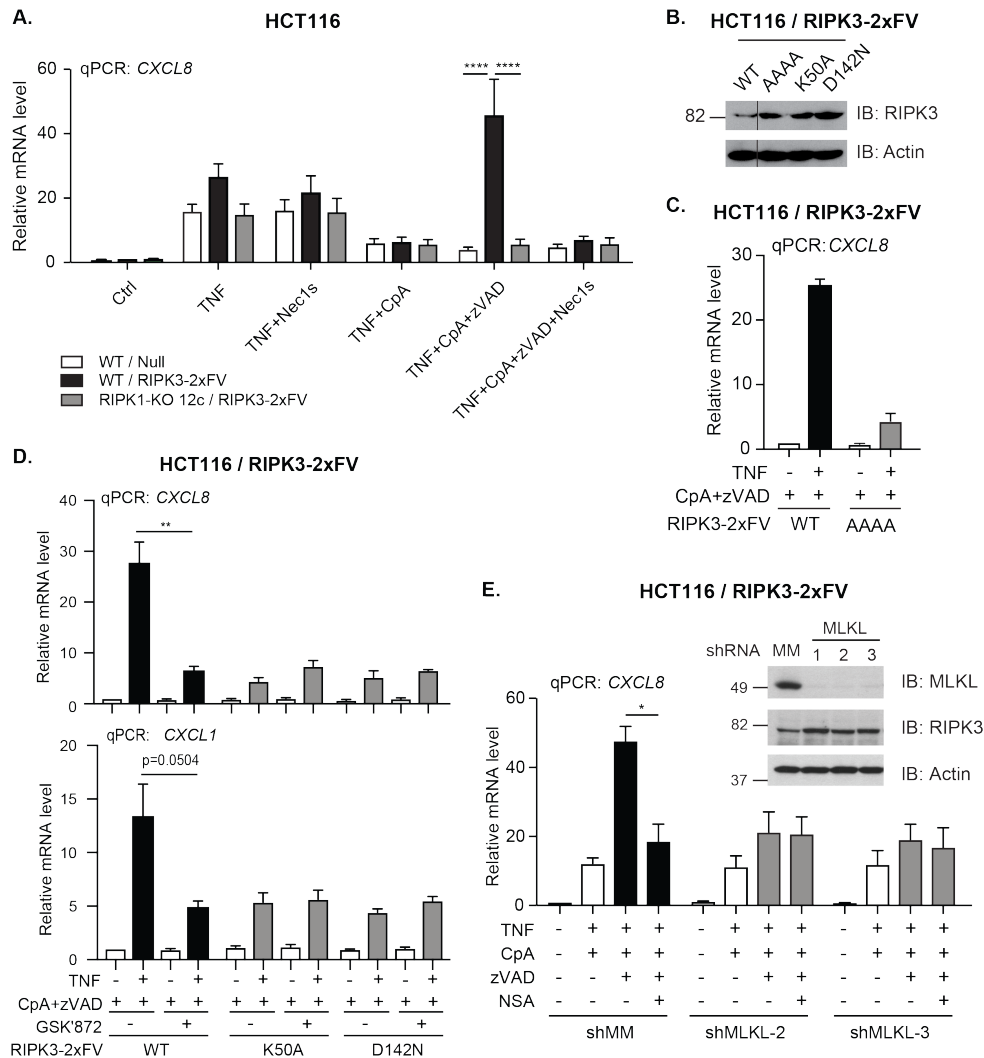


Figure 3.5: TNF+CpA+zVAD induces inflammatory signalling dependent on RIPK1, RIPK3 and MLKL. (A) HCT116, HCT116/RIPK3-2xFV or HCT116/RIPK1-KO clone 12c/RIPK3-2xFV cells were treated as indicated for 3h. TNF, 2ng/ml, CpA, 2 μ M. Ordinary two-way ANOVA and Tukey's multiple comparison test were used to test the difference between indicated conditions. (B) Expression levels of HCT116/RIPK3-RHIM^{AAAA}-2xFV, HCT116/RIPK3-K50A-2xFV and HCT116/RIPK3-D142N-2xFV compared to HCT116/RIPK3-2xFV. Results are from the same exposure of the same blot. (C-E) Different stable cell lines were treated as indicated for 3h for qPCR (n=3). (C) HCT116/RIPK3-2xFV and HCT116/RIPK3-RHIM^{AAAA}-2xFV cells. (D) HCT116/RIPK3-2xFV, HCT116/RIPK3-K50A-2xFV and HCT116/RIPK3-D142N-2xFV cells. Unpaired t-test was used to test for the statistical difference between indicated conditions. (E) HCT116/RIPK3-2xFV/shMM, HCT116/RIPK3-2xFV/shMLKL-2 and HCT116/RIPK3-2xFV/shMLKL-3 cells. Unpaired t-test was used to test for the statistical difference between indicated conditions. Equal number of cells from each line were collected for western blot analysis of the protein expression levels. *, p<0.05, **, p<0.01, ****, p<0.0001.

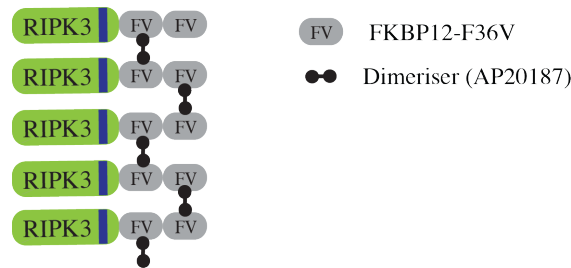


Figure 3.6: Chemical-induced oligomerisation of RIPK3. RIPK3 is fused with 2 copies of FKBP12-F36V (FV), a mutant form of the human 12kDa FK506-binding protein (FKBP12). Each FV has 1 binding site of the bivalent dimeriser (AP20187). Application of the B/B homodimeriser (Dimeriser/AP20187) results in the formation of RIPK3 oligomers.

could bring its kinase domains into close proximity for its activation (Figure 3.6) (Orozco et al. 2014). The system has proved effective in the murine system for both *in vitro* and *in vivo* studies (Yatim et al. 2015; Daniels et al. 2018).

3.3.2 Chemical-induced oligomerisation of human RIPK3 drives inflammatory signalling without cell death

We obtained the human RIPK3 oligomerisation system along with NIH3T3/mRIPK3-2xFV and NIH3T3/mRIPK3dC-2xFV cells from Professor Andrew Oberst (University of Washington, Seattle) (Yatim et al. 2015). As reported, induced oligomerisation of mouse RIPK3 (mRIPK3) in NIH3T3 cells resulted in both cell death and inflammatory responses (Figure 3.7) (Yatim et al. 2015). The loss of RHIM did not have an effect on cell death but delayed inflammatory signalling (Figure 3.7) (Ren et al. 2017).

In HCT116 cells, induced oligomerisation of RIPK3 led to RHIM-dependent activation of inflammatory responses through TAK1 and IKKs (Figures 3.8). Unlike in the mouse system, oligomerisation of the dC version of RIPK3 did not lead to cell death or inflammatory signalling in human cells. The same was confirmed in HT29, U2OS and HEK293FT cells (Figure 3.9). Moreover, the viability of HCT116 and HT29 cells did not change over 24h after RIPK3 oligomerisation (Figure 3.10). Although this is in contrast with the system in murine cells, it mimics the HT29/Tet-On-ZBP1 system with low levels of ZBP1 induction in that RIPK3 activation induces inflammatory signalling in the absence of cell death.

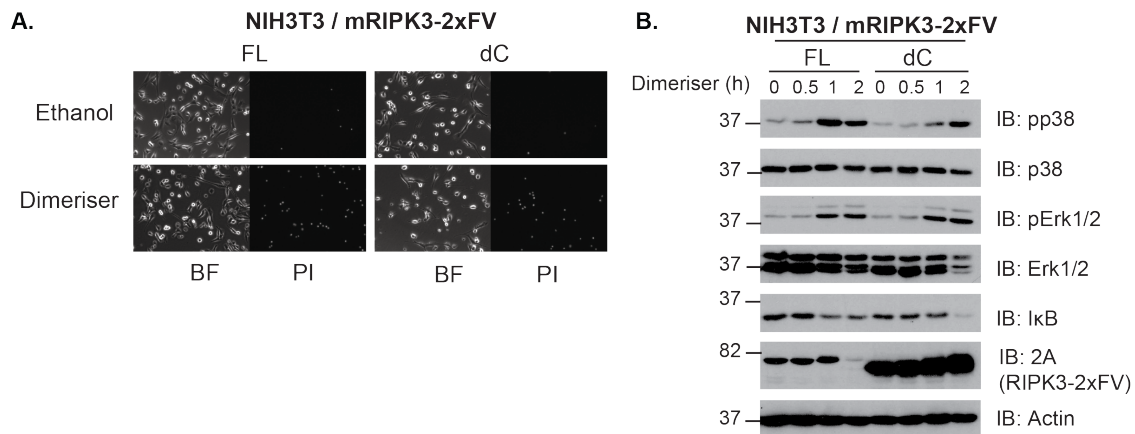


Figure 3.7: Chemical-induced RIPK3 oligomerisation induces inflammatory signalling and cell death in NIH3T3/mRIPK3-2xFV cells. NIH3T3/mRIPK3-2xFV or NIH3T3/mRIPK3dC-2xFV cells were treated with (A) ethanol or 100nM dimeriser for 4h or (B) 100nM dimeriser for indicated length of time. (A) 5µg/ml propidium iodide (PI) was added to stain dead cells. BF, bright field. PI, Green channel. Pictures were taken with the Inverted Olympus Fluorescent Microscope.

3.3.3 The role of RIPK1, RIPK3 kinase activity and MLKL in RIPK3-mediated inflammatory signalling

ZBP1-induced inflammatory signalling does not depend on the kinase activity of RIPK1, RIPK3 or the oligomerisation of MLKL, while TNF-induced RIPK3-mediated inflammatory signalling does. Next, I investigated the requirement of RIPK1, RIPK3 kinase activity and MLKL in RIPK3 oligomerisation-induced inflammatory signalling.

RIPK1

To investigate if RIPK1 is required for RIPK3-mediated inflammatory signalling, I knocked out RIPK1 in HCT116 cells using the CRISPR/Cas9 system and stably expressed RIPK3-2xFV in RIPK1-knockout (RIPK1-KO) clones (Figures 3.11A and 3.11B). In multiple HCT116/RIPK1-KO clones, RIPK3 oligomerisation induced TAK1- and IKK-dependent inflammatory responses to similar levels as WT (Figures 3.11A-D). Notably, oligomerisation of RHIM-truncated RIPK3 did not result in inflammatory responses regardless of the presence or absence of RIPK1, indicating that the lack of response in RIPK3dC-2xFV cells is not due to the lack of interaction with RIPK1. Instead, it could be due to structural abnormalities of the C-terminal truncated human RIPK3 or its oligomer, but the possibility requires further investigation.

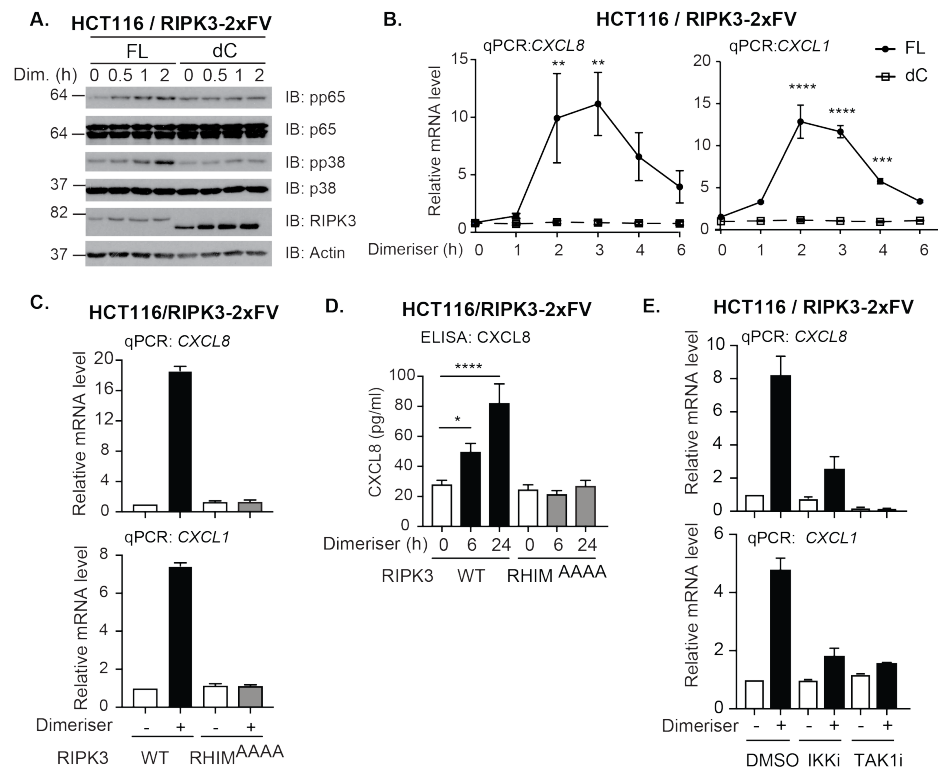


Figure 3.8: Chemical-induced RIPK3 oligomerisation induces inflammatory signalling in HCT116/RIPK3-2xFV cells. (A, B) HCT116/RIPK3-2xFV and HCT116/RIPK3dC-2xFV cells were treated as indicated. (B) $n=3$. Ordinary two-way ANOVA and Tukey's multiple comparison test were used to test the difference between indicated time points and 0h in FL cells. (C, D) HCT116/RIPK3-2xFV and HCT116/RIPK3-RHIM^{AAAA}-2xFV were treated as indicated for (C) 3h ($n=3$) or (D) indicated length of time ($n=4$). Supernatant was collected for ELISA. Ordinary one-way ANOVA and Sidak's multiple comparison test were used to test for statistical differences between indicated conditions. (E) HCT116/RIPK3-2xFV cells were treated as indicated for 3h ($n=3$). *, $p<0.05$, **, $p<0.01$, ****, $p<0.0001$.

RIPK1 is known to restrict RIPK3-mediated necroptosis in mice (Lin et al. 2016; Newton et al. 2016). Similarly, oligomerisation of RIPK3 in RIPK1-KO cells induced around 10% loss of viability, corresponding to around 8% Sytox Green-stained dead cells, which were not seen in WT cells (Figures 3.11E and 3.11F). Due to the low cell death levels in HCT116/RIPK3-2xFV cells even in response to TNF-induced necroptosis, whether RIPK1 restrains RIPK3-induced cell death in human cells requires further validation. Nevertheless, the low cell death level indicates that RIPK3-mediated inflammatory signalling in RIPK1-KO cells is unlikely due to the release of DAMPs during cell death.

RIPK3 can interact with TRIF via its RHIM, which can in turn activate the TBK1-IRF pathway (Kaiser et al. 2013; Guo and Cheng 2007). To investigate if TRIF mediates

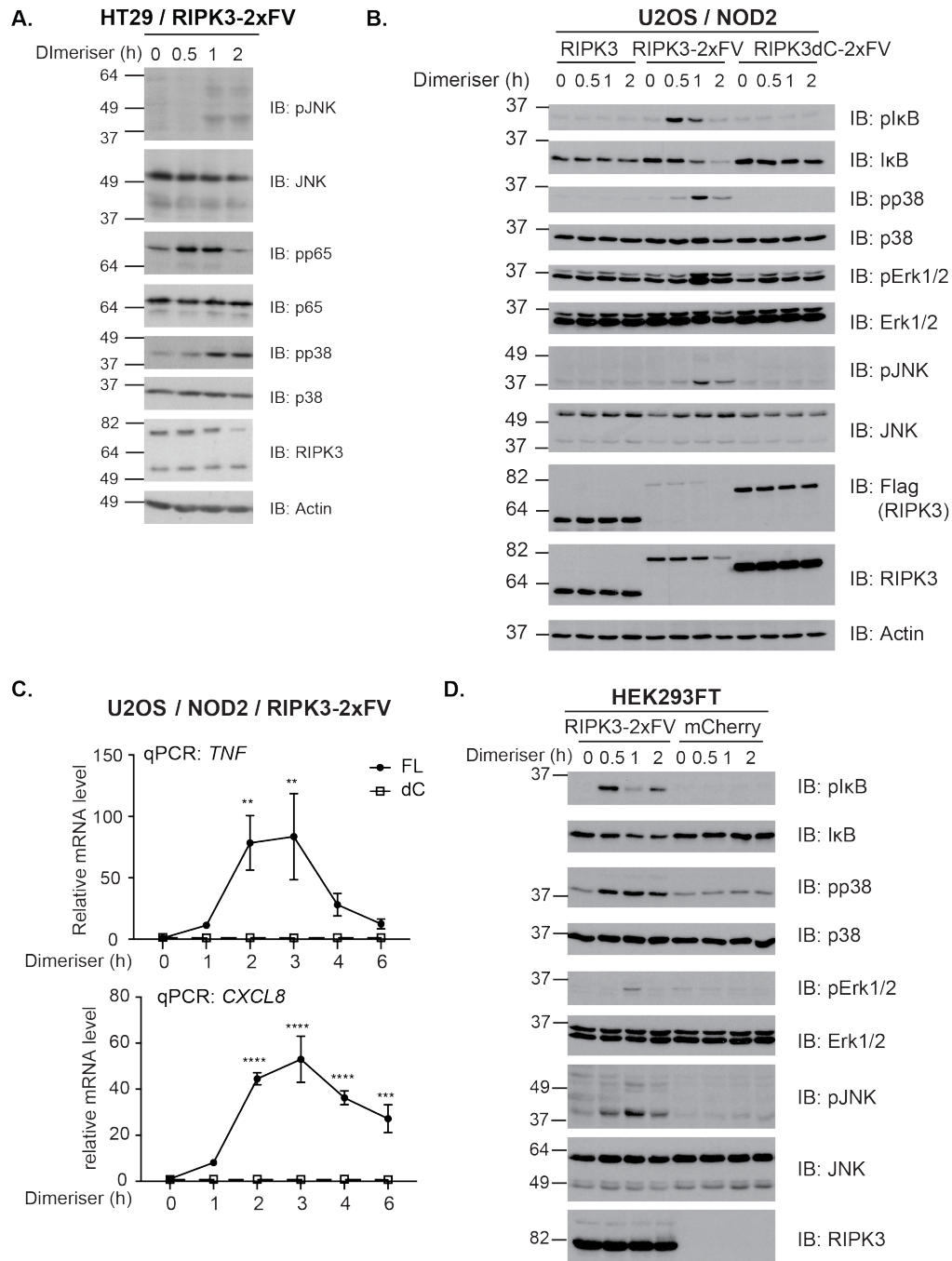


Figure 3.9: Chemical-induced RIPK3 oligomerisation drives inflammatory signalling in multiple cell lines. (A) HT29/RIPK3-2xFV, (B) U2OS/NOD2/RIPK3, U2OS/NOD2/RIPK3-2xFV, U2OS/NOD2/RIPK3dC-2xFV, (C) U2OS/NOD2/RIPK3-2xFV, U2OS/NOD2/RIPK3dC-2xFV, (D) HEK293FT/RIPK3-2xFV and HEK293FT/mCherry are treated as indicated. (C) Ordinary two-way ANOVA and Tukey's multiple comparisons test were used to test the difference between indicated time points and 0h in FL cells. **, $p < 0.01$, ***, $p < 0.001$, ****, $p < 0.0001$.

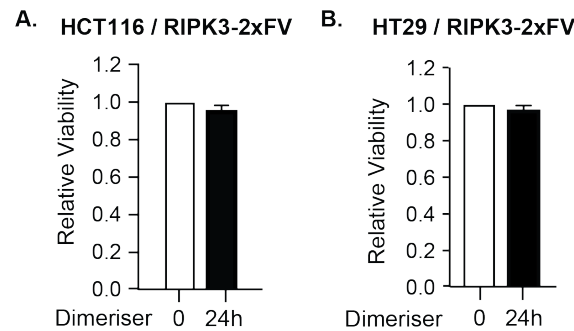


Figure 3.10: Chemical-induced RIPK3 oligomerisation does not affect viability in human cells. (A) HCT116/RIPK3-2xFV and (B) HT29/RIPK3-2xFV cells were treated as indicated. n=3.

RIPK3-induced inflammatory signalling in the absence of RIPK1, I knocked down TRIF with siRNA and found that it did not affect the level of RIPK3-mediated inflammatory signalling (Figure 3.12A). In addition, TNF neutralising antibody did not affect the levels of RIPK3 oligomerisation-induced inflammatory response in RIPK1-KO cells, indicating that RIPK3 oligomerisation-induced inflammatory signalling in RIPK1-KO cells is not dependent on TNF autocrine (Figure 3.12B).

However, the RIPK1 kinase inhibitor Nec1s inhibits RIPK3 oligomerisation-induced inflammatory signalling in an on-target way, suggesting the involvement of RIPK1 in RIPK3-mediated inflammatory signalling when it is present (Figure 3.13).

In summary, RIPK1 is not essential for RIPK3 to induce inflammatory signalling, but could be involved in it with unresolved function.

RIPK3 kinase activity and MLKL

RIPK3 kinase activity and MLKL oligomerisation do not affect RIPK3-mediated inflammatory signalling in ZBP1-induced activation, but are required for TNF-induced RIPK3-mediated inflammatory signalling (Figures 3.2, 3.3C, 3.5D and 3.5E). With the induced oligomerisation of RIPK3, the inhibition of RIPK3 kinase activity and MLKL oligomerisation both partially inhibited RIPK3 oligomerisation-induced inflammatory signalling (Figure 3.14).

To further investigate the requirement for the kinase activity of RIPK3, inflammatory signalling levels were compared between WT RIPK3 and two kinase dead mutants, one in the ATP-binding lysine, K50A, and the other in the catalytic aspartate, D142N (Sun

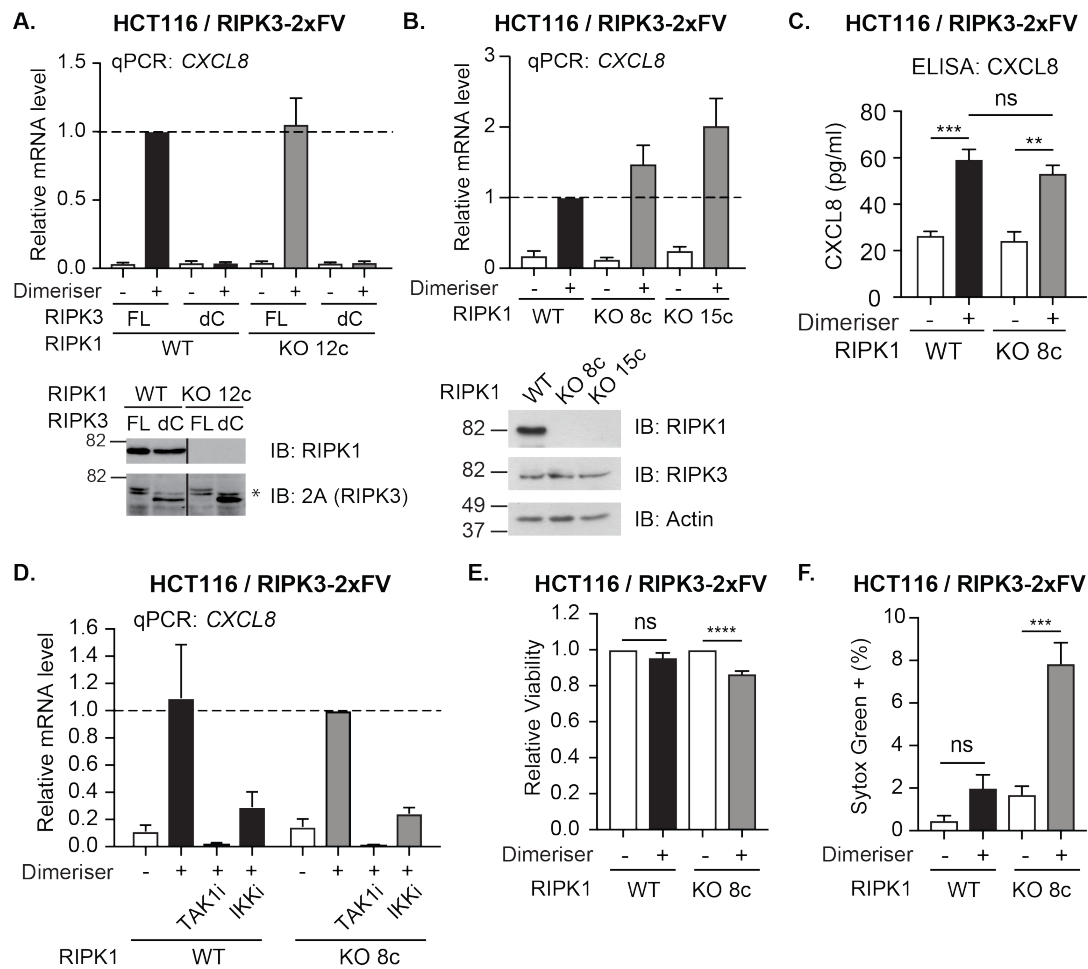


Figure 3.11: RIPK1 is not essential for RIPK3 oligomerisation-induced inflammatory signalling. (A) HCT116/RIPK3-2xFV, HCT116/RIPK3dC-2xFV, HCT116/RIPK1-KO clone 12c/RIPK3-2xFV and HCT116/RIPK1-KO clone 12c/RIPK3dC-2xFV cells were stimulated with 0 or 100nM dimeriser for 3h (n=3). Equal number of cells were collected for western blots. Asterisk indicates antibody background. (B) HCT116/RIPK3-2xFV, HCT116/RIPK1-KO clone 8c/RIPK3-2xFV and HCT116/RIPK1-KO clone 15c/RIPK3-2xFV were stimulated with 0 or 100nM dimeriser for 3h (n=3). Equal number of cells were collected for western blots. (C-F) HCT116/RIPK3-2xFV and HCT116/RIPK1-KO clone 8c/RIPK3-2xFV were treated with 0 or 100nM dimeriser for (C, E, F) 24h or (D) 3h (n=3). Ordinary one-way ANOVA and Sidak's multiple comparison test were used to test for statistical differences between indicated conditions. ns, not significant, **, p<0.01, ***, p<0.001, ****, p<0.0001.

et al. 2012). While neither RIPK3 mutant supported TNF-induced RIPK3-mediated inflammatory signalling (Figures 3.5B and 3.5D), they responded differently to dimeriser stimulation (Figure 3.14A). The K50A mutant reduced RIPK3 oligomerisation-mediated inflammatory signalling to similar levels as GSK'872, while the D142N mutant did not reduce the inflammatory signalling levels as much (Figure 3.14A). The difference in the

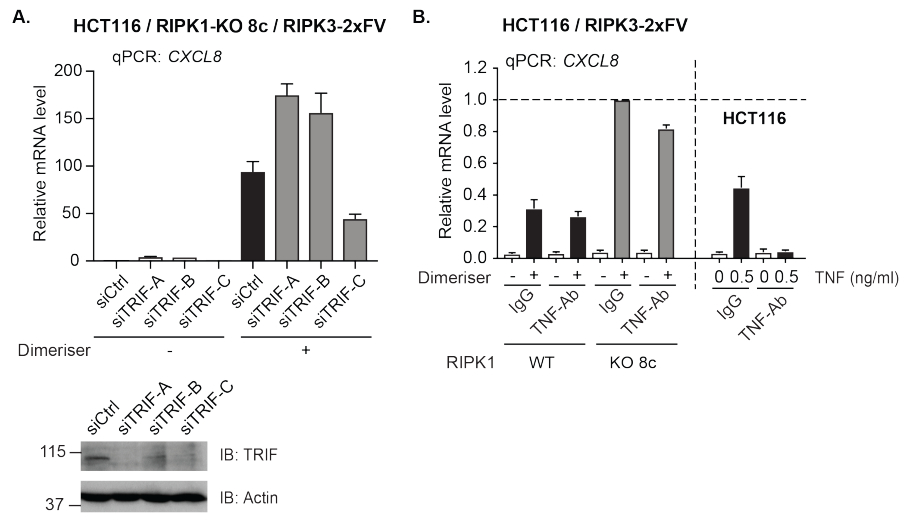


Figure 3.12: RIPK3 oligomerisation-induced inflammatory signalling in the absence of RIPK1 is not mediated by TRIF or TNF autocrine. (A) HCT116/RIPK1-KO clone 8c/RIPK3-2xFV were transfected with siRNA against mismatch (Ctrl) or 3 different sites of TRIF. 72h post transfection, cells were collected for western blots or treated with 0 or 100nM dimeriser for 3h for qPCR (n=3). (B) HCT116/RIPK3-2xFV, HCT116/RIPK1-KO clone 8c/RIPK3-2xFV and HCT116 cells were treated as indicated for 3h (n=3).

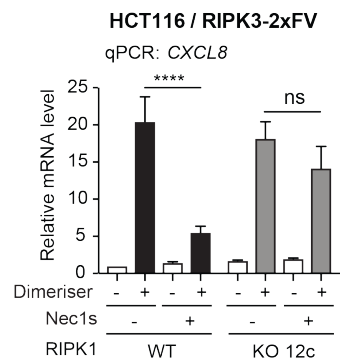


Figure 3.13: The RIPK1 kinase inhibitor Nec1s inhibits RIPK3 oligomerisation-induced inflammatory response in an on-target way. Nec1s, 30 μ M. n=4. Ordinary two-way ANOVA and Tukey's multiple comparison tests were used to test the difference between indicated conditions. ns, not significant, ****, p<0.0001.

inflammatory outcome may arise from a difference in the conformation of the kinase domain between GSK'872-bound, K50A and D142N mutants of human RIPK3.

According to studies on murine RIPK3, both K50A and D142N mutations lock RIPK3 in its inactive conformation and prevent its interaction with RIPK1, while GSK'872-bound RIPK3 should be locked in its active conformation, allowing it to interact with RIPK1

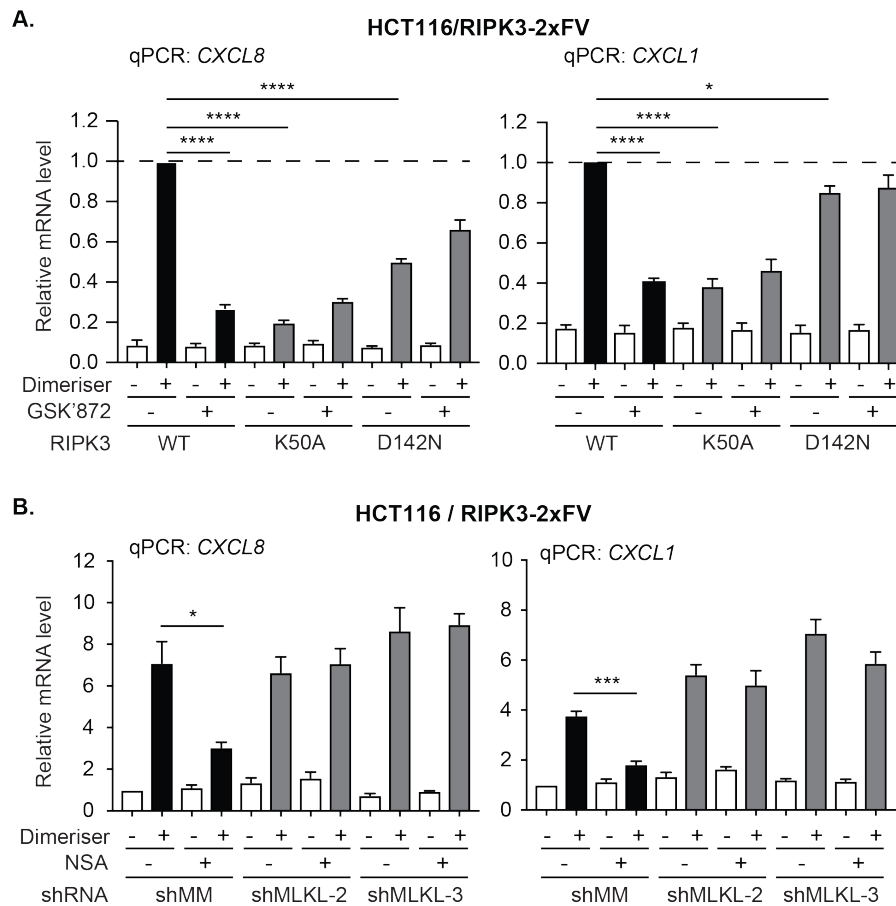


Figure 3.14: The role of RIPK3 kinase activity and MLKL in RIPK3 oligomerisation-induced inflammatory signalling. (A) HCT116/RIPK3-2xFV, HCT116/RIPK3-K50A-2xFV and HCT116/RIPK3-D142N-2xFV cells were treated as indicated for 3h. Ordinary one-way ANOVA and Sidak's multiple comparison test were used to test for statistical differences between indicated conditions. (B) HCT116/RIPK3-2xFV/shMM, HCT116/RIPK3-2xFV/shMLKL-2 and HCT116/RIPK3-2xFV/shMLKL-3 were treated as indicated for 3h. Unpaired t-test was used to test for statistical differences between indicated conditions. *, $p < 0.05$, ***, $p < 0.001$, ****, $p < 0.0001$.

(Mandal et al. 2014; Raju et al. 2018). However, GSK'872-bound human RIPK3 and K50A mutant have similar effects on chemical-induced RIPK3 oligomerisation-induced inflammatory signalling, but the effect of RIPK3-D142N mutant is different from them. This may indicate structural differences between human and murine RIPK3 in their kinase domain conformation. For further investigation, a coimmunoprecipitation experiment could be employed to investigate the interaction of different forms of RIPK3 with RIPK1 and MLKL. In addition, since murine RIPK3 carrying D161N kinase-inactive mutation is proposed to interact with RIPK1, a mutant on the same site, D160, of human RIPK3

could be made to test its function in interacting with RIPK1 and promoting inflammatory responses (Mandal et al. 2014; Raju et al. 2018).

To investigate the role of MLKL in RIPK3-mediated inflammatory signalling, I compared RIPK3-mediated inflammatory signalling levels between control and MLKL knockdown cells. Strikingly, although the MLKL oligomerisation inhibitor NSA partially inhibited RIPK3 oligomerisation-induced inflammatory signalling in an on-target manner, knockdown of MLKL did not (Figure 3.14B). As previous structural studies predict MLKL-bound murine RIPK3 to be locked in an inactive conformation, the difference between MLKL oligomerisation inhibitor-treated cells and MLKL-knockdown cells may arise from differences in the conformation of RIPK3 in the steady state, so that RIPK3 oligomerisation may induce inflammatory signalling in different ways in the presence or absence of MLKL.

In summary, neither MLKL nor RIPK3 kinase activity is required for RIPK3-oligomerisation-induced inflammatory signalling, although both the RIPK3 kinase inhibitor and the MLKL oligomerisation inhibitor partially inhibits it.

3.4 Discussion

In this chapter, I showed ZBP1-, TNF- and chemical oligomerisation-induced RIPK3 activation all lead to inflammatory signalling, while the inhibition of RIPK1 kinase activity, RIPK3 kinase activity and MLKL oligomerisation have different effects on the three ways of activation (Table 3.1). In general, TNF-induced RIPK3-mediated inflammation requires the same set of machinery as would be expected for necroptosis. In sharp contrast, ZBP1-induced RIPK3-mediated inflammatory signalling does not require any of them. Observation from the chemical-induced RIPK3 oligomerisation system falls between the two conditions.

Several models of RIPK3-mediated inflammatory signalling have been proposed before. Zhao et al. 2012 reported the phosphorylation of MAPK JNK by the residual kinase activity of the pseudokinase MLKL in HT29 cells treated with TNF+SMAC mimetics+zVAD. Najjar et al. 2016 showed that LPS+zVAD induces the production of NF- κ B-target genes in BMDMs dependent on TRIF, RIPK3, RIPK1 kinase activity,

Table 3.1: Different effects of RIPK1, RIPK3 kinase activity and MLKL on RIPK3-mediated inflammatory signalling

Conditions	ZBP1 expression	TNF+CpA+zVAD	Dimeriser
Nec1s	No inhibition	Complete inhibition	Partial inhibition
RIPK1 knockout	- (Not tested)	Complete inhibition	No inhibition
GSK'872	No inhibition	Complete inhibition	Partial inhibition
RIPK3-K50A	- (Not tested)	Complete inhibition	Partial inhibition
RIPK3-D142N	- (Not tested)	Complete inhibition	No inhibition
NSA	No inhibition	Complete inhibition	Partial inhibition
MLKL knockdown	- (Not tested)	Complete inhibition	No inhibition

MAPK ERK and NF- κ B p65 but not MLKL, and proposed a TLR4-TRIF-RIPK1-RIPK3-ERK axis in RIPK3-mediated inflammatory signalling, although it is not clear how ERK is activated by RIPK3. Different from them, RIPK3 oligomerisation-induced NF- κ B activation was shown to be separated from its necroptosis-inducing function, and proposed to be dependent on the RHIM-mediated recruitment of RIPK1 in NIH3T3 cells (Yatim et al. 2015). Taken together, two pathways can be summarised from previous reports, the RIPK3-RIPK1 pathway and the RIPK3-(MLKL-)-MAPK pathway. While the latter is shown to be dependent on the kinase activity of RIPK1 and RIPK3, the former likely requires their scaffolding function and not kinase activity (Figure 3.15).

Observations in this chapter fit the two-way model of RIPK3-mediated inflammatory signalling. ZBP1 expression could induce RIPK3-mediated inflammatory signalling through scaffolding components recruited by RIPK1. TNF+CpA+zVAD induces inflammatory signalling through MLKL, which also requires the kinase activity of RIPK1 and RIPK3. Dimeriser stimulation could be able to induce both pathways: it may induce a mixture of two pathways under native status, only the MLKL-dependent pathway in the absence of RIPK1, and only the RIPK1-mediated pathway in the absence of MLKL.

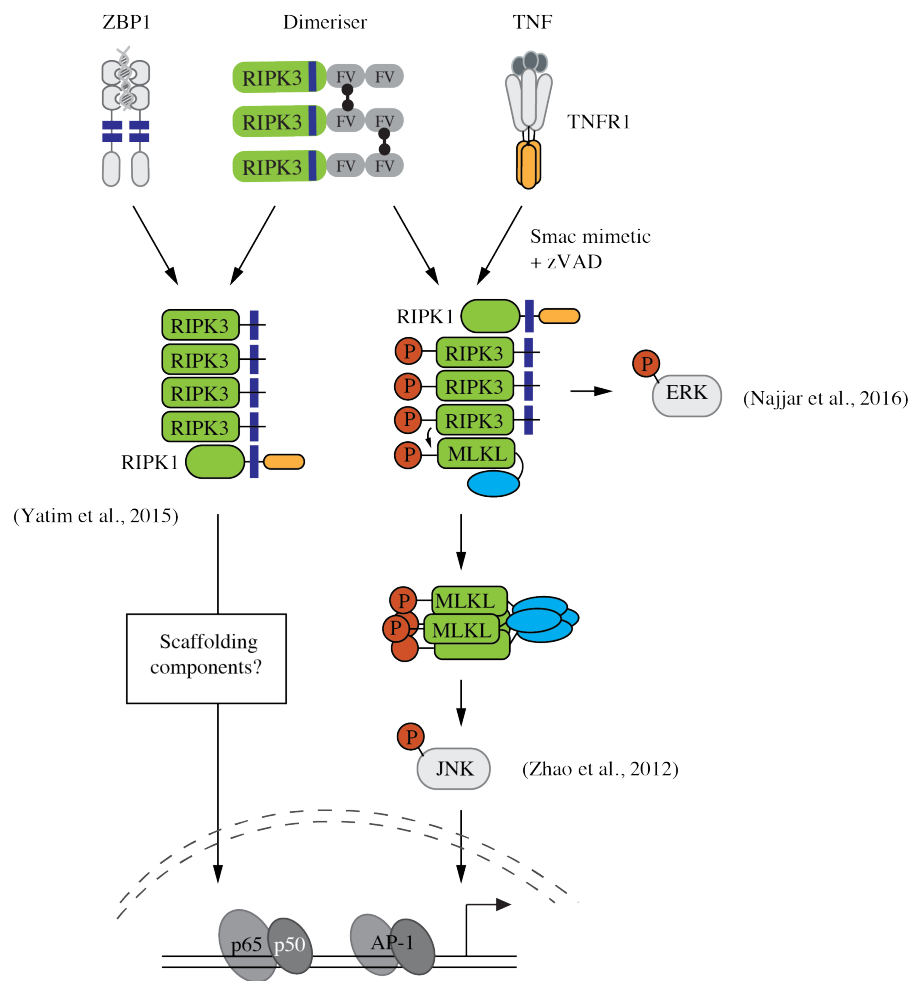


Figure 3.15: RIPK3 mediates inflammatory signalling in two ways.

4

The role of ubiquitin and caspase-8 in RIPK3-mediated inflammatory signalling

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4.1 Ubiquitylation controls RIPK3-mediated inflammatory signalling

Ubiquitylation is central to the activation of MAPK- and NF- κ B-mediated inflammatory signalling by mediating the recruitment of inflammatory kinase complexes (Hrdinka and

Gyrd-Hansen 2017). Specifically, K63- and M1-linked polyubiquitin in inflammatory complexes provide docking sites for the TAK1-TAB complex and the NEMO-IKK complex (Kanayama et al. 2004; Rahighi et al. 2009). As TAK1 and IKKs mediate RIPK3-induced inflammatory signalling, I investigated the function of ubiquitylation in this process.

Linkage-specific ubiquitin binders (SUBs) have been developed in the Gyrd-Hansen lab to study ubiquitylation-mediated inflammatory signalling (Hrdinka et al. 2016). SUBs are recombinant proteins containing ubiquitin binding domains that specifically bind certain linkage types of polyubiquitin to block their function when expressed (Figure 4.1A). I generated HCT116 cells that express the K63-SUB, the M1-SUB or the GFP control in response to doxycycline and stably expressed RIPK3-2xFV in them (Figure 4.1). The expression of SUBs significantly decreased RIPK3 oligomerisation-induced inflammatory signalling, indicating that K63- and M1-linked polyubiquitin are both required for this process (Figure 4.1B).

The ubiquitin ligases cellular inhibitor of apoptosis protein (cIAP) 1 and cIAP2 are known to assemble K63-linked polyubiquitin on RIPK1 in TNFR1-mediated inflammatory signalling (Bertrand et al. 2008). As RIPK1 is hypothesised to scaffold RIPK3-mediated inflammatory signalling, I investigated whether cIAPs are responsible for K63-linked ubiquitylation in RIPK3-mediated inflammatory signalling. SMAC mimetic compounds like Compound A (CpA) induces the auto-ubiquitylation and degradation of cIAPs, providing a convenient way to deplete them (Vince et al. 2007). CpA efficiently blocked RIPK3-mediated inflammatory signalling both from induced oligomerisation and from ZBP1 expression, indicating that cIAPs are required for RIPK3-mediated inflammatory response (Figures 4.2A-C).

At high concentrations, CpA binds another ubiquitin ligase, X-linked inhibitor of apoptosis protein (XIAP), and could block its recruitment to inflammatory complexes (Damgaard et al. 2013). To investigate whether the inhibitory effect of CpA comes from XIAP, I stably expressed RIPK3-2xFV in a CRISPR-mediated HCT116/XIAP-KO

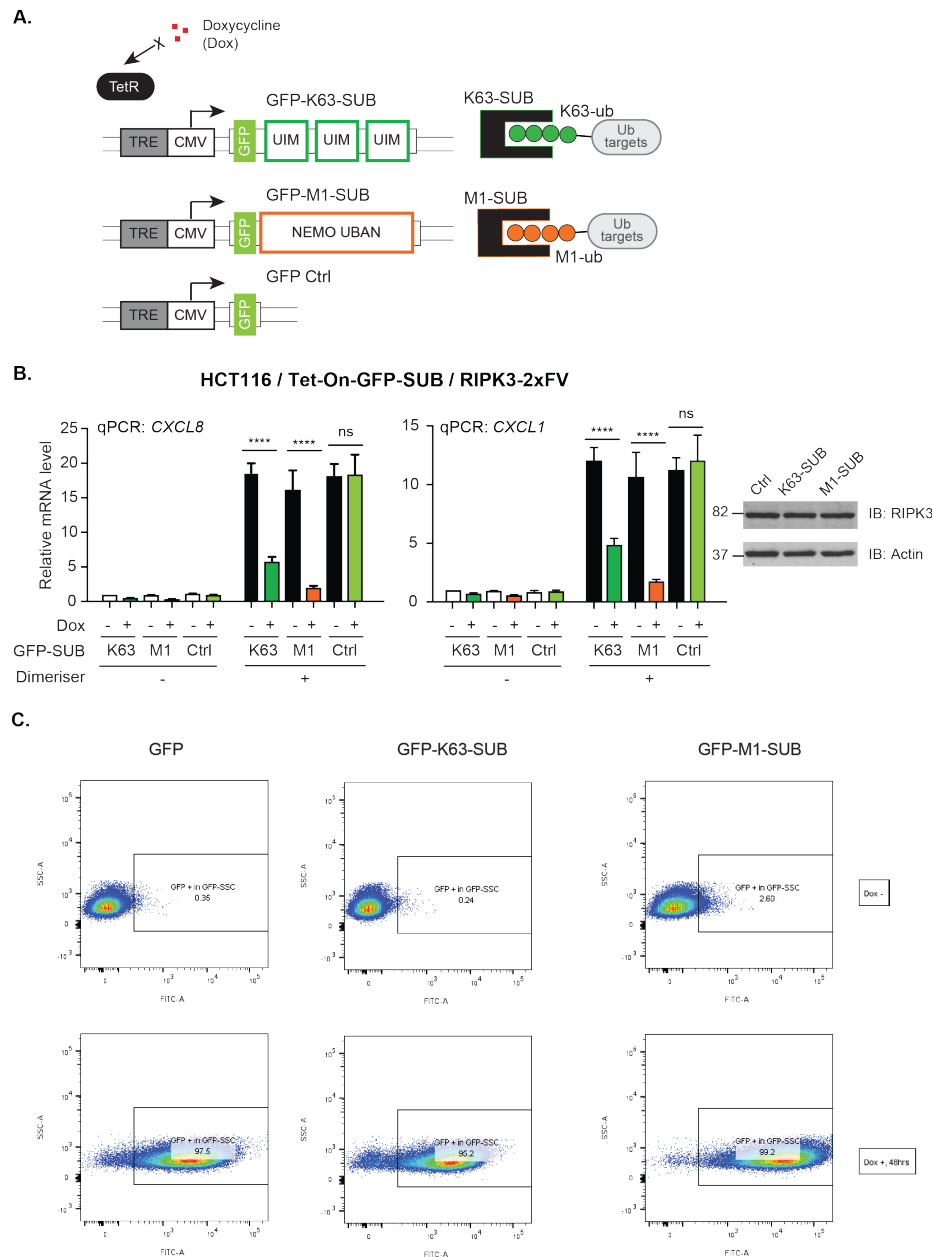


Figure 4.1: K63- and M1-SUBs inhibit RIPK3 oligomerisation-induced inflammatory signalling. (A) Dox-induced expression of GFP-SUBs. The ubiquitin-binding domains (UBDs) in the K63-SUB are three tandem ubiquitin-interacting motifs (UIMs) taken from the BRCA1-A complex subunit RAP80. The UBD in the M1-SUB is the ubiquitin-binding domain in A20 binding and inhibitor of NF- κ B proteins and NEMO (UBAN) domain of the NEMO protein. TRE, Tet-response element. CMV, cytomegalovirus promoter. (B) HCT116/Tet-On-GFP-K63-SUB/RIPK3-2xFV, HCT116/Tet-On-GFP-M1-SUB/RIPK3-2xFV and HCT116/Tet-On-GFP/RIPK3-2xFV cells were treated with 0 or 100ng/ml Dox for 48h before stimulated for qPCR (n=4). Ordinary one-way ANOVA and Sidak's multiple comparison test were used to test for the statistical differences as indicated. ns, not significant, ****, $p < 0.0001$. (C) HCT116/Tet-On-GFP-K63-SUB, HCT116/Tet-On-GFP-M1-SUB and HCT116/Tet-On-GFP cells were treated with 0 or 100ng/ml Dox for 48h before flow cytometry analysis of GFP expression levels.

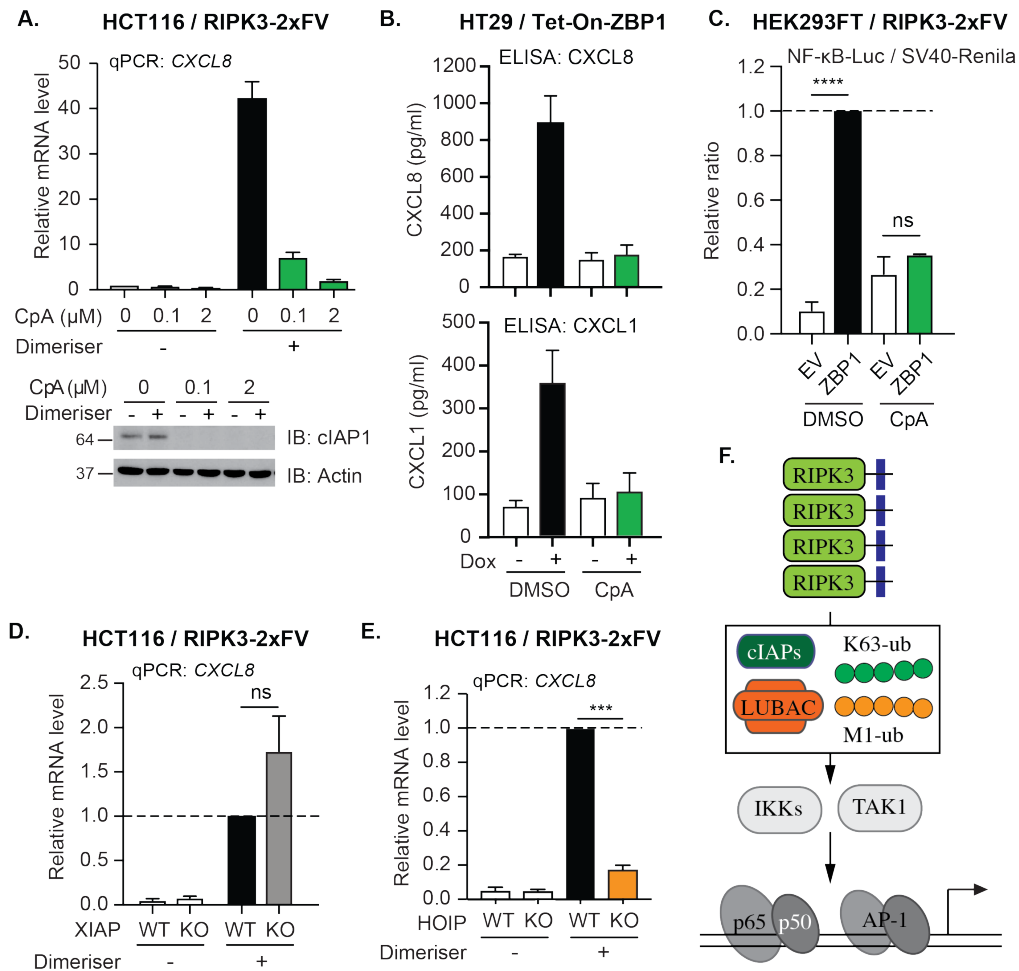


Figure 4.2: cIAPs and LUBAC are required for RIPK3 oligomerisation-induced inflammatory signalling. (A) HCT116/RIPK3-2xFV cells were pretreated with 0, 0.1 or 2 μ M CpA for 0.5h and collected for western blot analysis or treated with 0 or 100nM dimeriser for 3h for qPCR (n=3). (B) HT29/Tet-On-ZBP1 cells were treated as indicated for 24h. Supernatant was collected for ELISA analysis (n=3). CpA, 100nM, Dox, 50ng/ml. (C) HEK293FT/RIPK3-2xFV cells were transfected with empty vector (EV) or ZBP1-expressing vector along with NF- κ B luciferase reporter and SV40 Renilla control and treated with 0 or 100nM CpA immediately after transfection. Luciferase activities were measured 24h after transfection (n=3). Ordinary 1-way ANOVA and Sidak's multiple comparison test were used to test for the statistical differences as indicated. (D) HCT116/RIPK3-2xFV and HCT116/XIAP-KO/RIPK3-2xFV cells were treated with 0 or 100nM dimeriser for 3h (n=3). Welch's t-test was used to test for the statistical difference as indicated. (E) HCT116/RIPK3-2xFV and HCT116/HOIP-KO/RIPK3-2xFV cells were treated with 0 or 100nM dimeriser for 3h (n=3). Welch's t-test was used to test for the statistical difference as indicated. (F) Schematic illustration of the role of ubiquitylation in RIPK3-mediated inflammatory signalling. ns, not significant, ***, $p < 0.001$, ****, $p < 0.0001$.

clone (Cummins et al. 2004). The loss of XIAP did not affect RIPK3 oligomerisation-induced inflammatory signalling, suggesting that cIAPs but not XIAP is required for RIPK3-mediated inflammatory signalling (Figure 4.2D).

LUBAC is the only known E3 ligase that assembles M1-linked polyubiquitin (Fiil and Gyrd-Hansen 2014). To confirm the involvement of LUBAC in RIPK3-mediated inflammatory signalling, RIPK3-2xFV was stably expressed in HCT116 cells knocked out for HOIP, the catalytic subunit of LUBAC using the CRISPR/Cas9 system (Hrdinka et al. 2016). Similar to the expression of the M1-SUB, RIPK3 oligomerisation in HOIP-KO cells did not lead to inflammatory signalling, indicating that LUBAC is required for RIPK3 oligomerisation-induced inflammatory signalling (Figure 4.2E). Experiments are still ongoing to investigate the role of LUBAC and M1-linked polyubiquitin in ZBP1-induced inflammatory signalling.

In summary, RIPK3-mediated inflammatory signalling is dependent on K63- and M1-linked polyubiquitin as well as the ubiquitin ligases cIAPs and LUBAC (Figure 4.2F).

4.2 Caspase-8 determines the requirement of ubiquitylation in RIPK3-mediated inflammatory signalling

4.2.1 Caspase-8 determines the requirement of cIAPs

Although cIAPs are required in ZBP1- and dimeriser-induced RIPK3-mediated inflammatory signalling, TNF-induced RIPK3-mediated inflammatory signalling occurs in their absence, as CpA was used to compromise TNFR1-induced inflammatory Complex I (Figure 3.4C). I hypothesised that the inhibition of caspase activity during TNF-induced RIPK3 activation could underlie the lack of dependency on cIAPs, and tested the hypothesis by co-applying SMAC mimetics with caspase inhibitors in dimeriser-induced inflammatory signalling.

Consistent with the role of caspase-8 in suppressing the necroptosis pathway, the pan-caspase inhibitor zVAD greatly enhanced dimeriser-induced inflammatory signalling (Figures 4.3A and 4.3B) (Micheau et al. 2002; Feng et al. 2007; O'Donnell et al. 2011; Newton et al. 2019). Moreover, zVAD pretreatment rescued the inhibition by CpA in RIPK3-mediated inflammatory responses (Figures 4.3A and 4.3B). CpA treatment still reduced dimeriser+zVAD-induced inflammatory signalling when measured by chemokine transcript levels in 3 hours, but did not result in a significant difference in the protein

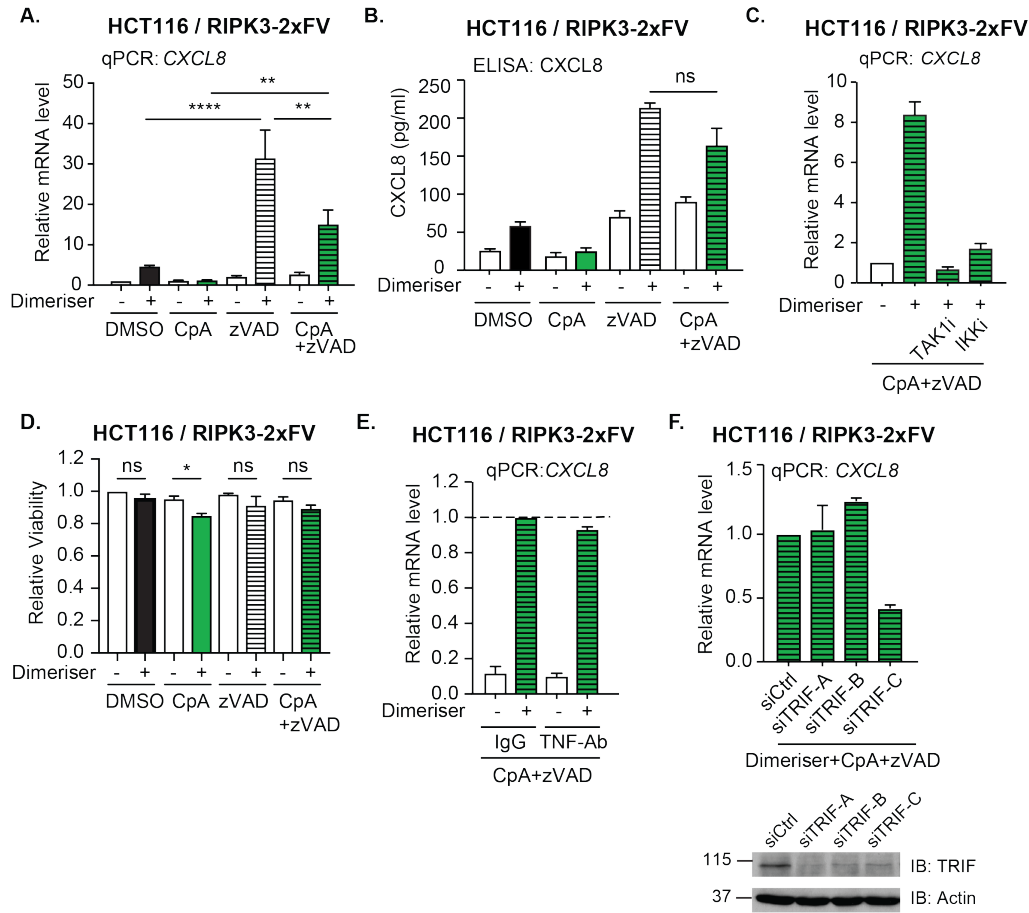


Figure 4.3: zVAD rescues RIPK3 oligomerisation-induced inflammatory signalling in the absence of cIAPs. HCT116/RIPK3-2xFV were treated as indicated (n=3). (A) 3h. CpA, 2 μ M. Ordinary 1-way ANOVA and Sidak's multiple comparison test were used to test for the statistical differences as indicated. (B) 24h. CpA, 100nM. Unpaired t-test were used to test the difference between indicated conditions. (C) 3h. CpA, 2 μ M. (D) 24h. CpA, 100nM. Ordinary 1-way ANOVA and Sidak's multiple comparison test were used to test for the statistical differences as indicated. (E) 3h. CpA, 2 μ M. (F) Cells were transfected with indicated siRNA. 72h after transfection, cells were collected for western blot or stimulated for qPCR for 3h. ns, not significant, *, p<0.05, **, p<0.01, ****, p<0.0001.

amount over 24h (Figures 4.3A and 4.3B). In addition, RIPK3-mediated inflammatory signalling in the absence of cIAPs and caspase activity was still blocked by TAK1 and IKK inhibition, did not correlate with an increased percentage of cell death, and was not dependent on TNF autocrine or TRIF (Figures 4.3C-F). Taken together, RIPK3-mediated inflammatory signalling in the absence of cIAPs and caspase activity is still

mediated by MAPK and NF- κ B pathways.

The rescue effect of caspase inhibition on the loss of cIAPs was validated in many ways. First, instead of depleting cIAPs, K63-SUB was used to block the function of K63-linked polyubiquitin, and zVAD treatment still rescued the blocked effect (Figure 4.4A). Second, instead of zVAD, treatment with another pan-caspase inhibitor quinolyl-valyl-O-methylaspartyl-[-2, 6-difluorophenoxy]-methyl ketone (Q-VD-Oph, QVD) also rescued CpA-inhibited RIPK3-mediated inflammatory signalling (Figure 4.4B). Third, in addition to HCT116 cells, zVAD treatment rescued RIPK3-mediated inflammatory signalling in the absence of cIAPs in HT29/RIPK3-2xFV cells, showing that the rescue effect is not cell type-specific (Figure 4.4C). Lastly, in addition to dimeriser-induced RIPK3 activation, zVAD also rescued ZBP1-induced NF- κ B activation in HEK293FT cells in the absence of cIAPs (Figure 4.4D).

To investigate if caspase-8 is responsible for the rescue effect, I stably knocked down caspase-8 in HCT116/RIPK3-2xFV cells to compare the effect of cIAPs depletion in cells with and without caspase-8. The loss of caspase-8 did not enhance the inflammatory signalling level as much as caspase inhibitors, but greatly compromised the inhibitory effect of CpA, indicating that the requirement of cIAPs in RIPK3-mediated inflammatory signalling depends on the presence of caspase-8 (Figure 4.5).

Taken together, in the absence of caspase-8 or its activity, cIAPs and K63-linked polyubiquitin are not required for RIPK3 to induce inflammatory signalling. Two hypotheses could be made to explain the observation: (A) K63-linked polyubiquitin could antagonise the activity of caspase-8 in suppressing RIPK3-mediated inflammatory signalling, or (B) the loss of caspase-8 activity could convert RIPK3-mediated inflammatory signalling from the ubiquitylation scaffolding-dependent pathway to the kinase activity-dependent pathway, where cIAPs are not required (Figure 4.6).

4.2.2 Caspase-8 determines the requirement of M1-linked ubiquitylation

Like K63-linked polyubiquitin, M1-linked polyubiquitin is required for RIPK3-mediated inflammatory signalling. I tested whether the loss of caspase-8 could also rescue the

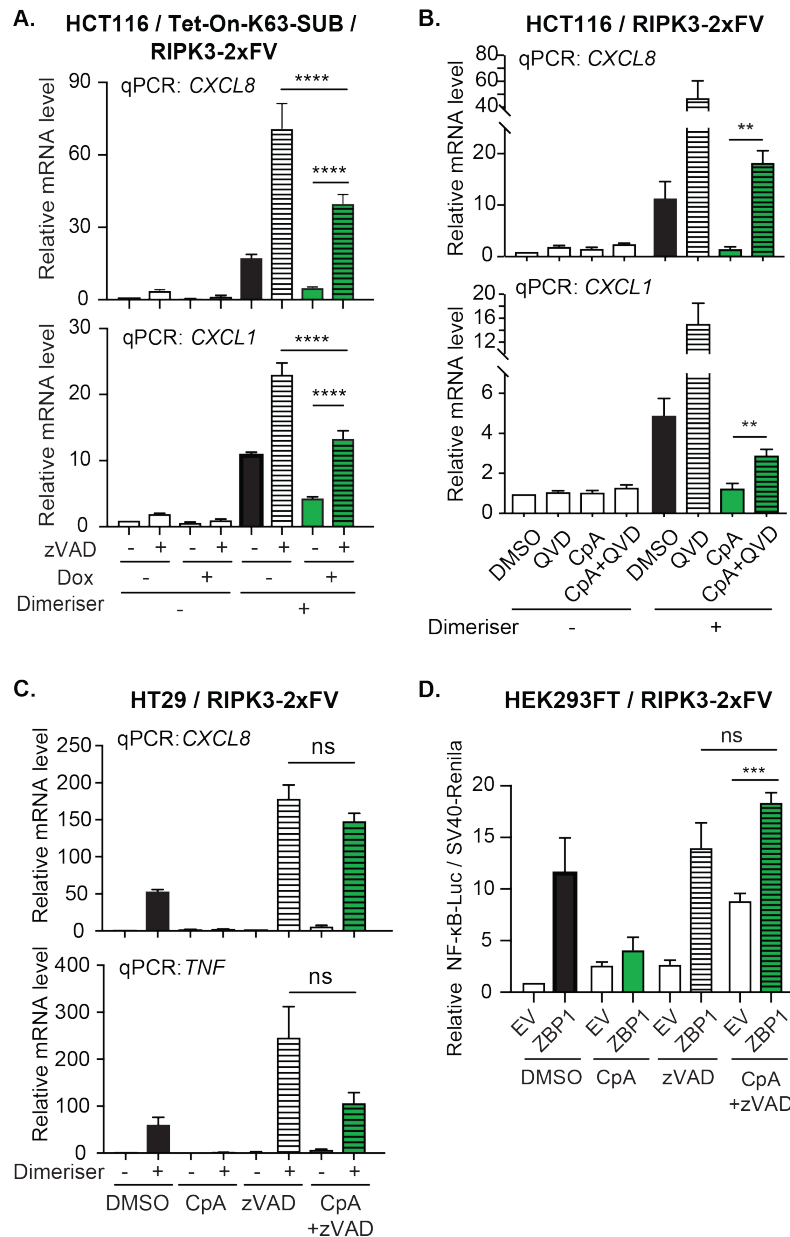


Figure 4.4: Caspase inhibition rescues RIPK3 oligomerisation-induced inflammatory signalling in the absence of K63-linked polyubiquitin. (A) Cells were treated with 0 or 100ng/ml Dox for 48hrs before stimulated as indicated for 3h (n=3). Ordinary one-way ANOVA and Sidak's multiple comparison test were used to test for the statistical differences between indicated conditions. (B, C) Cells were treated as indicated for 3h (n=3). CpA, 2μM. Unpaired t-tests were used to test for the statistical differences between indicated conditions. (D) Cells were transfected with 0.5ng empty vector (EV) or ZBP1-expressing vector along with NF-κB luciferase reporter and SV40 Renilla reporter. Immediately after transfection, cells were treated as indicated. CpA, 100nM. Luciferase activities were measured 24h after transfection (n=3). Ordinary one-way ANOVA and Sidak's multiple comparison test were used to test for the statistical differences between indicated conditions. ns, not significant, **, p<0.01, ***, p<0.001, ****, p<0.0001.

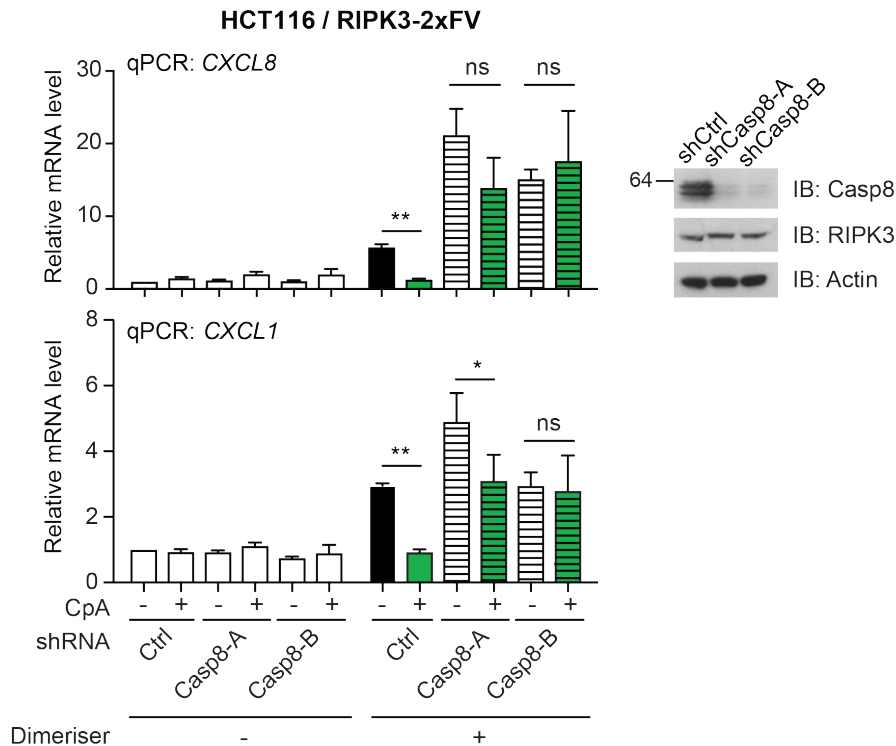


Figure 4.5: CpA does not inhibit RIPK3-mediated inflammatory signalling in the absence of caspase-8. HCT116/RIPK3-2xFV/shMM, HCT116/RIPK3-2xFV/shCasp8-A and HCT116/RIPK3-2xFV/shCasp8-B cells were treated as indicated for 3h for qPCR (n=4). Equal numbers of cells were collected for western blot analysis of caspase-8 knockdown efficiency. RM one-way ANOVA and Sidak's multiple comparison tests were used to test the difference between indicated conditions. ns, not significant. *, $p < 0.05$, **, $p < 0.01$.

inhibited signalling from the loss of M1-linked polyubiquitin. Both caspase inhibition and siRNA-mediated caspase-8 knockdown rescued RIPK3 oligomerisation-induced inflammatory signalling in HOIP-KO cells (Figures 4.7A and 4.7B). Similarly, conclusions were validated using M1-SUB and QVD (Figures 4.7C and 4.7D).

Moreover, in the absence of both cIAPs and HOIP, QVD treatment still showed a tendency towards rescuing RIPK3-mediated inflammatory signalling, though more replicates need to be performed for statistical significance (Figure 4.7D). This indicates that caspase-8 may still rescue RIPK3-mediated inflammatory signalling even in the absence of both K63- and M1-linked polyubiquitin. Consistent with this hypothesis, TNF+CpA+zVAD –induced inflammatory signalling was not affected by the loss of HOIP, indicating that the RIPK3 kinase activity-mediated inflammatory signalling pathway does not require cIAPs or M1-linked polyubiquitin (Figure 4.8).

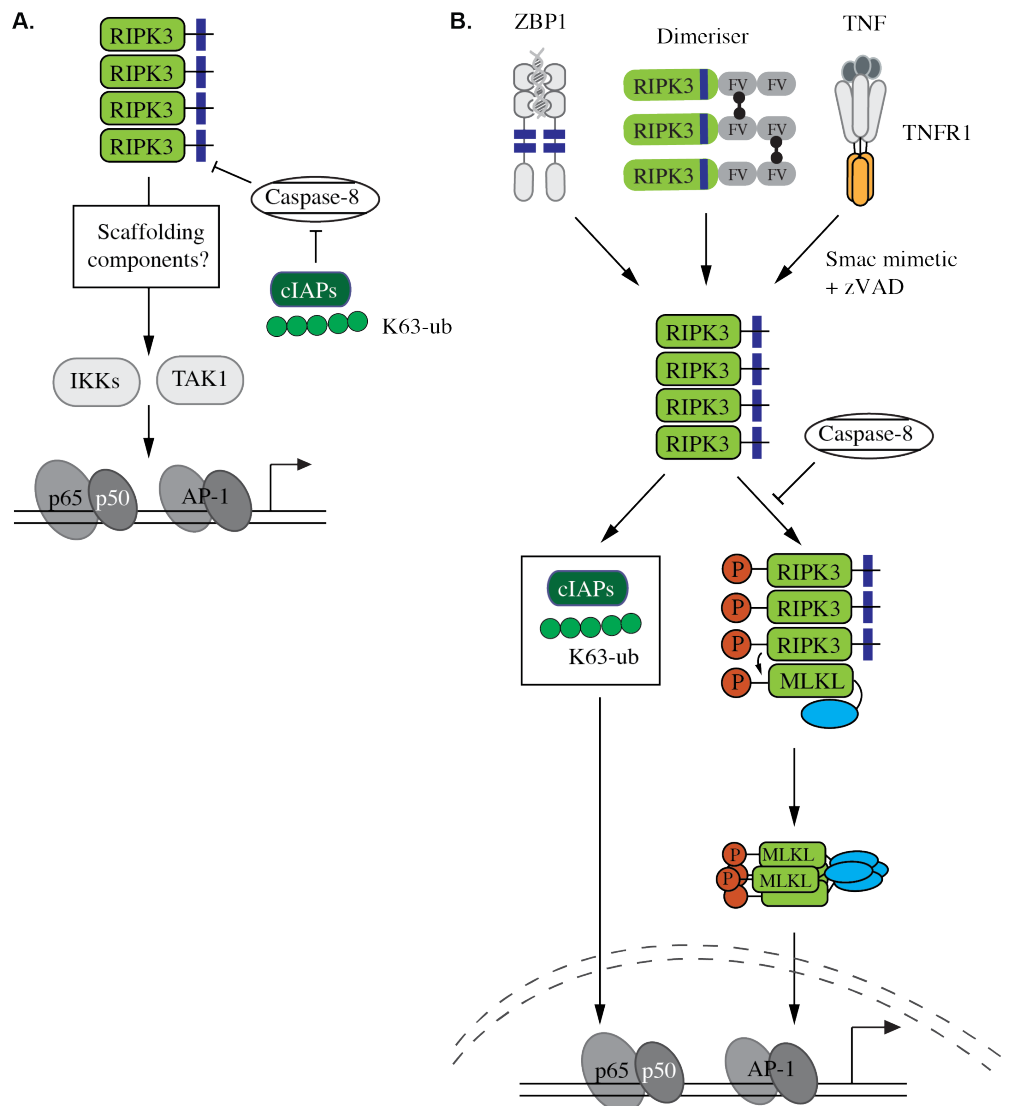


Figure 4.6: Two possible ways that caspase-8 antagonises the requirement of cIAPs in RIPK3-mediated inflammatory signalling. (A) cIAPs and K63-linked polyubiquitin could positively regulate RIPK3-mediated inflammatory signalling by antagonising the inhibitory effects of caspase-8. This could be achieved by ubiquitylating substrates to limit the access of caspase-8 to its cleavage sites, or by ubiquitylating caspase-8 to regulate its activity. (B) Continuing from the model in Figure 3.15, cIAPs and K63-linked ubiquitylation could be essential components of the scaffolding-mediated pathway, while the inhibition of caspase-8 could be required for the activation of the RIPK3 kinase activity-dependent pathway. Thus, with the loss of both cIAPs and caspase inhibition, RIPK3-mediated inflammatory signalling could be converted from the scaffolding-mediated pathway to the RIPK3 kinase activity-dependent pathway, similar to the situation in TNFR1-induced RIPK3-mediated inflammatory signalling.

In summary, in the absence of caspase-8 or its activity, M1-linked polyubiquitin is dispensable for RIPK3-mediated inflammatory signalling.

Similar to the situation with K63-linked polyubiquitin, two hypotheses could be

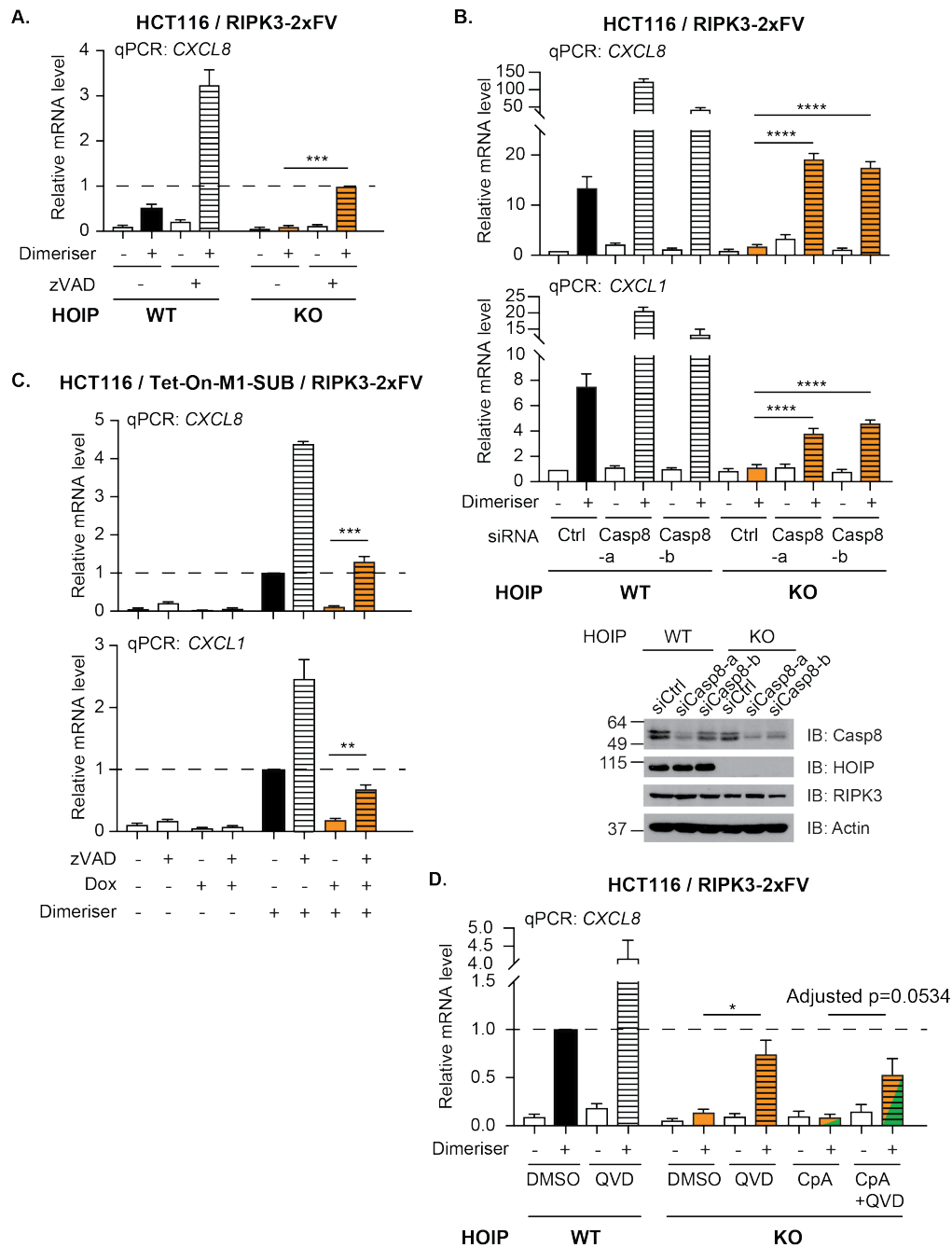


Figure 4.7: Caspase-8 inhibition rescues RIPK3-mediated inflammatory signalling in HOIP-KO cells. (A) HCT116/RIPK3-2xFV and HCT116/HOIP-KO/RIPK3-2xFV cells were stimulated as indicated for 3h (n=3). Welch's t-test was used to test for the statistical difference between indicated conditions. (B) HCT116/RIPK3-2xFV and HCT116/HOIP-KO/RIPK3-2xFV cells were transfected with siRNA against control or 2 different sites of caspase-8 for 72h before stimulated for 3h or collected for western blot analysis (n=3). Ordinary one-way ANOVA and Sidak's multiple comparison test were used to test for statistical differences between indicated conditions. (C) Cells were treated with doxycycline for 48h before stimulated as indicated for 3h (n=3). Unpaired t-test was used to test for the statistical difference between indicated conditions. (D) HCT116/RIPK3-2xFV and HCT116/HOIP-KO/RIPK3-2xFV cells were stimulated as indicated for 3h (n=3). Ordinary one-way ANOVA and Sidak's multiple comparison test were used to test for statistical differences between indicated conditions. *, p<0.05, **, p<0.01, ***, p<0.001, ****, p<0.0001.

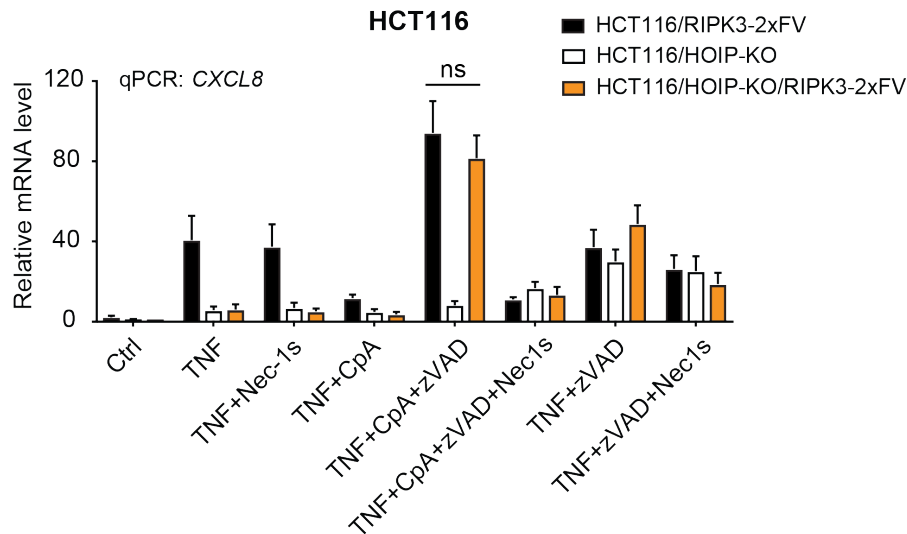


Figure 4.8: HOIP is not required for TNF+CpA+zVAD-induced inflammatory signalling. Cells were stimulated as indicated for 3h (n=3). Ordinary two-way ANOVA and Tukey's multiple comparisons test were used to test the significance between indicated conditions. ns, not significant.

proposed like in Figure 4.6, that M1-linked polyubiquitin could antagonise the activity of caspase-8 in suppressing RIPK3-mediated inflammatory signalling, or that the loss of caspase-8 activity could open up the RIPK3 kinase activity-dependent signalling pathway that does not require M1-linked polyubiquitin. According to the former hypothesis, the loss of M1-linked polyubiquitin should not further inhibit the inflammatory signalling level in the absence of caspase activity. However, the loss of HOIP or M1-linked polyubiquitin greatly reduced the inflammatory levels in the absence of caspase activity under all tested conditions (Figure 4.7). Therefore, the observations support the two-way model in the second hypothesis, where K63- and M1-linked polyubiquitin mediate the scaffolding-dependent pathway of RIPK3-mediated inflammatory signalling in the presence of caspase activity, while they are not required for the RIPK3 kinase activity-dependent pathway which is opened up by the inhibition of caspase activity (Figure 4.9). I will focus on the validation and development of this model in the rest of the thesis. Notably, the observations do not exclude the existence of regulation of caspase-8 activity by K63- and M1-linked polyubiquitin in the two-way model, which requires further investigation.

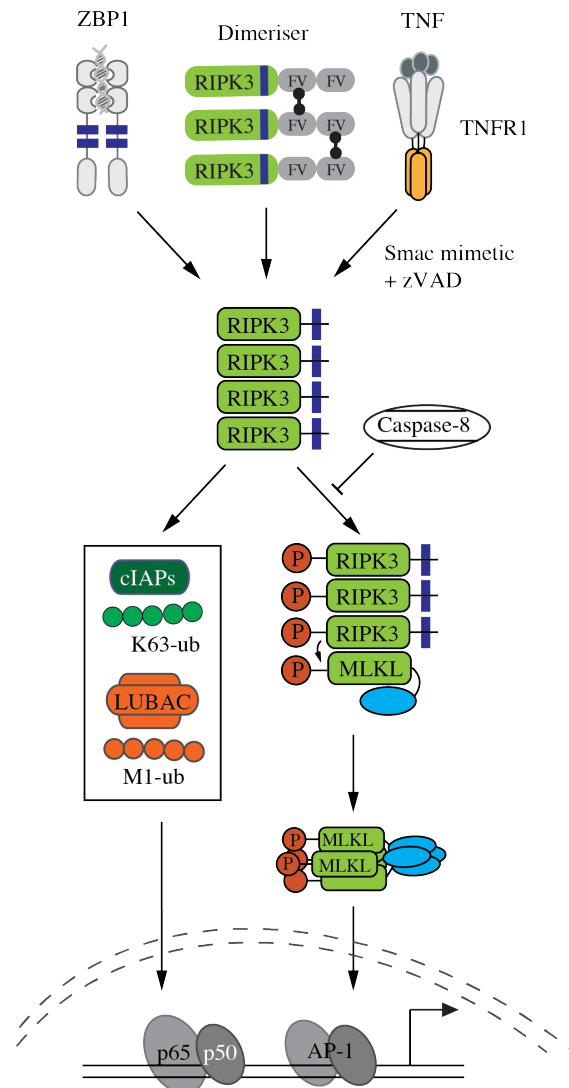


Figure 4.9: Working model for the interplay between ubiquitylation and caspase-8 in RIPK3-mediated inflammatory signalling. RIPK3 mediates inflammatory signalling in two ways, one dependent on its scaffolding function which is mediated by K63- and M1-linked polyubiquitin, and the other dependent on its kinase activity which is only activated with the loss of caspase-8 or its catalytic activity.

4.3 cIAPs and caspase-8 regulate the stability of activated RIPK3

According to the proposed model, the inhibition of caspase-8 during RIPK3 oligomerisation should induce the activation of RIPK3 kinase activity that is not affected by the loss of cIAPs or LUBAC (Figure 4.9). I next investigated if this is the case.

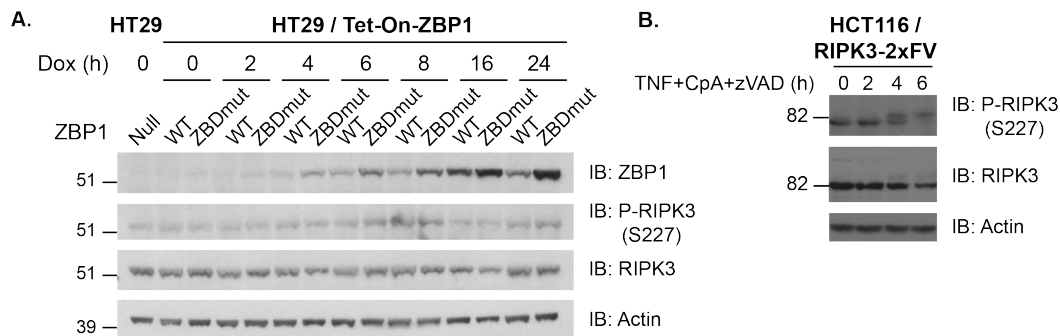


Figure 4.10: Activation of RIPK3 kinase activity correlates with the appearance of a slow-migrating form of RIPK3. (A) HT29, HT29/Tet-On-ZBP1 and HT29/Tet-On-ZBP1mut was treated with 50ng/ml Dox for indicated time. (B) Cells were treated as indicated. CpA, 100nM. TNF, 20ng/ml.

4.3.1 The appearance of slow-migrating RIPK3

The activation of RIPK3 kinase activity was reported to be associated with the appearance of a slow-migrating form of RIPK3 on western blots, which is dependent on the phosphorylation of the T182 residue on its activation loop (Choi et al. 2018). Consistent with the observations that TNF-induced RIPK3-mediated inflammatory signalling is dependent on RIPK3 kinase activity, TNF+CpA+zVAD induced an upshift of RIPK3 in HCT116/RIPK3-2xFV cells. In contrast, ZBP1 expression did not lead to the appearance of slow-migrating RIPK3, which correlates with the activation of the scaffolding-dependent pathway (Figure 4.10).

Consistent with the working model, dimeriser-induced RIPK3 activation did not induce the appearance of activated RIPK3 unless in the absence of caspase-8 or its catalytic activity (Figures 4.11 and 4.12). In addition, the depletion of cIAPs or LUBAC did not affect the appearance of slow-migrating RIPK3 (Figures 4.12D-F).

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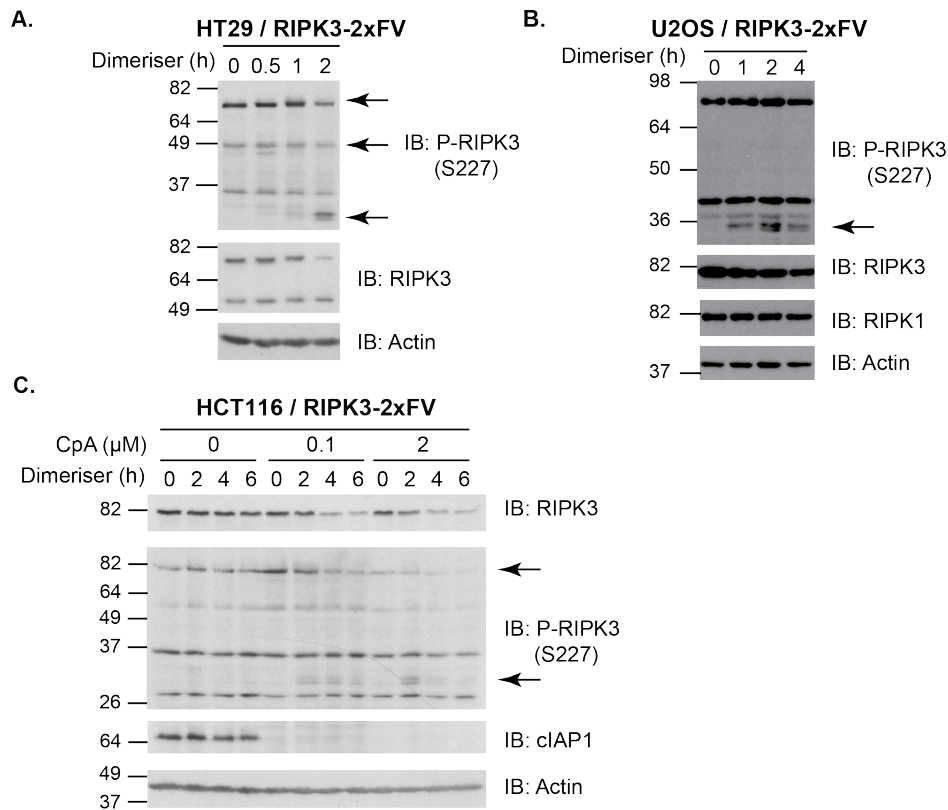


Figure 4.11: Dimeriser-induced oligomerisation does not lead to the appearance of slow-migrating RIPK3. (A) HT29/RIPK3-2xFV cells were treated as indicated. Arrows indicate (up to down) RIPK3-2xFV, endogenous RIPK3 and the cleaved N-terminal fragment of RIPK3. (B) U2OS/RIPK3-2xFV cells were treated as indicated. Arrow indicates cleaved RIPK3. (C) HCT116/RIPK3-2xFV cells were pretreated with 0, 0.1 or 2 μ M CpA for 0.5h before stimulated with dimeriser for indicated length of time. Arrows indicate RIPK3-2xFV and the cleaved N-terminal fragment.

4.3.2 cIAPs antagonise caspase-8-mediated cleavage of slow-migrating RIPK3

Caspase-8 was reported to cleave human RIPK3 at D328A (Feng et al. 2007). Dimeriser stimulation in HT29 and U2OS cells led to the appearance of a lower band around 30-35kDa, the size of which matches the aa 1-328 N-terminal cleavage product (Figures 4.11A and 4.11B). In HT29 cells, the appearance of the lower band correlated with a

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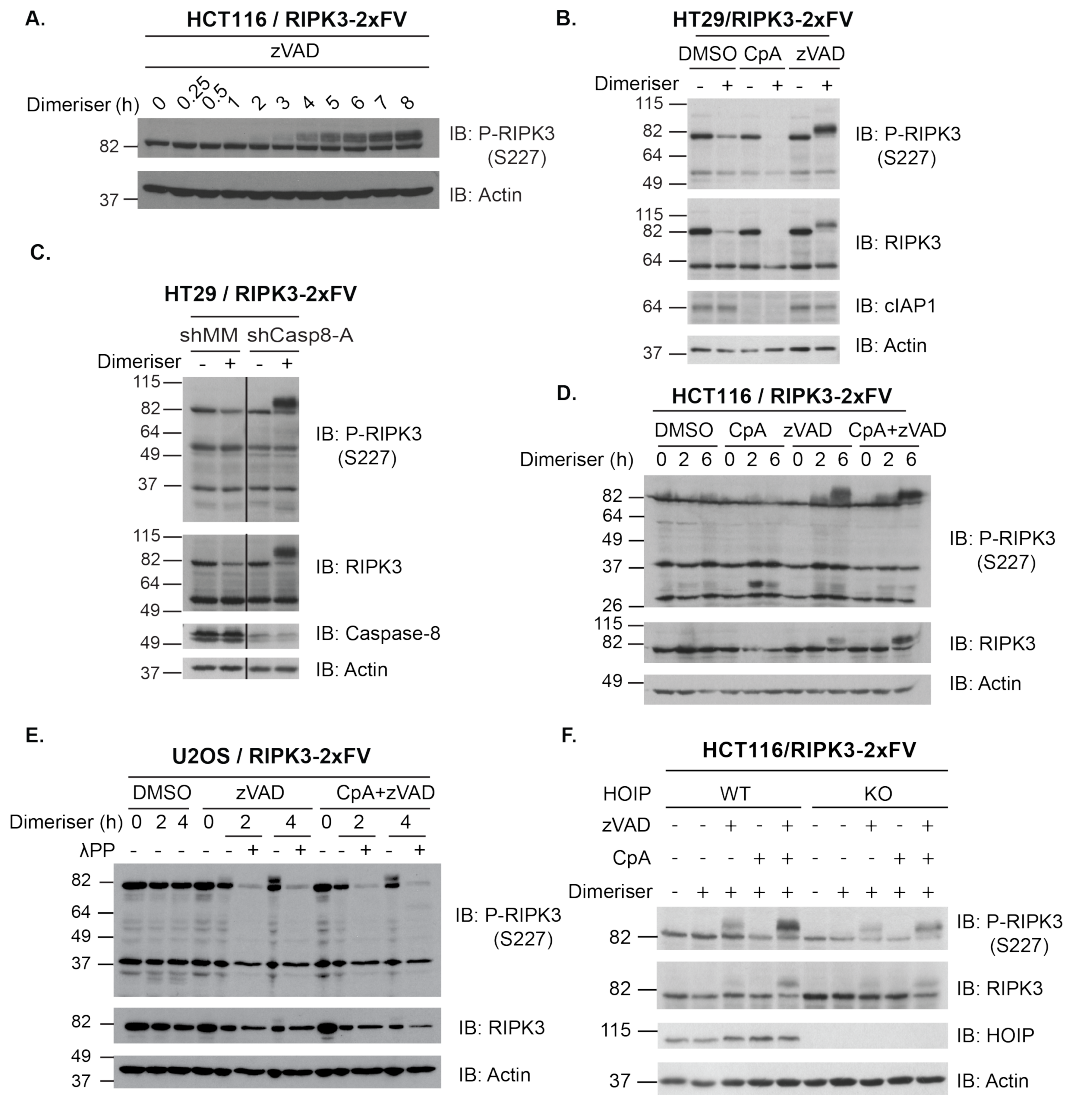


Figure 4.12: Inhibition of caspase-8 stabilises the slow-migrating form of RIPK3 (A, D) HCT116/RIPK3-2xFV cells were treated as indicated. CpA, 100nM. (B) HT29/RIPK3-2xFV cells were treated as indicated for 24h. CpA, 100nM. (C) HT29/RIPK3-2xFV/shMM and HT29/RIPK3-2xFV/shCasp8-A cells were treated as indicated for 6h. Lanes were taken from the same exposure in the same blot. (E) U2OS/RIPK3-2xFV cells were treated as indicated. Indicated conditions were treated with phosphatase after lysis. CpA, 100nM. (F) HCT116/RIPK3-2xFV and HCT116/HOIP-KO/RIPK3-2xFV cells were treated as indicated for 6h. CpA, 100nM.

decrease in the level of RIPK3-2xFV (Figure 4.11A). In HCT116 cells, the cleavage band and the decrease in RIPK3-2xFV levels were not observed with dimeriser stimulation alone, but were observed with dimeriser stimulation in the absence of cIAPs, which

happened at about the same time as the appearance of slow-migrating RIPK3 when caspase activity was inhibited (Figures 4.11C and 4.12A).

As caspase-8 is known to suppress the necroptosis pathway where RIPK3's kinase activity is activated, the observations could indicate that caspase-8 constantly cleaves activated RIPK3 that arises from dimeriser-induced proximity, to suppress the activation of its kinase activity. The loss of cIAPs accelerated the loss of RIPK3, suggesting that cIAPs could antagonise the cleavage of RIPK3 by caspase-8 (Figures 4.11C and 4.12B). This could be through assembling K63-linked polyubiquitin on RIPK3 to prevent the activation of its kinase activity, similar to the suppression of RIPK1 kinase activity in the TNFR1 pathway, which requires further investigation (Bertrand et al. 2008).

Taken together, the appearance of slow-migrating RIPK3 during inflammatory processes correlates with the conditions that are proposed to induce RIPK3 kinase-dependent inflammatory signalling, supporting the working model (Figure 4.9). To back up the model with more evidences, future work will need to 1) identify the ubiquitylation substrates in the scaffolding-dependent pathway, 2) elucidate the mechanisms of the RIPK3 kinase activity-dependent inflammatory signalling pathway, and 3) test whether the increased inflammatory signalling from the inhibition of caspase activity can be reduced by RIPK3 kinase inhibitor or MLKL oligomerisation inhibitor.

4.4 CYLD suppresses RIPK3-mediated inflammatory signalling in the absence of caspase activity

CYLD is a deubiquitinase that cleaves K63- and M1-linked polyubiquitin from inflammatory complexes (Hrdinka et al. 2016). As K63- and M1-linked polyubiquitin are both required for RIPK3 scaffolding-mediated inflammatory signalling, I investigated whether CYLD could suppress the process. Contrary to the expectation, shRNA-mediated knockdown of CYLD did not result in an increase in RIPK3-mediated inflammatory signalling (Figure 4.13A).

CYLD promotes TNFR1-induced cell death by suppressing the prosurvival ubiquitylation on RIPK1 (Hitomi et al. 2008). To antagonise this, caspase-8 is reported to constantly cleave CYLD for its proteolysis (O'Donnell et al. 2011). Consistent with caspase-8's

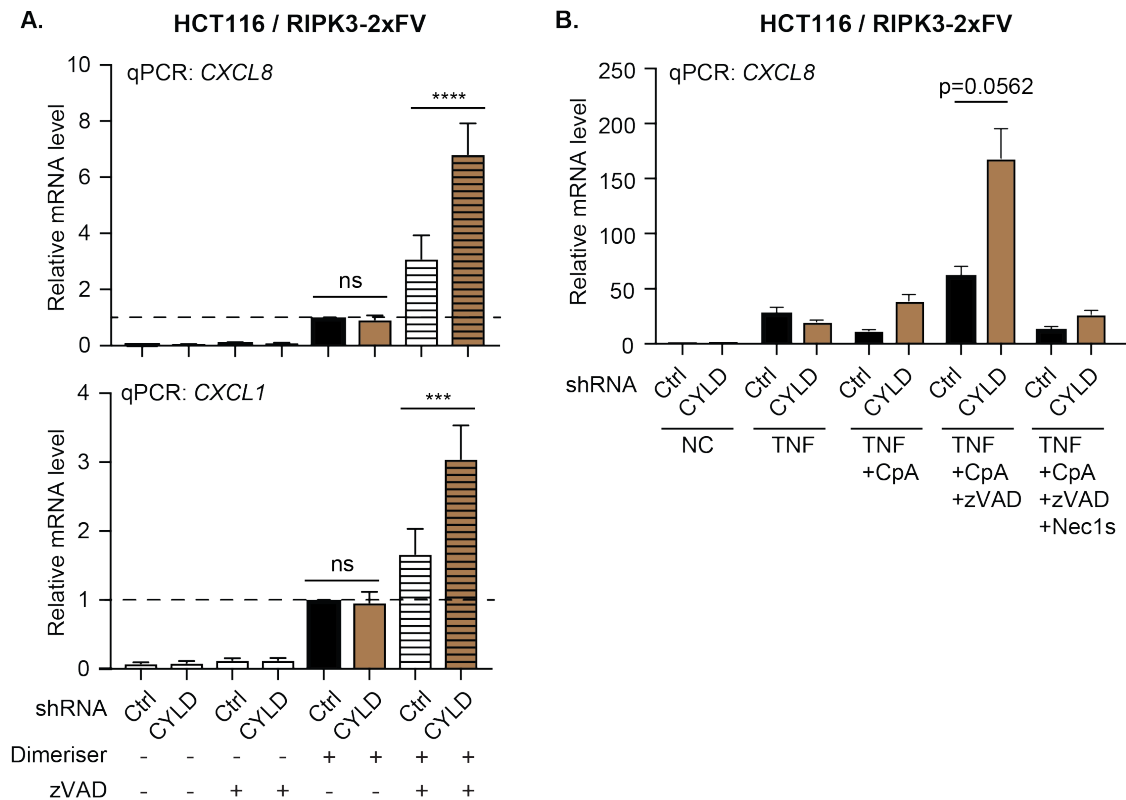


Figure 4.13: CYLD negatively regulates RIPK3-mediated inflammatory signalling in the absence of caspase activity. HCT116/shCtrl/RIPK3-2xFV and HCT116/shCYLD/RIPK3-2xFV cells were treated as indicated for 3h. (A) $n=4$. Ordinary one-way ANOVA and Sidak's multiple comparison test were used to test for the statistical differences between indicated conditions. (B) $n=3$. Unpaired t-test was used to test for statistical differences between indicated conditions. ns, not significant, ***, $p<0.001$, ****, $p<0.0001$.

role in cleave-inactivating CYLD, the knockdown of CYLD resulted in an increase in RIPK3 oligomerisation-induced inflammatory signalling in the absence of caspase activity (Figure 4.13A). In addition, the knockdown of CYLD also increased the level of TNF-induced RIPK3-mediated inflammatory signalling, but more technical replicates are required to see if the increase would be statistically significant (Figure 4.13B).

However, as cIAPs and HOIP are not required for TNF-induced RIPK3-mediated inflammatory signalling, it is not clear how CYLD could suppress it. One explanation could be that other ubiquitin ligases like the Pellinos could assemble K63-linked polyubiquitin to regulate TNF-induced RIPK3-mediated inflammatory signalling, which requires further investigation.

Taken together, the results indicate that CYLD is recruited to RIPK3-mediated

inflammatory signalling complexes but its activity is constantly suppressed by caspase-8. In the absence of caspase activity, CYLD suppresses RIPK3 kinase activity-mediated inflammatory signalling. Considering that CYLD could be recruited by HOIP in the LUBAC complex, CYLD could possibly also suppress the RIPK3 scaffolding-dependent pathway in the absence of caspase activity (Figure 4.14). To further investigate the involvement of CYLD in RIPK3-mediated inflammatory signalling, a coimmunoprecipitation experiment could be performed to see if CYLD exists in a RIPK3-associated complex after its activation, and whether caspase inhibition impacts the level of associated CYLD. To further investigate the role of caspases to control CYLD activity, caspase-uncleavable CYLD could be compared with its wild-type control on their effects on RIPK3-mediated inflammatory signalling.

4.5 Caspase substrates in RIPK3-mediated inflammatory signalling

From the results above, caspase-8 is a potent regulator of RIPK3-mediated inflammatory signalling. On the one hand, it could cleave slow-migrating RIPK3 to suppress the kinase activity-dependent pathway. On the other hand, it could cleave CYLD to antagonise its suppressive function of RIPK3-mediated inflammatory signalling.

RIPK3 was reported to be cleaved by caspase-8 at D328 (Feng et al. 2007). To investigate the role of caspase-8-mediated cleavage of RIPK3 in regulating RIPK3-mediated inflammatory signalling, I made RIPK3-D328A-2xFV and stably expressed it in HCT116/shCtrl and HCT116/shCYLD cells (Hrdinka et al. 2016) (Figure 4.15). If CYLD and RIPK3 are the only two caspase substrates important for controlling RIPK3-mediated inflammatory signalling, caspase inhibition in HCT116/shCYLD/RIPK3-D328A-2xFV would have no effect on RIPK3-mediated inflammatory signalling.

Intriguingly, oligomerisation of RIPK3-D328A-2xFV induced inflammatory signalling to similar levels as that of RIPK3-2xFV, and caspase inhibition increased the inflammatory responses of all four cell lines to similar levels (Figure 4.15). This suggests that RIPK3 and CYLD may not be the major proinflammatory targets of caspase-8 in RIPK3-mediated inflammatory signalling. However, as other caspase cleavage sites

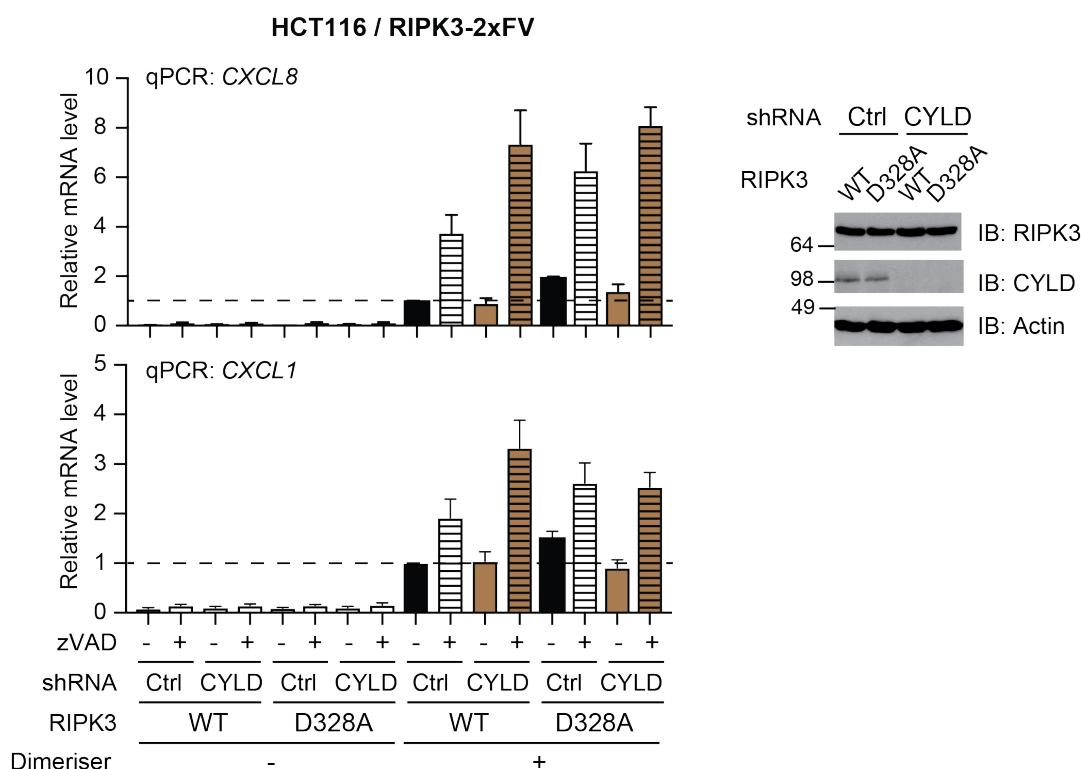


Figure 4.15: RIPK3 and CYLD are not the only substrates for caspase-8 to regulate RIPK3-mediated inflammatory signalling. HCT116/shCtrl/RIPK3-2xFV, HCT116/shCtrl/RIPK3-D328A-2xFV, HCT116/shCYLD/RIPK3-2xFV and HCT116/shCYLD/RIPK3-D328A-2xFV cells were treated as indicated for 3h (n=3). Equal number of cells are collected for western blots.

could happen in RIPK3-mediated inflammatory signalling (Lafont et al. 2017). More investigation needs to be made to study the cleavage of RIPK1 and HOIP in this process.

4.6 Summary and discussion

In this chapter, I showed the roles of ubiquitylation and caspases in regulating RIPK3-mediated inflammatory responses. The loss of K63-linked polyubiquitin and M1-linked polyubiquitin both inhibited RIPK3-induced inflammatory signalling, while the loss of caspase-8 or its activity not only increased the level of RIPK3-induced inflammatory signalling, but also at least partially rescued the inhibitory effects from the loss of cIAPs and LUBAC. In addition, TNF-induced RIPK3-mediated inflammatory signalling does not require cIAPs or LUBAC (Figures 4.1-4.8). Taken together, cIAPs and LUBAC are required for RIPK3-mediated inflammatory signalling in the presence of caspase activity, but are not required in the absence of caspase activity.

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Based on the observations, the two-way model proposed in Figure 3.15 could be developed further. In the presence of caspase activity, scaffolding elements including cIAPs-assembled K63-linked polyubiquitin and LUBAC-assembled M1-linked polyubiquitin mediate the activation of inflammatory signalling. The inhibition of caspase-8 unleashes the RIPK3 kinase-dependent inflammatory signalling pathway, which occurs independently of cIAPs or M1-linked polyubiquitin (Figure 4.9). Consistent with the model, the appearance of slow-migrating RIPK3 was specific to caspase-inhibited conditions (Figures 4.10-4.12).

Contrary to the ubiquitin ligases, the deubiquitinase CYLD suppresses RIPK3-mediated inflammatory signalling only under conditions where caspases are inhibited (Figure 4.13), which is consistent with previous reports that caspase-8 constantly processes the proteolysis of CYLD to antagonise its deubiquitinase activity (O'Donnell et al. 2011). However, the involvement of CYLD and its caspase-8-mediated cleavage in RIPK3-mediated inflammatory signalling require further investigation.

5

The role of RIPK1 in RIPK3-mediated inflammatory signalling

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One key question to be addressed in RIPK3-mediated inflammatory signalling is the involvement of RIPK1. RIPK1 was proposed to mediate the scaffolding pathway of RIPK3-mediated inflammatory signalling (Yatim et al. 2015) (Figure 3.15). In addition, RIPK1 is able to recruit caspase-8 through FADD and RIPK3 through RHIM, indicating that RIPK1 may also be involved in the suppression of RIPK3 kinase activity-dependent pathway by recruiting caspase-8 (Micheau et al. 2002). I next investigated the role of RIPK1 in RIPK3-mediated inflammatory signalling.

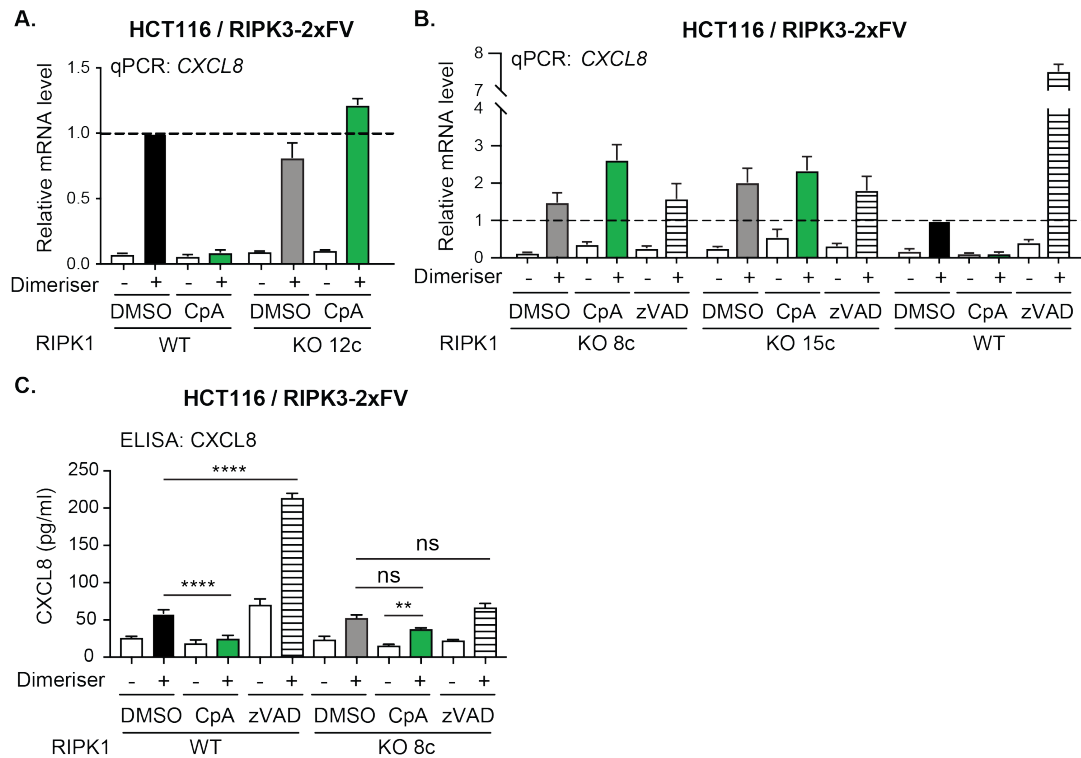


Figure 5.1: RIPK1 is required for cIAPs and caspase-8 to regulate RIPK3-mediated inflammatory signalling. (A) HCT116/RIPK3-2xFV and HCT116/RIPK1-KO clone 12c/RIPK3-2xFV were pretreated with 2 μ M CpA for 0.5h before treated with 0 or 100nM dimeriser for 3h (n=3). (B) HCT116/RIPK3-2xFV, HCT116/RIPK1-KO clone 8c/RIPK3-2xFV and HCT116/RIPK1-KO clone 15c/RIPK3-2xFV were treated as indicated for 3h (n=3). CpA, 2 μ M. (C) HCT116/RIPK3-2xFV and HCT116/RIPK1-KO clone 8c/RIPK3-2xFV were treated as indicated for 24h. Supernatant was collected for ELISA (n=3). CpA, 100nM. Ordinary one-way ANOVA and Sidak's multiple comparison test were used to test for statistical differences between indicated conditions. ns, not significant, **, p<0.01, ****, p<0.0001.

5.1 RIPK1 mediates the regulation by cIAPs and caspase-8 in RIPK3-mediated inflammatory signalling

5.1.1 RIPK1 is required for cIAPs and caspase-8 to regulate RIPK3-mediated inflammatory signalling

I first tested the hypothesis that RIPK1 mediates the recruitment of ubiquitylation elements and caspase-8 to RIPK3 to regulate its inflammatory signalling. In multiple HCT116/RIPK1-KO clones, depletion of cIAPs and inhibition of caspase activity did not affect RIPK3 oligomerisation-induced inflammatory responses, indicating that RIPK1 plays a scaffolding role between RIPK3 and its regulatory components, cIAPs and caspase-8 (Figure 5.1).

If caspase-8 cannot be recruited to RIPK3 in the absence of RIPK1, it would not be able to cleave oligomerisation-induced slow-migrating RIPK3. Indeed, RIPK3 oligomerisation in the absence of RIPK1 resulted in the appearance of slow-migrating RIPK3 even in the presence of caspase activity, which was not further affected by the depletion of cIAPs, the inhibition of caspase activity or the combination of both (Figure 5.2). In summary, RIPK1 is the link between RIPK3 and its regulatory components, cIAPs and caspase-8 in the two different pathways (Figure 5.3).

In TNFR1-mediated inflammatory signalling, cIAPs are proposed to ubiquitylate RIPK1 to suppress its autophosphorylation and subsequent association with caspase-8 (Bertrand et al. 2008; Newton et al. 2016). This prompts me to think that cIAPs may suppress the interaction between RIPK1 and caspase-8 in RIPK3-mediated inflammatory signalling in a similar fashion. Next, I investigated how RIPK1 coordinates the recruitment of cIAPs and caspase-8 in RIPK3-mediated inflammatory pathways.

5.1.2 RIPK1 mediates the recruitment of slow-migrating RIPK3 to the Ripoptosome complex

The Ripoptosome is proposed to be a 2MDa complex that dynamically forms and disassembles to control diverse cellular functions including cell death and division (Tenev et al. 2011; Feoktistova et al. 2011; Lippardi et al. 2019). RIPK1, FADD and caspase-8 are the central components of the Ripoptosome, while cIAPs promote its disassembly by ubiquitylating RIPK1 (Tenev et al. 2011; Feoktistova et al. 2011). Once formed, the Ripoptosome could promote apoptosis or necroptosis depending on the activity of caspase-8 in the complex, which is regulated by FLIP (Tenev et al. 2011; Feoktistova et al. 2011).

As the Ripoptosome and the RIPK3-mediated inflammatory complex share the same regulatory components of RIPK1, caspase-8 and cIAPs, I investigated whether the Ripoptosome is involved in the regulation of RIPK3-mediated inflammatory signalling. Caspase-8 was found to exist in a complex with FLIP and FADD at steady state in HCT116/RIPK3-2xFV cells (Figure 5.4A). As reported, the combination of cIAPs depletion and caspase inhibition stabilised the interaction between RIPK1 and the FADD-caspase-8-FLIP complex in the absence of extracellular signals (Tenev et al. 2011)

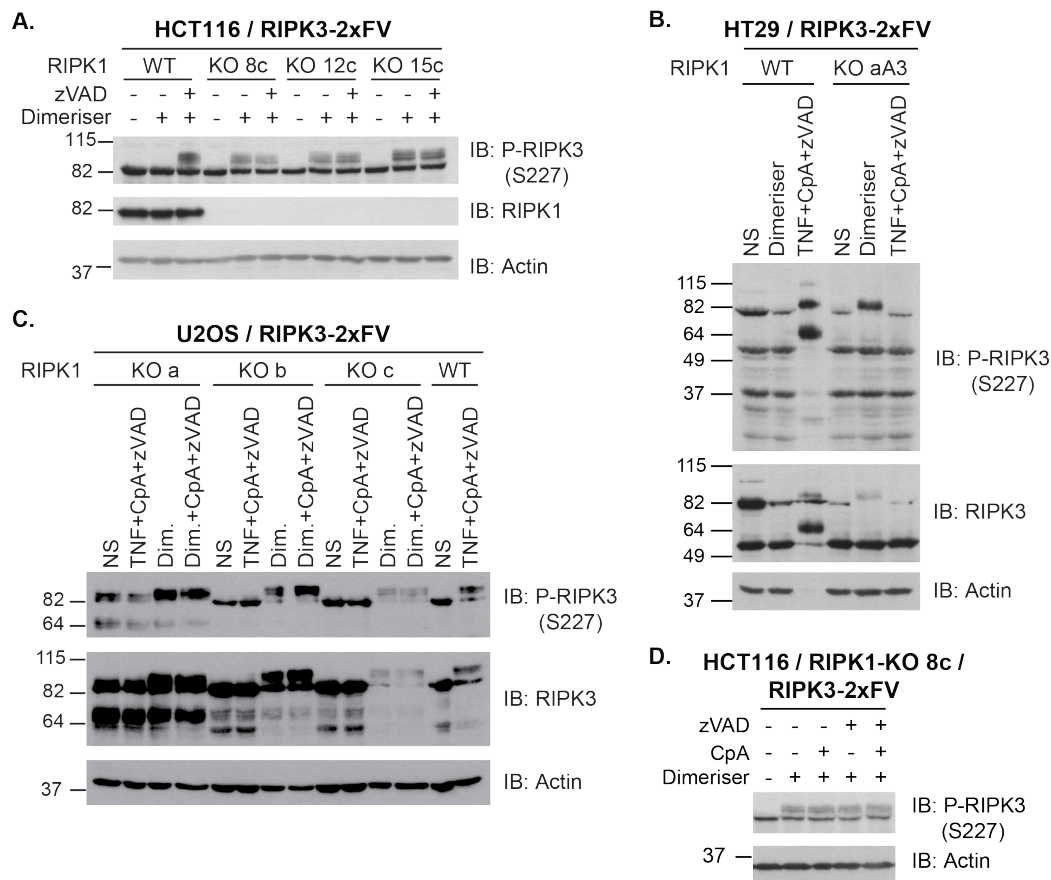


Figure 5.2: RIPK3 oligomerisation in the absence of RIPK1 leads to the appearance of its slow-migrating form. (A) HCT116/RIPK3-2xFV, HCT116/RIPK1-KO clone 8c/RIPK3-2xFV, HCT116/RIPK1-KO clone 12c/RIPK3-2xFV and HCT116/RIPK1-KO clone 15c/RIPK3-2xFV were treated as indicated for 6h. (B) HT29/RIPK3-2xFV and HT29/RIPK1-KO clone aA3/RIPK3-2xFV cells were treated as indicated for 24h. CpA, 100nM, TNF, 2ng/ml. (C) U2OS/RIPK1-KO clone a/RIPK3-2xFV, U2OS/RIPK1-KO clone b/RIPK3-2xFV, U2OS/RIPK1-KO clone c/RIPK3-2xFV and U2OS/RIPK3-2xFV were treated as indicated for 4h. CpA, 100nM, TNF, 20ng/ml. The expression of endogenous RIPK3 appears to differ between clones. (D) Cells were treated as indicated for 6h.

(Figure 5.4A). Under this condition, the oligomerisation-induced slow-migrating RIPK3 coimmunoprecipitated with RIPK1 and the FADD-caspase-8-FLIP complex (Figure 5.4A). Sucrose gradient assays further validated the formation of the Ripoptosome and its specific association with the slow-migrating RIPK3 (Figure 5.4B). Consistent with the role of RIPK1 in recruiting caspase-8 to RIPK3, the slow-migrating RIPK3 did not

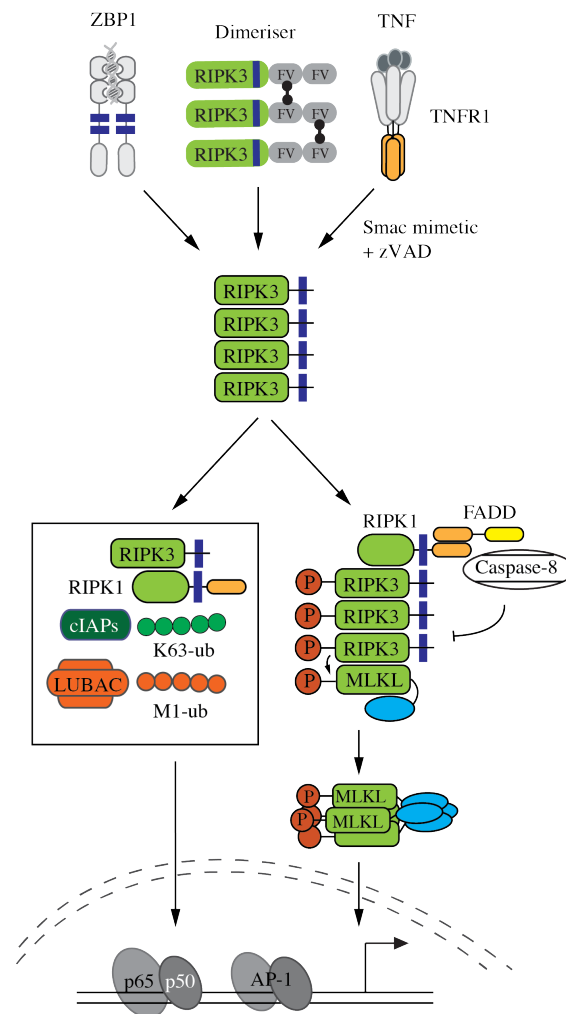
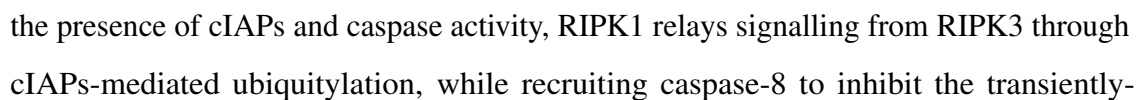


Figure 5.3: RIPK1 mediates the recruitment of cIAPs and caspase-8 for the regulation of RIPK3-mediated inflammatory signalling.

coimmunoprecipitate with caspase-8 in RIPK1-KO cells (Figure 5.5).

The stabilisation of the Ripoptosome requires the loss of cIAPs, suggesting that cIAPs-mediated ubiquitylation could suppress the association of caspase-8 with RIPK1. In addition, the specific recruitment of the slow-migrating RIPK3 to the Ripoptosome suggests that only the activated RIPK3 is cleaved by caspase-8. In support of this hypothesis, Choi et al. 2018 reported the specific degradation of slow-migrating RIPK3 mediated by Pellino 1. Further experiments can be carried out to determine whether caspase-8 and Pellino 1 cooperates to downregulate the level of slow-migrating RIPK3.

In summary, RIPK1 could coordinate the scaffolding- and RIPK3 kinase activity-dependent pathways of RIPK3-mediated inflammatory signalling in the following way. In



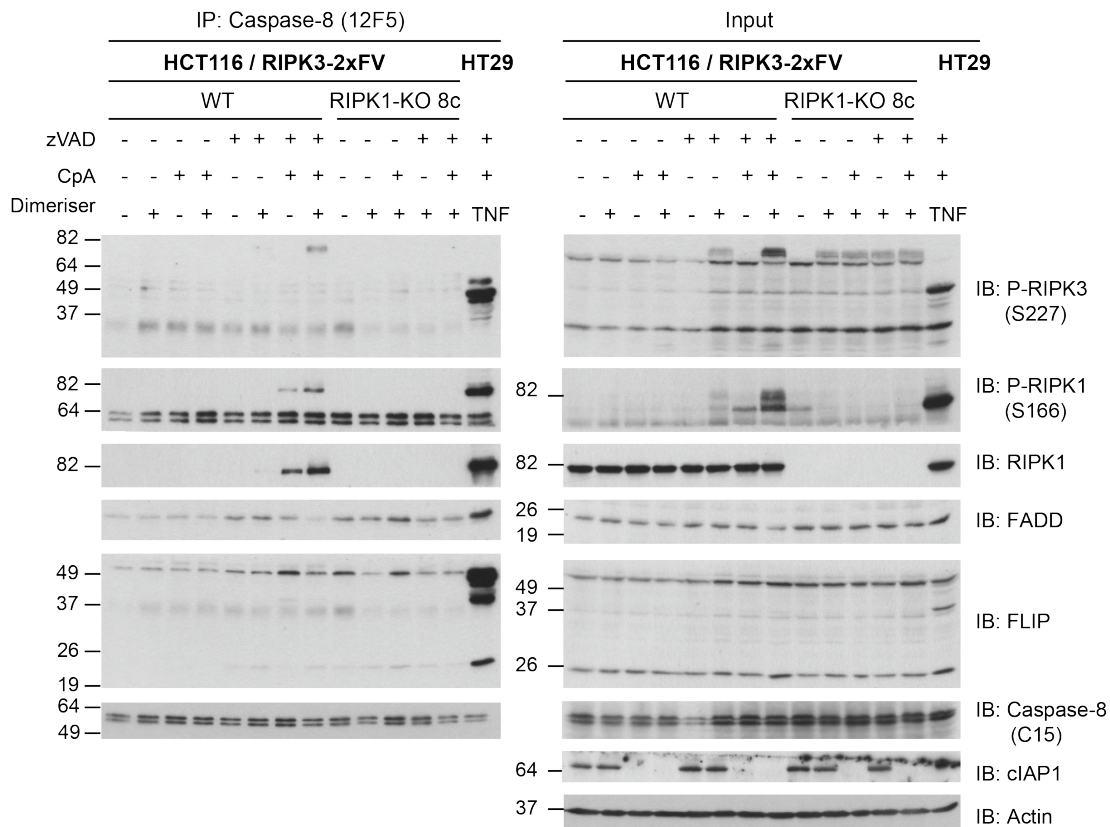


Figure 5.5: RIPK1 mediates the recruitment of RIPK3 to the Ripoptosome. HCT116/RIPK3-2xFV, HCT116/RIPK1-KO clone 8c/RIPK3-2xFV and HT29 cells were treated as indicated for 6h for immunoprecipitation with caspase-8 antibody. CpA, 100nM.

forming activated RIPK3 to suppress the kinase activity-dependent pathway (Figure 5.6A). In the absence of cIAPs, the association between RIPK1 and caspase-8 is stronger, which could potentially lead to enhanced cleavage of RIPK3 to suppress the kinase activity-dependent pathway, so that it is only activated in the absence of caspase activity (Figures 5.6B-D).

In the absence of RIPK1, the scaffolding-dependent pathway could not be activated, and caspase-8 could not be recruited to suppress activated RIPK3 either. Therefore, RIPK3 oligomerisation leads to the activation of the kinase activity-dependent inflammatory signalling pathway, as supported by the appearance of slow-migrating RIPK3 after its

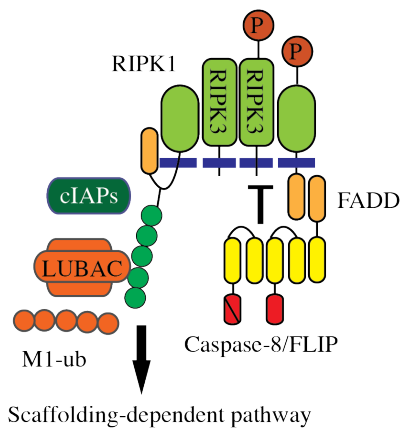
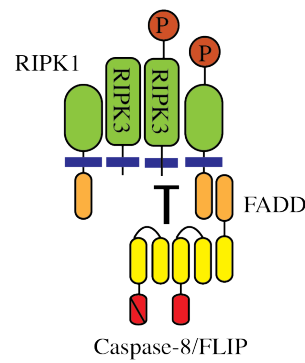
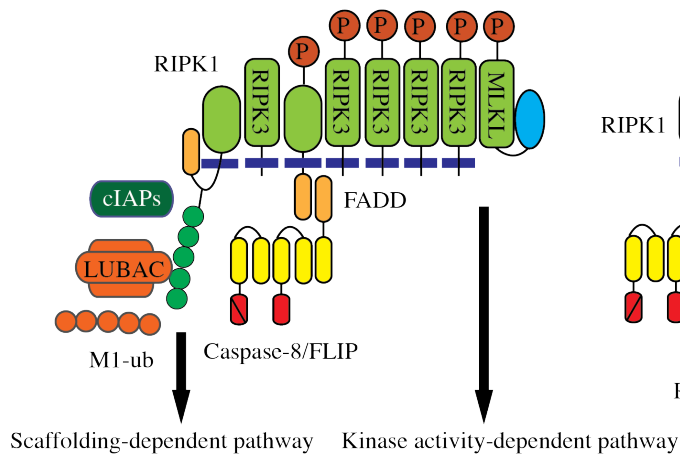
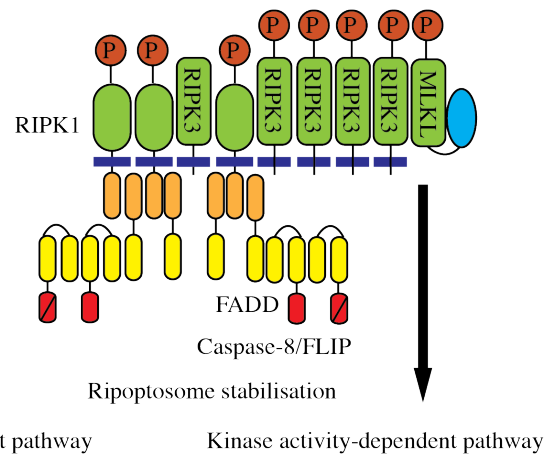
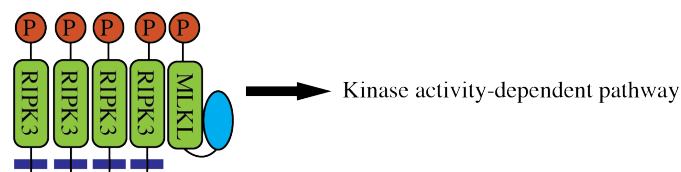
A. WT condition**B. Loss of cIAPs****C. Caspase inhibition****D. Loss of cIAPs + caspase inhibition****E. Loss of RIPK1**

Figure 5.6: RIPK1 coordinates RIPK3-mediated inflammatory signalling pathways by recruiting cIAPs and caspase-8.

oligomerisation in RIPK1-KO cells (Figures 5.2 and 5.6E).

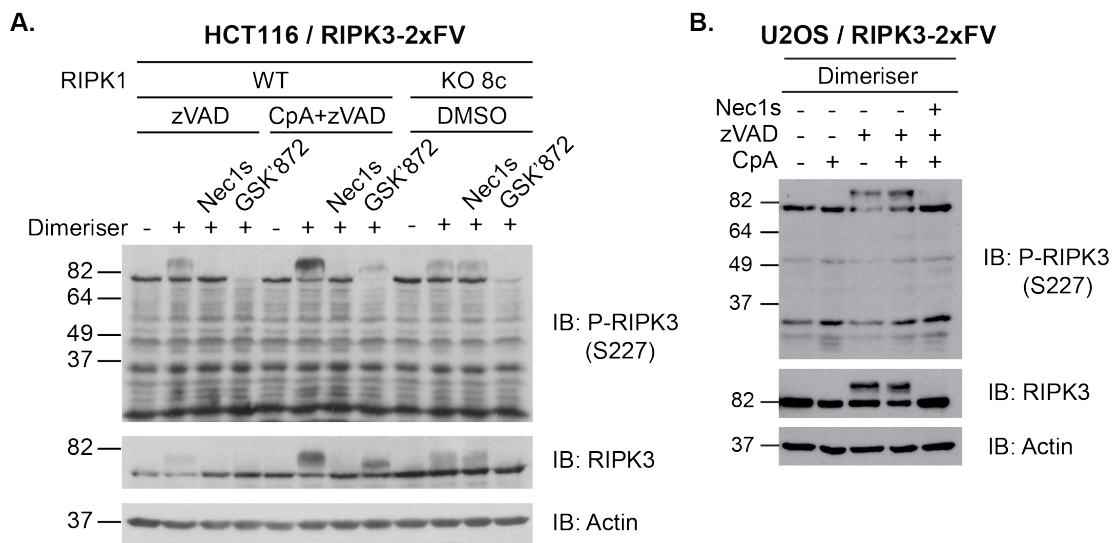


Figure 5.7: The role of RIPK1 and RIPK3 in the activation of RIPK3 kinase activity. Cells were treated as indicated for 6h. CpA, 100nM.

5.2 The role of RIPK1 kinase activity in RIPK3-mediated inflammatory signalling

According to the proposed model, the kinase activity of RIPK1 would not be required for either the scaffolding-dependent pathway or the RIPK3 kinase activity-dependent pathway. However, the RIPK1 kinase inhibitor Nec1s partially inhibited dimeriser-induced inflammatory signalling (Figure 3.13). One potential explanation is that there could be a dominant negative effect of Nec1s-bound RIPK1 in suppressing the activation of RIPK3's kinase activity. Consistent with the hypothesis, the appearance of the slow-migrating RIPK3 was inhibited by Nec1s in an on-target manner, but not inhibited by the loss of RIPK1 (Figure 5.7).

Intriguingly, the response of RIPK3-mediated inflammatory signalling to Nec1s differs among different models of activation. Nec1s had no effect on RIPK3-mediated inflammatory signalling in the absence of cIAPs and caspase activity, despite being able to inhibit the appearance of slow-migrating RIPK3 under this condition (Figures 5.7 and

5.8A-B). In contrast, Nec1s completely inhibited dimeriser-induced inflammatory signalling in caspase-8 knockdown cells in the absence of cIAPs, and reduced inflammatory signalling in K63/M1-SUB-expressing cells and HOIP-KO cells (Figures 5.8C-D and 5.9). It is not clear why Nec1s has different effects in different models of activating the kinase-dependent pathway. As Nec1s binding could impose structural changes on RIPK1, different kinase-inactive RIPK1 mutants could be made to investigate whether the effects arise from the kinase activity of RIPK1. However, experiments are limited by the difficulty in reexpressing RIPK1 in the knockout cells.

Notably, Nec1s inhibited the appearance of slow-migrating RIPK3, but not RIPK3-mediated inflammatory signalling in the absence of cIAPs and caspase activity, dissociating the appearance of slow-migrating RIPK3 from the activation of the kinase activity-dependent pathway. Further experiments are required to address the relationship between the appearance of slow-migrating RIPK3 and the activation of RIPK3's kinase activity.

5.3 Summary and discussion

In this chapter, I investigated the role of RIPK1 in RIPK3-mediated inflammatory signalling. Despite not being essential, RIPK1 links the RIPK3 scaffolding-mediated inflammatory signalling pathway and the kinase activity-mediated pathway through the recruitment of cIAPs and caspase-8, as summarised in Figure 5.6.

Taken together, RIPK3 is able to induce inflammatory signalling in both scaffolding-dependent and kinase activity-dependent ways, while the latter is constantly suppressed by caspase-8. ZBP1-induced RIPK3-mediated inflammatory signalling occurs in the presence of caspase activity, and therefore adopts the scaffolding-dependent way. In contrast, TNF-induced RIPK3 activation requires the loss of caspase activity and scaffolding ubiquitylation, and therefore is completely dependent on the kinase activity-mediated pathway. A RIPK1-centred Ripoptosome complex senses the presence of cIAPs and caspase-8 to modulate in which pathway RIPK3-mediated inflammatory signalling takes place. Given that RIPK3 activation could take place with the infection of viruses that often encode inhibitors of the ubiquitin machinery and caspase-8, the existence of two

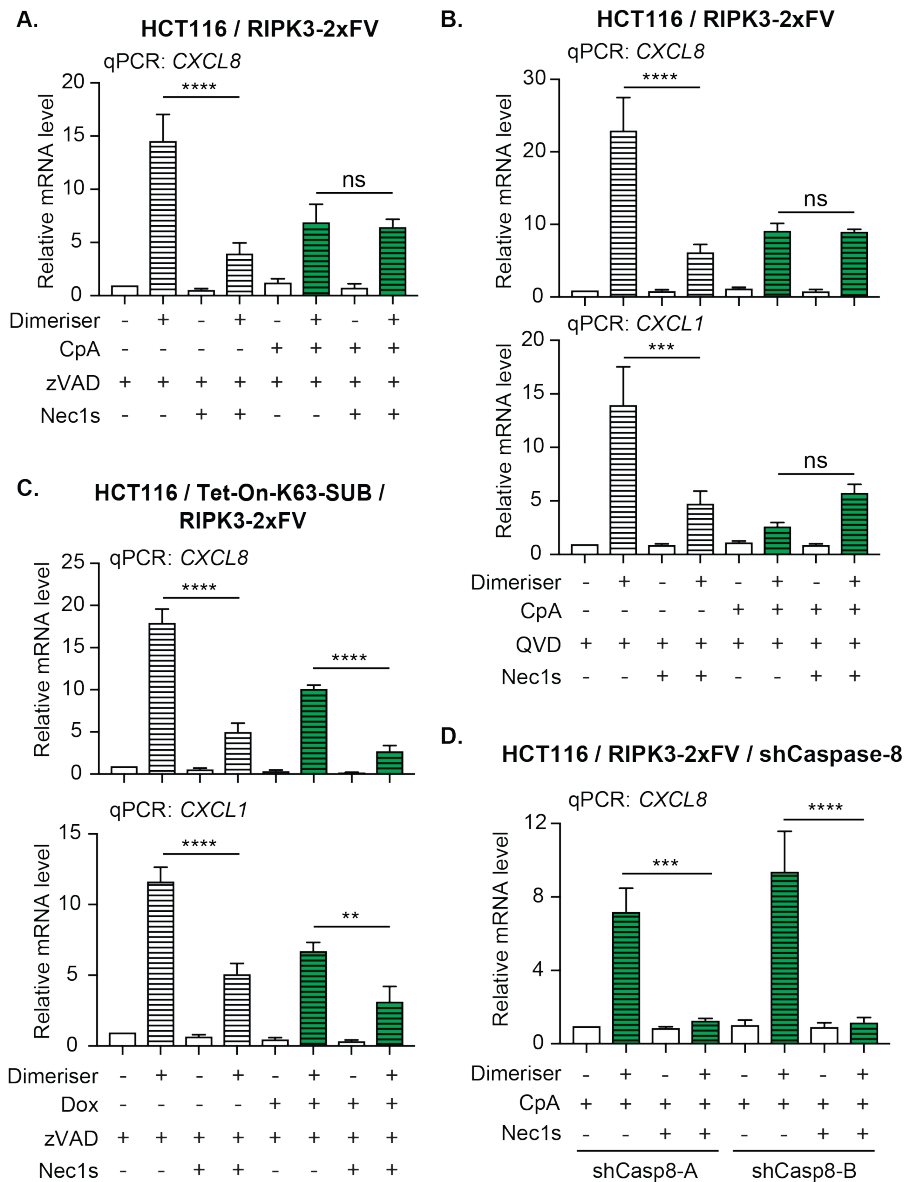


Figure 5.8: Nec1s has different effects in different models of RIPK3-mediated inflammatory signalling in the absence of K63-linked polyubiquitin and caspase activity. (A, B) Cells were stimulated as indicated for 3h (n=3). (C) Cells were treated with Dox for 48h before stimulated for 3h (n=3). (D) HCT116/RIPK3-2xFV/shCasp8-A and HCT116/RIPK3-2xFV/shCasp8-B were treated as indicated for 3h (n=4). Ordinary one-way ANOVA and Sidak's multiple comparisons test were used for all plots. ns, not significant, **, $p < 0.01$, ***, $p < 0.001$, ****, $p < 0.0001$.

different ways of RIPK3-mediated inflammatory signalling could better ensure the onset of inflammation that is required for the host to tackle the infection.

The activation of ZBP1-RIPK3-mediated cell death is also implicated during embryonic development. In RIPK1 epidermis-conditional knockout mice, ZBP1 levels were found to be upregulated, which leads to skin inflammation through binding RIPK3

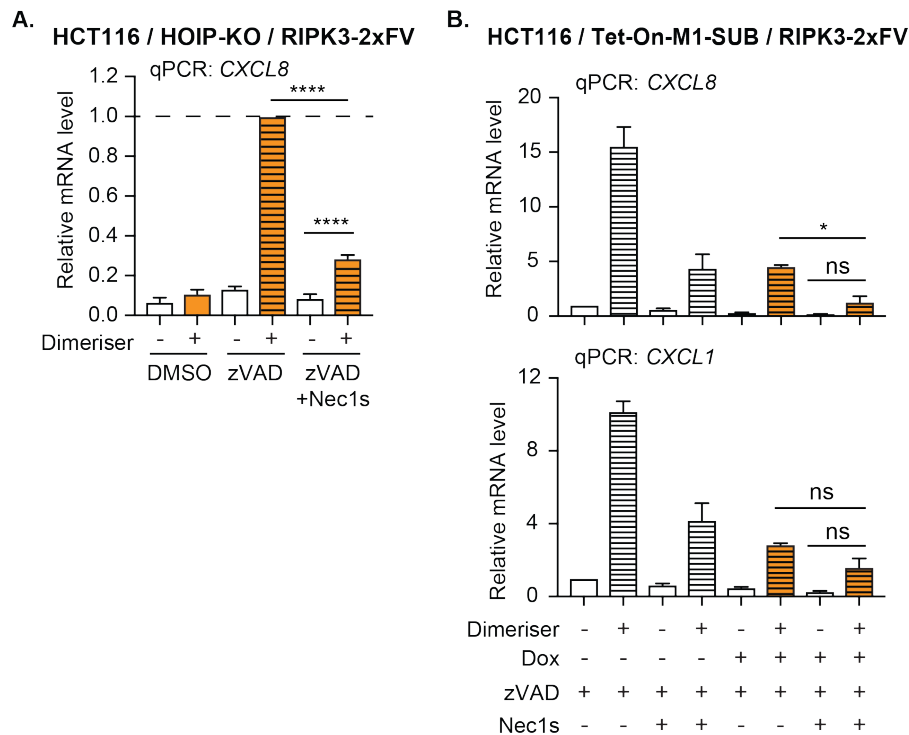


Figure 5.9: Nec1s partially inhibits RIPK3-mediated inflammatory signalling in the absence of M1-linked polyubiquitin and caspase activity. (A) Cells were stimulated as indicated for 3h (n=3). (B) Cells were treated with Dox for 48h before stimulated for 3h (n=3). Ordinary one-way ANOVA and Sidak's multiple comparison test were used to test for statistical differences between the indicated conditions. ns, not significant, *, $p < 0.05$, ****, $p < 0.0001$.

and activating its kinase activity, while RIPK1 was proposed to suppresses this process through competing with RIPK3 for binding the RHIM in ZBP1 (Lin et al. 2016; Newton et al. 2016). According to the model proposed here, the role of RIPK1 in ZBP1-RIPK3-mediated signalling during embryonic development could be both promoting the scaffolding-dependent inflammatory signalling and inhibiting the activation of RIPK3's kinase activity through recruiting caspase-8. Moreover, it remains unclear whether in the presence of RIPK1, low levels of ZBP1 expression may exist to promote RIPK3-mediated signalling during embryonic development. Further investigation could focus on the role of ZBP1-RIPK3-mediated inflammatory signalling in embryonic development, and the potential function of RIPK1-recruited caspase-8 in restricting this process.

6

Discussion

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6.1 Conclusions

Cell death and inflammatory signalling are both key aspects in innate immune defence. In this study, I showed that RIPK3, the necroptosis-promoting protein, is able to activate TAK1- and IKK-mediated inflammatory signalling independent of its ability to promote cell death in both kinase activity-dependent and -independent ways.

Whereas ZBP1-induced RIPK3-mediated inflammatory signalling does not require its kinase activity, TNF-induced RIPK3-mediated inflammatory signalling does. The

difference between the two ways of RIPK3-mediated inflammatory signalling was explored using a chemical-induced oligomerisation system to directly activate RIPK3.

RIPK3 was shown to promote inflammatory signalling in two different ways, through the scaffolding ability of K63- and M1-linked polyubiquitin assembled by cIAPs and LUBAC, and through its kinase activity and its substrate MLKL. The latter is only activated with the inhibition of caspase activity.

RIPK1 is the regulator between the scaffolding-dependent and the kinase activity-dependent RIPK3-mediated inflammatory pathways. On the one hand, RIPK1 recruits cIAPs for the scaffolding pathway. On the other hand, RIPK1 specifically recruits the slow-migrating form of RIPK3 to caspase-8 for its cleavage and elimination, leading to the suppression of RIPK3 kinase activity which is otherwise activated upon oligomerisation.

Further experiments need to be performed to elucidate the RIPK3-mediated two-way inflammatory signalling model. This includes the identification of ubiquitylation substrates in the scaffolding pathway, the investigation on how RIPK3 promotes inflammatory signalling through its kinase activity, and the validation of the mechanisms in ZBP1-promoted RIPK3 activation. In addition, since the activation of RIPK3 kinase activity is a priming condition for RIPK3-mediated necroptosis, caspase activity likely represents one of the checkpoints for RIPK3-mediated inflammation and cell death. More investigation needs to be carried out in a suitable system to address the life-death checkpoints in RIPK3-mediated signalling. Finally, the current study is limited to cancer cell lines, thus the conclusions remains to be validated in primary cells and *in vivo* systems.

Taken together, RIPK3, like its family members RIPK1 and RIPK2, is a scaffolding protein kinase that mediates inflammatory signalling pathways through kinase-dependent and -independent ways. While its ability to promote necroptosis has been extensively characterised, to promote inflammatory signalling may be the more prevalent function of RIPK3 in the human system. The identification of RIPK3 as an inducer of inflammatory signalling provides another means of crosstalk between cell death and inflammation in innate immune defence.

6.2 Physiological relevances of RIPK3-mediated inflammatory signalling

6.2.1 RIPK3-mediated inflammatory signalling during infection

The ZBP1-RIPK3 axis has been implicated in defense against many types of viruses, including cytomegaloviruses, influenza viruses, herpesviruses, the West Nile Virus (WNV) and the Zika virus (ZIKV) (Upton et al. 2012; Kuriakose et al. 2016; Thapa et al. 2016; Guo et al. 2015; Daniels et al. 2017; Daniels et al. 2018). However, it remains unclear how the ZBP1-RIPK3 axis confers antiviral activity. Studies found that CMVs and HSVs encode inhibitors of the necroptosis pathway, pointing out the importance of ZBP1-RIPK3-induced cell death in helping the clearance of viruses (Upton et al. 2012; Kuriakose et al. 2016; Thapa et al. 2016; Guo et al. 2015). Recently, the RIPK3-mediated inflammatory process was shown to have protective effects independently of cell death in WNV- and ZIKV-infected neurons (Daniels et al. 2017; Daniels et al. 2018). However, it is not clear whether the same process is specific to neurons or whether it takes place in the human system.

In this study, I showed that the ZBP1-RIPK3 axis promotes inflammatory signalling in human cell lines at a lower ZBP1 expression level than what is required to compromise cell viability. Given that ZBP1 is an interferon-stimulated gene, different ZBP1 expression levels may represent the physiological condition of different concentrations of IFN in different distances from the virus-infected site within the same tissue. Within the same tissue, far from the infected cell, low IFN levels induce low levels of ZBP1 expression to stimulate the production of chemokines to attract immune cells. Close to the site of infection, high levels of ZBP1 production for long time periods induces programmed cell death in addition to the production of chemokines to eliminate the surviving niche of the virus. Considering that proinflammatory cell death could lead to tissue damage that may be costly to repair, the proposed threshold-dependent mechanism of ZBP1-induced inflammatory signalling versus cell death pathway may represent a cost-effective means of antiviral defence. Future investigation could be directed to address how the threshold-dependent mechanism takes place.

Many viruses encode caspase inhibitors (Best 2008). RIPK3 activation in the absence of caspase activity induces its kinase-dependent way of inflammation, further enhancing the levels of inflammatory response. Although not observed in the cell lines used, it is possible that the activation of RIPK3 kinase activity would translate into cell death response *in vivo*, which would represent a life-death checkpoint downstream of RIPK3 activation. Further investigation needs to be done to address whether additional life-death checkpoints exist in the ZBP1-RIPK3-mediated signalling process.

6.2.2 Targeting RIPK3-mediated inflammatory signalling in pathological conditions

Due to its strong ability to promote inflammation, necroptosis underlies many pathological conditions that feature uncontrolled inflammation. Since RIPK3 promotes both inflammatory signalling and necroptosis, the strong inflammation-inducing ability of a necroptotic cell could come from RIPK3-induced *de novo* synthesised cytokines as well as constitutive DAMPs released from the dying cell. Targeting RIPK3-mediated inflammatory signalling pathway in addition to cell death may be effective in treating chronic inflammatory conditions that feature necroptosis.

The existence of many different ways that RIPK3 promotes inflammatory signalling may represent a challenge in targeting RIPK3-mediated inflammatory signalling. Inhibition of RIPK3-mediated inflammatory signalling requires deprivation of the scaffolding pathway while leaving the caspase-8 activity intact for suppressing the RIPK3 kinase-dependent pathway (Figure 5.6B). SMAC mimetics, which have been developed as therapeutic drugs that sensitise tumour cells to apoptosis, completely inhibit RIPK3-mediated inflammatory signalling, and could be a possible candidate for targeting pathological necroptosis conditions (Dubrez 2018).

6.2.3 Harnessing RIPK3-mediated inflammatory signalling for cancer therapy

Recently, RIPK3-mediated inflammatory signalling was shown to have antitumour values. RIPK3-promoted inflammatory signalling during necroptosis was found to be essential

for the dead cell to activate the adaptive immune system, and the injection of necroptotic cells with RIPK3-mediated inflammatory signalling was found to augment the anti-PD-1 therapy for melanoma in mice (Yatim et al. 2015; Snyder et al. 2019). Although it awaits validation in more tumour models, the proinflammatory cellular contents released by necroptotic cells appear to be valid reagents to convert the tumour microenvironment to induce the immune cells into an antitumour state.

Enhancing RIPK3-mediated inflammatory signalling in the injected necroptotic cell would therefore be expected to enhance the antitumour immune effect. A possible way is to inhibit caspase activity to unleash the kinase activity-dependent pathway while preserving the scaffolding complex to mediate the kinase activity-independent pathway (Figure 5.6C). For example, this could be done by expressing ZBP1 in combination with caspase inhibition.

It is not clear whether the antitumour effect is specific to RIPK3-mediated inflammatory signalling. It is possible that the molecular features of induced genes in RIPK3-mediated inflammatory signalling are optimal for the stimulation of the conversion of the tumour microenvironment. Future investigation could be directed to identifying which or the combination of which molecules being promoted by RIPK3-mediated inflammatory signalling contributes to the antitumour effect to develop more effective therapies.

6.3 Future perspectives

6.3.1 Addressing the prevalence of RIPK3-mediated inflammatory signalling

Necroptosis has been implicated in many physiological and pathological conditions, where RIPK3 is activated in all of them. However, it is not clear to what extent RIPK3-mediated inflammatory signalling contributes to the antiviral effect or pathological inflammation. This study points out the existence of RIPK3-mediated inflammatory signalling in necroptotic conditions. Future investigation could investigate its relevance in necroptosis-related conditions *in vivo*.

Taken further, this study also shows that conditions that activate RIPK3-mediated inflammatory signalling are more prevalent than those that induce necroptosis, indicating

that RIPK3-mediated inflammatory signalling may be more prevalent than necroptosis. Future investigation should also consider the involvement of RIPK3-mediated inflammatory signalling where RIPK3 could be involved, even if no cell death phenotype is observed.

6.3.2 The many ways towards inflammatory signalling

Since the discovery of non-degradative ubiquitylation in the recruitment and activation of MAPK- and NF- κ B pathways, ubiquitylation has been placed in the centre of the activation of inflammatory signalling. However, the discovery of RIPK3-mediated inflammatory signalling in the absence of cIAPs or M1-linked polyubiquitin may indicate the presence of ubiquitin-independent ways of activating inflammatory signalling.

RIPK3-mediated inflammatory signalling under certain conditions is dependent on its kinase activity, as shown by the kinase-inactive mutants in addition to RIPK3 kinase inhibitors (Figures 5.6D and 3.5D). This was proposed to be due to the direct phosphorylation of inflammatory kinases by RIPK3 or its substrate pseudokinase MLKL (Najjar et al. 2016; Zhao et al. 2012). If the mechanism holds, this would represent a novel way that scaffolding protein kinases activate inflammatory signalling.

In addition, although it has not been shown to be involved in RIPK3-mediated inflammatory signalling, the scaffolding activity of caspase-8-FLIP heterodimers was also shown to activate inflammatory signalling pathways, and FLIP was reported to directly interact with NEMO of the IKK complex (Baratchian et al. 2016; Ivanisenko et al. 2019). If the mechanism holds, it could provide another way of activating inflammatory signalling in the absence of scaffolding polyubiquitin.

Considering the central role of inflammation in combating pathogens, the diversified ways to promote inflammatory signalling would be beneficial for the host to prevent pathogens from inhibiting it. Future investigation can look into the different ways of activating inflammatory signalling, which could provide insight into improving the treatment of inflammation-related diseases.

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- 1.9 **The TNFR1 signalling pathway.** Activated TNFR1 recruits TRADD and RIPK1 through its DD. TRADD recruits TRAF2 in a complex with ubiquitin ligases cIAP1 and cIAP2. cIAPs assemble K63-linked polyubiquitin on RIPK1, TNFR1 and other components. K63-linked polyubiquitin recruits the TAK1-TAB complex to activate the MAPK pathway and the LUBAC complex to assemble M1-linked polyubiquitin on components of the complex, which in turn recruits the IKK complex to activate the NF- κ B pathway. Under apoptosis conditions, RIPK1 undergoes autophosphorylation and promote apoptosis in a cytosolic complex Complex II through interaction among DDs and DEDs. Several layers of restraints prevent TNFR1-induced cell death. Kinases in the inflammatory pathway, including MK2, IKKs and TBK1, put on inhibitory phosphorylation on RIPK1 to prevent its autophosphorylation. A20, a product of NF- κ B pathway, put on K48-linked polyubiquitin on RIPK1, leading to its degradation. Another target of NF- κ B, FLIP, counteracts the activation of caspase-8 by forming heterodimers with it. Defects in the inflammatory pathway license the initiation of apoptosis. RIPK3 is recruited to the apoptotic Complex II, but its activity is limited by caspase-8 and the FLIP-caspase-8 heterodimer. If caspase-8 is blocked, RIPK1 and RIPK3 enucleate the necrosome, mediating TNFR1-induced necroptosis. 26

- 1.10 **The TLR3/TLR4 signalling pathways.** TLR4 is activated on the cell surface by LPS, and recruits MyD88 and MAL via their TIR domains. MyD88 recruits IRAK4 and IRAK2 via DDs, resulting in the formation of the Myddosome that further recruits IRAK1. The IRAKs are activated by crossphosphorylation to recruit TRAF6 and phosphorylate Pellino 1, which assemble K63-linked polyubiquitin to recruit LUBAC and the TAK1-TAB complex to for the activation of MAPK- and NF- κ B pathways. After its internalisation, TLR4 recruits another 2 adaptors, TRIF and TRAM via its TIR domain. TLR3, localised on endosomes, is activated by engulfed dsRNA and directly recruits TRIF through TIR domains. TRIF can interact with TANK to activate TBK1 and IKK ϵ for the IRF pathway, resulting in the production of Type I IFNs. TRIF also recruits RIPK1 and RIPK3 via RHIMs. RIPK1 is modified with K63-linked polyubiquitin by Pellino 1, which recruits LUBAC for the activation of MAPK and NF- κ B pathway. Compromisation of the inflammatory signalling pathway sensitises cells to TRIF-mediated cell death, where a complex forms consisting of RIPK1, FADD, procaspase-8 and FLIP to regulate caspase-8 activation. Depending on the cell type, activated caspase-8 cleaves caspases-3 and -7 to activate apoptosis or GSDMD for pyroptosis. Activation of executioner caspases inhibits pyroptosis. The p43/41-caspase-8-p43-FLIP heterodimer antagonises necroptosis. When caspase-8 is inhibited, TRIF mediates necroptosis through RIPK3 activation. 29
- 1.11 **The ZBP1 signalling pathway.** Activation of ZBP1 can activate the TBK1-IRF pathway in a process that is so far not clear. Activated ZBP1 recruits RIPK1 and RIPK3 to mediate inflammatory signalling and cell death responses. ZBP1 overexpression-induced NF- κ B activation requires both RIPK1 and RIPK3. ZBP1-induced necroptosis in mice is mediated by its interaction with RIPK3 and antagonised by the RHIM of RIPK1. ZBP1-induced apoptosis depends on the RIPK1-FADD-caspase-8 complex. The mechanism of ZBP1-induced pyroptosis is not clear but likely similar to TLR-induced pyroptosis. 33
- 3.1 **High levels of ZBP1 expression in HT29 cells reduce cell viability.** (A) HT29/Tet-On-ZBP1 cells were treated with indicated concentrations of Dox for 24h. HT29 cells were plated and collected at the same time without treatment as control. (B, C) HT29, HT29/Tet-On-ZBP1 and HT29/Tet-On-ZBP1-ZBDmut cells were treated with indicated concentrations of Dox for the indicated length of time. (B) Arrow indicates the P-RIPK3 signal band. Asterisk indicates the antibody background band. (C) Cell viability was measured at the end of treatment with ATP-based CellTitre-Glo Assay (n=3). Ordinary 2-way ANOVA and Tukey's multiple comparison test were used to test the differences between HT29/Tet-On-ZBP1 and HT29 (Null) cells at 48h and 72h. *, p<0.05, **, p<0.01. *HT29/Tet-On-ZBP1 and HT29/Tet-On-ZBP1-ZBDmut cells were generated by Dr Jonathan Maelfait in Ghent University, Belgium.* 68
- 3.2 **Induced ZBP1 expression leads to TAK1- and IKK-dependent chemokine production independently of cell death.** HT29/Tet-On-ZBP1 cells were treated with indicated inhibitors in combination with 0 or 50 ng/ml Dox for 24h (n=3). Nec1s, RIPK1 kinase activity inhibitor, GSK'872, RIPK3 kinase activity inhibitor, NSA, MLKL oligomerisation inhibitor. See Table 2.9 for compound concentrations. (A) ELISA measurements of chemokine levels in the supernatant. Ordinary 1-way ANOVA and Sidak's multiple comparison tests were used to test the difference between Dox-induced samples treated with corresponding inhibitors and treated with DMSO. ns, not significant, *, p < 0.05, **, p < 0.01, ****, p<0.0001. (B) Cell viability assay performed under the same conditions in (A). 70

- 3.3 **RIPK3 mediates ZBP1-induced activation of NF- κ B reporters.** (A) Cells were transfected with titrated amounts of ZBP1-expressing plasmids along with the NF- κ B luciferase reporter and control SV40-Renilla reporter plasmids. The total DNA amount in each condition is topped up to the same with empty vector (n=3). 24h later, lysates were measured for luciferase activity and blotted for indicated proteins. (B) HEK293FT, HEK293FT/RIPK3-2xFV and HEK293FT/RIPK3dC-2xFV cells were transfected with 0.5ng empty vector, WT or ZBDMut ZBP1-expressing plasmids along with the NF- κ B luciferase reporter and control SV40-Renilla reporter plasmids (n=3). 24h later, lysates were measured for luciferase activity and blotted for indicated proteins. (C) Cells were transfected with 0.5ng empty vector or ZBP1-expressing plasmids along with the NF- κ B luciferase reporter and control SV40-Renilla reporter plasmids and treated with indicated compounds (n=3). 24h later, lysates were measured for luciferase activity. Ordinary 1-way ANOVA and Sidak's multiple comparison test were used to test the difference between ZBP1-transfected conditions treated with DMSO and with inhibitors. **, p<0.01, ns, not significant. 71
- 3.4 **TNF+CpA+zVAD induces compromised necroptosis response in HCT116/RIPK3-2xFV cells.** (A) Cells were treated as labelled for 6h. CpA, 100nM, TNF, 20ng/ml. (B) Cells were treated as indicated for 24h. TNF, 2ng/ml, CpA, 100nM. (C) HCT116, HCT116/RIPK3-2xFV or HCT116/RIPK1-KO clone 8c/RIPK3-2xFV cells were treated as indicated for 24h. TNF, 1ng/ml. 2-way ANOVA and Tukey's multiple comparison test were used to test the difference between indicated conditions and DMSO-treated condition of the same cell line. (D) HCT116/RIPK3-2xFV and HCT116/RIPK1-KO clone 8c/RIPK3-2xFV cells were treated for 24h and stained with Sytox Green and Hoechst 33342. Ordinary one-way ANOVA and Sidak's multiple comparison test was used to test for the statistical difference between indicated conditions. TNF, 1ng/ml, CpA, 100nM. ns, not significant, *, p<0.05, **, p<0.01, ****, p<0.0001. . . . 73
- 3.5 **TNF+CpA+zVAD induces inflammatory signalling dependent on RIPK1, RIPK3 and MLKL.** (A) HCT116, HCT116/RIPK3-2xFV or HCT116/RIPK1-KO clone 12c/RIPK3-2xFV cells were treated as indicated for 3h. TNF, 2ng/ml, CpA, 2 μ M. Ordinary two-way ANOVA and Tukey's multiple comparison test were used to test the difference between indicated conditions. (B) Expression levels of HCT116/RIPK3-RHIM^{AAAA}-2xFV, HCT116/RIPK3-K50A-2xFV and HCT116/RIPK3-D142N-2xFV compared to HCT116/RIPK3-2xFV. Results are from the same exposure of the same blot. (C-E) Different stable cell lines were treated as indicated for 3h for qPCR (n=3). (C) HCT116/RIPK3-2xFV and HCT116/RIPK3-RHIM^{AAAA}-2xFV cells. (D) HCT116/RIPK3-2xFV, HCT116/RIPK3-K50A-2xFV and HCT116/RIPK3-D142N-2xFV cells. Unpaired t-test was used to test for the statistical difference between indicated conditions. (E) HCT116/RIPK3-2xFV/shMM, HCT116/RIPK3-2xFV/shMLKL-2 and HCT116/RIPK3-2xFV/shMLKL-3 cells. Unpaired t-test was used to test for the statistical difference between indicated conditions. Equal number of cells from each line were collected for western blot analysis of the protein expression levels. *, p<0.05, **, p<0.01, ****, p<0.0001. 74
- 3.6 **Chemical-induced oligomerisation of RIPK3.** RIPK3 is fused with 2 copies of FKBP12-F36V (FV), a mutant form of the human 12kDa FK506-binding protein (FKBP12). Each FV has 1 binding site of the bivalent dimeriser (AP20187). Application of the B/B homodimeriser (Dimeriser/AP20187) results in the formation of RIPK3 oligomers. . . . 75

- 3.7 **Chemical-induced RIPK3 oligomerisation induces inflammatory signalling and cell death in NIH3T3/mRIPK3-2xFV cells.** NIH3T3/mRIPK3-2xFV or NIH3T3/mRIPK3dC-2xFV cells were treated with (A) ethanol or 100nM dimeriser for 4h or (B) 100nM dimeriser for indicated length of time. (A) 5 μ g/ml propidium iodide (PI) was added to stain dead cells. BF, bright field. PI, Green channel. Pictures were taken with the Inverted Olympus Fluorescent Microscope. 76
- 3.8 **Chemical-induced RIPK3 oligomerisation induces inflammatory signalling in HCT116/RIPK3-2xFV cells.** (A, B) HCT116/RIPK3-2xFV and HCT116/RIPK3dC-2xFV cells were treated as indicated. (B) n=3. Ordinary two-way ANOVA and Tukey's multiple comparison test were used to test the difference between indicated time points and 0h in FL cells. (C, D) HCT116/RIPK3-2xFV and HCT116/RIPK3-RHIM^{AAAA}-2xFV were treated as indicated for (C) 3h (n=3) or (D) indicated length of time (n=4). Supernatant was collected for ELISA. Ordinary one-way ANOVA and Sidak's multiple comparison test were used to test for statistical differences between indicated conditions. (E) HCT116/RIPK3-2xFV cells were treated as indicated for 3h (n=3). *, p<0.05, **, p<0.01, ****, p<0.0001. 77
- 3.9 **Chemical-induced RIPK3 oligomerisation drives inflammatory signalling in multiple cell lines.** (A) HT29/RIPK3-2xFV, (B) U2OS/NOD2/RIPK3, U2OS/NOD2/RIPK3-2xFV, U2OS/NOD2/RIPK3dC-2xFV, (C) U2OS/NOD2/RIPK3-2xFV, U2OS/NOD2/RIPK3dC-2xFV, (D) HEK293FT/RIPK3-2xFV and HEK293FT/mCherry are treated as indicated. (C) Ordinary two-way ANOVA and Tukey's multiple comparisons test were used to test the difference between indicated time points and 0h in FL cells. **, p<0.01, ***, p<0.001, ****, p<0.0001. 78
- 3.10 **Chemical-induced RIPK3 oligomerisation does not affect viability in human cells.** (A) HCT116/RIPK3-2xFV and (B) HT29/RIPK3-2xFV cells were treated as indicated. n=3. 79
- 3.11 **RIPK1 is not essential for RIPK3 oligomerisation-induced inflammatory signalling.** (A) HCT116/RIPK3-2xFV, HCT116/RIPK3dC-2xFV, HCT116/RIPK1-KO clone 12c/RIPK3-2xFV and HCT116/RIPK1-KO clone 12c/RIPK3dC-2xFV cells were stimulated with 0 or 100nM dimeriser for 3h (n=3). Equal number of cells were collected for western blots. Asterisk indicates antibody background. (B) HCT116/RIPK3-2xFV, HCT116/RIPK1-KO clone 8c/RIPK3-2xFV and HCT116/RIPK1-KO clone 15c/RIPK3-2xFV were stimulated with 0 or 100nM dimeriser for 3h (n=3). Equal number of cells were collected for western blots. (C-F) HCT116/RIPK3-2xFV and HCT116/RIPK1-KO clone 8c/RIPK3-2xFV were treated with 0 or 100nM dimeriser for (C, E, F) 24h or (D) 3h (n=3). Ordinary one-way ANOVA and Sidak's multiple comparison test were used to test for statistical differences between indicated conditions. ns, not significant, **, p<0.01, ***, p<0.001, ****, p<0.0001. 80
- 3.12 **RIPK3 oligomerisation-induced inflammatory signalling in the absence of RIPK1 is not mediated by TRIF or TNF autocrine.** (A) HCT116/RIPK1-KO clone 8c/RIPK3-2xFV were transfected with siRNA against mismatch (Ctrl) or 3 different sites of TRIF. 72h post transfection, cells were collected for western blots or treated with 0 or 100nM dimeriser for 3h for qPCR (n=3). (B) HCT116/RIPK3-2xFV, HCT116/RIPK1-KO clone 8c/RIPK3-2xFV and HCT116 cells were treated as indicated for 3h (n=3). 81
- 3.13 **The RIPK1 kinase inhibitor Nec1s inhibits RIPK3 oligomerisation-induced inflammatory response in an on-target way.** Nec1s, 30 μ M. n=4. Ordinary two-way ANOVA and Tukey's multiple comparison tests were used to test the difference between indicated conditions. ns, not significant, ****, p<0.0001. 81

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- 4.5 **CpA does not inhibit RIPK3-mediated inflammatory signalling in the absence of caspase-8.** HCT116/RIPK3-2xFV/shMM, HCT116/RIPK3-2xFV/shCasp8-A and HCT116/RIPK3-2xFV/shCasp8-B cells were treated as indicated for 3h for qPCR (n=4). Equal numbers of cells were collected for western blot analysis of caspase-8 knockdown efficiency. RM one-way ANOVA and Sidak's multiple comparison tests were used to test the difference between indicated conditions. ns, not significant. *, p<0.05, **, p<0.01. 94
- 4.6 **Two possible ways that caspase-8 antagonises the requirement of cIAPs in RIPK3-mediated inflammatory signalling.** (A) cIAPs and K63-linked polyubiquitin could positively regulate RIPK3-mediated inflammatory signalling by antagonising the inhibitory effects of caspase-8. This could be achieved by ubiquitylating substrates to limit the access of caspase-8 to its cleavage sites, or by ubiquitylating caspase-8 to regulate its activity. (B) Continuing from the model in Figure 3.15, cIAPs and K63-linked ubiquitylation could be essential components of the scaffolding-mediated pathway, while the inhibition of caspase-8 could be required for the activation of the RIPK3 kinase activity-dependent pathway. Thus, with the loss of both cIAPs and caspase inhibition, RIPK3-mediated inflammatory signalling could be converted from the scaffolding-mediated pathway to the RIPK3 kinase activity-dependent pathway, similar to the situation in TNFR1-induced RIPK3-mediated inflammatory signalling. 95

- 4.7 **Caspase-8 inhibition rescues RIPK3-mediated inflammatory signalling in HOIP-KO cells.** (A) HCT116/RIPK3-2xFV and HCT116/HOIP-KO/RIPK3-2xFV cells were stimulated as indicated for 3h (n=3). Welch's t-test was used to test for the statistical difference between indicated conditions. (B) HCT116/RIPK3-2xFV and HCT116/HOIP-KO/RIPK3-2xFV cells were transfected with siRNA against control or 2 different sites of caspase-8 for 72h before stimulated for 3h or collected for western blot analysis (n=3). Ordinary one-way ANOVA and Sidak's multiple comparison test were used to test for statistical differences between indicated conditions. (C) Cells were treated with doxycycline for 48h before stimulated as indicated for 3h (n=3). Unpaired t-test was used to test for the statistical difference between indicated conditions. (D) HCT116/RIPK3-2xFV and HCT116/HOIP-KO/RIPK3-2xFV cells were stimulated as indicated for 3h (n=3). Ordinary one-way ANOVA and Sidak's multiple comparison test were used to test for statistical differences between indicated conditions. *, p<0.05, **, p<0.01, ***, p<0.001, ****, p<0.0001. 96
- 4.8 **HOIP is not required for TNF+CpA+zVAD-induced inflammatory signalling.** Cells were stimulated as indicated for 3h (n=3). Ordinary two-way ANOVA and Tukey's multiple comparisons test were used to test the significance between indicated conditions. ns, not significant. 97
- 4.9 **Working model for the interplay between ubiquitylation and caspase-8 in RIPK3-mediated inflammatory signalling.** RIPK3 mediates inflammatory signalling in two ways, one dependent on its scaffolding function which is mediated by K63- and M1-linked polyubiquitin, and the other dependent on its kinase activity which is only activated with the loss of caspase-8 or its catalytic activity. 98
- 4.10 **Activation of RIPK3 kinase activity correlates with the appearance of a slow-migrating form of RIPK3.** (A) HT29, HT29/Tet-On-ZBP1 and HT29/Tet-On-ZBP1mut was treated with 50ng/ml Dox for indicated time. (B) Cells were treated as indicated. CpA, 100nM. TNF, 20ng/ml. 99
- 4.11 **Dimeriser-induced oligomerisation does not lead to the appearance of slow-migrating RIPK3.** (A) HT29/RIPK3-2xFV cells were treated as indicated. Arrows indicate (up to down) RIPK3-2xFV, endogenous RIPK3 and the cleaved N-terminal fragment of RIPK3. (B) U2OS/RIPK3-2xFV cells were treated as indicated. Arrow indicates cleaved RIPK3. (C) HCT116/RIPK3-2xFV cells were pretreated with 0, 0.1 or 2µM CpA for 0.5h before stimulated with dimeriser for indicated length of time. Arrows indicate RIPK3-2xFV and the cleaved N-terminal fragment. 100
- 4.12 **Inhibition of caspase-8 stabilises the slow-migrating form of RIPK3** (A, D) HCT116/RIPK3-2xFV cells were treated as indicated. CpA, 100nM. (B) HT29/RIPK3-2xFV cells were treated as indicated for 24h. CpA, 100nM. (C) HT29/RIPK3-2xFV/shMM and HT29/RIPK3-2xFV/shCasp8-A cells were treated as indicated for 6h. Lanes were taken from the same exposure in the same blot. (E) U2OS/RIPK3-2xFV cells were treated as indicated. Indicated conditions were treated with phosphatase after lysis. CpA, 100nM. (F) HCT116/RIPK3-2xFV and HCT116/HOIP-KO/RIPK3-2xFV cells were treated as indicated for 6h. CpA, 100nM. 101
- 4.13 **CYLD negatively regulates RIPK3-mediated inflammatory signalling in the absence of caspase activity.** HCT116/shCtrl/RIPK3-2xFV and HCT116/shCYLD/RIPK3-2xFV cells were treated as indicated for 3h. (A) n=4. Ordinary one-way ANOVA and Sidak's multiple comparison test were used to test for the statistical differences between indicated conditions. (B) n=3. Unpaired t-test was used to test for statistical differences between indicated conditions. ns, not significant, ***, p<0.001, ****, p<0.0001. 103

- 4.14 **Working model for the interplay between ubiquitylation and caspase-8 in RIPK3-mediated inflammatory signalling.** The deubiquitinase CYLD suppresses RIPK3-mediated inflammatory signalling in the absence of caspase activity. 105
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- 5.1 **RIPK1 is required for cIAPs and caspase-8 to regulate RIPK3-mediated inflammatory signalling.** (A) HCT116/RIPK3-2xFV and HCT116/RIPK1-KO clone 12c/RIPK3-2xFV were pretreated with 2 μ M CpA for 0.5h before treated with 0 or 100nM dimeriser for 3h (n=3). (B) HCT116/RIPK3-2xFV, HCT116/RIPK1-KO clone 8c/RIPK3-2xFV and HCT116/RIPK1-KO clone 15c/RIPK3-2xFV were treated as indicated for 3h (n=3). CpA, 2 μ M. (C) HCT116/RIPK3-2xFV and HCT116/RIPK1-KO clone 8c/RIPK3-2xFV were treated as indicated for 24h. Supernatant was collected for ELISA (n=3). CpA, 100nM. Ordinary one-way ANOVA and Sidak's multiple comparison test were used to test for statistical differences between indicated conditions. ns, not significant, **, p<0.01, ****, p<0.0001. 109
- 5.2 **RIPK3 oligomerisation in the absence of RIPK1 leads to the appearance of its slow-migrating form.** (A) HCT116/RIPK3-2xFV, HCT116/RIPK1-KO clone 8c/RIPK3-2xFV, HCT116/RIPK1-KO clone 12c/RIPK3-2xFV and HCT116/RIPK1-KO clone 15c/RIPK3-2xFV were treated as indicated for 6h. (B) HT29/RIPK3-2xFV and HT29/RIPK1-KO clone aA3/RIPK3-2xFV cells were treated as indicated for 24h. CpA, 100nM, TNF, 2ng/ml. (C) U2OS/RIPK1-KO clone a/RIPK3-2xFV, U2OS/RIPK1-KO clone b/RIPK3-2xFV, U2OS/RIPK1-KO clone c/RIPK3-2xFV and U2OS/RIPK3-2xFV were treated as indicated for 4h. CpA, 100nM, TNF, 20ng/ml. The expression of endogenous RIPK3 appears to differ between clones. (D) Cells were treated as indicated for 6h. 111
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- 5.9 **Nec1s partially inhibits RIPK3-mediated inflammatory signalling in the absence of M1-linked polyubiquitin and caspase activity.** (A) Cells were stimulated as indicated for 3h (n=3). (B) Cells were treated with Dox for 48h before stimulated for 3h (n=3). Ordinary one-way ANOVA and Sidak's multiple comparison test were used to test for statistical differences between the indicated conditions. ns, not significant, *, $p<0.05$, ****, $p<0.0001$ 119