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Regulation of Inflammatory Signalling by Caspases and M1-linked Ubiquitin Chains in *Drosophila melanogaster*

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To my beloved family

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ABSTRACT

The NF-κB family of transcription factors are master regulators of innate immune responses and their dysregulation can lead to chronic inflammation and cause diseases like inflammatory bowel disease and cancer. To treat inflammatory diseases, a deeper understanding of regulation of the inflammatory NF-κB pathway at the molecular level is needed. As the signalling mechanisms regulating the NF-κB pathway are highly conserved, the fruit fly *Drosophila melanogaster* serves as an excellent model to understand the basic principles of the regulatory machinery. The NF-κB pathway activity is tightly regulated by ubiquitin signalling, as E3 ligases conjugate ubiquitin chains to important mediators of the pathway, regulating their function.

In this thesis, I have studied how caspases and ubiquitin E3 ligases activate and restrain NF-κB signalling. We described a *Drosophila* interleukin 1β-converting enzyme (Drice)-mediated regulation of the *Drosophila* inhibitor of apoptosis 2 (Diap2), an E3 ligase that generates K63-linked ubiquitin chains, and a potent inducer of NF-κB. Drice functions by restraining Diap2 function under basal conditions in order to avoid unnecessary activation of NF-κB pathway induced by commensal bacteria. We also described the formation of M1-linked ubiquitin chains in flies upon infection, and identified Linear ubiquitin E3 ligase (LUBEL) as the enzyme responsible for formation of these chains. We found that M1-linked ubiquitin chains are important for activation of the NF-κB pathway in the intestinal epithelial tissue, showcasing the tissue specific regulation of NF-κB activation. We found previously unknown targets of M1-ubiquitination in the *Drosophila* Imd pathway, the Inhibitor of κB kinase γ (IKKγ) Kenny, and Death related ced-3/Nedd2-like caspase (Dredd). Similar to mammalian IKKγ, we also found that Kenny associated with M1-linked Ub chains, showing the conservation of function between mediators of mammalian and *Drosophila* NF-κB pathway. Finally, we found a novel mode of regulation of Kenny, where cleavage by Dredd protected Kenny from autophagosomal degradation, and this process was dependent on M1-ubiquitination. Our findings from this thesis have improved the understanding of the *Drosophila* NF-κB pathway by uncovering new mechanisms of regulation of NF-κB signalling by ubiquitination and caspases.

SAMMANFATTNING (ABSTRACT IN SWEDISH)

NF-κB-transkriptionsfaktorfamiljen är nyckelreglerare av det medfödda immunförsvaret och okontrollerad reglering av NF-κB:s aktivitet kan leda till kronisk inflammation och förorsaka sjukdomar som inflammatorisk tarmsjukdom och cancer. För att behandla inflammatoriska sjukdomar krävs en djupare förståelse av de molekylära mekanismer som reglerar den inflammatoriska NF-κB-signaleringsräckan. Eftersom dessa signaleringsmekanismer är välbevarade, fungerar bananflugan, *Drosophila melanogaster* som en utmärkt modellorganism för att förstå de grundläggande principerna för NF-κB-reglering. Aktiveringen av NF-κB-signaleringsräckan regleras noggrant av en proteinmodifikation som kallas ubikvitinering, eftersom E3-ubikvitinligaser konjugerar ubikvitinkedjor till viktiga NF-κB-signalförmedlare, vilket därmed kontrollerar deras funktion.

I denna avhandling har jag studerat hur kaspaser och E3- ubikvitinligaser aktiverar och hämmar NF-κB-signalering. Vi har beskrivit hur kaspaset *Drosophila* interleukin-1β-converting enzyme (Drice) kontrollerar *Drosophila* inhibitor of apoptosis 2 (Diap2), som är ett E3-ligas som genererar K63-kopplade ubikvitinkedjor och är en effektiv aktiverare av NF-κB. Drice fungerar genom att hämma Diap2-funktionen under basala förhållanden för att undvika att kommensala bakterier aktiverar NF-κB i onödan. Vi har också beskrivit att M1-kopplade ubikvitinkedjor syntetiseras vid bakteriell infektion i bananflugan, och har identifierat att Linear ubiquitin E3-ligase (LUBEL) är enzymet ansvarigt för bildandet av dessa kedjor. Vi har funnit att M1-kopplade ubikvitinkedjor är viktiga för aktiveringen av NF-κB-signaleringsräckan i tarmepitelvävnad, vilket påvisar en vävnadsspecifik reglering av NF-κB-aktiveringen. Vi har även funnit tidigare okända målproteiner för M1-ubikvitinering i bananflugans Imd-signaleringsräcka; kinaset Inhibitor of κB kinase γ (IKKγ) Kenny och kaspaset Death related ced-3/Nedd2-like caspase (Dredd). Vi har funnit att Kenny binder till M1-kopplade ubikvitinkedjor på samma sätt som IKKγ-proteinet i däggdjur, vilket antyder att NF-κB-signalförmedlare fungerar liknande i däggdjur och bananflugan. Slutligen har vi funnit en ny regleringsmekanism för Kenny, där Dredd skyddar Kenny från autofagosomal nedbrytning genom att klyva bort degraderingssignalsekvensen i Kenny. Dessutom har vi visat att denna process är beroende av M1-ubikvitinering. Sammanfattningsvis har resultaten från denna avhandling förbättrat förståelsen av de mekanismer som styr Drosophilas NF-κB-signalering och avslöjat nya regleringsmekanismer för NF-κB-signalering genom ubikvitinering och kaspaser.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications and manuscript, which are referred to in the text by Roman numerals (I-III). The original publications have been reproduced with the permission of the copyright holders.

I. Kietz C, Mohan AK, Pollari V, Tuominen I-E, Ribeiro PS, Meier P, Meinander A. (2021) Drice restrains Diap2-mediated inflammatory signalling and intestinal inflammation. Cell Death and Differentiation, doi:10.1038/s41418-021-00832-w.

II. Aalto AL*, Mohan AK*, Schwintzer L, Kupka S, Kietz C, Walczak H, Broemer M, Meinander A. (2019) M1-linked ubiquitination by LUBEL is required for inflammatory responses to oral infection in Drosophila. Cell Death and Differentiation, doi: 10.1038/s41418-018-0164-x. *Equal contribution

III. Mohan AK, Aalto AL, Hermanson E, Dahlström A, Kietz C, Meinander A. M1-ubiquitination of the initiator caspase Dredd promotes NF-κB activation by protection of the IKK complex. Manuscript.

AUTHOR CONTRIBUTIONS

I. The author planned and performed transfection of S2 cells, ubiquitin pulldown assays from S2 cells and analysed data. CK performed transfection of S2 cells, ubiquitin pulldowns from S2 cells and fly lysates, qPCR from S2 cells and flies, X-Gal staining, immunofluorescence staining of fly midguts, 16S rRNA sequencing, caspase activity assay of fly midguts and S2 cells, survival assays, pathogen clearance assays, cell viability and reared flies under axenic conditions. VP reared flies under axenic conditions and performed qPCR, I-ET performed survival assays and qPCR. PSR and AM performed survival assays. PSR, AM, PM designed experiments, analysed data and contributed to the writing of the manuscript.

II. The author and ALA contributed equally to experimental design, execution and analysis of most of the experiments and writing of the manuscript. The author performed the following experiments in this study: transfection of S2 cells to overexpress proteins, immunoprecipitations, LPS treatments, generated point mutations, X-gal staining of dissected fly guts, cloning to generate transgenic fly by PhiC31-mediated integration of RBR-LDD in the fly genome and verification by PCR. ALA performed survival assays, qPCR, ubiquitin pulldowns from S2 cells and fly lysates, immunofluorescence staining of dissected guts, pathogen clearance, and structural modelling of the catalytic domain of LUBEL. The author and ALA prepared samples and SK performed deubiquitination assays on fly and cell samples, LS performed all *in vitro* ubiquitination assays, CK performed immunofluorescence staining on dissected fly guts. MB, HW and AM contributed to designing experiments, writing and data analysis of this manuscript.

III. The author contributed by experimental design, execution and analysis of most of the experiments and writing of the manuscript. The author performed the following experiments for this study: transfection of S2 cells, immunoprecipitations, ubiquitin pulldown from S2 cells, sub-cloning and generation of point mutations. ALA performed transfection of S2 cells and V5-immunoprecipitations. EH performed transfection and Western blotting of S2 cell lysates. AD performed transfection of S2 cells and immunoprecipitations. CK performed inhibitor treatment, dissection and Western blotting of fly guts. AM contributed to experimental design, data analysis, structural modelling and writing of this manuscript.

ABBREVIATIONS

ABIN	A20-binding inhibitors
AMP	Antimicrobial peptide
AMSH	Associated molecule with the SH3 domain of STAM
APAF-1	Adaptor protein apoptotic protease activating factor-1
BIR	Baculovirus IAP Repeat
CARD	Caspase recruitment domain
CAP-Gly	Cytoskeleton-associated protein Gly-rich domain
c-FLIP	Cellular FLICE-like inhibitory protein
cIAP	Cellular inhibitor of apoptosis
CC	coiled coil
CoZi	Coil-zipper
Cpdm	Chronic proliferative dermatitis
CrmA	Cowpox virus protein cytokine response modifier A
CYLD	Cylindromatosis
daGal4	DaughterlessGal4
DAMP	Damage-associated molecular patterns
Damm	Damm Death associated molecule related to Mch2
DAP	diaminopimelic acid
Dark	Death-associated APAF1-related killer
dBruce	*Drosophila* BIR repeat containing ubiquitin-conjugating enzyme
Dcp-1	*Drosophila* effector caspase-1
DD	Death domain
Decay	Death executioner caspase related to apopain/yama
DED	Death effector domain
Diap	*Drosophila* inhibitor of apoptosis
Dif	Dorsal-related immunity factor
Dipt	Diptericin
DISC	Death-induced signalling complex

DR	Death receptor
Dredd	Death related ced-3/Nedd2-like caspase
Drice	*Drosophila* interleukin 1β-converting enzyme
Dronc	Death regulator Nedd2-like caspase
DUB	Deubiquitinating enzyme
FADD	Fas-associated death domain
FLIP	FLICE-like inhibitory protein
GNBP	Gram-negative binding proteins
HECT	Homologous to the E6-AP carboxyl terminus
Hid	Head involution defective
HOIL	Heme-oxidized IRP2 Ub ligase
HOIP	HOIL-1-interacting protein
IAP	Inhibitor of apoptosis protein
IBD	Inflammatory bowel disease
IBM	IAP-binding motif
IκB	Inhibitor of κB
IKK	Inhibitor of κB kinase
Imd	Immune deficiency
Ird5	Immune response deficient
JNK	c-Jun N-terminal kinase
LC3	Microtubule-associated protein 1 light chain 3
LDD	Linear ubiquitin chain determining domain
LIR	LC3-interacting region
LPS	Lipopolysaccharide
LRR	leucine-rich repeat
LTM	LUBAC-tethering motif
LUBAC	Linear ubiquitin chain assembly complex
LUBEL	Linear ubiquitin E3 ligase
MAPK	Mitogen-activated protein kinase
MDP	Muramyldipeptide

M1-linked	Methionine1-linked
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIK	NF-κB inducing kinase
NLR	NOD-like receptors
NLS	Nuclear localisation signal
NOD	Nucleotide-binding oligomerization domain
NZF	Npl4-type zinc finger
Optineurin	Optic neuropathy inducing
ORAS	OTULIN-related inflammatory syndrome
OTU	Ovarian tumour protease
PAMP	Pathogen-associated molecular patterns
PGRP	Peptidoglycan recognition protein
PGN	Peptidoglycan
PH	Pleckstrin homology
PIM	PUB domain-interacting motif
PUB	Peptide:N-glycanase/UBA- or UBX-containing proteins
PNG	Peptide:N-glycanase
PRR	Pattern-recognition receptor
qPCR	Quantitative reverse transcriptase PCR (qPCR)
RBR	RING-in between-RING
RHD	Rel homology domain
RIG-I	Retinoic acid-inducible gene I
RING	Really interesting new gene
RIPK	Receptor interacting protein kinase
RNAi	RNA interference
SAMP	Stress-associated molecular patterns
SHARPIN	Shank-associated RH domain-interacting protein
SMAC	Second mitochondrial-derived activator of caspases
SPATA2	Spermatogenesis-associated 2

SPE	Spätzle processing enzyme
Strica	Ser/Thr-rich caspase
TAB2	TAK binding protein 2
TAD	Transactivation domain
TAK1	TGF-β-activated kinase 1
TGFβ	Transforming growth factor β
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFR	TNF receptor
TRADD	TNFR1-associated death domain
TRAF	TNF receptor associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TUBE	Tandem ubiquitin binding entity
UAS	Upstream activating sequence
Ub	Ubiquitin
UBA	Ubiquitin-associated
UBAN	Ubiquitin binding in ABIN and NEMO proteins
UBD	Ubiquitin binding domain
UCH	Ubiquitin C-terminal hydrolases
XIAP	X-chromosome-linked inhibitor of apoptosis
ZnF	Zinc finger

INTRODUCTION

The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) family of transcription factors are master regulators of inflammatory responses, as they control the expression of several inflammatory genes. Their activation by pattern recognition receptors (PRRs), cytokines and antigen receptors is important for innate and adaptive immune function. Dysregulation of NF-κB is associated with inflammatory and autoimmune diseases, and can lead to cancer (Q. Zhang et al., 2017). In order to treat inflammatory diseases, a deeper understanding of regulation of the inflammatory NF-κB pathway at the molecular level is needed. A common feature of NF-κB signalling activated by different receptors is their reliance on ubiquitin (Ub) chains to transmit the signals from the receptor to downstream mediators (Iwai, 2014). E3 ligases are enzymes that catalyse ubiquitination, a highly conserved posttranslational modification, where ubiquitin is covalently conjugated to target proteins, thereby altering the function of those proteins. Polyubiquitin chains are formed when additional ubiquitin molecules are attached using one of the seven internal lysines (K6, K11, K27, K29, K33, K48, K63) or the N-terminal methionine (M1) residue of the proximal ubiquitin, forming polyubiquitin chains of varying topology. Ubiquitin chains of different topology can be bound by different ubiquitin binding proteins, providing versatile signalling outcomes (Komander & Rape, 2012; Swatek & Komander, 2016). While ubiquitination is commonly known to induce proteasomal degradation of substrate proteins, non-degradative Ub chains like K63- and M1-linked Ub chains are known to function as recruitment platforms for mediators of the NF-κB pathway.

Caspases have been extensively studied for their role in apoptosis, but they are also involved in other important functions such as regulation of the NF-κB pathway, and dysregulation of caspases can also lead to inflammatory diseases (McIlwain et al., 2013). How caspases are activated during inflammation, diverting from their apoptotic function is not fully understood. The activity of caspases is tightly controlled by inhibitor of apoptosis proteins (IAPs), and it is known that caspases are targeted with both degradative and non-degradative Ub chains (Dumétier et al., 2020). The role of Diap2 in generating K63-linked Ub chains in flies has been described (Meinander et al., 2012; Paquette et al., 2010). Diap2 targets the *Drosophila* caspase-8, Dredd, with K63-linked Ub chains, which is required for activation of Dredd catalytic activity. However, M1-linked Ub chains were first described in mammals in 2006 (Kirisako et al., 2006), and studies to understand their physiological function in mice by using genetic ablation or catalytic mutation of the E3 ligase responsible found them to be lethal (Emmerich et al., 2013; Peltzer et al., 2014; Sasaki et al., 2013). We aimed to identify the M1-ubiquitination machinery in the common biological model organism, *Drosophila melanogaster*, in order to study the physiological function

and mechanism of regulation of the NF-κB pathway by M1-linked Ub chains. The fruit fly, *Drosophila melanogaster* is a versatile model organism that has been used to study various biological processes including innate immune responses. As the mediators of the inflammatory NF-κB pathway and their mechanisms of regulation are well conserved, the fruit fly is an attractive model to investigate the regulation of the NF-κB pathway by ubiquitination and caspases (Myllymäki et al., 2014).

This thesis aims to further our knowledge of caspase-mediated regulation of NF-κB activation by describing new mechanisms of regulation of the Imd pathway, one of the *Drosophila* NF-κB pathways, by the effector caspase, Drice. Drice inhibits the function of Diap2, an E3 ligase that is important for activation of the Imd pathway, by inducing degradation of Diap2, restraining inflammation induced by commensal bacteria during basal conditions. Furthermore, it describes the M1-linked Ub chain generating machinery in *Drosophila*, LUBEL, and its importance in regulating the Imd pathway in the intestinal epithelial tissue. We have identified new targets of M1-ubiquitination in the Imd pathway, the *Drosophila* IKKγ, called Kenny, and an initiator caspase, called Dredd. Finally, it aims to describe a novel mode of regulation of Kenny, whereby Dredd-mediated cleavage protects Kenny from autophagy-mediated degradation.

REVIEW OF THE LITERATURE

1 *Drosophila* as a model organism

The fruit fly *Drosophila melanogaster* has been used in biological research for over a century. It has been used as a model in innate immunity since 1970s, when Boman and others described the humoral nature of the antibacterial defence using fruit flies (Boman et al., 1972). Notably, the discovery of the Toll pathway being responsible for innate immune response against fungi (Lemaitre et al., 1996), led to the identification of Toll-like receptor 4 (TLR4) as the receptor responsible for recognising bacterial lipopolysaccharide (LPS) in mammals (Poltorak et al., 1998). These discoveries were later awarded the Nobel prize in Physiology or Medicine in 2011. As the fruit fly lacks an adaptive immune system, combined with the host of genetic and molecular biology techniques made available in *Drosophila*, it serves as a valuable model to study innate immune signalling pathways.

The fruit fly is a versatile model organism that has many advantages over vertebrate model organisms. Easy and inexpensiveness to maintain in the laboratory, having a shorter lifecycle, while producing a large number of offspring are some of the characteristics that makes the fruit fly an efficient model organism. The fruit fly has been used to study a wide range of biological processes like genetics and inheritance, development and aging. It has been established that the fundamental biological processes and signalling pathways controlling development and survival are well conserved between flies and eukaryotes, including humans (Jennings, 2011). The genome of the fruit fly has been sequenced and can be accessed via the online database ''Flybase'' (Gramates et al., 2022). The fruit fly has around 14000 genes and 75% of human disease genes were found to have a counterpart in the genome of fruit fly (Bier, 2005; Reiter et al., 2001).

The lifecycle of fruit fly lasts about 10 days at 25°C. The eggs undergo embryonic development for about 24 hours, after which larva hatches from the eggs. The larvae eat and grow in three instar stages before forming pupae. The flies undergo metamorphosis during the pupal stage, after which the adult fly hatches. A wide range of genetic tools are available to manipulate gene expression in *Drosophila.* The upstream activating sequence (UAS)-Gal4 system is a binary expression system consisting of the components, the yeast transcriptional activator Gal4 and a transgene, that is under the control of UAS promoter, which is silent in the absence of Gal4. The UAS-Gal4 system can be used to overexpress or reduce expression of genes in a cell or tissue specific manner by using a specific Gal4 promoter element (Brand & Perrimon, 1993). The expression of genes can be reduced by RNA interference (RNAi), by expressing double-stranded RNA corresponding to a gene's sequence and it can

be targeted to a specific cell-type or tissue. The P-element transposon mediated integration of DNA into germline genome is commonly used as a mutagen to generate mutagenic flies. Alternatively, the P-element-based vectors can be cloned to contain a transgene of interest. It can then be injected to *Drosophila* germline cells along with a plasmid that codes for P transposase, which will cut the P-element backbone from the first plasmid and paste it into the germline genome at a random location. Insertion at a specific location is possible by several techniques, and one example is by using the bacteriophage ϕC31 integrase, which can insert a transgene at specific recognition sites in the genome called *attP* sites. *Drosophila* lines with specific landing sites (*attP* sites) have been generated, so that the ϕC31 integrase can integrate a segment of desired DNA flanked by the *attB* sites via recombination to the *attP* sites in the *Drosophila* genome (Groth et al., 2004). Both P-element and ϕC31 mediated insertion can be combined with other tools such as the UAS-Gal4 system to manipulate gene expression. In addition, the CRISPR-Cas9 system can be used to disrupt or edit genetic elements. Transgenic flies expressing the nuclease Cas9 are available, which can be injected with guide RNAs to induce a double-stranded break (DSBs) at a specific genomic locus. The target locus then undergoes DNA damage repair via nonhomologous end joining or homology-directed repair, both of which can be utilised to generate a desired editing outcome (Gratz et al., 2015; Ran et al., 2013).

2 Ubiquitination

Ubiquitination is a post-translational modification that regulates a wide variety of biological functions including apoptosis, protein processing, trafficking, immune responses, and DNA repair (Swatek & Komander, 2016). Ubiquitin (Ub) is a protein made up of 76 amino acids and ubiquitously expressed in all eukaryotes. Ub is synthesized either as single Ub fused to other proteins or tandem repeats of Ub, which are cleaved by deubiquitinating enzymes (DUBs) to generate Ub monomers. Ubiquitination refers to the covalent attachment of a Ub molecule to a substrate protein, in a three-step enzymatic reaction catalysed by a multicomponent enzymatic machinery, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3) (Figure 1 A) (Hershko et al., 1983). The first step is the ATP dependent formation of a thioester bond between Ub and the active cysteine residue of E1. Ub is then trans-thiolated to the active cysteine of E2. Finally, the Ub molecule is conjugated to a substrate protein by additional contribution from E3 ligase (Hershko & Ciechanover, 2003). Really interesting new gene (RING) type E3 ligases function by acting as an intermediate between E2 and the substrate by facilitating direct transfer of Ub from E2 to the substrate. Homologous to the E6-AP carboxyl terminus (HECT) type E3 ligases function by forming a thioester intermediate with the active cysteine of E3 ligase before transferring Ub to the substrate.

RING-between-RING (RBR) type ligases combine the properties of both RING and HECT type ligases, where the RING1 recognises the E2-Ub conjugate, followed by transfer of Ub from E2 to an active cysteine in RING2, after which the Ub is conjugated to the substrate (Yang et al., 2021). The human genome codes for two E1s, around 50 E2s and around 700 E3s (S. Liu & Chen, 2011), and there are 9 E1 Ub-like activating enzymes, 27 E2s and 151 E3s encoded by the *Drosophila* genome, according to Flybase gene groups ((FB2023_03, released June 13, 2023, (FlyBase Classification of D. Melanogaster Enzymes. 2017)). Ubiquitination starts when the C-terminus of a single ubiquitin molecule is attached to a lysine residue of a substrate protein through an isopeptide bond, leading to monoubiquitination. However, studies have recently shown that ubiquitin can also be attached to serine and threonine residues on target proteins, leading to the formation of an oxyester bond (Kelsall, 2022). The E3 ligases provide specificity by selecting the substrate protein to be ubiquitinated. It is possible that a substrate protein can be monoubiquitinated at multiple lysine residues. Ubiquitin itself contains seven internal lysine residues (K6, K11, K27, K29, K33, K48, K63) and an N-terminal methionine (M1) that can be used to attach additional ubiquitin molecules to form polymeric ubiquitin chains. Structurally distinct Ub chains are formed depending on which residues are utilized to conjugate Ub moieties (Komander & Rape, 2012; Peng et al., 2003). Homotypic Ub chains are formed when the same residue in Ub is used to elongate the chain, mixed chains are formed when distinct residues are used in succeeding positions, and branched Ub chains are formed when a single Ub molecule in a Ub chain is conjugated to two Ub molecules through different residues (Figure 1B). These Ub chains can be short, containing only two Ub molecules or long, containing more than ten Ub moieties. Distinct Ub chains formed using different residues give rise to distinct Ub chain conformations. K48-, K6-, and K11-linked Ub chains adopt a compact conformation and M1- and K63-linked Ub chains adopt an open conformation (Akutsu et al., 2016; Ye et al., 2012). Finally, ubiquitin can also undergo post-translational modifications such as acetylation and phosphorylation on their lysine and serine/threonine residues respectively. Each of these modifications on ubiquitin can provide additional means of regulation and has the potential to influence the signalling outcome (Swatek & Komander, 2016).

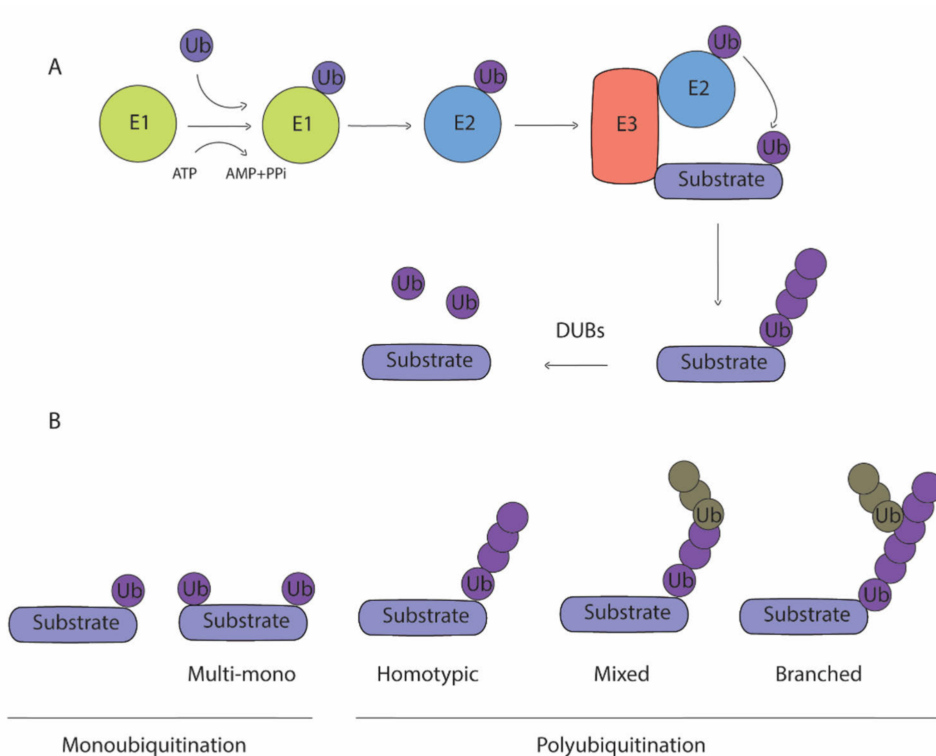


Figure 1. Schematic representation of the ubiquitination reaction. A. Ubiquitination is the enzymatic process of conjugating ubiquitin to a substrate protein by the combined action of E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme and E3 ubiquitin ligase. Ubiquitination is a reversible process and deubiquitinating enzymes (DUBs) hydrolyse ubiquitin molecules from the substrates. **B.** Substrates can be monoubiquitinated or polyubiquitinated, and polyubiquitination can give rise to homotypic, mixed or branched ubiquitin chains. Differently coloured balls represent ubiquitin chains of different linkages. Figure adapted from (Dikic & Schulman, 2022; Komander & Rape, 2012; Swatek & Komander, 2016).

3 *Drosophila* host defence

Insects and microorganisms coexist in nature, and insects have evolved mechanisms to recognize and defend themselves against bacteria, fungi, viruses and parasites. The fruit fly does not possess an adaptive immune system and relies solely on its innate immune system to defend itself from pathogens. The *Drosophila* innate immune response can be divided into humoral and cellular responses.

The cellular response in *Drosophila* is carried out by blood cells or hemocytes, several thousands of which are present in the body cavity of *Drosophila* larva.

Hemocytes can be divided into three cell types, plasmatocytes, lamellocytes and crystal cells, each responsible for performing a specialised function (Banerjee et al., 2019; Rizki & Rizki, 1984). Plasmatocytes are the most abundant, making up 90-95% of the hemocytes, and they function by removing dead cells and pathogens by phagocytosis. Lamellocytes are not found in healthy larva, but they can be induced to differentiate from hemocyte precursors upon infection with parasitoid wasp eggs. The crystal cells that constitute about 5% of the hemocytes are non-phagocytic and involved in melanisation. Crystal cells express pro-phenoloxidase (ProPO) which is the inactive zymogen form of phenoloxidase (PO) (Dudzic et al., 2015; Rizki et al., 1980). They are fragile cells that act as storage cells for ProPO and readily disrupt, releasing their contents into hemolymph when activated. PO can then oxidize phenols to quinones, which polymerize to form melanin, that ultimately sequesters the microorganism to the wound site (Vlisidou & Wood, 2015).

The humoral response of the innate immune system is characterized by signal dependent activation of the inflammatory NF-κB pathway (Gay & Keith, 1991; Lemaitre, Kromer-Metzger, et al., 1995). The NF-κB family of transcription factors are evolutionarily conserved and play a crucial role in regulating critical processes such as cell survival, proliferation and immune responses (Ghosh et al., 2003). Apart from NF-κB, other signalling pathways involved in host defence are also evolutionarily conserved, like the Janus kinase protein and signal transducer and activator of transcription (JAK-STAT) signalling pathway and the c-Jun N-terminal kinase (JNK) pathway, which are involved in wound healing and stress responses (S. Yu et al., 2022). NF-κB factors are sequestered in the cytoplasm during basal conditions. They are activated upon stimulus resulting in their translocation to the nucleus where they control expression of immune responsive genes encoding proteins and peptides including antimicrobial peptides (AMPs), which have direct antimicrobial activity (Lemaitre et al., 1997). Several aspects of the humoral response including sensing of the pathogens by pattern recognition receptors, mediators of the NF-κB signalling pathway, and their regulation are conserved between *Drosophila* and higher organisms (Myllymäki et al., 2014).

3.1 *Drosophila* NF-κB pathways

The *Drosophila* genome encodes three NF-κB family members, Dorsal, dorsal-related immunity factor (DIF) and Relish (Belvin & Anderson, 2003; Dushay et al., 1996; Hedengren et al., 1999). Activation of NF-κB upon microbial challenge results in the expression of AMP genes. The expression of AMP genes is controlled by two NF-κB pathways, the immune deficiency (Imd) and Toll pathway (Lemaitre et al., 1996; Lemaitre, Kromer-Metzger, et al., 1995). These pathways are triggered by sensing of pathogens via interactions of peptidoglycan recognition proteins (PGRPs) and Gram-negative binding proteins (GNBPs) with

microbial surface proteins (Kurata, 2014). There are 13 PGRP genes found in the *Drosophila* genome, of which 7 are small-sized and secreted, while 6 are large-sized and transmembrane receptors. PGRPs are related to bacteriophage type-II amidases, some of which have lost their catalytic activity and function as recognition PGRPs, whereas the ones that possess amidase activity degrade peptidoglycan (PGN) and reduce its ability to induce immune responses (Lemaitre & Hoffmann, 2007). While it was believed that PGRPs can structurally discriminate different bacterial PGNs, it was recently shown that the accessibility to PGNs on the cell wall of bacteria forms the basis of discrimination between different classes of bacteria (Vaz et al., 2019). In addition to pathogens, both the *Drosophila* and mammalian NF-κB pathways can be activated in response to sterile stresses such as damage-associated molecular patterns (DAMP) (Asri et al., 2019; Rider et al., 2017).

3.2 The Imd pathway

In flies, upon infection by Gram-negative bacteria, the receptors PGRP-LC or PGRP-LE detect and bind to meso-diaminopimelic acid (DAP)-type PGN found on the cell wall of Gram-negative bacteria (Choe et al., 2002, 2005; Takehana et al., 2002). Following receptor activation, a death domain containing protein called Imd, which has sequence similarity to the death domain of mammalian Receptor interacting protein kinase (RIPK) 1, is recruited (Georgel et al., 2001). Imd further interacts with Fas-associated death domain (FADD) through a homotypic interaction between death domains (DD) in these proteins. FADD then interacts with the *Drosophila* homologue of mammalian caspase-8, called Dredd, through homotypic interactions between death effector domains (DED) in these proteins (Hu & Yang, 2000; Naitza et al., 2002). Upon PGN stimulation, Diap2, via its RING domain, is known to conjugate Ub chains to mediators of the Imd pathway. The *Drosophila* TAK binding protein 2 (Tab2)/TGF-β-activated kinase 1 (Tak1) (dTab2/dTak1) complex is thought to be recruited to the Imd signalling complex through the ubiquitin chain binding property of the Ubiquitin-associated (UBA) domain of dTab2. Once recruited, dTak1 activates the inhibitor of κB (IκB) kinase (IKK) complex by phosphorylating IKKβ or Immune response deficient 5 (Ird5) (Kanayama et al., 2004; Kulathu et al., 2009; Skaug et al., 2009). In addition, Tak1 is also an important activator of the JNK pathway, which is an important stress-sensing pathway in eukaryotic cells. JNK is involved in a variety of biological processes in *Drosophila* including embryonic development, apoptosis, cell proliferation and differentiation, stress response and innate immune responses (Tafesh-Edwards & Eleftherianos, 2020). While the JNK pathway is required for the Imd pathway-mediated AMP release and epithelial shedding (Kallio et al., 2005; Zhai et al., 2018), activation of the NF-κB factor Relish is known to terminate JNK

signalling (J. M. Park et al., 2004), highlighting the role of Relish in controlling both the Imd and JNK signalling simultaneously.

K63-ubiquitination of Imd and Dredd by Diap2 is required for activation of Dredd, and the Imd pathway (Meinander et al., 2012; Paquette et al., 2010). The IKK complex comprised of a kinase, IKKβ/Ird5, and a regulatory subunit IKKγ, called Kenny functions downstream of Dredd. The NF-κB precursor Relish has an N-terminal Rel homology domain (RHD) and C-terminal ankyrin repeats. Relish is normally sequestered in the cytoplasm, as the ankyrin repeats mask the nuclear localisation signal. Cleavage of Relish by Dredd results in Relish translocating to the nucleus, and, expression of hundreds of genes, some of which encode AMPs (Figure 2). Relish activity inside the nucleus was shown to be inhibited by an IκB family member called Pickle, which also contains ankyrin repeats (Morris et al., 2016). Pickle resides in the nucleus and inhibits Relish activity possibly by recruiting the histone deacetylase dHDAC1, which is known to negatively affect transactivation of Relish (Lark et al., 2007). The IKK complex has two important functions in activating Relish. It has been shown that Ird5 phosphorylates Relish, which is not required for cleavage of Relish, but is necessary for a robust induction of Imd pathway target genes (Ertürk-Hasdemir et al., 2009). Curiously, the IKK complex is also required, independent of its kinase activity, for Dredd-mediated cleavage of Relish (Silverman et al., 2000; Stöven et al., 2003).

Although the *Drosophila* Imd pathway is normally compared to Tumour necrosis factor (TNF) receptor 1 (TNFR1) signalling pathway for the similarities in the components of the signalling pathway and their mechanisms of regulation, it also has many similarities to the Nucleotide-binding and oligomerization domain-like receptor 2 (NOD2) signalling pathway. Apart from having conserved components and mechanisms of regulation, both Imd and NOD2 signalling pathways are activated by components of the bacterial cell wall. Importantly, they are essential for innate immune responses in intestinal epithelia.

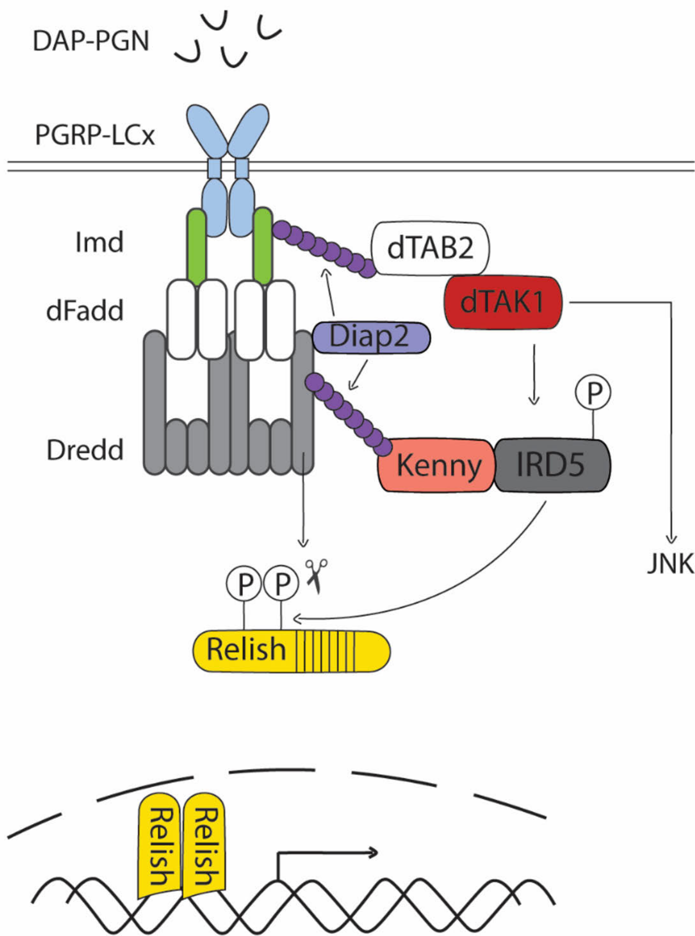


Figure 2. Schematic representation of the *Drosophila* Imd pathway. The Imd pathway is activated by PGRP-LCx receptor upon recognition of DAP-type peptidoglycan from Gram-negative bacteria. A complex comprising of Imd, dFadd and Dredd is recruited, after which Imd and Dredd are ubiquitinated by Diap2. The kinase complexes, Tab2/Tak1 and the IKK complex are recruited via ubiquitin binding, which culminates in phosphorylation of the NF-κB transcription factor, Relish. Finally, Dredd, which gets activated after ubiquitination, cleaves Relish to release it from inhibition by its ankyrin repeats. Relish is then free to translocate to the nucleus where it activates expression of its target genes.

3.3 The Toll pathway

The Toll receptor in *Drosophila* does not directly interact with microbial surface determinants. Infection by Gram-positive bacteria is detected in the flies by circulating receptor proteins PGRP-SA, PGRP-SD and GNBP1, whereas a fungal infection is detected by GNBP3 (Valanne et al., 2011). A protease called Modular Serine Protease (ModSP) integrates signalling upon fungal and Gram-positive bacterial infection and initiates a serine protease cascade that proceeds through Grass to finally activate Spätzle processing enzyme (SPE) (Buchon et al., 2009). Activated SPE cleaves a secreted cytokine-like molecule Spätzle, which binds the Toll receptor to induce receptor homodimerization and activates the intracellular part of the signalling (Jang et al., 2006). Alternatively, some virulence factors like proteases released by bacteria and fungi can activate a *Drosophila* serine protease called Persephone, which can again activate SPE, thus activating the intracellular Toll signalling. Activation of Toll leads to the formation of a signalling complex proximal to the receptor containing MyD88, Tube and Pelle. Toll contains a Toll/interleukin (IL)-1R (TIR) domain, similar to the mammalian TLR and IL-1R through which it interacts with MyD88. MyD88, Tube and Pelle contain death domains (DD) through which a heterotrimeric complex is formed (H. Sun et al., 2002). Pelle is a kinase, which gets activated upon recruitment to the complex which results in phosphorylation, ubiquitination and subsequent degradation of Cactus, the IκB-like protein that sequesters Dorsal and Dif in the cytoplasm. Dorsal and Dif are then free to translocate to the nucleus where they can transcribe Toll target genes (Figure 3) (Ip et al., 1993; Lemaitre, Meister, et al., 1995).

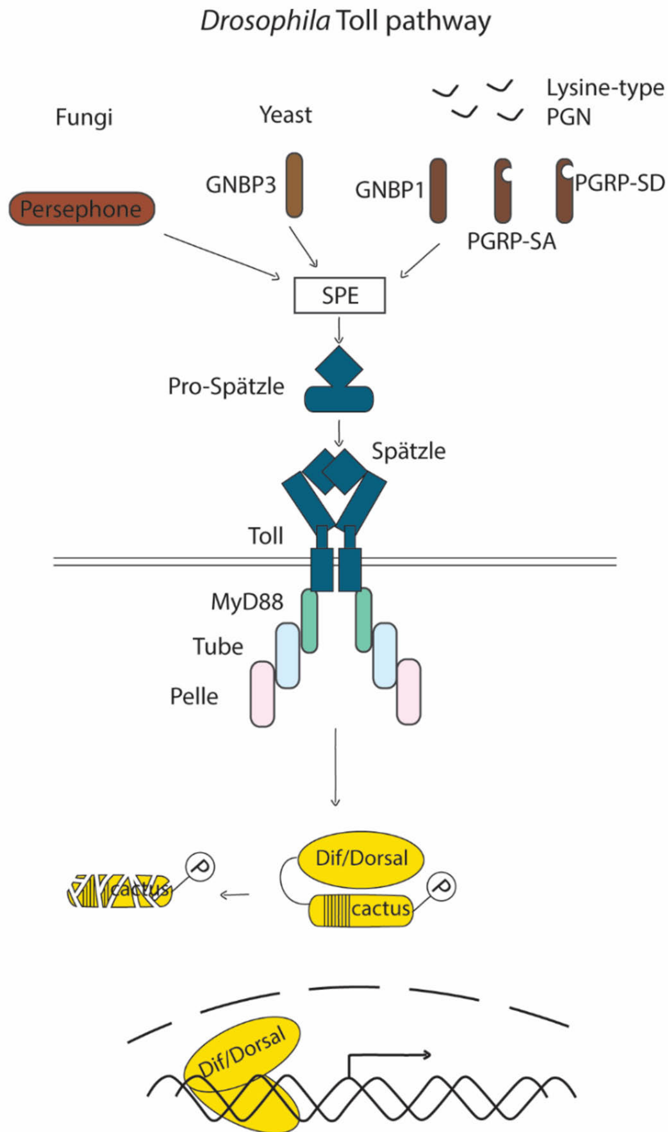


Figure 3. Schematic representation of the *Drosophila* Toll pathway. The Toll pathway is activated by Gram-positive bacteria and fungi, which are recognised by secreted recognition molecules PGRP-SA, PGRP-SD, GNBP1 and GNBP3. This is followed by a protease cascade to activate SPE which cleaves Spätzle, after which mature Spätzle binds to the Toll receptor. Three intracellular proteins with death domains, MyD88, Tube and Pelle are then recruited, after which the IκB like protein, cactus is phosphorylated and subsequently degraded by the proteasome. The transcription factors Dif and Dorsal then translocate to the nucleus where they activate expression of their target genes. Figure adapted from (Valanne et al., 2011).

3.4 Tissue-specific regulation of *Drosophila* NF-κB activity

Constitutive activation of the Imd pathway is detrimental to flies, and it is under tight control by several negative regulators that act at different levels of the signalling cascade. Some negative regulators act during basal conditions and others are activated by the Imd pathway providing a negative feedback loop. PGRPs that have catalytic activity, like PGRP-LB and PGRP-SC, degrade PGN to reduce the levels of immunostimulatory compounds. Pirk, which is expressed in response to Imd pathway activation negatively regulates the Imd pathway by acting at the level of Imd and PGRP-LC (Kleino et al., 2008). Plenty of SH3 (POSH) is an E3 ligase that reduces the stability of Tak1. Similarly, Defense repressor 1 (Dnr1) is thought to affect stability of Dredd (K. Aggarwal & Silverman, 2008; Lee & Ferrandon, 2011). Caspar, which is homologous to the human Fas associated factor 1 (FAF1), contains a UBA domain and a DED-interacting region, and blocks Dredd-mediated cleavage of Relish (Kim et al., 2006). Another layer of regulation is provided by compartmentalization of expression of AMPs. The homeobox transcription factor called Caudal is expressed in the posterior midgut and acts by repressing the expression of a subset of NF-κB target genes like AMPs, but not negative regulators like PGRP-LB (Ryu et al., 2008). Recently, Kenny was described to contain a LC3-interacting region (LIR), through which it interacts with the autophagosomal membrane protein microtubule-associated protein 1 light chain 3 (Atg8/LC3) (Tusco et al., 2017). Atg8/LC3 are important for formation of autophagosome and serve as scaffolding proteins to recruit cargo molecules to autophagosomes (Shpilka et al., 2011). It was shown that the IKK complex in the fly is selectively degraded by autophagy via the LIR domain of Kenny, to prevent constitutive activation of the Imd pathway in response to commensal microbiota (Tusco et al., 2017). Another protein that contributes to immune tolerance against commensal bacteria is PGRP-LC-interacting inhibitor of Imd signalling (PIMS). PIMS interacts with PGRP-LC causing its depletion from the plasma membrane, thus shutting down the Imd pathway (Lhocine et al., 2008).

The Toll and Imd pathways can be activated in the fatbody, the functional equivalent of the liver, to mount a systemic immune response resulting in the secretion of a large amount of AMPs in the body cavity. The epithelial tissue, like in the intestine, forms a barrier and protects the host from pathogenic microorganisms, at the same time tolerating beneficial gut microbiota. The peritrophic matrix comprised of chitin polymers and peritrophins makes up a mixed grid-like structure to prevent contact between intestinal epithelium and bacteria, potentially limiting the immune reactivity to commensal bacteria (Hegedus et al., 2008). The local immune response to pathogens in the intestine is under the control of the Imd pathway and not the Toll pathway (Basset et al., 2000; Ferrandon et al., 1998; Liehl et al., 2006). In the gut, the Imd pathway is

activated by PGRP-LC, which is functional in the foregut, anterior midgut and the hindgut, and PGRP-LE which acts in the midgut.

4 Mammalian host defence

Humans encounter a diverse array of microorganisms, both non-pathogenic and pathogenic that pose challenge to our health. The mammalian host defence is comprised of the innate and adaptive immune responses. When microorganisms breach the epithelial surface of the body, the inflammatory cells, neutrophils and macrophages, of the innate immune response are the first line of defence against pathogens. Neutrophils and macrophages contain surface receptors through which they can recognize invading bacteria by binding to constituents of bacterial surfaces. This is followed by phagocytosis of the invading bacteria and the secretion of chemokines and cytokines. Secretion of these biologically active molecules helps recruit other players of the immune system to the site of infection and initiate local inflammation (Medina, 2016). The inflammatory cells express PRRs including toll-like receptors (TLRs), NOD-like receptors (NLRs) and Retinoic acid-inducible gene I (RIG-I)-like receptors, which recognise pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMP) (Takeuchi & Akira, 2010). The innate immune response cannot always eliminate infections and that is when the lymphocytes of the adaptive immune response are activated and help neutralize the threat. Induction of the adaptive immune response starts with phagocytosis of the pathogen by specialized cells called dendritic cells, after which the pathogen is degraded intracellularly. They then carry the antigen from the pathogen to lymphoid organs and present them to lymphocytes. Clonal selection of lymphocytes containing antigen specific receptors forms the basis for characteristics of adaptive immune system to detect all pathogens specifically, neutralize them and remember the antigen to protect against reinfection (Marshall et al., 2018). Lymphocytes that have encountered its antigen then proliferates and differentiate to effector cells that eliminate the pathogen. A subset of these proliferating lymphocytes differentiates into memory cells that can respond rapidly if they encounter the same pathogen again. However, there is a delay of 4-7 days for the adaptive response to take effect and the innate immune system has a critical role during this period (Chaplin, 2010).

4.1 Mammalian NF-κB pathways

The NF-κB proteins were first discovered as a B-cell specific transcription factor that had a sequence-specific DNA binding property. It is now evident that NF-κB is a major regulator of transcriptional responses in almost all cell types in response to a broad range of stimuli (Sen & Baltimore, 1986; Q. Zhang et al., 2017). There are five different NF-κB proteins, p50, p52, Rel A (p65), Rel B, and

c-Rel, all of which contain a conserved Rel-homology domain (RHD) and they can form a variety of homodimers and heterodimers (Ghosh et al., 2003). The RHD is made up of about 300 amino acids performing three functions, sequence specific DNA binding, dimerization and binding to inhibitory proteins. The NF-κB proteins contain a nuclear localisation signal (NLS) following the RHD. The NF-κB proteins can be divided into two classes. First, p50 and p52 are generated from their respective precursor proteins p105 and p100, both of which contain C-terminal ankyrin repeats. Second, Rel-A, Rel-B and C-Rel do not contain ankyrin repeats but possess transcription transactivation domains (TADs) and, therefore the ability to initiate transcription. NF-κB proteins are mainly cytoplasmic due to their binding to the IκB family of proteins, IκBα, IκBβ, IκBε, IκBζ, BCL-3 (B-cell lymphoma 3), and IκBns. The IκB proteins contain multiple ankyrin repeats that can bind to NF-κB and mask their NLS, thereby localising them to the cytoplasm. Activation of NF-κB is achieved by phosphorylation of IκB proteins followed by ubiquitination and proteasome-mediated degradation of IκBs, which releases the NF-κB proteins to translocate to the nucleus where they can regulate transcriptional responses. The p100 and p105 proteins undergo partial proteasomal processing to remove the ankyrin repeats, releasing them for nuclear translocation (Hayden & Ghosh, 2008, 2012).

The NF-κB signalling pathways can be classified into canonical and non-canonical. The canonical pathway (Figure 4) is characterised by the inducible degradation of IκB. The pathway is activated upon recognition of the ligand by receptors like the TNF receptor (TNFR), IL-1R, PRRs such as the Toll-like receptors and antigen receptors (Hayden & Ghosh, 2014; Schulze-Luehrmann & Ghosh, 2006), that initiate formation of a signalling cascade that culminates in activation of the IKK complex by phosphorylation. The IKK complex, comprised of kinases IKKα, IKKβ and the regulatory subunit IKKγ, also called NF-κB essential modulator (NEMO), in-turn phosphorylates IκB to facilitate its ubiquitination and degradation by the proteasome, after which the NF-κB dimers are free to translocate to the nucleus (Wertz & Dixit, 2010).

The non-canonical pathway (Figure 4) is mediated by a subset of the TNFR superfamily members. It relies on the inducible processing of p100 and the nuclear translocation of the RelB/p52 dimer (S. C. Sun, 2017). Important components of the non-canonical pathway include a MAP kinase kinase kinase (MAP3K) member called NF-κB inducing kinase (NIK) and the downstream kinase, IKKα. Under normal conditions, NIK is ubiquitinated and constantly degraded by the proteasome (Liao et al., 2004). Upon receptor activation, ubiquitination and degradation of NIK is hindered, and leads to an accumulation of NIK. NIK then stimulates phosphorylation, ubiquitination and processing of p100, to give rise to p52 by activating the downstream kinase IKKα (Xiao et al., 2001). Upon processing of p100, the RelB/p52 dimer is free to translocate to the nucleus where it can induce expression of target genes.

The NF-κB family of transcription factors are major regulators of host defence and control expression of a number of genes that regulate immune responses, inflammation, cell survival and apoptosis, stress responses, cell growth and proliferation. Dysregulation of the NF-κB pathway can contribute to development of inflammatory, autoimmune, cardiovascular and neurodegenerative diseases (Fiordelisi et al., 2019; Kaltschmidt et al., 2022; Karin, 2006). Inflammation has been recognised as a hallmark of cancer, and plays an important role in development and progression of cancer (Taniguchi & Karin, 2018). As NF-κB is the master regulator of inflammatory responses, their activity must be tightly regulated, as their aberrant activation can have severe consequences for human health. A key feature of the NF-κB pathways is their reliance on formation of ubiquitin chains to transmit the signals from activated receptor to the nucleus for NF-κB mediated target gene expression (Wertz & Dixit, 2010). As ubiquitination fine tunes NF-κB signalling at multiple levels, dysregulation of ubiquitination can lead to diseases of the immune system and inflammatory diseases (Q. Zhang et al., 2017).

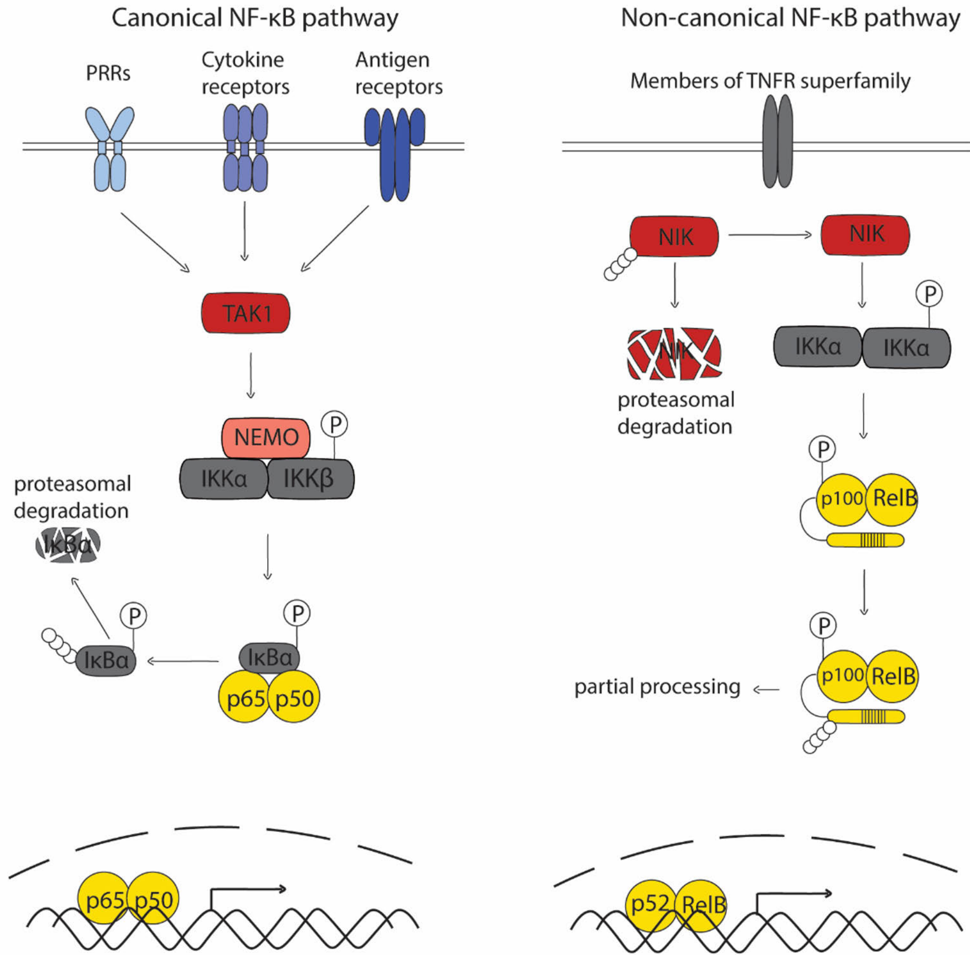


Figure 4. Schematic representation of the NF-κB pathway. The canonical NF-κB pathway, activated by pattern-recognition receptors, cytokine receptors, and antigen receptors culminate in the activation of the IKK complex, comprised of NEMO, IKKα and IKKβ, by phosphorylation. IKK complex further phosphorylates IκBα after which IκBα is degraded by the proteasome, releasing the NF-κB transcription factor dimer to translocate to the nucleus where it activates expression of its target genes. The non-canonical NF-κB pathway, activated by members of the TNFR superfamily results in the stabilisation of NIK, which is otherwise degraded in the proteasome. NIK can then phosphorylate IKKα, which in turn phosphorylates the NF-κB transcription factor p100 resulting in partial processing of p100 to give rise to p52. The p52/RelB dimer then translocates to the nucleus to activate target gene expression. Figure adapted from (Falschlehner & Boutros, 2012).

4.2 The TNFR1 signalling pathway

TNFR1 pathway is induced by its ligand, the tumour necrosis factor α (TNF- α), a master cytokine, which belongs to the TNF superfamily of cytokines. While the default mode of TNFR1 activity is to induce the NF-κB pathway, it also possesses an intrinsic cell death inducing property (Figure 5). It can affect any cell type and can cause a variety of cellular responses like induce the inflammatory NF-κB pathway or activate programmed cell death through apoptosis or necroptosis (B. B. Aggarwal et al., 2012). TNFR1 is a death receptor (DR) and contains a death domain (DD) in its cytoplasmic part, which upon ligand binding, trimerizes and recruits TNFR1-associated death domain (TRADD) and RIPK1 (Hsu et al., 1995; Hsu, Huang, et al., 1996). TRADD further recruits TNF receptor associated factor (TRAF) 2 or 5 to the complex (Hsu, Shu, et al., 1996). TRAF2 in-turn serves as a platform to recruit cellular inhibitor of apoptosis proteins (cIAPs) cIAP1 and cIAP2, followed by recruitment of the Linear ubiquitin chain assembly complex LUBAC (Haas et al., 2009; Rothe et al., 1995). TNFR1 signalling activates NF-κB pathway by parallel recruitment of two kinase complexes, comprising of the TAK1-TAB2 and, the IKK complex (Kanayama et al., 2004; Rahighi et al., 2009). TAK1 then activates the IKK complex by phosphorylation (Wang et al., 2001). Activation of IKK complex leads to phosphorylation and degradation of IκB, which releases the NF-κB dimer for nuclear translocation and transcription of its target genes. This membrane bound complex described above is referred to as complex I of the TNFRI pathway, which activates the NF-κB pathway. But, upon incomplete ubiquitination of RIPK1, it can lead to the assembly of a cytoplasmic complex II resulting in apoptosis or necroptosis (Annibaldi et al., 2018; Moquin et al., 2013; Tu et al., 2021). Incompletely ubiquitinated or unmodified RIPK1 dissociates from complex I and associates with FADD through interaction of their DDs (Annibaldi & Meier, 2018). FADD further recruits pro-caspase-8 or its catalytically inactive homologue FLICE-like inhibitory protein (FLIP) through interaction of their DEDs, to form a death platform, where caspase-8 gets activated to initiate apoptosis. Improper activation of caspase-8 in complex II can result in autophosphorylation of RIPK1 and recruitment of RIPK3 through their RIP homology interaction motifs (RHIMs) (X. Sun et al., 2002). RIPK3 undergoes autophosphorylation and further phosphorylates mixed lineage kinase domain-like protein (MLKL) (Murphy et al., 2013). Activated MLKL then translocates to the plasma membrane where it binds phosphoinositides to cause membrane permeabilization (Dovey et al., 2018; Quarato et al., 2016; Witt & Vucic, 2017).

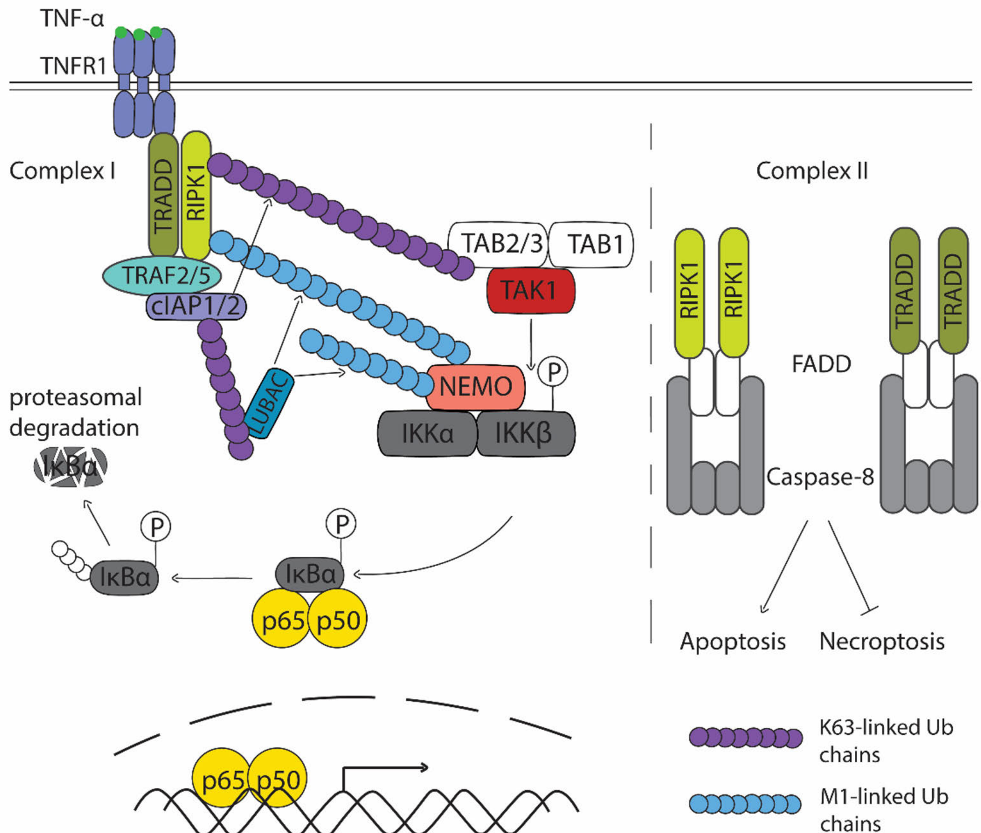


Figure 5. Schematic representation of the TNFR1 signalling pathway. The TNFR1 signalling is activated upon binding of TNF-α to TNFR1. Complex I of the TNFR1 is formed upon recruitment of TRADD, RIPK1, TRAF2/5. cIAP1/2 is recruited to the complex via TRAF2. cIAP1/2 ubiquitinates mediators of the pathway leading to Ub dependent recruitment of the TAK1/TAB2/3, LUBAC and IKK complex. LUBAC ubiquitinates mediators of the pathway and further promotes recruitment of the IKK complex. Activation of the IKK complex induces the phosphorylation and subsequent degradation of IκB, thus leading to activation of the NF-κB pathway. Incomplete ubiquitination of RIPK1 or absence of NF- κB activation leads to formation of complex II, comprising of RIPK1, FADD and caspase-8 or TRADD, FADD and caspase-8, inducing apoptosis by the extrinsic pathway, while inhibition of caspase-8 activity induces necroptotic cell death. Figure adapted from (Gyrd-Hansen & Meier, 2010).

4.3 The NOD2 signalling pathway

NOD2 is a member of NOD, leucine-rich repeat (LRR)- containing protein (NLR). It is an intracellular PRR that recognizes muramyldipeptide (MDP), a component of peptidoglycan from Gram-negative and Gram-positive bacteria (Girardin et al.,

2003). Mutations in NOD2 receptors are associated with an increased risk of Crohn's disease and colorectal cancer (Al Nabhani et al., 2017; Ferrand et al., 2019). NOD2 contains a NOD domain through which NOD2 oligomerizes upon detection of MDP through its LRR domain, which is a microbe-associated molecular pattern recognition region, similar to LRR domain of TLRs. They also possess a caspase recruitment domain (CARD) through which they recruit the CARD containing adaptor RIPK2 (J.-H. Park et al., 2007). Upon recruitment, RIPK2 undergoes autophosphorylation and ubiquitination (Hasegawa et al., 2008; Pellegrini et al., 2017). RIPK2 has been associated with a number E3 ligases like cIAP1,2, X-chromosome-linked (XIAP), TRAF2,5, and 6. The ubiquitin chains on RIPK2 is believed to function as scaffold to recruit the kinase complexes TAK1, TAB1, TAB2/3 and the IKK complex. Activation of the IKK complex results in phosphorylation and proteasomal degradation of IκB and subsequent nuclear translocation of NF-κB dimer followed by expression of its target genes (Caruso et al., 2014).

4.4 Linear ubiquitin chain assembly complex (LUBAC)

LUBAC is a 600kDa complex, composed of three subunits, Heme-oxidized IRP2 Ub ligase-1 (HOIL-1), HOIL-1-interacting protein (HOIP) and Shank-associated RH domain-interacting protein (SHARPIN) (Figure 6) (Kirisako et al., 2006). It is the only known mammalian E3 ligase that can generate M1-linked ubiquitin chains. Both HOIP and HOIL-1 belong to the family of RBR-type E3 ligases. Even though HOIL-1 has E3 ligase activity, HOIP is considered as the catalytic subunit of LUBAC. HOIP functions by combining the mechanism of its RBR domain together with the linear ubiquitin chain determining domain (LDD)-dependent specificity to generate M1-linked Ub chains (Smit et al., 2012). HOIP is in an autoinhibited state due to the interaction between the N-terminal region, containing ubiquitin-associated (UBA) domain, and the RBR domain. HOIL-1 and SHARPIN function as co-factors to enable E3 ligase activity of HOIP (Stieglitz et al., 2012). The UBA domain of HOIP interacts with the ubiquitin-like (UBL) domain of HOIL-1, and the HOIP UBA and Npl4-type zinc finger (NZF) domains interact with the UBL domain of SHARPIN to form this trimeric complex. LUBAC is a core component of the NF-κB signalling pathways activated by cytokine receptors, Toll-like receptors (TLRs), NLRs, and antigen receptors. Activation of these receptors leads to recruitment of receptor-associated adaptor proteins like RIPK1 to TNFR1 and RIPK2 to NOD2, followed by the recruitment of E3 ligases like cIAP1/2, XIAP and Pellino3. K63-linked Ub chains are conjugated to components of this complex which facilitates recruitment of LUBAC through its NZF domains (Haas et al., 2009). Once recruited LUBAC conjugates M1-linked Ub chains on components of the signalling pathway, like RIPK1 in the TNFR1 and RIPK2 in the NOD2 signalling pathways (Damgaard et al., 2012; Draber et al., 2015; Fiil et al., 2013). Interestingly, NEMO, which has been shown to bind M1-

linked Ub chains has also been shown to be a substrate for LUBAC mediated M1-ubiquitination (Tokunaga et al., 2009). Furthermore, it has been shown that LUBAC preferentially generates M1-linked Ub chains on existing K63-linked Ub chains to form mixed/hybrid K63/M1-linked Ub chains (Emmerich et al., 2013, 2016; Hrdinka et al., 2016). These mixed K63/M1-linked Ub chains could colocalize the TAK1-TAB2/3 and the IKK complex to the same polyubiquitin chain, facilitating activation of IKKα and IKKβ by TAK1. Loss of LUBAC and M1-linked Ub chains can lead to aberrant activation of the TNFR1 pathway leading to cell death caused by increased formation of apoptosis-inducing complex II (Peltzer et al., 2014, 2018). Loss of the LUBAC component SHARPIN, which affects LUBAC function is known to cause chronic proliferative dermatitis (cpdm) in mice (Gerlach et al., 2011; Seymour et al., 2007; Tokunaga et al., 2011). HOIL-1 and HOIP mutations are reported to contribute to immunodeficiency and autoinflammation in humans (Boisson et al., 2012, 2015).

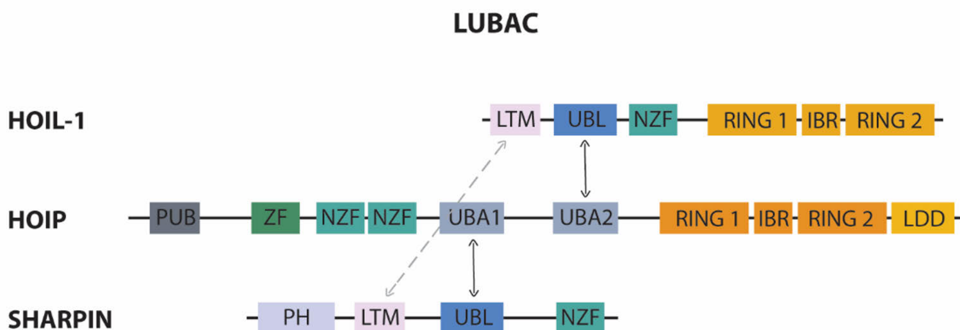


Figure 6. Schematic representation of the E3 ligase complex LUBAC. LUBAC is comprised of HOIL-1, HOIP and SHARPIN. Interactions between the subunits are mediated by LUBAC-tethering motif (LTM), UBL and UBA domains. NZF, RBR-LDD, ZF, PUB, Pleckstrin-homology (PH) are other domains of LUBAC. Figure adapted from (Fiil & Gyrd-Hansen, 2021).

Other targets of LUBAC-mediated M1-ubiquitination include MyD88, IRAK1/4, cellular FLIP (c-FLIP), and the initiator caspase, caspase-8. c-FLIP is a catalytically inactive homologue of caspase-8, and it was shown that LUBAC conjugates M1-linked Ub chains on c-FLIP during TNF signalling, which protects c-FLIP from proteasome-mediated degradation (Tang et al., 2018). c-FLIP is an anti-apoptotic protein that can form a complex with caspase-8, suppressing its catalytic activity and apoptosis. During TNF-related apoptosis-inducing ligand (TRAIL) signalling, it was shown that LUBAC conjugates M1-linked Ub chains on RIPK1 and caspase-8, facilitating the recruitment of IKK complex to the TRAIL

signalling complex, thereby promoting NF-κB activation and the production of cytokines (Lafont, Kantari-Mimoun, et al., 2017). It was suggested that HOIP suppressed the activity of caspase-8, but it is not known if this is a direct result of M1-ubiquitination of caspase-8.

A homologue of HOIP in the fly, called Linear ubiquitin E3 ligase (LUBEL), was described to catalyse the generation of M1-linked Ub chains *in vitro* (Asaoka et al., 2016), but its involvement in the *Drosophila* NF-κB pathway and the substrates that are ubiquitinated with M1-linked Ub chains was not previously described. The *Drosophila* DUB, called CYLD that was suggested to be a negative regulator of the Imd pathway, can interact with Kenny (Tsichritzis et al., 2007) and LUBEL (Asaoka et al., 2016). Similar to mammalian CYLD, it was also shown to hydrolyse both K63- and M1-linked Ub chains (Asaoka et al., 2016).

5 Ubiquitination-mediated regulation of NF-κB pathway

Distinct Ub chains can have distinct Ub chain conformations. K48, K6 and K11 Ub chains adopt a compact conformation, while M1- and K63-linked Ub chains adopt an open conformation (Akutsu et al., 2016; Ye et al., 2012). The message of the Ub chains is decoded by Ub receptors, which contain Ub binding domains (UBDs), that recognize and bind specific surface patches in Ub chains (Husnjak & Dikic, 2012). Ubiquitin receptors or UBD containing proteins vary widely in size, amino acid sequence and three-dimensional structure. By using the topology of ubiquitin chains, they are able to discriminate between ubiquitinated substrates. Hence, the UBDs of proteins can preferentially bind to Ub chains of specific linkage, which is utilized by the cells to regulate signalling pathways (Husnjak & Dikic, 2012). The conjugation of Ub chains can have different effects on the fate of the substrate, depending on the type of Ub chain linkage.

In the *Drosophila* Toll pathway, Cactus, the IκB-like protein that sequesters Drosal and Dif in the cytoplasm is known to be K48-ubiquitinated, resulting in its degradation, mediated by the proteasome. In the canonical NF-κB pathway, after the IκB proteins get phosphorylated by the IKK complex, they are recognized by the βTrCP E3 complex and conjugated with K48-linked Ub chains. The UBD of a receptor in the 26S proteasome binds K48-linked Ub chains, and the IκB proteins are subsequently degraded by the proteasome (Kanarek et al., 2010). In the non-canonical NF-κB pathway, cIAPs conjugate K48-linked Ub chains on TRAF3, which targets them for proteasomal degradation. This in turn hinders TRAF3-mediated K48-ubiquitination and degradation of NIK, resulting in NIK stabilization (Liao et al., 2004; Xiao et al., 2001). Similar to IκB, the p100 proteins also undergo K48-ubiquitination by the βTrCP E3 complex, but they are only targeted for partial proteasomal processing to form mature p52.

5.1 Non-degradative Ub chains promote NF-κB activation

While the K48-Ub chains usually target proteins for proteasome-mediated degradation, K63- and M1-linked Ub chains have non-degradative functions by serving as recruitment platforms for proteins with UBDs. Upon activation of the NF-κB pathway, receptor associated adaptor proteins are decorated with K63- and M1-linked Ub chains, which are bound by ubiquitin receptor proteins like the TAB/TAK and the IKK complex, which are key components of both the mammalian and *Drosophila* NF-κB pathways (Kleino & Silverman, 2014; Rutschmann et al., 2000; Silverman et al., 2000). The TAK1-TAB2/3 complex comprises of the kinase TAK1 and its accessory protein TAB2 or TAB3. Both TAB2 and TAB3 contain a N-terminal coupling of ubiquitin conjugation to endoplasmic reticulum degradation (CUE) domain, a C-terminal NZF ubiquitin binding domain, a coiled coil (CC) and a TAK1 binding domain. The NZF of TAB2 was shown to bind K63-linked Ub chains and it is thought that this binding mediates oligomerization and autophosphorylation of TAK1, after which TAK1 is able to phosphorylate and activate the IKK complex (Kanayama et al., 2004; Kulathu et al., 2009; Skaug et al., 2009). The IKK complex is comprised of the kinase IKKα, IKKβ and the regulatory subunit IKKγ, otherwise called NEMO. NEMO contains two coiled coil regions, CC1 and CC2, followed by a leucine zipper (LZ) and a zing finger (ZF). The CC2 and LZ domains, together termed as the coil-zipper (CoZi) region has been shown to be responsible for binding to Ub chains. The ubiquitin binding in ABIN and NEMO proteins (UBAN) domain, located within the CoZi region, has homology to other ubiquitin binding proteins like A20-binding inhibitor of NF-κB (ABIN) and optic neuropathy inducing (optineurin). Even though it was previously thought that NEMO bound K63-linked Ub chains, it was later shown that the CoZi region of NEMO binds M1-linked Ub chains with a 100-fold higher affinity than K63-linked Ub chains (Lo et al., 2009; Rahighi et al., 2009).

In the *Drosophila* Imd pathway, Dredd is targeted by Diap2 for K63-ubiquitination mediated by its RING-domain. After K63-ubiquitination, Dredd cleaves Imd on its N-terminus that harbours a caspase cleavage site (LEKD), which exposes a consensus IAP-binding motif (IBM) (AAPV). The baculovirus IAP repeat (BIR) 1 and 2 domains of Diap2 can then interact with IBM of Imd, after which Diap2 targets Imd with K63-linked Ub chains (Meinander et al., 2012; Paquette et al., 2010). The K63-linked Ub chains attached to Imd and Dredd are believed to recruit dTab2/dTak1 complex and the IKK complex via their ubiquitin binding properties (Kleino & Silverman, 2014). In addition, the cleavage of Relish by Dredd was shown to be dependent on Diap2 mediated K63-ubiquitination of Dredd, although how ubiquitination affects the catalytic activity of Dredd is not known. The involvement of M1-linked Ub chains in the Imd pathway and whether Kenny is recruited to the Imd signalling complex via binding to M1-linked Ub chains has been studied in this thesis project.

Mammalian LUBAC is recruited to the TNFR1 signalling complex by the action of E3 ligases TRAF2, cIAP1, and cIAP2 that modify themselves and RIPK1 with K63-linked ubiquitin chains (Varfolomeev et al., 2008). These K63-linked Ub chains serve as recruitment platform for LUBAC (Haas et al., 2009). Addition of M1-linked Ub chains, mediated by LUBAC, on its targets RIPK1 and NEMO was suggested to stabilize the signalling complex by retaining the mediators, resulting in full activation of downstream signalling (Draber et al., 2015; Tokunaga et al., 2009). Similarly, in the NOD2 pathway, K63-ubiquitination of RIPK2 by XIAP2 is an important signalling event as it is required for recruitment of LUBAC, which ubiquitinates RIPK2 with M1-linked Ub chains (Damgaard et al., 2012; Fiil et al., 2013). Together, the K63- and M1-linked Ub chains function as recruitment platforms for the TAK1-TAB2/3 complex and the IKK complex. Recruitment of the TAK1-TAB2/TAB3 complex and the IKK complex by K63- and M1-linked Ub chains respectively brings them in close proximity and enables sequential phosphorylation and activation of these kinase complexes, finally resulting in the phosphorylation, K48-ubiquitination and proteasome-mediated degradation of IκB (Emmerich et al., 2013, 2016). Importantly, the recognition and binding of NEMO to M1-linked Ub chains is required for activation of the NF-κB pathway (Tokunaga et al., 2009).

5.2 Regulation of NF-κB activity by deubiquitinases (DUBs)

Ubiquitination is a reversible process, and this reversal is performed by enzymes called deubiquitinases (DUBs). While the E1s, E2s and E3s combine to add ubiquitin moieties to a target protein, DUBs are ubiquitin proteases, responsible for breaking down ubiquitin chains by removing the ubiquitin moieties from target proteins or polyubiquitin chains. It is estimated that the human genome codes for around 95 DUBs (Nijman et al., 2005). Several DUBs, like A20, the ovarian tumour protease (OTU) deubiquitinase with linear linkage specificity (OTULIN) and the cylindromatosis tumour suppressor (CYLD) regulate NF-κB signalling by modulating Ub chains. A20, which contains seven zinc fingers and an OTU-type DUB domain, is an important negative regulator of NF-κB signalling, as inactivation of the A20 gene is associated with inflammatory diseases (Catrysse et al., 2014; Musone et al., 2008). A20 can bind to M1-and K63-linked Ub chains through its Zinc finger (ZnF) 7 and 4 respectively (Bosanac et al., 2010; Tokunaga et al., 2012). Additionally, A20 hydrolyses K63-linked Ub chains but stabilizes M1-linked Ub chains (Mevissen et al., 2013). It has been suggested that A20 suppresses NF-κB activation by competing with other activators of NF-κB, like the IKK complex for binding to M1-linked Ub chains (Draber et al., 2015; Skaug et al., 2011).

LUBAC associates with two DUBs, OTULIN and CYLD, and exits in the cell as two fractions, one that associates exclusively with CYLD and another with OTULIN (Draber et al., 2015; Elliott et al., 2016; Hrdinka & Gyrd-Hansen, 2017).

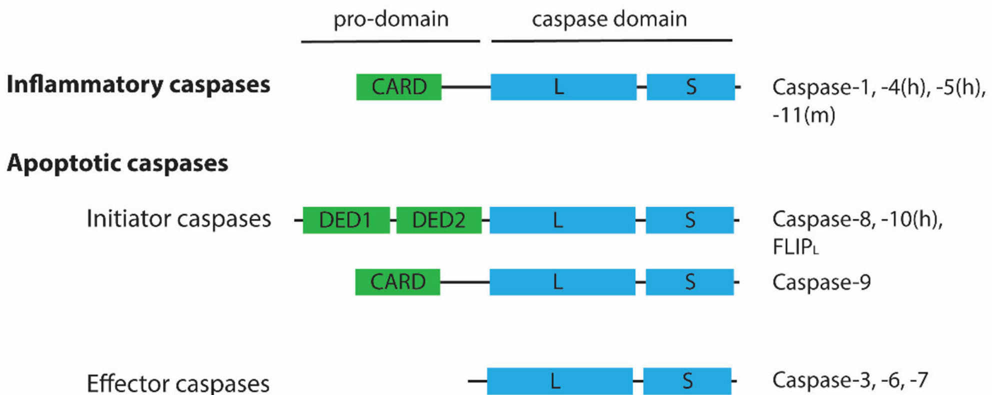
OTULIN contains a OTU domain and a peptide:N-glycanase/UBA- or UBX-containing proteins (PUB)-interacting motif (PIM), through which it interacts with the PUB domain of HOIP (Elliott et al., 2014; Schaeffer et al., 2014). The cylindromatosis tumour suppressor CYLD is a Ub-specific protease (USP)-type DUB that can hydrolyse both K63- and M1-linked Ub chains (Hrdinka et al., 2016). CYLD interacts with the PUB domain of HOIP indirectly through a bridging protein called Spermatogenesis-associated 2 (SPATA2) (Elliott et al., 2016). OTULIN restricts M1-ubiquitination of both LUBAC targets and autoubiquitination of LUBAC components (Heger et al., 2018). In contrast to OTULIN, CYLD is co-recruited along with LUBAC to the TNF and NOD2 signalling complex, where CYLD possibly functions by trimming K63-linked Ub chains, which is a target for LUBAC-mediated ubiquitination to generate mixed K63/M1-linked Ub chains (Hrdinka et al., 2016). Mutations in DUBs CYLD and OTULIN are known to cause cylindromatosis (Bignell et al., 2000; J. Zhang et al., 2006), characterized by development of benign tumours in the skin, and OTULIN-related inflammatory syndrome (ORAS) or otulipenia, characterized by severe inflammation of the skin and joints respectively (Damgaard et al., 2016; Zhou et al., 2016).

6 Caspases

Caspases are evolutionarily conserved cysteine-aspartic proteases that have important functions in regulating programmed cell death and inflammation. The function of caspases in cell death was discovered in *Caenorhabditis elegans* (Ellis & Horvitz, 1986). Caspases hydrolyse peptide bonds mediated by the catalytic cysteine residue in its active site and cleaves after certain aspartic acid residues on its substrates. Caspases contain a prodomain followed by a caspase domain comprised of large and small catalytic subunits (Figure 7) (Lamkanfi et al., 2002). They are produced as inactive zymogens, and often require dimerization and cleavage for activation. Dimerization depends on specific interaction regions in the prodomain of caspases that can bind to various adaptor proteins, which recruit caspases to signalling complexes. Mammalian caspases can be classified into inflammatory (caspase-1, -4, -5, -11) and apoptotic caspases. Depending on the presence of interaction domains at their N-terminus, the apoptotic caspases can be sub-divided into initiator (caspase-8, -9, -10) and effector caspases (caspase-3, -6, -7). Initiator caspases possess a death effector domain (DED) or a caspase recruitment domain (CARD) that mediate their dimerization and/or recruitment to signalling complexes where they can proteolytically cleave their substrates (Van Opdenbosch & Lamkanfi, 2019). The activation of the initiator caspase zymogen to active protease is induced by a process called proximity-induced autoactivation, where dimerization of the caspase zymogen induces a conformation change that leads to proteolytic cleavage of the flexible linker region between the prodomain and

the caspase domain (Salvesen & Dixit, 1999). Effector caspases on the other hand do not possess a prodomain or have a very short prodomain. They exist as inactive homodimers and get activated upon cleavage of the linker region between their large and small catalytic domains by activated initiator or effector caspases (Ramirez & Salvesen, 2018).

In *Drosophila*, three caspases, Dredd, Death regulator Nedd2-like caspase (Dronc) and Ser/Thr-rich caspase (Strica), which are similar to mammalian initiator caspases, and four caspases, *Drosophila* interleukin 1β-converting enzyme (Drice), Death-associated molecule related to Mch2 (Damm), Death executioner caspase related to apopain/yama (Decay) and *Drosophila* effector caspase-1 (Dcp-1), which are similar to mammalian effector caspases have been identified (Figure 7) (Kumar & Doumanis, 2000; Lamkanfi et al., 2002). Dronc is the only fly caspase that contains a CARD. Dronc is recruited to the apoptosome by homotypic CARD interaction to the adaptor protein Death-associated APAF1-related killer (Dark), which is similar to the recruitment of mammalian caspase-9 to Adaptor protein apoptotic protease activating factor-1 (APAF-1) (X. Yu et al., 2006). After activation of Dronc in the apoptosome, it proteolytically cleaves and activates effector caspases Drice and Dcp-1, which cleaves downstream targets to execute apoptosis. Dredd contains two DEDs in its prodomain, which are important for its recruitment to signalling complexes to conduct its catalytic activity. While Dredd has been described to have functions during apoptosis (P. Chen et al., 1998), it has also been shown to be an important mediator of the inflammatory NF-κB pathway (Leulier et al., 2000). The DED1 of Dredd has been shown to be important for binding to *Drosophila* Fadd (dFadd) and Diap2 upon activation of the Imd pathway, although the interaction between Dredd and Diap2 is not through IBM (Hu & Yang, 2000; Meinander et al., 2012). Strica contains a unique serine/threonine rich prodomain, however not much is known about the functions of Strica, Damm and Decay.



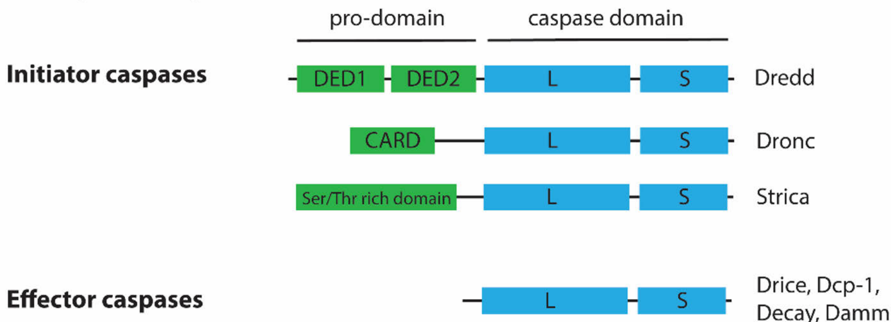


Figure 7. Domain composition of mammalian and *Drosophila* caspases. Mammalian inflammatory and initiator caspases contain a prodomain, comprised of recruitment domains like the DED or CARD, and a caspase domain comprised of a large (L)(~20kDa) and a small subunit (S)(~10kDa). Effector caspases contain a very short prodomain or do not contain a recruitment domain. m stands for murine and h for human. The *Drosophila* initiator caspases Dredd and Dronc have long prodomains containing two DEDs and a CARD respectively. Strica is a atypical caspase harbouring a Ser/Thr rich prodomain. The effector caspases Drice, Dcp-1, Decay and Damm have short prodomains that does not contain any recruitment domains. Figure adapted from (Kumar & Doumanis, 2000; Van Opdenbosch & Lamkanfi, 2019).

6.1 Caspase activity at crossroads of inflammation and cell death

Caspases are responsible for the induction of programmed cell death by apoptosis and pyroptosis (Taylor et al., 2008; Vande Walle & Lamkanfi, 2016). Apoptosis is a non-lytic mode of cell death, and as the apoptotic bodies are efficiently removed, they do not release damage-associated molecular patterns (DAMPs) and thus do not result in recruitment of inflammatory cells. Apoptosis

can be induced via the intrinsic pathway by UV- or γ-irradiation, DNA damage, chemotherapeutic drugs, and cytokine deprivation, or via extrinsic pathways by signalling through death receptors (DR). Some examples of the DR family include TNFR1, CD95, DR3, TRAILR-1, -2, and DR6. Signalling through DRs leads to the formation of the Death-inducing signalling complex (DISC) consisting of FADD, caspase-8 and c-FLIP, after which caspase-8 gets activated, initiating the pro-apoptotic caspase cascade (Taylor et al., 2008). However, inhibition of caspase activity upon activation of the extrinsic mode of cell death usually does not inhibit cell death but leads to a lytic mode of cell death, necroptosis (Holler et al., 2000). Pyroptosis is another major lytic mode of cell death mediated by inflammatory caspases, and is associated with the release of inflammatory cytokines IL-1β and IL-18. While apoptosis is considered to be immunologically silent, necroptosis and pyroptosis are inflammatory in nature. Dysregulated caspases have been associated with diseases involving tumorigenesis, autoimmunity, and autoinflammation (McIlwain et al., 2013).

Even though the DRs are well known for their role in induction of apoptosis, they can also activate inflammatory pathways, namely the NF-κB pathway (Cullen & Martin, 2015; Lavrik et al., 2007). During TRAIL-R1 signalling, it was shown that a complex comprised of FADD, caspase-8/-10 and RIPK1 is formed, which led to the recruitment of the IKK complex and subsequent NF-κB activation. In addition, caspase-8 was shown to play a non-enzymatic, scaffolding role for the assembly of this complex of proteins (Henry & Martin, 2017). In another study, it was shown that during TRAIL-R1 signalling, LUBAC was recruited to the TRAIL-R associated complex I where LUBAC targets caspase-8 for M1-ubiquitination to suppress its catalytic activity and facilitating recruitment of the IKK complex and NF-κB activation (Lafont, Kantari-Mimoun, et al., 2017).

6.2 Regulators of caspases

As dysregulated apoptosis and caspase activity can be detrimental to health, the activity of caspases needs to be tightly regulated. Caspase activity is regulated by c-FLIP and members of the inhibitor of apoptosis (IAPs) like c-IAP1 and -2 and XIAP that can directly bind to inhibit caspase-3, -7 and – 9, and inhibit their activity.

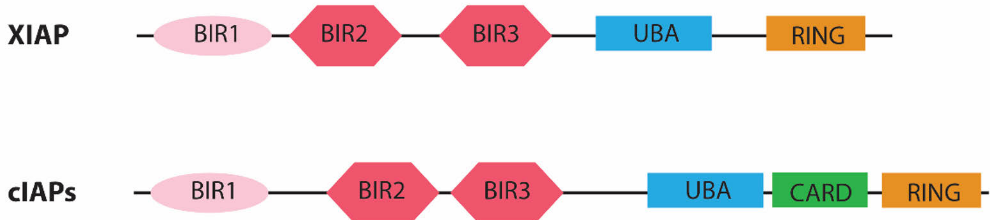
6.2.1 Inhibitor of apoptosis (IAP) proteins

IAP proteins, as their name suggests, are potent inhibitors of apoptosis and they act by modulating the activity of caspases. IAPs are also important mediators of signalling cascades during innate immune and NF-κB responses (Gyrd-Hansen & Meier, 2010). IAPs were first identified in baculoviruses for their ability to inhibit apoptosis (Bump et al., 1995). There are eight IAP members in mammals including X-linked IAP (XIAP), cellular IAP1 (cIAP1) and

cellular IAP2 (cIAP2). Four IAP proteins have been identified in flies, namely, *Drosophila* iap1 (Diap1), *Drosophila* iap2 (Diap2), Deterin and *Drosophila* BIR repeat containing ubiquitin-conjugating enzyme (dBruce). IAPs are characterized by the presence of interaction domains called BIR domains. XIAP, cIAP1, and cIAP2 contain three BIR domains, UBA and a RING domain. Diap1 contains two BIR domains and a RING domain, whereas Diap2 contains three BIR domains, a UBA domain and a RING domain, and is the closest homologue to mammalian IAPs on the basis of domain architecture (Figure 8). BIR domain containing IAPs can associate with proteins that possess IAP-binding motif (IBM) (Z. Liu et al., 2000; Srinivasula et al., 2001). IBMs can be found in proteins like the mammalian caspase-3, -7, -9 and second mitochondrial-derived activator of caspases (SMAC), the *Drosophila* Imd, the *Drosophila* caspases Drice and Dcp-1, and apoptotic proteins Head involution defective (Hid), Grim and Reaper (Scott et al., 2005; Vaux & Silke, 2003; Wilson et al., 2002; Yan et al., 2004). XIAP has been shown to bind to caspase-3 and caspase-7 via BIR2, and caspase-9 via BIR3 and inhibit their catalytic activity. cIAP1 and cIAP2 contain a CARD domain, which generally functions as an interaction surface. However, it was shown that CARD mediates autoinhibition of E3 ligase activity of cIAP1 by keeping it in a closed configuration and preventing dimerization of its RING domain (Lopez et al., 2011). In flies, Diap1 was shown to bind to Dronc, Dcp-1, and Drice and ubiquitinate them to inhibit their catalytic activity (Ditzel et al., 2008; Wilson et al., 2002). While Diap1 is the key inhibitor of apoptosis protein in the fly, Diap2 has its main function as a mediator of the NF-κB pathway (Goyal et al., 2000; Leulier et al., 2006).

Diap2 can also inhibit Drice by a mechanism that resembles caspase inhibition by the viral 'suicide substrate', the baculovirus protein p35. Diap2 and Drice form a covalent adduct between the catalytic cysteine (C211) of Drice and aspartic acid (D100) of Diap2, and through the binding between IBM of Drice and BIR3 of Diap2 (Ribeiro et al., 2007). In addition, Diap2 is cleaved and Drice is ubiquitinated as a consequence of their interaction but how this interaction affects the activity and function of these proteins is not known. Furthermore, IAPs possess E3 ligase activity via the RING domain, and can interact with ubiquitinated proteins via the UBA domain. IAPs have been shown to induce autoubiquitination and ubiquitination of their binding partners that target the substrate proteins with both degradative and non-degradative ubiquitin chains (Bertrand et al., 2008; Huang et al., 2000). Diap2 was shown to be important for expression of NF-κB target genes upon infection by Gram-negative bacteria (Leulier et al., 2006). Importantly, it was shown that Diap2 functions as a E3 ligase to target Imd and Dredd with K63-linked Ub chains (Meinander et al., 2012; Paquette et al., 2010).

Mammalian IAPs



Drosophila IAPs

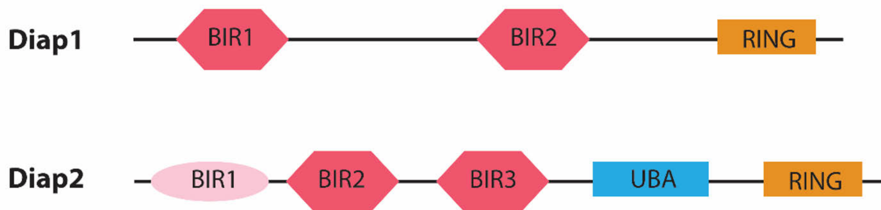


Figure 8. Domain composition of mammalian IAPs XIAP, cIAP1/2 and *Drosophila* IAPs, Diap1 and Diap2. Mammalian IAPs, XIAP and cIAPs contain three BIR domains, a UBA domain and a RING domain. In addition, cIAPs contain a CARD which normally functions as a recruitment domain, but whose function is not known in cIAPs. While Diap1 contains two BIR domains, Diap2 contains three. Both Diap1 and Diap2 have a RING domain. In addition, Diap2 contains a UBA domain. Figure adapted from (Gyrd-Hansen & Meier, 2010).

6.2.2 FLIPs

FLIPs are DED containing proteins that were first discovered in virus (v-FLIP) as inhibitors of apoptosis induced by death receptors (Thome et al., 1997). It was later described that human cellular FLIP (c-FLIP) in addition to having anti-apoptotic property, also had an important role in activating NF-κB signalling (Golks et al., 2006; Irmler et al., 1997; Kataoka et al., 2000; Krueger et al., 2001). While v-FLIPs contain only the two DEDs, c-FLIPs exist in three isoforms, c-FLIP long (c-FLIP_L), c-FLIP short (c-FLIP_S) and c-FLIP Raji (c-FLIP_R). While all c-FLIP isoforms contain two DEDs which are structurally similar to DEDs in the prodomain of caspase-8, only c-FLIP_L contains the catalytic domain. But c-FLIP_L does not possess catalytic activity due to several amino acid substitutions, hence functioning as a pseudo-caspase (Öztürk et al., 2012). c-

FLIPs block apoptosis by their recruitment to the DISC via DED interactions and inhibit caspase-8 processing and activation. On the other hand, they were also observed to facilitate activation of caspase-8 at the DISC (Chang et al., 2002). Importantly, it was described that c-FLIP is a target of M1-ubiquitination mediated by LUBAC. It was found that M1-ubiquitination of c-FLIP protects it from proteasomal degradation, stabilizing c-FLIP, thereby protecting the cells from TNF-α induced apoptosis (Tang et al., 2018). Apart from regulating apoptosis, both c-FLIPs and v-FLIPs also regulate NF-κB activation by DED-mediated binding to NEMO (Bagnéris et al., 2022; Golks et al., 2006; Neumann et al., 2010).

6.2.3 Other regulators of caspases

The first caspase inhibitors to be identified were viral gene products, cytokine response modifier A (CrmA) and p35. CrmA was first shown to inhibit caspase-1 activity and prevent the production of active cytokines IL-1β and IL-18 (Ray et al., 1992). However, it was later shown that CrmA inhibits apoptosis induced by DRs, by inhibiting caspase-8 (Miura et al., 1995; Tewari & Dixit, 1995). It is thought that while some CrmA molecules can be cleaved and released, other CrmA molecules form an irreversible stable complex with its substrate caspase, thereby inhibiting them. p35, another viral gene product was identified in a virus that affects moths (Clem et al., 1991). Similar to CrmA, p35 can also block apoptosis induced by viral infection. It has been shown that p35 can inhibit mammalian caspase-1, -3, -6, -7, -8, and -10. p35 inhibits substrate caspases by functioning as a suicide substrate. Once p35 get cleaved by the caspase, it forms a covalent linkage with the active site and does not dissociate from the caspase (Best, 2008). Ectopic expression of p35 and CrmA, along with synthetic caspase inhibitors like Z-VAD-FMK, Z-DEVD-FMK, Emricasan and others have been used in studies to understand the functions of caspases.

OUTLINE AND KEY AIMS OF THESIS

The NF-κB family of transcription factors are master regulators of the inflammatory responses and their activity is regulated by a highly conserved post-translational modification called ubiquitination. We used the fruit fly *Drosophila* as our model to further our understanding of the molecular mechanisms of regulation of the NF-κB pathway. The fly is a suitable model for this research, as the NF-κB signalling mechanisms are conserved from flies to humans, and as *Drosophila* provides excellent genetic and molecular tools needed to investigate NF-κB and innate immune responses.

This thesis aims to elucidate the role of an effector caspase, called Drice and an initiator caspase, called Dredd, in regulating the *Drosophila* NF-κB pathway. While K63-linked Ub chains have previously been shown to be involved in regulating the *Drosophila* NF-κB pathway, the role of M1-linked Ub chains has not been described. We investigated how the function of Diap2, the E3 ligase that conjugates K63-linked Ub chains upon activation of the *Drosophila* Imd pathway, is regulated by Drice. Furthermore, we identified LUBEL as the M1-linked Ub chain generating machinery in *Drosophila* and aimed to uncover the role of LUBEL and M1-linked Ub chains in regulating the *Drosophila* NF-κB pathway by studying how it affects the functions of Dredd and the IKK complex.

Key aims of this thesis

- To understand how the E3 ligase Diap2 is regulated in the *Drosophila* Imd pathway.
- To describe the role of the effector caspase, Drice as a regulator of the *Drosophila* Imd pathway.
- To identify the M1-linked Ub chain machinery in *Drosophila.*
- To identify mediators of the *Drosophila* Imd pathway that are targeted for M1-ubiquitination.
- To describe the functional consequence of M1-ubiquitination of the caspase Dredd and the IKK complex in the Imd pathway.

EXPERIMENTAL PROCEDURES

The experimental procedures used in this thesis work are presented briefly in this section. More detailed information can be found in the original publications.

Table 1. Experimental procedures used in this thesis work.

Experimental procedure	**Study**
16S rRNA sequencing	I
Axenic flies	I
Cell culture	I, II, III
Computational modelling	II, III
Drosophila dissections	I, II, III
Drosophila maintenance and crossing	I, II, III
Generate transgenic flies	I, II
Image analysis	I, II
Immunofluorescence	I, II
Immunoprecipitation	I, II, III
Infection and survival assays	I, II, III
In vitro ubiquitination assay	II
Light microscopy	I, II
Pathogen clearance assay	I, II
Polymerase chain reaction (PCR)	I, II
Purification of recombinant proteins	I, II, III
Purification of ubiquitin chains	I, II, III
Quantitative reverse transcriptase PCR (qPCR)	I, II
SDS-PAGE and Western blotting	I, II, III
Statistical analysis	I, II
Transfection	I, II, III
Ubiquitin chain restriction (UbiCRest) analysis	II
X-gal staining	I, II

1 Fly husbandry

Drosophila melanogaster were maintained at 25 °C on Nutri-fly BF (Dutscher Scientific) in a 12 h light–dark cycle. Adult flies were used for all experiments described. *CantonS* or Gal4 driver lines were used as controls. The list of fly lines used in this thesis work can be found in Table 2.

Table 2. *Drosophila* fly lines used in this thesis work.

Fly strain	Study
Canton^{S}	I, II, III
DaughterlessGal4 (DaGal4)	I, II, III
Diap2^{7c}	I
Diptericin-LacZ (Dipt-LacZ)	I, II
dredd^{D44}	III
dredd^{L23}	II, III
Drice^{17}	I
LUBEL^{Mi}	II
LUBEL^{MiMic}	II
NP1Gal4	I, III
Rel^{E20}	II, III
Spätzle^{RM7}	II
UAS-Drice^{C211A}*; DaGal4*	I
UAS-Drice^{WT}*; DaGal4*	I
UAS-Kenny-GFP	III
UAS- Lubel-RNAi	III
UAS-p35	I
UAS-RBR-LDD C>A, WT	II
UbiquitousGal4 (UbiGal4)	II
UbiGal4; Drice-RNAi	I, III
UbiGal4; UAS-Diap2$^{\Delta 100}$	I
UbiGal4; UAS-Diap2^{WT}	I
Yellow white (yw)	I

2 Cloning and generation of transgenic flies

Synthetic codon optimised LUBEL RBR-LDD obtained from Genescript was subcloned into pMT insect expression vector for expression in cells, pGEX vector for expression in *E. coli*, and pUAST-attB for ϕC31-mediated integration of RBR-LDD, V5-tagged Drice and untagged Diap2 into the fly genome. UAS-RBR-LDD^{WT} and UAS-RBR-LDD$^{C>A}$ were introduced to the landing site line #24749 and their expression was verified in cDNA using PCR. Fly egg injections for insertion of gene of interest at specific landing sites were performed by Bestgene Inc. Site-directed mutagenesis to generate point mutations in Kenny (F281A, R285A/R288A/E289A, D21E, D27E, D67E, D88E mutants) and LUBEL (RBR-LDD C2704A) was performed using QuikChange Lightning Site-directed Mutagenesis Kit (Agilent technologies).

3 Infection and survival assays

The Gram-negative bacteria *Erwinia carotovora carotovora 15* (*Ecc15*) and Gram-positive bacteria *Micrococcus luteus* (*M. luteus*) were grown in Luria-Bertani (LB) medium at 29 °C under rotation for 16 hours and concentrated (optical density 0.2). The ability of flies to survive infection with bacteria was studied by infecting 20 flies with Gram-negative bacteria *Ecc15*, *Escherichia coli (E. coli)* or Gram-positive bacteria *Micrococcus luteus* (*M. luteus*) and monitoring their survival over time. Septic infections were performed by pricking the flies in their lateral thorax with a thin needle dipped in concentrated *Ecc15* or *M. luteus.* Oral infections were performed by feeding the flies with a 1:1 solution of bacteria and 5% sucrose. The infected flies were then used to measure survival, NF-κB target gene expression, pathogen clearance, or purify Ub chains.

4 Quantitative RT-PCR

Drosophila adult flies were homogenised using QIAshredder (Qiagen). Total RNA was extracted using RNeasy Kit (Qiagen) according to the manufacturer's protocol from which cDNA synthesized. rp49 was used as a housekeeping gene for calculating ΔΔCt. The expression of the following genes *AttacinA*, *Diptericin*, *IM1*, *Drosomycin*, *RBR-LDD, ZnF*, *Ub* and *rp49* was measured by qPCR using specific primers, the sequences of which can be found from the publications in this thesis.

5 Purification of GST-TUBE and GST-NEMO-UBAN

The expression of GST-TUBE and GST-NEMO-UBAN grown in *E. coli BL21* overnight in LB medium at 18 °C under rotation, were induced by adding 0.2 mM IPTG. The bacteria was centrifuged and lysed by sonication in a buffer containing

50 mM Tris (pH 8.5), 150 mM NaCl, 3 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, and 0.2 mg/ml lysozyme. GST-TUBE and GST-NEMO-UBAN were purified from the lysate using Glutathione Sepharose™ 4B beads and washed in a buffer containing 50 mM Tris (pH 8.5) and 150 mM NaCl. GST-TUBE was eluted in 50 mM Tris (pH 8.5), 150 mM NaCl, 10% glycerol, 3 mM DTT, and 50 mM glutathione. The recombinant proteins were then concentrated from the eluate using Amicon® Ultra-4 30 K centrifugal filter devices.

6 X-gal staining of *Drosophila* intestines

Local NF-κB activity was measured in dissected intestines of flies from *Diptericin-LacZ (Dipt-LacZ)* reporter fly line, combined with mutants of interest. Intestines from female adult flies were dissected in cold PBS and fixed for 15 min with PBS containing 0.4% glutaraldehyde and 1 mM $MgCl_2$. The samples were washed with PBS and incubated with a freshly prepared staining solution containing 5 mg/ml X-gal, 5 mM potassium ferrocyanide trihydrate, 5 mM potassium ferrocyanide crystalline and 2 mg/ml $MgCl_2$ in PBS at 37 °C. After washing with PBS, the samples were mounted using Mowiol (Sigma) and imaged with a brightfield microscope (Leica).

7 Cell culture

Drosophila Schneider S2 cells (Invitrogen) were grown at 25 °C using Schneider medium supplemented with 10% fetal bovine serum, 1% l-glutamine and 0.5% penicillin/streptomycin. Effectene transfection reagent (Qiagen) was used to transfect indicated constructs according to manufacturer's instructions. Expression of constructs in pMT plasmids was induced using 0.5mM $CuSO_4$ for 16 hours before lysis. Lipopolysaccharide (LPS) (Sigma) was added at a concentration of 80 μg/ml for the indicated times and 1 μM of 20-hydroxyecdysone (Sigma) was added 24 h prior to LPS treatment.

8 Immunoprecipitation

Immunoprecipitations were performed in lysates from transfected S2 cells using α-HA or α-V5 agarose beads. S2 cells were lysed in a buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 5 mM NEM, 5 mM chloroacetamide and Pierce™ Protease Inhibitor and PhosSTOP. For immunoprecipitations under denaturing conditions, lysis was performed with a SDS concentration of 1% followed by sonication, after which it was diluted to 0.1% before clearing. The lysates were then cleared at 12,000 rpm for 10 min at 4 °C, and incubated with α-HA or α-V5 agarose beads (Sigma) for 2 h under rotation at 4 °C. The beads were washed in a buffer containing 10 mM Tris (pH

7.5), 150 mM NaCl, 0.1% Triton X-100 and 5% glycerol. HA- or V5-conjugated proteins were eluted using Laemmli sample buffer.

9 His-ubiquitin pulldowns

S2 cells were lysed in a buffer containing 6 M guanidinium-HCl, 0.1 M Na_2HPO_4, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl (pH 8), 5 mM imidazole, and 10 mM β-mercaptoethanol (β-ME). The lysates were incubated with Ni-NTA agarose beads (Qiagen) at 4 °C overnight. The beads were washed once with a buffer containing 6 M guanidinium-HCl, 0.1 M Na_2HPO_4, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl (pH 8), and 10 mM β-ME, and twice with a buffer containing 8 M urea, 0.1 M Na_2HPO_4, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl (pH 8), 10 mM β-ME, and 0.1% Triton X-100. His-Ub-conjugated proteins were eluted using a buffer containing 200 mM imidazole, 0.15 M Tris (pH 6.7), 30% glycerol, 0.72 M β-ME, and 5% SDS.

10 Purification of endogenous ubiquitin chains from cells and fly lysates

Ubiquitin chains from lysates of adult flies or S2 cells were purified using recombinant GST-Tandem ubiquitin binding entity (TUBE) protein, that binds ubiquitin chains with high affinity. M1-linked Ub chains were purified using a recombinant GST-tagged protein containing the UBAN region (residues 257–346) of NEMO (GST-NEMO-UBAN), that has high affinity towards M1-linked Ub chains. These recombinant proteins were first expressed in and purified from *E. coli,* after which they were used to purify ubiquitin chains from fly and cell lysates. Forty adult flies or S2 cells were lysed in a buffer containing 20 mM NaH_2PO_4, 1% NP-40, 2 mM EDTA supplemented with 1 mM DTT, 5 mM *N*-ethylmaleimide (NEM), Pierce™ Protease Inhibitor, PhosSTOP, 5 mM chloroacetamide. For denaturing conditions lysis buffer with 1% SDS was used and later diluted to 0.1% before incubation with Glutathione Sepharose™ 4B beads and GST-TUBE or GST-NEMO-UBAN (30–100 mg/ml) for at least 2 h under rotation at 4 °C. The beads were then washed three times with ice-cold 0.1% phosphate-buffered saline-Tween-20 (PBS-Tween-20) and eluted using Laemmli sample buffer. Ubiquitin chain restriction (UbiCRest) analysis of the GST-NEMO-UBAN-purified ubiquitin chains from S2 cells and flies was performed in a buffer containing 25 mM HEPES (pH 7.6), 150 mM NaCl and 2 mM DTT. The samples were treated with 1 µM of recombinant deubiquitinating enzymes (DUBs), OTULIN, vOTU or AMSH and incubated for 1 h at 37 °C.

11 Treatment of flies with inhibitors

Treatment of adult flies with inhibitors was performed by feeding them with 50 µM MG-132, 50 µM Z-DEVD-FMK, 250 µM Emricasan, or 100 µM Chloroquine

diluted in a 1:1 solution of LB media and 5% sucrose for 16 hours after starving them first for 2 hours. Adult flies or dissected intestines or carcasses from adult flies were homogenized and lysed in a buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA and 10% Glycerol for 10 minutes on ice. The lysates were then cleared by centrifugation before the addition of Laemmli sample buffer.

12 Antibodies

The expression of proteins in the purified/immunoprecipitated/lysate samples from S2 cells and adult flies were analysed by SDS-PAGE and Western blotting using antibodies listed in Table 3.

Table 3. List of antibodies used in this thesis.

Antibody	Company/reference
Actin (clone C-11)	Santa Cruz
DIAP2	(Leulier et al., 2006)
Drice	(Tencho et al., 2004)
GFP	Abcam
HA (clone 3F10)	Roche
K48 (clone Apu2)	Millipore
K63 (clone Apu3)	Millipore
M1 (clone IE3)	Millipore
Phospho-histone H3	Cell Signalling
Ubiquitin (clone FK2)	Enzo
Ubiquitin (clone Ubi-1)	Novus
V5 (clone SV5-Pk1)	Bio-Rad

13 Immunofluorescence of *Drosophila* intestines

Intestines from female adult flies were dissected in cold PBS and fixed using 4% paraformaldehyde. The tissues were then permeabilised using PBS-0.1% Triton X-100 for 1 h and incubated overnight at 4 °C with rabbit anti-phospho-Histone

H3 primary antibody in 1:1000 dilution. They were incubated with Alexa Fluor 488 donkey anti-rabbit IgG secondary antibody in 1:600 dilution for 2 hours at room temperature. DNA was stained using DAPI (4′,6-diamidino-2-phenylindole). The tissues were mounted using Mowiol and imaged using a spinning disk confocal microscope (Zeiss Axiovert-200M microscope, Yokogawa CSU22 spinning disk confocal unit) using ×20 objectives. The acquisition and processing was done using 3i SlideBook6 and image processing was done using Image J software.

14 Pathogen clearance assay

E. coli transformed with pMT/Flag-His was cultivated overnight in LB medium at 37 °C for 16 h on rotation and concentrated by centrifugation (optical density 0.150). Adult flies were fed with 1:1 solution of transformed *E. coli* in 5% sucrose after starving them for 2 hours. The flies were then surface cleaned with ethanol and distilled H_2O and homogenised in 150 µl PBS. The samples were then cleared by centrifugation and diluted 1:100, after which they were plated on LB plates with 50 µg/ml ampicillin. The number of bacterial colonies were counted 24 hours after plating.

RESULTS

1 Drice is a negative regulator of the Imd pathway in the intestine (I)

Intestinal immune responses need to be tightly regulated, as their dysregulation can cause chronic inflammation, leading to diseases like inflammatory bowel disease (IBD) and cancer (Garrett et al., 2009; Grivennikov & Karin, 2010). Apart from being regulators of caspase activity during apoptosis, IAP proteins are important mediators of the inflammatory NF-κB pathway. They ubiquitinate target proteins to induce NF-κB activation (Gyrd-Hansen & Meier, 2010), but how they are regulated during inflammation is unknown. Diap2 is in an important mediator of the *Drosophila* Imd pathway that is known to target Imd pathway mediators for K63-ubiquitination (Meinander et al., 2012; Paquette et al., 2010). As the caspase-3 homologue, Drice, is known to form a complex with Diap2 (Ribeiro et al., 2007), we investigated the role of the Drice-Diap2 complex in the NF-κB pathway.

1.1 Transgenic expression of Diap2 induces chronic inflammation in the intestine of flies

Diap2 is an important mediator of one of the *Drosophila* NF-κB pathways, the Imd pathway, upon both local and septic infection with Gram-negative bacteria (Kleino et al., 2005; Leulier et al., 2006). To understand the function of Diap2, we used a transgenic fly that overexpresses Diap2 via the UAS-Gal4 system. By using the *Diptericin-LacZ* reporter flies, we found that Diap2 overexpression spontaneously induced the expression of *Diptericin* in the guts, but not the fatbody (I, Figure 1 A, B, C, D). While we were able to detect full-length Diap2 in the fatbody, we detected both full-length and a truncated version of Diap2 in the guts (I, Figure 1 G,H). Diap2 and Drice have been shown to form a complex that results in ubiquitination of Drice and proteolytic cleavage of Diap2 after aspartic acid (D100) (Ribeiro et al., 2007). Interestingly, the size of this truncated form of Diap2 detected in the guts of flies expressing transgenic Diap2 corresponds to the Drice-cleaved form of Diap2. This indicates that Diap2 is regulated differently in the guts of flies, and Drice possibly has a role in this regulation. To investigate this, we measured the expression of NF-κB target genes in *Drice^{17}* mutant flies and *Drice-RNAi* flies, and found that they had a higher expression of NF-κB target genes (I, Figure 2 A, B), whereas an overexpression of Drice led to a lower basal expression (I, Figure 2 C). This indicates that Drice functions as a negative regulator of the Imd pathway. Similar to transgenic flies that overexpressed Diap2, loss of Drice mediated by *Drice-RNAi* induced *Diptericin* expression in the gut, but not in the fatbody (I, Figure 2 D, E).

1.2 Drice´s catalytic activity is required for regulating Diap2 and the Imd pathway

To investigate the role of catalytic activity of Drice in regulating Diap2, we expressed Drice^{WT} or the catalytically inactive Drice^{C211A} mutant in a Drice^{RNAi} background. Upon measuring the expression of NF-κB target genes, *Drosocin* and *Diptericin*, we saw that Drice^{WT} but not Drice^{C211A} was able to restrain the expression of AMPs induced by the loss to Drice (I, Figure 3A). By expressing the viral caspase inhibitor p35, which acts as a suicide substrate by blocking the catalytic machinery of Drice, we were able to show that inhibition of Drice activity in the intestinal enterocytes induced a local induction of *Diptericin* in the guts (I, Figure 3C, D), indicating that Drice activity is required for regulating Diap2.

Our results showed that the expression of AMPs upon overexpression of Diap2 and inhibition of Drice was induced specifically in the gut, and not the fatbody. The bacterial presence is constant in the gut due to resident gut microbiota, whereas the fatbody is exposed to bacteria only during a systemic infection. Therefore, we hypothesized that the commensal bacteria are responsible for Diap2-mediated Imd pathway activation, which in the absence of Drice, leads to excess activation of the Imd pathway. To test this, we reared the flies in axenic conditions to eliminate the commensal microbiome, and as expected, found that the expression of AMPs was no longer elevated upon both overexpression of Diap2 and downregulation of Drice (I, Figure 6A, C).

1.3 Drice modulates ubiquitination of Dredd and Kenny by regulating Diap2 levels

In order to investigate if Drice activity can modulate Diap2-mediated ubiquitination, we fed the flies with cell permeable caspase-3 inhibitor Z-DEVD-FMK, and found that it led to accumulation of full-length Diap2 levels and a Diap2-mediated increase in K63-linked Ub chains in fly lysates (I, Figure 4 A, B). As Dredd and Kenny are key mediators of the Imd pathway that are targeted by Diap2-mediated K63-ubiquitination, we investigated if Drice interferes with K63-ubiquitination of Dredd and Kenny. We overexpressed Diap2 together with Dredd or Kenny in *Drosophila* S2 cells to induce their ubiquitination and co-expressed Drice^{WT} or Drice^{C211A}. Inducing Drice activity by expression of Drice^{WT} resulted in a reduction in Diap2 levels and K63-ubiquitination of Dredd and Kenny (I, Figure 4C, D). Importantly, inhibiting Drice activity by co-expressing Drice^{C211A} or by treatment with Z-DEVD-FMK resulted in stabilization of full-length Diap2 and an increase in K63-ubiquitination of Dredd and Kenny (I, Figure 4C, D) showing that Drice catalytic activity restrains the ubiquitination function of Diap2. Our results thus shows that Drice, by regulating Diap2 levels, modulates

the E3 ligase function of Diap2. As a result, Drice affects the ability of Diap2 to ubiquitinate its substrates Dredd and Kenny, which is required for the Imd signalling pathway to proceed.

2 LUBEL regulates local inflammatory responses in the intestine of flies (II)

M1-linked Ub chains are induced upon various stress promoting stimuli (Asaoka et al., 2016; Damgaard et al., 2012; Haas et al., 2009; Lafont, Kantari-Mimoun, et al., 2017; Tang et al., 2018; Tokunaga et al., 2009), and we wanted to study the physiological role of M1-linked Ub chains *in vivo* using *Drosophila melanogaster* as the model organism. K63-ubiquitination of Imd and Dredd mediated by Diap2 was shown to be important for activation of the Imd pathway, but the role of M1-linked Ub chains in the *Drosophila* NF-κB pathway has not been described. The NF-κB pathway is activated in the fatbody when a pathogen enters the hemocoel and the hemolymph, which is usually achieved by delivering the bacteria by pricking or injecting the body. However, the epithelial surfaces of the gut and trachea also encounter pathogens associated with food and their environment. We analysed the role of M1-linked Ub chains using both routes of infection.

2.1 LUBEL catalyses the synthesis of M1-linked Ub chains upon infection

To study and understand the importance of M1-Ub chains in the *Drosophila* Imd pathway, we wanted to analyse if M1-linked Ub chains are generated when the Imd pathway is activated. Using a bioinformatics approach, we identified the *Drosophila* gene CG11321 to contain RBR and UBA domain similar to HOIP and a NZF found in all LUBAC components. Subsequently, we and others, described that CG11321, named LUBEL, as the orthologue of mammalian HOIP, the catalytic subunit of LUBAC (Asaoka et al., 2016). To study the role of LUBEL, we used a mutant fly line (yw;Mi{ET1}LUBELMB00197, labelled *lubel*^{Mi}) that has a 7.5 kb Minos transposable element inserted between the UBA1 and UBA2 region of LUBEL, which disrupts the gene transcription before the catalytic region. We then induced the Imd pathway in wild-type *Canton*^{S} and *lubel*^{Mi} mutant flies by both septic injury and feeding with Gram-negative bacteria *Ecc15*. M1-linked Ub chains from fly lysates were then enriched using a recombinant high-affinity binder, UBD of IKKγ or NEMO tagged with GST (GST-NEMO-UBAN) (Fiil et al., 2013; Keusekotten et al., 2013). We found that M1-linked Ub chains are induced in *Canton*^{S} flies upon both septic and oral infection, but completely abolished in *lubel*^{Mi} mutant flies (II, Figure 1A, B). In order to confirm that the enriched chains were linked via M1-Ub, we treated them with recombinant DUBs OTULIN and vOTU. Treatment with OTULIN,

which cleaves only M1-linked Ub chains, completely removed signal from M1-linked Ub chains, while treatment with vOTU, which cleaves all chains except M1-linked Ub chains, gave rise to only a ladder of free M1-linked Ub chains (II, Figure 1C). This is because vOTU can cleave the first ubiquitin that links M1-linked Ub chains to their substrates.

Similar to HOIP, LUBEL contains zinc fingers, UBA domains and a C-terminal RBR-LDD region. Structural modelling of RING2 and LDD indicates that the catalytic pocket including the catalytic cysteine, and the positioning around the donor and acceptor ubiquitin are conserved between HOIP and LUBEL (II, Figure 2 B). *In vitro*, LUBEL catalyses the formation of M1-linked Ub chains that are hydrolysed only by the M1-linked Ub chain specific DUB, OTULIN, but not the Associated molecule with the SH3 domain of STAM (AMSH) and otubain-1 (OTUB1), which are specific for K63- and K48-linked Ub chains respectively (I, Supplementary Figure 2B). Furthermore, we overexpressed wild-type and catalytically inactive C2704A RBR-LDD of LUBEL in *Drosophila* Schneider S2 cells, and found that only wild-type RBR-LDD generated M1-linked Ub chains (II, Figure 2C). Together, our results show that RBR-LDD is the catalytic region of LUBEL, and infection with Gram-negative bacteria induces the synthesis of LUBEL-mediated M1-linked Ub chains in *Drosophila*.

2.2 CYLD hydrolyses M1-linked Ub chains

In mammals, OTULIN and CYLD have deubiquitinase activity towards K63- and M1-linked Ub chains (Komander et al., 2009). Although no homologue of mammalian OTULIN has been described in the flies, *Drosophila* CYLD was shown to interact with Kenny in S2 cells (Tsichritzis et al., 2007). We analysed if CYLD expression can hydrolyse M1-linked Ub chains generated by LUBEL RBR-LDD. By co-expressing CYLD with LUBEL-RBR-LDD, we observed that CYLD completely hydrolysed all M1-linked Ub chains generated by LUBEL RBR-LDD, although having only a small effect on the levels of K63-linked Ub chains (II, Figure 2D). It was also shown *in vitro* that *Drosophila* CYLD can interact with LUBEL RBR-LDD (Asaoka et al., 2016) and have deubiquitinase activity towards K63- and M1-linked Ub chains (Asaoka et al., 2016).

2.3 LUBEL is important for activation of the Imd pathway in the intestine upon oral infection

In order to understand the role of M1-linked Ub chains in activation of the *Drosophila* NF-κB pathway, we performed survival assays after infecting the flies by septic injury with Gram-negative bacteria *Ecc15* or Gram-positive bacteria *M. luteus.* We also measured the expression of Toll and Imd pathway specific target genes by qPCR. Upon septic injury with *M. luteus*, we found that

both *Canton*^{S} and *lubel*^{Mi} mutant flies were able to tolerate the infection and upregulate the expression of Toll-specific AMPs *IM1* and *Drosomycin* (II, Supplementary Figure 4A, B). Similarly, they were able to survive septic injury with *Ecc15* and upregulate expression of *Drosocin*, a Imd pathway specific AMP (II, Figure 6A, B). This shows that even though M1-linked Ub chains are formed upon septic injury, they are not required for systemic activation of the Imd pathway in the fatbody, the organ which responds to septic injury by activating expression of AMP. However, upon infecting the flies by feeding them with *Ecc15*, the *lubel*^{Mi} mutant flies succumbed, while most of the *Canton*^{S} flies survived the infection (II, Figure 6C). Accordingly, the expression of *Drosocin* in the *lubel*^{Mi} mutant flies was significantly reduced upon oral infection (II, Figure 6D). By analysing the expression of *Diptericin* using *Diptericin-LacZ* reporter flies, we showed that the intestines of control flies, but not the *lubel*^{Mi} mutant flies, induced expression of *Diptericin* in the midgut upon oral infection with *Ecc15*, indicating that M1-linked Ub chains are important for local activation of the Imd pathway and expression of AMPs in the epithelia of the intestine (II, Figure 6E). Finally, to test if LUBEL is required for clearing pathogens upon ingestion, we fed wildtype *Canton*^{S} and *lubel*^{Mi} mutant flies with ampicillin-resistant *E. coli*. We then homogenised the flies and plated in agar plates containing ampicillin, and counted the colonies formed. We found that the number of colony-forming bacteria was significantly higher in *lubel*^{Mi} mutant flies than in wildtype flies (II, Figure 6G), indicating that LUBEL is required for clearing ingested pathogens.

2.4 Transgenic expression of LUBEL RBR-LDD drives NF-κB activation

We generated transgenic flies expressing wildtype and catalytically inactive LUBEL RBR-LDD under the control of UAS-Gal4 system. Upon driving the expression of transgenes with a ubiquitous driver (II, Figure 7A), DaGal4, we found that the expression of Imd pathway specific AMPs *Attacin*, *Drosocin,* and *Diptericin*, were induced in flies expressing wildtype, but not catalytically inactive RBR-LDD even without infection (II, Figure 7B). By analysing the expression of *Diptericin* in the intestine using *Diptericin-LacZ* reporter flies, we found that wildtype RBR-LDD induced expression of *Diptericin* in the midgut (II, Figure 7C). Intestinal inflammation is associated with hyperplasia in the midgut of flies (Amcheslavsky et al., 2009), and we found that there were more phospho-histone H3-positive proliferating cells in the midguts of flies expressing wildtype RBR-LDD (II, Figure 7D, E), showing that constitutive activity of LUBEL drives the Imd pathway specific NF-κB, Relish, mediated chronic inflammation.

3 Identifying targets of M1-ubiquitination in the Imd pathway (II, III)

To identify targets of M1-ubiquitination, we used *Drosophila* S2 cells to transfect constructs to express mediators of the Imd pathway. We checked the ability of LUBEL RBR-LDD to generate M1-linked Ub chains in S2 cells, and found that expression of wildtype, but not catalytically inactive RBR-LDD was able to generate M1-linked Ub chains (II, Figure 2C). We over-expressed important mediators of the Imd pathway along with RBR-LDD, and we identified targets of LUBEL-mediated M1-ubiquitination by performing immunoprecipitations under denaturing conditions.

3.1 Kenny is a target of M1-ubiquitination

It is known that NEMO, the mammalian IKKγ, regulates NF-κB signalling by both being a target of M1-ubiquitination, and via UBAN domain mediated binding to M1-linked Ub chains. To analyse if Kenny is similarly ubiquitinated, we purified M1-linked Ub chains using recombinant GST-NEMO-UBAN and found that over-expression of Kenny alone induced its M1-ubiquitination (II, Figure 3A). Co-expressing LUBEL RBR-LDD induced a further increase in M1-ubiquitination of Kenny. To understand if M1-ubiquitination of Kenny is signal dependent, we co-expressed PGRP-LCx or treated the cells with lipopolysaccharide (LPS) to induce the Imd pathway. We found that activation of the Imd pathway induced an increase in M1-ubiquitination of Kenny (II, Figure 3B, C). Interestingly, we found that CYLD hydrolyses M1-linked Ub chains induced by overexpression of Kenny. The LPS-induced increase of M1-ubiquitination of Kenny was also not seen upon CYLD expression (II, Figure 3C).

3.2 Kenny is modified by mixed K63- and M1-linked Ub chains

It has been reported that mixed K63-/M1-linked Ub chains are commonly formed upon activation of the TNFR1, MyD88, TLR3 and NOD1 signalling pathways (Emmerich et al., 2013, 2016). The M1-linked Ub chains were found to be attached to pre-formed K63-linked Ub chains, indicating that pre-formed K63-linked Ub chains generated by other E3 ligases are targeted by LUBAC for M1-ubiquitination to form hybrid K63-/M1-linked Ub chains. To analyse if mixed K63/M1-linked Ub chains are generated on Kenny, we first determined if Kenny is modified by K63-linked Ub chains. It has been previously shown that Diap2 generates K63-linked Ub chains on mediators of the Imd pathway (Meinander et al., 2012; Paquette et al., 2010). Therefore, we checked if Diap2 generates K63-linked Ub chains on Kenny by performing immunoprecipitation under denaturing conditions, using a His-tagged ubiquitin mutant where all other lysines except K63 were mutated. We found that co-expressing Diap2 not only

induced K63- but also boosted M1-ubiquitination of Kenny (II, Figure 4B, C). Importantly, the amount of M1-ubiquitinated Kenny, purified by GST-NEMO-UBAN, was decreased upon treatment with AMSH, indicating that M1-linked Ub chains attached to Kenny are likely attached indirectly through K63-linked Ub chains (II, Figure 4D).

Similar to NEMO, Kenny has a UBAN domain, and structural modelling suggested that their UBAN domains are conserved (II, Figure 5A). The strong binding surfaces of NEMO for M1-linked Ub chains formed by the amino acids F312, R316, R319 and E320, corresponding to F281 and R285, R288A, E289 of Kenny are also conserved, as the mutant of the binding surfaces (F281A and R285A/R288A/E289A) was no longer able to stabilize M1-linked Ub chains (II, Figure 5B). Our results thus indicate that Kenny is not only conjugated by M1-linked Ub chains, but also associates with M1-linked Ub chains via UBAN domain leading to stabilisation of M1-linked Ub chains.

3.3 The caspase-8 homologue, Dredd is a target of M1-ubiquitination

Using our over-expression system, we further analysed if Imd and Dredd are targets of LUBEL RBR-LDD-mediated M1-ubiquitination. While we were not able to detect LUBEL-mediated ubiquitination of Imd, we found that Dredd was targeted for M1-ubiquitination by wildtype, but not the catalytically inactive C2704A mutant form of RBR-LDD (III, Figure 1A). Upon co-expressing PGRP-LCx, we found that M1-ubiquitination of Dredd further increased, showing that Dredd is M1-ubiquitinated in a signal dependent manner (III, Figure 1B). Furthermore, from our ubiquitination assays, we found that the DED1 domain of Dredd is the region where Dredd is M1-ubiquitinated (III, Figure 1D). It has been previously shown that a point mutation in the DED1 domain, G98R (III, figure 3A), affected Diap2-mediated K63-ubiquitination of Dredd. This mutation was previously described as G120R, but the Dredd protein that is transcribed is found to be 22 amino acids shorter than the previously reported sequence. We have now found that this mutation also abrogates M1-ubiquitination of Dredd (III, Figure 3B). It is possible that mixed K63/M1-linked Ub chains are formed on Dredd and the decrease in M1-ubiquitination of G98R mutant of Dredd is due to the fact that the G98R mutation affects its K63-ubiquitination.

4 Dredd and Kenny interact leading to cleavage of Kenny (III)

It is known that interaction between NEMO and M1-linked Ub chains forms an activation hub for NF-κB signalling to proceed (Rahighi et al., 2009; Tokunaga et al., 2009). Interestingly, DED-mediated interactions to NEMO or its homologue

optineurin can affect NF-κB responses (Bagnéris et al., 2008; Nakazawa et al., 2016; Sadek et al., 2020). In order to test if Dredd can interact with Kenny, we performed immunoprecipitations and found that Dredd indeed can bind to Kenny (III, Figure 2A). Furthermore, we found that this interaction is mediated through the prodomain and specifically the DED1 domain of Dredd (III, Figure 2B, C). Surprisingly, we found that Dredd not only binds to Kenny, but also cleaves Kenny on its N-terminus. By generating point mutations of aspartic acid residues on the N-terminus of Kenny (D21E, D27E, D66E, D88E) (Figure 2E), we found that Dredd cleaves Kenny at D21, as only D21E mutation abrogated Dredd-mediated cleavage (Figure 2F).

In order to predict if the M1-ubiquitination-abrogating G98R mutation of Dredd distorts the interaction with Kenny, we did modelling based on the crystal structure of the complex of Kaposi's sarcoma herpes virus (KSHV)-FLIP and NEMO (PDB: 3CL3). We used the AlphaFold model of Dredd and aligned it to the structure of KSHV-FLIP bound to NEMO (III, Figure 3C). Our modelling suggested that DED1 of Dredd can interact with NEMO similarly as DED1 of KSVH-FLIP, and we saw that the G98 surface would phase the interaction surface. However, upon performing immunoprecipitation experiments, we found that Dredd G98R mutation did not significantly decrease the ability of Dredd to interact with Kenny (III, Figure 3D). The catalytically inactive Dredd (C408A) was also able to bind Kenny, similarly as wildtype Dredd (III, Figure 3D). Our results thus suggest that Dredd binds to Kenny via DED1 domain regardless of Dredd ubiquitination. We then studied if Dredd ubiquitination affects its ability to cleave Kenny. As expected, catalytically inactive Dredd (C408A) was not able to cleave Kenny. Importantly, G98R mutation of Dredd significantly affected the cleavage of Kenny compared to wildtype Dredd, indicating that Dredd ubiquitination affects its catalytic activity (III, Figure 3E).

We have previously shown that Kenny stabilizes M1-linked Ub chains through the UBAN domain, and overexpression of Kenny leads to stabilisation of M1-linked Ub chains in S2 cells. To investigate if Kenny can stabilize M1-linked Ub chains after cleavage by Dredd, we performed GST-NEMO-UBAN pulldowns after expressing Kenny alone and together with Dredd. We found that expressing Kenny alone and together with Dredd led to stabilisation of M1-linked Ub chains, and both full length and cleaved Kenny associated with M1-linked Ub chains. We then analysed if co-expressing Kenny with Dredd can stabilize M1-linked Ub chains on Dredd. Interestingly, we found that co-expression of Kenny increased the amount of M1-ubiquitinated wildtype but not the G98R mutant of Dredd (III, Figure 4A), indicating that Kenny and Dredd form a complex where Kenny stabilizes Ub chains on Dredd (III, Figure 4B).

5 Dredd-mediated cleavage protects Kenny from degradation (III)

In the N-terminal region of Kenny that is cleaved by Dredd, there is a LC3-interaction region (LIR) motif, which is required for interaction with the autophagosomal membrane protein microtubule-associated protein 1 light chain 3 (Atg8/LC3) (Pankiv et al., 2007). In fact, it was shown that the IKK complex via Kenny interacts with autophagosomes to undergo selective autophagosomal degradation to prevent constitutive activation of the Imd pathway in the intestine of the flies (Tusco et al., 2017). To study the cleavage of Kenny in flies, we used a transgenic fly that expresses N-terminally GFP-tagged Kenny under the control of UAS-Gal4 system (III, Figure 5A). We were able to detect full-length Kenny in the intestine of flies upon treatment with the lysosomal inhibitor chloroquine, indicating that Kenny undergoes autophagic degradation in the gut (III, Figure 5B). The IKK complex is an important mediator of the Imd pathway, apart from phosphorylating Relish, it is also required independent of its kinase activity for Dredd mediated cleavage of Relish and ultimately for activation of the Imd pathway (Ertürk-Hasdemir et al., 2009; Silverman et al., 2000; Stöven et al., 2003). By feeding the flies with *Ecc15*, we were able to see a stabilization of the N-terminal cleaved fragment of Kenny but not full-length (III, Figure 5C), suggesting that cleavage of Kenny protects it from autophagy-mediated degradation upon activation of the Imd pathway. To confirm that the cleavage of Kenny is mediated by Dredd, we treated the flies with the caspase inhibitor Emricasan, which prevented Kenny cleavage (III, Figure 5D). Upon expressing GFP-Kenny in a Dredd wildtype, $\textit{Dredd}^{G98R}$ or $\textit{Dredd}^{W430R}$ mutant background, we detected infection-induced cleavage of Kenny only in the presence of wildtype Dredd, indicating that abrogation of Dredd-ubiquitination and catalytic activity results in a lack of processing of its target, Kenny (III, Figure 5E). In order to assess the role of LUBEL, we expressed GFP-Kenny in a $\textit{Lubel}^{RNAi}$ background and found that Kenny is cleaved significantly less in the absence of LUBEL, showing that M1-linked Ub chains are important for infection induced cleavage and stabilization of Kenny (III, Figure 5F).

DISCUSSION

Caspases are well known for their role in regulating apoptosis, but they also have important functions in regulating inflammation. Dysregulated caspases have been associated with diseases involving tumorigenesis, autoimmunity and autoinflammation (McIlwain et al., 2013). While the role of IAP proteins in regulating caspases and the inflammatory NF-κB pathway has already been described, the role of caspases in regulating IAP function has not been established. In study I of this thesis, we reported the role of the *Drosophila* homologue of caspase-3, Drice, as a negative regulator of the Imd pathway. We suggest that Drice functions by restraining NF-κB responses to commensal bacteria by binding Diap2 and inducing degradation of the Drice-Diap2 complex. We showed that Drice regulates the Imd pathway by controlling the levels of Diap2. Only the catalytically active Drice and not inactive Drice^{C211A} mutant was able to induce degradation of Diap2, and restrain the NF-κB target gene expression, indicating that the degradation of Diap2 is preceded by cleavage of Diap2 by Drice. It has been established that activation of the Imd pathway through the PRRs results in Diap2-mediated K63-ubiquitination of Imd, Dredd and Kenny (II, Figure 4B; Meinander et al., 2012; Paquette et al., 2010). In line with this, co-expression of Drice abrogated Diap2-mediated K63-ubiquitination of Dredd and Kenny, and inhibition of Drice activity led to enhanced ubiquitination of Dredd and Kenny.

In addition, we found that the excess activation of the Imd pathway by overexpression of Diap2 or inhibition of Drice was dependent on the presence of gut microbiota. We propose that the commensal microbiota can induce formation of a complex comprised of the receptor PGRP-LC or PGRP-LE, Imd, dFadd and Dredd. For downstream signalling to proceed, members of this complex must undergo ubiquitination mediated by Diap2, and Drice may compete with this complex for interaction to Diap2. Under basal conditions, Drice binds to Diap2 to form a complex that gets degraded in a proteasome-dependent manner, thus restraining Diap2 from interacting with its targets (Meinander et al., 2012; Paquette et al., 2010). In the absence of Drice, Diap2 is free to interact and ubiquitinate its target proteins, Imd, Dredd and Kenny, thereby activating downstream signalling that results in activation of the Imd pathway and expression of Relish target genes, causing chronic inflammation and hyperplasia.

DIAP2 is a K63-linked Ub synthesizing E3 ligase, and one of the main functions of K63-linked Ub chains in the NF-κB pathway is the recruitment of the TAK1-TAB2/3 kinase complex (Kanayama et al., 2004; Kulathu et al., 2009; Skaug et al., 2009). The K63-linked Ub chains also function as targets for the LUBAC complex to form mixed K63/M1-linked Ub chains (Emmerich et al., 2013, 2016). The

formation of both K63- and M1-linked Ub chains is important, as they co-recruit and bring the TAK1-TAB2/3 and the IKK complex into close proximity. This results in efficient activation of the IKK complex, followed by phosphorylation of IκB, which is required for its degradation and activation of the NF-κB pathway. While a role of Diap2 and K63-linked Ub chains in the *Drosophila* Imd pathway was already established (Leulier et al., 2006; Meinander et al., 2012; Paquette et al., 2010), the role of M1-linked Ub chains was not known. In study II of this thesis, we described LUBEL as the E3 ligase that generates M1-linked Ub chains in flies.

While the LUBAC complex that generates M1-linked Ub chains in mammals, is made up of three subunits HOIP, HOIL-1L, and SHARPIN, only a HOIP orthologue called LUBEL was identified in flies (Asaoka et al., 2016). We and others found that RBR-LDD, the catalytic domain of LUBEL is highly conserved (Asaoka et al., 2016). Our structural modelling of the RING2 and LDD region showed that the catalytic pocket including the catalytic cysteine of LUBEL is similar to HOIP. LUBEL contains two UBAs, UBA1 and UBA2, similarly to HOIP. LUBAC was shown to be recruited to the NF-κB activating signalling complex via K63-linked Ub chains (Damgaard et al., 2012; Haas et al., 2009), and it was shown that LUBEL UBA2 can associate with K63-linked Ub chains (Asaoka et al., 2016). It is likely that in flies, LUBEL is recruited via K63-linked Ub chains that are pre-formed on its targets by Diap2, as both Dredd and Kenny, which are targeted for M1-ubiquitination are known targets of Diap2-mediated K63-ubiquitination.

Mammalian HOIP is in an autoinhibited state through an intramolecular interaction between the N-terminal and RBR regions (Stieglitz et al., 2012). HOIL-1 and SHARPIN, through their UBL domains interact with UBA domains of HOIP to release it from autoinhibition (Smit et al., 2012; Stieglitz et al., 2012). In addition, both HOIL-1 and SHARPIN have a LUBAC-tethering motif (LTM) through which they interact, and the LTMs of two proteins fold into a single globular domain (Fujita et al., 2018). Interaction between the three components HOIL-1, HOIP and SHARPIN provides stability of LUBAC. HOIL-1, which has limited E3 ligase activity, also belongs to the RBR family of E3 ligases. It was shown that upon TNFR1 signalling, HOIL-1 generates monoubiquitin on LUBAC subunits, which results in LUBAC autoubiquitination, while attenuating M1-ubiquitination of RIPK1 (Fuseya et al., 2020). Furthermore, upon TLR signalling, HOIL-1 was also shown to catalyse ubiquitination on serine and threonine residues, instead of lysine residues on IRAK1, IRAK2, and MyD88, adding further heterogeneity and complexity to ubiquitin signalling (Kelsall et al., 2019). While LUBEL (2892 amino acids) is considerably larger than HOIP (1072 amino acids), no other conserved motifs were found other than the ones mentioned (II, Figure 2 A). It is not known whether LUBEL is autoinhibited in a similar way as HOIP. As no homologues of HOIL-1 and SHARPIN can be found in *Drosophila*, studies

conducted by us and others describing LUBEL can help understand the specific physiologic function of HOIP *in vivo* (Asaoka et al., 2016).

DUBs, by disassembling Ub chains from target proteins, play an essential role in resolution and regulation of the NF-κB pathway. While the mammalian DUBs OTULIN and CYLD were shown to hydrolyse M1-linked Ub chains, the activity of CYLD seems to be particularly important in TNFR1 and NOD2 signalling complexes (Draber et al., 2015). The CYLD catalytic region, ubiquitin carboxyl-terminal hydrolase (UCH) domain, can hydrolyse both M1- and K63-linked Ub chains *in vitro*, however the N-terminal cytoskeletal-associated protein-glycine-conserved (CAP-Gly) domains enable CYLD to preferentially hydrolyse K63-linked Ub chains (Elliott et al., 2021). *Drosophila* CYLD contains a CAP-Gly domain and a UCH domain, whereas mammalian CYLD contains three CAP-Gly domains, a phosphorylation region and a UCH domain (Asaoka et al., 2016). As no homologue of OTULIN has been found in *Drosophila*, and as we found that CYLD hydrolyses M1-linked Ub chains in *Drosophila* S2 cells, it is possible that CYLD is the DUB responsible for hydrolysing M1-linked Ub chains in flies. However, as both mammalian and *Drosophila* CYLD can hydrolyse K63-linked Ub chains, it cannot be excluded that the loss of M1-linked Ub chains is a consequence of CYLD-mediated hydrolysis of K63-linked Ub chains, to which M1-linked Ub chains are conjugated. While LUBAC associates with OTULIN and CYLD via PUB-PIM mediated interaction, no PUB domain has been found in LUBEL. It is possible that *in vivo*, the interaction between CYLD and LUBEL is facilitated by a homologue in flies for the bridging protein SPATA2.

Target proteins that are M1-ubiquitinated upon activation of the TNFR1 and NOD2 signalling pathways have been described, and RIPK1 and RIPK2 were found to be decorated with mixed K63/M1-linked Ub chains (Emmerich et al., 2016; Fiil et al., 2013). Furthermore, NEMO, was shown to be both targeted for M1-ubiquitination and associated with M1-linked Ub chains (Rahighi et al., 2009; Tokunaga et al., 2009). In the *Drosophila* Imd pathway, Dredd and Imd are known to be targeted for K63-ubiquitination (Meinander et al., 2012; Paquette et al., 2010). While we were not able to detect M1-ubiquitination of Imd, we found that Dredd, was modified by M1-linked Ub chains. Whether K63/M1-linked mixed chains are formed on Dredd, similar to RIPK1 and RIPK2 in TNFR1 and NOD2 pathways needs more investigation. Our results further shows that the NEMO homologue in flies, Kenny is also a target of M1-ubiquitination. In addition, it is also targeted for K63-ubiquitination mediated by Diap2, forming mixed K63/M1-linked Ub chains. Interestingly, expression of Kenny also stabilizes M1-linked Ub chains, which is dependent on the UBAN domain of Kenny.

M1-ubiquitination of mammalian DED-containing proteins have been reported to drive NF-κB activation. During TRAIL signalling, caspase-8 was M1-

ubiquitinated promoting recruitment of NEMO and activation of the inflammatory NF-κB pathway, which was accompanied by a reduction in caspase-8 activity (Lafont, Kantari-Mimoun, et al., 2017). During TNFR1 signalling, c-$FLIP_L$ was M1-ubiquinated, which stabilized $FLIP_L$ so that it can compete with caspase-8 to promote activation of NF-κB (Tang et al., 2018). While the catalytic activity of caspase-8 is required to inhibit necroptosis, an inflammatory mode of cell death, the prodomain of caspase-8 is known to activate NF-κB signalling (Chaudhary et al., 2000; Henry & Martin, 2017). The recognition and binding of M1-linked Ub chains by NEMO is required for activation of the NF-κB pathway (Tokunaga et al., 2009), and we have found that Kenny associates with M1-linked Ub chains and stabilized M1-linked Ub chains on Dredd possibly by protecting Ub chains from DUB activity. We also found that Kenny and Dredd interact via the prodomain and specifically the DED1 domain of Dredd. Interestingly, Optineurin, a homologue of NEMO, was shown to bind caspase-8 and NEMO was shown to interact with FLIP, both interactions promoting cell survival. This suggests that the interaction between IKKγ homologues and DED-containing proteins is conserved (Bagnéris et al., 2008; Nakazawa et al., 2016; Sadek et al., 2020). In the *Drosophila* Imd pathway, DED functions of Dredd and the catalytic activity are both required for activating Relish, indicating that the functions DEDs of caspase-8 and FLIP, and the catalytic activity of caspase-8 are performed by Dredd in flies.

An efficient immune response in barrier epithelium is vital, as this prevents penetration of pathogens that can cause systemic infections. Our results showed that LUBEL is important for activation of the Imd pathway in the intestinal epithelia, but not in the fatbody. Septic injury induces production and secretion of AMPs from the fatbody, whereas infection by ingestion of bacteria induces release of AMPs from the intestinal epithelial cells. While both Imd and Toll pathways, are functional in the fatbody by activating Relish and Dif respectively, only the Imd pathway is functional in the epithelial tissues of the gut (Liehl et al., 2006). It is possible that Relish/Dif heterodimers are able to activate expression of AMPs in the fatbody upon septic injury even in the absence of M1-linked Ub chains, explaining the resistance of *lubel^{Mi}* mutant flies to septic injury. Alternatively, it is possible that Diap2-mediated K63-ubiquitination of Kenny and Dredd is sufficient for activation of Relish. Our results nevertheless show that the generation of M1-linked Ub chains, and their role in activating inflammatory NF-κB responses is conserved in *Drosophila*. Furthermore, transgenic expression of RBR-LDD led to expression of Relish target genes and intestinal stem cell proliferation in the midgut in the absence of infection, indicating that M1-linked Ub chains can activate Relish, and emphasizes the need for tight regulation of the Imd pathway in epithelial surfaces. The excess activation of the Imd pathway by transgenic expression of both Diap2 and

LUBEL, leading to chronic inflammation also highlights the suitability of *Drosophila* as a model to study disease conditions in epithelial tissues.

As aberrant activation of NF-κB signalling is associated with inflammatory diseases, it needs to be a strictly regulated and autophagy is one of the mechanisms through which NF-κB signalling is regulated. However, autophagy is most notable for its role as a survival response upon nutrient scarcity, where it compensates for the lack of intracellular energy stores by degrading non-vital intracellular components. It also takes part in removal of protein aggregates, misfolded proteins, damaged, and long-lived organelles (Mizushima, 2007). Perturbations in autophagy functions can also result in inflammatory diseases. Autophagy can capture various cargo in the cell including inflammation inducing PAMPs and DAMPs (Deretic et al., 2013; Rubinsztein et al., 2015). In addition, autophagy was shown to dampen inflammation by removing components of NLRP3 inflammasome, cGAS-STING pathway via selective autophagy receptors (M. Chen et al., 2016; Mehto et al., 2019). Some examples of selective autophagy receptors include p62 and optineurin. They are characterized by their ability to recognise and bind ubiquitin chains on substrate proteins and associate with autophagosomal membrane proteins. In *Drosophila*, it was shown that Kenny via a LIR motif in its N-terminus interacts with autophagosome, and selectively degraded under basal conditions to prevent constitutive activation of the Imd pathway by commensal bacteria in the intestine of flies (Tusco et al., 2017). Recently, other important mediators of the *Drosophila* Imd pathway, dTak1 and its coactivator dTab2, were also found to be degraded by autophagy to prevent constitutive activation of the Imd pathway, providing further evidence of autophagy mediated regulation of the Imd pathway (Tsapras et al., 2022).

Kenny, the regulatory subunit of the IKK complex, is required for Dredd-mediated cleavage of Relish and subsequently its translocation to the nucleus (Silverman et al., 2000). Intriguingly, we found that Dredd cleaves Kenny on its N-terminus that harbours the LIR motif. Importantly, this cleavage was dependent on LUBEL and M1-ubiquitination of Dredd. While degradation of Kenny prevents overactivation of the Imd pathway under basal conditions, upon infection, it is important that Kenny is stabilized in order to function in the Imd pathway. We propose that the cleavage of N-terminus of Kenny, harbouring the LIR motif, by Dredd is an important signalling event to prevent degradation of Kenny upon infection. A limitation in study III of this thesis is the lack of a Kenny mutant that cannot be cleaved by Dredd. This uncleavable mutant of Kenny would enable us to investigate the importance of cleavage of Kenny during infection, as we speculate that a lack of cleavage of Kenny would lead to degradation of Kenny by autophagy, and hence these flies won't be able to survive upon oral infection. Our results from studies II and III thus reveal a particular role of LUBEL, M1-linked Ub chains and caspase activation in regulating NF-κB activation especially in the barrier epithelia.

CONCLUSIONS

In order to treat inflammatory diseases such as chronic inflammation and cancer, we need to understand the underlying molecular mechanisms behind disease conditions. The aim of this thesis was to advance our knowledge of the regulatory mechanisms of the inflammatory NF-κB pathway. *Drosophila melanogaster*, due to low redundancy in its genome, a high degree of conservation of cell signalling pathways, in combination with the availability of a range of molecular biology tools has proved to be an excellent model organism to study and understand the molecular mechanisms of regulation of the NF-κB pathway. Intestinal epithelial cells are in constant contact with commensal bacteria and encounter foodborne pathogens. While they should be able to rapidly respond to infection, they must also tolerate beneficial commensals. Hence, there is a need for tight control of the inflammatory NF-κB pathway in order to avoid dysregulated immune response.

In this thesis, we have shown that the effector caspase, Drice, functions as a negative regulator of the *Drosophila* Imd pathway by restraining the function of the E3 ligase Diap2 (Figure 10). We have found that Drice is involved specifically in the intestine of flies to counter commensal bacteria induced NF-κB activation. While the role of K63-linked Ub chains was already described, we and others identified and described the M1-ubiquitination machinery, LUBEL in flies (Asaoka et al., 2016). We found that the IKKγ and caspase-8 homologues in the fly, Kenny and Dredd to be targets of M1-ubiquitination in the Imd pathway, showing that conjugation of M1-Ub chains to mediators of the NF-κB pathway is conserved. Importantly, we found that M1-ubiquitination was important for mounting an immune response in the epithelial tissue of the gut upon local infection, showing a tissue specific regulation of NF-κB activation.

Finally, we describe a novel mode of regulation of Kenny in the intestine of flies. It was reported that Kenny undergoes autophagy-mediated degradation in order to avoid constitutive activation of the Imd pathway. We found Kenny to be a novel target of Dredd-mediated cleavage upon activation of the Imd pathway in the gut. Based on this we propose a model where cleavage of Kenny by Dredd, which is dependent on LUBEL and ubiquitination of Dredd, protects Kenny from autophagosomal degradation (Figure 10). However, this model can only be verified by generating transgenic flies expressing a cleavage resistant D>E mutant of Kenny. Overall, this thesis has shown that *Drosophila* is a relevant and convenient model to study mechanisms of regulation of the NF-κB signalling pathway. Importantly, this thesis describes the role of M1-

ubiquitination and caspase activation in fine tuning inflammatory responses especially in the intestinal epithelial barrier.

Host-microbe interactions in epithelial barriers determine health, both the interactions with commensals and those with pathogens. As we have found the generation of M1-linked Ub chains, Dredd-activation and Kenny cleavage to be particularly important in the intestine, it will be intriguing to investigate the role of commensal bacteria in these processes, both in the intestine and in other barriers. We have found some mediators of the Imd pathway to be targets of caspase-mediated cleavage, showing that caspase activation is part of inflammatory processes. Hence, it will be important to identify also which other targets are cleaved by Drice and Dredd both during basal conditions and during exposure to NF-κB activating stimuli. As our studies indicate that M1-linked Ub chains are important for Dredd-mediated Imd pathway activity, it will be important to study how M1-ubiquitination of Dredd affects its recruitment to signalling complexes, catalytic activity, and selection of substrates for cleavage. This could provide clues about the non-apoptotic functions of caspase-8 upon engagement of DRs that normally induce apoptosis but can be rewired to support tumorigenesis.

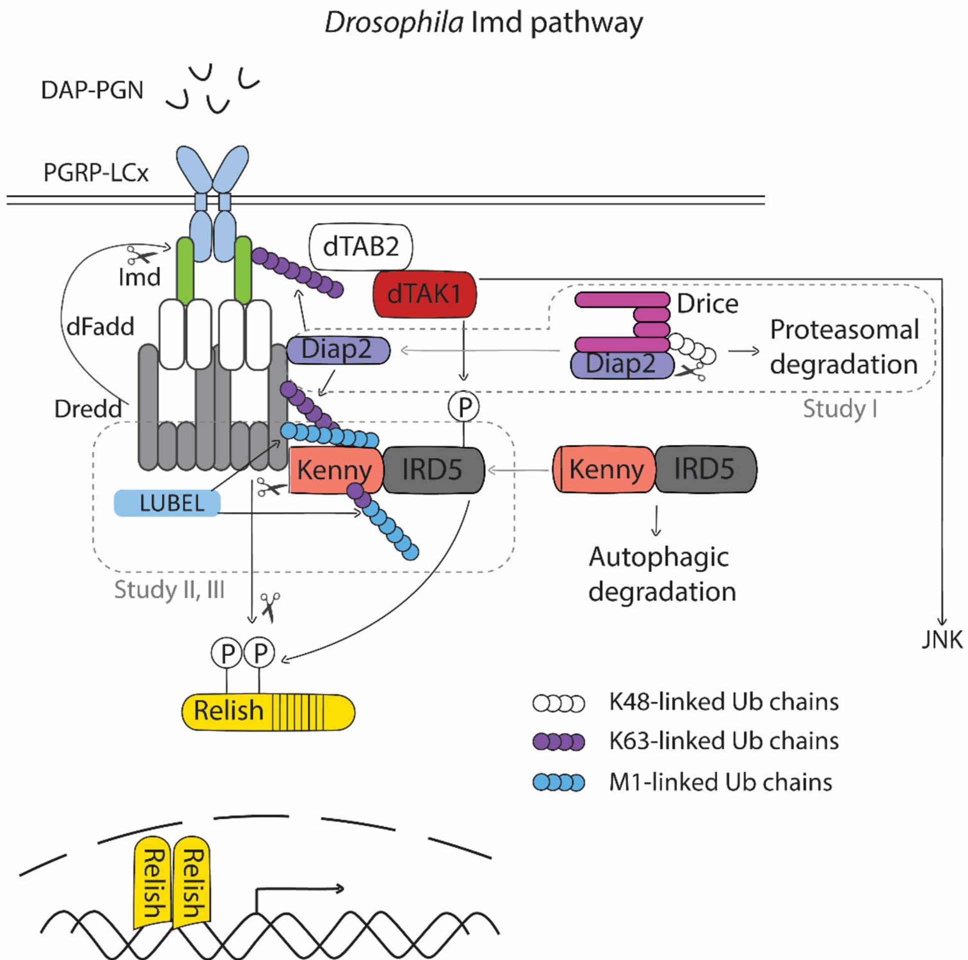


Figure 10. Model of Drice, Dredd and LUBEL mediated regulation of the Imd pathway. Upon basal conditions, Drice restrains the Imd pathway by forming a complex with Diap2 that results in Diap2-mediated ubiquitination, Drice-mediated cleavage of Diap2 and proteasomal degradation of the Drice-Diap2 complex. Absence of Drice results in unrestrained Diap2 functioning by ubiquitinating its targets, Imd, Dredd and Kenny (Study I). LUBEL generates M1-linked Ub chains which is important for activation of the Imd pathway in the epithelial tissue of the intestine, and specifically targets Kenny (Study II) and Dredd (Study III) for M1-ubiquitination. Kenny, which under basal conditions is degraded by autophagy, can interact with Dredd upon infection to stabilize M1-linked Ub chains on Dredd. Dredd on the other hand cleaves Kenny on its N-terminus in a LUBEL dependent manner to prevent Kenny from autophagosomal degradation, indicating the cleavage of Kenny is an important signalling event (Study III).

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Drice restrains Diap2-mediated inflammatory signalling and intestinal inflammation

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The *Drosophila* IAP protein, Diap2, is a key mediator of NF-κB signalling and innate immune responses. Diap2 is required for both local immune activation, taking place in the epithelial cells of the gut and trachea, and for mounting systemic immune responses in the cells of the fat body. We have found that transgenic expression of Diap2 leads to a spontaneous induction of NF-κB target genes, inducing chronic inflammation in the *Drosophila* midgut, but not in the fat body. Drice is a *Drosophila* effector caspase known to interact and form a stable complex with Diap2. We have found that this complex formation induces its subsequent degradation, thereby regulating the amount of Diap2 driving NF-κB signalling in the intestine. Concordantly, loss of Drice activity leads to accumulation of Diap2 and to chronic intestinal inflammation. Interestingly, Drice does not interfere with pathogen-induced signalling, suggesting that it protects from immune responses induced by resident microbes. Accordingly, no inflammation was detected in transgenic Diap2 flies and Drice-mutant flies reared in axenic conditions. Hence, we show that Drice, by restraining Diap2, halts unwanted inflammatory signalling in the intestine.

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INTRODUCTION

Innate immune responses are initiated by pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns and danger-associated molecular patterns. Activation of PRRs leads to the induction of microbicidal and pro-inflammatory responses, and culminates in elimination of the activating molecule and subsequent return to cellular homeostasis [1]. Proper regulation of inflammatory signalling is crucial as de-regulation at any step can be detrimental for the organism. One of the key players in the inflammatory response is the NF-κB family of transcription factors that regulate the expression of numerous inflammatory genes. Constitutively active NF-κB signalling is characteristic of chronic inflammation and increased NF-κB activity has been connected to irritable bowel diseases, such as ulcerative colitis and Crohn's disease [2, 3].

Inhibitor of apoptosis proteins (IAPs) influence ubiquitin-dependent pathways that modulate innate immune signalling by activation of NF-κB [4]. IAPs were first identified in insect baculoviruses as potent inhibitors of cell death [5, 6] and have subsequently been identified in both vertebrates and invertebrates. Cellular and viral IAPs are characterized by the presence of one or more caspase-binding Baculovirus inhibitor of apoptosis protein repeat (BIR) domains that are essential for their anti-apoptotic properties [6, 7], as well as a Really interesting new gene (RING) domain, providing them with E3 ligase activity [8]. *Drosophila* carries two *bona fide* IAP genes, *Drosophila* IAP (Diap) 1 and 2 [9]. Diap1 functions mainly as a suppressor of cell death, whereas Diap2, although also able to decrease the apoptotic threshold of the cell, has its main function in inflammatory signalling [10–13]. The most extensively studied mammalian IAPs, i.e. cellular IAP1/2 and X-chromosome-linked IAP (XIAP), and *Drosophila* Diap2 also harbour a Ubiquitin-associated (UBA) domain enabling them to interact with poly-ubiquitin chains [14, 15].

In contrast to mammals, *Drosophila* relies solely on an innate immune defence when combating pathogenic infections. One of the key components of the fly's immune system is the production and secretion of antimicrobial peptides (AMPs). AMP production is regulated by two NF-κB pathways, namely, the Toll pathway and the Immune deficiency (Imd) pathway [16]. Upon a gram-negative or gram-positive systemic infection, the Imd and Toll pathways, respectively, activate the production of AMPs in the fat body [17, 18]. However, during local immune responses in the gut, the Imd pathway solely controls the generation of AMPs [19, 20]. The Imd pathway is activated by PGRP-LCx receptors recognizing diaminopimelate (DAP)-type peptidoglycans, which are components of the cell wall of gram-negative bacteria [21–23]. Activation of PGRP-LCx leads to the recruitment of the adaptor proteins Imd and dFadd, and the initiator caspase Dredd [24]. Dredd-mediated cleavage of Imd exposes a conserved IAP-binding motif that recruits Diap2 to the complex, stimulating Diap2-mediated K63-linked ubiquitination (K63-Ub) of Imd, Dredd and the IKKγ Kenny [25–27]. Ubiquitination of Dredd is needed for cleavage and nuclear localization of the NF-κB protein Relish [26, 28], whereas the ubiquitination of Imd has been suggested to recruit the *Drosophila* mitogen-activated protein kinase kinase kinase dTak1 and the Relish kinase complex Ird5/Kenny to the Imd-signalling complex [29].

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Vertebrate and invertebrate organisms are in continuous contact with a diverse array of resident microorganisms [30]. One key interface for host–microbe interactions, in both humans and *Drosophila*, is the epithelial layer of the gut [30, 31]. In addition to being a platform for beneficial host-microbe interactions, the gut epithelium serves as the first line of defence against pathogens entering the body. In order for the organism to mount an efficient immune response against pathogenic bacteria, and simultaneously allow commensal bacteria to interact with the host, inflammatory signalling needs to be carefully regulated. Here, we report a novel role of the *Drosophila* caspase-3 homologue Drice as a negative regulator of the Imd pathway in the intestinal epithelium. Our results demonstrate that Drice restrains inflammatory signalling induced by commensal bacteria in the fly intestine by forming a complex with Diap2. This triggers proteasomal degradation of the Drice-Diap2 complex. We also show that transgenic expression of Diap2 leads to chronic inflammation only in the presence of commensal bacteria, which elucidates the need of Diap2 to be regulated in microbiotic environments.

MATERIAL AND METHODS

Fly husbandry, treatments and strains

Drosophila melanogaster was maintained at 25 °C with a 12-h light-dark cycle on Nutri-fly BF (Dutscher Scientific, Essex, UK) food. The *Canton^S* strain was used as wild type. In experiments with flies expressing genes under the UAS-Gal4 system, the Gal4 driver line was used as internal control. The driver lines *DaughterlessGal4* (*DaGal4*) and *NP1-Gal4*, the *Diptericin-LacZ* reporter line, and *Diap2^{7c}* mutants and transgenes were provided by Dr. François Leulier. The *UbiquitousGal4* (*UbiGal4*) strain was provided by Dr. Ville Hietakangas, and *Drice^{17}* mutants by Prof. Andreas Bergmann. *PGRP-LCΔ5* (stock #36323) and *UAS-p35* (stock #5073) strains were obtained from Bloomington *Drosophila* stock centre and *Drice-RNAi* (stock #28064) flies were from the Vienna *Drosophila* Resource Centre. V5-tagged *Drice^{WT}* and *Drice^{C211A}*, as well as untagged *Diap2^{WT}* and *Diap2$^{\Delta 100}$* were cloned into pUAST, and transgenic flies were generated by BestGene Inc. Axenic flies were reared germ-free according to the previously published protocol [32]. In short, fly embryos were de-chorionated using 2% active hypochlorite, and washed twice in 70% ethanol and sterile H_2O. After removal of the chorion, eggs were placed in autoclaved food and left to develop in a sterile environment. The hatched flies were confirmed to be axenic by 16S PCR and by growing fly homogenates on Luria Bertani (LB) plates and checking for bacterial growth. Inhibitor treatments in flies were done by feeding female adult flies for 16 h with 50 µM MG-132 (Sigma, St. Louis, Missouri, USA) or 50 µM Z-DEVD-FMK (BD Pharmingen, Franklin Lakes, New Jersey, USA) diluted in a 1:1 solution of LB media and 5% sucrose after a 2-h starvation.

Bacterial strains, infection and survival experiments

The gram-negative bacteria *Erwinia carotovora carotovora 15* (*Ecc15*) was kindly provided by Dr. François Leulier and the *Escherichia coli* (*E. coli*) Top10 strain was purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). *Ecc15* used for septic infection was cultivated in LB media at 29 °C for 16-18 h on agitation and concentrated (optical density of 200). To induce septic injury, adult flies were pricked in the lateral thorax with a needle previously dipped in concentrated *Ecc15* solution. For quantitative PCR (qPCR) 10 adult flies and for TUBE pulldowns 20 adult flies, were incubated 5 h at 25 °C after infection. For survival assays at least 20 adult flies were counted at indicated time points after infection. Infection experiments were excluded if more than 25% of the negative control strains survived bacterial infection or if AMP gene expression was significantly enhanced in these flies. In these cases, the bacterial potency was considered too low. Survival experiments in which wild-type flies survived to a lesser extent than 75%, were also excluded. These criteria were pre-established. For bacterial colony count, *E. coli* was transformed with pMT/Flag-His-amp and cultivated in ampicillin-containing LB medium at 37 °C for 16-18 h on agitation and concentrated by centrifugation (optical density of 100). After a 2-h starvation, adult flies were fed for 24 h with a 1:1 solution of transformed *E. coli* in 5% sucrose at 25 °C. Four flies were cleaned with ethanol and sterile H_2O, and homogenised in 300 µl PBS. Samples were diluted 1:1000 and plated on LB agar plates containing 50 µg/ml ampicillin and incubated 24 h at 37 °C, where after the colonies were counted.

Cell culture and transfection of *Drosophila* S2 cells

Drosophila Schneider S2 cells (Invitrogen, Waltham, Massachusetts, USA) were grown at 25 °C using Schneider's cell medium supplemented with 10% fetal bovine serum, 1% L-glutamine and 0.5% penicillin/streptomycin. S2 cells were transfected with indicated constructs using Effectene transfection reagent (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. For qPCR, western blotting and for GST-pulldown assays, respectively, 2×10^6 and 0.7×10^7 cells were seeded prior to transfection and the expression of pMT plasmids was induced with 500 µM $CuSO_4$ 16 h before lysis. For western blotting, transfected cells were harvested in ice cold PBS and lysed in Laemmli sample buffer. The caspase inhibitor Z-DEVD-FMK was used at 20 µM, 16 h before lysis.

Plasmids and antibodies

The plasmids pMT/V5His and pAc5/V5His (Invitrogen) were used as backbones for tag insertions and removals, and for subcloning of the constructs pMT/FlagHis, pMT/HAFlag, pAc5/Diap2, pMT/Dredd-V5His, pMT/Dredd-HAFlag, pMT/PGRP-LCx-Myc, pMT/Kenny-HA, pMT/Drice and pMT/ALG(p20/p10)Drice-V5Flag. The point mutation Drice^{C211A} was made by site-directed mutagenesis (Agilent Technologies, Santa Clara, California, USA or Stratagene, San Diego, California, USA). GST-TUBE (tandem ubiquitin entity) was provided by Dr. Mads Gyrd-Hansen. The following antibodies were used: α-K63 (clone Apu3, #05-1308, Millipore, Burlington, Massachusetts, USA), α-Diap2 [33], α-Drice [34], α-HA (clone 3F10, #11867423001, Roche, Basel, Switzerland), α-V5 (Clone SV5-Pk1, #MCA1360, Bio-Rad, Hercules, California, USA), α-Myc (#M4439, Sigma), α-phosphohistone H3 (PHH-3) (Ser10, #9701, Cell Signalling Technology, Danvers, Massachusetts, USA) and α-Actin (C-11, sc-1615, Santa Cruz Biotechnology, Dallas, Texas, USA).

Purification of GST-TUBE-fusion protein

GST-TUBE expression was induced in *E. coli* BL21 by the addition of 0.2 mM IPTG to an overnight culture of bacteria in LB medium at 18 °C. Bacteria were lysed by sonication in lysis buffer containing 50 mM Tris (pH 8.5), 150 mM NaCl, 3 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride and 0.2 mg/ml lysozyme. The lysate was added to a column with Glutathione Sepharose™ 4B (GE Healthcare, Chicago, Illinois, USA) and then washed with wash buffer containing 50 mM Tris (pH 8.5) and 150 mM NaCl. GST-TUBE was eluted in 50 mM Tris (pH 8.5), 150 mM NaCl, 10% glycerol, 3 mM DTT and 50 mM glutathione. The proteins were concentrated from the eluate using Amicon® Ultra-4 30 K centrifugal filter devices (Merck Millipore, Burlington, Massachusetts, USA).

Purification of ubiquitin conjugates from cells and fly lysates

Cells were lysed in a buffer containing 20 mM NaH_2PO_4, 20 mM Na_2HPO_4 1% NP-40 and 2 mM EDTA, and fly lysates in buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA and 10% glycerol, supplemented with 1 mM DTT, 5 mM NEM, Pierce™ Protease and Phosphatase Inhibitor, 5 mM chloroacetamide and 1% SDS. Lysates were sonicated, diluted to 0.1% SDS, and cleared before incubation with Glutathione Sepharose™ 4B (GE Healthcare) and GST-TUBE for a minimum of 2 h under rotation at 4 °C. The beads were washed four times with ice cold PBS-0.1% Tween-20 and eluted using Laemmli sample buffer.

Lysis of whole flies or fly organs for western blotting

Ten adult *Drosophila* flies, or twelve dissected intestines or carcasses from adult female flies, were homogenized and lysed 10 min on ice in a buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA and 10% Glycerol. The lysates were cleared before the addition of Laemmli sample buffer.

Quantitative RT-PCR (qPCR)

Ten *Drosophila* adult flies or transfected *Drosophila* S2 cells were homogenised using QIAshredder (QIAGEN) and total RNA was extracted with RNeasy Mini Kit (QIAGEN) according to the manufacturer's protocol. cDNA was synthesised with iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's protocol. qPCR was performed using SensiFAST™ SYBR Hi-ROX Kit (Bioline, London, UK). *rp49* was used as a housekeeping gene for ΔCt calculations. The following gene-specific primers were used

to amplify cDNA: *Diptericin* (5′-ACCGCAGTACCCACTCAATC-3′, 5′-ACTTTC CAGCTCGGTTCTGA-3′), *Drosocin* (5′-CGTTTTCCTGCTGCTTGC-3′, 5′-GGCAG CTTGAGTCAGGTGAT-3′), *rp49* (5′-GACGCTTCAAGGGACAGTATCTG-3′, 5′-AA ACGCGGTTCTGCATGAG-3′).

Immunofluorescence of *Drosophila* intestines

Intestines from three or more female adult flies were dissected in PBS and fixed for 10 min in 4% paraformaldehyde. Samples were permeabilised with PBS-0.1% Triton X-100, 1 h at room temperature, washed with PBS and incubated over night at 4 °C with primary antibody PHH-3 (Ser10, #9701, Cell Signalling Technology) at 1:1000. After washing, the intestines were incubated 2 h at room temperature with secondary antibody Alexa Fluor 488 donkey anti-rabbit IgG (#A21206, Invitrogen) at 1:600. Both primary and secondary antibodies were diluted in PBS and 1% bovine serum albumin. DNA was stained with DAPI (4′,6-diamidino-2-phenylindole) (Invitrogen). After washing with PBS, the samples were mounted using Mowiol (Sigma). Counting of PHH-3 positive cells was done using fluorescent microscopy (Zeiss Axiovert-200M microscope, Oberkochen, Germany)

X-gal staining of *Drosophila* intestines and carcasses

Intestines or carcasses from three or more adult female flies were dissected in PBS and fixed 15 min at room temperature with PBS containing 0.4% glutaraldehyde and 1 mM $MgCl_2$. The samples were washed with PBS and incubated with fresh staining solution containing 5 mg/ml X-gal, 5 mM potassium ferrocyanide trihydrate, 5 mM potassium ferrocyanide crystalline and 2 mg/ml $MgCl_2$ in PBS 1 h at 37 °C. After washing with PBS, the samples were mounted in Mowiol and imaged with bright field microscopy (Leica, Wetzlar, Germany).

Fluorometric measurement of caspase-3/7 activity and WST-1 assay

For fluorometric measurement of caspase-3/7 activity in flies, three dissected intestines or carcasses from adult female flies were lysed in buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA and Pierce™ Protease Inhibitor. The lysate was cleared at 12,000 rpm for 10 min at 4 °C and protein concentration adjusted with Bradford assay (Bio-Rad). For fluorometric measurement of caspase-3/7 activity in *Drosophila* S2 cells, 2×10^6 cells were transfected as described above. The caspase-3/7 activity of the cells and fly lysates was analysed using Apo-ONE® Homogenous Caspase-3/7 Assay (Promega, Madison, Wisconsin, USA) according to the manufacturer's protocol. Fluorescence was measured at 499/521 with the plate reader HIDEX sense (HIDEX, Turku, Finland). To measure cell viability, the WST-1 agent (Roche) was added to transfected cells in a ratio of 1:10. The absorbance was measured at 450 nm with the HIDEX plate reader after a 2-h incubation.

Sequencing of the 16S rRNA gene

Genomic DNA was isolated from 40 adult flies using a modified protocol for the QIAamp DNA mini kit (QIAGEN) [35]. Flies were surface sterilized by vortexing them twice in 2% active hypochlorite and sterile H_2O. The efficiency of the washes was confirmed by 16S PCR of water from the last wash step. Flies were homogenized in lysis buffer containing 20 mM Tris, pH 8.0, 2 mM EDTA, 1.2% Triton X-100 and 20 mg/ml lysozyme, and incubated 90 min at 37 °C. Overall, 200 µl AL buffer (QIAamp DNA mini kit) with 20 µl proteinase K were added and the lysate was incubated 90 min at 56 °C. Subsequent extraction was performed according to the manufacturer's protocol. Amplification and Illumina MiSeq sequencing of the V1-V3 region of the 16S rRNA gene, as well as selection of operational taxonomic units (OTUs) and taxonomy assignment of OTUs was done using Eurofins Genomics InView Microbiome Profiling 3.0 service. In order to ensure similar *Wolbachia* status of both control and mutat fly line, the *Wolbachia* positive or negative state of the sequenced fly lines was tested by PCR with the *Wolbachia* specific primers: 5′-GWATTACCGCGGCKGCTG-3′ and 5′-AGAGTTTGATCCTGGCTCAG-3′ prior to sequencing. *Canton^S* and *UbiGal4 > Diap2^{WT}* flies were positive for *Wolbachia*. The proportion of *Wolbachia* species have been omitted in Fig. 1F for easier comparison of bacterial species residing in the gut lumen.

Statistical analysis

Results from survival assays were analysed by two-way analysis of variance with Tukey's post hoc test for 95% confidence intervals and results from qPCR by two-tailed Student's *t*-test on the ΔCt value, graphs depict relative fold induction of the target gene compared to a normalised control sample. The number of PHH-3 positive cells, relative fluorescence units, and the relative protein expression from western blots measured with ImageJ, were analysed by two-tailed Student's *t*-test. In comparison to normalised control values, the Mann–Whitney U test was applied. In figures, ns stands for $p > 0.05$, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$. Error bars in figures specify ±SEM from the indicated number of independent experiments. Experiments were repeated at least three times.

RESULTS

Diap2 induces chronic inflammation and hyperplasia in the fly intestine

The *Drosophila* IAP protein Diap2 is required for inducing the Relish-dependent Imd pathway and mounting immune responses upon gram-negative bacterial infection, both locally in the epithelial layers of the gut and trachea, and upon systemic infections in the cells of the fat body [10, 12]. However, it is still unknown how the activity of Diap2 is regulated during basal conditions and upon pathogen-induced infections. To investigate how Diap2-mediated inflammatory responses are regulated, we analysed the impact of Diap2 on inflammatory NF-κB activation. For this purpose, we made transgenic flies, in which expression of Diap2 was induced via the UAS-Gal4 system (Fig. 1A). Analysis of the expression of the Relish target genes *Drosocin* and *Diptericin* showed that these AMPs are induced in the Diap2-expressing flies compared to *Canton^S* and *Ubiquitin-Gal4* (*UbiGal4*) control flies (Fig. 1B). To investigate the origin of Diap2-induced inflammation, we used the *Diptericin-LacZ* reporter system to compare *Diptericin* expression in the two major immune organs of the fly, the gut and the fat body [17]. We found that transgenic expression of Diap2 leads to a spontaneous induction of *Diptericin* in the *Drosophila* midgut, but not in the fat body (Fig. 1C, D).

Intestinal inflammation is associated with midgut hyperplasia and proliferating cells can be detected in *Drosophila* by staining the proliferation marker PHH-3 [36]. We found that Diap2-expressing flies have a significantly increased number of proliferating cells in the midgut compared to control flies (Fig. 1E). Another factor associated with chronic inflammation is dysbiosis of the gut microbiome. When profiling the bacterial composition of wild type and Diap2 transgenic flies by 16S sequencing, we found the Diap2-expressing flies to have a higher ratio of *Proteobacteria* to *Firmicutes* compared to control flies (Fig. 1F), a notion that has been associated with increased gut inflammation in both flies and humans [37, 38].

Finally, as Diap2 overexpression leads to different inflammatory phenotypes in gut and fat body, we compared the endogenous expression of Diap2 in these organs of *Canton^S* wild type flies. We were able to detect Diap2 in fat body samples, but not in the gut (Fig. 1G). However, when examining the protein levels of Diap2 in Diap2 transgenic flies, we were able to detect, in addition to full-lenght Diap2 in the fat body, both a full-length and a truncated version of Diap2 in the intestine (Fig. 1H), indicating that increased expression of Diap2 induces inflammation only in the intestine.

Drice restrains intestinal activation of the Imd pathway and gut hyperplasia

Caspases belong to a family of conserved cysteine-dependent endoproteases that cleave their substrates after specific aspartic residues [39]. The *Drosophila* effector caspase Drice is one of the key inducers of apoptosis in the fly [40]. Diap2 and Drice have been shown to interact and form a stable complex, wherein Diap2 inhibits the activity of Drice by binding to, and ubiquitinating the caspase. This inhibition is mechanism-based, requiring Drice activity, and results in cleavage of Diap2 [41]. As the truncated

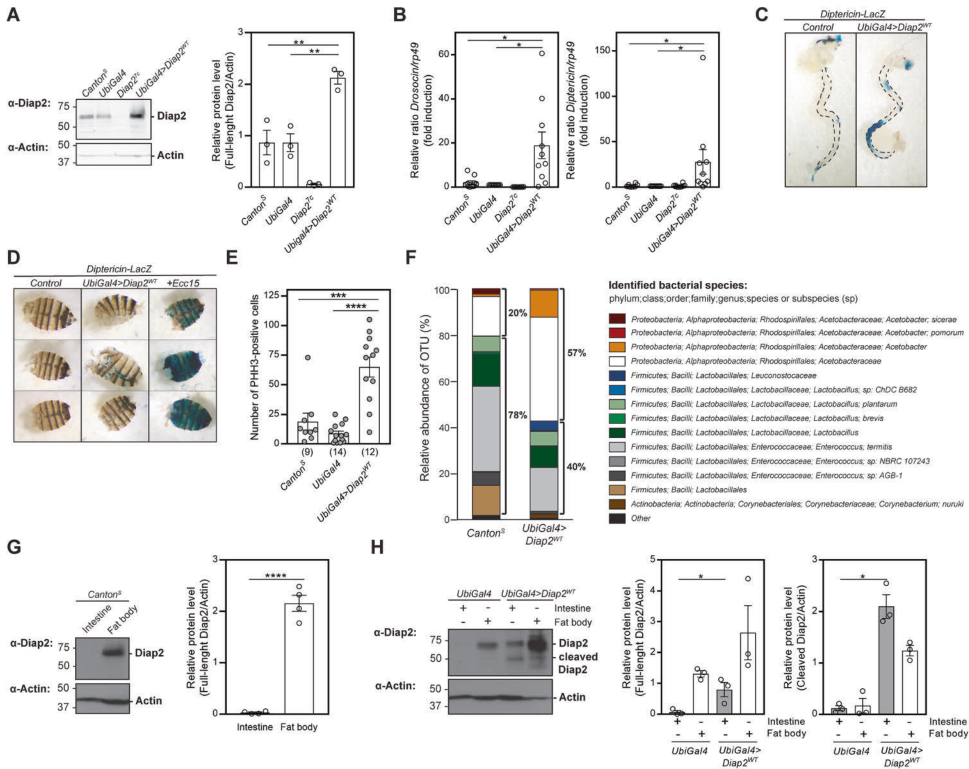


Fig. 1 Diap2 induces chronic inflammation and hyperplasia in the fly intestine. **A** Whole fly lysates from *Canton^S*, *UbiGal4*, *Diap2^7c* and *UbiGal4;UAS-Diap2^WT* flies were analysed by western blotting with α-Diap2 and α-Actin antibodies, and the relative protein level of Diap2 was quantified, $n = 3$. **B** Relative *Drosocin* and *Diptericin* mRNA levels analysed with qPCR in adult *Canton^S*, *UbiGal4*, *Diap2^7c* and *UbiGal4;UAS-Diap2^WT* flies, $n = 10$. Adult female intestines (**C**) and carcasses (**D**) from *Diptericin-LacZ* and *UbiGal4;UAS-Diap2^WT/Diptericin-LacZ* flies were dissected and stained for β-galactosidase activity, $n = 3$. The last lane in **D** shows a positive control for fat body activation induced by septic infection with *Ecc15*. **E** Intestines from adult *Canton^S*, *UbiGal4* and *UbiGal4;UAS-Diap2^WT* flies were dissected and stained for phosphohistone H3 (PHH-3), and the number of positive cells were counted. The number of intestines analysed is indicated in brackets. **F** Bacterial 16S rRNA metagenomics analysis of the 1V-3V region in *Canton^S* and *UbiGal4;UAS-Diap2^WT* flies. Colours indicate identified operational taxonomic units (OTUs). Black brackets indicate proportions of *Proteobacteria* and *Firmicutes*, $n = 1$. Intestines and fat bodies from adult female *Canton^S* (**G**) or *UbiGal4* and *UbiGal4;UAS-Diap2^WT* (**H**) flies were dissected and lysed, and analysed by western blotting with α-Diap2 and α-Actin antibodies, $n \geq 3$. The relative protein levels of full-length Diap2 (**G**) and full-length and cleaved Diap2 (**H**) were quantified. Data represent mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

form of Diap2 expressed in the intestine of Diap2 transgenic flies corresponds in size to the Drice-cleaved form of Diap2, we wanted to investigate a possible role for Drice in the regulation of Diap2-mediated inflammatory signalling. For this purpose, we measured the expression of NF-κB target genes in *Drice^17* mutant flies and in *Drice-RNAi* flies. *Drice^17* flies encode an unstable form of the Drice protein, reported to express less than 5% of the levels of Drice in wild-type flies [42], and neither *Drice^17* nor *Drice-RNAi* flies have detectable expression of the protein in our study (Fig. S1A, B). We detected a significantly higher expression of the Relish target genes *Drosocin* and *Diptericin* during basal conditions in whole fly lysates from both *Drice^17* (Fig. 2A) and *Drice-RNAi* flies (Fig. 2B) and, accordingly, a lower basal *Drosocin* and *Diptericin* expression in flies overexpressing Drice (Fig. 2C), suggesting that Drice acts as a negative regulator of Imd signalling.

To investigate if the Drice-mediated regulation of the Imd pathway is tissue-specific, we examined *Diptericin* expression in the gut and fat body of *Drice-RNAi* flies carrying the *Diptericin-LacZ* reporter gene. Similarly to Diap2 overexpression, loss of *Drice* induced *Diptericin* expression in the midgut but not in the fat body (Fig. 2D, E). Interestingly, active Drice, assessed by measuring DEVD-activity, was also detected only in dissected fly guts and not in the fat body (Fig. 2F). As in the Diap2-expressing flies, we found *Drice^17* mutant flies to have a significantly increased number of proliferating cells in the midgut, compared to *Canton^S* control flies (Fig. 2G). Further, *Drice-RNAi* flies have a higher ratio of *Proteobacteria* to *Firmicutes* compared to *UbiGal4* driver flies (Fig. 2H). Taken together, these results show that Drice is a negative regulator of Imd signalling, needed for maintaining gut homeostasis.

Finally, Diap2 is stabilised in the intestine of *Drice-RNAi* flies, suggesting that Drice and the formation of the Drice-Diap2 complex regulate the levels of Diap2 in the *Drosophila* intestine (Fig. 2I). In addition, we found that inhibiting the proteasome by

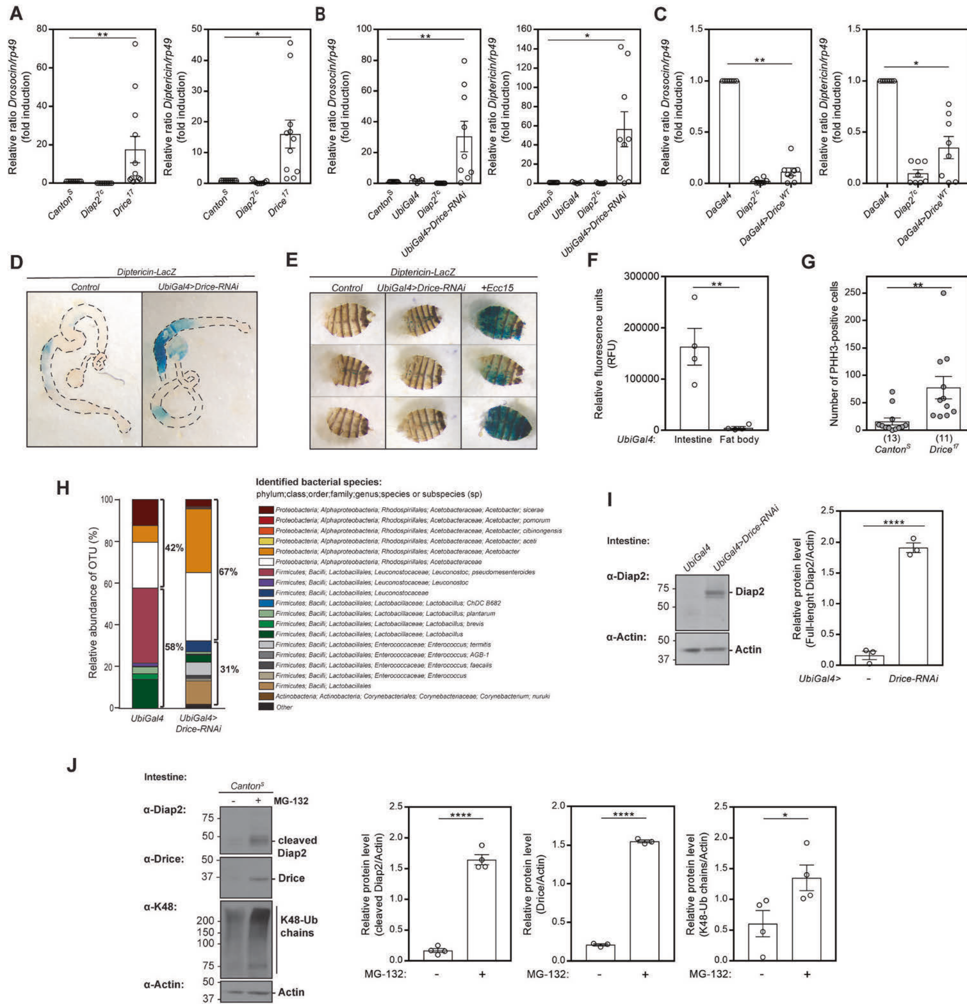


Fig. 2 Drice restrains intestinal activation of the Imd pathway and gut hyperplasia. Relative *Drosocin* and *Diptericin* mRNA levels analysed with qPCR in adult *Canton*^{S}, *Diap2*^{7c} and *Drice*^{17} flies (**A**), in *Canton*^{S}, *UbiGal4*, *Diap2*^{7c} and *UbiGal4;UAS-Drice-RNAi* (**B**) flies or in *DaGal4*, *Diap2*^{7c} and *UAS-Drice*^{WT}*;DaGal4* flies (**C**), $n \geq 8$. Adult female intestines (**D**) and carcasses (**E**) from *Diptericin-LacZ* and *UbiGal4;UAS-Drice-RNAi/Diptericin-LacZ* flies were dissected and stained for β-galactosidase activity, $n = 3$. The last lane in **E** shows a positive control for fat body activation induced by septic infection with *Ecc15*. **F** Adult female intestines or fat bodies from *UbiGal4* flies were dissected and lysed, and the caspase-3/7 activity was assessed after addition of Apo-ONE reagent by measuring fluorescence at 499/521 nm, $n = 4$. **G** Intestines from adult *Canton*^{S} and *Drice*^{17} flies were dissected and stained for phosphohistone H3 (PHH-3), and the PHH-3 positive cells were counted. The number of intestines analysed is indicated in brackets. **H** Bacterial 16S rRNA metagenomics analysis of the 1V-3V region in *UbiGal4* and *UbiGal4;Drice-RNAi* flies. Colours indicate identified operational taxonomic units (OTUs). Black brackets indicate proportions of *Proteobacteria* and *Firmicutes*, $n = 1$. **I** Intestines from adult *UbiGal4* and *UbiGal4;UAS-Drice-RNAi* were dissected and lysed, and analysed by western blotting with α-Diap2 and α-Actin antibodies, $n = 3$, and the relative protein level of full-length Diap2 was quantified. **J** Adult *Canton*^{S} flies were fed 50 µM MG-132, their intestines were dissected and analysed by western blotting with α-Diap2, α-Drice, α-K48 and α-Actin antibodies, $n \geq 3$. The relative protein levels of cleaved Diap2, Drice and K48-Ub chains were quantified. Data represent mean ± SEM, $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$.

feeding flies MG-132 leads to a stabilization of Drice, the cleaved form of Diap2, as well as an increase in K48-linked chains in the intestine (Fig. 2J). This indicates that formation of the Drice-Diap2 complex induces degradation of both proteins in a manner that is preceded by cleavage of Diap2.

The catalytic activity of Drice is required for Imd pathway regulation in the intestine

To investigate whether the regulation of Diap2 is reliant of the catalytic activity of Drice, we generated transgenic flies expressing Drice^{WT} or the catalytically inactive Drice^{C211A} mutant. While *Drice-*

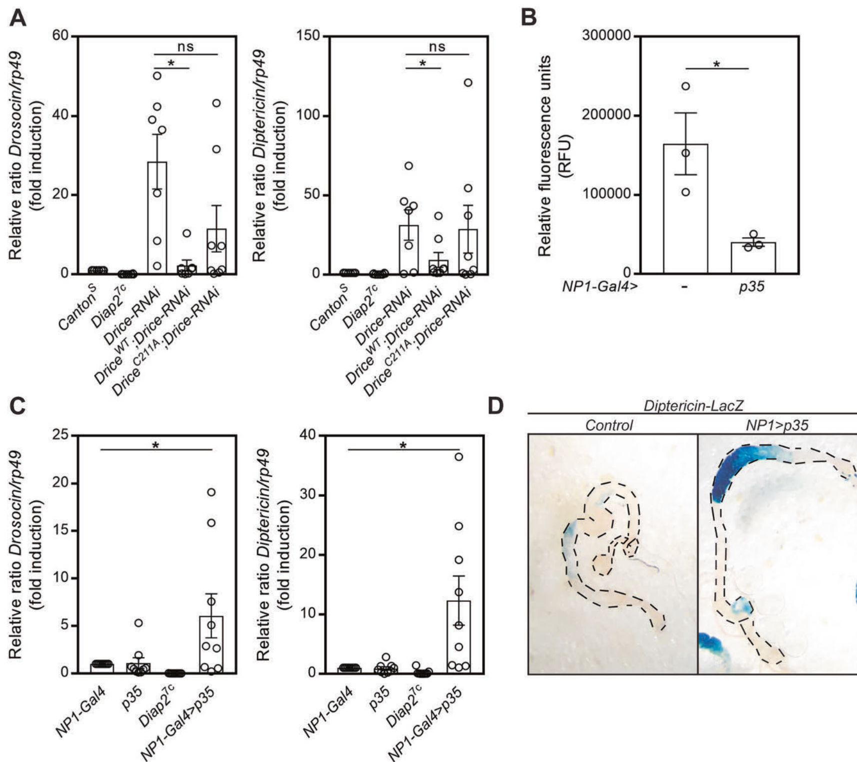


Fig. 3 The catalytic activity of Drice is required for Imd pathway regulation in the intestine. A Relative *Drosocin* and *Diptericin* mRNA levels analysed with qPCR in adult *Canton*^{S}, *Diap2*^{7c}, *UbiGal4;UAS-Drice-RNAi*, *UAS-Drice*^{WT}*/UbiGal4;UAS-Drice-RNAi* and *UAS-Drice*^{C211A}*/UbiGal4;UAS-Drice-RNAi* flies, $n = 7$. **B** Adult female intestines from *NP1-Gal4* and *NP1-Gal4;UAS-p35* flies were dissected and lysed, and the caspase-3/7 activity was assessed after addition of Apo-ONE reagent by measuring fluorescence at 499/521 nm, $n = 3$. **C** Relative *Drosocin* and *Diptericin* mRNA levels analysed with qPCR in adult *NP1-Gal4*, *UAS-p35*, *Diap2*^{7c} and *NP1-Gal4;UAS-p35* flies, $n = 9$. **D** Adult female intestines from *DaGal4, Diptericin-LacZ* and *NP1-Gal4;UAS-p35/Diptericin-LacZ* flies were dissected and stained for β-galactosidase activity, $n = 3$. Data represent mean ± SEM, $*p < 0.05$.

RNAi eliminated the endogenous Drice, the transgenic expressed Drice remained high in a *Drice-RNAi* background (Fig. S2). When we analysed the expression of the Relish target genes *Drosocin* and *Diptericin* in whole fly lysates during basal conditions, we found that only Drice^{WT}, but not Drice^{C211A}, is able to restrain the AMP expression induced by loss of Drice (Fig. 3A).

To verify that Drice is the caspase regulating Diap2, we expressed the effector caspase inhibitor p35 in the intestinal epithelial cells with the enterocyte-specific driver *NP1-Gal4*. When cleaved by a caspase, the viral caspase inhibitor p35 acts as a suicide substrate by trapping the catalytic machinery of the caspase via a covalent thioacyl linkage [43]. p35 has been shown to inhibit the *Drosophila* effector caspases Drice and Dcp-1 [44], which both recognise the DEVD amino acid sequence [34, 40]. However, Drice is shown to be the only *Drosophila* effector caspase to interact with Diap2 [45]. We found that expression of p35 in intestinal enterocytes led to reduced effector caspase activity (Fig. 3B) and increased expression of the Relish target genes *Drosocin* and *Diptericin* (Fig. 3C). Likewise, a local induction of *Diptericin* expression in the intestine was detected in *Diptericin-LacZ* flies expressing the p35 caspase inhibitor (Fig. 3D). Our results indicate, thus, that the catalytic cysteine and, thereby, the covalent bond formed between Diap2 and Drice is needed for Drice to regulate the activity of the Imd pathway, and that p35, by trapping the catalytic cysteine of Drice, interferes with the ability of Drice to restrain Imd signalling. Although the initiator caspases Dredd and Dronc have been shown to interact with Diap2 [46, 47], neither Dredd nor Dronc is inhibited by p35 [44, 48], suggesting that Drice alone inhibits Diap2-mediated activation of Relish target genes by interacting with Diap2.

Drice regulates Diap2 levels and ubiquitination of Dredd and Kenny

Diap2-mediated K63-Ub of the Imd pathway components Imd and Dredd has been shown to be required for Imd signalling, additionally the *Drosophila* NEMO, IKKγ or Kenny, has recently been identified as a target of K63-Ub mediated by Diap2 [25–27]. To investigate if inhibition of Drice in the intestine affects Diap2-mediated ubiquitination, we fed flies with the cell-permeable caspase-3 inhibitor Z-DEVD-FMK. Indeed, a local intestinal inhibition of Drice led to the accumulation of full-length Diap2 in gut samples (Fig. 4A) and to a Diap2-dependent increase in K63-linked ubiquitin chains pulled down with recombinant GST-TUBE from whole fly lysates (Fig. 4B). To investigate further whether Drice interferes with the ability of Diap2 to ubiquitinate Dredd and Kenny, we co-transfected S2

Fig. 4 Drice regulates Diap2 levels and ubiquitination of Dredd and Kenny. A Adult *Canton^S* flies were fed 50 μM Z-DEVD-FMK, their intestines were dissected, lysed and subjected to western blotting analysis with α-Diap2, α-K63 and α-Actin antibodies, $n = 3$. The relative protein levels of full-length Diap2 and K63-linked ubiquitin chains were quantified. **B** *Canton^S* and *Diap2^{7c}* flies were fed 50 μM Z-DEVD-FMK and lysed. Ubiquitin chains were isolated with GST-TUBE under denaturing conditions and the samples were analysed by western blotting with α-K63, α-Diap2, α-Drice and α-Actin antibodies, $n = 3$. The relative protein level of K63-Ub chains was quantified. *Drosophila* S2 cells were transfected with empty vector, Diap2^{WT}, Drice^{WT}, Drice^{C211A}, V5-tagged Dredd (**C**) or with HA-tagged Kenny (**D**), where after the cells were treated with 20 μM Z-DEVD-FMK. Ubiquitin chains were isolated with GST-TUBE at denaturing conditions and the samples were analysed by western blotting with α-V5, α-HA, α-Diap2, α-K63 and α-Actin antibodies, $n = 3$. The relative protein level of ubiquitinated Dredd or Kenny was quantified. Data represent mean ± SEM, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

cells with Diap2^{WT} and Dredd-V5 or Kenny-HA, while inducing Drice activity by overexpressing Drice, or interefering with the catalytic activity by overexpressing Drice^{C211A} or treating with the inhibitor Z-DEVD-FMK (Fig. S3A, B). Importantly, by measuring the activity of mitochondrial dehydrogenases with the WST-1 assay, we verified that cell viability was not affected by overexpression of Drice^{WT} or Drice^{C211A} in S2 cells (Fig. S3C). Ubiquitin chains were pulled down with recombinant GST-TUBE from cell lysates under denaturing conditions. When overexpressing Diap2^{WT} alone, we could detect ubiquitin smears on

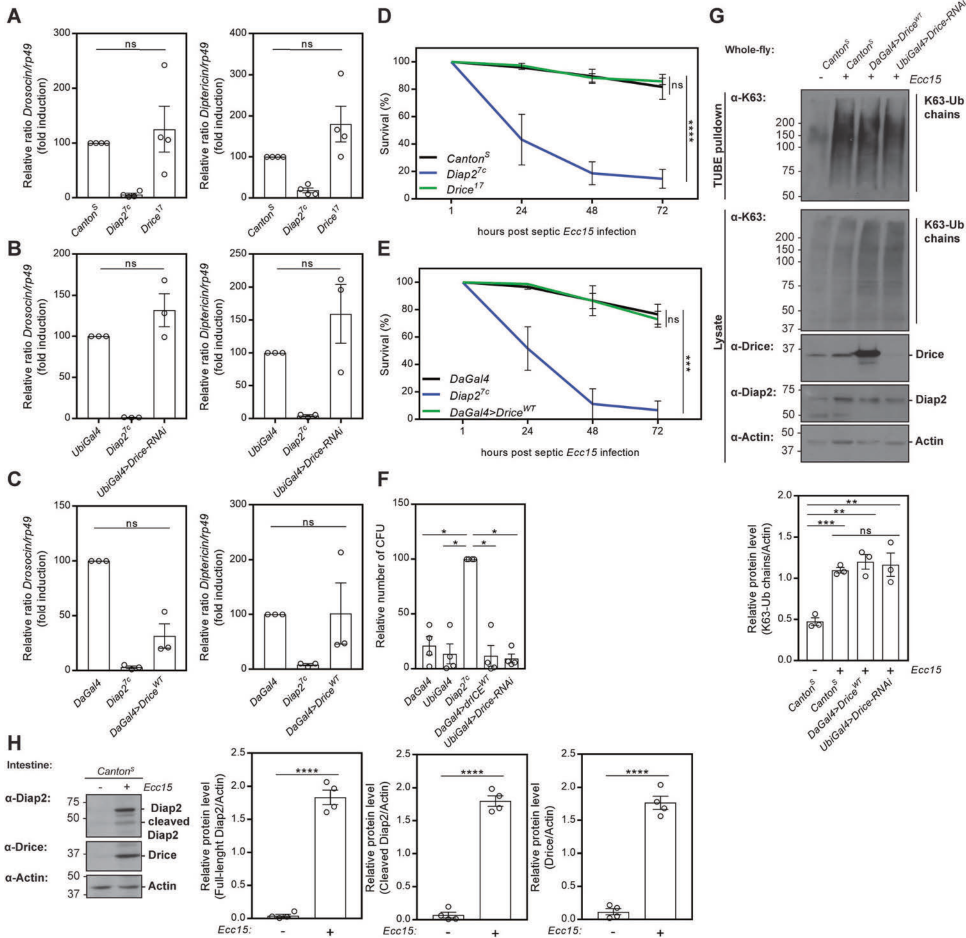
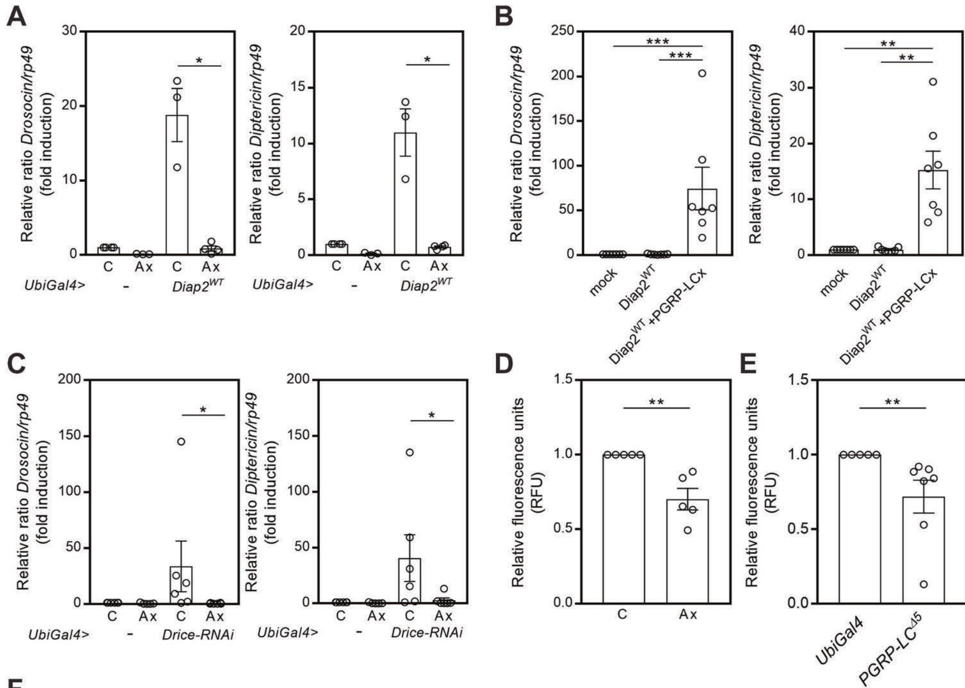


Fig. 5 Drice does not restrain pathogen-induced inflammatory signalling. Relative *Drosocin* and *Diptericin* mRNA levels analysed with qPCR in *Canton^S*, *Diap2^{7c}*, *Drice^{17}* (**A**) *UbiGal4;UAS-Drice-RNAi* (**B**) and in *DaGal4*, *Diap2^{7c}* and *UAS-Drice^{WT};DaGal4* (**C**) flies 5 h after septic infection with the gram-negative bacteria *Ecc15*, $n \geq 3$. Adult *Canton^S*, *Diap2^{7c}* and *Drice^{17}* (**D**) or *DaGal4*, *Diap2^{7c}* and *UAS-Drice^{WT};DaGal4* (**E**) flies were subjected to septic injury with *Ecc15* and their survival was monitored over time, $n = 3$. **F** *DaGal4*, *UbiGal4*, *Diap2^{7c}*, *UAS-Drice^{WT};DaGal4* and *UbiGal4 > Drice-RNAi* flies were infected by feeding with *E. coli* for 24 h and the bacterial load was assessed by counting colony-forming units (CFU), $n = 4$. **G** The induction of K63-Ub chains was analysed in *Canton^S*, *UAS-Drice^{WT};DaGal4* and *UbiGal4 > Drice-RNAi* flies 5 h after septic infection with *Ecc15*. Ubiquitin chains were isolated with GST-TUBE under denaturing conditions and samples analysed by western blotting with α-K63, α-Diap2, α-Drice and α-Actin antibodies, $n = 3$. The relative protein level of K63-Ub chains was quantified. **H** Adult flies were infected by feeding *Ecc15* 16 h, their guts were dissected, lysed and used in western blot analysis with α-Drice, α-Diap2 and α-Actin antibodies, $n = 4$. The relative protein levels of full-length Diap2, cleaved Diap2 and Drice were quantified. Data represent mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

both Dredd and Kenny (Fig. 4C, D, lanes 2). Co-expression with Drice^{WT} reduced the amount of Diap2, and also the ubiquitination of Dredd and Kenny (Fig. 4C, D, lanes 3). Likewise, overexpression of catalytically inactive Drice^{C211A}, and treatment with Z-DEVD-FMK that stabilized full-length Diap2, resulted in enhanced ubiquitination of Dredd and Kenny (Fig. 4C, D, lanes 4 and 5). This indicates that the levels of Diap2 can be regulated by exogenous manipulation of Drice activity in S2 cells, restraining the ability of overexpressed Diap2 to ubiquitinate the Imd pathway inducers Dredd and Kenny.

Drice does not restrain pathogen-induced inflammatory signalling

As Drice seems to restrain Diap2 from inducing inflammatory signal transduction, the immune response upon septic pathogen-infection was tested in Drice-overexpressing and in Drice-mutant flies. Interestingly, *Canton^S* control flies, *Drice^{17}*, *Drice-RNAi* flies, as well as flies overexpressing wild-type Drice (Fig. 5A–C) showed a similar induction of *Drosocin* and *Diptericin* 5 h after septic infection with *Ecc15*. Furthermore, when monitoring the survival of flies, neither control flies, *Drice^{17}* flies, nor *Drice*-overexpressing



flies, succumb to septic infection with *Ecc15* (Fig. 5D, E). To investigate if the Drice-mediated regulation of Diap2 affected local immune signalling in the intestine, we analysed the response to ingested pathogen. By performing a bacterial colony count after a 24-h feeding on *E. coli*, we found that both Drice-overexpressing flies and *Drice-RNAi* flies were able to fend off pathogens ingested with food, like control flies (Fig. 5F). In addition, K63-Ub was induced to similar level in Drice-overexpressing flies, *Drice-RNAi* flies and wild type *Canton^{S}* flies upon septic infection with *Ecc15* (Fig. 5G). Likewise, no reduction of Diap2 levels nor a significant decrease in Diap2-mediated ubiquitination of Dredd and Kenny could be detected upon Drice^{WT} overexpression after activating the Imd pathway by overexpression of PGRP-LCx in S2 cells (Fig. S4A, B). These results suggest that Drice does not inhibit the

Fig. 6 Drice restrains inflammatory signalling induced by the resident microbiome. A Relative *Drosocin* and *Diptericin* mRNA levels were analysed by qPCR in conventionally reared (C) or axenic (Ax) adult *UbiGal4* and *UbiGal4;UAS-Diap2*^{WT} flies, $n = 3$. **B** Relative *Drosocin* and *Diptericin* mRNA levels were analysed by qPCR from *Drosophila* S2 cells transfected with empty vector, Diap2^{WT} and PGRP-LCx, $n = 7$. **C** Relative *Drosocin* and *Diptericin* mRNA levels analysed with qPCR in conventionally reared (C) or axenic (Ax) adult *UbiGal4* and *UbiGal4;UAS-Drice-RNAi* flies, $n = 6$. **D** Caspase-3/7 activity in adult female guts of conventionally reared (C) or axenic (Ax) *yellow white* control flies was analysed by measuring fluorescence at 499/521 nm after addition of Apo-ONE reagent, $n = 5$. **E** Caspase-3/7 activity in adult female guts of *PGRP-LC*$^{\Delta 5}$ mutant flies and *UbiGal4* control flies was analysed by measuring fluorescence at 499/521 nm after addition of Apo-ONE reagent, $n = 5$. Data represent ∓ SEM, **p* < 0.05, ***p* < 0.01, ****p* < 0.001. **F** Proposed model for Drice-mediated regulation of Imd signalling. PGN from the cell wall of commensal bacteria induces a low activation of the PGRP-LCx receptor, leading to the recruitment of Imd, dFadd and Dredd to the receptor complex. To regulate NF-κB activation, active Drice binds to Diap2 to form the Drice-Diap2 complex, which is subsequently targeted for proteasomal degradation. As a consequence, ubiquitination of Kenny and Dredd is decreased. In the absence of Drice, uncontrolled activation of intestinal Imd-signalling leads to excessive levels of AMPs in the gut lumen and a disturbed gut homeostasis. Unrestrained Diap2 drives Imd sigalling by ubiquitinating Imd, Dredd and Kenny. Ubiquitination of Imd enables recruitment of the dTab2-dTak1 complex, and dTak1-mediated activation of Ird5 by phosphorylation. Ird5 in turn phosphorylates Relish. Ubiquitination-dependent activation of Dredd and Dredd-mediated cleavage of Relish precedes translocation of the Relish dimer to the nucleus and subsequent activation of Relish target genes.

induction of Diap2-mediated ubiquitination and activation of Imd signalling during infection. Supporting this, pathogenic infection coincides with stabilization of full-length Diap2, cleaved Diap2 and Drice in the gut, indicating that the presence of pathogens leads to the disruption of the intestinal Drice-Diap2 complex, freeing both proteins (Fig. 5H). This would indicate that Drice is able to regulate only basal immune responses.

While Diap2 cleavage is a consequence of interaction with Drice, we wanted to examine if cleavage of Diap2, and, thereby, separation of the BIR1 domain, also reduces its activity in the Imd pathway. However, both flies expressing Diap2^{WT} and flies expressing constitutively cleaved Diap2$^{\Delta 100}$ in a *Diap2*^{7c} mutant background were able to induce AMPs and survive upon septic infections, and clear pathogens locally in the intestine similarly to control flies (Fig. S4C, D, E). These results indicate that the Drice-cleaved form of Diap2, indeed still harbouring BIR2 and BIR3, mediating Imd and Dredd binding [25, 26], a UBA and a RING domain, is a functional mediator of Imd signalling. Hence, we suggest that the role of Drice is to induce cleavage-mediated degradation of Diap2 in the absence of infection.

Drice restrains inflammatory signalling induced by the resident microbiome

As the bacterial presence is constant in the gut, and the fat body only encounters bacteria during a systemic infection [49], we hypothesized that the commensal microbiome activates Diap2-mediated Imd-signalling, which in the absence of Drice, leads to an excessive inflammatory response. To eliminate the commensal intestinal microbiome, we reared flies under axenic conditions. As expected, the expression of *Drosocin* and *Diptericin* was no longer elevated in axenic Diap2^{WT} expressing flies (Fig. 6A). Likewise, overexpression of Diap2^{WT} in S2 cells was not able to induce AMP expression in the absence of receptor activation (Fig. 6B). The increased expression of *Drosocin* and *Diptericin* detected in *UbiGal4 > Drice-RNAi* flies was, as in Diap2^{WT} expressing flies, significantly decreased by rearing the flies under axenic conditions (Fig. 6C), indicating a link between commensal bacteria and the requirement for Drice-mediated regulation of Imd signalling.

Interestingly, Diap2 is undetectable in the guts of axenic wild-type flies (Fig. S5), indicating that degradation of the Diap2-Drice complex is continuous also in a bacteria-free environment. This is further supported by a reduction in caspase activity in the intestines of both wild-type axenic and PGRP-LCx mutant flies, compared to conventionally reared flies or control flies, respectively (Fig. 6D, E). Taken together, we propose that commensal bacteria trigger the formation of an initial PGRP-LCx receptor complex, competing for Diap2-recruitment to further activate the Imd pathway. The inflammatory response is counteracted by Drice binding to, and forming a complex with Diap2 that is subsequently targeted for degradation. Hence, Drice interferes with the ability of Diap2 to induce downstream signalling and NF-κB target gene activation under basal conditions (Fig. 6F).

DISCUSSION

Caspases were first identified as regulators of apoptosis, but their regulatory functions now extend to other cellular processes, such as immune signalling and development. Dysregulation of caspases has been implied in tumorigenesis, autoimmunity, autoinflammation and infectious pathologies [39, 50, 51]. Caspases are known to be regulated by IAP proteins, however, caspase-mediated regulation of IAP proteins during inflammatory signalling has not been established before. Here, we report that the *Drosophila* caspase Drice functions as a negative regulator of intestinal immune responses regulated via the *Drosophila* NF-κB Relish. While Drice does not seem to impact pathogen-induced immune signalling, it restrains responses induced by commensal bacteria by binding Diap2, leading to the degradation of both proteins. This indicates that Drice halts NF-κB signalling by trapping Diap2 to a degradation complex during basal conditions, and that a pathogenic infection leads to complex disruption and freeing of a functional Diap2. The constitutive interaction between Diap2 and Drice in the intestinal epithelia may also contribute to the regulation of caspase activation to avoid apoptosis-induced cell proliferation [52].

We suggest that the low activation of PGRP-LCx induced by commensal bacteria is sufficient to induce Diap2-mediated Relish activation in the absence of a brake. In the intestinal epithelia, the brake is provided by the effector caspase Drice, halting unwanted inflammatory responses. Interestingly, while Drice transcription is moderate or low in most *Drosophila* tissues during normal conditions, including the fat body, transcription of Drice is high in the adult *Drosophila* intestine [53]. This indicates a specific need for continuous expression of Drice to overcome the constant Drice turnover, protecting from unwanted Diap2-induced inflammatory responses in the intestinal cells. As Diap2 has been shown to ubiquitinate Drice [41], and itself [26], we speculate that formation of a Diap2-Drice complex induces Diap2-mediated K48-linked ubiquitination of both proteins, targeting them for proteasomal degradation. Further characterization of this mechanism and identification of the E2 ubiquitin-conjugating enzymes involved is, however, needed.

While Drice and Diap2 are targeted for degradation during normal conditions, they both are stabilised upon activation of the PGRP-LCx receptor. We suggest that the receptor stimulation leads to disruption of a pre-existing Drice-Diap2 complex, as also the Drice-cleaved form of Diap2 is stabilised. However, it cannot be excluded that Diap2 is released from Drice due to competition by the activated receptor complex. Regardless, we suggest that the released Diap2 is redirected for ubiquitination of new target substrates during infection. A similar IAP-induced shift in target

ubiquitination upon receptor activation has been shown in non-canonical NF-κB signalling, where cIAPs switch from degradation-inducing ubiquitination of the NF-κB-inducing kinase (NIK) to ubiquitination of TRAF2/3 upon receptor activation, releasing NIK to activate downstream signalling [54].

While the Drice-Diap2 complex formation leads to the degradation of both proteins, Diap2 cleavage, separating the BIR1 domain is also a consequence of this interaction [41]. The Diap2 homologue XIAP is a key regulator of NOD2-induced inflammatory signalling in the mammalian intestine, shown to induce the pathway by ubiquitinating RIPK2 [55, 56]. Interestingly, a nonsense mutation E99X in XIAP that introduces a stop codon after the BIR1 domain was found in an early onset Crohn's disease patient. This mutation induced a severe and selective defect in intestinal NOD signalling, without affecting immune signalling in T cells and peripheral blood mono-nuclear cells [57]. This suggests that a separated BIR1 domain is associated with impaired NF-κB signalling also in mammalian intestinal cells. The BIR1 domain has been shown to regulate XIAP-mediated NF-κB signalling, by bringing TAB1 and XIAP together, leading to activation of TAK1 [58]. Hence, it will be interesting to study if the separated BIR1 of Diap2 mediates NF-κB responses via, the yet unidentified, *Drosophila* TAB1-homologue in the epithelial cells of the fly intestine.

Intestinal epithelial cells coexist with commensal bacteria and need to develop tolerance to pattern recognition to allow for healthy host–microbe interactions. However, these epithelial cells also need to maintain responsiveness to foodborne pathogens. Hence, proper regulation of NF-κB signalling is crucial to avoid deregulated immune responses and chronic inflammation deleterious for the host [59]. We propose that IAP regulation may provide a mechanism of restraining unwanted inflammatory responses in cells of epithelial tissues exposed to non-pathogenic microbiota. Interestingly, the caspase-mediated restriction in IAP activity, we describe, does not affect pathogen-induced responses. Hence, it may allow for specific regulation of malfunctional epithelial responses in chronic inflammatory disease.

DATA AVAILABILITY

All data generated or analysed during this study are included in this published article and in its supplementary information file.

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AUTHOR CONTRIBUTIONS

CK designed and executed most of the experiments, data analysis and writing of the manuscript. VP planned and performed axenic fly experiments. AKM performed TUBE pulldowns. I-ET, PR and AM performed survival and AMP analyses of Diap2 and Drice mutants. PR, PM and AM contributed to the design of the experiments, writing and data analysis of this manuscript.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS STATEMENT

All use of transgenic flies and genetically modified material are approved by the Finnish Board for Gene Technology (Reg. Nr. 003/S/18).

ADDITIONAL INFORMATION

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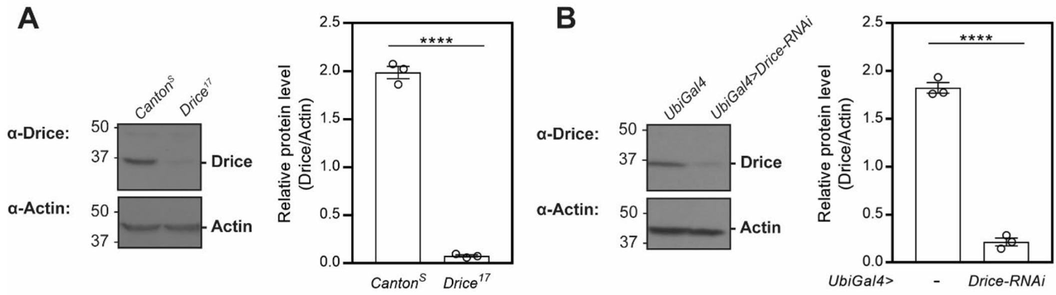
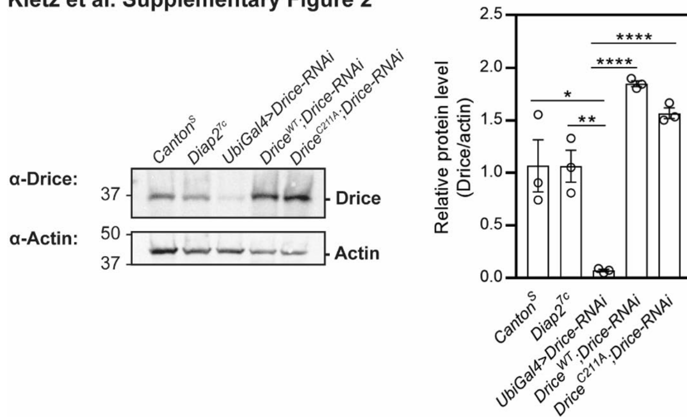
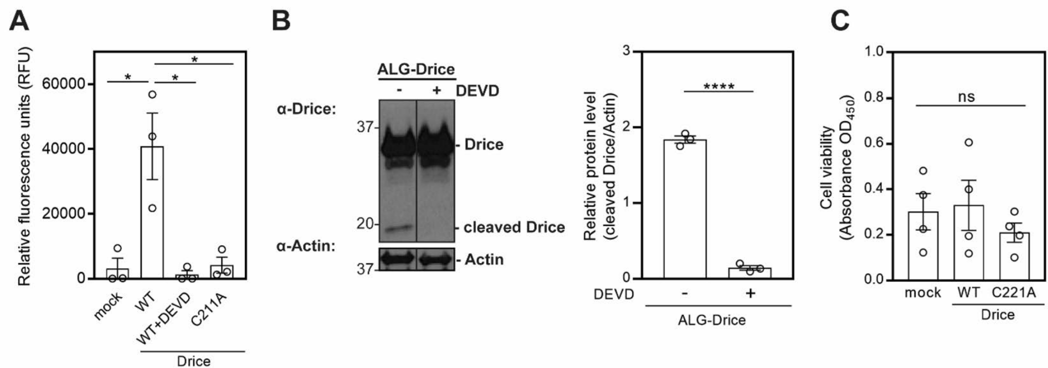
Supplementary figures

Drice restrains Diap2-mediated inflammatory signalling and intestinal inflammation

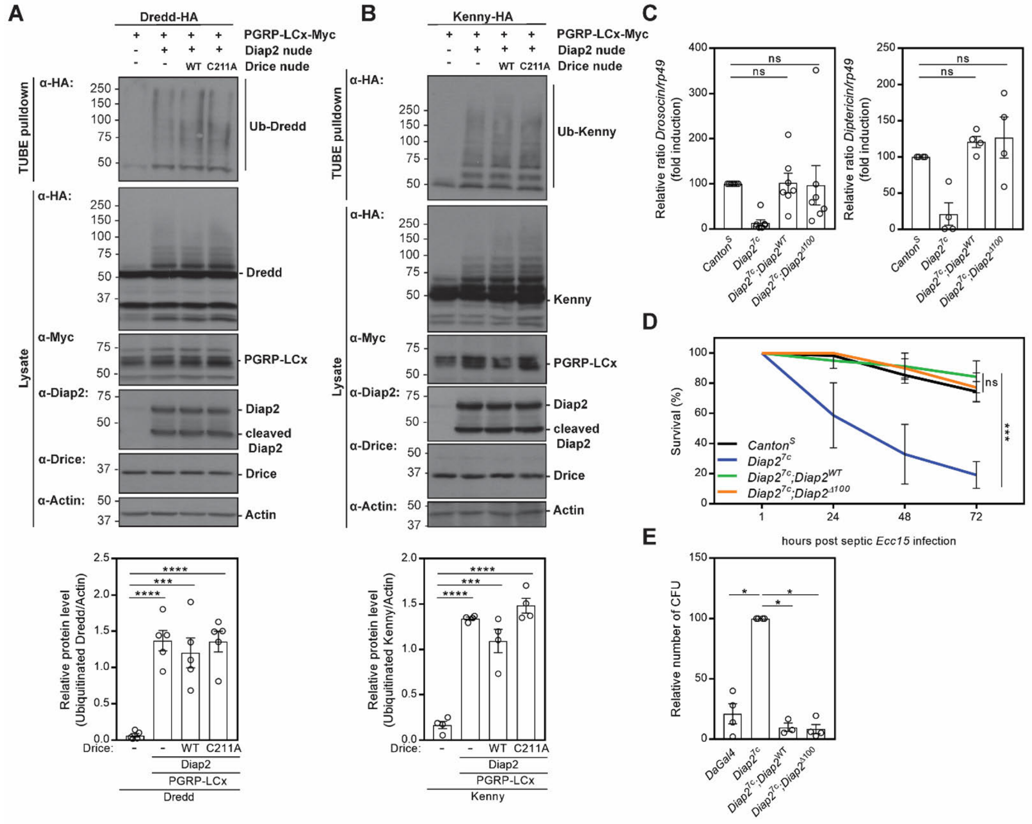
Christa Kietz, Aravind K Mohan, Vilma Pollari, Ida-Emma Tuominen, Paulo S Ribeiro, Pascal Meier, Annika Meinander



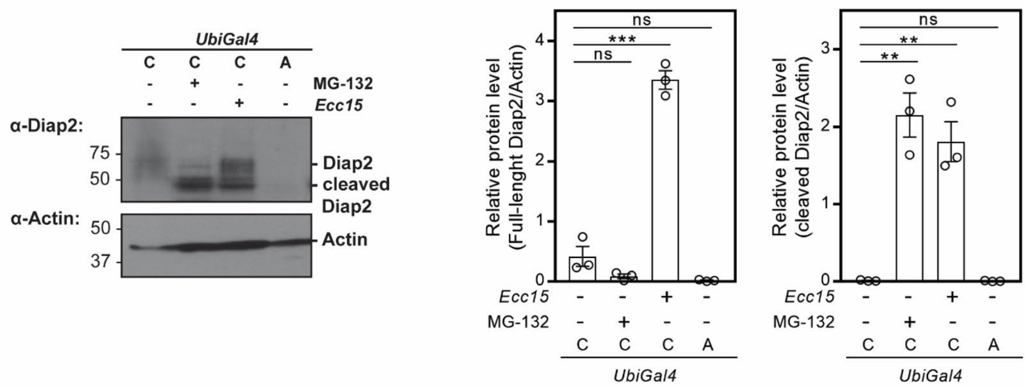




Kietz et al. Supplementary Figure 4



Kietz et al. Supplementary Figure 5



Supplementary figure legends

Drice restrains Diap2-mediated inflammatory signalling and intestinal inflammation

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Supplementary Figure 1. Drice protein levels in Drice mutants A, B) Whole fly lysates from *Canton*^{S} and *Drice*^{17} (A) or *UbiGal4* and *UbiGal4;UAS-Drice-RNAi* (B) flies were analysed by Western blotting with α-Drice and α-Actin antibodies, n = 3. The relative protein level of Drice was quantified. Data represent mean ± SEM and **** $p < 0.0001$.

Supplementary Figure 2. Drice protein level in flies expressing Drice^{WT} and Drice^{C211A} in *Drice-RNAi* background. Whole fly lysates from *Canton*^{S}, *Diap2*^{7c}, *UbiGal4*;*UAS-Drice-RNAi*, *UAS-Drice*^{WT}*/UbiGal4;UAS-Drice-RNAi* and *UAS-Drice*^{C211A}*/UbiGal4;UAS-Drice-RNAi* were analysed by Western blotting with α-Drice and α-Actin antibodies, and the relative protein level of Drice quantified, n = 3. Data represent mean ± SEM. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

Supplementary Figure 3. Verification of Drice inhibition by DEVD-treatment, and of cell viability after Drice overexpression in S2-cells. A) *Drosophila* S2-cells were transfected with empty vector, Drice^{WT} or Drice^{C211A}, where after the cells were treated with 20 μM Z-DEVD-FMK 16 h. The caspase-3/7 activity was analysed by adding Apo-ONE reagent to plated cells and measuring fluorescence at 499/521 nm, n = 3. B) *Drosophila* S2-cells were transfected with ALG-Drice and treated with Z-DEVD-FMK for 16 h. Cells were lysed and Drice cleavage was analysed by Western blotting with α-Drice and α-Actin antibodies, n = 3. The relative protein level of cleaved Drice was quantified. C) S2-cells were transfected with empty vector, Drice^{WT} or Drice^{C211A} and the cell viability assessed by addition of WST-1 reagent and measurement of absorbance at 450 nm, n = 4. Data represent mean ± SEM. ns stands for non-significant, * $p < 0.05$, **** $p < 0.0001$.

Supplementary Figure 4. Ubiquitination of Dredd and Kenny upon PGRP-LCx overexpression, and immune response in *Diap2*$^{\Delta100}$ expressing flies. A, B) *Drosophila* S2 cells were transfected with empty vector, Diap2^{WT}, PGRP-LCx-Myc, Drice^{WT}, Drice^{C211A} and HA-tagged Dredd (A) or HA-tagged Kenny (B). Ubiquitin chains were isolated with GST-TUBE at denaturing conditions and the samples were analysed by Western blotting with α-HA, α-Diap2, α-Drice, α-Myc and α-Actin antibodies, n ≥ 4. The relative protein levels of ubiquitinated Dredd or Kenny were quantified. C) Relative *Drosocin* and *Diptericin* mRNA levels analysed with qPCR in adult *Canton*^{S}, *Diap2*^{7c}, *Diap2*^{7c}*;UAS-Diap2*^{WT}*/DaGal4* and in *Diap2*^{7c}*;UAS-Diap2*$^{\Delta100}$*/DaGal4* flies 5 h after septic infection with *Ecc15*, n ≥ 4. D) Adult *Canton*^{S}, *Diap2*^{7c}, *Diap2*^{7c}*;UAS-Diap2*^{WT}*/DaGal4* and *Diap2*^{7c}*;UAS-Diap2*$^{\Delta100}$*/DaGal4* flies were subjected to septic injury with *Ecc15* and their survival was monitored over time, n = 4. E) *DaGal4, Diap2*^{7c}, *Diap2*^{7c}*;UAS-Diap2*^{WT}*/DaGal4 Diap2*^{7c}*;UAS-Diap2*$^{\Delta100}$*/DaGal4* flies were infected by feeding with *E. coli* for 24 h and the bacterial load was assessed by counting colony-forming units (CFU), n = 4. The same *DaGal4* and *Diap2*^{7c} controls were used as in Figure 5F. Data represent mean ± SEM. ns stands for non-significant, * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

Supplementary Figure 5. Diap2 protein levels in axenic flies. The intestines from adult female conventionally reared (C) *UbiGal4* flies treated with 50 μM MG-132 or orally infected with *Ecc15*, and of axenic (A) *UbiGal4* flies were dissected and lysed and analysed by Western blotting with α-Diap2 and α-Actin antibodies, n = 3. The relative protein levels of full-length and cleaved Diap2 were quantified. Data represent mean ± SEM. ns stands for non-significant and ** $p < 0.01$, *** $p < 0.001$.

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ARTICLE

M1-linked ubiquitination by LUBEL is required for inflammatory responses to oral infection in *Drosophila*

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Abstract

Post-translational modifications such as ubiquitination play a key role in regulation of inflammatory nuclear factor-κB (NF-κB) signalling. The *Drosophila* IκB kinase γ (IKKγ) Kenny is a central regulator of the *Drosophila* Imd pathway responsible for activation of the NF-κB Relish. We found the *Drosophila* E3 ligase and HOIL-1L interacting protein (HOIP) orthologue linear ubiquitin E3 ligase (LUBEL) to catalyse formation of M1-linked linear ubiquitin (M1-Ub) chains in flies in a signal-dependent manner upon bacterial infection. Upon activation of the Imd pathway, LUBEL modifies Kenny with M1-Ub chains. Interestingly, the LUBEL-mediated M1-Ub chains seem to be targeted both directly to Kenny and to K63-linked ubiquitin chains conjugated to Kenny by DIAP2. This suggests that DIAP2 and LUBEL work together to promote Kenny-mediated activation of Relish. We found LUBEL-mediated M1-Ub chain formation to be required for flies to survive oral infection with Gram-negative bacteria, for activation of Relish-mediated expression of antimicrobial peptide genes and for pathogen clearance during oral infection. Interestingly, LUBEL is not required for mounting an immune response against systemic infection, as Relish-mediated antimicrobial peptide genes can be expressed in the absence of LUBEL during septic injury. Finally, transgenic induction of LUBEL-mediated M1-Ub drives expression of antimicrobial peptide genes and hyperplasia in the midgut in the absence of infection. This suggests that M1-Ub chains are important for Imd signalling and immune responses in the intestinal epithelia, and that enhanced M1-Ub chain formation is able to drive chronic intestinal inflammation in flies.

Introduction

Ubiquitination is a reversible process, involving addition of ubiquitin, a 76-amino acid-long polypeptide, to the target substrate through a three-step enzymatic process carried out by E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligases [1]. Poly-ubiquitin chains are created when lysine residues (K6, K11, K27, K29, K33, K48, K63) or the N-terminal methionine (M1) of ubiquitin itself are ubiquitinated. M1-linked ubiquitin (M1-Ub) chains are formed through linkage of the C-terminal glycine of the incoming ubiquitin to the N-terminal methionine of the preceding ubiquitin, instead of to a lysine residue. M1-Ub chain formation is catalysed by the linear ubiquitin chain assembly complex (LUBAC) consisting of HOIL-1L interacting protein (HOIP), HOIL-1 and SHARPIN [2–7] in mammals and by the recently described E3 ligase LUBEL (linear ubiquitin E3 ligase) in *Drosophila* [8]. The really interesting new gene (RING)-in-between-RING (RBR) domains of HOIP and LUBEL carry the respective catalytic activity for M1-linkage-specific ubiquitination [4, 8]. Deubiquitinating enzymes (DUBs) provide an important level of regulation of ubiquitin chain formation by breaking down ubiquitin chains and removing the ubiquitin moieties from substrates [9]. CYLD and

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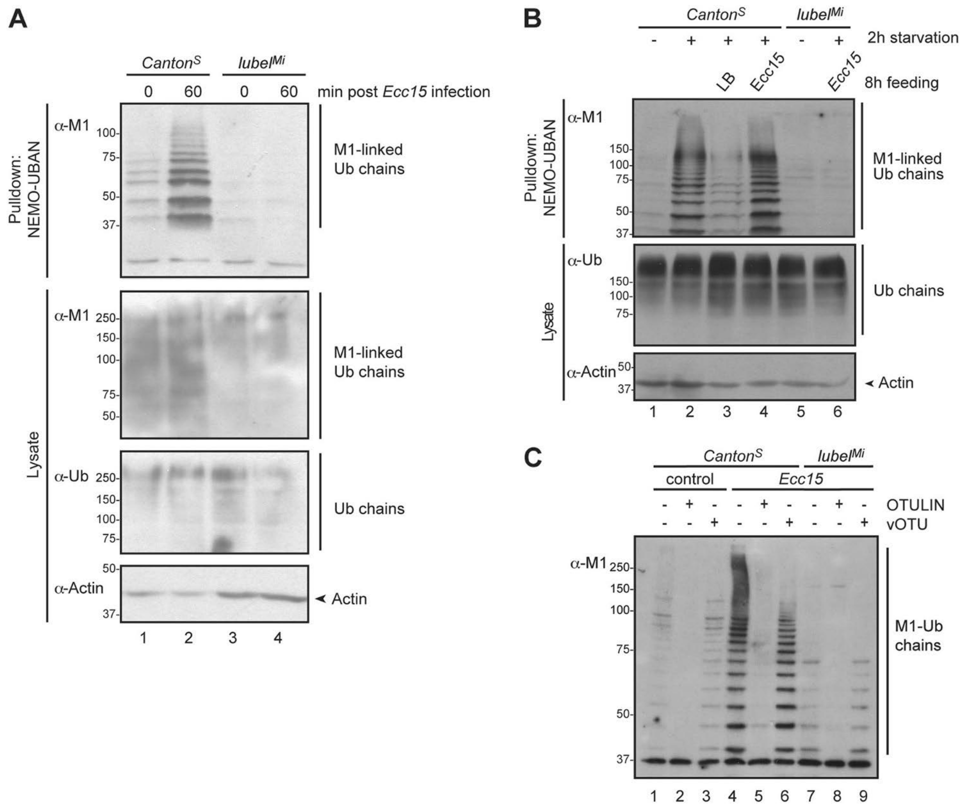


Fig. 1 LUBEL is required for signal-dependent M1-Ub chain formation in flies. **a** Adult wild-type *Canton*^{S} and *lubel*^{Mi} mutant flies were infected by septic injury with the Gram-negative bacteria *Ecc15*. M1-Ub chains were isolated at denaturing conditions from fly lysates with GST-NEMO-UBAN. Ubiquitin chains from lysates and pulldown samples were analysed by Western blotting with α-M1 and pan-ubiquitin antibodies and equal loading was controlled with α-Actin antibody, $n = 9$. **b** Adult wild-type *Canton*^{S} and *lubel*^{Mi} flies were starved for 2 h before infection by feeding with the Gram-negative bacteria *Ecc15*. *Canton*^{S} flies fed with LB were used as controls. M1-Ub chains were isolated from fly lysates with GST-NEMO-UBAN at denaturing conditions. Ubiquitin chains from lysates and pulldown samples were analysed by Western blotting with α-M1 and pan-ubiquitin antibodies and equal loading was controlled by immunoblotting with α-Actin antibodies, $n = 3$. **c** Adult wild-type *Canton*^{S} and *lubel*^{Mi} mutant flies were infected by septic injury with the Gram-negative bacteria *Ecc15*. M1-Ub chains were isolated from fly lysates with GST-NEMO-UBAN. The GST-NEMO-UBAN-isolated samples were subjected to ubiquitin chain restriction (UbiCRest) with recombinant OTULIN and vOTU and M1-Ub chains were analysed by Western blotting with an α-M1 antibody, $n = 3$

OTULIN are DUBs shown to be able to degrade M1-Ub chains [10–15]. Ubiquitin conjugation to target proteins may regulate proteins through conformational changes. However, the most common mode of regulation involves specific "ubiquitin receptors" that recognise ubiquitinated proteins via their ubiquitin-binding domains (UBDs). This ubiquitin binding allows for recognition of the ubiquitin modification and decoding of the ubiquitin message [16]. K48-linked ubiquitin chains have for long been known as the main signal for proteasomal degradation of target substrates [1], due to recognition by ubiquitin receptors in the proteasome lids [17]. However, it has also been established that ubiquitination, particularly with K63-linked ubiquitin (K63-Ub) and M1-Ub chains, plays an important role in regulation of nuclear factor-κB (NF-κB) activation and cell death induction in signalling complexes [2, 5, 6, 18–21].

Inflammation is induced by cells that recognise and respond to danger signals such as damage-associated or pathogen-associated molecular patterns and is essential for survival of organisms. Members of the NF-κB family of transcription factors are found to be chronically active in many inflammatory diseases, including in intestinal

bowel disease, and to be involved in colitis-associated carcinogenesis [22, 23]. The fly intestine is structurally and functionally reminiscent of the mammalian, and similarly as in mammals, the NF-κB family of transcription factors are major mediators of inflammatory signalling in flies. In addition to the inflammatory signalling pathways controlling NF-κB, also the enzymatic cascades regulating ubiquitination, the ubiquitin-binding receptors, and the ubiquitin chains themselves are well conserved through evolution [24, 25]. K63-Ub chains induced by the *Drosophila* inhibitor of apoptosis protein 2 (DIAP2) are important for activation of the *Drosophila* Imd pathway [26–28]. This *Drosophila* NF-κB pathway is rapidly activated by PGRP-LCx receptors recognising diaminopimelate-type peptidoglycans, which are components of the cell wall of Gram-negative bacteria. The Imd pathway activation leads to expression of hundreds of genes, some of which encode antimicrobial peptides (AMPs) required for fending off intruding pathogens [25, 29–32]. PGRP-LCx activation leads to recruitment of the protein Imd and formation of a signalling complex including FADD and the *Drosophila* caspase-8 homologue Dredd. Dredd-mediated cleavage of Imd leads to exposure of an inhibitor of apoptosis (IAP)-binding motif, recruiting the *Drosophila* inhibitor of apoptosis protein DIAP2 to the complex [26, 32]. For signalling to proceed, DIAP2-mediated K63-linked ubiquitination of Imd and Dredd is necessary [26, 27]. While the ubiquitination of Dredd is required for cleavage and nuclear localisation of the Imd pathway-specific NF-κB protein Relish [27, 33], Imd ubiquitination has been suggested to promote recruitment of the *Drosophila* mitogen-activated protein kinase kinase kinase dTAK1/TAB2 and the Relish kinase complex IRD5/Kenny (IκB kinase β/γ (IKKβ/IKKγ)) to the Imd signalling complex [25].

We have now studied the contribution of M1-Ub chains to *Drosophila* NF-κB signalling, which adds another layer of complexity to the established role for K63-linked ubiquitination in the Imd pathway [26–28]. We found that the *Drosophila* E3 ligase LUBEL catalyses formation of M1-Ub chains upon bacterial challenge. We show that the *Drosophila* IKKγ Kenny is a target for LUBEL, suggesting that M1-linked ubiquitination in IKK complex regulation is conserved. Importantly, LUBEL-mediated M1 ubiquitination is required for the flies to mount an immune response to oral infection with Gram-negative bacteria and clearing out the pathogen. Finally, transgenic expression of the catalytic domain of LUBEL drives Relish-mediated activation of AMP genes in the absence of receptor stimulation and leads to intestinal inflammation in flies.

Results

M1-Ub chains are formed upon bacterial infection in *Drosophila*

M1-Ub chains have been shown to be induced by a plethora of inflammation and stress promoting stimuli [2, 5, 6, 8, 19, 34, 35] and to have an important function in preventing cell death and in the activation of the pro-inflammatory IKK complex in mammals [2, 5, 6, 19, 34]. However, their role in IKK regulation in non-mammalian species has not yet been studied. To investigate M1-Ub chains in flies, we used a recombinant (GST)-tagged UBD of IKKγ or NF-κB essential modulator (NEMO) (GST-NEMO-UBAN), which is a high-affinity M1-Ub chain binder [10, 11], to pull down M1-Ub chains from whole fly lysates. We were able to detect only traces of M1-Ub chains in wild-type $Canton^S$ flies under basal conditions. However, when inducing inflammation by septic injury (Fig. 1a) or oral feeding (Fig. 1b) with the Gram-negative bacteria *Ecc15*, an increase in M1-Ub chain formation was observed. In contrast, infection did not induce any changes in overall ubiquitin chain formation in flies (Fig. 1a, b). Interestingly, also starvation induced a transient M1-Ub chain formation (Fig. 1b, lane 2) that was lost after 2 h recovery without bacteria feeding (Fig. 1b, lane 3).

LUBEL (CG11321) was recently reported to be a homologue of the mammalian LUBAC component and E3 ligase HOIP [8]. The *lubel* gene gives rise to nine splicing variants of messenger RNA (mRNA) encoding for seven different translated isoforms. Among these, only four contain the catalytic RBR domain (Supplementary Fig. 1A). To study the role of LUBEL in formation of M1-Ub chains in flies, we used a Minos transposable element fly line *yw;Mi{ET1}LUBELMB00197*. These $lubel^{Mi}$ flies carry a 7.5 kb insertion between the UBA1 and UBA2 in the *lubel* gene (Supplementary Fig. 1A). The Minos element disrupts gene transcription before the catalytic part of LUBEL, as mRNA transcripts of the N-terminal zinc-finger (ZnF) domains of LUBEL can be detected, but mRNA transcripts including the C-terminal catalytic RBR region are not present in these transgenic flies (Supplementary Fig. 1B). Importantly, the M1-Ub chain formation induced upon infection in $Canton^S$ flies was almost completely abolished in the $lubel^{Mi}$ flies (Fig. 1a–c), indicating that the induced M1-Ub chains are formed by LUBEL. To confirm that the identified ubiquitin chains are M1-linked, GST-NEMO-UBAN-purified ubiquitin chains from fly lysates were treated with recombinant OTULIN. OTULIN treatment led to a complete removal of the M1-specific signal. In contrast, only a ladder of free ubiquitin chains was found after treating samples with

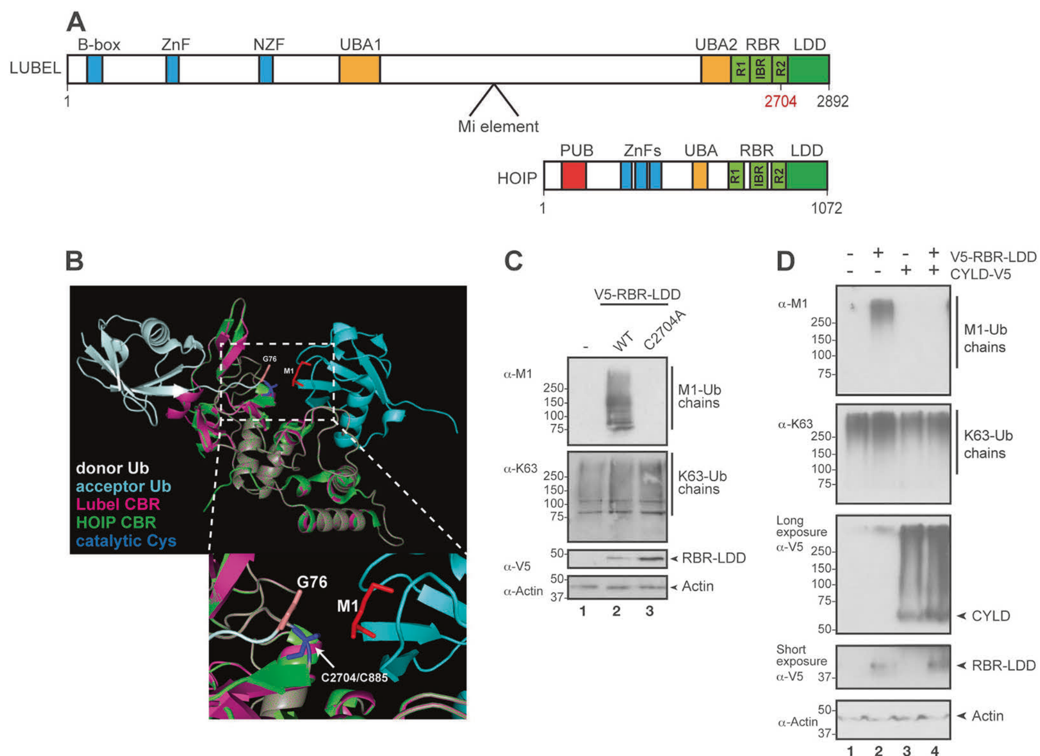


Fig. 2 LUBEL synthesises and CYLD breaks down M1-Ub chains. **a** Schematic comparison of the different domains of LUBEL encoded by the *lubel (CG11321)* gene and human HOIP. The indicated cysteine residue marks the ubiquitin acceptor site in RING2. The *lubel*^{Mi} fly line carries a 7.5 kb Minos transposable element (Mi element) inserted between UBA1 and UBA2 in the *lubel* gene, as indicated in the figure (B-box, ZnF and NZF: zinc fingers; UBA: ubiquitin-associated domain; R: RING, really interesting new gene; IBR: in-between-RING; RBR: RING-in-between-RING; LDD: linear ubiquitin chain determining domain; PUB: peptide *N*-glycanase/UBA-containing or UBX-containing protein). **b** Structural modelling of the catalytic in-between-RING (CBR) consisting of RING2 and the LDD of LUBEL (Phyre2). The CBR of LUBEL (magenta) is modelled on human HOIP (green) associated with donor (white) and acceptor (light blue) ubiquitins (PDB: 4LJO). The catalytic cysteines are indicated in blue (LUBEL: C2704, HOIP: C885), the donor C-terminal glycines are shown in beige, while the acceptor M1 is shown in red. The lower panel is a zoom-in of the catalytic site in the upper panel. **c** *Drosophila* S2 cells were transfected with empty vector, V5-tagged wild-type or catalytically inactive C2704A mutant LUBEL RBR-LDD and lysates were analysed by Western blotting using α-M1, α-K63, α-V5 and α-Actin antibodies, $n = 3$. **d** *Drosophila* S2 cells were transfected with empty vector, V5-tagged wild-type LUBEL RBR-LDD and V5-tagged *Drosophila* CYLD and lysates were analysed by Western blotting with α-M1, α-K63, α-V5 and α-Actin antibodies, $n = 3$

vOTU (Fig. 1c), which cleaves all ubiquitin chain types except M1-Ub [36], and hence also the ubiquitin moieties through which the M1-Ub chains are linked to their substrates. As genes encoding for ubiquitin concatemers are present in the *Drosophila* genome, we also wanted to exclude that the M1-Ub chains detected upon infection were a result of enhanced ubiquitin gene expression. While there was no significant difference in ubiquitin-p5E mRNA expression in wild-type and *lubel* mutant flies, and as this ubiquitin mRNA expression was not significantly altered upon infection (Supplementary Fig. 1C), we conclude that the M1-Ub chains induced in *Drosophila* are synthesised de novo by LUBEL.

LUBEL catalyses formation of M1-Ub chains in *Drosophila* cells

Like HOIP, LUBEL contains N-terminal ZnF domains, a ubiquitin-associated (UBA) domain and a C-terminal RBR (Fig. 2a) [2–7, 37]. In addition, a second UBA is localised immediately before the RBR. In mammals, HOIP interacts with both HOIL-1 and SHARPIN through ubiquitin-like domains, UBA domains and ZnF domains [2–7, 37]. Interestingly, no homologues of SHARPIN or HOIL-1 can be found in the *Drosophila* genome. Consisting of 2892 amino acids, the size of the *Drosophila* LUBEL is significantly larger than the 1072 amino acids large human

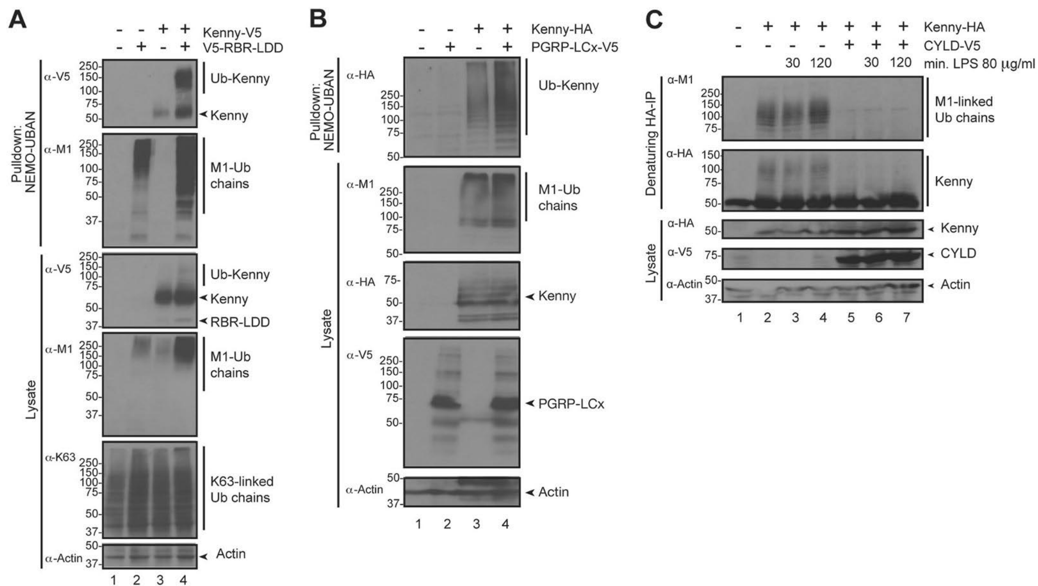
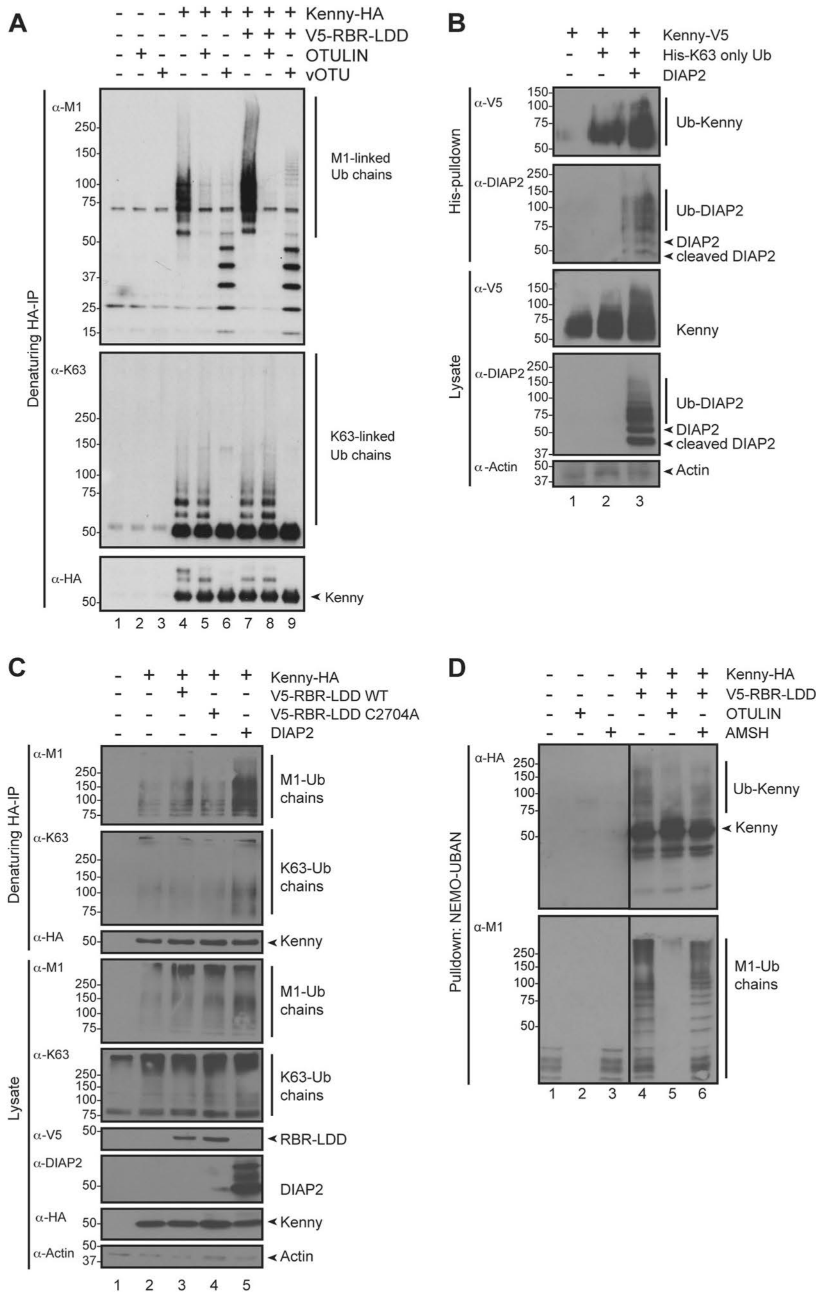


Fig. 3 Kenny is targeted with M1-Ub chains upon activation of Imd signalling. **a** *Drosophila* S2 cells were transfected with empty vector, V5-tagged wild-type Kenny and V5-tagged wild-type RBR-LDD. M1-Ub chains were isolated from cell lysates with GST-NEMO-UBAN. Ubiquitin chains from lysates and pulldown samples were analysed by Western blotting with α-V5, α-M1, α-K63 and α-Actin antibodies, $n = 4$. **b** *Drosophila* S2 cells were transfected with empty vector, V5-tagged PGRP-LCx and HA-tagged Kenny. M1-Ub chains were isolated from cell lysates with GST-NEMO-UBAN at denaturing conditions and the samples were analysed by Western blotting with α-M1, α-HA, α-V5 and α-Actin antibodies, $n = 3$. **c** *Drosophila* S2 cells were transfected with empty vector and HA-tagged Kenny, V5-tagged *Drosophila* CYLD and treated with 80 μg/ml LPS for 0.5 and 2 h. HA immunoprecipitations were performed at denaturing conditions and the samples were analysed by Western blotting with α-M1, α-HA, α-V5 and α-Actin antibodies, $n = 3$

HOIP (Fig. 2a); however, no conserved motives have been found in the large connecting sequences between the mentioned domains.

The catalytic RBR domain in HOIP is responsible for positioning the proximal and distal ubiquitin into close vicinity of one another and for ligation of the moieties. The RBR consists of three ZnF structures, RING1, IBR (in-between-RING) and RING2 with a linear ubiquitin chain determining region (LDD) (Fig. 2a, Supplementary Fig. 1A) [38, 39]. Structural modelling of the RING2 and the LDD together with M1-linked di-ubiquitin indicates that the catalytic pocket including the positioning of the catalytic cysteine of LUBEL is similar to the one in mammalian HOIP, referred to as the catalytic in-between-RING (CBR). Also, the positioning around the donor and acceptor ubiquitin moieties in the catalytic core of HOIP and LUBEL seems conserved (Fig. 2b). The RBR-LDD of LUBEL is able to form M1-Ub chains (Supplementary Fig. 2A), which are sensitive to in vitro treatment with the M1-specific DUB OTULIN, but not to treatment with the K48-specific DUB otubain-1 (OTUB1) or the K63-specific DUB-associated molecule with the SH3 domain of STAM (AMSH) (Supplementary Fig. 2B). Furthermore, the RBR-LDD of LUBEL seems to specifically synthesise M1-Ub chains, as it cannot use N-terminally His-tagged ubiquitin to build ubiquitin chains (Supplementary Fig. 2C, D).

To study whether the E3 ligase activity of RBR-LDD is able to induce M1-Ub chain formation in cells, we expressed wild-type LUBEL RBR-LDD and a catalytically inactive C2704A mutation in RING2 in *Drosophila* S2 cells. Importantly, the wild-type but not the C2704A mutant form of the LUBEL RBR-LDD was able to induce formation of M1-Ub chains (Fig. 2c), showing that the catalytic function of RING2 mediates M1-Ub chain assembly in fly cells. The *Drosophila* CYLD has been shown to interact with the LUBEL RBR-LDD and degrade M1-Ub chains in vitro [8]. To study whether *Drosophila* CYLD is able to cleave M1-Ub chains formed by LUBEL, we overexpressed CYLD in S2 cells. Indeed, CYLD co-expression removed all M1-Ub chains induced by overexpression of the LUBEL RBR-LDD (Fig. 2d), indicating that CYLD can regulate LUBEL-induced M1-linked ubiquitination in *Drosophila* cells. In addition, overexpression of CYLD had a small effect on the amount of K63-Ub chain in S2 cells.


A
Kenny-HA
V5-RBR-LDD
OTULIN
vOTU
Denaturing HA-IP
α-M1
M1-linked
Ub chains
α-K63
K63-linked
Ub chains
α-HA
Kenny
B
Kenny-V5
His-K63 only Ub
DIAP2
His-pulldown
α-V5
Ub-Kenny
α-DIAP2
Ub-DIAP2
DIAP2
cleaved DIAP2
Lysate
Kenny
α-Actin
Actin
C
Kenny-HA
V5-RBR-LDD WT
V5-RBR-LDD C2704A
DIAP2
M1-Ub
chains
K63-Ub
chains
RBR-LDD
D
Kenny-HA
V5-RBR-LDD
OTULIN
AMSH
Pulldown: NEMO-UBAN

◀ **Fig. 4** Kenny is modified by mixed K63-Ub and M1-Ub chains. **a** *Drosophila* S2 cells were transfected with empty vector, HA-tagged Kenny and V5-tagged wild-type LUBEL RBR-LDD. HA immunoprecipitations were performed at denaturing conditions and the samples were subjected to ubiquitin chain restriction (UbiCRest) with OTULIN and vOTU. Samples were analysed by Western blotting with α-M1, α-K63 and α-HA antibodies, $n = 2$. **b** *Drosophila* S2 cells were transfected with DIAP2, His-tagged K63-only Ub and V5-tagged Kenny. His-Ub pulldowns were performed at denaturing conditions and the samples were analysed by Western blotting with α-V5, α-DIAP2 and α-Actin antibodies, $n = 3$. **c** *Drosophila* S2 cells were transfected with empty vector, DIAP2, V5-tagged wild-type or C2704A LUBEL RBR-LDD, and HA-tagged Kenny. HA immunoprecipitations were performed at denaturing conditions and the samples were analysed by Western blotting with α-M1, α-K63, α-V5, α-HA, α-DIAP2 and α-Actin antibodies, $n = 3$. **d** *Drosophila* S2 cells were transfected with empty vector, HA-tagged Kenny and V5-tagged wild-type LUBEL RBR-LDD. M1-Ub chains were isolated from cell lysates with GST-NEMO-UBAN at denaturing conditions and the samples were subjected to UbiCRest with OTULIN and AMSH. Samples were analysed by Western blotting with α-M1 and α-HA antibodies, $n = 3$

The regulatory IKK Kenny is a target for M1-Ub chains

M1-Ub chains have been implicated to regulate NF-κB signalling via the regulatory IKK NEMO both via UBAN-mediated binding and by NEMO ubiquitination [5, 6, 40]. The *Drosophila* NEMO homologue Kenny is an important mediator of Imd signalling and Relish activation. To test whether Kenny is M1-ubiquitinated, we pulled down M1-Ub chains with recombinant GST-NEMO-UBAN from *Drosophila* S2 cell lysates made under denaturing conditions. Indeed, high-molecular weight smears of ubiquitinated Kenny were detected upon overexpression of LUBEL RBR-LDD (Fig. 3a). To test whether Kenny ubiquitination is signal-dependent, we induced the Imd pathway by overexpression of PGRP-LCx or by lipopolysaccharide (LPS) treatment [41]. Kenny expression alone induced M1 ubiquitination of Kenny, and, importantly, the M1 ubiquitination of Kenny was increased upon activation of the Imd pathway via PGRP-LCx (Fig. 3b) or LPS (Fig. 3c). As we found *Drosophila* CYLD to break down M1-Ub chains formed by the RBR-LDD of LUBEL (Fig. 2d), and as CYLD has been shown to function as a DUB and to interact with Kenny in *Drosophila* S2 cells [42], we wanted to test how CYLD affects Kenny ubiquitination. Indeed, ectopic expression of CYLD completely abolished the overexpression-induced M1 ubiquitination of Kenny, suggesting that CYLD is able to remove M1-Ub chains from Kenny. Similarly, CYLD upregulation reduced the LPS-induced M1 ubiquitination of Kenny (Fig. 3c). Imd, which is related to the mammalian HOIP target RIPK1 [43], is a signalling protein shown to be targeted by K63-linked ubiquitination by DIAP2. However, we could not detect any RBR-LDD-mediated M1-linked ubiquitination of Imd (Supplementary Fig. 3).

Kenny is modified by mixed K63-Ub and M1-Ub chains

To further analyse the Ub chains recruited to Kenny, we pulled down HA-tagged Kenny from lysates made under denaturing conditions (Fig. 4a) and treated the samples with recombinant chain-specific DUBs. The M1-Ub-specific OTULIN cleaved all M1-Ub chains bound to Kenny (Fig. 4a, lanes 5 and 8), confirming that the chains attached to Kenny were M1-linked. A ladder of free M1-Ub chains was left after treatment with vOTU (Fig. 4a, lanes 6 and 9), which cleaves all except M1-Ub chains [36], and hence also the link to the substrate.

It has been shown that M1-Ub chains can be conjugated to K63-Ub chains to form mixed or branched chains [44], and the pattern of M1-Ub chains seen in fly lysates is changed upon treatment with vOTU (Fig. 1c, lanes 4 and 6), indicating that lysine-linked ubiquitin chains may affect M1 ubiquitination. To know if mixed ubiquitin chains may be associated with Kenny, we first tested if Kenny is subjected to K63 ubiquitination. For this purpose, we co-expressed Kenny with a His-tagged ubiquitin mutant in which all lysines except K63 were mutated to arginines (His-K63-only Ub) and with DIAP2, which we previously showed catalyses K63-linked ubiquitination [27, 45]. Immunoprecipitations performed under denaturing conditions indeed showed that Kenny was modified by K63-linked ubiquitination with DIAP2, further increasing this modification (Fig. 4b). Importantly, we found DIAP2 not only to induce K63 ubiquitination of Kenny but also to boost M1 ubiquitination of Kenny (Fig. 4c). Furthermore, a reduction in Kenny M1 ubiquitination was detected, when the M1-Ub chains pulled down with GST-NEMO-UBAN were treated with the DUB AMSH, which specifically cleaves K63-linked chains [36], indicating that Kenny is M1-ubiquitinated both directly and indirectly on K63-Ub chains (Fig. 4d).

Molecular modelling of the UBANs of NEMO and Kenny indicates that the UBAN is structurally conserved throughout evolution (Fig. 5a), including the strong M1-Ub-binding surfaces formed by the amino acids F312, R316, R319 and E320 in human NEMO [46, 47]. Interestingly, expression of wild type Kenny, but not the ubiquitin-binding surface mutant forms of Kenny (F281A and R285A/R288A/E289A, corresponding to F312A and R316A/R319A/E320A) lead to accumulation of M1-Ub chains (Fig. 5b). These results indicate that in addition to being conjugated to M1-Ub chains, Kenny is associated with M1-Ub chains via its UBAN, which leads to ubiquitin chain stabilisation.

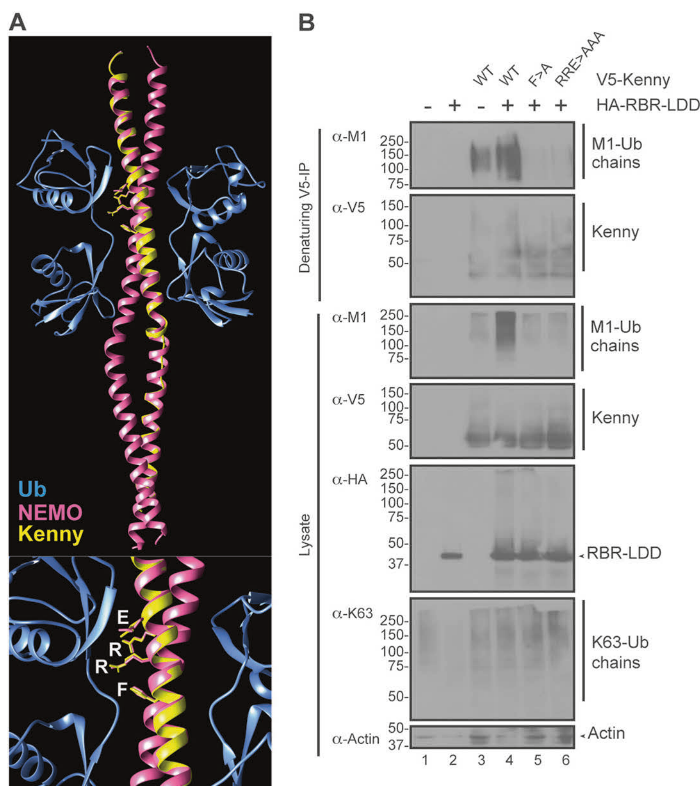
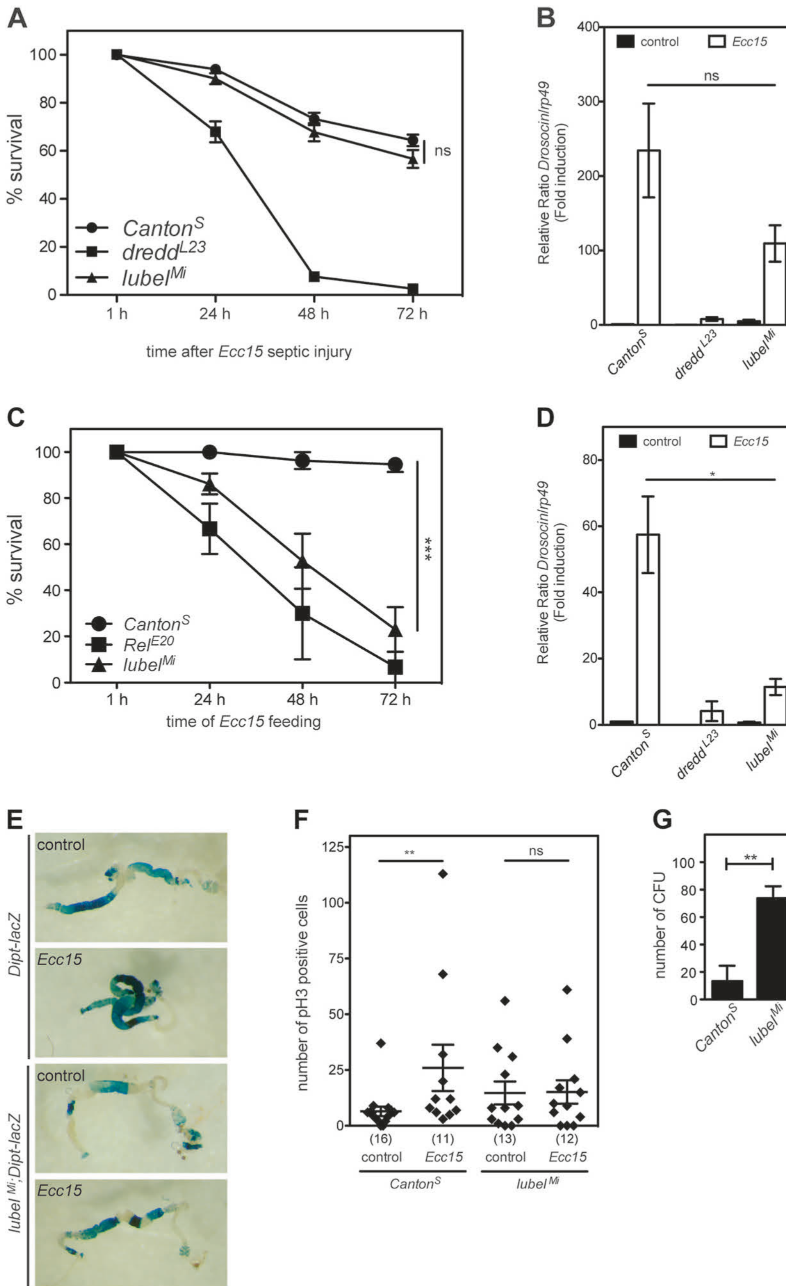


Fig. 5 Kenny UBAN binding stabilises M1-Ub chains. **a** Structural modelling of the UBAN of Kenny (Phyre2). The Kenny UBAN (yellow) is modelled on a di-Ub-bound dimer of human NEMO (pink), PDB: 2ZVN. Ubiquitin is shown in blue and the conserved F281, R285, R288 and E289 are indicated in white. The lower panel is a zoom-in of the catalytic site in the upper panel. **b** *Drosophila* S2 cells were transfected with empty vector, V5-tagged wild type, F281A, R285A/R288A/E289A mutant Kenny and HA-tagged RBR-LDD. V5 immunoprecipitations were performed at denaturing conditions and the samples were analysed by Western blotting with α-M1, α-K63, α-V5, α-HA and α-Actin antibodies, $n = 3$

LUBEL is required for mounting an immune response upon oral infection with Gram-negative bacteria

As M1-Ub chain formation is induced upon infection in *Drosophila*, we wanted to study whether LUBEL is important for mounting an immune response against bacteria in flies. However, we did not detect any significant differences in the survival of *Canton^S* and *lubel^{Mi}* flies infected with *Ecc15* by septic injury, whereas *dredd^{L23}* mutant flies, in which cleavage and activation of Relish is prohibited, succumbed upon septic infection (Fig. 6a). When analysing the infection-induced expression of Imd pathway-specific AMPs in *lubel^{Mi}* flies, only a slight, not significant reduction in expression of *Drosocin* was detected (Fig. 6b). Likewise, *lubel^{Mi}* flies tolerated septic infection with the Gram-positive bacteria *Micrococcus luteus* (*M. luteus*) and were able to upregulate expression of the Toll pathway-specific AMPs *IM1* and *Drosomycin* equally well as the wild-type *Canton^S* flies (Supplementary Fig. 4A, B). However, when infecting flies orally, by feeding them with *Ecc15*, most *lubel^{Mi}* flies succumbed, whereas most wild-type *Canton^S* flies survived the bacterial feed (Fig. 6c). Importantly, the *Mi{MIC}LUBELMI14859* mutant fly strain, with an insertion after the catalytic RBR-LDD region was not sensitive to oral infection (Supplementary Fig. 1A, 4C). Finally, a significant reduction in *Drosocin* expression could be detected after oral *Ecc15* infection in the *lubel^{Mi}* flies, correlating with their sensitivity (Fig. 6d). These results indicate that although M1-Ub chain formation is induced upon septic infection, it is not required for systemic activation of NF-κB in the fat body, which is the organ responsive for activation of AMP expression in response to septic infection [48].

To study if M1-Ub chains are required for induction of Imd signalling and local expression of AMPs as a response to infection in the epithelia of the intestine, we examined the expression of *Diptericin* in the midgut of control and bacteria-fed flies using *Diptericin*-LacZ reporter flies. Importantly, the *Diptericin* expression was enhanced only in the intestines of control *Diptericin*-LacZ and not in *lubel* mutant *Diptericin*-LacZ flies (Fig. 6e). Intestinal inflammation is associated with midgut hyperplasia in *Drosophila* [49] and can be detected by staining the proliferation marker



phospho-histone H3. To analyse the role of LUBEL in infection-induced inflammation in the intestine, we counted phospho-histone H3-positive cells in the midguts in control flies and in flies fed with *Ecc15*. While an increase in cell proliferation could be detected upon oral infection in wild-type *Canton*^{S} flies, no such increase could be detected in

◀ **Fig. 6** LUBEL is required for mounting an immune response upon oral infection with Gram-negative bacteria. **a** Adult wild-type *Canton^S*, *dredd^{L23}* and *lubel^{Mi}* mutant flies were subjected to septic injury with the Gram-negative bacteria *Ecc15* and their survival was monitored over time. Error bars indicate SEM from more than 10 independent experimental repeats using at least 20 flies per repeat. **b** Adult *Canton^S*, *dredd^{L23}* and *lubel^{Mi}* mutant flies were infected by septic injury with the Gram-negative bacteria *Ecc15*. Relish activation was studied by analysing the expression of *Drosocin* with qPCR. Error bars indicate SEM from more than 10 independent experimental repeats using at least 10 flies per repeat. **c** Adult *Canton^S*, *Rel^{E20}* and *lubel^{Mi}* mutant flies were infected by feeding with the Gram-negative bacteria *Ecc15* and their survival was monitored over time. Error bars indicate SEM from three independent experimental repeats using at least 20 flies per repeat. **d** Adult *Canton^S*, *dredd^{L23}* and *lubel^{Mi}* mutant flies were infected by feeding with the Gram-negative bacteria *Ecc15*. *Canton^S* flies fed with LB-sucrose were used as controls. Relish activation was studied by analysing the expression of *Drosocin* with qPCR. Error bars indicate SEM from three independent experimental repeats using at least 10 flies per repeat. **e** Adult female *DaGal4,Dipt-lacZ* and *lubel^{Mi}; DaGal4,Dipt-lacZ* mutant flies were infected by feeding with the Gram-negative bacteria *Ecc15* for 8 h. Intestines were dissected and stained for β-galactosidase activity, $n = 3$. **f** *Canton^S* and *lubel^{Mi}* mutant flies were infected by feeding with the Gram-negative bacteria *Ecc15* for 24 h. Intestines were dissected and stained for phospho-histone H3. All phospho-histone H3-positive cells in midguts prepared and stained were counted for statistics, error bars indicate SEM from four independent experimental repeats and the number of intestines analysed are indicated in brackets. **g** *Canton^S* and *lubel^{Mi}* mutant flies were infected by feeding with ampicillin-resistant *E. coli* for 24 h and the bacterial load was assessed by counting colony-forming units (CFU); error bars indicate SEM from three independent experimental repeats. ns nonsignificant, nonsignificant, * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$

lubel^{Mi} flies (Fig. 6f). This suggests that LUBEL is required for infection-induced inflammation in the *Drosophila* gut. To finally test the requirement of LUBEL in clearing ingested food-borne pathogens, we fed wild-type *Canton^S* and *lubel^{Mi}* mutant flies with ampicillin-resistant *Escherichia coli*. After feeding, we plated homogenised flies on ampicillin-containing agar plates and counted colonies. Interestingly, the amount of colony-forming bacteria was significantly higher in the *lubel^{Mi}* mutant flies than in wild-type flies, suggesting that LUBEL is required for clearing pathogens from the ingested food (Fig. 6g).

RBR-LDD-induced M1 chain formation drives NF-κB activation in flies

To investigate the role of M1-Ub chains in vivo, we generated transgenic flies to express wild-type and catalytically inactive RBR-LDD under the control of the UAS-Gal4 system. The transgenes were successfully incorporated in the genome and ubiquitous expression was driven by DaGal4 (Fig. 7a). To test the effect of M1-Ub chains on activation of Relish target genes induced via the Imd pathway, we studied the expression of *AttacinA*, *Diptericin* and *Drosocin*. Interestingly, all these inflammatory AMP genes were induced in the absence of infection in flies expressing wild-type RBR-LDD (Fig. 7b).

As M1-Ub chain formation was induced upon oral infection and required for immune responses (Figs. 1b, 6c–g), we wanted to study the consequences of the LUBEL-induced AMP expression in intestinal inflammatory signalling. To determine whether induction of M1-Ub chain formation by transgenic expression of RBR-LDD affected inflammatory signalling in the intestine, we examined the expression of *Diptericin* in the midgut of control and RBR-LDD-expressing flies using the *Diptericin*-LacZ reporter. Interestingly, *Diptericin* expression in the intestine was enhanced by wild-type RBR-LDD (Fig. 7c). Importantly, also the amount of phospho-histone H3-positive proliferating cells in the midguts of flies expressing wild-type RBR-LDD was significantly increased compared to control flies and flies expressing the catalytically inactive RBR-LDD-C > A (Fig. 7d, e), suggesting that constitutive LUBEL activity drives Relish-mediated chronic intestinal inflammation in flies.

Discussion

Both K63-linked and M1-linked ubiquitination have been described to have important roles in regulation of mammalian NF-κB signalling [2, 5, 6, 19, 34, 35] and the role for K63-linked ubiquitination in the *Drosophila* Imd pathway is well established [26–28]. Here, we show that the *Drosophila* ubiquitin E3 ligase LUBEL induces M1-linked ubiquitination upon activation of the Imd pathway. Our results indicate that the IKK Kenny, which is required for activation of Relish, is a target of M1-linked ubiquitination. In addition to being directly modified by M1-linked ubiquitination, K63-Ub chains conjugated to Kenny by DIAP2 seem to be modified by M1-linked ubiquitination, forming K63-M1-linked mixed heterotypic chains. Interestingly, the stability of M1-Ub chains depends on binding to the UBAN of Kenny, as no M1-Ub chains can be detected upon expression of Kenny UBAN mutants.

We also found the *Drosophila* CYLD to induce degradation of M1-Ub chains. In mammals, OTULIN and CYLD have been shown to cleave M1-Ub chains [10–15], and the M1-Ub chain-antagonising activity of CYLD seems to be particularly important in NF-κB activating signalling complexes [50]. As no OTULIN homologue has been found in the *Drosophila* genome, CYLD may have an important role in degrading M1-Ub chains in the fly. However, as we show here and as previously described [8, 13–15], both mammalian and *Drosophila* CYLD are able to degrade also K63-Ub chains. Hence, we cannot exclude that the loss of M1-Ub chains due to CYLD activity is a consequence of degradation of the K63-Ub chains to which M1-Ub

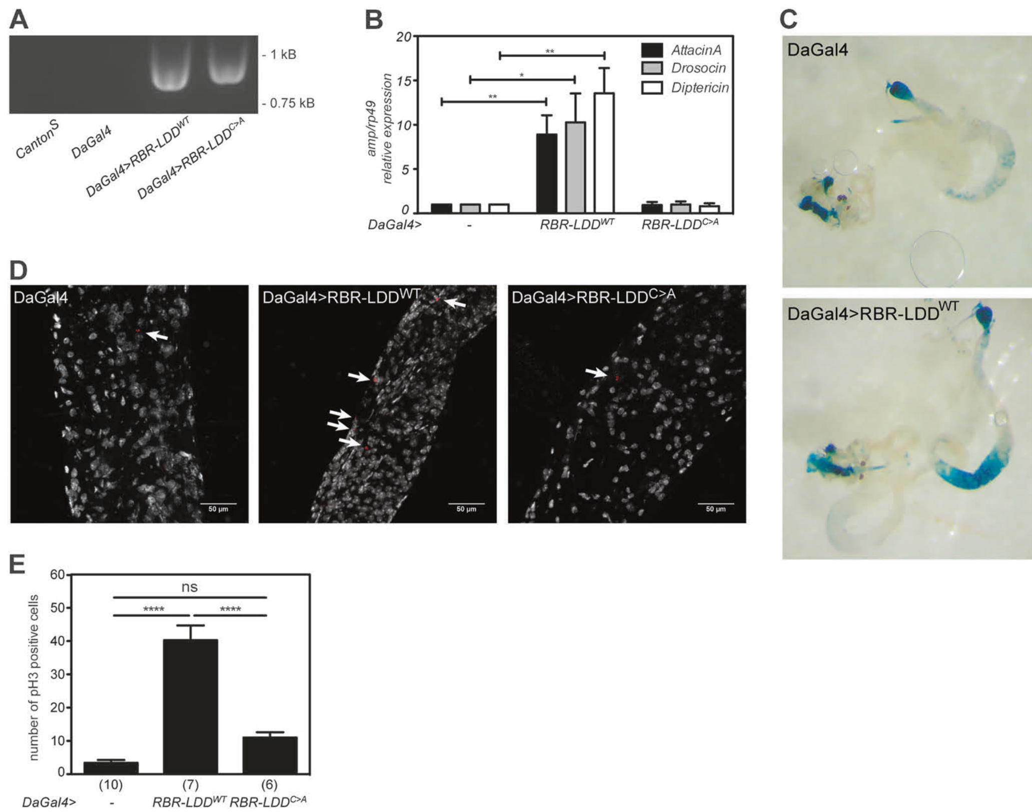


Fig. 7 RBR-LDD-induced M1 chain formation drives NF-κB activation in flies. Transgenic expression of wild-type or C2704A RBR-LDD was induced via the UAS-Gal4 system using the ubiquitous DaGal4 driver. **a** mRNA expression was analysed by PCR of cDNA. Fly lines: wild-type *Canton^S*, *DaGal4/TM6*, *UAS-RBR-LDD^{WT}/DaGal4*, *UAS-RBR-LDD$^{C>A}$/DaGal4*, $n = 3$. **b** Basal Relish activation was studied by analysing the expression of the AMPs *AttacinA*, *Drosocin* and *Diptericin* with qPCR. Error bars indicate SEM from three independent experimental repeats using at least 10 flies per repeat. Fly lines: control *DaGal4/TM6*, *UAS-RBR-LDD^{WT}/DaGal4*, *UAS-RBR-LDD$^{C>A}$/DaGal4*. **c** Transgenic expression of wild-type RBR-LDD was induced via the UAS-Gal4 system using the ubiquitous *DaGal4,Dipt-lacZ* driver. Intestines from adult female *DaGal4,Dipt-lacZ* driver flies and *DaGal4,Dipt-lacZ/UAS-RBR-LDD* flies expressing wild-type RBR-LDD were dissected and stained for β-galactosidase activity, $n = 3$. **d** Intestines from adult DaGal4 driver flies and flies expressing wild-type or C2704A RBR-LDD flies were dissected and stained for phospho-histone H3 (red) and DAPI (white). The phospho-histone H3-positive cells are marked with arrows. **e** All phospho-histone H3-positive cells in midguts prepared and stained were counted for statistics, error bars indicate SEM from at least three independent experimental repeats and the number of intestines analysed are indicated in brackets. ns nonsignificant, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$

chains are coupled. The association between LUBEL and CYLD may also not be conserved, as no PUB (peptide *N*-glycanase/UBA-containing or UBX-containing protein) domain responsible for DUB binding is found in LUBEL. Interestingly, CYLD has been shown to be able to bind directly to the RBR-LDD of LUBEL in vitro [8]. However, in vivo the connection may also be indirectly mediated via a connecting protein such as Tamo, which is a homologue of the mammalian HOIP-CYLD connector Spata2 [51–54].

Interestingly, LUBEL-mediated M1 ubiquitination is required for mounting an immune response upon oral infection, but not upon septic injury. Septic injury induces a systemic inflammation requiring activation of AMP expression and release from the fat body, whereas pathogen feeding induces a local inflammatory expression and release of AMPs from the intestinal epithelial cells [48]. While both the Imd and Toll pathways are able to induce Relish-mediated and Dif-mediated expression of AMP genes in the fat body of flies during systemic infection, only the Imd

pathway has been found to function in the intestinal epithelia [55]. Heterodimers of the NF-κB transcription factors Dif and Relish can activate AMP expression upon activation of both the Toll and Imd pathways, but with different specificity for different genes [43, 56]. Thus, it is possible that Dif-Relish heterodimers are activated upon systemic infection in the fat body also in the absence of M1-Ub chain formation, inducing the resistance to systemic infection with Gram-negative bacteria independently of M1-Ub chains. As we found Kenny to be strongly modified with K63-linked ubiquitin chains by DIAP2, it is also possible that the DIAP2-induced K63-linked ubiquitination is sufficient for activation of both Dredd and Relish in response to septic infection. Intriguingly, we found that transgenic expression of the RBR-LDD of LUBEL is able to induce M1-Ub chain formation and activation of Relish target genes, including *AttacinA*, *Drosocin* and *Diptericin* in the absence of infection, indicating that M1-Ub chains may be used for Relish activation. Furthermore, transgenic induction of LUBEL-mediated M1-Ub chain formation in the absence of infection induced both *Diptericin* expression and intestinal stem cell proliferation in the midgut of *Drosophila*, suggesting that M1-Ub chains may be linked to chronic intestinal inflammation.

Both K63-linked and M1-linked ubiquitination have been described to have important roles in regulation of inflammatory NF-κB signalling mediated via the mammalian TNFR1 and NOD2 signalling pathways [2, 5, 6, 19, 34, 35, 50, 57]. While LUBAC activates these canonical NF-κB pathways by M1-linked ubiquitination of NEMO, RIPK1, TRADD, TNFR1 and RIPK2, IAPs regulate the same signalling molecules by K63-linked ubiquitination [2, 5, 6, 35, 50, 57–59]. The similarities in the mammalian TNFR1 and NOD2 and the *Drosophila* Imd pathways include many conserved signalling mediators. IAP-mediated K63-Ub chain formation is already established in both mammalian and fly pathways [19, 26–28]. We have now found that also M1-Ub chain formation is induced upon activation of the Imd pathway and, importantly, that this is required for mounting an immune response against Gram-negative bacteria. In addition to the E3 ligases and DUBs regulating ubiquitination, also some of the targets for K63- and M1 ubiquitination seem to be conserved through evolution, making *Drosophila* a convenient organism to study the general principles of ubiquitin-mediated regulation of inflammatory signalling.

To be able to control unwanted inflammation that may cause diseases such as chronic inflammation and cancer, flexible but precise mechanisms are required to tune inflammatory signals in cells. As we show that uncontrolled induction of M1-linked ubiquitination drives intestinal inflammation in *Drosophila*, it would be important to further investigate whether ubiquitin modifications can be used as molecular switches to therapeutically target inflammatory signalling in chronic inflammation and cancer in the intestine. Hence, this critical knowledge of ubiquitin regulation in inflammation may open up possibilities for discovery of new drug targets and diagnostic markers.

Materials and methods

Fly husbandry and strains

Drosophila melanogaster were maintained at 25 °C with a 12 h light–dark cycle on Nutri-fly BF (Dutscher Scientific). *Canton*^{S} wild-type flies, *DaGal4* driver lines, *DaGal4,Dipt-LacZ* reporter lines, and balancer lines, as well as *dredd*^{L23} and *spätzle*^{RM7} mutant flies were kindly provided by Prof. Pascal Meier [60, 61]. The *Drosophila* fly lines *w:Rel*^{E20} (stock #9457), *yw;Mi{ET1}LUBELMB00197* (stock #22725 referred to as *lubel*^{Mi}) and *Mi{MIC}LUBELMI14859* (stock #59639 referred to as *lubel*^{MiMic}) were obtained from Bloomington stock centre. Fly egg injection for generation of LUBEL RBR-LDD transgenic flies was done by Bestgene Inc. UAS-RBR-LDD^{WT} and UAS-RBR-LDD$^{C>A}$ were both introduced to the landing site line #24749, and expression of the transgenes was verified by PCR on cDNA using Q5 High-Fidelity Polymerase Kit (NEB) according to the manufacturer's instructions (primers: 5′-CCTAACC CTCTCCTCGGTCT and 5′-CACATTCTGCTCCTTCA GCA).

Bacterial strains, infection and survival experiments

The Gram-negative bacteria *Erwinia carotovora carotovora 15* (*Ecc15*) and Gram-positive bacteria *Micrococcus luteus* (*M. luteus*) were kindly provided by Dr. François Leulier and the *E. coli* Top10 strain was purchased from ThermoFisher Scientific. The bacteria were cultivated in Luria-Bertani (LB) medium at 29 °C for 16–18 h on agitation and concentrated (optical density of 0.2). Septic injuries were performed by pricking 2–4-day-old adult flies in the lateral thorax with a thin needle previously dipped in a concentrated solution of *Ecc15* or *M. luteus*. For isolation of M1-Ub chains, 40 flies were incubated for 1 h after the septic injury, and for quantitative PCR (qPCR), 10 flies were incubated for 5 h at 25 °C. Oral feeding was performed by first starving adult flies for 2 h at 25 °C and thereafter feeding them with a 1:1 solution of bacteria and 5% sucrose. For isolation of M1-Ub chains, 40 flies were incubated for 8 h, and for qPCR, 10 flies were incubated for 8 h at 25 °C. For survival assays, 20 flies were counted at indicated time points after infection. Infection experiments were excluded if more than 25% of the negative control strains survived bacterial infection or if AMP gene expression was significantly enhanced in these flies. In these

cases, the bacterial potency was considered too low. Survival experiments, in which wild-type flies survived to a less extent than 75%, were also excluded. These criteria were pre-established.

Cell culture and transfection of *Drosophila* S2 cells

Drosophila Schneider S2 cells (Invitrogen) were grown at 25 °C using Schneider medium supplemented with 10% fetal bovine serum, 1% L-glutamine and 0.5% penicillin/streptomycin. S2 cells were transfected with indicated constructs using Effectene transfection reagent (Qiagen) according to the manufacturer's instructions. Fifty percent confluent 10 cm plates were used to prepare lysates for immunoprecipitations and GST-pulldown assays, and 6-well plates were used for lysates for Western blot analysis. Expression of pMT plasmids was induced with 500 µM $CuSO_4$ for 16 h before lysis. LPS (Sigma) was used at 80 µg/ml for the indicated times and 1 µM of 20-hydroxyecdysone (Sigma) was added 24 h prior to LPS treatment.

Plasmids and antibodies

Plasmids pMT/Flag-His, pMT/HA-Flag, pMT-V5-Kenny-V5, pAc-DIAP2, pMT-dCYLD-V5-His and pMT-PGRP-LCx-V5-His were kindly provided by Prof. Pascal Meier. Kenny-HA was subcloned from pMT-V5-Kenny-V5. Synthetic codon-optimised LUBEL RBR-LDD (GeneScript) was subcloned into pMT for expression in cells, pGEX for in vitro expression and pUAS-attB for PhiC31-mediated integration in the genome [62]. Site-directed mutagenesis to make Kenny F281A and R285A/R288A/E289A mutants and LUBEL RBR-LDD C2704A mutants was performed using QuikChange Lightning Site-directed Mutagenesis Kit (Agilent Technologies). GST-NEMO-UBAN [10, 11] was provided by Dr. Mads Gyrd-Hansen. The following antibodies were used: α-M1 (clone IE3, #MABS199, Millipore or LUB9 #AB130, Lifesensor), α-K63 (clone Apu3, #05-1308, Millipore), α-Ub (clone Ubi-1, #NB300-130, Novus or clone FK2, #BML-PW8810-0100, Enzo), α-DIAP2 [63], α-GST (#27-4577-50, GE Healthcare), α-HA (clone 3F10, #11867423001, Roche), α-V5 (Clone SV5-Pk1, #MCA1360, Bio-Rad), α-phospho-histone H3 (Ser10, #9701, Cell Signalling Technology) and α-Actin (C-11, sc-1615, Santa Cruz).

Purification of GST-fusion proteins

Expression of GST-RBR-LDD was induced in *E. coli* BL21 by the addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (o/n culture at 18 °C) in LB medium containing 50 µM $ZnCl_2$. Bacteria were lysed by sonication in lysis buffer containing 50 mM Tris (pH 8), 100 mM NaCl, 50 µM $ZnCl_2$, 1 mM EDTA, 1% Triton X-100, 1 mM dithiothreitol (DTT) and protease inhibitor Complete, EDTA-free (Roche). The lysate was incubated with Glutathione Sepharose™ 4B (GE Healthcare) for 2 h. Beads were washed in wash buffer containing 20 mM Tris (pH 8), 100 mM NaCl, 50 µM $ZnCl_2$, 1 mM EDTA and 0.1% Triton X-100. Elution of GST-RBR-LDD was performed in wash buffer without Triton X-100 containing 20 mM glutathione. The proteins were concentrated from the eluate using Amicon® Ultra-4 30K centrifugal filter devices (Merck Millipore). Glutathione was removed from the concentrated samples using Zeba™ spin desalting columns (Thermo Scientific). GST-NEMO-UBAN expression was induced in *E. coli* BL21 by the addition of 0.2 mM IPTG to an overnight culture of bacteria in LB medium at 18 °C. Bacteria were lysed by sonication in lysis buffer containing 50 mM Tris (pH 8.5), 150 mM NaCl, 3 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride and 0.2 mg/ml lysozyme. The lysate was added to a column with Glutathione Sepharose™ 4B (GE Healthcare) and then washed with wash buffer containing 50 mM Tris (pH 8.5) and 150 mM NaCl. GST-NEMO-UBAN was eluted in 50 mM Tris (pH 8.5), 150 mM NaCl, 10% glycerol, 3 mM DTT and 50 mM glutathione. The proteins were concentrated from the eluate using Amicon® Ultra-4 30K centrifugal filter devices (Merck Millipore).

In vitro ubiquitination assays

Ubiquitination reactions were carried out as described [38]. Samples contained 15 µM ubiquitin, 10 mM ATP, 0.1 µM hUBE1 (Boston Biochem), 0.6 µM UbcD1 [64], 1 µM wild-type or mutant GST-RBR-LDD fragment or GST in a buffer of 20 mM HEPES/HCl (pH 8), 150 mM NaCl, 10 mM $MgCl_2$ and 0.5 mM DTT. The reaction was incubated at 25 °C overnight. Sample separation was performed on NuPAGE Novex 4–12% Bis-Tris protein gels (Life Technologies) in 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer or TruPAGE 4–20% gels (Sigma-Aldrich) in TEA-Tricine sodium dodecyl sulfate (SDS) running buffer.

Purification of linear ubiquitin conjugates from flies and cells

M1-Ub conjugates were purified using a recombinant protein containing the UBAN region of NEMO (residues 257–346) fused to GST (GST-NEMO-UBAN) [10, 11]. Forty flies or one 10 cm confluent plate of cells were lysed using a buffer containing 20 mM NaH_2PO_4, 1% NP-40, 2 mM EDTA supplemented with 1 mM DTT, 5 mM *N*-ethylmaleimide (NEM), Pierce™ Protease Inhibitor, PhosSTOP, 5 mM chloroacetamide and 1% SDS. Lysates were

sonicated, diluted to 0.1% SDS and cleared before incubation with Glutathione Sepharose™ 4B (GE Healthcare) and GST-NEMO-UBAN (30–100 mg/ml) for a minimum of 2 h under rotation at 4 °C. The beads were washed three times with ice-cold phosphate-buffered saline-Tween-20 (PBS-Tween-20) (0.1%) and eluted using Laemmli sample buffer.

Ubiquitin chain restriction analysis

Deubiquitination of in vitro reactions were carried out with 1 µM of GST, 5 µM activated GST-OTULIN (Ubiquigent), GST-OTUB1 (Ubiquigent) or GST-AMSH (Ubiquigent) in a buffer of 50 mM Tris (pH 7.5), 50 mM NaCl and 5 mM DTT for 30 min at 37 °C. For activation, the cysteine protease DUBs were incubated in a buffer of 25 mM Tris (pH 7.5), 150 mM NaCl and 10 mM DTT for 15 min at room temperature [36]. Samples were separated on NuPAGE Novex 4–12% Bis-Tris protein gels (Life Technologies) in MES buffer. For deubiquitination of the GST-NEMO-UBAN-purified ubiquitin chains from pulldowns from fly lysates and S2 cell lysates, the washed beads were resuspended in 25 µl DUB buffer containing 25 mM HEPES (pH 7.6), 150 mM NaCl and 2 mM DTT. One micromole of recombinant OTULIN, vOTU or AMSH was added to the respective samples and incubated for 1 h at 37 °C. Lithium dodecyl sulfate sample buffer was added and samples were heated at 70 °C for 10 min.

His-ubiquitin pulldowns

S2 cells were lysed in a buffer containing 6 M guanidinium-HCl, 0.1 M Na_2HPO_4, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl (pH 8), 5 mM imidazole and 10 mM β-mercaptoethanol (β-ME). The lysates were incubated with Ni-NTA agarose beads (Qiagen) at 4 °C overnight. The beads were washed once with a buffer containing 6 M guanidinium-HCl, 0.1 M Na_2HPO_4, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl (pH 8) and 10 mM β-ME, and twice with a buffer containing 8 M urea, 0.1 M Na_2HPO_4, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl (pH 8), 10 mM β-ME and 0.1% Triton X-100. His-Ub-conjugated proteins were eluted using a buffer containing 200 mM imidazole, 0.15 M Tris (pH 6.7), 30% glycerol, 0.72 M β-ME and 5% SDS.

HA and V5 immunoprecipitations

S2 cells were lysed in a buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 5 mM NEM, 5 mM chloroacetamide and Pierce™ Protease Inhibitor and cleared at 12,000 rpm for 10 min at 4 °C. For denaturing conditions, lysates were sonicated after adding SDS to a final concentration of 1%. After sonication the lysates were diluted to 0.1% SDS before clearing. For immunoprecipitation, samples were incubated in α-HA or α-V5 agarose beads (Sigma) for 2 h. The beads were washed three times in a buffer containing 10 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Triton X-100 and 5% glycerol. HA-conjugated or V5-conjugated proteins were eluted using Laemmli sample buffer.

Quantitative RT-PCR

Drosophila S2 cells or adult flies were homogenised using QIAshredder (Qiagen) and total RNA was extracted with RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. cDNA was synthesised with iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's protocol. qPCR was performed using Kapa SYBR Fast ABI Prism qPCR Kit (Kapa Biosystems). *rp49* was used as a housekeeping gene for ΔΔCt calculations. The following gene-specific primers were used to amplify cDNA: *AttacinA* (5′-ATGCTCGTTTGGATCTGACC, 5′-GACCTTGGCAT CCAGATTGT), *Diptericin* (5′-ACCGCAGTACCCACT CAATC, 5′-ACTTTCCAGCTCGGTTCTGA), *Drosocin* (5′-CGTTTTCCTGCTGCTTGC, 5′-GGCAGCTTGAGT CAGGTGAT), *IM1* (5′-GTTTTTGTGCTCGGTCTGCT, 5′-CACCGTGGACATTGCACA), *Drosomycin* (5′-CGTG AGAACCTTTTCCAATATGATG, 5′-TCCCAGGACCA CCAGCAT), *RBR-LDD* (5′-CGGAACCCATGCAGATC AAG, 5′-CGCAGTCCGTCAGATCAAAG), *ZnF* (5′-TGC TCCATATGCTGCAAGAC, 5′-CGGATTTCTGACTGG GTTGT), *Ub* (5′-AGGAGTCGACCCTTCACTTG, 5′-CG AAGATCAAACGCTGCTGA), and *rp49* (5′-GACGCTT CAAGGGACAGTATCTG, 5′-AAACGCGGTTCTGCAT GAG).

Immunofluorescence of *Drosophila* intestines

Intestines from female adult flies were dissected in PBS and fixed for 10 min in 4% paraformaldehyde. Samples were permeabilised with PBS-0.1% Triton X-100 for 1 h at room temperature, washed with PBS and incubated overnight at 4 °C with primary antibody rabbit anti-phospho-Histone H3 1:1000 (S10, Cell Signalling Technology) and 2 h at room temperature with secondary antibody Alexa Fluor 488 donkey anti-rabbit IgG 1:600 (#A21206, Invitrogen). Both primary and secondary antibodies were diluted in PBS and 0.1% bovine serum albumin. DNA was stained with DAPI (4′,6-diamidino-2-phenylindole) (Invitrogen). After washing with PBS, the samples were mounted using Mowiol (Sigma). Imaging was performed with a spinning disk confocal microscope (Zeiss Axiovert-200M microscope, Yokogawa CSU22 spinning disk confocal unit) using ×20 objectives. The acquisition and processing software was 3i SlideBook6 and image processing was done with Image J.

X-gal staining of *Drosophila* intestines

Intestines from female adult flies were dissected in PBS and fixed for 15 min with PBS containing 0.4% glutaraldehyde and 1 mM $MgCl_2$. The samples were washed with PBS and incubated with a freshly prepared staining solution containing 5 mg/ml X-gal, 5 mM potassium ferrocyanide trihydrate, 5 mM potassium ferrocyanide crystalline and 2 mg/ml $MgCl_2$ in PBS at 37 °C. After washing with PBS, the samples were mounted using Mowiol (Sigma) and imaged with brightfield microscopy (Leica).

Bacterial colony count

Escherichia coli was transformed with pMT/Flag-His and cultivated in LB medium at 37 °C for 16–18 h on agitation and concentrated by centrifugation (optical density of 0.150). After a 2 h starvation, female adult flies were fed for 24 h with a 1:1 solution of transformed *E. coli* in 5% sucrose at 25 °C. Two flies were cleaned with ethanol and distilled H_2O, and homogenised in 150 µl PBS. The sample was cleared at 12,000 rpm for 10 min at 4 °C, and cleared samples were diluted 1:100 and plated on LB agar plates containing 50 µg/ml ampicillin. Colonies were counted 24 h after plating.

Structural modelling

The 3D structure of the Kenny UBAN and the LUBEL CBR was modelled with Phyre2 [65]. Molecular graphics and analyses were performed with PyMol or the UCSF Chimera package [66] using the indicated templates.

Statistical analysis

Results from survival assays were analysed by two-way analysis of variance (ANOVA) and results from AMP analysis with qPCR by one-way ANOVA, both with Bonferroni's post test for 95% confidence interval. In comparison to normalised control values and in analyses of qPCR results and in colony counts, one-sample *t* tests were applied if less than three genotypes were analysed. In figures, ns stands for $p > 0.05$, * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$ and **** for $p < 0.0001$. Error bars in figures specify SEM from the indicated number of independent experiments. The experiments were repeated at least three times. With smaller differences in detection, more repeats were done.

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Conflict of interest The authors declare that they have no conflict of interest.

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M1-linked ubiquitination by LUBEL is required for inflammatory responses to oral infection in *Drosophila*

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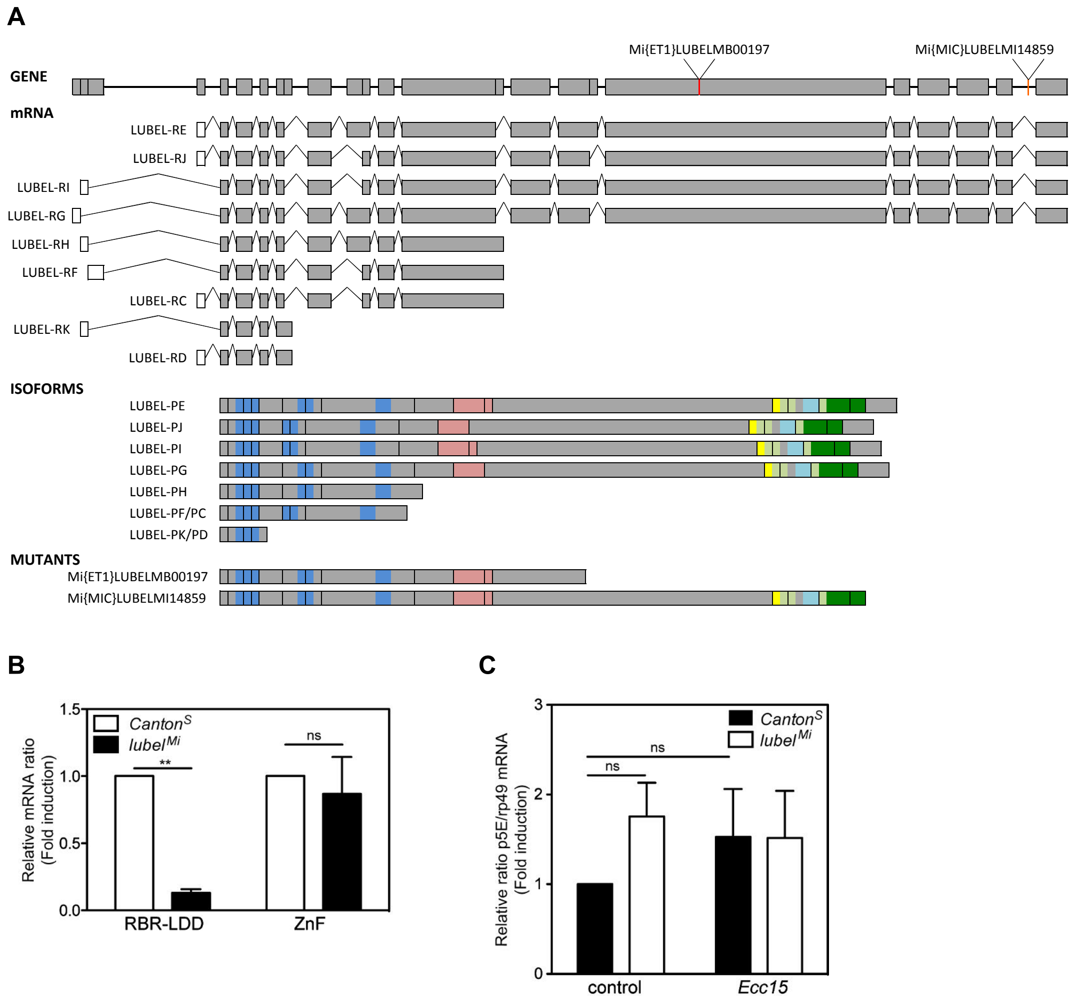
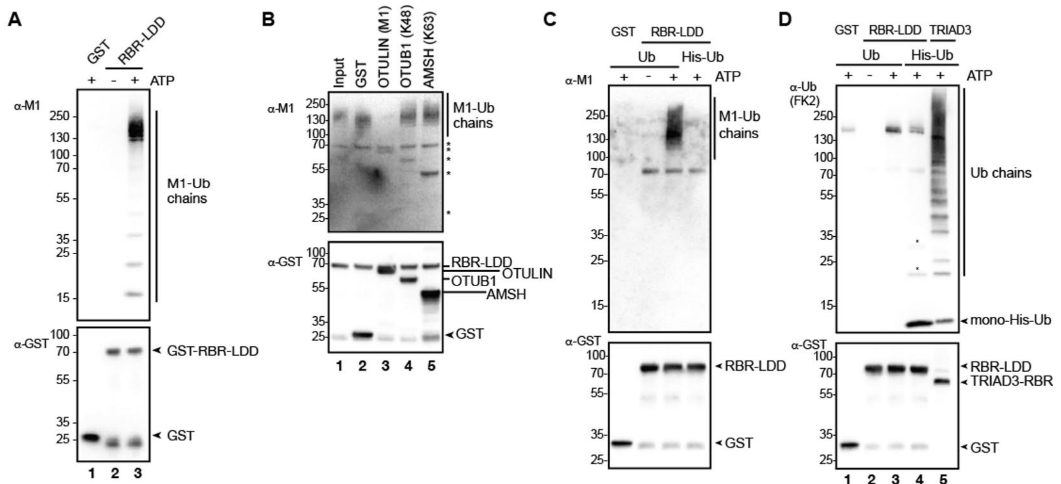
Supplementary Information

Supplementary Figure 1. Structure of the *lubel* gene and analysis of *lubel* mutant flies.



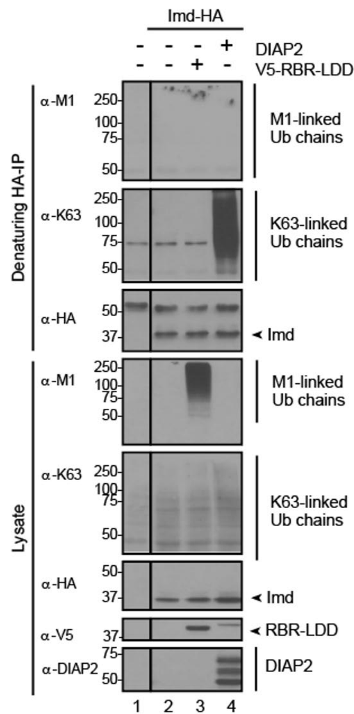
(A) Structure of the *lubel* gene, the transcribed mRNA variants, the expressed isoforms and the LUBEL proteins expressed in the *Mi{ET1}LUBELMB00197 and Mi{MIC}LUBELMI14859* mutant *Drosophila* strains. The ZnFs are indicated in blue, UBA1 in red, UBA2 in yellow, the RING domains in light green, the IBR in light blue and the LDD in green. (**B)** Comparison of mRNA transcripts of the C-terminal RBR and third ZnF in wild type *Canton*^{S} and *lubel*^{Mi} mutant flies by qPCR. Error bars indicate SEM from 3 independent experimental repeats using at least 10 flies per repeat. **(C)** Ubiquitin mRNA levels were compared in *Canton*^{S} and *lubel*^{Mi} mutant flies by qPCR. The flies were either non-treated or subjected to septic injury with the Gram-negative bacteria *Ecc15*. Error bars indicate SEM from 3 independent experimental repeats using at least 10 flies per repeat.

Supplementary Figure 2. The RBR-LDD of LUBEL selectively mediates M1-Ub chain formation.



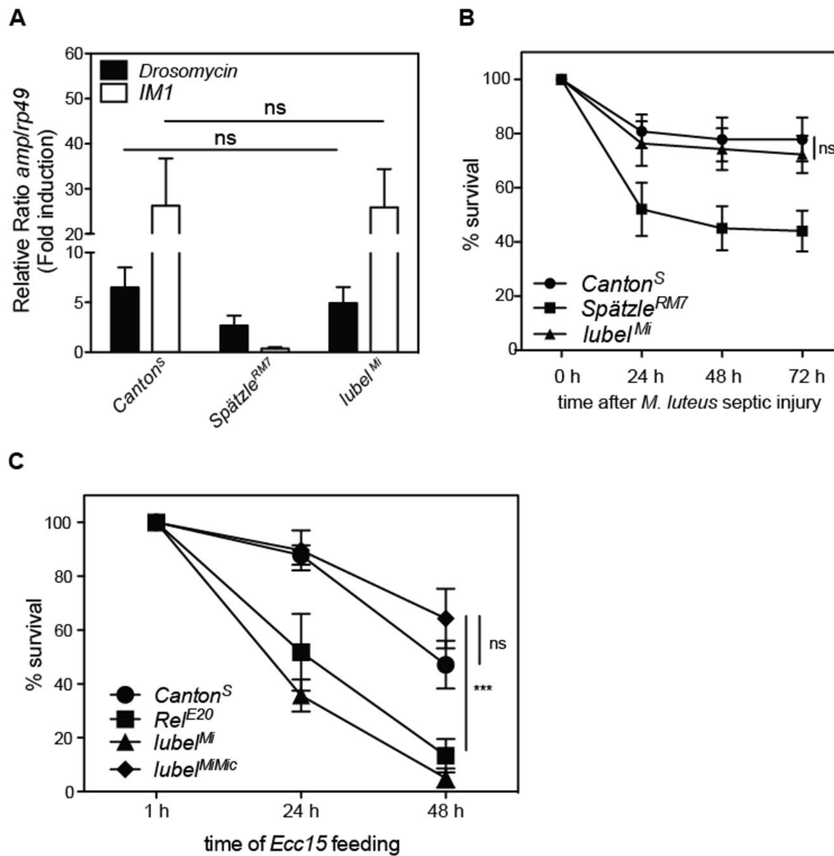
(A) GST-RBR-LDD fusion protein or GST protein were incubated in *in vitro*-ubiquitination reactions and samples were analysed by Western blotting using α-M1 and α-GST antibodies, n=3. **(B)** RBR-LDD-ligated Ub chains were analysed using UbiCRest and incubated with GST protein, OTULIN, OTUB1, or AMSH deubiquitinating enzyme, specific for M1, K48 or K63 chains, respectively. Western blot analysis with α-M1 and α-GST antibodies (* in upper panel indicates unspecific bands of GST-fusion proteins), n=3. **(C,D)** GST-RBR-LDD, GST-TRIAD3-RBR or GST were incubated with ubiquitin or N-terminally blocked His-ubiquitin (His-Ub). Chain formation was analysed by Western blotting using α-M1 (C) and FK2 α-Ub antibody (D). TRIAD3-RBR efficiently synthesised Lys-linked chains with His-Ub, while RBR-LDD only catalysed low level-formation of di- and tri-His-Ub, indicated with asterisks. Lower panels show Western blot analysis using α-GST antibody, n=7.

Supplementary Figure 3. The *Drosophila* Imd is not modified with M1-Ub chains.



Drosophila S2 cells were transfected with empty vector, HA-tagged Imd and V5-tagged wild type LUBEL RBR-LDD or DIAP2. HA-immunoprecipitations were performed at denaturing conditions and the samples were analysed by Western blotting with α-M1, α-K63, α-HA, α-V5 and α-DIAP2 antibodies, n=3.

Supplementary Figure 4. Analysis of *lubel* mutant flies and LUBEL transgenes.



(A) The Toll pathway was induced in adult *Canton*^{S}, *Spätzle*^{RM7} and *lubel*^{Mi} mutant flies by septic injury with the Gram-positive bacteria *M. luteus*. Dif activation was studied by analysing the expression of the AMPs *IM1* and *Drosomycin* with qPCR. *Spätzle*^{RM7} mutant flies were used as negative controls. Error bars indicate SEM from 3 independent experimental repeats using at least 10 flies per repeat. **(B)** Adult wild type *Canton*^{S} and *Spätzle*^{RM7} and *lubel*^{Mi} mutant flies were subjected to septic injury with the Gram-positive bacteria *M. luteus* and their survival was monitored over time. *Canton*^{S} flies were used as wild type controls and *Spätzle*^{RM7} mutant flies as negative controls. Error bars indicate SEM from 5 independent experimental repeats using at least 20 flies per repeat. **(C)** Adult *Canton*^{S}, *Rel*^{E20}, *lubel*^{Mi} and *lubel*^{MiMic} mutant flies were infected by feeding with the Gram-negative bacteria *Ecc15* and their survival was monitored over time. Error bars indicate SEM from 3 independent experimental repeats using at least 20 flies per repeat.

Aravind Kumar Mohan

Regulation of Inflammatory Signalling by Caspases and M1-linked Ubiquitin Chains in *Drosophila melanogaster*

The nuclear factor κ-light-chain enhancer of activated B cells (NF-κB) family of transcription factors are master regulators of inflammatory signalling, and they control the expression of several inflammatory genes. Uncontrolled activation of NF-κB can lead to chronic inflammation which is known to promote cancer progression. To treat inflammatory diseases, a deeper understanding of regulation of the NF-κB pathway at the molecular level is needed. The NF-κB pathway activity is tightly regulated by post-translational modifications such as ubiquitination. This thesis aims to understand the molecular mechanisms that control the activation and inhibition of the NF-κB pathway. Due to evolutionary conservation of signalling pathways and availability of molecular biology tools, the fruit fly *Drosophila melanogaster* serves as an excellent model to study molecular mechanisms of regulation of the NF-κB pathway. This thesis proposes new regulatory roles of caspases and M1-linked ubiquitin (M1-Ub) chains. It describes a mechanism by which the caspase, *Drosophila* interleukin 1β-converting enzyme (Drice), restrains the NF-κB pathway activity. Furthermore, it demonstrates the key role of M1-Ub chains and the caspase, Death related ced-3/Nedd2-like caspase (Dredd), in fine tuning the NF-κB pathway activity especially in the intestinal epithelial tissue.

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