Anna Aalto

Met1-ubiquitination determines inflammatory signalling in *Drosophila melanogaster*

Åbo Akademi University

Anna Aalto

Born 1987 in Åbo, Finland

Previous studies and degrees

M.Sc. degree in Cell Biology, Åbo Akademi University, 2012

B.Sc. degree in Cell Biology, Åbo Akademi University, 2011

Met1-ubiquitination determines inflammatory signalling in *Drosophila melanogaster*

Anna Aalto

Cell Biology
Faculty of Science and Engineering
Turku Doctoral Network in Molecular Biosciences
Åbo Akademi University,
Turku, Finland, 2022

From the Faculty of Science and Engineering, Cell Biology, Turku Doctoral Network in Molecular Biosciences, Åbo Akademi University

Supervised by

Docent Annika Meinander, PhD
Faculty of Science and Engineering, Cell Biology, Åbo Akademi University,
Turku, Finland

Reviewed by

Docent Susanna Valanne, PhD
Faculty of Medicine and Health Technology, Tampere University
Tampere, Finland

and

Maria de las Nieves Peltzer, PhD
Department of Translational Genomics, CECAD Cologne, University of Cologne
Cologne, Germany

Opponent

Professor Mads Gyrd-Hansen, PhD
Department of Immunology and Microbiology, University of Copenhagen
Copenhagen, Denmark

ISBN 978-952-12-4206-9 (printed)
ISBN 978-952-12-4207-6 (digital)
Painosalama, Turku Finland 2022

To my grandfather

Ukille

TABLE OF CONTENTS

ABSTRACT

The innate immune response is an immediate immune response facilitated by the host to combat pathogen invasion and other harmful stimuli, such as damaged cells and environmental irritants. It is activated when pattern-recognising receptors (PRRs) on immune cells detect pathogen-associated molecular patterns (PAMPs) or stress/damage-associated molecular patterns (SAMPs/DAMPs) and respond to these stresses by activating rapid transcription of inflammatory cytokines and chemokines. Upon receptor triggering one of the main pathways induced is the nuclear factor κ-light-chain enhancer of activated B cells (NF-κB) signalling pathway leading to activation of the transcription factor NF-κB. During basal conditions, these pathways are tightly regulated as unwanted activation of NF-κB is associated with chronic inflammation and promotes cancer progression. Post-translational modifications (PTMs), such as ubiquitination, play a key role in the regulation of the inflammatory NF-κB signalling. In my thesis, my main objective is to improve our understanding of ubiquitin-mediated regulation of NF-κB, with a specific focus on Met1-linked ubiquitination. Conjugated Met1-linked polyubiquitin (Met1-Ub) chains function as scaffolds for other NF-κB signalling mediators and are essential for proper NF-κB signalling.

To further study the role of Met1-ubiquitination in NF-κB signalling, we have taken advantage of the highly efficient and conserved NF-κB signalling pathways in the fruit fly, *Drosophila melanogaster*. By using the fruit fly as a model organism, we have found the E3 ligase LUBEL to synthesise Met1-Ub chains in *Drosophila* when the NF-κB activating pathway is induced by bacterial infection. Furthermore, the fruit fly IκB kinase γ (IKKγ), a known mammalian substrate for Met1-linked ubiquitination, was similarly identified as a substrate in the flies. This indicates a conserved signalling outcome in response to pathogenic invasion in flies. We have also found that Met1-Ub chains are required and sufficient to induce NF-κB activation and defence against pathogens in the intestinal epithelium. Met1-Ub chain formation is also augmented by sterile stresses such as hypoxia, oxidative stress, starvation and mechanical stress. During sterile inflammation, Met1-Ub chains are required for survival and this protective action seems to be driven by stress-induced activation of NF-κB in a PRR-independent manner. Finally, we show that the stress-induced upregulation of Met1-Ub chains in response to hypoxia, oxidative and mechanical stress is also induced in mammalian cells and protects from stress-induced cell death. In addition, we have optimised a model for detection and induction of intestinal inflammation in *Drosophila*. We have used this model to study the anti-inflammatory properties of stilbenoid-compounds *in vivo*. Taken together, we have used the fruit fly to study the molecular regulatory mechanisms of inflammatory signalling in response to a wide range of noxious stresses and provided us with new tools to manipulate and regulate inflammatory signalling.

SAMMANFATTNING (ABSTRACT IN SWEDISH)

Det medfödda immunsvaret är en omedelbar immunrespons som förmedlas av värdorganismen för att bekämpa skadliga stimuli, såsom patogener, skadade celler och andra irriterande molekyler. Immunresponsen aktiveras när receptorer på immunceller påträffar patogenassocierade molekylära mönster eller stress-/skadeassocierade molekylära mönster och svarar på dessa påfrestningar genom att aktivera snabb transkription av inflammatoriska cytokiner och kemokiner. Vid receptorstimulering aktiveras NF-κB-signaleringsräckan, som leder till aktivering av transkriptionsfaktorn NF-κB. Under basala förhållanden är dessa signaleringsräckor hämmade eftersom överaktiv NF-κB associeras med kronisk inflammation och gynnar uppkomst av tumörer. Post-translationella modifieringar, såsom ubikvitinering, har en nyckelroll i regleringen av den inflammatoriska NF-κB-signaleringen. I min avhandling är mitt huvudmål att förbättra vår förståelse av ubikvitin-medierad reglering av NF-κB, med ett specifikt fokus på Met1-ubikvitinering. Met1-kopplade ubikvitinkedjor fungerar som en rekryterings-plattform för andra NF-κB-signalförmedlare och har därmed en essentiell funktion i NF-κB-signaleringen.

För att vidare studera betydelsen av Met1-ubikvitinering i NF-κB-signaleringen, har vi utnyttjat de mycket effektiva och välbevarade NF-κB-signaleringsräckorna hos bananflugan, Drosophila melanogaster. Genom att använda bananflugan som modellorganism, har vi funnit att E3-ligaset LUBEL syntetiserar Met1-kopplade polyubikvitinkedjor i bananflugan vid bakteriell infektion. Dessutom har vi identifierat bananflugans IκB kinas γ (IKKγ), ett känt substrat för Met1-ubikvitinering i däggdjur, som substrat för Met1-ubikvitinering. Detta antyder att de molekylära mekanismerna, som reglerar NF-κB aktivering vid patogen invasion, är välbevarade. Vi har även funnit att LUBEL är viktigt för lokalt immunsvar i tarmepitelet. Met1-Ub-kedjebildning induceras också av steril stress, som hypoxiska förhållanden, oxidativ stress, svält och mekanisk stress. Vid steril inflammation krävs Met1-ubikvitinkedjor för överlevnad och denna skyddande effekt drivs av stressinducerad aktivering av NF-κB-signaleringen på ett receptoroberoende sätt. Vi har också funnit att den stressinducerade uppregleringen av Met1-Ub-kedjor induceras vid hypoxiska förhållanden, oxidativ och mekanisk stress i däggdjursceller och att kedjorna skyddar mot stressinducerad celldöd. Dessutom har vi optimerat en modell för att inducera och utforska förorsakare av tarminflammation hos bananflugan. Den optimerade tarminflammations-modellen har vi vidare utnyttjat för att studera de antiinflammatoriska egenskaperna av stilbenoidföreningar in vivo i bananflugan. Sammanfattningsvis har ovannämnda studier belyst den praktiska nyttan med att studera regleringen av inflammatorisk signalering, som respons på ett brett spektrum av skadliga stressförhållanden, i bananflugan och försett oss med nya verktyg för att manipulera och reglera inflammatorisk signalering.

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), and on additional unpublished data included in the results section. The original publications have been reproduced with the permission of the copyright holders.

I. **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

II. **Aalto AL**, Martínez-Chacón G, Kietz C, Tsyganova N, Kreutzer J, Kallio P, Broemer M, Meinander A (2022) M1-linked ubiquitination facilitates NF-κB activation and survival during sterile inflammation. *FEBS Journal*, doi: 10.1111/febs.16425.

III. **Aalto AL**, Saadabadi A, Lindholm F, Kietz C, Himmelroos E, Marimuthu P, Salo-Ahen OMH, Eklund P, Meinander A. Modulating DSS-induced intestinal inflammation with stilbenoid compounds in *Drosophila melanogaster*. *Manuscript.*

AUTHOR CONTRIBUTION

I. The author and AKM contributed equally to the design and execution of most of the experiments, data analysis and writing of the manuscript. For this study, the author performed following experimental procedures: oral and septic survival assays, qPCR of fly lysates, transfection of S2 cells, immunoprecipitations and recombinant protein pulldowns on S2 cell and fly lysates, immunofluorescence staining of dissected guts, colony counting of pathogenic bacteria and structural modelling of the catalytic domain of LUBEL. SK planned and performed deubiquitination assays on fly and cell samples, LS performed all *in vitro* ubiquitination assays, CK planned and performed the dissections and immunofluorescence staining. MB, HW and AM contributed to the design of the experiments, writing and data analysis of this manuscript.

II. The author designed and executed most of the experiments, data analysis and writing of the manuscript. The author performed following experimental procedures: hypoxia and paraquat survival assays, qPCR of fly lysates, recombinant protein pulldowns on fly lysates, X-Gal staining of dissected larvae, reared flies under axenic conditions. CK planned and performed caspase assays. NT planned and performed the mechanical stress experiments in flies and human cells. GMC planned and performed experiments with human cells and contributed to data analysis and writing of the manuscript. JK and PK planned and assembled the MiniHypoxy platform. MB contributed to the design of the experiments. AM contributed to the design of the experiments, writing and data analysis of this manuscript.

III. The author contributed to designing and executing the stilbene experiments, data analysis and writing of the manuscript. The author performed following experimental procedures: feeding DSS, stilbenoid compounds and TRPA1 antagonist to larvae, qPCR of larvae lysates, reared flies under axenic conditions. AS performed molecular modelling studies, isolation of natural compounds and writing of the manuscript. FL and CK planned and performed the optimisation of DSS feeding and 16S sequencing. EH performed axenic experiments. PM supervised the comparative modelling. OSA contributed to the design and analysis of the modelling studies and writing of the manuscript. PE contributed to the design of the experiments. AM contributed to the design of the experiments, writing and data analysis of this manuscript.

ABBREVIATIONS

ABIN	A20-binding inhibitors
AMP	Antimicrobial peptide
AMSH	Associated molecule with the SH3 domain of STAM
AnkR	Ankyrin repeat
ATG	Autophagy-related genes
BIR	Baculovirus IAP Repeat
Caco	Colon adenocarcinoma
CaMKII	calcium/calmodulin-dependent kinases 2
CAP-Gly	Cytoskeleton-associated protein Gly-rich domain
CARD	Caspase activation and recruitment domain
CD	Crohn's disease
c-FLIP	Cellular FLICE-like inhibitory protein
cIAP	Cellular inhibitor of apoptosis
CLR	Cullin-RING ligase
CoZi	coiled-coil and leucine zipper
cpdm	Chronic proliferative dermatitis
CRL	Cullin-RING ligase
CYLD	Cylindromatosis-associated
daGal4	Daughterless-Gal4
DAMP	Damage-associated molecular pattern
DAP	Diaminopimelic
DD	Death domain
DDR	DNA damage response
DED	Death effector domain
Diap	Drosophila inhibitor of apoptosis
Dif	Dorsal-related immunity factor
Dipt	Diptericin
Dredd	Death-related ced-3/Nedd2-like protein
Drice	Drosophila interleukin 1β-converting enzyme
Drs	Drosomycin
DSS	Dextran sodium sulphate
DUB	Deubiquitinating enzyme
DUOX	Dual oxidase
Eiger	Ectodysplasia-like cell death trigger
Fadd	Fas-associated death domain
Gint3	GDI interacting protein 3
GNBP	Gram-negative binding proteins
GRP	glucan recognition protein
HDAC	Histone deacetylase
HECT	Homologous to E6-AP carboxy terminus
HHARI	Human homologue of Ariadne
HIF	Hypoxia-inducible factor

HOIL	Heme-oxidized iron-responsive element- binding protein 2 ubiquitin ligase
HOIP	HOIL-1-interacting protein
HOIPIN	HOIP inhibitor
HPV	Human papilloma virus
Htt-polyQ	Huntingtin containing a pathogenic polyglutamine expansion
IAP	Inhibitor of apoptosis
IBD	Inflammatory bowel disease
IBM	IAP binding motif
IFN	Interferon
IKK	IκB kinases
IL-1β	Interleukin-1β
Imd	Immune deficiency
IRF	Interferon regulatory factor
ISG15	Interferon-stimulated gene product of 15
JAB1	C-Jun activation domain-binding protein 1
Jak/Stat	Janus kinases/signal transducer and activator of transcription proteins
JAMM	JAB1/MPN/MOV34 metalloprotease
JNK	c-Jun N-terminal kinase
LC3	Microtubule-associated protein 1A/1B-light chain 3
LDD	Linear chain determining domain
LIR	LC3-interacting region
LOF	Loss-of-function
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
LSCC	Lung squamous cell carcinoma cells
LTM	LUBAC-tethering motif
LUBAC	Linear ubiquitin chain assembly complex
LUBEL	Linear ubiquitin E3 ligase
MAPK	Mitogen-activated protein kinase
MDP	Muramyl dipeptide
Met1-Ub	Met1-linked linear ubiquitin
MHC	Major histocompatibility complex
MINDY	Motif interacting with ubiquitin (MIU)- containing novel DUB family
MPN	MPR1/PAD1 N-terminal
MPR1	Multistep phosphorelay regulator 1
MyD88	Myeloid differentiation primary-response protein
NACHT	NAIP, cIITA, HET-E, TP1
NBR1	Neighbor of BRCA1 receptor 1
Nedd	Neural precursor cell-expressed, developmentally downregulated
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor κ-light-chain enhancer of activated B cells

NLR	NOD-like receptor
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
PAD1	Peptidylarginine deiminase 1
PAMP	Pathogen-associated molecular pattern
Parkin	Parkinson juvenile disease protein 2
PGRP	Peptidoglycan recognition protein
PH	Pleckstrin homology
pH3	Phosho-histone3
PHD	Plant homeodomain
PIM	PUB domain-interacting motif
pirk	Poor Imd response upon knock-in
PM	Peritrophic matrix
PNG	Peptide:N-glycanase
PRR	Pattern-recognition receptor
PS	Pinosylvin
PSMME	Pinosylvinmonomethylether
PTM	Post-translational modification
PUB	UBA or UBX-containing protein
qPCR	Quantitative reverse transcriptase
RBCK1	Ran binding protein 2-Type and C3HC4-type Zinc Finger Containing 1
RBR	RING-in between-RING
RH	RBCK1 homology
RHD	Rel-homology domain
RIG-I	Retinoic acid-inducible gene I
RING	Really interesting new gene
RIPK	Receptor interacting protein kinases
RNAi	RNA interference
RNF	Ring Finger Protein
ROS	Reactive oxygen species
rRNA	ribosomal RNA
SA	Survival assay
SAMP	Stress-associated molecular pattern
SCF	Skp1-Cullin1-F-box
SH2/3	Src homology 2/3
SHANK	SH3 and multiple ankyrin repeat domains protein
SHARPIN	SHANK-associated RH-domain- interacting protein
Sima	Similar
SPATA2	Spermatogenesis-associated protein 2
SPE	Spätzle processing enzyme

SUMO	Small ubiquitin-related modifier
TAB2	TAK binding protein 2
TAD	Transactivation domain
TAK1	TGF-β-activated kinase 1
TANK	TRAF Family Member Associated NFKB Activator
TCR/BCR	T- and B-cell receptor
TER94	Transitional endoplasmic reticulum 94
TGFβ	Transforming growth factor β
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFR	TNF receptor
TP	Telomerase-associated protein
TRADD	TNFR1-associated death domain
TRAF	TNF receptor associated factors
TRAIL	TNF-related apoptosis-inducing ligand
TRIF	Toll-interleukin (IL)-1-resistance domain-containing adapter protein inducing IFNβ
TRPA1	Transient receptor potential ankyrin 1
TUBE	Tandem ubiquitin-binding entity
UAS	Upstream activating sequence
Ub	Ubiquitin
UBA	Ubiquitin-associated
UBAN	Ubiquitin-binding domain in ABINs and NEMO
UBC	Ubiquitin-conjugating
UBD	Ubiquitin-binding domain
UC	Ulcerative colitis
UCH	Ubiquitin C-terminal hydrolases
USP	Ubiquitin-specific protease
VCP	Valosin-containing protein
WB	Western blot
X-Gal	5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside
XIAP	X-chromosome-linked inhibitor of apoptosis
ZnF	Zinc finger
Wnt	Wingless/integration site 1 (Int1)

INTRODUCTION

Posttranslational modifications (PTMs) refer to modifications of proteins following protein synthesis. PTMs can be divided into two types of modifications, the addition of chemical groups and the addition of small proteins to substrates. In ubiquitination, the small ubiquitin protein is conjugated to a substrate via a three-step enzymatic cascade. Polyubiquitin chains are formed when a ubiquitin moiety is attached to another ubiquitin. These polyubiquitin chains vary in topology and can be differently recognised by various ubiquitin recognising proteins, resulting in a versatile signalling outcome that allows the cell to regulate its functions in a highly complex and accurate way. Ubiquitination was first discovered to target proteins for degradation, however later, it has been shown to have non-proteolytic functions in signal transduction, endocytosis, autophagy, cell cycle, DNA stability, metabolic pathways, transcription, and translation. Nowadays, ubiquitination is recognised as a dynamic and reversible PTM.

This thesis focuses mainly on the role of ubiquitination in the regulation of nuclear factor κ-light-chain enhancer of activated B cells (NF-κB) signalling, with a specific aim to advance our knowledge of Met1-linked linear ubiquitin (Met1-Ub)-mediated regulation. Met1-Ub polyubiquitin chains are formed when a ubiquitin moiety is conjugated to the N-terminal methionine of acceptor ubiquitin. These ubiquitin chains have been described to have an essential role in inflammatory NF-κB signalling by functioning as scaffolds for signalling mediators. The NF-κB signalling pathways, leading to activation of the transcription factor NF-κB, are activated by pattern-recognition receptors (PRRs). PRRs detect harmful irritants such as pathogens and damaged cells. Activated NF-κB subsequently results in production of cytokines and chemokines, required for restoring cellular homeostasis.

Drosophila melanogaster, or the fruit fly, is an exemplary model organism for research in innate immune signalling. Even though the fruit fly has a less complex immune signalling in comparison to mammalian biological models, the NF-κB signalling pathways are still highly efficient and conserved in the flies. The fruit fly has been extensively utilised as a model system for innate immunity and there is a treasure trove of biological tools for conducting elaborate *in vivo* studies in the flies. By activating the NF-κB pathway in flies by pathogen infection and various pathological stress conditions, such as hypoxia and oxidative stress, we have assessed the role of Met1-ubiquitination in the signalling leading to transcriptional activity of NF-κB. We have also refined protocols for inducing inflammation in the intestine, optimised the use of a novel hypoxia chamber for *Drosophila* experiments, and developed protocols for dissection of tissue-specific activation of NF-κB in response to distinct infectants and stresses in the intestine and trachea of flies.

The first study of this thesis examines the role of Met1-ubiquitination in bacterial infection. The study presents the key players for assembly, disassembly, and recognition of Met1-Ub chains in flies and presents that Met1-Ub chains have a protective role in response to bacterial challenges. The second study reveals that Met1-Ub chains are also required for protection against sterile inflammation. Concomitantly, the thesis aims to advocate the use of *Drosophila* as a model organism to study NF-κB-mediated immune signalling and provides with means to regulate the immune response with anti-inflammatory compounds. All in all, this thesis provides tools to probe possible novel NF-κB signalling mediators and general regulators of immune signalling and highlights the practicality of the fruit fly in studying the regulation of inflammatory signalling.

REVIEW OF THE LITERATURE

1 Ubiquitination is a versatile post-translational modification (PTM)

Ubiquitination is a well-studied PTM that covalently binds the small ubiquitin (Ub) protein to target proteins and is best known for its role in the signalling of target proteins to degradation. Ubiquitin is found ubiquitously in eukaryotic cells, hence the name ubiquitin (Goldstein *et al.*, 1975). Ubiquitin was first discovered to promote lymphocyte differentiation *in vitro* (Goldstein *et al.*, 1975). Thereafter, it was shown to be covalently bound to a lysine residue on histone 2A (H2A)(Schlesinger, Goldstein and Niall, 1975; Goldknopf and Busch, 1977; Hunt and Dayhoff, 1977; Levinger and Varshavsky, 1980, 1982). Concurrently, first discoveries were made on ubiquitin to be covalently bound to proteins targeted for ATP-dependent proteolysis (Ciehanover, Hod and Hershko, 1978; Hershko *et al.*, 1980). In 1984, two seminal papers showed that ubiquitination targets short-lived proteins for degradation *in vivo* and is required for cell survival, cell cycle progression and cell stress response (Ciechanover, Finley and Varshavsky, 1984; Finley, Ciechanover and Varshavsky, 1984). For a while, majority of the research done in the ubiquitin field were on how ubiquitination is involved in the proteasomal degradation of proteins, and was often called the UPS, for ubiquitin-proteasome system. However, the first indication of non-proteolytic role of ubiquitination came in 1989, when it was shown to have a co-translational chaperone properties in ribosomal biogenesis (Finley, Bartel and Varshavsky, 1989). Since then, ubiquitination has been shown to also have a plethora of other non-proteolytic functions, such as signal transduction, endocytosis, autophagy, cell cycle, DNA stability, trafficking, metabolic pathways, transcription, and translation. Nowadays, ubiquitination is recognised as a dynamic and reversible PTM, that allows the cell to regulate its functions in a highly complex and accurate way (Swatek and Komander, 2016).

The ubiquitin system is conserved throughout the evolution from eukaryotes to prokaryotes and archaea. It encompasses all the enzymes required for attaching and removing ubiquitin to/from a substrate, as well as all the proteins that bind ubiquitinated proteins and thus leading to the desired outcome (Hochstrasser, 2009; Humbard *et al.*, 2010). The addition of a small ubiquitin protein to a target protein is a three step reaction involving three enzymes; the ubiquitin activating enzyme (E1), the ubiquitin conjugating enzyme (E2) and the ubiquitin ligase (E3)(Ciechanover *et al.*, 1981, 1982; Hershko *et al.*, 1983). Polyubiquitin chains are created when ubiquitin itself is ubiquitinated (Hershko and Heller, 1985). These chains are then recognised by proteins with ubiquitin-binding domains (UBDs) and thus the signal is forwarded. Ubiquitination is a reversible modification and the removal of the

ubiquitin code from target proteins is performed by deubiquitinating enzymes or deubiquitinases (DUBs).

1.1 The ubiquitin gene

Ubiquitin is a highly stable 76 amino acid protein and is encoded as a multigene family in all eukaryotes. The ubiquitin gene is expressed as different ubiquitin precursors, either as a polyubiquitin or as a fusion protein with other proteins (Dworkin-Rastl, Shrutkowski and Dworkin, 1984; Özkaynak, Finley and Varshavsky, 1984; Bond and Schlesinger, 1985; Lund *et al.*, 1985; Wiborg *et al.*, 1985; Arribas, Sampedro and Izquierdo, 1986; Özkaynak *et al.*, 1987; Finley, Bartel and Varshavsky, 1989; Poch, Arribas and Lzquierdo, 1990). The polyubiquitin genes have been identified from yeast to mammals and are organised as head-to-tail repeating tandem units, with the last ubiquitin terminating with additional residues (Dworkin-Rastl, Shrutkowski and Dworkin, 1984; Özkaynak, Finley and Varshavsky, 1984; Lund *et al.*, 1985; Arribas, Sampedro and Izquierdo, 1986). The polyubiquitin gene is usually inducible and essential for cellular resistance to high temperatures, starvation, and other stresses (Finley, Ciechanover and Varshavsky, 1984; Bond and Schlesinger, 1985, 1986; Finley, Özkaynak and Varshavsky, 1987; Fornace *et al.*, 1989).

The ubiquitin fusion proteins, also called ubiquitin-extension proteins, are followed by in-frame fused proteins, usually ribosomal proteins (Özkaynak *et al.*, 1987; Finley, Bartel and Varshavsky, 1989; Redman and Rechsteiner, 1989; Poch, Arribas and Lzquierdo, 1990). The ubiquitin acts like a co-translational chaperone in front of a ribosomal protein moiety and is required for efficient biogenesis of ribosomes (Finley, Bartel and Varshavsky, 1989). The ubiquitin precursors are processed by DUBs, yielding mature ubiquitin, in this way the cell generates large numbers of ubiquitin molecules in response to an activating cue. Polyubiquitin precursors can be cleaved at the ribosome, however, a fraction of the precursors escape the co-translational cleavage and remain in partially processed or unprocessed forms (Grou *et al.*, 2015).

1.2 The ubiquitin structure

The ubiquitin structure is a highly conserved structure from eukaryotes to bacteria (Schlesinger, Goldstein and Niall, 1975; Lenkinski *et al.*, 1977; Gavilanes *et al.*, 1982; Dye and Schulman, 2007). Ubiquitin is an extremely compact and tightly hydrogen-bonded structure consisting of 3.5 turns of an amphipathic α-helix, a short 3_{10}-helix, a mixed β-sheet that contains five strands and seven reverse turns (Figure 1A). Ubiquitin belongs to the superfamily of β-grasp fold proteins and the hydrophobic core formed between the β-sheet and α-helix gives this structure stability. The ubiquitin structure is shared by a conserved family of proteins called ubiquitin-like

(UBL) modifiers. UBL proteins are similar in structure and conjugation mechanism to ubiquitin but differ in the DNA sequence (Hochstrasser, 2009; Cappadocia and Lima, 2018). In addition to ubiquitin, at least eight other eukaryotic UBL proteins are conjugated to their substrates and known to possess the β-grasp fold partially wrapping around a central α-helix. These conjugating UBLs are small ubiquitin-related modifier (SUMO), neural precursor cell-expressed, developmentally downregulated 8 (Nedd8), autophagy-related genes 8 and 12 (Atg8/12), Ub-related modifier 1 (Urm1), ubiquitin-fold modifier 1 (Ufm1), major histocompatibility complex, class I F (HLA-F) adjacent transcript 10 (FAT10), and interferon-stimulated gene product of 15 kDa (ISG15) and are referred to as type I UBLs. Type II UBLs are not conjugated to substrates and are usually found in multiprotein complexes (Cappadocia and Lima, 2018).

The protruding carboxyl (C-) terminus, consisting of six residues, is the only flexible part of ubiquitin. Thus, this portion of the molecule is accessible by enzymes involved in formation of the isopeptide bond between ubiquitin and substrate (Vijay-Kumar *et al.*, 1985; Vijay-kumar, Bugg and Cook, 1987). Apart from the flexible C-terminal tail, the first loop (β1/β2) containing Leu8, shows some flexibility and can adopt different conformations, which is used by proteins binding to ubiquitin (Lange *et al.*, 2008). The main functionality of the ubiquitin modification is that the ubiquitin moieties are recognised by other proteins. These interactions are formed through the ubiquitin surface residues and mainly through two hydrophobic patches, the Ile44 patch, consisting of Ile44, Leu8, Val70, and His68, and the Ile36 patch, which involves Leu71 and Leu73 of the ubiquitin tail. In addition, the Phe4 patch surface with Gln2, Phe4 and Thr12 can also interact with ubiquitin binding proteins (Figure 1B) (Komander and Rape, 2012).

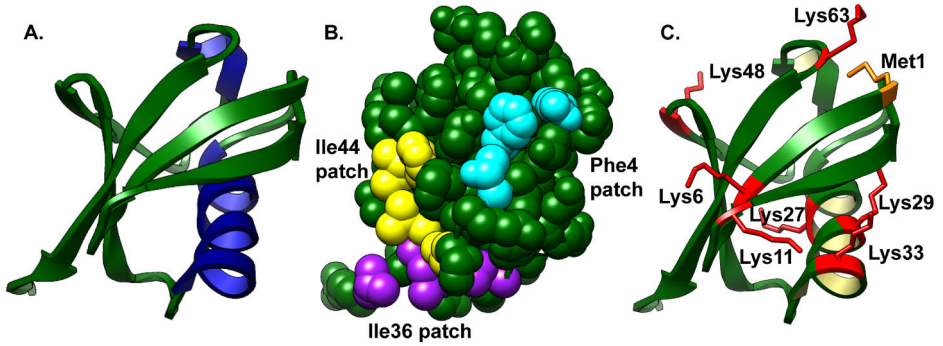

Figure 1. Ubiquitin structure. A. Ubiquitin structure, with beta-strands in green and alpha-helices as blue. B. Ubiquitin with the hydrophobic patches marked. C. Ubiquitin with the lysine residues marked as red and the N-terminal methionine as orange. Ubiquitin PDB: 1TBE.

Ubiquitin moieties can be attached to one another and create polyubiquitin chains. These ubiquitin moieties are attached either to one of the lysine residues Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63 or the N-terminal methionine of acceptor ubiquitin. These seven lysine residues and Met1 are pointing to different directions on the ubiquitin surface (Figure 1C). Therefore, when different lysine residues are used to attach ubiquitin moieties to one another, the chain conformation varies depending on which lysine was used. Ubiquitin chains can have either compact conformation, where ubiquitin moieties interact with each other, or open conformation where the only point of interaction is the linkage site. Structural characterisation shows that ubiquitin chains linked via Lys6, Lys11, Lys27 and Lys48 adopt a compact conformation, while linking via Lys29, Lys33, Lys63 and Met1 results in an open conformation (Figure 2) (Tenno *et al.*, 2004; Komander *et al.*, 2009; Komander and Rape, 2012; Castañeda *et al.*, 2016). In the Lys48-linked polyubiquitin structures ubiquitin moieties interact predominantly with their Ile44 patches, however some interactions with the Ile36 is also possible, leaving the important Ile44 patch open for other proteins to recognise (Eddins *et al.*, 2007). Lys11-chains also have a closed conformation, however the Ile44 patch is always exposed and ready to interact in Lys11-Ub chains (Bremm, Freund and Komander, 2010). Interestingly, Lys27-Ub chains exhibit unique properties among other polyubiquitin chain types. This is to be expected as the Lys27 is the only lysine residue buried in the ubiquitin structure and requires chemical shift in the hydrophobic residues in order for it to be used for ubiquitination (Castañeda *et al.*, 2016).

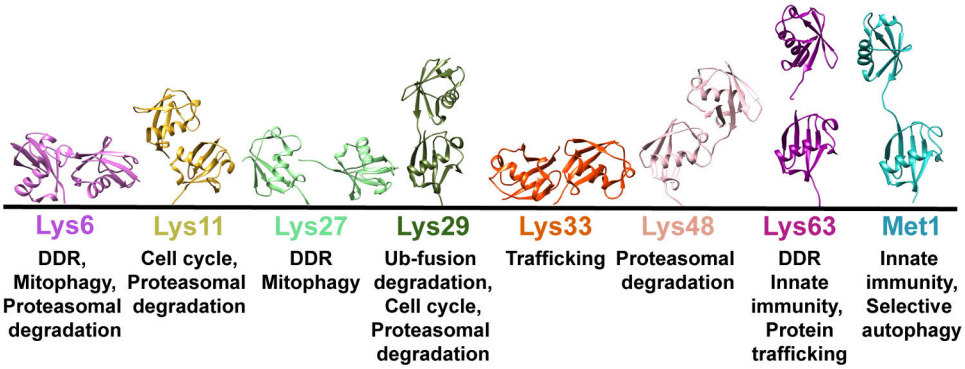

Figure 2. Structure and biological relevance of the different ubiquitin linkages. Individual polyubiquitin chains are represented by their di-Ub crystal structures (PDB from left, 2XK5, 2XEW, 6QML, 4S22, 4XYZ, 5GOI and 2JF5 and 2W9N). Biological relevance of each polyubiquitin chain type are shown below (described more in section 1.6), DDR=DNA damage response (Based on Franklin and Pruneda, 2021).

The ubiquitin tail used for linkage is flexible, resulting in conformational variability of ubiquitin chains. This feature is more prominent in the chain types with more open conformation, such as Lys63- and Met1-linked, because they have less interactions with other ubiquitin moieties. In these chains the

binding partners are more likely to determine binding specificity by utilising the distance and flexibility between ubiquitin moieties, instead of binding surfaces (Komander *et al.*, 2009). Recognition of the structural differences between these chain types enables cells to build a complex signalling platform to maintain cellular homeostasis (Rahighi and Dikic, 2012).

1.3 The ubiquitin conjugating pathway

The addition of a small ubiquitin protein to a target protein is a three step reaction involving three enzymes; the ubiquitin activating enzyme (E1), the ubiquitin conjugating enzyme (E2) and the ubiquitin ligase (E3)(Ciechanover *et al.*, 1981, 1982; Hershko *et al.*, 1983). Ubiquitin is mainly conjugated to lysine residues on substrates by forming a peptide bond between the ubiquitin C-terminal glycine and the lysine residue of the substrate. However, ubiquitin can also be conjugated to serine, threonine, cysteine or the N-terminal amine in proteins (Wang, Herr and Hansen, 2012). Ubiquitin can be conjugated to a substrate as monoubiquitination or at multiple sites (multi-monoubiquitination). In addition, polyubiquitin chains are created when a lysine residue (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, Lys63) or the N-terminal methionine (Met1) on ubiquitin itself are ubiquitinated. This is facilitated through multiple E2 cycles of E1-mediated ubiquitin loading and subsequent unloading. These chains can consist of only one linkage type (homotypic) or multiple linkage types (heterotypic), thus generating chains with mixed conformations and branched structures (Figure 3)(Hershko and Heller, 1985; Hershko and Ciechanover, 1998; Swatek and Komander, 2016).

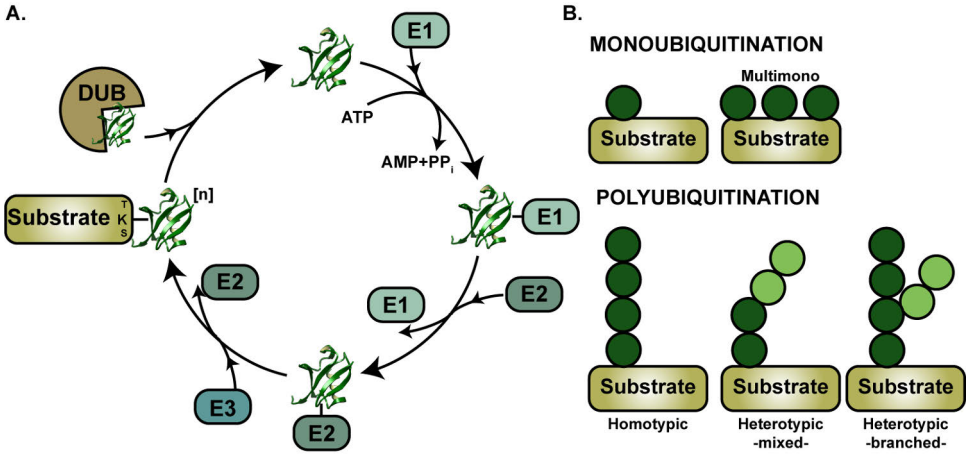

Figure 3. The ubiquitin conjugating pathway. A. The ubiquitin activating enzyme (E1) activates ubiquitin in an ATP-dependent manner. Next ubiquitin is transferred to the ubiquitin conjugating enzyme (E2). The third and final step is the formation of a bond between ubiquitin and substrate, this is mediated by the ubiquitin ligase (E3). Ubiquitination is a reversible PTM that can be removed from substrates by deubiquitinating enzymes (DUBs). B. Substrates can be monoubiquitinated or polyubiquitinated by the ubiquitin conjugation pathway, the green balls represent ubiquitin and shades of green represent different ubiquitin linkage types.

1.3.1 Ubiquitin activation and conjugation

The first step in the ubiquitin pathway is mediated by the ubiquitin-activating enzyme, E1, in an ATP and Mg^{2+}-dependent manner (Figure 4). E1s are coded in most species by only one gene and by two genes in humans. E1 is essential for cell vitality and mutations in the E1 gene cause cell cycle arrest, highlighting the importance of the first activating step in the ubiquitination machinery (Hershko and Ciechanover, 1992; Jin *et al.*, 2007). The first ubiquitin reacts with MgATP, leading to formation of the ubiquitin adenylate intermediate, in which the glycine residue of ubiquitin and AMP are in an acyl-phosphate linkage resulting in the release of PP_i. E1 has minimal affinity for ubiquitin prior to the binding of ATP, which suggests that an ATP-dependent conformation change may help to increase the accessibility of a ubiquitin binding site. Thereafter, the sulfhydryl group of the catalytic cysteine on E1 forms a covalent thioester linkage with ubiquitin, resulting in the release of the AMP. At the same time, E1 catalyses the non-covalent adenylation of a second ubiquitin molecule, thus the E1 becomes asymmetrically loaded with two ubiquitin molecules. E1 is a highly efficient enzyme, allowing sufficient pool of activated ubiquitin for downstream signalling, furthermore individual steps of the E1 reaction are reversible (Ciechanover *et al.*, 1981, 1982; Haas and Rose, 1982; Haas *et al.*, 1982; Haas, Warms and Rose, 1983).

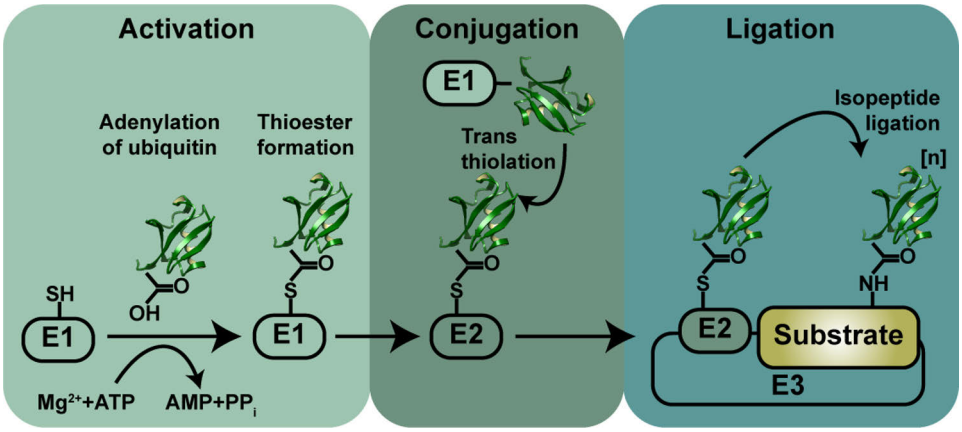

Figure 4. Ubiquitin ligation to substrate. The ubiquitin activating enzyme (E1) activates ubiquitin in an ATP-dependent manner by forming a thioester linkage with ubiquitin. Next, a thioester transfer reaction occurs, where the C-terminus of ubiquitin is transferred to the catalytic cysteine on the ubiquitin conjugating enzyme (E2). The third and final step is the formation of an isopeptide bond between ubiquitin and substrate, this is mediated by the ubiquitin ligase (E3).

In the next step of the Ub conjugating pathway, the E1 physically associates with the E2 conjugating enzyme and a thioester transfer reaction occurs. In this step, the C-terminus of ubiquitin is transferred to the catalytic cysteine on E2 (Figure 4)(Hershko *et al.*, 1983). The E1 associates with E2 through the negatively charged groove within the ubiquitin fold domain and interacts

with two highly conserved lysine residues present in an α-helix on E2 (Lee and Schindelin, 2008). The E2 only binds E1 with significant affinity if the E1 is bound to ubiquitin, this is due to a conformational change in E1 triggered by bound ubiquitin, which reveals a cryptic E2-binding site and allows formation of the E1-E2 complex (Haas, Bright and Jackson, 1988; Huang *et al.*, 2007; Lee and Schindelin, 2008).

In humans, the E2s represent a super family that is encoded by 38 genes and can be classified into 17 subfamilies based on phylogenetic analyses (Michelle *et al.*, 2009). E2s can regulate the switch from ubiquitin chain initiation to elongation, regulate the processivity of chain formation and determine the topology of assembled chains (Pickart and Rose, 1985; Ye and Rape, 2009). However, all active E2s share a conserved core domain, called the ubiquitin-conjugating (UBC) domain, which contains the catalytic cysteine residue that interacts with the E1 and adopts a structure consisting of four α-helices, an anti-parallel β-sheet formed by four strands, and a short 3_{10}-helix (Pickart, 2001). Interestingly, this domain also interacts with E3 ligases, suggesting that dissociation of E2s from E3 ligases is required for E2s to be 'reloaded' with ubiquitin by E1 (Eletr *et al.*, 2005). In some cases, E2s are able to ubiquitinate a substrate in a ligase-independent fashion, such as E2s involved with ubiquitination of the histones, but mostly the transfer of ubiquitin to a target substrate is mediated by the E3 ligases (Haas, Bright and Jackson, 1988).

1.3.2 Ubiquitin ligation to substrate

The third and final step, in the ubiquitin conjugation pathway, is the formation of an isopeptide-bond between ubiquitin and substrate (Figure 4). This is mediated by the ubiquitin E3 ligases, that are responsible for bringing together the E2 and substrate (Hershko *et al.*, 1983). The biological diversity of proteins being targeted for ubiquitination can be mostly explained by the sheer amount of E3 ligases, counting to over 600 genes encoding for E3 ligases in humans (Li *et al.*, 2008). The E3 ligases can be divided into three classes; homologous to E6-associated protein (AP) carboxy terminus (HECT) E3 ligases, really interesting new gene (RING) and U-box fold E3 ligases and RING-in between-RING (RBR) E3 ligases (Figure 5).

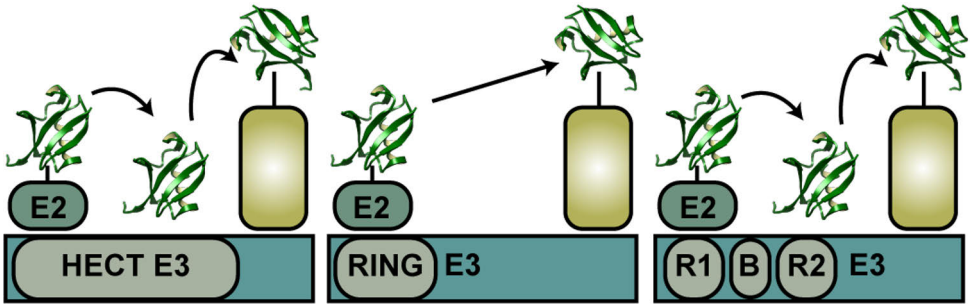

Figure 5. Ubiquitin E3 ligases. From left to right: HECT E3 ligases, RING and U-box fold E3 ligases, and RBR E3 ligases and how the ubiquitin (green structure) is transferred from the E2 and E3 ligase to the substrate (graded gold).

1.3.2.1 *Homologous to E6-AP carboxy terminus (HECT) type E3 ligases*

HECT E3 ligases form a thioester-linked intermediate with ubiquitin, before moving the ubiquitin to the substrate (Figure 5). The HECT domain, of ~350 amino acids with the catalytic cysteine needed for ubiquitin transfer, was first described in human papilloma virus (HPV) E6-AP. Today, the protein family consists of 28 genes encoded in humans (Huibregtse *et al.*, 1995; Huang *et al.*, 1999; Li *et al.*, 2008). The C-terminal catalytic domain consists of a bilobal conformation with a short hinge (Huang *et al.*, 1999; Verdecia *et al.*, 2003). The flexible interlobe configuration brings the catalytic cysteine in C-lobe (towards the C-terminus) to close vicinity of the E2 enzyme bound N-lobe (towards the N-terminus). Before the ubiquitin is transferred to the catalytic cysteine in the HECT domain, the donor ubiquitin interacts non-covalently with the C-lobe of HECT, which primes the ubiquitin bound E2 conformation for thioester transfer (Kamadurai *et al.*, 2009). The ubiquitin remains bound to HECT even after the ubiquitin tail has been transferred to the E3. Upon transfer, the ubiquitin tail morphs into a fully extended conformation (Maspero *et al.*, 2013). The substrate binding interface is coupled to the N-lobe of HECT, opposite from the E2 binding domain. By dynamic conformational rotations, the C-lobe, with donor ubiquitin, is moved away from the E2 and closer to the substrate. In this way, the ubiquitin is presented to the lysine residue, in the right orientation, to mediate a nucleophilic attack (Kamadurai *et al.*, 2013). The substrate binding interface, coupled to the N-lobe of HECT, is also a ubiquitin binding domain, which binds to the Ile44 hydrophobic pocket on ubiquitin. This non-covalent interaction orients the distal end of the growing polyubiquitin chain. When polyubiquitin chains are made by HECT domain ligases, the chain linkage specificity is determined solely by the C-lobe of the HECT domain (Kim and Huibregtse, 2009).

1.3.2.2 *Really interesting new gene (RING) and U-box type E3 ligases*

The RING E3 ligases are characterised by their RING or U-box fold catalytic domain, which promotes direct transfer of ubiquitin from E2 to substrate in a E2-dependent manner (Figure 5)(Lorick *et al.*, 1999; Aravind and Koonin, 2000; Zheng *et al.*, 2000). The RING type of E3 ligases are a huge family being coded by over 600 genes in the human genome (Li *et al.*, 2008). Unlike the HECT E3 ligases, the catalytic activity of the RING domain does not involve an E3-linked ubiquitin thioester intermediate. Instead, the RING proteins function as a scaffold that brings the E2 and the substrate together. With RING E3s the substrate specificity can also be dictated by the E2 interacting with E3, this is opposite from HECT domain ligases, where the chain specificity is solely determined by the E3 (Stewart *et al.*, 2016).

The RING domain has similarities with zinc finger (ZnFs) domains. Both have high content of conserved cysteines and histidine, a conserved spacing, and a ability to bind two zinc molecules at its core (Freemont, Hanson and Trowsdale, 1991). A rigid hydrophobic globular structure coordinates the zinc binding sites and enables a platform for protein-protein interactions (Barlow *et al.*, 1994; Borden *et al.*, 1995). The zinc-binding residues are required for RING domain activity, whereas zinc-binding sites in the U-box domain are replaced by charged and polar residues (Barlow *et al.*, 1994; Borden *et al.*, 1995; Aravind and Koonin, 2000). RING and U-box proteins interact with E2s with the help of two loop-like regions that surround a shallow groove formed by the central α-helix. This platform interacts with the UBC domain of E2s (Metzger *et al.*, 2014).

When the ubiquitin-bound E2 interacts with RING E3 ligases a conformational change occurs, from an open flexible topology to a more compact and closed conformation (Özkan, Yu and Deisenhofer, 2005; Pruneda *et al.*, 2011, 2012). The flexible tail of donor ubiquitin is in the catalytic site of E2, while the globular domain is packed against the central α-helix of E2 via its Ile44 surface. All of this is supported by a donor ubiquitin-RING interface and a conserved residue (usually arginine, lysine or asparagine) that positions the C-terminus of ubiquitin for a nucleophilic attack by the substrate (Dou *et al.*, 2012; Plechanovov *et al.*, 2012). Without the conformational change the E2 is not positioned correctly for an aminolysis, but instead is poised for transthiolation reaction. Therefore, the HECT-type E3 ligases do not induce conformational changes when bound to E2-ubiquitin (Walden and Rittinger, 2018). The RING-E2 interaction is generally of low affinity, however there are exceptions where the E3 ligases contain regions outside the RING motif that bind to E2s through distinct interfaces, resulting in high affinity (Deshaies and Joazeiro, 2009). Apart from the RING domain, RING proteins have usually one or more other signalling domains, such as ZnFs, src homology 2 and 3 (SH2 and SH3), baculovirus IAP repeat (BIR), ankyrin repeats (AnkRs) or UBLs (Li *et al.*, 2008).

An interesting feature of the RING proteins is that not all RING domains have intrinsic E3 ligase activity. RING domains can be found as monomers, dimers or multimers. In homodimers both RING domains have intrinsic E3 ligase activity, but this is not the case with some heterodimers. In these cases, they interact with a second RING protein, usually either enhancing or enabling the E3 ligase activity of the catalytically active E3. Some RING type E3 ligases function in multi-subunit complexes and have high specificity and adaptation to target substrate. In the Cullin-RING ligase (CRL) superfamily, the cullin proteins are associated with RING proteins and other adaptor protein(s) that bind(s) target recognition interfaces. The Skp1-Cullin1-F-box (SCF) family is one of the members in the CRL superfamily that has several interchangeable F-box proteins with different target specificity. Some multi-subunit E3s exhibit great complexity, for example the anaphase promoting complex/cyclosome complex, required for cell cycle progression, which consist of 13 core subunits in humans (Metzger *et al.*, 2014).

1.3.2.3 *RING-in between-RING (RBR) type E3 ligases*

The third class of E3s is the RING-Between-RING (RBR) E3 ubiquitin ligases. The class was named after having three conserved cysteine-rich zinc binding domains sequentially ordered after one another; two of them were sequenced as RING domains (RING1 and RING2) and the one between the RING domains was named in-between RING (IBR) domain (Figure 5)(Morett and Bork, 1999; Van Der Reijden *et al.*, 1999). However, later structural and functional studies have shown that RBR E3 ligases are more like RING-HECT hybrids (Wenzel *et al.*, 2011; Stieglitz *et al.*, 2012). The RBR family consist of 14 members in humans, but only Parkinson juvenile disease protein 2 (Parkin), human homologue of Ariadne (HHARI), heme-oxidized iron-responsive element-binding protein 2 ubiquitin ligase-1L (HOIL-1L, also known as Ran binding protein 2-Type and C3HC4-type Zinc Finger Containing 1 (RBCK1)) and HOIL-1-interacting protein (HOIP, also known as Ring Finger Protein 31 (RNF 31)) have been studied in detail (Marín *et al.*, 2004; Walden and Rittinger, 2018).

All E3 ligases in the RBR family have a RING1 domain with a canonical RING fold that binds to the E2 and a RING2 domain with a conserved cysteine needed for thioester intermediate (Figure 5)(Wenzel *et al.*, 2011). An interesting and distinct feature of RBR E3 ligases is that the RBR E3 ligases are autoinhibited. How the different members in the RBR E3 ligase family are autoinhibited varies, however it is a domain outside the RBR domain that physically separates the RING2 from RING1-IBR and buries the active cysteine interface (Walden and Rittinger, 2018). The mode of activation also varies, but when activated the RBR E3 ligases undergo major topological reorganisation. How exactly the conformational changes required for activation are induced is still unclear. The RBR E3 ligases have multiple binding domains for ubiquitin-like proteins, and these have been shown to have different roles in RBR E3 ligase activation. For example, the linear chain

determining domain (LDD) of HOIP has separate binding domains for acceptor and donor ubiquitin and the donor ubiquitin has been shown to interact with the outside of the helix leading into the IBR domain (Stieglitz *et al.*, 2013; Lechtenberg *et al.*, 2016). In addition, RBR E3 ligases are usually multidomain enzymes, and other proteins have been shown to be important for RBR E3 ligase activation. Best known example of this is HOIP that is in an autoinhibited state when not interacting with its binding partners HOIL-1L and SH3 and multiple AnkR domains protein (SHANK)-associated RBCK1 homology (RH)-domain-interacting protein (SHARPIN)(see section 1.7.1).

As mentioned before, RBR E3 ligases are RING-HECT hybrids. RBR E3 ligases form thioester intermediates and are therefore like HECT E3 ligases. However, the RING1 domain of the RBR E3 ligase also has similarities to the RING E3 ligases that favours aminolysis. Therefore, the aminolysis by RING1 is prevented in RBR E3 ligases. Structural evidence shows that RING1 stabilises ubiquitin bound E2 into an open conformation, which favours transthiolation (Dove *et al.*, 2016, 2017; Lechtenberg *et al.*, 2016). This is due to a second longer Zn-binding loop in the RING1 domain or extensive non-covalent contacts with ubiquitin along the entire RBR domain, that acts like a steric wedge that prevents the E2 from adopting a closed formation (Lechtenberg *et al.*, 2016; Dove *et al.*, 2017)(Yuan 2017). When HOIP and HHARI RBR cores are opened the core wraps around donor ubiquitin tail, leading to multiple contacts to E2, as well as an extended conformation of the ubiquitin tail, which favours thioester transfer (Maspero *et al.*, 2013; Lechtenberg *et al.*, 2016; Dove *et al.*, 2017).

For the time being HOIP is the only RBR E3 ligase that has known structural features for substrate selection and chain specificity for Met1-Ub chains (Stieglitz *et al.*, 2013). In contrast little is known how Parkin substrates are recognised or how HHARI selects CRL substrates. With HECT and RING type E3 ligases the rule for substrate and chain specificity is determined by the last thioester-forming enzyme of the ubiquitination. With HECT and RBR E3 ligases the chain specificity is mostly determined by the E3 and in RINGs the specificity is determined by E2s. However, it is known that Parkin can function with multiple E2s and can catalyse the formation of multiple polyubiquitin chain types (Martino *et al.*, 2018). HHARI has been shown to form a tag team with CRL E3 ligases, by primarily monoubiquitinating CRL client substrates, which can then be polyubiquitinated by the traditional CRL ubiquitination machinery (Scott *et al.*, 2016).

1.4 Readers of the ubiquitin code

Ubiquitin itself is a versatile signalling messenger, as the various ubiquitin modifications on substrates are recognised differently by a class of proteins with ubiquitin binding domains (UBDs). These UBD-containing proteins mediate most of the effects of protein ubiquitination. One or several UBDs can be found in enzymes that catalyse ubiquitination or deubiquitination, or in ubiquitin receptors that recognize and interpret the ubiquitin signal. The non-covalent interactions between UBDs and ubiquitin are usually weak and reversible, but highly specific and controlled. All UBDs can interact with the exposed hydrophobic patch, which includes Leu8, Ile44 and Val70 and is in the β-sheet on ubiquitin (Figure 1), however the structural fold and specific binding interactions of UBDs affect the overall specificity. More than 20 different families of UBDs have been characterised based on structural fold. The UBDs have been divided into four main groups: α-helical structures, zinc fingers (ZnFs), the UBC domains present in E2 enzymes and pleckstrin homology (PH) folds. The largest of these four groups is the α-helical structures with members such as the ubiquitin-associated (UBA), ubiquitin-interacting motif, ubiquitin-binding motif and ubiquitin-binding domain in A20-binding inhibitors (ABINs) and the NF-κB essential modulator (NEMO)(UBAN). The second largest group of UBDs contain ZnFs in their structures such as Npl4 ZnF (NZF), A20 ZnF domains, ubiquitin-specific processing protease, and ubiquitin-binding ZnF (Dikic, Wakatsuki and Walters, 2009; Rahighi and Dikic, 2012).

Most UBDs recognise monoubiquitin via a single α-helix on UBD, which binds to the hydrophobic patch on ubiquitin. The UBA domain is an exception and binds to ubiquitin through two α-helices. When only one region on the ubiquitin is used for recognition, the monoubiquitin is mutually exclusive to one UBD and thus might add one more level of control to the ubiquitin signalling (Dikic, Wakatsuki and Walters, 2009). However, the hydrophobic patch on the ubiquitin surface is not the only domain interacting with UBDs. In some cases, two different proteins with different interacting interfaces with ubiquitin can bind a monoubiquitin, thus leading to desired outcome. An example of this is ZnF of A20 binding a monoubiquitin at another site from the hydrophobic patch, leaving it free for the UBA on p62 to bind the hydrophobic patch (Garner *et al.*, 2011).

How the UBDs determine specificity for different polyubiquitin chains is affected by many factors. The structural fold and binding surfaces on UBDs require a specific orientation of the ubiquitin, leading to selectivity toward a specific polyubiquitin chain type. Thus, the relative orientation of the ubiquitin moieties and the spacing of the linker region between them can determine the specificity of the UBDs. For example, Lys63- and Met1-Ub chains both adopt an extended chain conformation, where ubiquitin moieties lack interaction with one another (Komander *et al.*, 2009). On the opposite,

the Lys48-linked ubiquitin chains have interaction sites between the ubiquitin moieties and alternates between closed, packed structure and open (Figure 2)(Eddins *et al.*, 2006, 2007). Another factor that contributes to UBD specificity is when there are several UBDs, from the same UBD family or different families on the same protein, co-operating (Rahighi and Dikic, 2012). In some cases, co-operation of UBDs increases affinity of proteins for more variety of ubiquitin chains rather than providing selectivity toward a specific ubiquitin chain type. One example would be the NEMO protein that is the key regulator of the canonical NF-κB signalling pathway. The UBAN domain on NEMO has a 100 fold higher affinity for Met1-Ub chains compared to Lys63-Ub chains, and the C-terminal ZnF domain can bind to both Lys63- and Lys11-Ub chains (Laplantine *et al.*, 2009; Lo *et al.*, 2009; Rahighi *et al.*, 2009; Dynek *et al.*, 2010; Ngadjeua *et al.*, 2013).

Depending on which UBDs ubiquitin chains are bound to, they can have slightly different conformational changes. This indicates that when bound to UBDs, Ub chains with different linkages exert flexibility and adaptation to some degree. This further enables another level in determining specificity for each ubiquitin-UBD interaction (Dikic, Wakatsuki and Walters, 2009). Also, some UBDs can recognise several ubiquitin moieties, which, as a result, determines specificity for a polyubiquitin chain topology. For example, the NFZ UBD in the TAB2, in the NF-κB signalling pathway, binds specifically to Lys63-linked ubiquitin chains. The NFZ interacts with the hydrophobic patch of two ubiquitin moieties, but this is only possible with the conformational orientation of Lys63-linked ubiquitin chains (Kulathu *et al.*, 2009; Sato *et al.*, 2009). Some UBDs interact with the linker between two ubiquitin moieties, which determines the polyubiquitin chain linkage specificity. For instance, the DUB associated molecule with the SH3 domain of STAM (AMSH) recognises the isopeptide bond between two ubiquitin moieties in a Lys63-Ub chain (Sato *et al.*, 2008). Also, the UBAN motif on NEMO tightly interacts with Met1-linked ubiquitin chain linkages (Rahighi *et al.*, 2009).

1.5 Disassembly of ubiquitin chains

Similarly, as other PTMs, ubiquitination is a reversible process. The addition of ubiquitin on a target protein is counter-regulated by deubiquitinating enzymes or deubiquitinases (DUBs). The complex nature of the ubiquitin code on substrates, dictates the manner in which DUBs recognize different ubiquitin chains with their UBDs.

1.5.1 Mechanisms of deubiquitination

DUBs can be classified into two mechanistic classes: cysteine proteases and metalloproteases. The cysteine proteases rely on a papain-fold consisting of a catalytic diad or triad of amino acid residues. A histidine amino acid in close vicinity of a catalytic cysteine enables a nucleophilic attack of the isopeptide linkage. In most cases a third residue, which is usually an Asn or Asp, aligns

and polarises the catalytic histidine. A catalytic acyl intermediate is formed when the carboxyl group of ubiquitin is covalently bound to the catalytic cysteine of DUB. The negatively charged transition state is then stabilised by hydrogen-donating residues. In the next step a water molecule hydrolyses the acyl intermediate to complete the catalytic cycle (Storer and Ménard, 1994; Komander, Clague and Urbé, 2009). The metalloproteases coordinate a Zn^{2+} ion in the active site of DUB and nucleophilic water is ligated to the metal ion to hydrolyse the isopeptide bond (Sato *et al.*, 2008).

All DUBs have at least one ubiquitin binding site, the S1 site (Figure 6). The S1 site guides the C-terminus and the scissile bond into the active site of the DUB and thus enables hydrolysis of the isopeptide bond. An important feature of the ubiquitin DUBs is their specificity for ubiquitin over other ubiquitin-like proteins such as Nedd8, SUMO, ISG15. This specificity comes from the amino acid residues in the surface of ubiquitin that interacts with the S1 site and from residues in the flexible C-terminus (Ronau, Beckmann and Hochstrasser, 2016). The interface between ubiquitin and S1 is extensive, consisting of 20-40 % of the bound ubiquitin molecule. When bound to S1, the flexible C-terminus of ubiquitin extends maximally and is engulfed by the DUB. For this to happen, more compact polyubiquitin chain conformations, such as Lys48-Ub chains, have to partially unfold. When a DUB cleaves polyubiquitin chains the distal ubiquitin occupies the S1 site while the proximal ubiquitin occupies a second binding site, called the S1' site (Figure 6A). When the S1' site is in the catalytic domain it determines the linkage specificity of the DUB, but the S1' site can also be outside the catalytic domain or on another protein in a complex with the DUB. The proximal ubiquitin is positioned by the S1' site in the catalytic domain so that the lysine is presented to the active site of the DUB. Thus, it is both the dynamic nature of ubiquitin chains and the ubiquitin binding sites on DUBs that determine the activity and specificity of DUBs (Mevissen and Komander, 2017).

Some DUBs have additional ubiquitin binding sites, S2, S2', S3, S3', that may contribute to linkage specificity and additional binding to long polyubiquitin chains. In addition to the S1 sites, the substrate or the ubiquitin providing the lysine residue for hydrolysis can interact with the catalytic domain of the DUB, however this is not necessary for hydrolysis. With monoubiquitin, some catalytic domains on DUBs recognise specific ubiquitin sites directly on their substrates. Some of the most abundant DUBs work in multimeric complexes, which recruit the proteins to be deubiquitinated. In these complexes there might be no interaction between the DUB and the substrate (Mevissen and Komander, 2017).

DUBs can cleave off monoubiquitin and polyubiquitin chains either through endo- or exo-cleavage (Figure 6B). In endo-cleavage the monoubiquitin and ubiquitin chains are cleaved off the substrate, leading to released unanchored ubiquitin chains. These unanchored chains need then to be cleaved further to

regenerate monoubiquitin. Exo-cleavage directly produces monoubiquitin but needs several rounds of DUB activity to remove polyubiquitin chains. The topology and the DUBs specificity to different polyubiquitin conformations, determines if the DUB has endo- or exo-cleavage properties. This means that in some cases a DUB can have endo-cleavage activity with one chain type and exo-cleavage with another chain type (Mevissen and Komander, 2017).

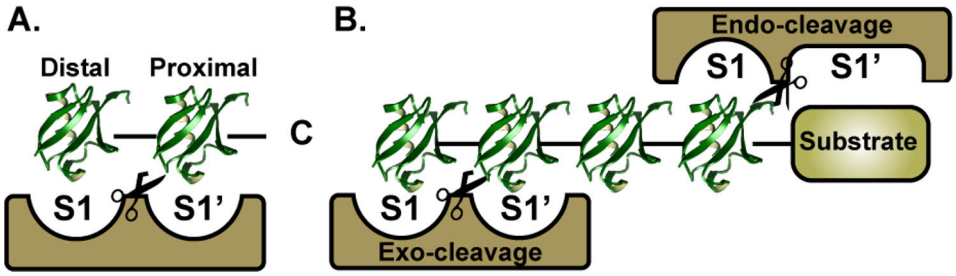

Figure 6. Basic nomenclature for DUB function. A. DUB-ubiquitin-binding sites (S1 and S1') and a model of a di-ubiquitin substrate. The DUB active site is indicated with a scissor. B. Polyubiquitin chains can be cleaved from the distal or the proximal end (exo-cleavage) or within a chain (endo-cleavage).

1.5.2. DUB families

There are six structurally different DUB families and the Ub-linkage specificities vary between these families (Ritorto *et al.*, 2014; Mevissen and Komander, 2017). The DUB family JAMM, for C-Jun activation domain-binding protein 1 (JAB1), Multistep phosphorelay regulator 1 (MPR1)/Peptidylarginine deiminase 1 (PAD1) N-terminal (MPN) and MOV34, is the only metalloprotease family of the DUBs. The JAMM/MPN+ motif coordinates two zinc ions, one of which activates a water molecule to attack the isopeptide bond. AMSH was the first JAMM DUB for which linkage specificity for Lys63-Ub chains was noted. Thereafter, many JAMMs have been shown to be Lys63-specific (McCullough *et al.*, 2006; Sato *et al.*, 2008; Ritorto *et al.*, 2014).

The rest of the DUB families are cysteine proteases: the ubiquitin-specific proteases (USPs), the ovarian tumour proteases (OTUs), the ubiquitin C-terminal hydrolases (UCHs), the Josephin family and the motif interacting with ubiquitin (MIU)-containing novel DUB family [MINDYs)]. The MINDY DUB family is the newest member in the DUB families and structurally distinct from the other DUB members. MINDY DUBs are specific for Lys48-Ub chains, because they have a tandem-MIU UBD domain that is highly specific for Lys48-Ub chains. By binding three ubiquitin moieties in an open conformation, that is accommodated by Lys48-linked tri-ubiquitin, only one of MIU domain on MINDY is required for Lys48-linkage specificity (Abdul Rehman *et al.*, 2016; Kristariyanto *et al.*, 2017). As the name ubiquitin C-terminal hydrolases (UCH) indicates, UCHs catalyse the removal of adducts from the C-terminal end of ubiquitin. UCH DUBs preferentially cleave small

leaving groups such as amino acids and oligopeptides from the C-terminus of ubiquitin, but not larger leaving groups such as proteins (Larsen, Krantz and Wilkinson, 1998). The larger leaving groups and proteins are unable to fit the active site due to a crossover loop covering the active site of UCH (Johnston *et al.*, 1997, 1999). Therefore, it is not surprising that UCH family members have only weak or no activity toward ubiquitin chains (Ritorto *et al.*, 2014). The Josephin family shows only weak or no activity toward diubiquitin. Instead Josephin DUBs have preference for longer ubiquitin chains consisting of four or more ubiquitin moieties, and mixed linkage polyubiquitin chains (Nicastro *et al.*, 2009; Ritorto *et al.*, 2014). Ataxin-3, which is the most studied DUB in this family, binds to both Lys48- and Lys63-Ub chains, but preferentially cleaves Lys63-Ub chains. Interestingly, Ataxin-3 cleaves more efficiently if it is bound to mixed polyubiquitin chains (Winborn *et al.*, 2008). Ataxin-3 has an interesting structure that allows Ataxin-3 to bind two distal ubiquitin moieties, which permits it to bind long mixed polyubiquitin chains (Nicastro *et al.*, 2009).

The USP family is the largest family of DUBs. UPSs vary both in size and domain composition, however they have a common papain-like fold. USPs are often found in multimeric complexes and have many identified interaction partners. For most part, the function of these interaction partners are unclear, but in some cases the interaction partners activate the USPs (Faesen *et al.*, 2011). USPs bind usually directly to their targets, through additional protein-protein interaction domains and have been shown to cleave off the substrate bound ubiquitin, resulting in an unmodified protein (Mevissen and Komander, 2017). Although most USPs show little or no linkage preference, some such as cylindromatosis-associated (CYLD), a tumour suppressor implicated in NF-κB signalling, prefers both Lys63- and Met1-Ub chains (Komander *et al.*, 2008, 2009; Faesen *et al.*, 2011; Ritorto *et al.*, 2014; Sato *et al.*, 2015). Finally, the OTU family members are regulators of important signalling cascades, such as NF-κB signalling, interferon signalling and DNA damage response (Mevissen *et al.*, 2013). The OTU family is interesting in how members of this family have distinct preferences towards one or a small subset of linkage types. OTUB1 prefers Lys48-linked chains, Cezanne prefers Lys11-linked chains, TRABID is Lys29- and Lys33-specific and OTULIN is Met1-specific (Keusekotten *et al.*, 2013; Mevissen *et al.*, 2013; Rivkin *et al.*, 2013; Ritorto *et al.*, 2014). As OTULIN is able to cleave linear polyubiquitin chains it is also able to cleave polyubiquitin precursors (Grou *et al.*, 2015). The linkage specificity of OTU family is determined by the use of an additional UBD, specific recognition of a ubiquitin sequence, the use of a conserved S1' ubiquitin binding site on OTU itself and the use of an S2 site that enables OTU DUBs to bind longer chains in a linkage-specific manner (Mevissen *et al.*, 2013).

DUBs are regulated in the same way as other proteins: through translation, transcription and degradation. The abundance of specific DUBs is also varying

in a cell type- and tissue-dependent manner. DUB amounts can be induced by various stimuli and DUBs can undergo proteolytic processing to inactivate them. DUBs are also regulated by the localisation in the cell, this is also mediated in the same manner as with other proteins through localisation signals and protein-protein interaction. The catalytic activity of DUBs can be regulated by other PTMs, as well as with oxidation and allosteric regulation. The majority of the DUBs are cysteine proteases, which means they can be susceptible to oxidation. Oxidation by reactive oxygen species (ROS) have been shown to inactive members of OTU, USP and UCH families (Cotto-Rios *et al.*, 2012; Kulathu *et al.*, 2013). Modifications of a cysteine residue to a sulfenic acid (-SOH) can be reversed in the presence of reducing agents, but further oxidation to sulfinic acid (- SO_2) or sulfonic acid (-SO_3) is irreversible. The oxidation is favoured when the catalytic triad is in close vicinity. Therefore, some DUBs counter the oxidation by keeping the catalytic amino acid residues physically separated when the DUB is not bound to its substrate. Substrate binding induces a conformational change resulting in assembly of the catalytic triad (Ronau, Beckmann and Hochstrasser, 2016). Other DUBs stabilise the unstable hydroxylation intermediate, SOH, from further oxidation (Kulathu *et al.*, 2013).

1.6 Biological relevance of ubiquitination

Ubiquitin receptors can recognize different ubiquitin chains with their UBDs. In this way the message is recognized and translated into a signal, which can lead to different physiological functions in the cells. The most common chain types in the cells are Lys48- and Lys63-Ub chains, together amounting for almost half of the chain type abundance in a living cell and are thus referred as the canonical ubiquitin chains (Xu *et al.*, 2009). Extensive studies have been performed in understanding the biological relevance of Lys48- and Lys63-Ub chains, however, more and more studies have been conducted in understanding the biological relevance of the so-called atypical chain types, Lys6, Lys11, Lys27, Lys29, Lys33 and Met1 (Figure 2)(Akutsu, Dikic and Bremm, 2016; Swatek and Komander, 2016). The most important function of Lys48-Ub chains is to target proteins for proteasomal degradation (Chau *et al.*, 1989). Both Met1-linked and Lys63-Ub chains are crucial for various immune signalling pathways, NF-κB mediated signalling in particular (Fiil and Gyrd-Hansen, 2014; Zinngrebe *et al.*, 2014; Shimizu, Taraborrelli and Walczak, 2015). Lys63-Ub chains have been shown to be involved in endocytosis and autophagy, in DNA-damage response and in NF-κB signalling. For the atypical chains, Lys6-Ub chains are involved with mitochondrial homeostasis and DNA repair (Akutsu, Dikic and Bremm, 2016; Swatek and Komander, 2016). Lys11-Ub chains are the second abundant chain in living cells and are involved in proteasomal degradation (Xu *et al.*, 2009; Akutsu, Dikic and Bremm, 2016; Swatek and Komander, 2016). Lys27-Ub chains are involved in DNA damage response, chromatin ubiquitination and innate immunity (Gatti *et al.*, 2015). Lys29-Ub chains have been described to have a

role as a negative regulator in Wingless/integration site 1 (Int1) (Wnt) signalling and epigenetic regulation. Lys33-Ub chains are regulating Golgi membrane protein trafficking (Akutsu, Dikic and Bremm, 2016; Swatek and Komander, 2016). The complexity of ubiquitin signalling is further enhanced by mixed and branched ubiquitin chains (Figure 3). For example, in proteasomal degradation, Lys48/Lys11 chains have been shown to enhance the efficiency of the proteasome (Meyer and Rape, 2014b). Similarly, Met1/Lys63-hybrid chains have been implicated to have a role in inflammatory signalling (Emmerich *et al.*, 2013, 2016).

1.6.1 Protein degradation by the proteasome

Lys48-Ub chains were the first chain type to be discovered (Chau *et al.*, 1989). Lys48-Ub chains are the most abundant chain type in cells, however, upon proteasomal inhibition the Lys48-Ub chains are even further induced, indicating that Lys48-Ub chains are needed for proteasomal degradation (Xu *et al.*, 2009). The 26S proteasome degrades unneeded or damaged proteins through an ATP-dependent proteolysis. The 26S proteasome consists of the 20S core subunit and one or two 19S regulatory subunits. The proteolytic activity is in the 20S core subunit. The 19S subunit is responsible for recognition of Lys48-tagged proteins. Polyubiquitin recognition occurs in several steps. First, there is an initial binding of high affinity between ubiquitin and UBDs of ubiquitin binding proteins. This step is ATP-activated. Thereafter, some chains are more tightly bound and thus the target protein is committed for proteolysis. Before the protein is translocated to the 20S subunit, the ubiquitin tag is removed by DUBs in the 19S subunit. Ubiquitin is recognised by intrinsic ubiquitin receptors, adaptor proteins, in the 19S subunit as well by extrinsic proteins that transiently associate with the 19S (Bard *et al.*, 2018). The extrinsic proteins have both UBL and UBD domains. The UBD binds to ubiquitin and UBL binds with high affinity to ubiquitin receptors in the 19S subunit (Grice and Nathan, 2016). Interestingly, the intrinsic proteins in the 19S can also recognise Lys63-Ub chains, however, the extrinsic proteins are Lys48-specific and, therefore, Lys63-Ub chains are not a signal for proteasomal degradation (Nathan *et al.*, 2013). The proteasome also recognises Lys11/Lys48 heterotypic chains, multi-monoubiquitinated proteins and other ubiquitin chain types, and therefore, these can also act as signal for proteasomal degradation (Meyer and Rape, 2014a; Bard *et al.*, 2018).

1.6.2 Protein degradation by the autophagosome

Long lived proteins, organelles and proteins that are resistant to proteasomal degradation, such as aggregate-prone proteins or aggregates, are targeted for lysosomal degradation. In autophagy, cytosolic cargo is engulfed in a specialised double-membrane organelle, called autophagosome, that is later fused with the lysosome. Autophagy can be highly selective for a specific

substrate, for example specific for aggregates (aggrephagy), mitochondria (mitophagy) or pathogens (xenophagy). The specificity is dictated by adaptor proteins in the autophagosome. Ubiquitination has been shown to have a role in autophagosome formation, substrate recognition and termination of autophagosome activity. Many key members of the autophagosome machinery are activated when Lys63-Ub chains are conjugated to them (Grumati and Dikic, 2018). In the same way as adaptor proteins in the proteasome recognise ubiquitin conjugated substrates for proteasomal degradation, adaptor proteins in the autophagosome recognise ubiquitin tagged proteins for autophagy. The adaptor proteins bind ubiquitin through UBDs and the microtubule-associated protein 1A/1B-light chain 3 (LC3) protein on the autophagic vacuole through their LC3-interacting region (LIR) domains (Ji and Kwon, 2017; Grumati and Dikic, 2018). In ubiquitin-dependent selective autophagy, the adaptor proteins recognise mainly Lys63-Ub chains, but other chain types are also recognised depending on the substrate. In aggrephagy, the adaptor proteins p62, neighbour of BRCA1 (NBR1) and histone deacetylase 6 (HDAC6) recruit Lys63-linked conjugated substrates. In mitophagy, the E3 ligase Parkin mediates ubiquitination of many mitochondrial proteins leading to recruitment of adaptor proteins, such as p62, NBR1, optic neuropathy inducing (Optineurin), and progression of mitophagy. In a host cell, bacterial infection leads to ubiquitination of the bacterial surface with Lys6-, Lys11-, Lys48- and Met1-Ub chains. These chains are then recognised by the adaptor proteins in the autophagosome (Grumati and Dikic, 2018). Met1-Ub chains have been shown to decorate the surface of cytosolic *Salmonella typhimurium* leading to activation of NF-κB and restriction of bacterial proliferation (Fiskin *et al.*, 2016; Noad *et al.*, 2017; Van Wijk *et al.*, 2017). The Met1-specific E3 ligase HOIP binds to the already existing ubiquitin coat on bacteria via its N-terminal NZF domain. The catalytic activity of HOIP is required for the Met1-linked ubiquitin chains to recruit NEMO and Optineurin to the cytosolic bacteria. This leads to two events: local activation of NF-κB mediated by NEMO and stimulation of xenophagy mediated by Optineurin (Noad *et al.*, 2017).

1.6.3. Endocytosis

Ubiquitin also plays a major role in endocytosis of membrane proteins. In endocytosis, the cell wall is engulfed around the cargo and buds into the cytosol of the cell. The engulfed cargo is then fused with the lysosome for degradation of the cargo. Ubiquitin works either as an internalisation signal for substrates from the plasma membrane to the clathrin-dependent internalisation pathway or modifies the players in the endocytic machinery (Shih *et al.*, 2000; Piper, Dikic and Lukacs, 2014). The adaptor proteins in the endocytic pathway are also equipped with UBDs, however the interaction of adaptor proteins with a single ubiquitin moiety in the endocytic pathway is weak. Therefore, to enhance the interaction affinity, the internalisation signal is usually a polyubiquitin chain or multiple monoubiquitination on a

substrate. For the most part, multi-monoubiquitination and Lys63-Ub chains predominate as sorting signals in the endocytic pathway, although other linkages such as Lys11, Lys29, and Lys48 can also be used (Piper, Dikic and Lukacs, 2014).

1.7 The Met1-ubiquitination machinery

Met1-linked linear ubiquitin (Met1-Ub) chains differ from the other ubiquitin chains by not being linked by lysine residues. Instead, Met1-linked linear chains are produced when a peptide bond is formed between the carboxyl-terminal glycine of the incoming ubiquitin and the amino-terminal methionine of the preceding ubiquitin. Met1-Ub chains are crucial for NF-κB activating pathways, induced by proinflammatory cytokines, pathogen-associated molecular patterns (PAMPs), T cell receptor agonists, genotoxic stress and inflammasome activation (Sasaki and Iwai, 2015; Rittinger and Ikeda, 2017). In addition, Met1-ubiquitination has been shown to have a role in selective autophagy (see section 1.6.2).

1.7.1 Linear ubiquitin chain assembly complex (LUBAC)

In 2006, an E3 ligase specific for forming Met1-Ub chains was described (Kirisako *et al.*, 2006). To date, the linear ubiquitin chain assembly complex (LUBAC) is the only mammalian E3 ligase found to be able to catalyse Met1-chains. LUBAC is a complex consisting of three proteins: HOIP, HOIL-1L and SHARPIN (Kirisako *et al.*, 2006; Gerlach *et al.*, 2011; Ikeda *et al.*, 2011; Tokunaga *et al.*, 2011). LUBAC exists as monomers and dimers with a 1:1:1 stoichiometry between HOIP, HOIL-1L, and SHARPIN (Fujita *et al.*, 2018; Carvajal *et al.*, 2021). Both HOIL-1L and HOIP are RBR E3 ligases, however the RBR of HOIP is responsible for the Met1-specific ubiquitination (Figure 7)(Yamanaka *et al.*, 2003; Kirisako *et al.*, 2006; Smit *et al.*, 2012; Stieglitz *et al.*, 2012, 2013; Lechtenberg *et al.*, 2016).

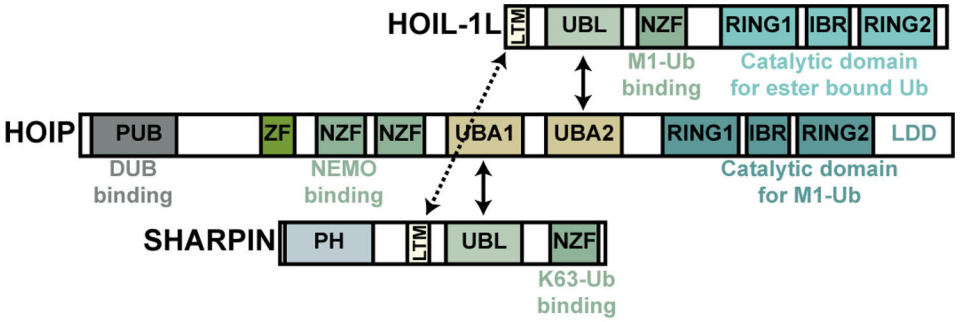

Figure 7. Domain structure and functional regions of the LUBAC subunits, HOIL-1L, HOIP, and SHARPIN. LTM, LUBAC-tethering motif; UBL, ubiquitin-like; NZF, Npl4-type zinc finger; RING, really interesting new gene; IBR, in-between RING; PUB, PNGase/UBA or UBX; ZF, zinc finger; UBA, ubiquitin-associated; LDD, linear ubiquitin chain determining domain; PH, Pleckstrin-homology (Based on Oikawa *et al.*, 2020).

In vitro, the minimal domain required for Met1-Ub chain formation is the RING2 with the catalytic cysteine and a C-terminal extension called linear ubiquitin chain determining domain (LDD) (Smit *et al.*, 2012). The LDD domain consists of a ZnF domain, which is integrated into the RING2 domain. The LDD domain determines the chain specificity and is required to position the two ubiquitin moieties into close vicinity for ubiquitin transfer (Smit *et al.*, 2012; Stieglitz *et al.*, 2013). The N-terminus of acceptor ubiquitin is positioned in close vicinity of the catalytic cysteine, with the help of surface interactions with the RING2 and LDD. In this way, the other lysine residues on ubiquitin are positioned away from the catalytic cysteine promoting Met1-linkage. The C-terminus of the donor ubiquitin is interacting through hydrophobic interactions with the RING2, and a β-hairpin in the LDD domain further guides the C-terminal tail into the catalytic site (Stieglitz *et al.*, 2013). For Met1-ubiquitination, HOIP requires priming of a first ubiquitin to a target lysine, followed by LUBAC attaching a new ubiquitin moiety to N-terminus of the existing ubiquitin. HOIP assembles Met1-Ub chains preferentially on Lys63-Ub substrates, thus forming Met1/Lys63-heterotypic Ub chains (Emmerich *et al.*, 2013, 2016; Fiil *et al.*, 2013; Hrdinka *et al.*, 2016).

For efficient and stable complex formation, all the LUBAC members are necessary and have been shown to interact with one another. *In vivo,* the catalytic RBR site is autoinhibited by the N-terminal UBA domain and peptide:N-glycanase (PNG)/UBA- or UBX-containing proteins (PUB) domain of HOIP (Stieglitz *et al.*, 2012). The catalytic activity of HOIP is restored when HOIP interacts with HOIL-1L and SHARPIN. HOIL-1L and SHARPIN binding to HOIP induces a conformational change in the HOIP UBA, which causes allosteric changes in both the UBA and RBR-LDD, which is required for the E2 loading and the catalytic activity of HOIP (Smit *et al.*, 2012; Stieglitz *et al.*, 2012; Liu *et al.*, 2017; Fujita *et al.*, 2018). The interaction of HOIP and HOIL is essential for LUBAC formation. This interaction is mediated via the HOIP UBA2 and the UBL of HOIL-1L (Yagi *et al.*, 2012). Simultaneously, the UBL on SHARPIN interacts with HOIP UBA1. In addition, HOIL-1L and SHARPIN interact with one another through a LUBAC-tethering motif (LTM) located N-terminally of the UBL domains of both proteins. These LTM domains form a globular structure and further stabilise the complex (Fujita *et al.*, 2018). Besides interacting with other LUBAC members, HOIP, HOIL and SHARPIN have other UBDs, with various chain specificities. Both HOIL and SHARPIN have NZFs that bind preferentially to Met1- and Lys63-Ub chains (Figure 7)(Haas *et al.*, 2009; Gerlach *et al.*, 2011; Ikeda *et al.*, 2011; Sato *et al.*, 2011).

Although HOIL-1L is unable to catalyse Met1-Ub chains, HOIL-1L has recently been shown to have a catalytic role in regulating LUBAC signalling. By automonoubiquitinating itself, as well as HOIP and SHARPIN, the ubiquitinated LUBAC members become preferred targets for HOIP-mediated Met1-Ub chains, which attenuates Met1-Ub chain formation on target proteins. This means that the minor catalytic activity of HOIL-1L, negatively

regulates the main ligase activity of HOIP (Fuseya *et al.*, 2020). In addition, to conjugating ubiquitin to lysine residues, HOIL-1L is an atypical E3 ligase that forms an oxyester bond between C-terminal ubiquitin and serine and threonine residues on a substrate. Therefore, in addition to the previously mentioned Met1/Lys63- heterotypic chains, HOIL together with HOIP have been described to form branched ester/Met1-heterotypic chains (Cohen *et al.*, 2019; Kelsall *et al.*, 2019; Carvajal *et al.*, 2021). However, as the catalytic action of HOIP precedes that of HOIL-1L, the assembly of Met1- Ub chains precedes the appearance of the branches. Therefore, HOIL-1L interacts with a Met1-Ub chain as a substrate for branching. This interaction is via the Met1-Ub-specific binding domain NZF of HOIL (Carvajal *et al.*, 2021). Besides stabilising the LUBAC complex and formation of heterotypic chains, the functional NZF domain of HOIL is required for optimal recruitment of LUBAC to signalling complexes, such as the TNF signalling complex (Peltzer *et al.*, 2018).

1.7.2 Recognition of Met1-Ub chains

Met1-Ub chains are recognised by specific UBDs. To date, seven proteins have been identified in mammals with specific chain specificity for Met1-Ub chains (Fennell, Rahighi and Ikeda, 2018). The first and best characterised Met1-Ub specific UBD discovered is the UBAN domain of NEMO (Laplantine *et al.*, 2009; Lo *et al.*, 2009; Rahighi *et al.*, 2009). The UBAN domain is capable of binding both Lys63- and Met1-Ub chains, however, the affinity for Met1-Ub chains is 100 times stronger for Met1-Ub chains. The UBAN domain forms a homodimeric coiled-coil structure and is also called coiled-coil and leucine zipper (CoZi) domain. The coiled-coil structure enables binding of two Met1-Ub chains simultaneously. The UBAN domain of NEMO associates with both distal and proximal ubiquitin on the Met1-Ub chain via distinct surfaces focused on Ile44 and Phe4, respectively. Furthermore, the specificity for Met1-Ub chains is enhanced through a special linker formed between two ubiquitin moieties, through a tight interaction of the C-terminal tail of the distal ubiquitin and the UBAN (Lo *et al.*, 2009; Rahighi *et al.*, 2009). The amino acids required for the interaction have been mapped and are Val300, Tyr308, Lys309, Phe312, Arg316, Arg319 and Glu320 in humans (Figure 8) (Gautheron and Courtois, 2010). By disrupting these amino acids, as well as other amino acids required for the interaction between NEMO and Met1-Ub chains, have been shown to impact the downstream signalling of NEMO. The mutations Asp311Asn, Glu315Ala, and Arg319Gln of the UBAN domain have been shown to disrupt the interaction between NEMO and Met1-Ub chains, which leads to decreased NF-κB activation, causing diseases such as incontinentia pigmenti and Ectodermal dysplasia with immunodeficiency (EDA-ID)(Rahighi *et al.*, 2009; Hadian *et al.*, 2011).

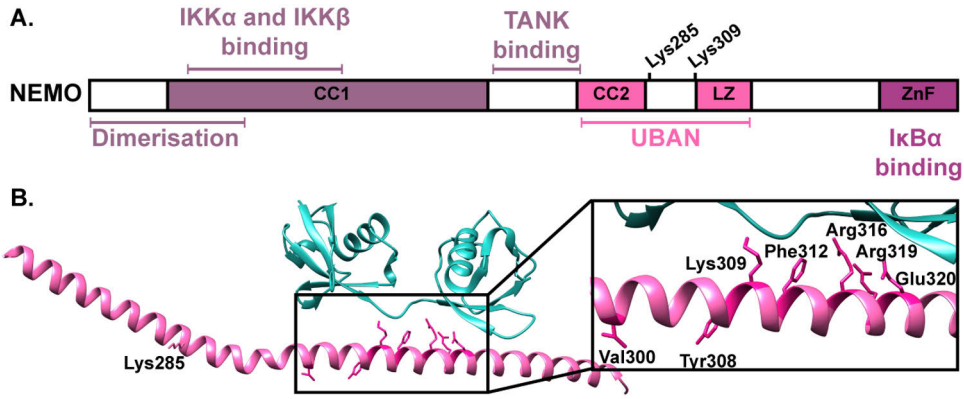

Figure 8. Domain structure and functional regions of NEMO and structural modelling of the UBAN domain. A. NEMO harbours coiled-coil domains 1 (CC1), a dimerization domain, a IKK binding domain, TRAF family member associated NF-κB activator (TANK) binding domain, UBAN domain consisting of a CC2 domain and a leucine zipper (LZ) domain, and an inhibitor of κB α (IκBα) binding domain/Zinc finger domain (ZNF). B. The NEMO UBAN (pink) is modelled with a Met1-Ub dimer (cyan), PDB: 2ZVN. The amino acids (Lys285, Val300, Tyr308, Lys309, Phe312, Arg316, Arg319 and Glu320) required for Met1-Ub interaction are indicated in the zoom-in to the right.

Other proteins with a known UBAN domain are Optineurin and ABIN1-2. Both Optineurin and ABIN proteins have been shown to inhibit the NF-κB pathway and the UBAN domain of ABIN is important for this action. Furthermore, Optineurin has been shown to have an important role in interferon production pathways and autophagy. Also, mutations in the Optineurin and its ability to bind ubiquitin have been linked to primary open-angle glaucoma and familial amyotrophic lateral sclerosis (Wagner *et al.*, 2008; Nakazawa *et al.*, 2016; Fennell, Rahighi and Ikeda, 2018). Apart from the UBAN domains the Met1-Ub chains can also be specifically recognised by ZnF domains. One of these domains are found on HOIL-1L, where the Met1-Ub binding is mediated by the Npl4 ZnF domain and C-terminal α-helical tail extension, called NZF-tail, of HOIL-1L. The NZF domain binds to the Ile44 patch on distal ubiquitin, while the NZF-tail binds to the Phe4 patch on proximal ubiquitin (Sato *et al.*, 2011).

Also, the DUBs recognise Met1-Ub chains and can thus facilitate the hydrolysis of the chains. The main Met1-specific DUBs are OTULIN and CYLD, however, the DUB A20 also binds to linear ubiquitin chains. This interaction is through one of the seven ZnFs, ZnF7, in A20 (Figure 9)(Tokunaga *et al.*, 2012; Verhelst *et al.*, 2012). The ZnF7 of A20 binds simultaneously to the Ile44 patch on the distal ubiquitin and forms a hydrogen bonding network with the proximal ubiquitin (Fennell, Rahighi and Ikeda, 2018). However, A20 does not hydrolyse the Met1-Ub chains, but by binding to Met1-Ub chains protects the chains from removal and, consequently, inhibits cell death (Tokunaga *et al.*,

2012; Verhelst *et al.*, 2012; Mevissen *et al.*, 2013; Draber *et al.*, 2015). Interestingly, A20 binding to Met1-Ub chains seems to compete with the NEMO/IKK complex and in this way, suppresses NF-κB activation (Draber *et al.*, 2015).

1.7.3 Disassembly of Met1-Ub chains

As HOIP is constitutively bound to other LUBAC members *in vivo*, the LUBAC-mediated chain formation is kept in check by the DUBs associating with LUBAC. To date, two DUBs, OTULIN and CYLD, have been shown to interact with LUBAC and to hydrolyse Met1-Ub chains (Figure 9). OTULIN cleaves exclusively Met1-Ub chains, whereas CYLD preferentially cleaves both Met1-Ub and Lys63-Ub chains (Keusekotten *et al.*, 2013; Rivkin *et al.*, 2013; Ritorto *et al.*, 2014; Takiuchi *et al.*, 2014; Sato *et al.*, 2015; Fujita *et al.*, 2018). The DUBs interact with LUBAC, and this interaction is facilitated via the PUB domain on HOIP, which associates with the PUB-interacting motif (PIM). As OTULIN and CYLD bind the same PIM pocket, they are mutually excluding one another, and therefore, give rise to two distinct LUBAC-DUB complexes (Elliott *et al.*, 2014; Draber *et al.*, 2015). The functional significance of these different LUBAC-DUB complexes is still elusive, however, there is a clear difference, as the lack of either OTULIN or CYLD results in different levels of Met1-Ub chains. In the absence of OTULIN, a strong increase in Met1-Ub chains is observed, however, this is not the case in the absence of CYLD (Rivkin *et al.*, 2013; Draber *et al.*, 2015; Damgaard *et al.*, 2016). This has been suggested to be due to CYLD trimming Lys63-Ub chains and thus may also influence Lys63/Met1-hybrid chains (Emmerich *et al.*, 2013, 2016; Hrdinka *et al.*, 2016). As expected, also a mutation in the PUB domain of HOIP leads to induced activation of the NF-κB pathway when the DUBs are not recruited to LUBAC (Takiuchi *et al.*, 2014).

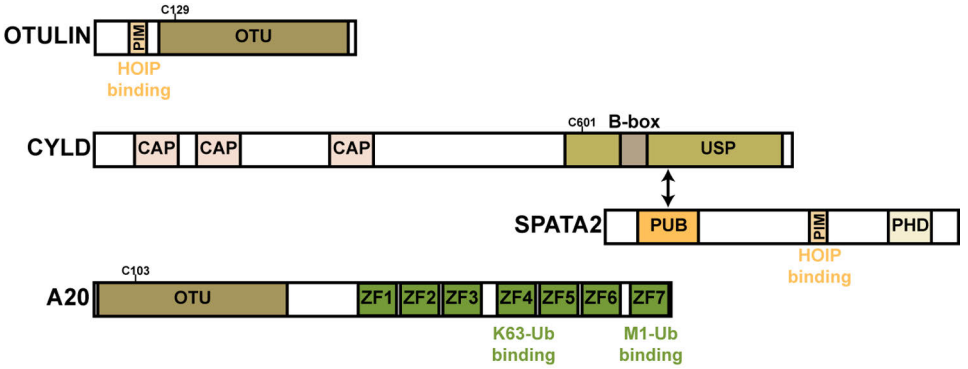

Figure 9. Domain structure and functional regions of the DUBs involved in the regulation of the Met1-ubiquitination. PIM, PUB domain-interacting motif; OTU, ovarian tumour protease; CAP-Gly, cytoskeleton-associated protein Gly-rich domain; B box, B-box-type zinc finger domain; PUB, PNGase/UBA or UBX; PHD, plant homeodomain; ZF, Zinc finger. The catalytic cysteines on CYLD, OTULIN and A20 are also indicated.

Similarly, as other UBD proteins, OTULIN binds to the Ile44 patch of the distal ubiquitin and the proximal ubiquitin via Phe4 patch. OTULIN is unable to hydrolyse isopeptide bonds, but instead cleaves the peptide bond linking two ubiquitin moieties. This specificity of OTULIN is due to a ubiquitin-assisted hydrolysis. In ubiquitin-assisted hydrolysis, extensive contact between OTULIN and Glu16 in the proximal ubiquitin positions correctly the active catalytic triad required for hydrolysis (Keusekotten *et al.*, 2013). Furthermore, OTULIN prevents auto-ubiquitination of LUBAC (Fiil *et al.*, 2013; Keusekotten *et al.*, 2013; Hrdinka *et al.*, 2016; Heger *et al.*, 2018). Mutations in the catalytic OTU domain of OTULIN have been identified in autoinflammation and dermatosis syndrome patients (Damgaard *et al.*, 2016; Zhou *et al.*, 2016). Formation of the LUBAC-OTULIN complex is regulated by OTULIN phosphorylation in the PIM. Phosphorylation of OTULIN prevents HOIP binding, whereas unphosphorylated OTULIN is part of the endogenous LUBAC complex (Elliott *et al.*, 2014).

The USP domain of CYLD interacts, similarly to other UBDs and OTULIN, with the Ile44 patch of the distal ubiquitin and Phe4 patch of proximal ubiquitin. Similarly to the OTU domain, the Glu16 of the proximal ubiquitin assists the hydrolysis and the C-terminal tail of ubiquitin interacts with the USP domains, enabling the hydrolysis (Fennell, Rahighi and Ikeda, 2018). CYLD is also recruited to LUBAC, but CYLD is unable to interact directly with HOIP. Instead, CYLD associates with HOIP through a PIM domain containing protein Spermatogenesis-associated protein 2 (SPATA2) and thus exerts its effect on the LUBAC activity indirectly (Elliott *et al.*, 2016; Kupka *et al.*, 2016; Schlicher *et al.*, 2016; Wagner *et al.*, 2016).

1.7.4 Biological relevance of Met1-ubiquitination

In addition, to the already mentioned roles in innate immune signalling and xenophagy (see section 1.6 and next sections), LUBAC is also recruited to cytosolic protein aggregates. LUBAC modifies the misfolded Huntingtin protein containing a pathogenic polyglutamine expansion (Htt-polyQ) with Met1-Ub chains. HOIP is recruited to the aggregates by Valosin-containing protein (VCP)/p97 a triple A-type quality control ATPase that can extract ubiquitinated proteins from macromolecular complexes or lipid membranes. The PUB domain of HOIP interacts with VCP/p97 through the PIM domain on VCP/p97. The recruitment of LUBAC facilitates two outcomes. Firstly, the interactive surface of the misfolded Huntingtin is shielded from unwanted interactions, and secondly, LUBAC facilitates degradation of misfolded Htt-polyQ in a VCP/p97-dependent manner (van Well *et al.*, 2019). The accumulation of misfolded, aggregated and ubiquitinated proteins is a common mechanism for the progression of neurodegenerative diseases.

2 NF-κB-mediated innate immune response

The innate immune response is an immediate immune response facilitated by the innate immune cells to combat infection, tissue injury and other harmful stressors. The immune cells detect and respond to these stresses by activating rapid transcription of inflammatory cytokines and chemokines, such as tumour necrosis factor (TNF) and interleukin 1β (IL-1β). The innate immune response is activated when PRRs detect PAMPs or stress/damage-associated molecular patterns (SAMP/DAMPs). PAMPs include various bacterial cell wall components, such as lipopolysaccharide (LPS), peptidoglycan (PGN) and lipopeptides, as well as flagellin, bacterial DNA and viral double-stranded RNA. SAMPs/DAMPs include radicals, intracellular proteins, such as heat shock proteins, as well as protein fragments from the extracellular matrix. The innate immune response can also be activated by cytokines binding to their corresponding receptors. A properly regulated immune response clears the threat to the tissue and normal tissue homeostasis is acquired. In normal conditions, when the cell is not challenged, the signalling pathways are tightly controlled in order to avoid uncontrolled activation. A chronic inflammation is induced when cytokines and other inflammatory factors are constantly activated and leads to diseases such as chronic intestinal inflammation and cancer. The main signalling pathways activated, as a response against inflammation, are the NF-κB, mitogen-activated protein kinase (MAPK) and Janus kinases/signal transducer and activator of transcription proteins (Jak/Stat) signalling pathways. In the NF-κB signalling pathway, the cytosolic transcription factor NF-κB is released from inhibition and is translocated to the nucleus, where they can activate transcription of specific target genes (Rubartelli and Sitia, 2009; Newton and Dixit, 2012; Rider *et al.*, 2017; Asri *et al.*, 2019)

2.1 NF-κB/Rel family of transcription factors

In 1986, the transcription factor NF-κB was discovered to bind to a specific conserved DNA sequence required for activation of B lymphocytes, hence, the name nuclear factor binding near the κ light-chain gene in B cells, NF-κB (Sen and Baltimore, 1986; Zhang, Lenardo and Baltimore, 2017). Since then, NF-κB factors have been shown to be key regulators in a plethora of cellular processes and are considered as main regulators of the innate immune response (Figure 10)(Taniguchi and Karin, 2018).

The NF-κB family of transcription factors are also called Rel proteins, due to their conserved N-terminal Rel-homology domain (RHD) with the oncogene v-Rel. The RHD mediates the sequence-specific interaction with DNA and dimerisation to other Rel proteins as well as interaction with the inhibitory proteins. The RHD domain, consisting of two subdomains joined by a hinged loop, clamps around the κB consensus site, 5'-GGGRNWYYCC-3' (N=any base; R=purine; W=adenine or thymine; Y=pyrimidine), on the DNA. The κB

consensus sequence can be found in enhancers or promoters of hundreds of NF-κB target genes (Zhang, Lenardo and Baltimore, 2017). Next to the RHD domain, the NF-κB proteins have a nuclear localisation signal (NLS), which is required for nuclear translocation and transcriptional activity (Hayden and Ghosh, 2008, 2012; Zhang, Lenardo and Baltimore, 2017).

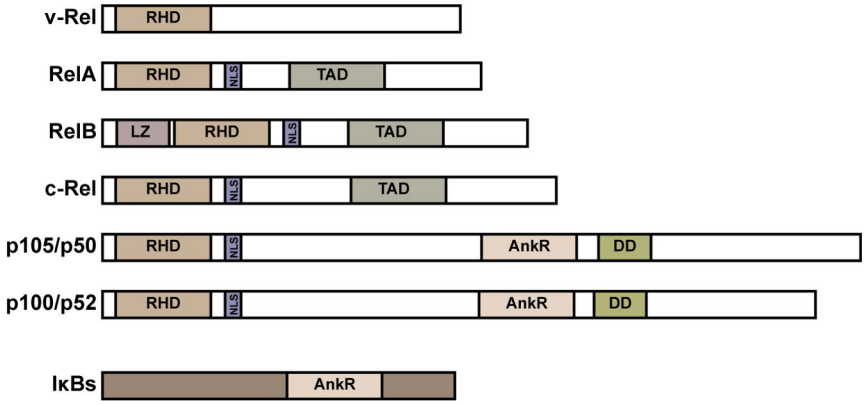

Figure 10. Domain structure and functional regions of the NF-κB family members and IκBs. All NF-κB proteins harbour a Rel homology domain (RHD). In addition, a nuclear localisation signal (NLS), a transactivation domain (TAD), ankyrin repeats (AnkR), a leucine zipper domain (LZ) and a death domain (DD) can be found on NF-κB proteins.

The mammalian NF-κB family consists of five members, p50, p52, p65 (RelA), RelB, and c-Rel, which can be subdivided into two groups: mature and precursor proteins. Of the five NF-κBs, p50 and p52 are produced as precursor proteins NF-κB1 (p105) and NF-κB 2 (p100), respectively. The precursor proteins contain ankyrin repeats (AnkRs) that autoinhibit the RHD by masking the NLS, thus retaining them in the cytosol. The precursor proteins of p50 and p52 undergo a limited proteolysis to reach their active forms. p65 (RelA), RelB, and c-Rel are synthesised as mature proteins and, in addition to the precursor proteins, they contain a transactivation domain (TAD), which enables initiation of transcription. NF-κB proteins can form homo- and heterodimers, which adds to the complexity of NF-κB-mediated transcription of target genes. The composition of the dimers can affect their DNA-binding affinity and sequence motif specificity for certain genes. The most common mammalian dimers are the p50/p65 and p52/RelB. Furthermore, by forming dimers, the lack of TAD in p50 and p52 is compensated through dimerising with TAD containing Rel proteins.

To regulate the transcriptional activity of mature Rel proteins, the NLS signal is masked by AnkR containing inhibitory of κB (IκB) proteins (Figure 10). Therefore, the activation of NF-κB depends on the degradation of the IκBs. This degradation is initiated by the phosphorylation of IκBs by the IκB kinases (IKK) complex (Hayden and Ghosh, 2008, 2012; Zhang, Lenardo and Baltimore, 2017).

2.2 Activation of the IKK and TAK/TAB complexes

PTMs, such as ubiquitination and phosphorylation, play a vital role in the signalling outcome leading to activation of NF-κB. The NF-κB signalling pathways are further divided into canonical and non-canonical signalling (Figure 11). The common outcome for the canonical NF-κB signalling pathways is the activation of the kinase complexes, IκB kinases (IKK) and transforming growth factor β (TGFβ)-activated kinase 1 (TAK1)/TAK binding protein (TAB). In the canonical pathway the IKK complex consists of the regulatory subunit IKKγ, also called NEMO, and the two catalytic subunits IKKα and IKKβ. When activated, the IKK complex catalyses the phosphorylation of IκBs, which serves as a degradation signal. The phosphorylated IκB is recognised by the E3 ligase complex $SCF^{\beta TrCP}$, leading to conjugation of Lys48-Ub chains on IκB, subsequently targeting IκBs for degradation by the 26S proteasome (Häcker and Karin, 2006; Kanarek *et al.*, 2010). The catalytic activity is predominantly acting through IKKβ, which is in turn activated by TAK1-mediated phosphorylation (Kanayama *et al.*, 2004; Ea *et al.*, 2006; Wu *et al.*, 2006; Kulathu *et al.*, 2009). Apart from phosphorylation, ubiquitination also plays an important role in the activation of the kinase complexes. The non-catalytic IKKγ/NEMO is essential for recruitment of the catalytic subunits to the signalling hubs through recognition of ubiquitin chains. The UBAN domain of NEMO recognises Met1-Ub chains with high affinity, whereas C-terminal ZnF domain can bind to both Lys63- and Lys11-Ub chains (Figure 8)(Lo *et al.*, 2009; Rahighi *et al.*, 2009; Dynek *et al.*, 2010; Ngadjeua *et al.*, 2013). Furthermore, recent structural studies have shown that NEMO adopts a more open conformation when bound to longer Met1-Ub chains, facilitating a more efficient signalling (Hauenstein *et al.*, 2017). Similarly, TAK1 is recruited through the TAB2/3 binding to Lys63-Ub chains. Furthermore, Lys63-ubiquitination of TAK1 has been suggested to be important for proper activation of TAK1 (Fan *et al.*, 2010).

In addition to the above-mentioned canonical NF-κB signalling pathways, the non-canonical NF-κB pathway predominantly targets activation of the NF-κB heterodimer p52/RelB in an IKKβ and IKKγ independent manner. Instead, the non-canonical pathway is dependent on a IKKα dimer, where the IKKα dimer is phosphorylated by the NF-κB-inducing kinase (NIK) protein. The target for the IKKα homodimer is predominantly the NF-κB2, which is phosphorylated at two C-terminal sites. The phosphorylation is essential for p100 to p52 maturation (Figure 11). Whereas the canonical NF-κB signalling pathway is a rapid response to cellular changes, the non-canonical signalling pathway is slow and persistent (Sun, 2017).

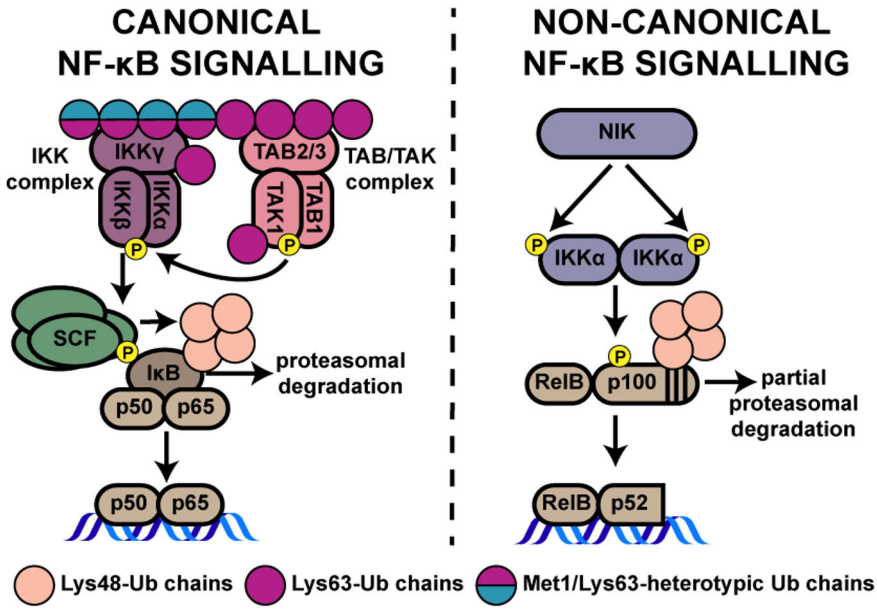

Figure 11. The canonical and non-canonical activation of the IKK and TAK/TAB complexes. In canonical NF-κB signalling, TAK1 is recruited to the signalling complex by binding to Lys63-Ub chains. TAK1 activates the IKK complex consisting of NEMO, IKKβ and IKKα, by phosphorylation. The IKK complex is recruited to the signalling complex by binding to Met1- and Lys63-Ub homo- or heterotypic chains. The IKKβ phosphorylates the IκBα and subsequently targets it for proteasomal degradation. The released NF-κB is translocated to the nucleus and activates target gene expression. In non-canonical NF-κB signalling, stabilised NIK activates IKKα homodimer by phosphorylation, which in turn phosphorylates p100, the precursor for p52. p100 is partially degraded to mature p52. The free p52/RelB dimer is translocated to the nucleus and induces gene expression.

2.3 Receptor activation and recruitment of signalling complex

The canonical NF-κB signalling cascades are activated downstream from extracellular PRRs, such as Toll-like receptors (TLRs)/interleukin receptors (IL-R), proinflammatory cytokine receptors, such as the TNF receptor (TNFR) superfamily, and T- and B-cell receptors (TCR/BCR). Also, the intracellular PRR receptors such as NOD-like receptors (NLRs) and retinoic acid-inducible gene I (RIG-I), activate canonical NF-κB signalling cascades (Figure 12). In addition to being activated upon recognition of PAMPs, NF-κB activation can also be induced in the absence of pathogens. Such sterile inflammation may be induced by molecules leaking out from necrotic cells or by materials and compounds that are recognised as DAMPs. These molecules can be found in the extracellular or intracellular milieu for example during pathological conditions triggered by mechanical trauma, hypoxia, radiation and chemicals (Rider *et al.*, 2017; Asri *et al.*, 2019). In addition, it has been suggested that

cells exposed to stressful conditions release SAMPs, which may lead to similar inflammatory responses (Rubartelli and Sitia, 2009).

Upon receptor activation, a common signalling event is the formation/recruitment of the initial receptor complex, consisting of receptor-associated adaptor proteins, kinases and E3 ligases. The adaptor proteins contain signalling domains, required for precise recruitment of desired signal mediators. Kinases serve to activate these mediators and the recruitment of E3 ligases facilitates the conjugation of ubiquitin chains creating more scaffolds for the signalling hub and clearance of undesired proteins. The non-degradative Ub chains function as signalling platforms for recruitment of signalling mediators, such as the TAK/TAB and IKK complexes, as well as in stabilising the complex for efficient NF-κB activation (Figure 12)(Kanayama *et al.*, 2004; Ea *et al.*, 2006; Wu *et al.*, 2006; Kulathu *et al.*, 2009).

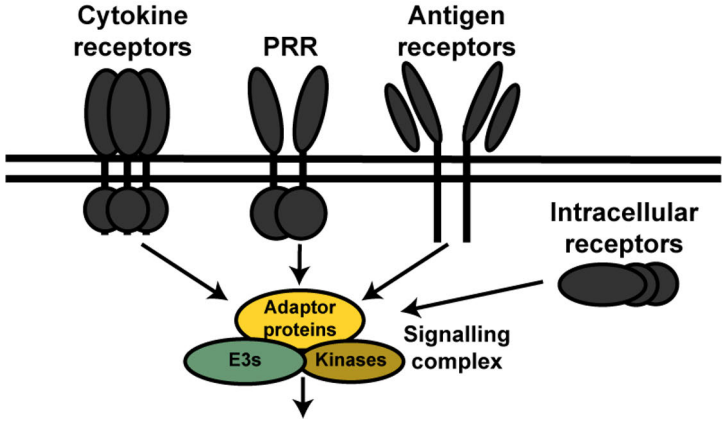

Figure 12. General activators and mediators of canonical NF-κB signalling pathways. Examples of receptors that are involved in activating the canonical NF-κB pathways. Transmembrane cytokine receptors, pattern-recognition receptors (PRR) and antigen receptors as well as intracellular PRR receptors such as NLRs and RIG-1 recruit a signalling complex consisting of adaptor proteins (yellow), kinases (light brown) and E3 ligases (green). All the canonical NF-κB signalling pathways lead to recruitment of the IKK and TAB/TAK complexes, subsequently leading to NF-κB activation.

2.3.1 The TNF receptor signalling pathway

The TNF receptor (TNFR) signalling pathway in humans is activated when one of the 19 TNF superfamily members binds to one of the 29 TNF superfamily of receptors, resulting in a plethora of physiological outcomes, including inflammation, proliferation, and cell death. As can be expected with the combination of TNF ligands and receptors, the signalling outcomes vary to a great deal from NF-κB activation to apoptosis pathways, and activation of the MAPK and c-Jun N-terminal kinase (JNK) signalling pathways. The best known and studied activator of the TNFR signalling is the cytokine TNFα, which leads to activation of the TNFR1 and also in some degree TNFR2 (Aggarwal, Gupta and Kim, 2012). TNF ligand binding to TNFR1 can lead to

two signalling complexes being formed with opposite signalling outcomes: pro-inflammatory and induction of cell death (Micheau and Tschopp, 2003). The pro-inflammatory complex is called complex I, which leads to activation of NF-κB and subsequent cell survival. If complex I formation is destabilised, a second complex, called complex II, is formed, which induces either apoptosis or necroptosis (Figure 13)(Micheau and Tschopp, 2003; Vandenabeele *et al.*, 2010). The dual role of the signalling pathway is efficient for the cells, however at the same time the regulation of the pathway is of utmost importance.

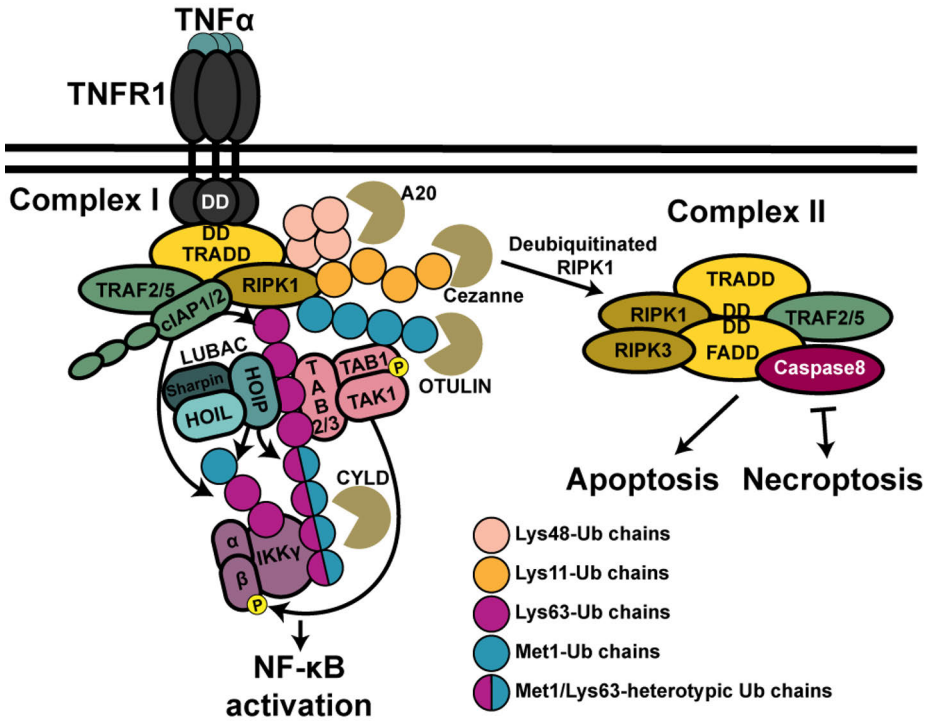

Figure 13. The TNFR1 signalling pathway. Binding of TNFα to TNF-R1 stimulates the formation of complex I consist of TNFR1, TRADD, RIPK1, TRAF2, TRAF5 and cIAP1/2. TRAF2 recruits the E3 ligases cIAP1/2 to complex I. Subsequently, cIAPs ubiquitinate several components of this complex, which is required to recruit the IKK and TAB/TAK complexes. In addition, LUBAC is recruited, which promotes further ubiquitination resulting in further stabilisation of complex I. Complex II is formed when complex I is disrupted or RIPK1 is deubiquitinated. Complex II consists of RIPK1, TRADD, TRAF2, FADD and RIPK3. If caspase 8 is recruited to complex II, activation of complex II leads to apoptosis. If caspase 8 is inhibited, the cell undergoes necroptosis.

The cytokine TNFα binds to the trimeric transmembrane protein TNF receptor 1 (TNFR1), leading to conformational change in the intracellular domain of TNFR1. Complex I is initiated by the recruitment of TNFR1-associated death domain (DD)(TRADD) and receptor interacting protein kinases 1 (RIPK1), with a subsequent recruitment of the E3s TNF receptor

associated factors 2 (TRAF2) and cellular inhibitor of apoptosis 1/2 (cIAP1/2)(Hsu, Xiong and Goeddel, 1995; Rothe *et al.*, 1995; Hsu, Huang, *et al.*, 1996; Hsu, Shu, *et al.*, 1996; Shu, Takeuchi and Goeddel, 1996). The E3 ligases TRAF2 and cIAP1/2 catalyse the addition of Lys63, Lys48- and Lys11-Ub chains on RIPK1 and themselves (Bertrand *et al.*, 2008; Varfolomeev *et al.*, 2008; Dynek *et al.*, 2010; Darding and Meier, 2012). The Lys63-Ub chains on RIPK1 are recognised by UBDs of TAB2/3, leading to recruitment of the TAK/TAB-complex (Ea *et al.*, 2006; Wu *et al.*, 2006). The recruitment of TAK1 then leads to the activation of the NF-κB via the IKKs and the MAPK pathway, which together promote proinflammatory and pro-survival gene expression.

The E3 ligase LUBAC is recruited to the TNF signalling complex via HOIP interaction with Lys63-Ub chains catalysed by cIAP (Haas *et al.*, 2009; Gerlach *et al.*, 2011; Walczak, 2011). Similarly, as the Lys63-Ub chains, the Met1-Ub chains function as recruitment platforms for other proteins. The Met1-Ub chains generated by LUBAC decorate RIPK1, TRADD, TNFR1 and NEMO. The Met1-Ub chains stabilise complex I by retaining RIPK1, TRAF2, cIAP and TAK1 in the complex, and function as a scaffold for IKK complex recruitment (Tokunaga *et al.*, 2009; Gerlach *et al.*, 2011; Peltzer, Darding and Walczak, 2016). This extends the half-life of complex I and thus enhances IKK activation (Haas *et al.*, 2009; Tokunaga *et al.*, 2009). In the absence of the E3s LUBAC and cIAP, complex I is destabilised and complex II is formed, leading to cell death signalling (Moulin *et al.*, 2012; Peltzer *et al.*, 2014; Annibaldi and Meier, 2018). Similarly, the activity of DUBs are required for maintenance of the signalling complexes. DUBs involved in the NF-κB pathways are A20, CYLD, Cezanne, USP11, USP15 and USP21. For instance, deubiquitination of RIPK1 by CYLD appears to be a prerequisite for complex II formation, as RIPK1 ubiquitination protects from cell death (O'Donnell *et al.*, 2007, 2011; Wang, Du and Wang, 2008; Peltzer, Darding and Walczak, 2016). CYLD is recruited to the signalling complex through the Lys63-Ub chain and by interacting with LUBAC via SPATA2. Interestingly, the Met1-specific DUB OTULIN is not recruited to the TNFR1 signalling complex, but it does regulate the TNF signalling by modulating the amount of cytosolic Met1-ubiquitinated proteins, including the LUBAC components themselves (Draber *et al.*, 2015; Damgaard *et al.*, 2016; Heger *et al.*, 2018).

When RIPK1 dissociates from complex I, it forms complex II, together with Fas-associated protein with DD (FADD). FADD in turn recruit caspase 8, via its death effector domain (DED), resulting in the activation of the apoptosis signalling pathway. If caspase 8 is inhibited or absent from complex II, another complex II is formed with the kinases RIPK1 and RIPK3, and the pseudokinase Mixed lineage kinase domain-like protein (MLKL), which leads to necroptosis (Figure 13). RIPK1 and RIPK3 are kept inactive by a caspase 8/cellular FLICE-like inhibitory protein (c-FLIP) heterodimer, which blocks necroptosis (Peltzer and Walczak, 2019).

2.3.2 The Toll-like receptor signalling pathway

The Toll-like receptors (TLRs) were the first PRRs identified, and to date, the best characterised of them. The human TLRs comprise of 10 members, and they are localised either to the cell surface or to intracellular compartments such as the ER, endosome, lysosome, or endolysosome. TLRs are single transmembrane proteins, with leucine-rich repeats (LRRs) responsible for recognising PAMPs/DAMPs such as lipid, lipoprotein, protein, and nucleic acids. Upon ligand binding, the TLRs dimerise, which induces an intracellular conformational change of the receptors. The cytoplasmic Toll/IL-1 receptor (TIR) domains dimerise, which creates a new scaffold for recruitment of specific TIR-domain-containing adaptor proteins. This result in a signalling cascade that culminates in the activation of NF-κB, interferon regulatory factors (IRFs), or MAPK to regulate the expression of cytokines, chemokines, and type-I interferons (IFNs)(Kawai and Akira, 2007, 2010). Depending on the adaptor protein recruited to the receptor, the signalling outcome varies. All TLRs, except TLR3, recruits the TIR-domain and DD containing adaptor protein myeloid differentiation primary-response protein (MyD88). TLR3 recruits the Toll-interleukin (IL)-1-resistance domain-containing adapter protein inducing IFNβ (TRIF). TLR4 can interact with both MyD88 and TRIF, depending on its cellular localisation (Figure 14).

In the MyD88-dependent signalling, MyD88, together in various combinations with other TIR-containing adaptor proteins, recruits the DD containing Il-1R-associated kinases (IRAKs). Thereafter, TNFR-associated factor 6 (TRAF6), TRAF3, cIAP1 and cIAP2 are recruited to the IRAKs. The E3 ligase TRAF6 promotes Lys63-ubiquitination of itself and other signalling mediators, including cIAPs (Deng *et al.*, 2000; Dhillon *et al.*, 2019). The Lys63-Ub chains are recognised by UBDs of TAB1/2, leading to recruitment of the TAK1/TAB-complex and further activation of downstream signalling. LUBAC is recruited to the MyD88 signalling complex by TRAF6, and catalyses Met1-Ub chains on pre-existing Lys63-Ub chains. The Met1-Lys63 heterotypic Ub chains, are required for efficient NF-κB activation (Cohen and Strickson, 2017).

In MyD88-independent TLR signalling, TRIF binds directly to TRAF6 by replacing MyD88 with the RIPK1. Also, the E3 ligase Pellino1 is recruited to the RIPK1 signalling complex. Pellino1 decorates RIPK1 with Lys63-Ub chains, resulting in further activation of the TAB/TAK- and IKK-complexes and transcription of cytokines (Chang, Jin and Sun, 2009). For IFN production, TRAF3 is Lys63-ubiquitinated, leading to recruitment of the IKKε/TRAF family member associated NF-κB activator (TANK) binding kinase 1 (TBK1)-complex, leading to IRF3 phosphorylation and induction of type-I interferon production (Tseng *et al.*, 2010; Häcker, Tseng and Karin, 2011). Other members of the ubiquitin machinery have also been implicated to have a role in TLR signalling. The E3 ligases cIAPs have been shown to ubiquitinate RIPK1, leading to apoptotic outcomes and the LUBAC component SHARPIN is

vital in protection against TLR3-induced cell death (Zinngrebe *et al.*, 2014, 2016).

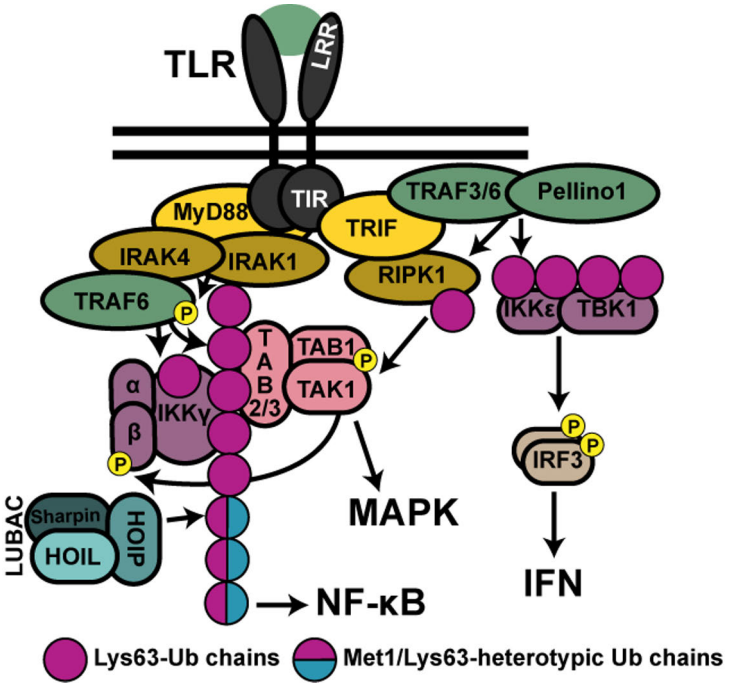

Figure 14. TLR signalling pathway. Ligand binding (green) to TLR stimulates the formation of the signalling complex consisting of MyD88, IRAK1/4, TRAF6. TRAF6 Lys63-ubiquitinates several components of this complex, which in turn recruits the IKK and TAB/TAK complexes. TRAF6 also recruits LUBAC to the complex. LUBAC catalyses Met1-Ub chains on pre-existing Lys63-chains. Alternatively, TRIF is recruited to TLR resulting in a signalling complex consisting of RIPK1 and the E3 ligases TRAF3/6 and Pellino1. Pellino1 decorates RIPK1 with Lys63-Ub chains, resulting in further activation of the TAB/TAK- and IKK-complexes and activation of NF-κB and MAPK pathways. For IFN production, TRAF3 is Lys63-ubiquitinated, leading to recruitment of the IKKε/TBK1-complex, leading to IRF3 phosphorylation and induction of type-I interferon production.

2.3.3 The NOD-like receptor signalling pathway

Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are cytosolic PRRs essential for detecting non-self-components, such as invading pathogens. Engagement of the NLRs lead to recruitment of a NOD-signalling complex and activation of cytokines, chemokines and antimicrobial peptides (Akira, Uematsu and Takeuchi, 2006). The human NLR family consists of 22 members. The founding members, NOD1 and NOD2 are composed of 3 domains. The leucine-rich repeat (LRR) is important for recognition of ligands. The effector domain caspase activation and recruitment domain (CARD) is required for linking NLR to downstream adaptor proteins and effector molecules. Finally, the NACHT domain, named after the protein family

consisting of the neuronal apoptosis inhibitory protein (NAIP), major histocompatibility complex (MHC) class II transcription activator (cIITA), incompatibility locus protein from *Podospora anserina* HET-E and telomerase-associated protein (TP1), is important for oligomerisation and activation of NLRs. NOD1 and NOD2 recognise peptidoglycan motifs, which consist of N-acetylglucosamine and N-acetylmuramic acid from the bacterial cells. NOD1 is expressed both in hematopoietic and non-hematopoietic cells, and recognises a molecule called meso-diaminopimelic acid (meso-DAP), which is mostly found in Gram-negative bacteria. NOD2 is mostly expressed only in hematopoietic cells and some specialised cells in the small intestine and is known to recognise intracellular muramyl dipeptide (MDP) (Girardin *et al.*, 2003; Ogura *et al.*, 2003). Mutations in NOD2 have been shown to be associated with higher susceptibility to the intestinal inflammatory disease, Crohn's disease (CD)(Hugot *et al.*, 2001; Ogura *et al.*, 2001).

After pattern recognition, NOD1 and NOD2 self-oligomerise, undergo a conformational change and recruit the CARD-containing serine-threonine kinase RIPK2 (Kobayashi *et al.*, 2002; Fridh and Rittinger, 2012). RIPK2 is then autophosphorylated and ubiquitinated, but the autophosphorylation has later been shown to be redundant for RIPK2 activity, whereas, the ubiquitination of RIPK2 is essential for signalling (Hasegawa *et al.*, 2008; Tigno-Aranjuez *et al.*, 2014; Pellegrini *et al.*, 2017; Goncharov *et al.*, 2018; Hrdinka *et al.*, 2018). RIPK2 associates with the E3 ligases cIAP1/2, X-chromosome-linked inhibitor of apoptosis (XIAP), TRAF2/5/6 and LUBAC, leading to various ubiquitin chains conjugated on RIPK2 (Yang *et al.*, 2007; Hasegawa *et al.*, 2008; Bertrand *et al.*, 2009; Krieg *et al.*, 2009). The Lys63-Ub chains catalysed by XIAP on RIPK2 are indispensable for NOD2-dependent signalling and responsible for the recruitment of LUBAC to NOD2. Similarly, LUBAC conjugates Met-Ub chains to RIPK2 (Damgaard *et al.*, 2012; Stafford *et al.*, 2018). Together, Lys63- and Met1-linkages are essential for the efficient recruitment and activation of TAB-TAK and IKK complexes (Kanayama *et al.*, 2004; Abbott *et al.*, 2007; Tokunaga *et al.*, 2009). Interestingly, recent studies show that endogenous levels of RIPK2 and the ubiquitin events of RIPK2 are redundant for NOD signalling. Instead, the interaction with XIAP is crucial for NOD signalling (Goncharov *et al.*, 2018; Heim *et al.*, 2020). Comparably with other NF-κB activating pathways, the DUBs A20 and CYLD function as negative regulators of the NLR signalling pathway by deubiquitinating RIPK2 (Hitotsumatsu *et al.*, 2008; Hrdinka *et al.*, 2016). Similarly, the Met1-specific DUB OTULIN negatively regulates NOD2-mediated signalling by preventing LUBAC autoubiquitination under basal conditions, as well as restricting the accumulation of Met1-Ub chains on RIPK2 and LUBAC upon stimulation (Figure 15)(Fiil *et al.*, 2013).

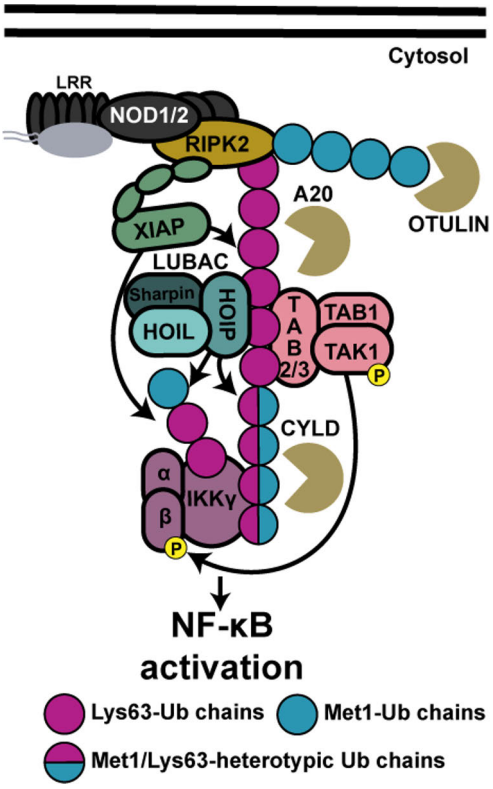

Figure 15. NLR signalling pathway. The intracellular NOD receptor is activated by binding via the LRR domain to the MDP derived from bacteria. Activated NOD binds to RIPK2, which is Lys63-ubiquitinated by XIAP. The Lys63-Ub chains are recognised by the IKK and TAB/TAK complexes. In addition, LUBAC is recruited to the Lys63-Ub chain, which promotes Met1-ubiquitination (cyan) of RIPK2. The DUBs OTULIN, CYLD and A20 have been implicated in the regulation of NLR-mediated NF-κB activation.

2.4 Met1-Ub mediated immune disorders

As LUBAC is required for several of the innate immune signalling pathways, it is not surprising that disabled LUBAC activity causes severe phenotypes in mice and humans. When either HOIP or the UBL domain of HOIL-1 are lacking in mice, the mice are not viable and die around E10,5. In both cases, the absence of LUBAC leads to aberrant TNFR1-induced endothelial cell death, which is caused by increased formation of complex II (Emmerich *et al.*, 2013; Sasaki *et al.*, 2013; Peltzer *et al.*, 2014, 2018; Fujita *et al.*, 2018). The similarities in the phenotype between HOIL-UBL and HOIP mutant mice are probably in some degree due to reduced levels of HOIP, as the HOIP levels are also decreased in HOIL mutant mice. However, when HOIL-1 deficient mouse embryonic fibroblasts are reconstituted with either HOIP or HOIP and SHARPIN, it does not restore the levels of Met1-Ub chains in the TNF signalling complex. Hence indicating, that the phenotype observed in HOIL-deficient mice is also due to lack of HOIP recruitment to the LUBAC and TNF signalling complex by HOIL (Tokunaga and Iwai, 2009; Peltzer *et al.*, 2018). Whereas the interaction of HOIP and HOIL is vital for mice, mice expressing a catalytically inactive HOIL-1 are still viable and are free of chronic inflammation, indicating that the catalytic activity of HOIL is not required for LUBAC activity. Even though the catalytically inactive HOIL mice are viable, they still exhibit some phenotypes, such as polyglucosan body myopathy in old age and are extremely susceptible to some pathogens. In addition, the

mice contained about twofold more B cells, T cells and resident macrophages activity (MacDuff *et al.*, 2015; Kelsall *et al.*, 2019). Mice with a loss-of-function (LOF) mutation in the *Sharpin* gene develop chronic proliferative dermatitis (cpdm), systemic inflammation and increased apoptosis in liver and skin (Seymour *et al.*, 2007; Gerlach *et al.*, 2011; Ikeda *et al.*, 2011; Tokunaga *et al.*, 2011). Ablation of TNF, TNFR1, TRADD or FADD rescues the skin phenotype in cpdm mice, indicating the importance of TNFR1 signalling mediators in SHARPIN-dependent anti-apoptosis signalling (Kumari *et al.*, 2014; Rickard *et al.*, 2014). Interestingly, loss of the catalytic activity of HOIL protects mice against apoptotic liver failure and cures dermatitis caused by the lack of SHARPIN. This clearly shows that augmentation of Met1-ubiquitination activity of HOIP following the loss of catalytic activity of HOIL-1L ameliorates cpdm. Moreover, these findings indicate that cpdm is mainly caused by attenuated HOIP E3 ligase activity rather than an altered composition of LUBAC subunits (Fuseya *et al.*, 2020).

Patients with a mutation in HOIP or HOIL have been described to have multiorgan autoinflammation, immunodeficiency, polyglucosan storage myopathy (muscular amylopectinosis), cardiomyopathy, lymphadenopathy, hepatosplenomegaly and die in early childhood (Figure 16). When analysing the patients, the recessively inherited diseases are caused by mutations resulting in truncated HOIL or affecting the PUB domain of HOIP. Both HOIP- and HOIL-deficient patients have been reported with muscular amylopectinosis, however this phenotype seems to be milder in HOIP patients. Interestingly, the severity of HOIL-deficiency, may be due to HOIL ubiquitinating branched glucoses, with a preference to less branched glucoses. This activity is enabled by HOIP binding to glucoses, but not necessary for HOIL activity. Therefore, HOIL-deficiency is more detrimental for the glucose homeostasis (Boisson *et al.*, 2012, 2015; Nilsson *et al.*, 2013; Wang *et al.*, 2013; Oda *et al.*, 2019; Kelsall *et al.*, 2022). Human patients with SHARPIN-deficiency have not been found in patients with LUBAC deficiency symptoms, instead, SHARPIN deficiency has been associated with late-onset Alzheimer's disease (Asanomi *et al.*, 2019).

Faulty disassembly of Met1-Ub chains also lead to severe consequences, as mice with OTULIN deficiency are embryonically lethal between E12.5 and E14, due to vascular defects and impaired Wnt signalling (Rivkin *et al.*, 2013; Heger *et al.*, 2018). In humans, a homozygous LOF mutation in the OTULIN gene causes an auto-inflammatory condition, called OTULIN-related inflammatory syndrome (ORAS) or otulipenia. The disease characteristics are fever, panniculitis, gastrointestinal inflammation/diarrhoea, and arthritis. In addition, OTULIN-deficiency causes spontaneous and progressive fatty liver disease (Figure 16). In contrast to patients with LUBAC deficiency, OTULIN-deficient patients have no obvious immunodeficiency. The inflammation in ORAS patients is driven by TNF signalling, which has been shown to lead to hyperactivation of LUBAC and NF-κB activation in myeloid cells. In other cell

types such as fibroblasts, loss of OTULIN leads to degradation of LUBAC and TNF-induced cell death. Whereas treatment with anti-TNF reduces inflammation in ORAS patients, the reported liver phenotype is independent of TNFR1 signalling. With the help of liver-specific deletion of OTULIN in mice, it was shown that OTULIN-deficiency is associated with aberrant mTOR activation, which leads to metabolic alterations, apoptosis, and inflammation in the liver. By inhibiting the mTOR signalling in these mice, the liver pathology was significantly reduces (Damgaard *et al.*, 2016, 2019, 2020; Zhou *et al.*, 2016).

When analysing the role of LUBAC in cancer progression, two studies have shown enhanced LUBAC activity in Diffuse large B cell lymphoma and human lung squamous cell carcinoma (LSCC) cells. In the first case, mutations in the UBA domain of HOIP lead to enhanced interactions between HOIP and HOIL-1L, subsequently leading to increased NF-κB activation and tumour progression. Similarly, the expression of LUBAC subunits is enhanced in LSCC, resulting in increased Met1-ubiquitination and NF-κB activation (Oikawa, Sato, Ito, *et al.*, 2020). Also, CYLD deficiency causes cylindromatosis in humans, a disease characterised by formation of benign tumours in the skin of affected individuals. In most cases, mutations leading to cylindromatosis result in truncated CYLD lacking DUB activity. CYLD has also been implicated in having a broader role in suppressing tumour progression (Harhaj and Dixit, 2010). Deficiency in A20, but interestingly not inactivation of its DUB activity, causes early death in mice due to severe inflammation, implying that A20 likely exerts major functions independently from its DUB activity (Lee et al., 2000; Lu et al., 2013).

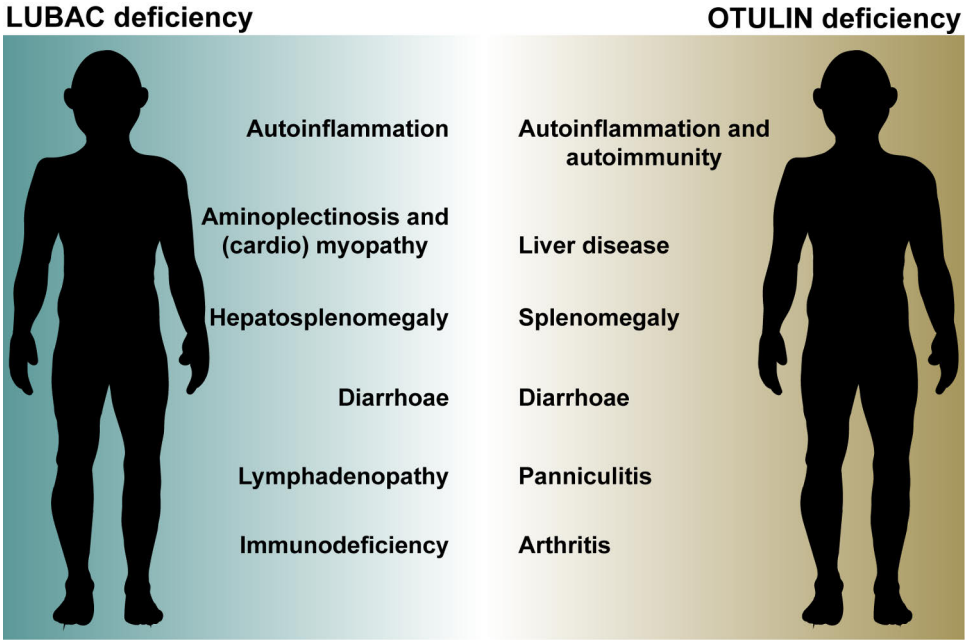

Figure 16. Comparison of clinical manifestations of LUBAC and OTULIN deficiency.

3 *Drosophila melanogaster* as a model organism to study immune response

Drosophila melanogaster, known colloquially as the fruit fly, has been used as a common model organism in biology and biomedical science for over 100 years. The break-through in using the fruit flies as a model organism to study genetics came when Thomas Morgan published in 1910 a paper describing the genetic inheritance of a mutation causing flies with white eyes (Morgan, 1910). Morgan was later awarded a Nobel Prize in Physiology and Medicine for his findings. Since then, seminal findings utilising the fruit fly have been undertaken in the field of development, inheritance, mutagenesis, and immunity, highlighting the advantage of using *Drosophila* as a biological model organism.

In 2011, Jules Hoffman was awarded a shared Nobel Prize for his discoveries in the activation of the innate immune response. The seminal findings were performed in the fruit fly, describing the fly Toll as receptor and activator for innate immune signalling (Lemaitre *et al.*, 1996). Since then, the findings have been confirmed and further studied both in flies and mammals. The innate immune response in the fly is well conserved and less redundant when compared to mammalian innate immune signalling cascades. The fruit flies lack lymphocytes, cells constituting the adaptive immune response in mammals and solely relies on the highly effective innate immune response. Therefore, the fruit fly is a good model organism to study general concepts of innate immune signalling/mediators. The innate immune response in the fly can be further divided into cellular and humoral immune responses. In response to infection, the fly upregulates a plethora of genes that are involved in the humoral immune response as well as the cellular immune response. These genes encode for proteins involved in microbial recognition, phagocytosis, coagulation, melanisation, reactive oxygen metabolism, iron metabolism and synthesis of antimicrobial peptides (AMPs)(De Gregorio *et al.*, 2001).

3.1 *Drosophila melanogaster* as a model organism

The main advantages in using the fruit fly as a biological model organism are the low maintenance costs, limited need of space, food and care, as well as abundant offspring and a short life cycle. The life cycle of the fruit fly lasts only 9-10 days at room temperature and consists of four developmental stages: embryo, larvae, pupa and adult (Figure 17A). The embryo has been used in studies to better understand first stages in development, whereas the larvae, especially the wandering third instar phase larvae, have been used for foraging studies and development of organs. Also, extensive studies have been performed on the extensive morphological changes when a larva is metamorphosing in the pupa. The flies have many similarities to humans, for instance, the heart, lungs, kidney, gut, and reproductive tract are functionally

similar from flies to humans. Similarly, the adult brain has been used in complex studies of behaviourism, such as wake and sleep circadian rhythms, learning and memory, feeding, aggression, courtship, and grooming (Figure 17B)(Yamaguchi and Yoshida, 2018).

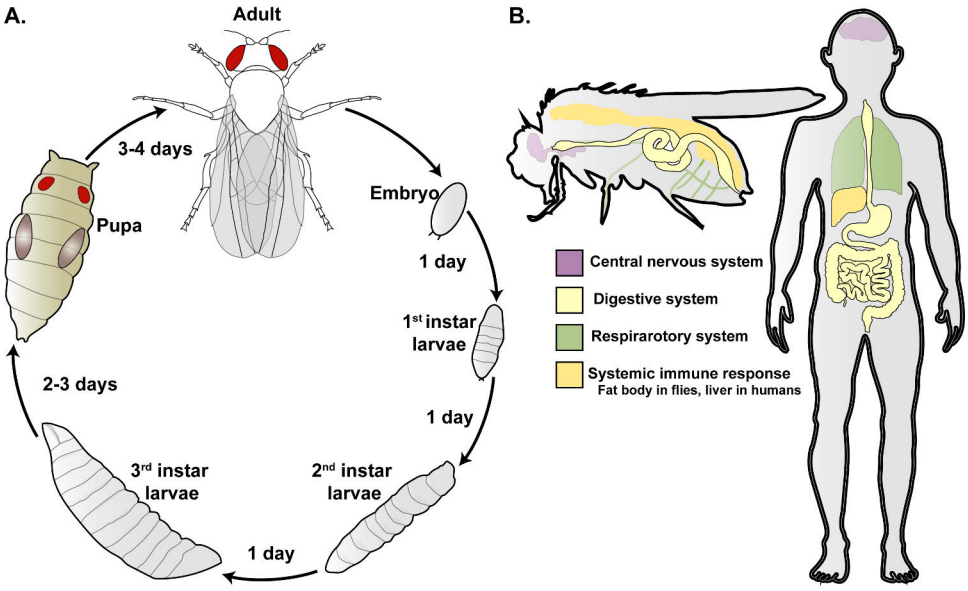

Figure 17. A. The life cycle of the fruit fly lasts approximately 10 days at 25°C and consists of four developmental stages: embryo, larvae, pupa and adult. B. Organ systems of *Drosophila melanogaster* analogous to those in humans.

Today, the genetics of the fruit fly are considered relatively simple, since the fly has only four chromosome pairs. The entire fruit fly genome is about 140 million base pairs (20 times smaller than humans) and encodes about 14,000 proteins (Adams *et al.*, 2000; Rubin *et al.*, 2000). Apart from the aforementioned advantages, the fruit fly is also highly conserved when comparing disease-causing genes in humans, as 75% of the disease-causing genes in humans have a counterpart in the fly (Reiter *et al.*, 2001). Furthermore, during the past 100 years of research, the tools for using the fruit flies have expanded considerably. Deletion and addition of genes, as well as manipulation and regulation of gene expression have been done for decades in the fly model (Venken and Bellen, 2007). For instance, the early development of P-element transposases, for landing genes at specific sites in the genome, and the Upstream activating sequence (UAS)-Gal-system, for inducing gene expression under the regulation of the UAS-promoter, have facilitated the use of *Drosophila* as an efficient model organism when elucidating gene functions. The fruit fly is also ideal for visualisation, staining and dissection of different organs and tissues. In addition, several of the organs and tissues have an equivalent in humans, and, therefore, ideal to research different disease models that cannot be researched in humans due

to ethical reasons. Also, due to the many similarities, the fly can be used as a model organism for screening therapeutic drugs in a more complex system than cell cultures (Chintapalli, Wang and Dow, 2007; Ugur, Chen and Bellen, 2016).

3.2 Cellular immune responses in *Drosophila*

In *Drosophila*, the cellular immune response is mediated by immune cells called haemocytes, and involves phagocytosis and encapsulation of foreign material, as well as activation of a proteolytic cascade that leads to melanisation of invading pathogens. Haemocytes can be found in the body cavity, hemocoel, in the circulating haemolymph. Haemocytes are equivalent to the blood cells in vertebrates and can be divided into plasmatocytes, crystal cells and lamellocytes. However, crystal cells and lamellocytes have only been reported in larvae (Vlisidou and Wood, 2015). Plasmatocytes constitute 90-95% of haemocytes in larvae, and they are responsible for the phagocytic clearance of microbial pathogens, apoptotic cells, and dendrite debris. In addition, plasmatocytes are involved in production of AMPs, cytokines, clotting factors, and extracellular matrix components. Upon ligand recognition by receptors on the surface, downstream signalling pathways are activated leading to uptake of the harmful particle (Melcarne, Lemaitre and Kurant, 2019). Lamellocytes are large, flat and adherent cells that are involved in encapsulation of particles too large for phagocytosis. The encapsulation process in flies has been extensively studied when wasps inject eggs to the haemolymph of *Drosophila* larvae. The wasp egg is recognised by plasmatocytes, consequently leading to lamellocyte recruitment and formation of a multi-layered capsule, with tight cellular junctions, around the invader. Thereafter, the encapsulation process is followed by melanisation, thus encapsulation requires all types of haemocytes. Within the capsule, the parasite is eventually killed, possibly by the local production of cytotoxic products such as ROS and intermediates of the melanisation cascade (Vlisidou and Wood, 2015; Kim-Jo, Gatti and Poirié, 2019). Melanisation is characterised as blackening of the surface of an invading pathogen or a wound site upon injury. The blackening is caused by localised synthesis of melanin, which facilitates wound closing. Crystal cells are non-phagocytic and involved in the melanisation process in larvae. Crystal cells contain the key enzyme in melanin biosynthesis called prophenoloxidase. The enzyme is stored in the form of crystalline inclusions and released upon rupture of the crystal cells (Vlisidou and Wood, 2015).

3.3 Humoral immune responses in *Drosophila*

The humoral immune response involves mainly secretion of immune effector proteins, such as antimicrobial peptides (AMPs), from the fly fat body, which is functionally analogous to the mammalian liver (Figure 17 and 18). The AMP expression in response to infection is regulated by one of the two NF-κB

activating signalling pathways in the fly, the immune deficiency (Imd) or the Toll signalling pathway (Valanne, Wang and Rämet, 2011; Myllymäki, Valanne and Rämet, 2014). The fly has three NF-κB proteins; Dif and Dorsal are p65-like factors and Relish is a NF-κB precursor protein. Dif and Dorsal have a conserved N-terminal RHD and their TAD is located in the C-terminus. Dorsal was first discovered to have an important role in early embryonic patterning, but later it was found to also function in larval humoral response. Dif and Dorsal have redundant effects on AMP expression in larval immune responses, however, the primary effect on adult flies comes from Dif-mediated immune responses. Dif and Dorsal are inhibited by the IκB protein Cactus in flies. In order for Dif and Dorsal to be released from inhibition, Cactus must be targeted for degradation by Lys48-Ub chains (Ganesan *et al.*, 2011). Relish differs from Dif and Dorsal by being a precursor protein, which needs to be cleaved in order to be active. The C-terminal portion of Relish contains ankyrin repeats that autoinhibit the RHD of Relish. Relish is endoproteolytically cleaved by a caspase, before the N-terminal domain (p68) can translocate to the nucleus, leaving the C-terminal domain (p48) in the cytosol (Stöven *et al.*, 2003; Ertürk-Hasdemir *et al.*, 2009). The fly NF-κB Dif and Dorsal are mainly activated by the Toll-mediated signalling pathway and Relish is activated by the Imd signalling pathway (Ganesan *et al.*, 2011).

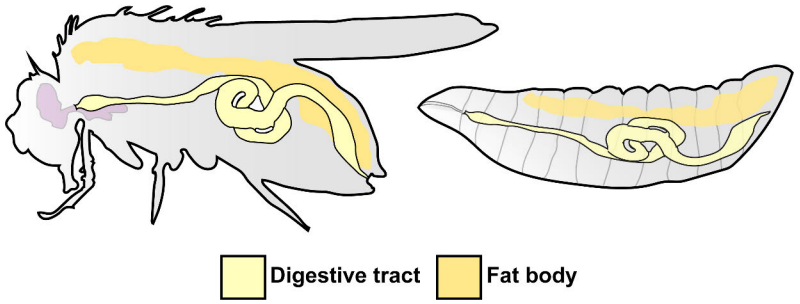

Figure 18. Immune responsive organs in *Drosophila*. A systemic immune response is activated in the fat body of flies and larvae. A local immune response can be activated in the epithelia of the digestive tract.

3.3.1 Immune effector proteins

The antimicrobial peptides (AMPs) are the main effector proteins produced in response to infection. AMPs are small and positively charged peptides that target negatively charged membrane of microbes. The AMPs are embedded into the hydrophobic regions of the lipid membranes of microbes, which leads to membrane destabilisation and cell death. In the fruit fly, there are 21 AMPs grouped into seven families: Drosomycin (seven genes), Metchnikowin, Cecropin (four genes), Defensin, Drosocin, Attacin (four genes) and Diptericin (two genes). Some of the AMPs have distinct activities directed against fungi, Gram-positive bacteria, or Gram-negative bacteria, whereas many of the AMPs, although induced only via one pathway, can have broad activities against many microbes. For instance, cecropins are considered “broad

spectrum" peptides (Hultmark, 2003; Ferrandon *et al.*, 2007; Hanson and Lemaitre, 2020). Most of the AMPs are induced in the fat body in response to systemic infection, however, a few of the AMPs are constitutively expressed locally in some epithelial tissue (Figure 18). For example, *Drosomycin* is constitutively expressed in the salivary glands and female spermatheca. Whereas the induced expression of AMPs in response to systemic infection is regulated by one of the two NFκB signalling pathways in the fly fat body, the local constitutive AMP expression is not. Upon local bacterial challenge, such as epithelial gut inflammation, the epithelium respond by local induction of AMPs. The local inducible AMP expression is only regulated by the Imd pathway, as the Toll pathway is not similarly required for local epithelial immune response (Ferrandon et al, 1998; Tzou et al, 2000). Likewise, as AMPs, the effector peptides Bomanins (Boms) are induced by Gram-positive bacteria and are regulated by the Toll pathway. The Boms are coded by 12 genes in the fly and have been shown to have an essential role in defence against Gram-positive bacteria and some fungal species (Lindsay and Wasserman, 2014; Clemmons, Lindsay and Wasserman, 2015; Lindsay, Lin and Wasserman, 2018).

3.3.2 Activators of humoral immune responses

The humoral immune response can recognize and respond accordingly between different PAMPs on pathogens. This is due to specific receptors recognising the peptidoglycan (PGN) present on most bacteria. The PGN forms a layer of conserved polymeric glycan chains of β-1,4-linked N-acetylglucosamine and N-acetylmuramic acid crosslinked by short stem peptides, which vary between different types of bacteria. In Gram-negative bacteria, the third amino acid in the PGN is a meso-DAP residue, whereas Gram-positive bacteria have a Lysine residue in this position. The difference between these two types of PGNs can be recognised by PGN-recognition protein (PGRPs) and Gram-negative binding proteins (GNBPs), also known as β-glucan recognition proteins (β-GRP). The PGRP family in flies is encoded by 13 genes coding for 19 proteins and can be divided into short (S) or long (L) depending on their transcript size. All the members have a common PGRP domain and can be divided into two subgroups; enzyme-activated amidases and activators of signalling pathways and proteolytic cascades (Werner *et al.*, 2000). The catalytic PGRPs (PGRP-SC1/2, LB, SB1/2) have a zinc-dependent amidase activity and can remove peptides from glycan chains. Catalytic PGRPs modulate the immune response, by degrading peptidoglycans that can activate the immune response (Paredes *et al.*, 2011; Zaidman-Rémy *et al.*, 2011) In contrast, the other group of PGRPs (PGRP-SA, SD, LA, LC, LD, LE, and LF) lack zinc-binding residues required for amidase activity. Instead, they bind and recognize PGN and function as PRRs, which leads to activation of signalling pathways and proteolytic cascades (Ferrandon *et al.*, 2007; Lemaitre and Hoffmann, 2007; Charroux and Royet, 2010).

3.3.3 The Imd signalling pathway

The Imd pathway of the fly resembles in many ways the mammalian NLR and TNFR1 signalling pathways. Whereas the cytokine TNFα is one of the major NF-κB activators in mammalian cells, the sole homolog for TNFα in flies, called Ectodysplasia-like cell death trigger (Eiger), induces the gene expression mainly through JNK signalling and not NF-κB (Igaki and Miura, 2014). Instead, the receptors PGRP-LC and PGRP-LE mediate the detection of Gram-negative bacteria and are the main receptors that activate the Imd pathway (Choe *et al.*, 2002; Gottar *et al.*, 2002; Rämet *et al.*, 2002). PGRP-LC and PGRP-LE bind directly to the bacteria and are activated by DAP-type PGNs or shorter PGN end fragments (muramyl peptides). PGRP-LC is a transmembrane protein and combines the function of a microbial binding protein, through its PGRP domain, and of a signalling receptor, through its intracellular domain. PGRP-LE is expressed both extra- and intracellularly, however, the extracellular PGRP-LE is a fragment that together with PGRP-LC enhances the PGN recognition. The PGN on the Gram-negative bacteria cell wall is masked by a lipopolysaccharide (LPS) layer, and therefore, not accessible directly for binding by the PGRP receptors. However, it is believed that short PGN fragments are released when the cell wall is remodelled during proliferation and growth. These short PGN fragments are then recognised and activate the immune response. In this way, the immune system recognises proliferating bacteria over the presence of non-proliferating bacteria, which could partly explain why the endogenous bacteria fail to induce the immune response (Takehana *et al.*, 2004; Kaneko *et al.*, 2006; Ferrandon *et al.*, 2007; Lemaitre and Hoffmann, 2007).

Upon Gram-negative bacterial recognition, by the oligomerised PGRP receptors, an initial signalling complex is recruited, consisting of Imd, *Drosophila* Fas-associated death domain (dFadd) and caspase 8 homolog death-related ced-3/Nedd2-like protein (Dredd)(Lemaitre *et al.*, 1995; Georgel *et al.*, 2001; Choe, Lee and Anderson, 2005; Zhou *et al.*, 2005). Dredd performs cleavage of Imd, which exposes a consensus inhibitor of apoptosis (IAP) binding motif (IBM) domain, which interacts with the RING E3 ligase *Drosophila* IAP 2 (Diap2)(Choe, Lee and Anderson, 2005; Gyrd-Hansen *et al.*, 2008; Paquette *et al.*, 2010). Diap2 conjugates Lys63-Ub chains on Imd and Dredd, which further activates Dredd (Kleino *et al.*, 2005; Leulier *et al.*, 2006; Paquette *et al.*, 2010; Meinander *et al.*, 2012). The ubiquitin chains on Imd are suggested to function as scaffold for recruitment of *Drosophila* Tak1 through the UBD of Tab2 (Kanayama *et al.*, 2004; Zhuang *et al.*, 2006). Upon recruitment, Tak1 phosphorylates and thus activates the IKK complex, consisting of the catalytic subunit Ird5 (IKKβ) and the regulatory subunit Kenny (IKKγ)(Vidai *et al.*, 2001; Silverman *et al.*, 2003; Kleino *et al.*, 2005). Relish is phosphorylated by the Ird5 (Rutschmann *et al.*, 2000; Silverman *et al.*, 2000; Lu, Wu and Anderson, 2001), however, the phosphorylation of Relish is not absolutely necessary for Relish transcriptional activity. Instead,

it is required for enhanced transcriptional activity of Relish in the nucleus (Ertürk-Hasdemir *et al.*, 2009). To release Relish from its autoinhibition, it is cleaved by Dredd and the RHD containing domain of Relish is translocated to the nucleus (Figure 19)(Stöven *et al.*, 2003).

DUBs have been shown to deubiquitinate Imd signalling mediators, leading to termination of the signalling. As an example, Imd is deubiquitinated by *Drosophila* Usp36 (dUSP36)/scny and fat facets (Thevenon *et al.*, 2009; Taillebourg *et al.*, 2012; Yagi *et al.*, 2013). These DUBs are required for proper ubiquitin-mediated regulation of Imd. First, Imd is rapidly Lys63-ubiquitinated by DIAP2, which leads to Tak1 activation. Tak1 phosphorylates Imd, triggering the removal of Lys63-Ub chains and the addition of Lys48-Ub chains. This in turn leads to proteasomal degradation of Imd and subsequent termination of signalling (Chen *et al.*, 2017). Besides Imd, Tak1 is deubiquitinated by the A20 homolog Trabid (Fernando, Kounatidis and Ligoxygakis, 2014), and the IKK complex is deubiquitinated by CYLD (Tsichritzis *et al.*, 2007).

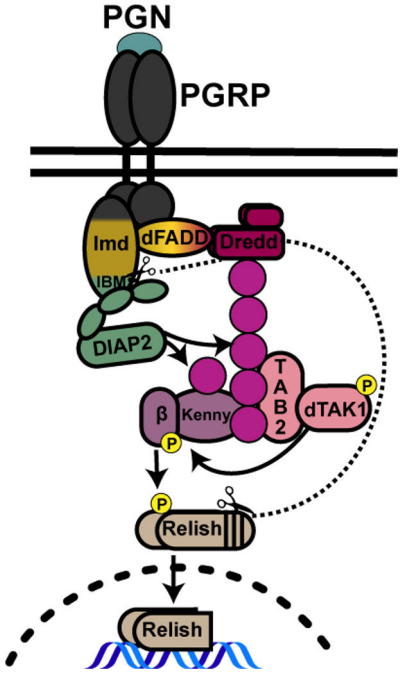

Figure 19. The Imd signalling pathway. The Imd pathway is activated when Gram-negative bacteria bind to the membrane receptor PGRP-LC. Upon receptor stimulation, the adaptor proteins Imd and dFadd, and the caspase Dredd, are recruited to the receptor complex. Dredd cleaves Imd, revealing an IBM motif. The IBM is recognised by Diap2, which ubiquitinates both Dredd and Imd. The *Drosophila* TAK/TAB and the IKK complexes are recruited to the receptor complex via the ubiquitin chains. TAK1 phosphorylates IKKβ/Ird5 of the IKK complex. The NF-κB Relish is phosphorylated by Ird5, whereas Dredd cleaves off the inhibitory ankyrin repeats. After activation, Relish is translocated to the nucleus and induces gene transcription.

3.3.4 The Toll signalling pathway

In flies, the Toll receptor functions more like a cytokine receptor and is not activated directly by binding to bacteria. Instead, Toll is activated by binding to the cytokine Spätzle. Spätzle is synthesised as an inactive dimer precursor (DeLotto and DeLotto, 1998), which is unable to bind and activate Toll. Spätzle is cleaved, when either Lys-type Gram-positive bacteria are sensed by secreted PGRP-SA, PGRP-SD and GNBP1, or glucans on the fungal cell wall are recognised by the GNBP3 (Michel *et al.*, 2001; Bischoff *et al.*, 2004; Gottar *et*

al., 2006). The cleaved Spätzle is a product of a proteolytic cascade, consisting of several serine proteases, that undergo zymogen activation. Two serine proteases have been identified to cleave Spätzle and activate Toll signalling in response to Gram-positive bacteria and fungi. Spätzle is cleaved by Spätzle processing enzyme (SPE) in response to infection by Gram-positive bacteria and by Persephone in response to fungi (Jang *et al.*, 2006; Ferrandon *et al.*, 2007; Lemaitre and Hoffmann, 2007; El Chamy *et al.*, 2008). The fly genome encodes a family of nine Toll receptors (Toll, Toll3-Toll9 and 18-wheeler). Toll, Toll7 and Toll8 have been shown to have a role in mediating the immune response, whereas the other Toll receptors in the fly are more involved in developmental processes (Tauszig *et al.*, 2000; Kambris *et al.*, 2002; Akhouayri *et al.*, 2011; Narbonne-Reveau, Charroux and Royet, 2011; Chowdhury *et al.*, 2019). As in the mammalian TLR signalling, the adaptor protein MyD88 is recruited to Toll, which further recruits the adaptor protein Tube and the kinase Pelle through death domain (DD)-mediated interactions (Valanne, Wang and Rämet, 2011). The highly conserved Pelle/IRAK-interacting protein RING E3 ligase Pellino is recruited and believed to ubiquitinate Pelle (Medvedev *et al.*, 2015). The signalling cascade continues to the phosphorylation of the fly IκB Cactus by Pelle. Cactus is consequently targeted to proteasomal degradation, and therefore, the fly NF-κB proteins Dorsal and/or Dif bound by Cactus are released and translocated to the nucleus (Figure 20)(Valanne, Wang and Rämet, 2011).

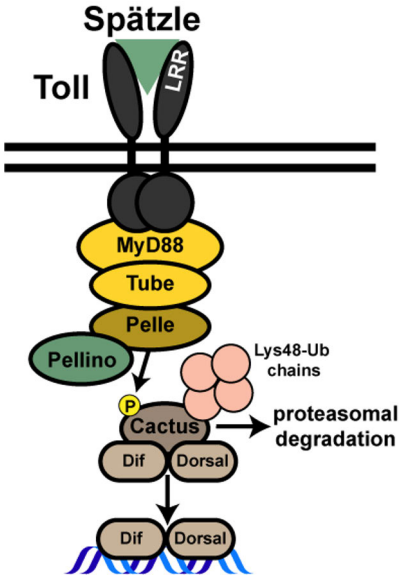

Figure 20. The Toll signalling pathway. The Toll pathway is activated when the cytokine Spätzle is cleaved and activated in response to Gram-positive bacteria and fungi. Spätzle binds to the membrane receptor Toll. Upon activation, the adaptor proteins MyD88 and Tube, as well as the kinase Pelle and E3 ligase Pellino, are recruited to the receptor complex. The IκB protein Cactus is phosphorylated and targeted for Lys48-Ub chain mediated proteasomal degradation resulting in the release of Dif and Dorsal from inhibition. Dif and/or Dorsal translocate to the nucleus and induce gene transcription.

3.4 Local epithelial immune responses

The epithelium, consisting of epithelial cells, forms a protective layer surrounding the internal organs and body. The epithelial cells are tightly

connected to one another and form either a simple layer, as in the intestine and respiratory organs, or a multilayer, as in the epidermis/cuticle. The tight physical contact between the cells, creates a protective barrier against invasive pathogens, and therefore, a balanced epithelial tissue homeostasis is of utmost importance in order to protect the integrity of the epithelial layer. The simple epithelial layer in the intestine and the airways constitute the first cellular border between the complex bacterial environment in the lumen and sterile haemolymph. The epithelia also act as a first line of defence against these irritants by activating the production of AMPs and other chemicals in order to protect the internal tissue (Lemaitre and Hoffmann, 2007). Interestingly, the local inducible AMP expression is only regulated by the Imd pathway, as the Toll pathway is not similarly required for local epithelial immune response (Ferrandon et al, 1998; Tzou et al, 2000). Moreover, reactive oxygen species generated by a dual oxidase is essential for the antimicrobial activity of the gastrointestinal tract (Ha et al., 2005).

3.4.1 Immune responses in the gut

The gut is the second major immune organ in the flies. However, much to the difference to the normally sterile environment of the fat body, the epithelium in the gut is in constant contact with commensal bacteria residing in the intestine. Therefore, the intestinal immune response must distinguish commensal and mutualistic bacteria from pathogenic bacteria, in order to activate the immune response in response to pathogens and pathobionts leaving the commensal bacteria unscathed (Ryu *et al.*, 2008). In mammals, acute and chronic dysregulation of the intestinal immune processes can lead to gastrointestinal infections, inflammatory bowel diseases (IBDs) and cancer. *Drosophila* is widely used to study intestinal immune responses, as the fly intestine resembles the mammalian in its function to digest and absorb ingested food, as well as the immune activated protection against food borne pathogens (Apidianakis and Rahme, 2011). The *Drosophila* gut consists of a low bacterial diversity (1–30 species), and the most commonly found species are members of two major families *Lactobacillaceae* and *Acetobacteraceae.* In addition, *Enterobacteriaceae* and yeasts such as *Hanseniaspora* or *Saccharomyces* are also commonly found (Miguel-Aliaga, Jasper and Lemaitre, 2018).

The first line of defence in flies is the peritrophic matrix (PM), which is a semi-permeable membrane that allows the passage of enzymes and nutrients, but not bacteria. In case the bacteria penetrates the PM, the immune response in the gut is mainly mediated by the secretion of AMPs and production of antimicrobial ROS, which is activated by the bacteria derived molecules PGNs and uracil, respectively (Apidianakis and Rahme, 2011; El Chamy *et al.*, 2015; Colombani and Andersen, 2020). Uracil is produced by pathogens and pathobionts, but not by symbionts, and is therefore a way for the epithelial immune response to distinguish between pathogens and commensal (Lee *et*

al., 2013). Uracil activates the NADPH dual oxidase (DUOX), which regulates the production of ROS and is the first line of defence in the gut (Ha, Oh, Bae, *et al.*, 2005; Lee *et al.*, 2013). The bacterial uracil also activates the Hedgehog signalling pathway, which leads to formation of cadherin-dependent endosomes. These endosomes are essential signalling platforms for PLCβ/PKC/Ca^{2+}-dependent DUOX activation (Ha *et al.*, 2009; Lee *et al.*, 2015). Upon infection, the intestinal enterocytes reprogram lipid metabolism, by enhancing lipolysis via lipophagy, which is also required for DUOX activity (Lee *et al.*, 2018). Excessive ROS production is detrimental for the host and therefore prevented by immune responsive catalase in gut epithelia (Ha, Oh, Ryu, *et al.*, 2005).

The Imd pathway is the second line of defence in the gut and is believed to act synergistically and complementarily with production of ROS (Ryu *et al.*, 2006). The Imd pathway is activated upon recognition of PGN either by the transmembrane receptor PGRP-LC or the intracellular receptor PGRP-LE (Bosco-Drayon *et al.*, 2012; Neyen *et al.*, 2012). Some of the released AMPs from the epithelia suppress pathogens, but also promote colonisation of beneficial microbes and thus Imd/AMPs are involved in shaping the intestinal microbiota. The Imd pathway also regulates other immune functions, such as enterocyte shedding, production of digestive enzymes and production of ROS (El Chamy *et al.*, 2015; Miguel-Aliaga, Jasper and Lemaitre, 2018).

As not only pathogens, but also gut symbionts release PGNs, several negative regulators have been described along the signalling cascade to prevent constitutive activation (Colombani and Andersen, 2020; Hanson and Lemaitre, 2020). The homeobox transcription factor Caudal has been shown to be a gut-specific negative regulator of the Imd pathway. Caudal regulates the expression of the AMPs such as *Diptericin* and *Cecropin* and in this way prevents Imd activation by commensal bacteria. When Caudal is knocked down specifically in the gut, the commensal bacterial composition is disturbed (Ryu *et al.*, 2008). Moreover, Caudal regulates the constitutive NF-κB-independent expression of *Drosomycin* and *Cecropin* in some local epithelial tissues (Ryu *et al.*, 2004). Similarly, the caspase 3 homolog *Drosophila* interleukin 1β-converting enzyme (Drice) has been shown to function as a negative regulator of Diap2-mediated inflammatory signalling in the gut, keeping the Imd pathway at bay in response to commensal bacteria (Kietz *et al.*, 2022). Furthermore, the negative regulator poor Imd response upon knock-in (Pirk) inhibits Imd interaction with the receptors PGRP-LC and PGRP-LE, which disrupts signalling complex formation. Also, hyperactivation of Pirk in response to pathogens, maintains bacterial homeostasis in the fly gut by preventing the induction of AMPs by the commensal bacteria (Aggarwal *et al.*, 2008; Kleino *et al.*, 2008; Lhocine *et al.*, 2008). Taken together, the Imd pathway needs to be tightly regulated in order to avoid unwanted activation in response to commensal bacteria, while an efficient and precise NF-κB activation is required in response to pathogenic invasion.

OUTLINE AND KEY AIMS OF THESIS

The NF-κB signalling pathway is regulated by various post-translational modifications and many key signalling mediators are either ubiquitinated or involved in the ubiquitination of other proteins in the pathway. Met1-ubiquitination has been shown to be important in the regulation of pathogen and cytokine-induced NF-κB signalling. However, the role of Met1-ubiquitination in cellular stress had not been studied.

The key aim of my thesis was to elucidate the role of Met1-ubiquitination in NF-κB signalling in response to bacterial infection and sterile stress-induced inflammation in the fruit fly, *Drosophila melanogaster*. Hence, I have used *Drosophila* as a model organism to study molecular mechanisms of inflammatory NF-κB signalling *in vivo*. I have developed methods to induce NF-κB-mediated immune signalling by bacterial infection and various stress-promoting stimuli and then analysed the role of Met1-ubiquitination in NF-κB-mediated signalling. In addition, I have developed a model system to induce chronic intestinal inflammation, which I have used to test anti-inflammatory properties of stilbenoid compounds.

The key aims for my doctoral thesis:

- Characterise LUBEL as an Met1-ubiquitinating E3 ligase in *Drosophila*
- Determine the role of Met1-ubiquitination in pathogen-induced and sterile stress-induced inflammation in *Drosophila*
- Determine the targets and consequences of Met1-ubiquitination in *Drosophila*
- Study Met1-ubiquitination during cell stress in mammalian cells
- Develop a model system to study NF-κB activation in chronic intestinal inflammation and use this to study how inflammatory signalling can be regulated by stilbenoid compounds

EXPERIMENTAL PROCEDURES

In this section, the experimental procedures (Table 1) used in this thesis work are briefly presented. More detailed information about specific methods can be found from original publications (I-III).

Table 1. An overview of the experimental procedures used in this thesis

Experimental procedures	**Study**
16S rRNA sequencing*	III
Axenic flies	I-III
Bacterial colony count	I
Caspase activity assay*	II
Cell culture (*Drosophila* S2)	II
Cell culture (mammalian Caco2*)	II
Computational modelling (LUBEL/Kenny*/TrpA1*)	I,III
Drosophila dissections	I-III
Drosophila maintenance and crossing	I-III
Fluorescence microscopy	I
Generate transgenic flies*	I
Hypoxia experiments	II
Immunofluorescence (IF)	I
Immunoprecipitation (IP)	I
In vitro ubiquitination assay*	I
Larvae feeding	III
Light microscopy	I-III
Polymerase chain reaction (PCR)	I
Purification of recombinant protein	I,II
Quantitative reverse transcriptase PCR (qPCR)	I-III
SDS-page and Western Blot (WB)	I,II
Statistical analysis	I-III
Stilbene treatment	III
Survival assays (SA)	I,II
TUBE pulldown (GST-NEMO-UBAN/ M1-TUBE)	I,II
Ubiquitin chain restriction (UbiCRest) analysis*	I
X-Gal staining	I-III

*Experiments performed by colleagues

1 Fly husbandry, stress treatments and survival experiments

Drosophila melanogaster were maintained at 20°C or 25°C with a 12 h light–dark cycle on Nutri-fly BF (Dutscher Scientific, Essex, UK). Either adult flies (study I and II) or early foraging 3rd instar larvae (study II and III) were used for experiments. *Canton^{S}*, *DaGal4* and *c564Gal4* fly lines were used as controls. Fly lines used in this thesis can be found in Table 2. To investigate the role of Met1-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 (study I). For this purpose, plasmids were designed and sent to Bestgene Inc. (thebestgene.com, California, USA) for fly egg injection.

To study NF-κB signalling, the immune response was activated by subjecting flies to pathogenic infection, hypoxia, oxidative stress, mechanical stress, and dextran sodium sulphate (DSS)-treatment (Table 3). In study I, flies were subjected to Gram-negative bacteria *Erwinia carotovora carotovora 15* (*Ecc15*), Gram-positive bacteria *Micrococcus luteus* (*M. luteus*) and Gram-negative bacteria *Escherichia coli* (*E. coli*). Septic injuries were performed by pricking 2-4 days old adult flies in the lateral thorax with a thin needle previously dipped in a concentrated solution of *Ecc15* or *M. luteus*. 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. In study II, hypoxia experiments were performed by placing adult flies or larvae in a modified portable MiniHypoxy-platform (Faculty of Medicine and Health Technology, Tampere University, Finland). In this study, flies were exposed to a gas-mixture of 5 % O_2 with 95 % N_2. To induce oxidative stress, adult flies were fed with paraquat mixed in 5 % sucrose pipetted on a Whatman paper and for mechanical stress, 3rd instar larvae were subjected to mechanical stress by vortexing them for 10 seconds at 3,200 rpm. For study III, 3rd instar larvae were fed with fly food mixed with 40 kDa DSS (TdB Consultancy AB, Uppsala, Sweden), to induce intestinal inflammation. After optimising the concentration and timepoints leading to induced NF-κB target gene expression, the DSS-treated larvae were treated with stilbenoid compounds (pinosylvin, pinosylvinmonomethylether, isorhapontin and astringin) or transient receptor potential ankyrin 1 (TrpA1) antagonist A-967079 (Sigma-Aldrich, Missouri, USA) and HC-030031 (Sigma-Aldrich) mixed in the fly food.

For isolation of Met1-Ub chains, 20-40 adult flies or 15 larvae per genotype were used and for qPCR, 10 adult flies and 5 larvae per genotype were used. For survival assays (SA), 10-20 flies per fly genotype were exposed to abovementioned insults and survival was monitored daily.

Table 2. *Drosophila* strains used during the thesis work.

Fly strains	**Provider**	**Study**
c564Gal4	Pascal Meier	II
c564Gal4>UAS-Dredd	Pascal Meier	II
c564Gal4>UAS-LUBEL-RNAi		II
Canton^{S}	Pascal Meier	I-III
DaGal4>UAS-Dredd	Pascal Meier	II
DaGal4>UAS- LUBEL-RNAi	*18055	II
DaGal4>UAS-RBR-LDDC>A	Bestgene Inc.	I
DaGal4>UAS-RBR-LDDWT	Bestgene Inc.	I
DaGal4	Pascal Meier	I, II
DaGal4;Dipt-lacZ	Pascal Meier	II
DaGal4>UAS-Duox-TRiP	#33975	II
diap2^{7c}	Pascal Meier	II
Dredd^{L23}	Pascal Meier	II
Drs-lacZ	#55708	II
fatiga^{0225}	#11561	II
key^{4}	Pascal Meier	II
LUBEL ^{Mi}/ LUBEL$^{\Delta RBR}$	#22725	I, II
LUBEL^{MiMic}	#59639	I
PGRP-LCx$^{\Delta 5}$	#36323	II, III
RelE20	#9457	II, III
sima^{KG607607}	#14640	II
Spätzle^{RM7}	Pascal Meier	I
tak1^{1}	#26272	II
TRPA1^{1}	#36342	II

Bloomington stock centre *Vienna Drosophila resource center

Table 3. An overview of the treatments and applications used on flies in this thesis

Treatments	Application	Study
Axenic (adult and larvae)	SA, qPCR	II
DSS (larvae)	qPCR, 16S,	III
E.coli	Colony counting	I
Ecc15 (adult and larvae)	SA, qPCR, X-Gal, TUBE, pH3	I
Hypoxia (adult and larvae)	SA, qPCR, X-Gal, TUBE	II
M.luteus	SA, qPCR	I
Mechanical stress (larvae)	TUBE	II
Oxidative stress	SA, TUBE	II
Stilbene (larvae)	qPCR	III
TRPA1 antagonist (larvae)	qPCR	III

2 Cell culture and stress treatments

In this thesis, cells were used to investigate protein levels, protein-protein interactions, and Met1-ubiquitination of specific proteins in the Imd pathway. The *Drosophila* S2 cell line was established by Imogen Schneider (S2 stands for Schneider's line 2) in 1970, from *Drosophila* embryos (Schneider, 1972). S2 cells are good for the study of innate immunity, as these cells share several properties with haemocytes. *Drosophila* 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, Hilden , Germany). 50% confluent 10 cm plates were used to prepare lysates for immunoprecipitation and pulldown assays of GST-tagged recombinant proteins, 6-well plates were used for lysates for Western blot analysis. Expression of pMT plasmids was induced with 500 μM CuSO4 for 16 h before lysis. Lipopolysaccharides (LPS, Sigma) was used at 80 μg/ml for the indicated times and 1 μM 20-hydroxyecdysone (Sigma) was added 24 hours prior to LPS treatment.

In study II, human epithelial colon adenocarcinoma (Caco2) cells (ACC 169, DSMZ, Leipzig, Germany) were grown in DMEM/F-12 (Gibco, ThermoFisher Scientific, Waltham, Massachusetts, USA) supplemented with 10 % heat inactivated fetal bovine serum (Biowest, Nuaillé, France), 100 IU/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich, Missouri, USA) at 37°C humidified atmosphere with 5 % CO_2 until use. For hypoxia and oxidative stress experiments, cells were plated at $1x10^6$ cells on 10 cm diameter dishes with serum free DMEM/F-12 supplemented with 0,1 % bovine serum albumin (Sigma-Aldrich) and 100 IU/ml penicillin and 100 μg/ml

streptomycin. Hypoxic conditions were achieved by exposing 80-90 % confluent cells to 5 % O_2, 5 % CO_2 and 90 % pure N_2 (AGA, Finland) by placing the plates in a hypoxic chamber (Galaxy 14S; Eppendorf, Hamburg, Germany) for 2 h at 37°C. Oxidative stress was induced by treating cells with 1 μM paraquat (Sigma-Aldrich) for 24 h. To inhibit Met1-ubiquitination, Caco2 cells were treated with 10 μM HOIP inhibitor 1 (HOIPIN1) (Axon Medchem BV, Groningen, Netherlands) for 3 h. For the shear stress experiments, Caco2 cells were seeded in 6-well plates. For mechanical shear stress, the plates with Caco2 cells were placed on an orbital shaker, 100 rpm, for maximum 2 h at 37°C. Only the cells from the outer part of the well were collected to ensure a flow with a smaller oscillatory shear index.

3 Protein expression and protein-protein interaction studies

Protein-protein interactions were studied on transfected S2 cells, stress exposed human Caco2 cells and fly samples. To study Met1-ubiquitination and possible targets of Met1-ubiquitination, Met1-Ub conjugates were purified using a recombinant protein containing the UBAN region of NEMO (residues 257–346) fused to GST (GST-NEMO-UBAN), also called Met1-tandem ubiquitin binding entity (M1-TUBE). First, the recombinant protein was expressed in and purified from *E.coli* bacteria, thereafter, interaction studies were performed either under denaturing conditions (detecting ubiquitin conjugated proteins) or non-denaturing (detecting both ubiquitin conjugated and ubiquitin-associated proteins) conditions. Protein-protein interactions were studied in transfected S2 cells by immunoprecipitation (IP) either by using HA or V5-tagged agarose beads (Sigma). Protein expression from the TUBE pulldowns as well as IP experiments from whole flies and cells were investigated by SDS-PAGE and Western blotting (WB) using the antibodies listed in Table 4.

To study intestinal inflammation induced midgut hyperplasia, proliferating cells were analysed by staining the proliferation marker phospho-histone3 (pH3). For this purpose, fly guts were dissected and stained with α-pH3 antibody. Then pH3-positive cells were detected and counted by imaging with a spinning disk confocal microscope, 20x (Zeiss Axiovert-200M microscope, Yokogawa CSU22 spinning disk confocal unit).

Table 4. Primary antibodies used, their source and their application.

Antibody	Company/reference	Application
Actin (clone C-11)	Santa Cruz	TUBE, IP, WB
DIAP2	Tenev et al., 2005	IP, WB
GST	GE Healthcare	TUBE, IP, WB
HA (clone 3F10)	Roche	IP, WB
Lys63 (clone Apu3)	Millipore	TUBE, IP, WB
Met1 (clone IE3)	Millipore	TUBE, IP, WB
Met1 (clone LUB9)	Lifesensor	TUBE, IP, WB
Phospho-Histone H3 (clone Ser10)	CST	IF
Ubiquitin (clone FK2)	Enzo	TUBE, IP, WB
Ubiquitin (clone Ubi-1)	Novus	TUBE, IP, WB
V5 (clone SV5-Pk1)	Bio-Rad	IP, WB

4 Bacterial colony count

To study bacterial clearance, flies were fed with ampicillin resistant *E. coli*. *E. coli* transformed with empty vector pMT/Flag-His were 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 water and homogenised in PBS. The sample was cleared and diluted 1:100 before plated on LB-agar plates containing 50 μg/ml ampicillin. Colonies were counted 24 h after plating.

5 Measurement of NF-κB target gene expression

Quantitative reverse transcriptase PCR (qPCR) was used to study the NF-κB target gene expression during basal conditions and in response to inflammation induction, as well as to control RNAi-efficiency silenced by the UAS-Gal4 system in flies. For qPCR, total RNA was extracted from whole fly lysates (QIAGEN). cDNA was synthesised and qPCR was performed by detecting SYBR Green fluorescence. The *ribosomal protein (rp49)* was used as a housekeeping gene for normalisation. The gene-specific primers used to amplify cDNA are listed in Table 5. To study local activation of NF-κB target genes, guts and trachea were dissected from *Diptericin-lacZ* or *Drosomycin-lacZ* reporter fly lines and stained with 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (abbreviated as X-Gal). Briefly, dissected organs were fixed for 15 min and then stained with a 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 imaged with brightfield microscopy.

Table 5. Gene-specific primers used for qPCR.

Primers	**Forward 5'**	**Reverse 5'**
attacinA	ATGCTCGTTTGGATCTGACC	GACCTTGGCATCCAGATTGT
diptericin	ACCGCAGTACCCACTCAATC	ACTTTCCAGCTCGGTTCTGA
dredd	ACATTGCCCTTCTCCACAGA	CATGGCGATGCTGTTGGATG
drosocin	CGTTTTCCTGCTGCTTGC	GGCAGCTTGAGTCAGGTGAT
drosomycin	CGTGAGAACCTTTTCCAATATGATG	TCCCAGGACCACCAGCAT
IM1	GTTTTTGTGCTCGGTCTGCT	CACCGTGGACATTGCACA
rbr-ldd	CGGAACCCATGCAGATCAAG	CGCAGTCCGTCAGATCAAAG
rp49	GACGCTTCAAGGGACAGTATCTG	AAACGCGGTTCTGCATGAG
ubiquitin	AGGAGTCGACCCTTCACTTG	CGAAGATCAAACGCTGCTGA
ZnF	TGCTCCATATGCTGCAAGAC	CGGATTTCTGACTGGGTTGT

6 Structural modelling

The 3D structure of the Kenny UBAN and the LUBEL CBR was modelled with Phyre265. Molecular graphics and analyses were performed with PyMol or the UCSF Chimera package using the indicated templates.

RESULTS AND DISCUSSION

1 LUBEL-mediated Met1-linked ubiquitination facilitates NF-κB activation in response to inflammation (I, II)

Both Lys63- and Met1-ubiquitination have been described to have important functions in regulation of mammalian NF-κB signalling (Haas *et al.*, 2009; Tokunaga and Iwai, 2009; Gerlach *et al.*, 2011; Damgaard *et al.*, 2012; Corn and Vucic, 2014; Shimizu, Taraborrelli and Walczak, 2015; Rittinger and Ikeda, 2017). Whereas the role for Lys63- ubiquitination in the *Drosophila* Imd pathway is well established (Zhou *et al.*, 2005; Paquette *et al.*, 2010; Meinander *et al.*, 2012), the role of Met1-ubiquitination in *Drosophila* NF-κB signalling has not been studied before. Hence, to elucidate the role of Met1-Ub chains in *Drosophila*, we studied Met1-Ub chains in the immune response to pathogen infection and sterile inflammation-inducing stresses.

1.1 Met1-ubiquitin chains are formed upon infection and cellular stress promoting stimuli

Met1-Ub chains have been shown to be induced by a plethora of inflammation promoting stimuli in mammals, whereas during basal conditions Met1-Ub chains are near undetectable (Haas *et al.*, 2009; Tokunaga and Iwai, 2009; Gerlach *et al.*, 2011; Damgaard *et al.*, 2012; Corn and Vucic, 2014; Shimizu, Taraborrelli and Walczak, 2015; Asaoka *et al.*, 2016). Therefore, we subjected the flies to bacterial infection and various pathological conditions to induce Met1-Ub chain formation. As ubiquitin is highly conserved throughout evolution, the tools used to study mammalian Met1-ubiquitination can also be used in *Drosophila*. With the help of a specific GST-tagged Met1-Ub chain binder (NEMO-UBAN/M1-TUBE), we were able to enrich and detect Met1-Ub chains from fly lysates and *Drosophila* S2 cells. As expected, in flies, during basal conditions, we were able to detect only traces of Met1-ubiquitin chains. However, upon exposure to bacterial infection (I, Fig. 1A, B), and also in response to sterile stresses such as starvation (I, Fig. 1B), hypoxia (II, Fig. 1C), oxidative stress (II, Fig. 6A) and mechanical stress (II, Fig. 6E), an increase in Met1-Ub chains can be detected. This is in line with the study done by Asaoka *et al*., showing that Met1-Ub chains are induced by heat stress (Asaoka *et al.*, 2016). To assure that the ubiquitin chains detected are not an enhanced expression of the gene encoding for ubiquitin concatemers, we also analysed the ubiquitin mRNA expression in response to bacterial infection. As ubiquitin mRNA expression was not significantly altered upon infection (I, Supplementary Fig. 1C) the regulation of Met1-Ub chains is done at the level of E3 ligase activity vs DUB activity. These experiments show that we can detect Met1-Ub chains also in flies in response to distinct stimuli and that

Drosophila can be used as a model organism to study Met1-ubiquitination. Moreover, we suggest that Met1-Ub chains may function as universal stress responders in flies to various cellular stress promoting stimuli.

1.2 Characterisation of the Met1-ubiquitination system in *Drosophila*

1.2.1 LUBEL catalyses Met-Ub chains in *Drosophila*

LUBAC is the only known mammalian E3 ligase complex able to catalyse Met1-Ub chains. We and others have reported LUBEL (fly gene number CG11321) to be a homologue of the mammalian LUBAC component HOIP (I, Fig. 2A)(Asaoka *et al.*, 2016). Like HOIP, LUBEL contains N-terminal ZnF domains, two UBA domains and a C-terminal RBR domain. In mammals, HOIP interacts with both HOIL-1 and SHARPIN through the UBA and ZnF domains (Smit *et al.*, 2012; Stieglitz *et al.*, 2012; Yagi *et al.*, 2012). Interestingly, no homologues of SHARPIN or HOIL-1 can be found in the *Drosophila* genome. However, LUBEL is significantly larger than HOIP and some of the UBA domains of LUBEL may function in the same way as mammalian SHARPIN and HOIL-1 (I, Fig. 2A). Structural modelling of the RING2 and the LDD together with Met1-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, and really highlights the similarities between LUBEL and HOIP (I, Fig. 2B). Furthermore, when transfecting fly S2 cells with the catalytic RBR-LDD domain of LUBEL, Met1-ubiquitin chains were formed, whereas a catalytically inactive C2704A mutation of LUBEL was unable to induce formation of Met1-Ub chains (I, Fig. 2C). Correspondingly, LUBEL LOF flies lacking the catalytic RBR domain ($LUBEL^{\Delta RBR}/LUBEL^{Mi}$) or LUBEL-RNAi flies are unable to catalyse Met1-ubiquitin chains in response to any of the stress conditions tested (I, Fig. 1A and II, Fig. 1C and 6A). Therefore, we conclude that LUBEL is a HOIP homologue and similarly catalyses the formation of Met1-Ub chains in flies.

1.2.2 CYLD hydrolyses Met-Ub chains in *Drosophila*

Mammalian Met1-Ub chains are constitutively catalysed by LUBAC and then hydrolysed by the DUBs OTULIN and CYLD (Komander *et al.*, 2009; Fiil *et al.*, 2013; Keusekotten *et al.*, 2013; Mevissen *et al.*, 2013; Ritorto *et al.*, 2014; Hrdinka *et al.*, 2016). Whereas, no OTULIN homologue has been found in the *Drosophila* genome, we and Asaoka *et al.* have shown that LUBEL-mediated Met1-Ub chains are hydrolysed by CYLD in fly S2 cells (I, Fig. 2D) (Asaoka *et al.*, 2016). Interestingly, CYLD mutant flies show increased Met1-ubiquitin chain formation during basal conditions, indicating that similarly as in mammals, LUBEL forms Met1-Ub chains constitutively (Figure 21). However, no PUB domain responsible for DUB binding is found in LUBEL. The mode of interaction between LUBEL and CYLD is still unknown. *In vitro,* LUBEL is able

to interact with CYLD directly (Asaoka *et al.*, 2016), however it is unclear if this is possible also *in vivo*. *In vivo* the LUBEL-CYLD interaction could, similarly as in mammals, be facilitated by the SPATA2 homologue Tamo. To determine if Tamo is required for LUBEL-CYLD interactions, further experiments need to be performed. Curiously, initial experiments with Tamo LOF flies show increased Met1-Ub chain levels during basal conditions (Aalto, Himmelroos, Meinander, unpublished), thus indicating that Tamo may function as a SPATA2 homologue in flies. Finally, similarly to the mammalian CYLD, *Drosophila* CYLD cleaves both Lys63- and Met1-Ub chains (Komander *et al.*, 2009; Ritorto *et al.*, 2014; Asaoka *et al.*, 2016; Hrdinka *et al.*, 2016). As Met1-Ub chains are known to be attached to Lys63-chains, CYLD may hydrolyse the Lys63-linkages leading to simultaneous release of Met1-Ub chains from target substrate. All in all, the Met1-Ub chain-antagonising activity of CYLD has been described to be particularly important in NF-κB activating signalling complexes (Draber *et al.*, 2015). Likewise, we hypothesised that the LUBEL-CYLD interaction is important for the fly NF-κB signalling.

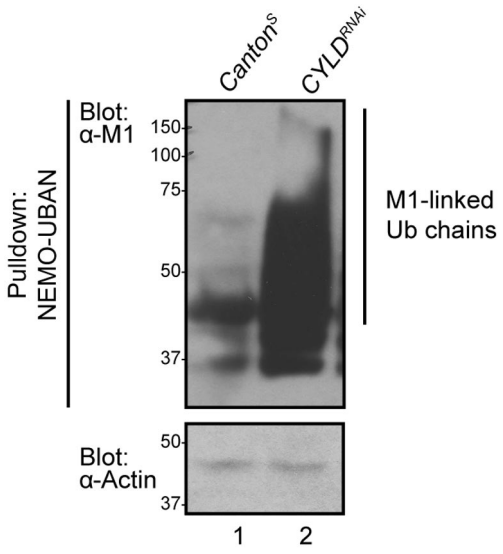

Figure 21. CYLD silencing leads to increased levels of Met1-Ub chains during basal conditions. Met1-Ub chains were isolated at denaturing conditions from fly lysates of adult control *Canton^S* flies and *UAS-CYLD-RNAi;daGal4* flies with recombinant GST-NEMO-UBAN. Met1-Ub chains from samples were analysed by Western blotting with α- Met1 and equal loading was controlled with α-Actin antibody, n=3 (Aalto, Himmelroos, Meinander, unpublished).

1.2.3 The *Drosophila* IKKγ Kenny is a conserved target of Met1-ubiquitination

Met1-Ub chains have been implicated to regulate NF-κB signalling via NEMO both through UBAN-mediated binding and by NEMO ubiquitination (Rahighi *et al.*, 2009; Tokunaga and Iwai, 2009; Gerlach *et al.*, 2011). To test whether the *Drosophila* NEMO homologue Kenny is Met1-ubiquitinated, we pulled down Met1-Ub chains from *Drosophila* S2 cell lysates made under denaturing conditions. Upon activation of the Imd pathway and overexpression of LUBEL RBR-LDD, high-molecular weight smears of ubiquitinated Kenny were detected (I, Fig. 3A-C), confirming that Kenny is similarly as NEMO

ubiquitinated by Met1-Ub chains. As expected, ectopic expression of CYLD completely abolished the overexpression-induced Met1-ubiquitination of Kenny, suggesting that CYLD is able to remove Met1-Ub chains from Kenny (I, Fig. 3C). When we further analysed the ubiquitin chains on Kenny, we discovered that Kenny is modified not only directly with Met1-ubiquitin chains, but in addition, also to Lys63-Ub chains conjugated to Kenny by the Diap2 (I, Fig. 4A-D). This is in line with NEMO being modified by Met1/Lys63-linked mixes of heterotypic chains (Emmerich *et al.*, 2013).

NEMO also binds to Met1-Ub chains through a Met1-specific binding domain called UBAN. Molecular modelling performed on the UBAN domains of NEMO and Kenny verifies that the strong Met1-Ub-binding surfaces of NEMO (Figure 8)(Lo *et al.*, 2009; Gautheron and Courtois, 2010), are structurally conserved in Kenny (I, Fig. 5A). Interestingly, by mutating the ubiquitin-binding surface of Kenny, no Met1-Ub chains were detected (I, Fig. 5B). This indicates that Kenny stabilises Met1-Ub chains and possibly protects ubiquitin chains from CYLD. This, we hypothesise, is mediated by the UBAN domain of Kenny interacting with the Met1-Ub chain, thus masking/protecting the chains from other UBD containing proteins. This interaction is similar to the interaction between overexpressed NEMO-UBAN or OTULIN in mammalian systems (Van Wijk *et al.*, 2012; Keusekotten *et al.*, 2013).

So far, we have only analysed the Kenny/Met1-Ub chain interaction in *Drosophila* S2 cells as transient transfections, and therefore, there could be some artefacts from overexpressing proteins. To confirm these data *in vivo* in flies, few experimental approaches could be tested. A fly line with corresponding Kenny amino acids mutated could be made by utilising the clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9) system. In this way, the stability of Kenny could be analysed *in vivo*. Unfortunately, the detection of the Kenny-Met1-Ub interaction is difficult without proper antibodies, and to date, no commercially available Kenny antibodies exist. However, the stability of Met1-Ub chains could be addressed in the mutant fly lines by performing Met1-Ub pulldown experiments. In addition, by knocking in a Kenny construct with mutated amino acids with a tag, could be used to study the interactions. A drawback with this method is that the tag could possibly alter the folding of Kenny, however, if the tag is a small peptide the possibility for this is lower. Moreover, we have not investigated the consequences of Kenny overexpression in S2 cells or flies. It may have similar adverse effects in NF-κB activation as overexpression of NEMO in the mammalian counterpart, which has been shown to reduce NF-κB activation in mammals (Van Wijk *et al.*, 2012). Taken all together, we have identified the key mediators required for Met1-conjugation in flies (Figure 22). We can additionally conclude that the Met1-ubiquitination machinery is conserved when comparing the E3 ligase and the DUB, and the IKKγ as a Met1-Ub chain reader and target.

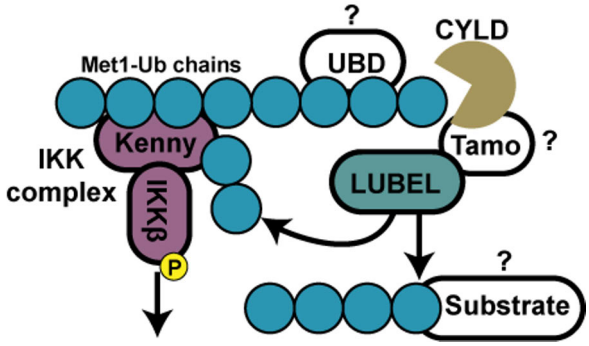

Figure 22. Key regulators and mediators of Met1-ubiquitination in *Drosophila*. LUBEL forms Met-Ub chains on Kenny and other targets. CYLD hydrolyses Met1-Ub chains and Kenny binds to the Met1-Ub chains for effective downstream signalling. The white ovals are unknown/suggested mediators, interactors, and targets of Met1-ubiquitination in flies. UBD, ubiquitin binding domain.

1.2.4 Other targets and regulators of Met1-ubiquitination

With the first two studies of this thesis, we have improved our knowledge and toolbox to study Met1-ubiquitination in flies. These methods can be used to further elucidate other Met1-ubiquitin targets and interactors. In addition to NEMO, mammalian LUBAC activates the canonical NF-κB pathways by Met1-ubiquitination of RIPK1, TRADD, and TNFR1 (see section 2.3). To date, no other Met1-Ub targets have been described in flies. When we investigated Met1-ubiquitination of Imd, a RIPK1 homolog (Georgel *et al.*, 2001), in S2 cells, we could not detect any RBR-LDD-mediated Met1-ubiquitination of Imd (I, Supplementary Fig. 3), indicating that the regulation by Met1-Ub chains varies to some degree between the fly and mammals. Further experiments on determining other possible targets of Met1-ubiquitination in the Imd pathway are currently ongoing.

When considering other possible LUBEL targets and interactors in flies, some exciting possibilities can be found from the mammalian system. For instance, the mammalian HOIP has been shown to be recruited indirectly to ubiquitin-marked molecules by its PUB domain interacting with the PIM of the evolutionarily conserved ubiquitin-binding chaperone Valosin-containing protein (VCP)/p97 (Schaeffer *et al.*, 2014; Takiuchi *et al.*, 2014; van Well *et al.*, 2019). The VCP/p97 homologue in flies is called the transitional endoplasmic reticulum 94 (TER94). However, an interaction between TER94 and LUBEL would most probably require, similarly to CYLD, a PUB-containing protein such as Tamo. Additionally, other PUB-containing proteins can be found with *in silico* approaches. For instance, GDI-interacting protein 3 (Gint3), a protein with a functional role in Wnt signalling, has a PUB domain, and it would be interesting to study if other PUB-containing proteins, such as Gint3, have a role in LUBEL-mediated signalling (flybase.org and uniprot.org).

In addition, HOIP has been shown to be recruited to intracellular bacteria marked by ubiquitin. This interaction is mediated via the NZF on HOIP (Noad *et al.*, 2017). Similarly to HOIP, LUBEL contains several NZF motifs in its N-terminus (I, Fig. 2A)(Asaoka *et al.*, 2016) that could potentially recognise ubiquitinated molecules during stress conditions in the fly. To gain a full understanding of Met1-ubiquitinome/interactome a mass spectrometry could be done on Met1-pulldown samples, which would elucidate the interactome of LUBEL-mediated Met1-ubiquitination in response to all the various stress conditions known to induce Met1-ubiquitination.

1.3 LUBEL is required for survival in response to bacterial infection and sterile insults

As Met1-Ub chain formation is induced upon various stress-conditions and LUBEL ubiquitinates Kenny, a key mediator of *Drosophila* immune response, we wanted to investigate whether LUBEL is required for survival to distinct stresses. For this purpose, we assessed the survival of LUBEL mutant flies to bacterial infection, under low oxygen conditions and oxidative stress. When exposing flies for prolonged periods to these stress conditions, we observed LUBEL-mediated Met1-Ub chains to be essential for resistance to the sterile stress conditions, hypoxia (II, Fig. 1F) and oxidative stress (II, Fig. 6B). Unexpectedly, we did not detect any significant differences in survival of LUBEL mutant flies in comparison to WT flies in response to septic infection (I, Fig. 6A). However, when we infected the flies orally, by feeding them with Gram-negative bacteria *Ecc15*, most of the LUBEL mutant flies succumbed, whereas most of WT flies survived (I, Fig. 6C). Similarly, LUBEL mutant flies were resistant to septic infection with the Gram-positive bacteria *Micrococcus luteus* (I, Supplementary Fig. 4B), but not when the flies were fed with Gram-positive bacteria (Sundén, Meinander, unpublished). This indicates that the biological relevance of Met1-Ub chains varies between stresses and the organ detecting the stress.

1.4 LUBEL regulates intestinal immune responses

1.4.1 LUBEL is required for maintaining intestinal homeostasis and local epithelial AMP expression in response to bacterial infection

To investigate if the susceptibility to bacterial infection and cell stress is reflected in the ability of LUBEL mutant flies to mount an immune response, we analysed NF-κB target gene expression in response to bacterial infection. In response to septic infection, only a slight, but not significant reduction in expression of AMP genes was detected in LUBEL mutant flies (I, Fig. 6B). This was unexpected, as Met1-Ub chains are essential for NF-κB activation in mammalian NF-κB signalling. On the contrary and corresponding to the survival experiments, a significant reduction in AMP expression could be

detected after oral *Ecc15* infection in the LUBEL mutant flies, correlating with their sensitivity to infection (I, Fig. 6D and II, Fig. 3A). Our results show that although Met1-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 (Charroux and Royet, 2010). Instead, Met1-Ub chains are required for mounting a local inflammatory expression and release of AMPs from the intestinal epithelial cells in response to pathogen feeding (Charroux and Royet, 2010).

To better understand the difference between survival to septic versus oral bacterial infection, we analysed the infection-induced expression of Imd pathway-specific AMPs in LUBEL mutant flies specifically in the intestine of the flies after oral infection. For this purpose, we utilised a *Diptericin-lacZ* reporter fly lines. By X-Gal staining dissected guts from control and infected flies, we observed that LUBEL-mutant flies were unable to mount an immune response in the intestinal epithelial tissue (I, Fig. 6E). Furthermore, intestinal inflammation is associated with midgut hyperplasia in *Drosophila* (Amcheslavsky, Jiang and Ip, 2009) and can be detected by staining the proliferation marker pH3. After counting pH3-positive cells in the midguts in control flies and in flies fed with *Ecc15*, we detected an increase in cell proliferation upon oral infection in wild type flies, whereas no such increase could be detected in LUBEL mutant flies (I, Fig. 6F). Correspondingly, LUBEL mutant flies were unable to clear ingested food-borne pathogens (I, Fig. 6G), supporting the notion that LUBEL-mediated immune responses are essential for intestinal immune responses.

Taken together, the abovementioned results indicate that LUBEL-mediated regulation of the immune response differs between organs responding to these stresses. A possible explanation may be that the intestine requires an additional level of regulation, as it is in constant interaction with the commensal microbiome. This additional regulatory level might be provided by LUBEL-mediated Met1-Ub chains. For instance, upon intestinal pathogen detection, the epithelial NF-κB signalling cascade is activated and the Met1-Ub chains are required for a more stable and efficient complex recruitment, thus leading to enhanced NF-κB activation in response to oral infection. On the contrary, in the fat body, the bacterial insults are more straightforward, as the haemolymph is normally a sterile environment. In the fat body, a robust septic infection activates the PRR, which induces a signalling cascade where Lys63-Ub chains induced by Diap2 are essential and sufficient for activation of both Dredd and Relish (Zhou *et al.*, 2005; Paquette *et al.*, 2010; Meinander *et al.*, 2012), even in the absence of Met1-Ub chains.

1.4.2 Transgenic RBR-LDD expression drives intestinal NF-κB activation

To investigate the role of LUBEL *in vivo* and the possible outcome of LUBEL overexpression, we generated transgenic flies to express wild type and catalytically inactive RBR-LDD under the control of the UAS-Gal4 system. Interestingly, when LUBEL activation is boosted by transgenic expression of wild type RBR-LDD, we can observe an induced activation of Relish target genes also in the absence of an infection in whole flies (I, Fig. 7B). When we specifically examined the expression of *diptericin* in the midgut of control and RBR-LDD-expressing flies using the *Diptericin-LacZ* reporter, we observed an enhanced *diptericin* expression by wild type RBR-LDD (I, Fig. 7C). Importantly, also the amount of pH3-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 (I, Fig. 7D, E). This suggests that constitutive LUBEL activity drives Relish-mediated chronic intestinal inflammation in flies and confirms that LUBEL activity needs to be properly regulated in order to maintain intestinal homeostasis. In study I, we have only overexpressed the RBR-LDD domain, however, it would be interesting to further investigate if the catalytic RBR-LDD-WT can catalyse chains and activate the immune response in a LUBEL knock-out background. These experiments would give an idea if the fly LUBEL requires additional domains for proper catalytic activity. Nevertheless, the RBR-LDD overexpression flies can be used as a genetically induced intestinal inflammation model, to further study ubiquitin-mediated dysregulation in the fly gut and possibly also in other tissues.

1.4.3 Loss of LUBEL leads to chronic inflammation and dysbiosis

While analysing our results on NF-κB-mediated AMP expression in whole adult flies, we observed that loss of LUBEL induces expression of AMP genes already in the absence of infection, indicating that loss of LUBEL activity induces chronic inflammation in flies. This induction is low compared to bacterial infection, but it is significant (I, Fig. 6B and Figure 23A). As LUBEL-mediated Met1-ubiquitination is required for mounting an immune response upon oral infection, but not upon septic injury, we further investigated whether lack of LUBEL activity interferes with the intestinal homeostasis. By investigating the microbiome in LUBEL mutant flies by 16S sequencing, LUBEL mutant flies were found to have altered microbiome compared to wild type flies. In LUBEL mutant flies, the proportion of *Firmicutes* to *Proteobacteria* was decreased when compared to wild type flies (Kietz, Meinander, unpublished). This shift in the bacterial ratio is associated with increased gut inflammation and is proposed as a marker for microbial instability (Matsuoka and Kanai, 2015; Shin, Whon and Bae, 2015). To further investigate if the chronic AMP expression is due to microbial dysbiosis, we reared larvae in axenic conditions and measured the AMP expression.

Interestingly, the induced AMP expression observed in the presence of commensal bacteria, is rescued by rearing larvae under axenic conditions (Figure 23B). This supports the notion that the induced AMP expression is caused by the commensal bacteria in the gut. However, when analysing the basal intestinal AMP expression, we observed that dissected guts of LUBEL mutant larvae had comparable AMP expression with guts dissected from wild type larvae. Therefore, the previously observed chronic inflammation in Figure 23A and 23B, does not originate from the gut. Instead, the increased AMP expression may originate from the immune cells in the fat body or the circling haemocytes (Figure 23C). This is supported by the X-Gal-stained guts in study I, Fig 6E, where no induction of *diptericin* could be observed in the LUBEL mutant flies compared to wild type flies during basal conditions. This indicates that the microbial dysbiosis in LUBEL mutant flies somehow causes a systemic increase in the basal AMP expression. Interestingly, when dissecting guts, the LUBEL mutant fly guts seemed more fragile than the wild type guts, which would support the notion that the epithelial barrier in LUBEL mutant flies is disrupted leading to invasion of commensal bacteria to the otherwise sterile haemolymph. This is in line with previous studies showing that peptidoglycan fragments can also cross the intestinal epithelial barrier and remotely induce the production of antimicrobial peptides by the fat body (Neyen *et al.*, 2012; Charroux *et al.*, 2018). To confirm if the epithelial barrier integrity in LUBEL mutant flies is compromised, further experiments need to be performed. Taken together, we suggest that LUBEL is required for protection of the intestine from microbial dysbiosis and chronic inflammation, both during pathogen infection and basal conditions.

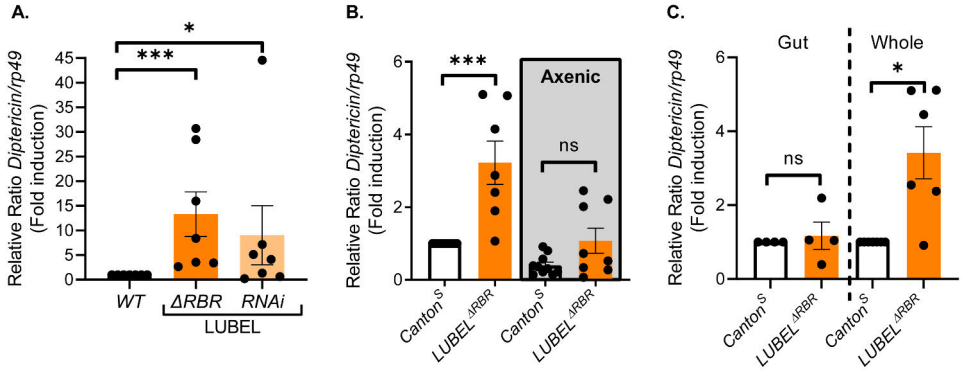

Figure 23. Basal AMP expression. A. Relish activation in adult wild type *Canton*^{S} and LUBEL mutant flies, *LUBEL*$^{\Delta RBR}$ and *LUBEL*-RNAi was studied by analysing the expression of *Diptericin* with qPCR. B. Relish activation in control or germ-free wild type *Canton*^{S} and *LUBEL*$^{\Delta RBR}$ larvae was studied by analysing the expression of *Diptericin* with qPCR. C. Relish activation in dissected and whole wild type *Canton*^{S} and *LUBEL*$^{\Delta RBR}$ larvae was studied by analysing the expression of *Diptericin* with qPCR. Error bars indicate SEM from more than 4 independent experimental repeats using at least 5 larvae per repeat, ns stands for nonsignificant, $p > 0.05$, * $p < 0.05$, *** $p < 0.001$.

1.5 LUBEL is required for mounting a NF-κB-mediated immune response upon sterile inflammation

NF-κB can also be induced in the absence of pathogens. In flies, it has been previously shown that the NF-κB expression is induced also in response to sterile stress, such as hypoxia (Liu, Roy and Johnson, 2006; Bandarra *et al.*, 2014). To investigate if LUBEL is required for mounting an immune response when flies are exposed to hypoxia, we analysed the NF-κB target gene expression. As expected, expression of the NF-κB target gene *diptericin* was induced during hypoxia in control flies, whereas ubiquitous RNAi silencing of LUBEL prevented the AMP induction (II, Fig. 3A). Interestingly, only the expression of the Imd/NF-κB Relish pathway-specific gene, *diptericin* (Boutros, Agaisse and Perrimon, 2002; Lemaitre and Hoffmann, 2007; Buchon *et al.*, 2009), was affected in LUBEL-silenced flies (II, Fig. 3A). In contrast, the expression of *drosomycin*, an AMP specific for the Toll pathway (Boutros, Agaisse and Perrimon, 2002; Lemaitre and Hoffmann, 2007; Buchon *et al.*, 2009), was similar in control and LUBEL-RNAi flies (II, Fig. 3B). These results indicate that LUBEL is exclusively required for Relish activation in response to hypoxia. The Relish-induced *diptericin* expression is shown to depend on Dredd in response to infection (Silverman *et al.*, 2000). To investigate if the LUBEL-mediated Relish activation during hypoxia is mediated via Dredd, we introduced transgenic expression of Dredd in LUBEL-RNAi flies (II, Fig. 3C and D). Dredd overexpression, as expected, rescued both the inability to induce *diptericin* expression (II, Fig. 3E) and the sensitivity to hypoxia (II, Fig. 3F) of LUBEL mutant flies. Even though we did not measure the NF-κB target gene expression in response to oxidative stress by qPCR, we could, similarly to hypoxia, observe an improved sensitivity to paraquat feeding when Dredd was ectopically expressed in the LUBEL mutant flies. Overall, these experiments indicate that LUBEL indeed regulates hypoxia- and oxidative stress induced Relish activation upstream of Dredd. In addition, in these experiments we have tested a newly developed portable hypoxia system to induce hypoxia in flies.

1.5.1 LUBEL is required for both local and systemic AMP expression in response to sterile hypoxia

To further analyse the origin of the immune response to hypoxia, we dissected *Drosophila* larvae after hypoxia treatment. To study the local hypoxia-induced NF-κB activation in the trachea, the *Drosophila* organ for oxygen uptake and distribution, we dissected 3rd instar larvae carrying either a *Drosomycin-LacZ* or *Diptericin-LacZ* reporter. On the contrary to the intestine and the fat body, it has been shown that *drosomycin*, but not *diptericin* is expressed upon activation of Relish via the Imd pathway in the trachea (Ferrandon *et al.*, 1998; Tzou *et al.*, 2000; Akhouayri *et al.*, 2011). Indeed, *drosomycin* was induced in the trachea of control *Drosomycin-LacZ* larvae, but not in *LUBEL$^{\Delta RBR}$* mutant *Drosomycin-LacZ* larvae during hypoxia (II, Fig. 5A). These

data indicate that LUBEL is required for activation of Relish in the trachea during hypoxia, which is similar to the epithelial immune response in the fly gut in response to bacterial infection (I, Fig. 6E-G). To analyse whether LUBEL is required also for systemic fat body-mediated NF-κB responses, we silenced LUBEL in the fat body by using a fat body expressing driver c564Gal4 (II, Fig. 5C). While *diptericin*, a specific Relish target gene in the fat body (Hedengren *et al.*, 1999; Tzou *et al.*, 2000), was induced in control flies during hypoxia, no hypoxia-induced *diptericin* expression could be detected when silencing LUBEL using c564Gal4 driver (II, Fig. 5D). On the other hand, *drosomycin* expression, which on the contrary to the trachea is mainly induced via the Toll pathway in the fat body, was not affected by loss of LUBEL in the fat body (II, Fig. 5E). These results suggest that LUBEL is specifically required for activation of Relish both in the trachea and the fat body during hypoxia. Curiously, this deviates from pathogenic infection, where LUBEL is required only for intestinal response. Altogether, the studies I and II indicate that the Imd pathway is activated in different ways in the fat body depending on the incoming noxious stimulus. In the case of hypoxia, the immune response activated is mediated solely through the Imd pathway. However, another example of differently regulated fat body response has been reported in response to mechanical stress. In mechanical stress, the Toll pathway and *Drosomycin* expression is activated in the fat body during sterile inflammation induced by mechanical pinching of *Drosophila* larvae (Kenmoku *et al.*, 2017). Therefore, when further elucidating the role of Met1-ubiquitination the immune responses in flies, both Imd and Toll pathway mediators and activators should be considered as possible regulators of Met-ubiquitination.

1.5.2 Intracellular, but not extracellular mediators are required for Relish activation in response to sterile noxious stimuli

As the NF-κB is activated in response to hypoxia, we investigated further how the Imd pathway is engaged during hypoxia. To assess this, we first examined whether the transmembrane pattern-recognition receptor PGRP-LCx is required for hypoxia-induced Relish activation. However, flies with LOF of PGRP-LCx were not sensitive to low oxygen levels (II, Fig. 4A). On the contrary, the intracellular, key Imd pathway mediators Tak1, Dredd, Diap2 and Kenny were required for flies to survive hypoxic conditions (II, Fig. 4B). Furthermore, PGRP-LCx, but not Tak1, Dredd, Diap2 or Kenny mutant flies were able to induce *diptericin* expression in response to hypoxia (II, Fig. 4C). Similarly, LOF mutants of the receptor PGRP-LCx did not affect the sensitivity to paraquat feeding (II, Fig. 6D). Taken together, this indicates that the Imd pathway is engaged downstream of the PGRP-LCx receptor during hypoxia and oxidative stress. Naturally, this raises the question how the NF-κB pathway is engaged in sterile inflammation and what activates LUBEL-mediated Met1-ubiquitination.

While PAMPs, DAMPs and SAMPs may be patterns of extracellular origin leading to receptor activation, the Imd pathway can, in addition, be activated by other means. The NF-κB activation in response to hypoxia has been suggested to be mediated by Tak1. Tak1 is activated by the Ca^{2+}/calcium/calmodulin-dependent kinases 2 (CaMKII) pathway in response to hypoxia and oxidative stress (Ermak and Davies, 2002; Culver *et al.*, 2010). Similarly, mammalian XIAP has been shown to be essential for Tak1-mediated NF-κB activation in response to hypoxia (Culver *et al.*, 2010). To investigate if these conserved key mediators are upstream of LUBEL and could be possible regulators of LUBEL-engagement in response to sterile stress, we analysed the Met1-Ub chains in Tak1 and Diap2 LOF mutant flies. In response to hypoxia, LUBEL-mediated ubiquitination was lost in the Tak1 (II, Fig. 4E) and Diap2 (II, Fig. 4F) mutant flies. This suggests that LUBEL is engaged in a Tak1 and Diap2-dependent manner during hypoxia. In addition to Tak1 activation by changes in intracellular calcium levels, Tak1 is also activated by the *Drosophila* TNF receptor Wengen (Geuking *et al.*, 2009; Palmerini *et al.*, 2021), and by the intracellular PRR PGRP-LE (Takehana *et al.*, 2004; Kaneko *et al.*, 2006; Bosco-Drayon *et al.*, 2012). Therefore, it would be interesting to analyse if these pathways and receptors contribute to LUBEL activation and Met1-ubiquitination during sterile cell stress.

Even though Dredd and Relish activation seem to be downstream from LUBEL in response to sterile stress (II, Fig. 4E, 4F and 6C), it would be interesting to properly evaluate the signalling events leading both to LUBEL activation and downstream Met1-ubiquitination of signalling targets. This could be done with epistatic analyses and Met1-TUBE pulldowns. Curiously, LUBEL-mediated Met1-Ub chains are required for survival and mounting an NF-κB immune response from the fat body to sterile insults, whereas Met1-Ub chains are not necessary for efficient NF-κB activation from the fat body in response to septic infection. The difference in LUBEL-dependency in the fat body in response to septic or sterile inflammation is enigmatic. The reason for this may be the degree of severity and occurrence of incoming insults. A sterile insult activating the Imd pathway in a PRR-independent manner, might require a more efficient signalling hub for NF-κB to be activated and this efficiency may be provided by Met1-Ub chains.

1.6 Met1-Ub chains are formed in response to stress in human intestinal epithelial cells and protect from stress-induced cell death

All the sterile stress conditions, applied in our studies in *Drosophila*, activate evolutionarily conserved stress-mediated inflammatory responses. Therefore, we also investigated if the stress-induced Met1-Ub chain formation is a conserved response present in mammals. When we exposed human intestinal Caco2 cells to the same stress conditions used in flies, all

these stress conditions led to increased Met1-Ub chain levels (II, Fig. 7A-C). Importantly, the commercial HOIP inhibitor 1 (HOIPIN1) (Oikawa, Sato, Ohtake, *et al.*, 2020) reduced Met1-Ub chain levels during hypoxia and oxidative stress (II, Fig. 7A and B). We also investigated changes in NF-κB activation during hypoxia and oxidative stress, however, we were unable to detect any significant changes. Instead, we were able to measure changes in apoptotic effector caspase-3 activity. While both hypoxia and oxidative stress lead to activation of caspase-3, HOIPIN1-treatment further induced caspase activation (II, Fig. 7D and E), indicating that Met1-Ub chains are required for cell survival. Taken together, our findings indicate that formation of Met1-Ub chains is an evolutionarily conserved stress response, important to protect the organism and its cells from stress-induced damage. As all the examined sterile stress conditions are present in the tumour environment and tumour cells profit from cell death evasion, it would be of great interest to study the role of Met1-ubiquitination in tumour progression.

2 *Drosophila* as a model organism for studying intestinal inflammation and anti-inflammatory compounds (III)

A well-functioning intestinal immune system monitors and maintains intestinal homeostasis. When this homeostasis is disturbed, as in IBD patients, the activation of the transcription factor NF-κB is markedly induced, which further promotes intestinal inflammation (Schreiber, Nikolaus and Hampe, 1998; Atreya, Atreya and Neurath, 2008). As the IBDs are idiopathic diseases, we need good representative model systems to study the disease progression and possible drug candidates. For this purpose, the fly gut is structurally and functionally highly similar to the mammalian gut and is therefore a good model system for studying intestinal inflammation (Apidianakis and Rahme, 2011). To study the molecular mechanisms of intestinal inflammation, and to find drugs that alleviate the disease, model systems resembling chronic disease are needed. A chronic intestinal inflammation can be induced by genetic approaches or chemically. One example for inducing intestinal inflammation chemically in murine models is by administering dextran sulphate sodium (DSS) in the drinking water. This induces excessive intestinal inflammation in the model, which mimics ulcerative colitis (UC) in humans. For study III, we have optimised a protocol to chemically induce intestinal inflammation with DSS in flies and characterised the NF-κB activation in this inflammation model. Thereafter, we have attempted to relieve the inflammatory response by treating flies with stilbenoids.

2.1 DSS-induced intestinal inflammation model

DSS has been used widely in murine models to induce UC-like inflammation (Perše and Cerar, 2012; Kiesler, Fuss and Strober, 2015; Eichele and Kharbanda, 2017). DSS is a sulphated polysaccharide, known to directly disrupt the epithelial barrier in experimental models (Okayasu *et al.*, 1990; Eichele and Kharbanda, 2017). In *Drosophila*, oral DSS treatment has been used to induce inflammation and has been reported to cause epithelial tissue damage, leading to intestinal stem cell proliferation and increased ROS levels (Amcheslavsky, Jiang and Ip, 2009; Wu *et al.*, 2012). However, the intestinal induction of *Drosophila* NF-κB responses via Relish has not been addressed to date. To study the inflammatory response to DSS feeding, we optimised a feeding protocol of DSS mixed in fly food. The larvae were chosen for their constant eating behaviour. The inflammatory response was measured by detecting expression of Relish-specific AMP target genes. We observed that a treatment with 5 % of DSS for three hours was sufficient to induce all the Relish target genes tested (III, Fig. 1A). We continued to confirm that the DSS-induced expression of Relish target genes is mediated by the Imd pathway. For this purpose, we used LOF mutants of Relish. In these larvae the

inducibility of *diptericin* expression was impaired in response to DSS-feeding (III, Fig. 1B).

As the fly immune response can be activated both as a local immune response in the gut, as well as a systemic immune response from the fat body (Figure 18), we wanted to use a model where responses from the intestine could be separated from systemic responses. For this purpose, we performed X-Gal staining on the dissected gut and fat body of *Diptericin-lacZ* reporter larvae fed with DSS. Interestingly, our short three-hour DSS-treatment induced *diptericin* expression in the larval gut, but not in the fat body (III, Fig. 2A). This indicates that the short timepoint we used only induced an acute intestinal inflammation, whereas a longer DSS feeding has been shown to activate NF-κB target gene expression in the fat body via organ-to-organ immunological communication (Wu *et al.*, 2012).

As DSS-treatment is known to alter the bacterial composition in mice (Okayasu *et al.*, 1990; Munyaka *et al.*, 2016), we wanted to study whether this is the case also in *Drosophila.* With the help of 16S sequencing, we found the bacterial composition to be different in DSS-treated larvae compared to control larvae. The DSS treatment led to a decrease of the proportion of *Firmicutes* to *Proteobacteria* (III, Fig. 2B), a notion associated with IBD, and a proposed marker for microbial instability (Matsuoka and Kanai, 2015; Shin, Whon and Bae, 2015). Whereas a DSS-induced bacterial dysbiosis was expected, we were taken aback by the swiftness of the change in the bacterial composition. This really awakens the curiosity to what could be the cause for this abrupt change in bacterial composition. DDS feeding increases ROS levels and changes the epithelial landscape of the intestine, which in the case of some of the commensal bacterial strains may be detrimental for survival, leading to reduction of these strains in comparison to the unaffected strains. IBDs are also characterised by diarrhoea as the mucosal damage caused by the prolonged intestinal inflammation causes dysregulated intestinal ion transport and impaired epithelial barrier function (Anbazhagan *et al.*, 2018). This, on the other hand, may lead to changes in bacterial composition if some of the bacterial strains are flushed out due to DSS-induced impairment of epithelial barrier functions. DSS-induced inflammation has been shown to be reduced in germ-free model organisms, indicating that the presence of luminal bacteria exacerbates the inflammation together with the DSS-mediated epithelial wall disruption (Hernández-Chirlaque *et al.*, 2016). Expectedly, flies reared under axenic conditions, did not induce Relish activation in germ-free flies compared to their conventionally reared counterparts in response to DSS-treatment (III, Fig. 2C). Finally, to assess if the NF-κB-mediated immune response is activated through conventional pattern-recognising receptors, flies lacking the pattern-recognising receptor PGRP-LCx were treated with DSS. In these flies, the inducibility of *diptericin* is impaired (III, Fig. 2D), indicating that DSS-induced *diptericin* expression is not

driven by disruption of the epithelial barrier. Rather, the immune response is mediated by receptor activation in response to a dysbiotic microbiome.

Taken together, we have optimised a method to study NF-κB-mediated activation upon DSS-induced intestinal inflammation. DSS-treatment induces activation of Relish in flies and can be used to induce inflammation in *Drosophila* by feeding. As the excessive inflammation induced by DSS can be compared to UC in patients, the fly can be used to study the general mechanism and treatment of intestinal inflammation in these patients.

2.2 Modulating intestinal inflammation with stilbenoid compounds

The ligand gated, transmembrane bound transient receptor potential ankyrin 1 (TrpA1) receptor is a sensory protein that can be activated by environmental stimuli such as noxious cold and mechanical stimuli, as well as by endogenous irritant and pungent compounds. Moreover, TrpA1 serves as an attractive target for analgesic and anti-inflammatory drugs, as it is triggered during inflammation, oxidative stress and tissue damage and is considered a key player in acute and chronic pain sensation (Koivisto *et al.*, 2018; Souza Monteiro de Araujo *et al.*, 2020). One strategy for targeting TrpA1 is the use of stilbenoid compounds that have been shown to function as TrpA1 antagonists. Stilbenoids are hydroxylated derivatives of polyphenolic compounds characterised by a 1,2-diphenylethylnucleus and are present in berries, fruits and grape vine, but also in roots and stumps of conifer trees, such as spruce and pine (Rivière, Pawlus and Mérillon, 2012; Routa *et al.*, 2017; Akinwumi, Bordun and Anderson, 2018). Stilbenoids exhibit antioxidant properties and some stilbenoid compounds, such as resveratrol (3,4′,5-trihydroxystilbene), pinosylvin (3,5-dihydroxystilbene, PS) and pinosylvin monomethyl ether (3-hydroxy-5-methoxystilbene, PSMME) have been described to have anti-inflammatory properties in animal models *in vivo* (Fauconneau *et al.*, 1997; Chong, Poutaraud and Hugueney, 2009; Quideau *et al.*, 2011; Laavola *et al.*, 2015; Moilanen *et al.*, 2016). Therefore, by taking advantage of our newly optimised DSS-induced intestinal inflammation model, we were able to assess the anti-inflammatory properties of stilbenoid compounds *in vivo* in flies. The tested stilbenoid compounds were PS and PSMME, and the stilbenoid glucosides isorhapontin (4,5'-dihydroxy-3-methoxy-3'-glucopyranosylstilbene) and astringin (3,4,3',5'-tetra-hydroxystilbene 3'-glucoside). Both PS and PSMME have been previously shown to have anti-inflammatory properties *in vivo* and are known to inhibit Ca^{2+}- influx through the TrpA1 ion channel in response to TrpA1 activators in mammalian cells (Yu *et al.*, 2013; Laavola *et al.*, 2015; Poulsen *et al.*, 2015; Moilanen *et al.*, 2016). The glucosides isorhapontin and astringin have never been studied *in vivo* before. To assess the anti-inflammatory effect of the stilbenoids *in vivo* in *Drosophila*, larvae were first fed with DSS, to induce inflammation, and then treated by feeding with stilbenoids. While DSS-fed

flies still expressed *diptericin* post DSS-treatment, PS and PSMME-treated larvae were able to alleviate the DSS-induced inflammation (III, Fig. 3F, G). Interestingly, isorhapontin only had a mild effect on *diptericin* expression, whereas astringin seemed to have an opposite effect (III, Fig. 3H, I). The reason for the mild and adverse effect of isorhapontin and astringin, respectively, could be due to the highly hydrophobic glucose group, which keeps them from passing through the membrane. Therefore, both isorhapontin and astringin would most likely need to be metabolised in order to pass the membrane and to exert any proper anti-inflammatory effects.

To determine if the anti-inflammatory effect of PS and PSMME is mediated through the *Drosophila* TrpA1, we utilised both *in silico* and *in vivo* approaches. With the *in silico* approach, the *Drosophila* TrpA1 structure was predicted based on human TrpA1. Thereafter, two TrpA1 antagonists, A-967079 and HC-030031, with known binding sites on TrpA1 were used as reference compounds, when comparing PS and PSMME interactions with the fly TrpA1. The tested stilbenoid compounds interacted with both the A-967079 and HC-030031 binding sites, with a slightly better preference to A-967079 site (III, Fig. 4A). This indicates that PS and PSMME are both able to bind *Drosophila* TrpA1. Finally, to assess if the anti-inflammatory effect of PS and PSMME are indeed mediated via the TrpA1 channel *in vivo*, we investigated the ability of the stilbenoids to alleviate inflammation in TrpA1 LOF larvae. When we treated the DSS-fed TrpA1 mutant flies with stilbenoid compounds, PS- and PSMME-fed larvae lost their anti-inflammatory properties observed in control flies (III, Fig. 5B and C), indicating that PS and PSMME function in a TrpA1-dependent manner.

2.3 Advantages, limitations and future prospects of testing stilbenoids in *Drosophila*

As we have shown, feeding flies with DSS leads to intestinal inflammation and microbial dysbiosis and can be used as a model for studying inflammatory diseases, such as IBDs. However, there are some limitations with the experiments conducted for study III. Our experiments were all performed in the larvae because they are easier to dissect and are sure to eat. However, this generates a time-constraint on the experimental setup. Whereas the larvae are excellent for oral feeding experiments, the larvae undergo metamorphosis to a pupa after only a few days. Because of this we could only perform short experiments. Luckily, three hours is enough to induce an acute intestinal inflammation, however, due to the time constraint caused by using larvae, the experimental setup was limited to only testing acute intestinal inflammation and to treat the inflammation with stilbenoids. To induce chronic inflammation, a longer and possibly milder DSS concentration would be more beneficial. Naturally, for this purpose, the larvae are not suitable, and adult flies should be used instead. Also, as mentioned above, we only used the

stilbenoids to treat DSS-induced acute intestinal inflammation. It would be interesting to test whether stilbenoids could be used also in preventing inflammation in response to DSS-induced inflammation. For this purpose, the flies could be fed with stilbenoids prior to DSS-treatment, to measure their protective properties against DSS-induced inflammation.

The four tested compounds are only a small set of known stilbenoids. Extensive studies have already been made with stilbenoids, such as resveratrol, and the therapeutic interest in other TrpA1 inhibitors/activators is vast. Whereas PS and PSMME have been used previously *in vivo* to treat inflammation, isorhapontin and astringin have only been tested *in vitro*. Both isorhapontin and astringin had only mild or no anti-inflammatory effects, respectively, on flies. Isorhapontin and astringin are both hydrophilic glucosides that most likely cannot diffuse through the lipophilic cell membrane to the lipid-surrounded binding site at TrpA1, and would hence need to be metabolised in order to function properly (Henry-Vitrac *et al.*, 2006). The reason for different immunological responses to isorhapontin and astringin may be due to the metabolic properties of the compounds. Also, we do not know how the stilbenoids are metabolised in flies and do not know if these compounds accumulate in flies. If the compound accumulates, we might get an overdose in some tissues, leading to similar adverse proinflammatory effects that we could observe with the stronger concentration of PS in flies (III, Fig. 3A). With longer experiments and survival experiments, these adverse effects should be studied before the tested stilbenoids can be considered as potential anti-inflammatory drugs.

Interestingly, some of the stilbenoid compounds were able to alleviate basal NF-κB activation (III, Fig. 3A-C). This may be due to the stilbenoid compounds themselves exhibiting antimicrobial effects (Välimaa *et al.*, 2007; Plumed-Ferrer *et al.*, 2013). As the microbial dysbiosis caused by DSS seems to be the main reason for Relish activation, the antimicrobial activities of stilbenoids may alleviate the bacterial burden on the intestinal epithelia, which also may contribute to the anti-inflammatory effect for PS and PSMME.

Finally, a limitation in this study is the lack of a mechanism on how treatment with stilbenoids known to interact with TrpA1 leads to reduced NF-κB target gene expression. To answer this question, a more extensive study on the crosstalk between the TrpA1-mediated signalling and NF-κB signalling could be conducted. However, our results clearly show a crosstalk between the two pathways and the already done *in vivo* studies are encouraging in manipulating intestinal inflammation with stilbenoid compounds.

CONCLUDING REMARKS

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. For this purpose, we need to properly understand the underlying mechanisms leading to uncontrolled immune responses. In my PhD thesis work, I have taken advantage of the highly efficient innate immune response of the fruit fly, *Drosophila melanogaster*, to further our understanding of the ubiquitin-mediated regulation of the NF-κB-mediated immune signalling and exploited fruit fly as a biological model to study anti-inflammatory compounds.

Non-degradative ubiquitination is a key regulatory mechanism of NF-κB activation both in flies and mammals. In the first two studies, we have studied the contribution of Met1-Ub chains in the *Drosophila* NF-κB signalling, which adds another layer of complexity to the already established role of Lys63-ubiquitination in the Imd pathway. We have shown that Met1-Ub chains are augmented in response to pathological conditions and Met1-ubiquitination is required for proper cellular stress resistance. We have discovered tissue specific regulation of NF-κB in response to bacterial infection and hypoxia. Finally, the Met1-ubiquitin machinery and some of the targets for Lys63- and Met1-ubiquitination seem to be conserved through evolution. However, even though Met1-ubiquitination has been extensively studied in PRR-induced immune signalling, more focus needs to be addressed into how Met1-ubiquitination is activated in PRR-independent activation during sterile inflammation. The work done in the second study is only the beginning of understanding the role of Met1-Ub chains in response to sterile inflammation. Further work is required to understand the mechanism leading to Met1-Ub chain formation and what the signalling outcome is. Especially the regulation of Met1-ubiquitination in response to various sterile noxious stresses should be elucidated further. For this purpose, the Met1-ubiquitinome, as well as the effects of Met1-ubiquitination on NF-κB-mediated transcription would be beneficial to explore. As sterile inflammation is prevalent in chronic inflammation and tumour environments, the need to unravel the details of ubiquitin-mediated induction of sterile inflammation is really heightened. In addition, this critical knowledge of ubiquitin-regulation in inflammation may open possibilities for discovery of new regulators, drug targets and diagnostic markers and ultimately may help us understand diseases involving innate immunity, such as cancer, immunodeficiency and inflammation.

Furthermore, we have optimised a model to study chemically induced intestinal inflammation and used it to examine the anti-inflammatory properties of stilbenoid compounds. The third study proves the convenience of using the fruit fly as a biological incubator for chemical interactions. It provides a new tool for studying intestinal inflammation in flies and means to alleviate the overly activated immune response.

Taken together, this thesis provides further evidence that *Drosophila* is a convenient organism to study the general principles of NF-κB-mediated inflammatory signalling. Moreover, the thesis expands our knowledge in how *Drosophila* can be used for screening potential new regulators of inflammation and for assessing novel anti-inflammatory compounds *in vivo*.

ACKNOWLEDGEMENTS

The thesis work was carried out at the Faculty of Science and Engineering, at the department of Cell Biology, at Åbo Akademi University (ÅAU) and was supported by the Turku Doctoral Network in Molecular Biosciences (MolBio). I want to thank John Eriksson, Cecilia Sahlgren, and Annika Meinander for their work as heads of the Cell Biology department. I would also like to thank all the group leaders, specifically John Eriksson, Lea Sistonen, Cecilia Sahlgren, Diana Toivola, Kid Törnquist, Malin Åkerfelt and Jacquemet Guillaume and all their past and present group members. The essence of science oozes in our corridors and is honed in all of these amazing lab groups and I am happy I have been able to bask in all of the scientific greatness throughout these years. I would like to thank the technical staff at ÅAU and Turku Bioscience for their assistance during my time as a doctoral candidate. I would especially like to mention Gunilla Henriksson, Sten Lindholm, Anne-Leena Gröning, Barbro Lindholm, Melissa Haga, Thomas Bymark and Kim Granholm for their help with ordering, travels and technical maintenance throughout the years. Thank you, Markku Saari, Jari Korhonen and Pasi Kankanpää for assisting me in all my image-acquisition questions. Thank you, Fredrik Karlsson, for helping with the official details regarding my PhD studies and the defence.

Foremost, I would like to express my deepest gratitude to Annika for giving me the opportunity to do my PhD in her laboratory. Thank you for choosing me to be your first solo PhD student. To be part of the group since the beginning, has been a very valuable lesson for me and I truly cherish all the scientific and non-scientific talks we have had during all these years. Your open-door policy is something I have admired throughout this process, and you have always found time for us. In your care, I have grown into a fine scientist, and I can proudly walk with my head up high thanks to your encouragements and pushing me to do my best. That being said, let's not share a bed again. Once is enough!

I wish to thank Susanna Valanne and Nieves Peltzer for taking the time to review my thesis and give constructive comments that significantly improved my work. I would also like to thank Mads Gyrd-Hansen for accepting the invitation to be my opponent. We have discussed my thesis work before in meetings and during your last visit to Turku, and I truly appreciate the chance to further discuss my research and thesis with you.

Jeroen Pouwels and Ville Hietakangas are acknowledged for being part of my thesis advisory committee. Your input in the beginning of my PhD career has been inspiring and your expertise and scientific background have truly helped me in improving my work.

Apart from the science, the collaborations are what makes science as fun as it is. I have been lucky to have been given the opportunity to work with amazing scientists throughout the years. Thank you for the work and joint efforts of all co-authors that have contributed to the publications in this thesis: Aravind Kumar Mohan, Christa Kietz, Lucas Schwintzer, Sebastian Kupka, Henning Walczak, Meike Broemer, Gabriela Martínez-Chacón, Nadezhda Tsyganova, Joose Kreutzer, Pasi Kallio, Atefeh Saadabadi, Fanny Lindholm, Emmy Himmelroos, Parthiban Marimuthu, Outi Salo-Ahen and Patrik Eklund.

I would be nothing today, without the supporting help and encouraging environment provided by the whole Meinander lab, both past and present members. Luckily, I have been there since the beginning and have therefore been blessed with many dear colleagues. Thank you, Ida-Emma, Mikaela, Binu, Josef, Ida, Vilma, Nadya, Jesper, Fanny, Anna x 2, Emmy, Emmi, Marc, Oliver, Sabrina, Nadya, Gaby and our adopted group member Pasi, for all your help in the lab and outside of it. I especially remember with warm and fuzzy feelings all our get-togethers outside the lab.

Aravind and Christa. Thank you! Even as I am writing this, tears are flowing down my chins. I think you know how much you mean to me and how much you have helped me throughout all these years. We really have been a dynamic trio and I hope to cherish our friendship throughout my life.

A special shoutout to my BYOSF peeps (yeah, we could have done better work with the acronym), and co-organisers of non-science events at the department. We have had a blast!

Luckily, there is also life outside the lab. I am grateful to be blessed with a group of friends since waaaaay back, that have been there, well, always! Thanks for the laughs and support! Love you to the moon and back! And Gulis06, where would I be without you?!? We had a blast during our studies, and fortunately, we have been keeping in touch even after those times.

Finally, my family, the biggest chunk of my heart goes to you. Even though you have no idea of what I am doing, you have been supporting me and given me a place to go to, always, no matter what! Tack för allt stöd och intresse under alla dessa år! Kiitos kaikesta tuesta ja kiinnostuksesta kaikkien näiden vuosien aikana! Last but not least, Nikke, elämäni ilo ja rakkaus, thank you for being my everyday support, I could not have done anything without you!

I am grateful for the financial support that enabled this research: MolBio, The Åbo Akademi foundation, Orion research foundation, Swedish cultural foundation in Finland, Ida Montin foundation, Magnus Ehrnrooth foundation, The Paulo foundation, Lounais-Suomen syöpäyhdistys and Victoriastiftelsen.

Turku, 2.5.2022

Anna

REFERENCE LIST

Abbott, D. W. *et al.* (2007) 'Coordinated Regulation of Toll-Like Receptor and NOD2 Signaling by K63-Linked Polyubiquitin Chains', *Molecular and Cellular Biology*. American Society for Microbiology (ASM), 27(17), pp. 6012–6025.

Abdul Rehman, S. A. *et al.* (2016) 'MINDY-1 Is a Member of an Evolutionarily Conserved and Structurally Distinct New Family of Deubiquitinating Enzymes', *Molecular Cell*. Cell Press, 63(1), pp. 146–155. doi: 10.1016/j.molcel.2016.05.009.

Adams, M. D. *et al.* (2000) 'The Genome Sequence of Drosophila melanogaster', *Science*. American Association for the Advancement of Science, 287(5461), pp. 2185–2195. doi: 10.1126/SCIENCE.287.5461.2185.

Aggarwal, B. B., Gupta, S. C. and Kim, J. H. (2012) 'Historical perspectives on tumor necrosis factor and its superfamily: 25 years later, a golden journey', *Blood*. American Society of Hematology, 119(3), pp. 651–665. doi: 10.1182/BLOOD-2011-04-325225.

Aggarwal, K. *et al.* (2008) 'Rudra interrupts receptor signaling complexes to negatively regulate the IMD pathway', *PLoS pathogens*. PLoS Pathog, 4(8). doi: 10.1371/JOURNAL.PPAT.1000120.

Akhouayri, I. *et al.* (2011) 'Toll-8/Tollo negatively regulates antimicrobial response in the Drosophila respiratory epithelium.', *PLoS pathogens*. Public Library of Science, 7(10), p. e1002319. doi: 10.1371/journal.ppat.1002319.

Akinwumi, B. C., Bordun, K. A. M. and Anderson, H. D. (2018) 'Biological activities of stilbenoids', *International Journal of Molecular Sciences*. MDPI AG. doi: 10.3390/ijms19030792.

Akira, S., Uematsu, S. and Takeuchi, O. (2006) 'Pathogen recognition and innate immunity', *Cell*, pp. 783–801. doi: 10.1016/j.cell.2006.02.015.

Akutsu, M., Dikic, I. and Bremm, A. (2016) 'Ubiquitin chain diversity at a glance', *Journal of Cell Science*. Company of Biologists Ltd, 129(5), pp. 875–880. doi: 10.1242/jcs.183954.

Amcheslavsky, A., Jiang, J. and Ip, Y. T. (2009) 'Tissue Damage-Induced Intestinal Stem Cell Division in Drosophila', *Cell Stem Cell*. NIH Public Access, 4(1), pp. 49–61. doi: 10.1016/j.stem.2008.10.016.

Anbazhagan, A. N. *et al.* (2018) 'Pathophysiology of IBD associated diarrhea', *Tissue barriers*. Tissue Barriers, 6(2). doi: 10.1080/21688370.2018.1463897.

Annibaldi, A. and Meier, P. (2018) 'Checkpoints in TNF-Induced Cell Death: Implications in Inflammation and Cancer', *Trends in Molecular Medicine*. Elsevier Ltd, 24(1), pp. 49–65. doi: 10.1016/j.molmed.2017.11.002.

Apidianakis, Y. and Rahme, L. G. (2011) 'Drosophila melanogaster as a model for human intestinal infection and pathology', *Disease Models & Mechanisms*. The Company of Biologists, 4(1), pp. 21–30. doi: 10.1242/DMM.003970.

Aravind, L. and Koonin, E. V. (2000) 'The U box is a modified RING finger - A common domain in ubiquitination [1]', *Current Biology*. Current Biology Ltd, pp. R132–R134. doi: 10.1016/S0960-9822(00)00398-5.

Arribas, C., Sampedro, J. and Izquierdo, M. (1986) 'The ubiquitin genes in D. melanogaster: Transcription and polymorphism', *BBA - Gene Structure and Expression*. Elsevier, 868(2–3), pp. 119–127. doi: 10.1016/0167-4781(86)90014-X.

Asanomi, Y. *et al.* (2019) 'A rare functional variant of SHARPIN attenuates the inflammatory response and associates with increased risk of late-onset Alzheimer's disease', *Molecular Medicine*. The Feinstein Institute for Medical Research, 25(1). doi: 10.1186/S10020-019-0090-5.

Asaoka, T. *et al.* (2016) ' Linear ubiquitination by LUBEL has a role in Drosophila heat stress response ', *EMBO reports*. EMBO, 17(11), pp. 1624–1640. doi: 10.15252/embr.201642378.

Asri, R. M. *et al.* (2019) 'Sterile induction of innate immunity in Drosophila melanogaster', *Frontiers in Bioscience - Landmark*. Frontiers in Bioscience, 24(8), pp. 1390–1400. doi: 10.2741/4786.

Atreya, I., Atreya, R. and Neurath, M. F. (2008) 'NF-κB in inflammatory bowel disease', *Journal of Internal Medicine*. John Wiley & Sons, Ltd, 263(6), pp. 591–596. doi: 10.1111/J.1365-2796.2008.01953.X.

Bandarra, D. *et al.* (2014) 'Hypoxia activates IKK-NF-κB and the immune response in Drosophila melanogaster', *Bioscience Reports*. Portland Press Ltd, 34(4), pp. 429–440. doi: 10.1042/BSR20140095.

Bard, J. A. M. *et al.* (2018) 'Structure and Function of the 26S Proteasome', *Annual review of biochemistry*. NIH Public Access, 87, p. 697. doi: 10.1146/ANNUREV-BIOCHEM-062917-011931.

Barlow, P. N. *et al.* (1994) 'Structure of the C3HC4 domain by 1H-nuclear magnetic resonance spectroscopy: A new structural class of zinc-finger', *Journal of Molecular Biology*. Academic Press, 237(2), pp. 201–211. doi: 10.1006/jmbi.1994.1222.

Bertrand, M. J. M. *et al.* (2008) 'cIAP1 and cIAP2 facilitate cancer cell survival by functioning as E3 ligases that promote RIP1 ubiquitination', *Molecular cell*. Mol Cell, 30(6), pp. 689–700. doi: 10.1016/J.MOLCEL.2008.05.014.

Bertrand, M. J. M. *et al.* (2009) 'Cellular inhibitors of apoptosis cIAP1 and cIAP2 are required for innate immunity signaling by the pattern recognition receptors NOD1 and NOD2', *Immunity*. Immunity, 30(6), pp. 789–801. doi: 10.1016/J.IMMUNI.2009.04.011.

Bischoff, V. *et al.* (2004) 'Function of the drosophila pattern-recognition receptor PGRP-SD in the detection of Gram-positive bacteria', *Nature immunology*. Nat Immunol, 5(11), pp. 1175–1180. doi: 10.1038/NI1123.

Boisson, B. *et al.* (2012) 'Immunodeficiency, autoinflammation and amylopectinosis in humans with inherited HOIL-1 and LUBAC deficiency', *Nature Immunology*, 13(12), pp. 1178–1186. doi: 10.1038/ni.2457.

Boisson, B. *et al.* (2015) 'Human HOIP and LUBAC deficiency underlies autoinflammation, immunodeficiency, amylopectinosis, and lymphangiectasia.', *The Journal of experimental medicine*. The Rockefeller University Press, 212(6), pp. 939–51. doi: 10.1084/jem.20141130.

Bond, U. and Schlesinger, M. J. (1985) 'Ubiquitin is a heat shock protein in chicken embryo fibroblasts.', *Molecular and cellular biology*. American Society for Microbiology, 5(5), pp. 949–56. doi: 10.1128/mcb.5.5.949.

Bond, U. and Schlesinger, M. J. (1986) 'The chicken ubiquitin gene contains a heat shock promoter and expresses an unstable mRNA in heat-shocked cells.', *Molecular and Cellular Biology*. American Society for Microbiology, 6(12), pp. 4602–4610. doi: 10.1128/mcb.6.12.4602.

Borden, K. L. *et al.* (1995) 'The solution structure of the RING finger domain from the acute promyelocytic leukaemia proto-oncoprotein PML.', *The EMBO Journal*. Wiley, 14(7), pp. 1532–1541. doi: 10.1002/j.1460-2075.1995.tb07139.x.

Bosco-Drayon, V. *et al.* (2012) 'Peptidoglycan Sensing by the Receptor PGRP-LE in the Drosophila Gut Induces Immune Responses to Infectious Bacteria and Tolerance to Microbiota', *Cell Host & Microbe*. Cell Press, 12(2), pp. 153–165. doi: 10.1016/J.CHOM.2012.06.002.

Boutros, M., Agaisse, H. and Perrimon, N. (2002) 'Sequential activation of signaling pathways during innate immune responses in Drosophila', *Developmental Cell*. Cell Press, 3(5), pp. 711–722. doi: 10.1016/S1534-5807(02)00325-8.

Bremm, A., Freund, S. M. V. and Komander, D. (2010) 'Lys11-linked ubiquitin chains adopt compact conformations and are preferentially hydrolyzed by the deubiquitinase Cezanne', *Nature Structural and Molecular Biology*. Nature Publishing Group, 17(8), pp. 939–947. doi: 10.1038/nsmb.1873.

Buchon, N. *et al.* (2009) 'Drosophila Intestinal Response to Bacterial Infection: Activation of Host Defense and Stem Cell Proliferation', *Cell Host and Microbe*. Cell Press, 5(2), pp. 200–211. doi: 10.1016/j.chom.2009.01.003.

Cappadocia, L. and Lima, C. D. (2018) 'Ubiquitin-like Protein Conjugation: Structures, Chemistry, and Mechanism', *Chemical Reviews*, pp. 889–918. doi: 10.1021/acs.chemrev.6b00737.

Carvajal, A. R. *et al.* (2021) 'The linear ubiquitin chain assembly complex (LUBAC) generates heterotypic ubiquitin chains', *eLife*, 10, pp. 1–28. doi: 10.7554/eLife.60660.

Castañeda, C. A. *et al.* (2016) 'Linkage via K27 Bestows Ubiquitin Chains with Unique Properties among Polyubiquitins', *Structure*. Cell Press, 24(3), pp. 423–436. doi:

10.1016/j.str.2016.01.007.
El Chamy, L. *et al.* (2008) 'Sensing of "danger signals" and pathogen-associated molecular patterns defines binary signaling pathways "upstream" of Toll', *Nature Immunology*. Nat Immunol, 9(10), pp. 1165–1170. doi: 10.1038/ni.1643.
El Chamy, L. *et al.* (2015) 'The multilayered innate immune defense of the gut', *Biomedical Journal*, 38(4), pp. 276–284. doi: 10.4103/2319-4170.158621.
Chang, M., Jin, W. and Sun, S. C. (2009) 'Peli1 facilitates TRIF-dependent Toll-like receptor signaling and proinflammatory cytokine production', *Nature immunology*. Nat Immunol, 10(10), pp. 1089–1095. doi: 10.1038/NI.1777.
Charroux, B. *et al.* (2018) 'Cytosolic and Secreted Peptidoglycan-Degrading Enzymes in Drosophila Respectively Control Local and Systemic Immune Responses to Microbiota', *Cell host & microbe*. Cell Host Microbe, 23(2), pp. 215-228.e4. doi: 10.1016/J.CHOM.2017.12.007.
Charroux, B. and Royet, J. (2010) 'Drosophila immune response: From systemic antimicrobial peptide production in fat body cells to local defense in the intestinal tract', *Fly*. Taylor and Francis Inc., 4(1), pp. 40–47. doi: 10.4161/fly.4.1.10810.
Chau, V. *et al.* (1989) 'A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein', *Science*. American Association for the Advancement of Science, 243(4898), pp. 1576–1583. doi: 10.1126/science.2538923.
Chen, L. *et al.* (2017) 'Innate immune signaling in Drosophila is regulated by transforming growth factor β (TGFβ)-activated kinase (Tak1)-triggered ubiquitin editing', *Journal of Biological Chemistry*. Elsevier, 292(21), pp. 8738–8749. doi: 10.1074/JBC.M117.788158.
Chintapalli, V. R., Wang, J. and Dow, J. A. T. (2007) 'Using FlyAtlas to identify better Drosophila melanogaster models of human disease', *Nature Genetics 2007 39:6*. Nature Publishing Group, 39(6), pp. 715–720. doi: 10.1038/ng2049.
Choe, K. M. *et al.* (2002) 'Requirement for a peptidoglycan recognition protein (PGRP) in Relish activation and antibacterial immune responses in Drosophila', *Science (New York, N.Y.)*, 296(5566), pp. 359–362. doi: 10.1126/science.1070216 [doi].
Choe, K. M., Lee, H. and Anderson, K. V. (2005) 'Drosophila peptidoglycan recognition protein LC (PGRP-LC) acts as a signal-transducing innate immune receptor', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 102(4), pp. 1122–1126. doi: 10.1073/pnas.0404952102.
Chong, J., Poutaraud, A. and Hugueney, P. (2009) 'Metabolism and roles of stilbenes in plants', *Plant Science*. Elsevier, 177(3), pp. 143–155. doi: 10.1016/J.PLANTSCI.2009.05.012.
Chowdhury, M. *et al.* (2019) 'Toll family members bind multiple Spätzle proteins and activate antimicrobial peptide gene expression in Drosophila', *The Journal of biological chemistry*. J Biol Chem, 294(26), pp. 10172–10181. doi: 10.1074/JBC.RA118.006804.
Ciechanover, A. *et al.* (1981) 'Activation of the heat-stable polypeptide of the ATP-dependent proteolytic system', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 78(2 II), pp. 761–765. doi: 10.1073/pnas.78.2.761.
Ciechanover, A. *et al.* (1982) '"Covalent affinity" purification of ubiquitin-activating enzyme.', *Journal of Biological Chemistry*, 257(5), pp. 2537–2542.
Ciechanover, A., Finley, D. and Varshavsky, A. (1984) *Ubiquitin Dependence of Selective Protein Degradation Demonstrated in the Mammalian Cell Cycle Mutant ts85*, *Cell*.
Ciehanover, A., Hod, Y. and Hershko, A. (1978) 'A heat-stable polypeptide component of an ATP-dependent proteolytic system from reticulocytes', *Biochemical and Biophysical Research Communications*. Academic Press, 81(4), pp. 1100–1105. doi: 10.1016/0006-291X(78)91249-4.
Clemmons, A. W., Lindsay, S. A. and Wasserman, S. A. (2015) 'An Effector Peptide Family Required for Drosophila Toll-Mediated Immunity'. doi: 10.1371/journal.ppat.1004876.
Cohen, P. *et al.* (2019) 'HOIL-1, an atypical E3 ligase that controls MyD88 signalling by forming ester bonds between ubiquitin and components of the Myddosome', *Advances in Biological Regulation*. Elsevier Ltd, p. 100666. doi: 10.1016/j.jbior.2019.100666.
Cohen, P. and Strickson, S. (2017) 'The role of hybrid ubiquitin chains in the MyD88 and other innate immune signalling pathways', *Cell Death and Differentiation*. Nature Publishing Group, 24(7), p. 1153. doi: 10.1038/CDD.2017.17.
Colombani, J. and Andersen, D. S. (2020) 'The Drosophila gut: A gatekeeper and coordinator of

organism fitness and physiology', *Wiley Interdisciplinary Reviews: Developmental Biology*, 9(6), pp. 1–19. doi: 10.1002/wdev.378.

Corn, J. E. and Vucic, D. (2014) 'Ubiquitin in inflammation: the right linkage makes all the difference', *Nature structural & molecular biology*. Nature Publishing Group, 21(4), pp. 297–300.

Cotto-Rios, X. M. *et al.* (2012) 'Deubiquitinases as a Signaling Target of Oxidative Stress', *Cell Reports*, 2(6), pp. 1475–1484. doi: 10.1016/j.celrep.2012.11.011.

Culver, C. *et al.* (2010) 'Mechanism of hypoxia-induced NF-kappaB.', *Molecular and cellular biology*. American Society for Microbiology (ASM), 30(20), pp. 4901–21. doi: 10.1128/MCB.00409-10.

Damgaard, R. B. *et al.* (2012) 'The Ubiquitin Ligase XIAP Recruits LUBAC for NOD2 Signaling in Inflammation and Innate Immunity', *Molecular Cell*. doi: 10.1016/j.molcel.2012.04.014.

Damgaard, R. B. *et al.* (2016) 'The Deubiquitinase OTULIN Is an Essential Negative Regulator of Inflammation and Autoimmunity', *Cell*. Cell Press, 166(5), pp. 1215-1230.e20. doi: 10.1016/j.cell.2016.07.019.

Damgaard, R. B. *et al.* (2019) 'OTULIN deficiency in ORAS causes cell type-specific LUBAC degradation, dysregulated TNF signalling and cell death', *EMBO molecular medicine*. EMBO Mol Med, 11(3). doi: 10.15252/EMMM.201809324.

Damgaard, R. B. *et al.* (2020) 'OTULIN protects the liver against cell death, inflammation, fibrosis, and cancer', *Cell Death & Differentiation*. Nature Publishing Group, pp. 1–18. doi: 10.1038/s41418-020-0532-1.

Darding, M. and Meier, P. (2012) 'IAPs: Guardians of RIPK1', *Cell Death and Differentiation*. Nature Publishing Group, pp. 58–66. doi: 10.1038/cdd.2011.163.

DeLotto, Y. and DeLotto, R. (1998) 'Proteolytic processing of the Drosophila Spätzle protein by Easter generates a dimeric NGF-like molecule with ventralising activity', *Mechanisms of Development*. Elsevier, 72(1–2), pp. 141–148. doi: 10.1016/S0925-4773(98)00024-0.

Deng, L. *et al.* (2000) 'Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain', *Cell*. Cell, 103(2), pp. 351–361. doi: 10.1016/S0092-8674(00)00126-4.

Deshaies, R. J. and Joazeiro, C. A. P. (2009) 'RING Domain E3 Ubiquitin Ligases', *Annual Review of Biochemistry*. Annual Reviews, 78(1), pp. 399–434. doi: 10.1146/annurev.biochem.78.101807.093809.

Dhillon, B. *et al.* (2019) 'The Evolving Role of TRAFs in Mediating Inflammatory Responses', *Frontiers in Immunology*. Frontiers Media SA, 10(FEB), p. 104. doi: 10.3389/FIMMU.2019.00104.

Dikic, I., Wakatsuki, S. and Walters, K. J. (2009) 'Ubiquitin-binding domains from structures to functions', *Nature Reviews Molecular Cell Biology*, pp. 659–671. doi: 10.1038/nrm2767.

Dou, H. *et al.* (2012) 'BIRC7-E2 ubiquitin conjugate structure reveals the mechanism of ubiquitin transfer by a RING dimer.', *Nature structural & molecular biology*, 19(9), pp. 876–83. doi: 10.1038/nsmb.2379.

Dove, K. K. *et al.* (2016) ' Molecular insights into RBR E3 ligase ubiquitin transfer mechanisms ', *EMBO reports*. EMBO, 17(8), pp. 1221–1235. doi: 10.15252/embr.201642641.

Dove, K. K. *et al.* (2017) 'Structural Studies of HHARI/UbcH7~Ub Reveal Unique E2~Ub Conformational Restriction by RBR RING1', *Structure*. Cell Press, 25(6), pp. 890-900.e5. doi: 10.1016/j.str.2017.04.013.

Draber, P. *et al.* (2015) 'LUBAC-Recruited CYLD and A20 Regulate Gene Activation and Cell Death by Exerting Opposing Effects on Linear Ubiquitin in Signaling Complexes', *Cell Reports*, 13(10), pp. 2258–2272. doi: 10.1016/j.celrep.2015.11.009.

Dworkin-Rastl, E., Shrutkowski, A. and Dworkin, M. B. (1984) *Multiple Ubiquitin mRNAs during Xenopus laevis Development Contain Tandem Repeats of the 76 Amino Acid Coding Sequence*, *Cell*.

Dye, B. T. and Schulman, B. A. (2007) 'Structural mechanisms underlying posttranslational modification by ubiquitin-like proteins', *Annual Review of Biophysics and Biomolecular Structure*. Annual Reviews, pp. 131–150. doi: 10.1146/annurev.biophys.36.040306.132820.

Dynek, J. N. *et al.* (2010) 'c-IAP1 and UbcH5 promote K11-linked polyubiquitination of RIP1 in TNF signalling', *The EMBO Journal*. John Wiley & Sons, Ltd, 29(24), pp. 4198–4209. doi:

10.1038/emboj.2010.300.
Ea, C.-K. *et al.* (2006) 'Activation of IKK by TNFalpha requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO.', *Molecular cell*. Elsevier, 22(2), pp. 245–57. doi: 10.1016/j.molcel.2006.03.026.
Eddins, M. J. *et al.* (2006) 'Mms2-Ubc13 covalently bound to ubiquitin reveals the structural basis of linkage-specific polyubiquitin chain formation', *Nature Structural and Molecular Biology*. Nature Publishing Group, 13(10), pp. 915–920. doi: 10.1038/nsmb1148.
Eddins, M. J. *et al.* (2007) 'Crystal Structure and Solution NMR Studies of Lys48-linked Tetraubiquitin at Neutral pH', *Journal of Molecular Biology*. Academic Press, 367(1), pp. 204–211. doi: 10.1016/j.jmb.2006.12.065.
Eichele, D. D. and Kharbanda, K. K. (2017) 'Dextran sodium sulfate colitis murine model: An indispensable tool for advancing our understanding of inflammatory bowel diseases pathogenesis', *World Journal of Gastroenterology*. Baishideng Publishing Group Co, pp. 6016–6029. doi: 10.3748/wjg.v23.i33.6016.
Eletr, Z. M. *et al.* (2005) 'E2 conjugating enzymes must disengage from their E1 enzymes before E3-dependent ubiquitin and ubiquitin-like transfer', *Nature Structural and Molecular Biology*. Nature Publishing Group, 12(10), pp. 933–934. doi: 10.1038/nsmb984.
Elliott, P. R. *et al.* (2014) 'Molecular basis and regulation of OTULIN-LUBAC interaction', *Molecular Cell*. Cell Press, 54(3), pp. 335–348. doi: 10.1016/j.molcel.2014.03.018.
Elliott, P. R. *et al.* (2016) 'SPATA2 Links CYLD to LUBAC, Activates CYLD, and Controls LUBAC Signaling', *Molecular cell*. Mol Cell, 63(6), pp. 990–1005. doi: 10.1016/J.MOLCEL.2016.08.001.
Emmerich, C. H. *et al.* (2013) 'Activation of the canonical IKK complex by K63/M1-linked hybrid ubiquitin chains', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 110(38), pp. 15247–15252. doi: 10.1073/pnas.1314715110.
Emmerich, C. H. *et al.* (2016) 'Lys63/Met1-hybrid ubiquitin chains are commonly formed during the activation of innate immune signalling', *Biochemical and biophysical research communications*. Biochem Biophys Res Commun, 474(3), pp. 452–461. doi: 10.1016/J.BBRC.2016.04.141.
Ermak, G. and Davies, K. J. A. (2002) 'Calcium and oxidative stress: from cell signaling to cell death', *Molecular Immunology*. Pergamon, 38(10), pp. 713–721. doi: 10.1016/S0161-5890(01)00108-0.
Ertürk-Hasdemir, D. *et al.* (2009) 'Two roles for the Drosophila IKK complex in the activation of Relish and the induction of antimicrobial peptide genes', *Proceedings of the National Academy of Sciences of the United States of America*, 106(24), pp. 9779–9784. doi: 10.1073/pnas.0812022106.
Faesen, A. C. *et al.* (2011) 'The differential modulation of USP activity by internal regulatory domains, interactors and eight ubiquitin chain types', *Chemistry and Biology*, 18(12), pp. 1550–1561. doi: 10.1016/j.chembiol.2011.10.017.
Fan, Y. *et al.* (2010) 'Lysine 63-linked polyubiquitination of TAK1 at lysine 158 is required for tumor necrosis factor alpha- and interleukin-1beta-induced IKK/NF-kappaB and JNK/AP-1 activation', *The Journal of biological chemistry*. J Biol Chem, 285(8), pp. 5347–5360. doi: 10.1074/JBC.M109.076976.
Fauconneau, B. *et al.* (1997) 'Comparative study of radical scavenger and antioxidant properties of phenolic compounds from Vitis vinifera cell cultures using in vitro tests', *Life Sciences*. Pergamon, 61(21), pp. 2103–2110. doi: 10.1016/S0024-3205(97)00883-7.
Fennell, L. M., Rahighi, S. and Ikeda, F. (2018) 'Linear ubiquitin chain-binding domains', *FEBS Journal*. Blackwell Publishing Ltd, pp. 2746–2761. doi: 10.1111/febs.14478.
Fernando, M. D. A., Kounatidis, I. and Ligoxygakis, P. (2014) 'Loss of Trabid, a New Negative Regulator of the Drosophila Immune-Deficiency Pathway at the Level of TAK1, Reduces Life Span', *PLoS Genetics*, 10(2), p. e1004117. doi: 10.1371/journal.pgen.1004117.
Ferrandon, D. *et al.* (1998) 'A drosomycin-GFP reporter transgene reveals a local immune response in Drosophila that is not dependent on the Toll pathway', *EMBO Journal*, 17(5), pp. 1217–1227. doi: 10.1093/emboj/17.5.1217.
Ferrandon, D. *et al.* (2007) 'The Drosophila systemic immune response: Sensing and signalling

during bacterial and fungal infections', *Nature Reviews Immunology*. Nature Publishing Group, pp. 862–874. doi: 10.1038/nri2194.

Fiil, B. K. *et al.* (2013) 'OTULIN Restricts Met1-Linked Ubiquitination to Control Innate Immune Signaling', *Molecular Cell*, 50(6), pp. 818–830. doi: 10.1016/j.molcel.2013.06.004.

Fiil, B. K. and Gyrd-Hansen, M. (2014) 'Met1-linked ubiquitination in immune signalling', *FEBS Journal*, pp. 4337–4350. doi: 10.1111/febs.12944.

Finley, D., Bartel, B. and Varshavsky, A. (1989) 'The tails of ubiquitin precursors are ribosomal proteins whose fusion to ubiquitin facilitates ribosome biogenesis', *Nature*. Nature Publishing Group, 338(6214), pp. 394–401. doi: 10.1038/338394a0.

Finley, D., Ciechanover, A. and Varshavsky, A. (1984) *Thermolability of Ubiquitin-Activating Enzyme from the Mammalian Cell Cycle Mutant ts85*, *Cell*.

Finley, D., Özkaynak, E. and Varshavsky, A. (1987) 'The yeast polyubiquitin gene is essential for resistance to high temperatures, starvation, and other stresses', *Cell*. Elsevier, 48(6), pp. 1035–1046. doi: 10.1016/0092-8674(87)90711-2.

Fiskin, E. *et al.* (2016) 'Global Analysis of Host and Bacterial Ubiquitinome in Response to Salmonella Typhimurium Infection', *Molecular Cell*. Cell Press, 62(6), pp. 967–981. doi: 10.1016/J.MOLCEL.2016.04.015/ATTACHMENT/5B1A9991-86D2-4FFE-B6F2-502BCB48BF7B/MMC8.XLSX.

Fornace, A. J. *et al.* (1989) 'Ubiquftin mRNA is a major stress-induced transcript in mammalian cells', *Nucleic Acids Research*, 17(3), pp. 1215–1230. doi: 10.1093/nar/17.3.1215.

Franklin, T. G. and Pruneda, J. N. (2021) 'Bacteria make surgical strikes on host ubiquitin signaling', *PLOS Pathogens*. Public Library of Science, 17(3), p. e1009341. doi: 10.1371/JOURNAL.PPAT.1009341.

Freemont, P. S., Hanson, I. M. and Trowsdale, J. (1991) 'A Novel Cysteine-Rich Sequence Motif', *Cell*, 64, pp. 483–484. doi: 10.3138/jvme.0514-051.

Fridh, V. and Rittinger, K. (2012) 'The tandem CARDs of NOD2: intramolecular interactions and recognition of RIP2', *PloS one*. PLoS One, 7(3). doi: 10.1371/JOURNAL.PONE.0034375.

Fujita, H. *et al.* (2018) 'Cooperative Domain Formation by Homologous Motifs in HOIL-1L and SHARPIN Plays A Crucial Role in LUBAC Stabilization', *Cell Reports*. Cell Press, 23(4), pp. 1192–1204. doi: 10.1016/J.CELREP.2018.03.112.

Fuseya, Y. *et al.* (2020) 'The HOIL-1L ligase modulates immune signalling and cell death via monoubiquitination of LUBAC', *Nature Cell Biology 2020 22:6*. Nature Publishing Group, 22(6), pp. 663–673. doi: 10.1038/s41556-020-0517-9.

Ganesan, S. *et al.* (2011) 'NF-κB/Rel Proteins and the Humoral Immune Responses of Drosophila melanogaster', *Current Topics in Microbiology and Immunology*, 349, pp. 25–60. doi: 10.1007/82_2010_107.

Garner, T. P. *et al.* (2011) 'Independent Interactions of Ubiquitin-Binding Domains in a Ubiquitin-Mediated Ternary Complex', *Biochemistry*, 50(42), pp. 9076–9087. doi: 10.1021/bi201137e.

Gatti, M. *et al.* (2015) 'RNF168 promotes noncanonical K27ubiquitination to signal DNA damage', *Cell Reports*. Elsevier, 10(2), pp. 226–238. doi: 10.1016/j.celrep.2014.12.021.

Gautheron, J. and Courtois, G. (2010) '"Without Ub I am nothing": NEMO as a multifunctional player in ubiquitin-mediated control of NF-kappaB activation.', *Cellular and molecular life sciences*. INSERM U781, Tour Lavoisier, Hôpital Necker-Enfants Malades and Université Paris-Descartes, 149, rue de Sèvres, 75015, Paris, France., 67(18), pp. 3101–3113.

Gavilanes, J. G. *et al.* (1982) 'Isolation, characterization, and amino acid sequence of a ubiquitin-like protein from insect eggs.', *Journal of Biological Chemistry*, 257(17), pp. 10267–10270.

Georgel, P. *et al.* (2001) 'Drosophila immune deficiency (IMD) is a death domain protein that activates antibacterial defense and can promote apoptosis', *Developmental cell*. Dev Cell, 1(4), pp. 503–514. doi: 10.1016/S1534-5807(01)00059-4.

Gerlach, B. *et al.* (2011) 'Linear ubiquitination prevents inflammation and regulates immune signalling', *Nature*, 471, pp. 591–596.

Geuking, P. *et al.* (2009) 'A Non-Redundant Role for Drosophila Mkk4 and Hemipterous/Mkk7 in TAK1-Mediated Activation of JNK', *PLOS ONE*. Public Library of Science, 4(11), p. e7709. doi: 10.1371/JOURNAL.PONE.0007709.

Girardin, S. E. *et al.* (2003) 'Nod2 Is a General Sensor of Peptidoglycan through Muramyl

Dipeptide (MDP) Detection', *Journal of Biological Chemistry*. Elsevier, 278(11), pp. 8869–8872. doi: 10.1074/JBC.C200651200.

Goldknopf, I. L. and Busch, H. (1977) 'Isopeptide linkage between nonhistone and histone 2A polypeptides of chromosomal conjugate protein A24', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 74(3), pp. 864–868. doi: 10.1073/pnas.74.3.864.

Goldstein, G. *et al.* (1975) 'Isolation of a polypeptide that has lymphocyte differentiating properties and is probably represented universally in living cells', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 72(1), pp. 11–15. doi: 10.1073/pnas.72.1.11.

Goncharov, T. *et al.* (2018) 'Disruption of XIAP-RIP2 Association Blocks NOD2-Mediated Inflammatory Signaling', *Molecular Cell*. Cell Press, 69(4), pp. 551-565.e7. doi: 10.1016/J.MOLCEL.2018.01.016.

Gottar, M. *et al.* (2002) 'The Drosophila immune response against Gram-negative bacteria is mediated by a peptidoglycan recognition protein', *Nature*. Nature Publishing Group, 416(6881), pp. 640–644. doi: 10.1038/nature734.

Gottar, M. *et al.* (2006) 'Dual detection of fungal infections in Drosophila via recognition of glucans and sensing of virulence factors', *Cell*. Cell, 127(7), pp. 1425–1437. doi: 10.1016/J.CELL.2006.10.046.

De Gregorio, E. *et al.* (2001) 'Genome-wide analysis of the Drosophila immune response by using oligonucleotide microarrays', *Proceedings of the National Academy of Sciences of the United States of America*, 98(22), pp. 12590–12595. doi: 10.1073/pnas.221458698.

Grice, G. L. and Nathan, J. A. (2016) 'The recognition of ubiquitinated proteins by the proteasome', *Cellular and Molecular Life Sciences*. Birkhauser Verlag AG, pp. 3497–3506. doi: 10.1007/s00018-016-2255-5.

Grou, C. P. *et al.* (2015) 'The de novo synthesis of ubiquitin: identification of deubiquitinases acting on ubiquitin precursors', *Nature Publishing Group*, 5, p. 12836. doi: 10.1038/srep12836.

Grumati, P. and Dikic, I. (2018) 'Ubiquitin signaling and autophagy', *Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology Inc., pp. 5404–5413. doi: 10.1074/jbc.TM117.000117.

Gyrd-Hansen, M. *et al.* (2008) 'IAPs contain an evolutionarily conserved ubiquitin-binding domain that regulates NF-κB as well as cell survival and oncogenesis', *Nature cell biology*. NIH Public Access, 10(11), p. 1309. doi: 10.1038/NCB1789.

Ha, E. M., Oh, C. T., Bae, Y. S., *et al.* (2005) 'A direct role for dual oxidase in Drosophila gut immunity', *Science*. American Association for the Advancement of Science, 310(5749), pp. 847–850. doi: 10.1126/science.1117311.

Ha, E. M., Oh, C. T., Ryu, J. H., *et al.* (2005) 'An antioxidant system required for host protection against gut infection in Drosophila', *Developmental Cell*. Dev Cell, 8(1), pp. 125–132. doi: 10.1016/j.devcel.2004.11.007.

Ha, E. M. *et al.* (2009) 'Regulation of DUOX by the Gαq-Phospholipase Cβ-Ca2+ Pathway in Drosophila Gut Immunity', *Developmental Cell*. Cell Press, 16(3), pp. 386–397. doi: 10.1016/j.devcel.2008.12.015.

Haas, A. L. *et al.* (1982) 'Ubiquitin-activating enzyme. Mechanism and role in protein-ubiquitin conjugation.', *Journal of Biological Chemistry*. Elsevier, 257(5), pp. 2543–2548. doi: 10.1016/S0021-9258(18)34958-5.

Haas, A. L., Bright, P. M. and Jackson, V. E. (1988) 'Functional diversity among putative E2 isozymes in the mechanism of ubiquitin-histone ligation', *Journal of Biological Chemistry*, 263(26), pp. 13268–13275.

Haas, A. L. and Rose, I. A. (1982) 'The Mechanism of Ubiquitin Activating Enzym.e', *The Journal of biological chemistry*, 257(17), pp. 10329–10337.

Haas, A. L., Warms, J. V and Rose, I. A. (1983) 'Ubiquitin adenylate: structure and role in ubiquitin activation.', *Biochemistry*, 22(19), pp. 4388–94. doi: 10.1021/bi00288a007.

Haas, T. L. *et al.* (2009) 'Recruitment of the Linear Ubiquitin Chain Assembly Complex Stabilizes the TNF-R1 Signaling Complex and Is Required for TNF-Mediated Gene Induction', *Molecular Cell*, 36(5), pp. 831–844. doi: 10.1016/j.molcel.2009.10.013.

Häcker, H. and Karin, M. (2006) 'Regulation and function of IKK and IKK-related kinases.', *Science's STKE : signal transduction knowledge environment*, 2006(357). doi: 10.1126/stke.3572006re13.

Häcker, H., Tseng, P. H. and Karin, M. (2011) 'Expanding TRAF function: TRAF3 as a tri-faced immune regulator', *Nature reviews. Immunology*. Nat Rev Immunol, 11(7), pp. 457–468. doi: 10.1038/NRI2998.

Hadian, K. *et al.* (2011) 'NF-κB essential modulator (NEMO) interaction with linear and Lys-63 ubiquitin chains contributes to NF-κB activation', *Journal of Biological Chemistry*, 286(29), pp. 26107–26117. doi: 10.1074/jbc.M111.233163.

Hanson, M. A. and Lemaitre, B. (2020) 'New insights on Drosophila antimicrobial peptide function in host defense and beyond', *Current Opinion in Immunology*. Elsevier Ltd, pp. 22–30. doi: 10.1016/j.coi.2019.11.008.

Harhaj, E. W. and Dixit, V. M. (2010) 'Deubiquitinases in the regulation of NF-κB signaling', *Cell Research 2011 21:1*. Nature Publishing Group, 21(1), pp. 22–39. doi: 10.1038/cr.2010.166.

Hasegawa, M. *et al.* (2008) 'A critical role of RICK/RIP2 polyubiquitination in Nod-induced NF-κB activation', *EMBO Journal*, 27(2), pp. 373–383. doi: 10.1038/sj.emboj.7601962.

Hauenstein, A. V. *et al.* (2017) 'Evidence for M1-linked Polyubiquitin-Mediated Conformational Change in NEMO', *Journal of molecular biology*. NIH Public Access, 429(24), p. 3793. doi: 10.1016/J.JMB.2017.10.026.

Hayden, M. S. and Ghosh, S. (2008) 'Shared Principles in NF-κB Signaling', *Cell*. Cell Press, 132(3), pp. 344–362. doi: 10.1016/J.CELL.2008.01.020.

Hayden, M. S. and Ghosh, S. (2012) 'NF-κB, the first quarter-century: remarkable progress and outstanding questions', *Genes & Development*. Cold Spring Harbor Laboratory Press, 26(3), p. 203. doi: 10.1101/GAD.183434.111.

Hedengren, M. *et al.* (1999) 'Relish, a central factor in the control of humoral but not cellular immunity in Drosophila', *Molecular Cell*, 4(5), pp. 827–837. doi: 10.1016/S1097-2765(00)80392-5.

Heger, K. *et al.* (2018) 'OTULIN limits cell death and inflammation by deubiquitinating LUBAC', *Nature*. Nature Publishing Group, 559(7712), pp. 120–124. doi: 10.1038/s41586-018-0256-2.

Heim, V. J. *et al.* (2020) 'A regulatory region on RIPK 2 is required for XIAP binding and NOD signaling activity', *EMBO reports*, 21(11). doi: 10.15252/embr.202050400.

Henry-Vitrac, C. *et al.* (2006) 'Transport, deglycosylation, and metabolism of trans-piceid by small intestinal epithelial cells', *European Journal of Nutrition 2006 45:7*. Springer, 45(7), pp. 376–382. doi: 10.1007/S00394-006-0609-8.

Hernández-Chirlaque, C. *et al.* (2016) 'Germ-free and Antibiotic-treated Mice are Highly Susceptible to Epithelial Injury in DSS Colitis', *Journal of Crohn's and Colitis*. Oxford Academic, 10(11), pp. 1324–1335. doi: 10.1093/ECCO-JCC/JJW096.

Hershko, A. *et al.* (1980) 'Proposed role of ATP in protein breakdown: conjugation of protein with multiple chains of the polypeptide of ATP-dependent proteolysis.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 77(4), pp. 1783–1786. doi: 10.1073/pnas.77.4.1783.

Hershko, A. *et al.* (1983) 'Components of Ubiquitin-Protein Ligase System', *The Journal of Biological Chemistry*, 258(13), pp. 8206–8214.

Hershko, A. and Ciechanover, A. (1992) *THE UBIQUITIN SYSTEM FOR PROTEIN DEGRADATION !* Available at: www.annualreviews.org (Accessed: 30 March 2020).

Hershko, A. and Ciechanover, A. (1998) 'The ubiquitin system', *Annual Review of Biochemistry*. Annual Reviews, 67(1), pp. 425–479. doi: 10.1146/annurev.biochem.67.1.425.

Hershko, A. and Heller, H. (1985) 'Occurrence of a polyubiquitin structure in ubiquitin-protein conjugates', *Biochemical and Biophysical Research Communications*. Academic Press, 128(3), pp. 1079–1086. doi: 10.1016/0006-291X(85)91050-2.

Hitotsumatsu, O. *et al.* (2008) 'The ubiquitin-editing enzyme A20 restricts nucleotide-binding oligomerization domain containing 2-triggered signals', *Immunity*. Immunity, 28(3), pp. 381–390. doi: 10.1016/J.IMMUNI.2008.02.002.

Hochstrasser, M. (2009) 'Origin and function of ubiquitin-like proteins', *Nature*, pp. 422–429. doi: 10.1038/nature07958.

Hrdinka, M. *et al.* (2016) 'CYLD Limits Lys63- and Met1-Linked Ubiquitin at Receptor Complexes to Regulate Innate Immune Signaling', *Cell Reports*, 14(12), pp. 2846–2858. doi: 10.1016/j.celrep.2016.02.062.

Hrdinka, M. *et al.* (2018) 'Small molecule inhibitors reveal an indispensable scaffolding role of RIPK2 in NOD2 signaling', *The EMBO Journal*. John Wiley & Sons, Ltd, 37(17), p. e99372. doi: 10.15252/EMBJ.201899372.

Hsu, H., Huang, J., *et al.* (1996) 'TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex', *Immunity*. Immunity, 4(4), pp. 387–396. doi: 10.1016/S1074-7613(00)80252-6.

Hsu, H., Shu, H. B., *et al.* (1996) 'TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways', *Cell*. Cell, 84(2), pp. 299–308. doi: 10.1016/S0092-8674(00)80984-8.

Hsu, H., Xiong, J. and Goeddel, D. V. (1995) 'The TNF receptor 1-associated protein TRADD signals cell death and NF-κB activation', *Cell*. Cell, 81(4), pp. 495–504. doi: 10.1016/0092-8674(95)90070-5.

Huang, D. T. *et al.* (2007) 'Basis for a ubiquitin-like protein thioester switch toggling E1-E2 affinity', *Nature*. Nature Publishing Group, 445(7126), pp. 394–398. doi: 10.1038/nature05490.

Huang, L. *et al.* (1999) 'Structure of an E6AP-UbcH7 complex: Insights into ubiquitination by the E2-E3 enzyme cascade', *Science*. American Association for the Advancement of Science, 286(5443), pp. 1321–1326. doi: 10.1126/science.286.5443.1321.

Hugot, J. P. *et al.* (2001) 'Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease', *Nature 2001 411:6837*. Nature Publishing Group, 411(6837), pp. 599–603. doi: 10.1038/35079107.

Huibregtse, J. M. *et al.* (1995) 'A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 92(7), pp. 2563–2567. doi: 10.1073/pnas.92.7.2563.

Hultmark, D. (2003) 'Drosophila immunity: Paths and patterns', *Current Opinion in Immunology*. Elsevier Ltd, 15(1), pp. 12–19. doi: 10.1016/S0952-7915(02)00005-5.

Humbard, M. A. *et al.* (2010) 'Ubiquitin-like Small Archaeal Modifier Proteins (SAMPs) in Haloferax volcanii Correspondence to HHS Public Access', *Nature*, 463(7277), pp. 54–60. doi: 10.1038/nature08659.

Hunt, L. T. and Dayhoff, M. O. (1977) 'Amino-terminal sequence identity of ubiquitin and the nonhistone component of nuclear protein A24', *Biochemical and Biophysical Research Communications*. Academic Press, 74(2), pp. 650–655. doi: 10.1016/0006-291X(77)90352-7.

Igaki, T. and Miura, M. (2014) 'The Drosophila TNF ortholog Eiger: Emerging physiological roles and evolution of the TNF system', *Seminars in Immunology*. Academic Press, 26(3), pp. 267–274. doi: 10.1016/J.SMIM.2014.05.003.

Ikeda, F. *et al.* (2011) 'SHARPIN forms a linear ubiquitin ligase complex regulating NF-κB activity and apoptosis', *Nature*, 471(7340), pp. 637–641. doi: 10.1038/nature09814.

Jang, I. H. *et al.* (2006) 'A Spätzle-Processing Enzyme Required for Toll Signaling Activation in Drosophila Innate Immunity', *Developmental Cell*. Cell Press, 10(1), pp. 45–55. doi: 10.1016/J.DEVCEL.2005.11.013.

Ji, C. H. and Kwon, Y. T. (2017) 'Crosstalk and interplay between the ubiquitin-proteasome system and autophagy', *Molecules and Cells*. Korean Society for Molecular and Cellular Biology, pp. 441–449. doi: 10.14348/molcells.2017.0115.

Jin, J. *et al.* (2007) 'Dual E1 activation systems for ubiquitin differentially regulate E2 enzyme charging', *Nature*. Nature Publishing Group, 447(7148), pp. 1135–1138. doi: 10.1038/nature05902.

Johnston, S. C. *et al.* (1997) *Crystal structure of a deubiquitinating enzyme (human UCH-L3) at 1.8 Å resolution E3 (ubiquitin ligase) enzymes then catalyze ligation of*, *The EMBO Journal*.

Johnston, S. C. *et al.* (1999) *Structural basis for the specificity of ubiquitin C-terminal hydrolases*, *The EMBO Journal*.

Kamadurai, H. B. *et al.* (2009) 'Insights into Ubiquitin Transfer Cascades from a Structure of a

UbcH5B~Ubiquitin-HECTNEDD4L Complex', *Molecular Cell*, 36(6), pp. 1095–1102. doi: 10.1016/j.molcel.2009.11.010.

Kamadurai, H. B. *et al.* (2013) 'Mechanism of ubiquitin ligation and lysine prioritization by a HECT E3', *eLife*. eLife Sciences Publications Ltd, 2013(2). doi: 10.7554/eLife.00828.001.

Kambris, Z. *et al.* (2002) 'Tissue and stage-specific expression of the Tolls in Drosophila embryos', *Gene Expression Patterns*. Elsevier, 2(3–4), pp. 311–317. doi: 10.1016/S1567-133X(02)00020-0.

Kanarek, N. *et al.* (2010) 'Ubiquitination and degradation of the inhibitors of NF-kappaB', *Cold Spring Harbor perspectives in biology*. Cold Spring Harb Perspect Biol, 2(2). doi: 10.1101/CSHPERSPECT.A000166.

Kanayama, A. *et al.* (2004) 'TAB2 and TAB3 Activate the NF-κB Pathway through Binding to Polyubiquitin Chains', *Molecular Cell*. Cell Press, 15(4), pp. 535–548. doi: 10.1016/J.MOLCEL.2004.08.008.

Kaneko, T. *et al.* (2006) 'PGRP-LC and PGRP-LE have essential yet distinct functions in the drosophila immune response to monomeric DAP-type peptidoglycan', *Nature Immunology 2006 7:7*. Nature Publishing Group, 7(7), pp. 715–723. doi: 10.1038/ni1356.

Kawai, T. and Akira, S. (2007) 'Signaling to NF-κB by Toll-like receptors', *Trends in Molecular Medicine*. Elsevier, 13(11), pp. 460–469. doi: 10.1016/J.MOLMED.2007.09.002.

Kawai, T. and Akira, S. (2010) 'The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors', *Nature immunology*. Nat Immunol, 11(5), pp. 373–384. doi: 10.1038/NI.1863.

Kelsall, I. R. *et al.* (2019) 'The E3 ligase HOIL-1 catalyses ester bond formation between ubiquitin and components of the Myddosome in mammalian cells', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 116(27), pp. 13293–13298. doi: 10.1073/PNAS.1905873116/-/DCSUPPLEMENTAL.

Kelsall, I. R. *et al.* (2022) 'HOIL-1 ubiquitin ligase activity targets unbranched glucosaccharides and is required to prevent polyglucosan accumulation', *The EMBO Journal*, 41(8), pp. 1–17. doi: 10.15252/embj.2021109700.

Kenmoku, H. *et al.* (2017) 'A novel mode of induction of the humoral innate immune response in Drosophila larvae', *DMM Disease Models and Mechanisms*, 10(3), pp. 271–281. doi: 10.1242/dmm.027102.

Keusekotten, K. *et al.* (2013) 'OTULIN antagonizes LUBAC signaling by specifically hydrolyzing met1-linked polyubiquitin', *Cell*, 153(6), p. 1312. doi: 10.1016/j.cell.2013.05.014.

Kiesler, P., Fuss, I. J. and Strober, W. (2015) 'Experimental Models of Inflammatory Bowel Diseases', *Cellular and Molecular Gastroenterology and Hepatology*. Elsevier, 1(2), pp. 154–170. doi: 10.1016/J.JCMGH.2015.01.006.

Kietz, C. *et al.* (2022) 'Drice restrains Diap2-mediated inflammatory signalling and intestinal inflammation', *Cell Death and Differentiation*. Cell Death Differ, 29(1), pp. 28–39. doi: 10.1038/s41418-021-00832-w.

Kim-Jo, C., Gatti, J.-L. and Poirié, M. (2019) 'Drosophila Cellular Immunity Against Parasitoid Wasps: A Complex and Time-Dependent Process', *Frontiers in Physiology*. Frontiers Media S.A., 10(MAY), p. 603. doi: 10.3389/fphys.2019.00603.

Kim, H. C. and Huibregtse, J. M. (2009) 'Polyubiquitination by HECT E3s and the Determinants of Chain Type Specificity', *Molecular and Cellular Biology*. American Society for Microbiology, 29(12), pp. 3307–3318. doi: 10.1128/mcb.00240-09.

Kirisako, T. *et al.* (2006) 'A ubiquitin ligase complex assembles linear polyubiquitin chains', *EMBO Journal*, 25(20), pp. 4877–4887. doi: 10.1038/sj.emboj.7601360.

Kleino, A. *et al.* (2005) 'Inhibitor of apoptosis 2 and TAK1-binding protein are components of the Drosophila Imd pathway', *The EMBO Journal*. John Wiley & Sons, Ltd, 24(19), pp. 3423–3434. doi: 10.1038/SJ.EMBOJ.7600807.

Kleino, A. *et al.* (2008) 'Pirk is a negative regulator of the Drosophila Imd pathway', *Journal of immunology (Baltimore, Md.: 1950)*. J Immunol, 180(8), pp. 5413–5422. doi: 10.4049/JIMMUNOL.180.8.5413.

Kobayashi, K. *et al.* (2002) 'RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems', *Nature 2002 416:6877*. Nature Publishing Group, 416(6877), pp. 194–199. doi: 10.1038/416194a.

Koivisto, A. *et al.* (2018) 'TRPA1 Antagonists for Pain Relief', *Pharmaceuticals*. Multidisciplinary Digital Publishing Institute (MDPI), 11(4). doi: 10.3390/PH11040117.

Komander, D. *et al.* (2008) 'The Structure of the CYLD USP Domain Explains Its Specificity for Lys63-Linked Polyubiquitin and Reveals a B Box Module', *Molecular Cell*, 29(4), pp. 451–464. doi: 10.1016/j.molcel.2007.12.018.

Komander, D. *et al.* (2009) 'Molecular discrimination of structurally equivalent Lys 63-linked and linear polyubiquitin chains', *EMBO Reports*. Nature Publishing Group, 10(5), pp. 466–473. doi: 10.1038/embor.2009.55.

Komander, D., Clague, M. J. and Urbé, S. (2009) 'Breaking the chains: Structure and function of the deubiquitinases', *Nature Reviews Molecular Cell Biology*. Nature Publishing Group, pp. 550–563. doi: 10.1038/nrm2731.

Komander, D. and Rape, M. (2012) 'The Ubiquitin Code', *Annual Review of Biochemistry*. Annual Reviews, 81(1), pp. 203–229. doi: 10.1146/annurev-biochem-060310-170328.

Krieg, A. *et al.* (2009) 'XIAP mediates NOD signaling via interaction with RIP2', *Proceedings of the National Academy of Sciences of the United States of America*. Proc Natl Acad Sci U S A, 106(34), pp. 14524–14529. doi: 10.1073/PNAS.0907131106.

Kristariyanto, Y. A. *et al.* (2017) ' A single MIU motif of MINDY -1 recognizes K48-linked polyubiquitin chains ', *EMBO reports*. EMBO, 18(3), pp. 392–402. doi: 10.15252/embr.201643205.

Kulathu, Y. *et al.* (2009) 'Two-sided ubiquitin binding explains specificity of the TAB2 NZF domain', *Nature Structural & Molecular Biology 2009 16:12*. Nature Publishing Group, 16(12), pp. 1328–1330. doi: 10.1038/nsmb.1731.

Kulathu, Y. *et al.* (2013) 'Regulation of A20 and other OTU deubiquitinases by reversible oxidation', *Nature Communications*. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved., 4, p. 1569. doi: 10.1038/ncomms2567.

Kumari, S. *et al.* (2014) 'Sharpin prevents skin inflammation by inhibiting TNFR1-induced keratinocyte apoptosis', *eLife*, 3. doi: 10.7554/eLife.03422.

Kupka, S. *et al.* (2016) 'SPATA2-Mediated Binding of CYLD to HOIP Enables CYLD Recruitment to Signaling Complexes', *Cell Reports*. Elsevier, 16(9), pp. 2271–2280.

Laavola, M. *et al.* (2015) 'Pinosylvin and Monomethylpinosylvin, Constituents of an Extract from the Knot of Pinus sylvestris, Reduce Inflammatory Gene Expression and Inflammatory Responses in Vivo', *Journal of Agricultural and Food Chemistry*, 63(13), pp. 3445–3453. doi: 10.1021/jf504606m.

Lange, O. F. *et al.* (2008) 'Recognition dynamics up to microseconds revealed from an RDC-derived ubiquitin ensemble in solution', *Science*. American Association for the Advancement of Science, 320(5882), pp. 1471–1475. doi: 10.1126/science.1157092.

Laplantine, E. *et al.* (2009) 'NEMO specifically recognizes K63-linked poly-ubiquitin chains through a new bipartite ubiquitin-binding domain', *The EMBO Journal*. John Wiley & Sons, Ltd, 28(19), pp. 2885–2895. doi: 10.1038/emboj.2009.241.

Larsen, C. N., Krantz, B. A. and Wilkinson, K. D. (1998) 'Substrate specificity of deubiquitinating enzymes: Ubiquitin C-terminal hydrolases', *Biochemistry*. American Chemical Society , 37(10), pp. 3358–3368. doi: 10.1021/bi972274d.

Lechtenberg, B. C. *et al.* (2016) 'Structure of a HOIP/E2~ubiquitin complex reveals RBR E3 ligase mechanism and regulation', *Nature*, 529(7587), pp. 546–550.

Lee, I. and Schindelin, H. (2008) 'Structural Insights into E1-Catalyzed Ubiquitin Activation and Transfer to Conjugating Enzymes', *Cell*. Cell Press, 134(2), pp. 268–278. doi: 10.1016/j.cell.2008.05.046.

Lee, K. A. *et al.* (2013) 'Bacterial-derived uracil as a modulator of mucosal immunity and gut-microbe homeostasis in drosophila', *Cell*. Cell Press, 153(4), pp. 797–811. doi: 10.1016/j.cell.2013.04.009.

Lee, K. A. *et al.* (2015) 'Bacterial uracil modulates drosophila DUOX-dependent Gut immunity via hedgehog-induced signaling endosomes', *Cell Host and Microbe*. Cell Press, 17(2), pp. 191–204. doi: 10.1016/j.chom.2014.12.012.

Lee, K. A. *et al.* (2018) 'Inflammation-Modulated Metabolic Reprogramming Is Required for DUOX-Dependent Gut Immunity in Drosophila', *Cell Host and Microbe*. Cell Press, 23(3), pp. 338-352.e5. doi: 10.1016/j.chom.2018.01.011.

Lemaitre, B. *et al.* (1995) 'A recessive mutation, immune deficiency (imd), defines two distinct control pathways in the Drosophila host defense', *Proceedings of the National Academy of Sciences of the United States of America*. Proc Natl Acad Sci U S A, 92(21), pp. 9465–9469. doi: 10.1073/PNAS.92.21.9465.

Lemaitre, B. *et al.* (1996) 'The dorsoventral regulatory gene cassette spatzle/Toll/Cactus controls the potent antifungal response in Drosophila adults', *Cell*. Cell Press, 86(6), pp. 973–983. doi: 10.1016/S0092-8674(00)80172-5.

Lemaitre, B. and Hoffmann, J. (2007) 'The Host Defense of Drosophila melanogaster', *Annual Review of Immunology*, 25(1), pp. 697–743. doi: 10.1146/annurev.immunol.25.022106.141615.

Lenkinski, R. E. *et al.* (1977) 'Nuclear magnetic resonance studies of the denaturation of ubiquitin', *BBA - Protein Structure*. Elsevier, 494(1), pp. 126–130. doi: 10.1016/0005-2795(77)90140-4.

Leulier, F. *et al.* (2006) 'The Drosophila inhibitor of apoptosis protein DIAP2 functions in innate immunity and is essential to resist gram-negative bacterial infection', *Molecular and cellular biology*, 26, pp. 7821–7831.

Levinger, L. and Varshavsky, A. (1980) 'High-resolution fractionation of nucleosomes: minor particles, "whiskers," and separation of mononucleosomes containing and lacking A24 semihistone.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 77(6), pp. 3244–3248. doi: 10.1073/pnas.77.6.3244.

Levinger, L. and Varshavsky, A. (1982) 'Selective arrangement of ubiquitinated and D1 protein-containing nucleosomes within the drosophila genome', *Cell*. Elsevier, 28(2), pp. 375–385. doi: 10.1016/0092-8674(82)90355-5.

Lhocine, N. *et al.* (2008) 'PIMS modulates immune tolerance by negatively regulating Drosophila innate immune signaling', *Cell host & microbe*. Cell Host Microbe, 4(2), pp. 147–158. doi: 10.1016/J.CHOM.2008.07.004.

Li, W. *et al.* (2008) 'Genome-wide and functional annotation of human E3 ubiquitin ligases identifies MULAN, a mitochondrial E3 that regulates the organelle's dynamics and signaling', *PLoS ONE*, 3(1), p. e1487. doi: 10.1371/journal.pone.0001487.

Lindsay, S. A., Lin, S. J. H. and Wasserman, S. A. (2018) 'Short-Form Bomanins Mediate Humoral Immunity in Drosophila', *Journal of Innate Immunity*. Karger Publishers, 10(4), p. 306. doi: 10.1159/000489831.

Lindsay, S. A. and Wasserman, S. A. (2014) 'Conventional and non-conventional Drosophila Toll signaling', *Developmental and Comparative Immunology*. doi: 10.1016/j.dci.2013.04.011.

Liu, G., Roy, J. and Johnson, E. A. (2006) 'Identification and function of hypoxia-response genes in Drosophila melanogaster', *Physiological Genomics*, 25(1). Available at: http://physiolgenomics.physiology.org/content/25/1/134.long (Accessed: 26 April 2017).

Liu, J. *et al.* (2017) 'Structural Insights into SHARPIN-Mediated Activation of HOIP for the Linear Ubiquitin Chain Assembly', *Cell reports*. Cell Rep, 21(1), pp. 27–36. doi: 10.1016/J.CELREP.2017.09.031.

Lo, Y. C. *et al.* (2009) 'Structural Basis for Recognition of Diubiquitins by NEMO', *Molecular Cell*, 33(5), pp. 602–615. doi: 10.1016/j.molcel.2009.01.012.

Lorick, K. L. *et al.* (1999) 'RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 96(20), pp. 11364–11369. doi: 10.1073/pnas.96.20.11364.

Lu, Y., Wu, L. P. and Anderson, K. V. (2001) 'The antibacterial arm of the Drosophila innate immune response requires an IκB kinase', *Genes and Development*. Cold Spring Harbor Laboratory Press, 15(1), pp. 104–110. doi: 10.1101/gad.856901.

Lund, P. K. *et al.* (1985) 'Nucleotide sequence analysis of a cDNA encoding human ubiquitin reveals that ubiquitin is synthesized as a precursor', *Journal of Biological Chemistry*, 260(12), pp. 7609–7613.

MacDuff, D. A. *et al.* (2015) 'Phenotypic complementation of genetic immunodeficiency by chronic herpesvirus infection', *eLife*. eLife Sciences Publications, Ltd, 4(4), p. 4494. doi: 10.7554/ELIFE.04494.

Marín, I. *et al.* (2004) 'Parkin and relatives: the RBR family of ubiquitin ligases', *Physiological Genomics*. American Physiological Society, 17(3), pp. 253–263. doi: 10.1152/physiolgenomics.00226.2003.

Martino, L. *et al.* (2018) 'Determinants of E2-ubiquitin conjugate recognition by RBR E3 ligases.', *Scientific reports*. Nature Publishing Group, 8(1), p. 68. doi: 10.1038/s41598-017-18513-5.

Maspero, E. *et al.* (2013) 'Structure of a ubiquitin-loaded HECT ligase reveals the molecular basis for catalytic priming.', *Nature structural & molecular biology*, 20(6), pp. 696–701. doi: 10.1038/nsmb.2566.

Matsuoka, K. and Kanai, T. (2015) 'The gut microbiota and inflammatory bowel disease', *Seminars in Immunopathology*. Springer Verlag, pp. 47–55. doi: 10.1007/s00281-014-0454-4.

McCullough, J. *et al.* (2006) 'Activation of the endosome-associated ubiquitin isopeptidase AMSH by STAM, a component of the multivesicular body-sorting machinery', *Current Biology*, 16(2), pp. 160–165. doi: 10.1016/j.cub.2005.11.073.

Medvedev, A. E. *et al.* (2015) 'E3 Ubiquitin Ligases Pellinos as Regulators of Pattern Recognition Receptor Signaling and Immune responses', *Immunological reviews*. NIH Public Access, 266(1), p. 109. doi: 10.1111/IMR.12298.

Meinander, A. *et al.* (2012) 'Ubiquitylation of the initiator caspase DREDD is required for innate immune signalling', *The EMBO journal*. The Breakthrough Toby Robins Breast Cancer Research Centre, Institute of Cancer Research, Chester Beatty Laboratories, London, UK.: Nature Publishing Group, 31(12), pp. 2770–2783. doi: 10.1038/emboj.2012.121 [doi].

Melcarne, C., Lemaitre, B. and Kurant, E. (2019) 'Phagocytosis in Drosophila: From molecules and cellular machinery to physiology', *Insect Biochemistry and Molecular Biology*. Elsevier Ltd, 109, pp. 1–12. doi: 10.1016/j.ibmb.2019.04.002.

Metzger, M. B. *et al.* (2014) 'RING-type E3 ligases: Master manipulators of E2 ubiquitin-conjugating enzymes and ubiquitination', *Biochimica et Biophysica Acta - Molecular Cell Research*, pp. 47–60. doi: 10.1016/j.bbamcr.2013.05.026.

Mevissen, T. E. T. *et al.* (2013) 'OTU deubiquitinases reveal mechanisms of linkage specificity and enable ubiquitin chain restriction analysis', *Cell*. Cell Press, 154(1), p. 169. doi: 10.1016/j.cell.2013.05.046.

Mevissen, T. E. T. and Komander, D. (2017) 'Mechanisms of Deubiquitinase Specificity and Regulation', *Annual Review of Biochemistry*. Annual Reviews, 86(1), pp. 159–192. doi: 10.1146/annurev-biochem-061516-044916.

Meyer, H. J. and Rape, M. (2014a) 'Enhanced protein degradation by branched ubiquitin chains', *Cell*. Cell Press, 157(4), pp. 910–921. doi: 10.1016/j.cell.2014.03.037.

Meyer, H. J. and Rape, M. (2014b) 'Enhanced Protein Degradation by Branched Ubiquitin Chains', *Cell*. Cell Press, 157(4), pp. 910–921. doi: 10.1016/J.CELL.2014.03.037.

Micheau, O. and Tschopp, J. (2003) 'Induction of TNF Receptor I-Mediated Apoptosis via Two Sequential Signaling Complexes', *Cell*. Cell Press, 114(2), pp. 181–190. doi: 10.1016/S0092-8674(03)00521-X.

Michel, T. *et al.* (2001) 'Drosophila Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein', *Nature*. Nature, 414(6865), pp. 756–759. doi: 10.1038/414756A.

Michelle, C. *et al.* (2009) 'What was the set of ubiquitin and ubiquitin-like conjugating enzymes in the eukaryote common ancestor?', *Journal of Molecular Evolution*, 68(6), pp. 616–628. doi: 10.1007/s00239-009-9225-6.

Miguel-Aliaga, I., Jasper, H. and Lemaitre, B. (2018) 'Anatomy and Physiology of the Digestive Tract of Drosophila melanogaster', *Genetics*. Oxford University Press, 210(2), p. 357. doi: 10.1534/GENETICS.118.300224.

Moilanen, L. J. *et al.* (2016) 'Pinosylvin Inhibits TRPA1-Induced Calcium Influx *In Vitro* and TRPA1-Mediated Acute Paw Inflammation *In Vivo*', *Basic & Clinical Pharmacology & Toxicology*. Wiley/Blackwell (10.1111), 118(3), pp. 238–242. doi: 10.1111/bcpt.12485.

Morett, E. and Bork, P. (1999) 'A novel transactivation domain in parkin', *Trends in Biochemical Sciences*. Elsevier Ltd, 24(6), pp. 229–231. doi: 10.1016/S0968-0004(99)01381-X.

Morgan, T. H. (1910) 'Sex Limited Inheritance in Drosophila Author (s): T . H . Morgan

Published by : American Association for the Advancement of Science Stable URL : http://www.jstor.org/stable/1635471', *Science*, 32(812), pp. 120–122.

Moulin, M. *et al.* (2012) 'IAPs limit activation of RIP kinases by TNF receptor 1 during development', *The EMBO Journal*, 31, pp. 1679–1691. doi: 10.1038/emboj.2012.18.

Munyaka, P. M. *et al.* (2016) 'Acute dextran sulfate sodium (DSS)-induced colitis promotes gut microbial dysbiosis in mice', *Journal of basic microbiology*. Wiley-VCH Verlag, 56(9), pp. 986–998. doi: 10.1002/jobm.201500726.

Myllymäki, H., Valanne, S. and Rämet, M. (2014) 'Imd Signaling Pathway Drosophila The The Drosophila Imd Signaling Pathway', *J Immunol References Northwestern Univ. on January*, 192(9), pp. 3455–3462. doi: 10.4049/jimmunol.1303309.

Nakazawa, S. *et al.* (2016) 'Linear ubiquitination is involved in the pathogenesis of optineurin-associated amyotrophic lateral sclerosis', *Nature Communications 2016 7:1*. Nature Publishing Group, 7(1), pp. 1–14. doi: 10.1038/ncomms12547.

Narbonne-Reveau, K., Charroux, B. and Royet, J. (2011) 'Lack of an Antibacterial Response Defect in Drosophila Toll-9 Mutant', *PLoS ONE*. Edited by A. Bergmann. Public Library of Science, 6(2), p. e17470. doi: 10.1371/journal.pone.0017470.

Nathan, J. A. *et al.* (2013) 'Why do cellular proteins linked to K63-polyubiquitin chains not associate with proteasomes?', *The EMBO Journal*. John Wiley & Sons, Ltd, 32(4), pp. 552–565. doi: 10.1038/emboj.2012.354.

Newton, K. and Dixit, V. M. (2012) 'Signaling in Innate Immunity and Inflammation', *Cold Spring Harbor Perspectives in Biology*. Cold Spring Harbor Laboratory Press, 4(3). doi: 10.1101/CSHPERSPECT.A006049.

Neyen, C. *et al.* (2012) 'Tissue- and Ligand-Specific Sensing of Gram-Negative Infection in Drosophila by PGRP-LC Isoforms and PGRP-LE', *The Journal of Immunology*. American Association of Immunologists, 189(4), pp. 1886–1897. doi: 10.4049/JIMMUNOL.1201022.

Ngadjeua, F. *et al.* (2013) 'Two-sided ubiquitin binding of NF-κB essential modulator (NEMO) zinc finger unveiled by a mutation associated with anhidrotic ectodermal dysplasia with immunodeficiency syndrome', *Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology, 288(47), pp. 33722–33737. doi: 10.1074/jbc.M113.483305.

Nicastro, G. *et al.* (2009) 'Josephin domain of ataxin-3 contains two distinct ubiquitin-binding sites', *Biopolymers*. John Wiley & Sons, Ltd, 91(12), pp. 1203–1214. doi: 10.1002/bip.21210.

Nilsson, J. *et al.* (2013) 'Polyglucosan body myopathy caused by defective ubiquitin ligase RBCK1', *Annals of Neurology*. John Wiley & Sons, Ltd, 74(6), pp. 914–919. doi: 10.1002/ANA.23963.

Noad, J. *et al.* (2017) 'LUBAC-synthesized linear ubiquitin chains restrict cytosol-invading bacteria by activating autophagy and NF-κB', *Nature Microbiology*. Nature Publishing Group, 2(7), pp. 1–10. doi: 10.1038/nmicrobiol.2017.63.

O'Donnell, M. A. *et al.* (2007) 'Ubiquitination of RIP1 regulates an NF-κB-independent cell death switch in TNF signaling', *Current biology : CB*. NIH Public Access, 17(5), p. 418. doi: 10.1016/J.CUB.2007.01.027.

O'Donnell, M. A. *et al.* (2011) 'CASPASE 8 inhibits programmed necrosis by processing CYLD', *Nature cell biology*. NIH Public Access, 13(12), p. 1437. doi: 10.1038/NCB2362.

Oda, H. *et al.* (2019) 'Second case of HOIP deficiency expands clinical features and defines inflammatory transcriptome regulated by LUBAC', *Frontiers in Immunology*. Frontiers Media S.A., 10(MAR), p. 479. doi: 10.3389/fimmu.2019.00479.

Ogura, Y. *et al.* (2001) 'A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease', *Nature*. Nature, 411(6837), pp. 603–606. doi: 10.1038/35079114.

Ogura, Y. *et al.* (2003) 'Expression of NOD2 in Paneth cells: a possible link to Crohn's ileitis', *Gut*. BMJ Publishing Group, 52(11), pp. 1591–1597. doi: 10.1136/GUT.52.11.1591.

Oikawa, D., Sato, Y., Ito, H., *et al.* (2020) 'Linear Ubiquitin Code: Its Writer, Erasers, Decoders, Inhibitors, and Implications in Disorders', *International Journal of Molecular Sciences*. Multidisciplinary Digital Publishing Institute, 21(9), p. 3381. doi: 10.3390/ijms21093381.

Oikawa, D., Sato, Y., Ohtake, F., *et al.* (2020) 'Molecular bases for HOIPINs-mediated inhibition of LUBAC and innate immune responses', *Communications Biology 2020 3:1*. Nature Publishing Group, 3(1), pp. 1–17. doi: 10.1038/s42003-020-0882-8.

Okayasu, I. *et al.* (1990) *A Novel Method in the Induction of Reliable Experimental Acute and Chronic Ulcerative Colitis in Mice*, *GASTROENTEROLOGY*.

Özkan, E., Yu, H. and Deisenhofer, J. (2005) 'Mechanistic insight into the allosteric activation of a ubiquitin-conjugating enzyme by RING-type ubiquitin ligases', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 102(52), pp. 18890–18895. doi: 10.1073/pnas.0509418102.

Özkaynak, E. *et al.* (1987) 'The yeast ubiquitin genes: a family of natural gene fusions.', *The EMBO Journal*, 6(5), pp. 1429–1439. doi: 10.1002/j.1460-2075.1987.tb02384.x.

Özkaynak, E., Finley, D. and Varshavsky, A. (1984) 'The yeast ubiquitin gene: Head-to-tail repeats encoding a polyubiquitin precursor protein', *Nature*, 312(5995), pp. 663–666. doi: 10.1038/312663a0.

Palmerini, V. *et al.* (2021) 'Drosophila TNFRs Grindelwald and Wengen bind Eiger with different affinities and promote distinct cellular functions', *Nature Communications 2021 12:1*. Nature Publishing Group, 12(1), pp. 1–12. doi: 10.1038/s41467-021-22080-9.

Paquette, N. *et al.* (2010) 'Caspase-Mediated Cleavage, IAP Binding, and Ubiquitination: Linking Three Mechanisms Crucial for Drosophila NF-κB Signaling', *Molecular Cell*. Division of Infectious Disease, Department of Medicine, University of Massachusetts Medical School, Worcester, MA 01605, USA.: Elsevier Ltd, 37(2), pp. 172–182. doi: 10.1016/j.molcel.2009.12.036.

Paredes, J. C. *et al.* (2011) 'Negative regulation by Amidase PGRPs shapes the drosophila antibacterial response and protects the Fly from innocuous infection', *Immunity*. Elsevier, 35(5), pp. 770–779. doi: 10.1016/J.IMMUNI.2011.09.018/ATTACHMENT/30EC8642-D321-40B0-B632-2891C58B1B1C/MMC1.PDF.

Pellegrini, E. *et al.* (2017) 'Structures of the inactive and active states of RIP2 kinase inform on the mechanism of activation', *PLOS ONE*. Public Library of Science, 12(5), p. e0177161. doi: 10.1371/JOURNAL.PONE.0177161.

Peltzer, N. *et al.* (2014) 'HOIP deficiency causes embryonic lethality by aberrant TNFR1-mediated endothelial cell death', *Cell Reports*. Elsevier, 9(1), pp. 153–165. doi: 10.1016/j.celrep.2014.08.066.

Peltzer, N. *et al.* (2018) 'LUBAC is essential for embryogenesis by preventing cell death and enabling haematopoiesis', *Nature*. Nature, 557(7703), pp. 112–117. doi: 10.1038/s41586-018-0064-8.

Peltzer, N., Darding, M. and Walczak, H. (2016) 'Holding RIPK1 on the Ubiquitin Leash in TNFR1 Signaling', *Trends in Cell Biology*. Elsevier Current Trends, pp. 445–461. doi: 10.1016/j.tcb.2016.01.006.

Peltzer, N. and Walczak, H. (2019) 'Cell Death and Inflammation – A Vital but Dangerous Liaison', *Trends in Immunology*. Elsevier Ltd, 40(5), pp. 387–402. doi: 10.1016/j.it.2019.03.006.

Perše, M. and Cerar, A. (2012) 'Dextran sodium sulphate colitis mouse model: Traps and tricks', *Journal of Biomedicine and Biotechnology*. doi: 10.1155/2012/718617.

Pickart, C. M. (2001) 'Mechanisms Underlying Ubiquitination', *Annual Review of Biochemistry*. Annual Reviews, 70(1), pp. 503–533. doi: 10.1146/annurev.biochem.70.1.503.

Pickart, C. M. and Rose, I. A. (1985) 'Functional heterogeneity of ubiquitin carrier proteins', *Journal of Biological Chemistry*, 260(3), pp. 1573–1581.

Piper, R. C., Dikic, I. and Lukacs, G. L. (2014) 'Ubiquitin-dependent sorting in endocytosis', *Cold Spring Harbor Perspectives in Biology*. Cold Spring Harbor Laboratory Press, 6(1). doi: 10.1101/cshperspect.a016808.

Plechanovov, A. *et al.* (2012) 'Structure of a RING E3 ligase and ubiquitin-loaded E2 primed for catalysis', *Nature*. Nature Publishing Group, 489(7414), pp. 115–120. doi: 10.1038/nature11376.

Plumed-Ferrer, C. *et al.* (2013) 'The antimicrobial effects of wood-associated polyphenols on food pathogens and spoilage organisms', *International Journal of Food Microbiology*, 164(1), pp. 99–107. doi: 10.1016/j.ijfoodmicro.2013.04.001.

Poch, H. l. cabrer. Y., Arribas, C. and Lzquierdo, M. (1990) 'Sequence of a Drosophila cDNA encoding a ubiquitin gene fusion to a 52-aa ribosomal protein tail', *Nucleic Acids Research*, 18(13), p. 3994. doi: 10.1093/nar/18.13.3994.

Poulsen, M. M. *et al.* (2015) 'Resveratrol and inflammation: Challenges in translating pre-clinical findings to improved patient outcomes', *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*. Elsevier, 1852(6), pp. 1124–1136. doi: 10.1016/J.BBADIS.2014.12.024.

Pruneda, J. N. *et al.* (2011) 'Ubiquitin in motion: Structural studies of the ubiquitin-conjugating enzyme~ubiquitin conjugate', *Biochemistry*. NIH Public Access, 50(10), pp. 1624–1633. doi: 10.1021/bi101913m.

Pruneda, J. N. *et al.* (2012) 'Structure of an E3:E2~Ub Complex Reveals an Allosteric Mechanism Shared among RING/U-box Ligases', *Molecular Cell*. Cell Press, 47(6), pp. 933–942. doi: 10.1016/j.molcel.2012.07.001.

Quideau, S. *et al.* (2011) 'Plant polyphenols: Chemical properties, biological activities, and synthesis', *Angewandte Chemie - International Edition*, pp. 586–621. doi: 10.1002/anie.201000044.

Rahighi, S. *et al.* (2009) 'Specific Recognition of Linear Ubiquitin Chains by NEMO Is Important for NF-κB Activation', *Cell*, 136(6), pp. 1098–1109. doi: 10.1016/j.cell.2009.03.007.

Rahighi, S. and Dikic, I. (2012) 'Selectivity of the ubiquitin-binding modules', *FEBS Letters*. No longer published by Elsevier, pp. 2705–2710. doi: 10.1016/j.febslet.2012.04.053.

Rämet, M. *et al.* (2002) 'Functional genomic analysis of phagocytosis and identification of a Drosophila receptor for E. coli', *Nature 2002 416:6881*. Nature Publishing Group, 416(6881), pp. 644–648. doi: 10.1038/nature735.

Redman, K. L. and Rechsteiner, M. (1989) 'Identification of the long ubiquitin extension as ribosomal protein S27a', *Nature*. Nature Publishing Group, 338(6214), pp. 438–440. doi: 10.1038/338438a0.

Van Der Reijden, B. A. *et al.* (1999) 'TRIADs: A new class of proteins with a novel cysteine-rich signature', *Protein Science*. Wiley, 8(7), pp. 1557–1561. doi: 10.1110/ps.8.7.1557.

Reiter, L. T. *et al.* (2001) 'A Systematic Analysis of Human Disease-Associated Gene Sequences In Drosophila melanogaster', *Genome Research*. Cold Spring Harbor Laboratory Press, 11(6), pp. 1114–1125. doi: 10.1101/GR.169101.

Rickard, J. A. *et al.* (2014) 'TNFR1-dependent cell death drives infammation in Sharpin-defcient mice', *eLife*. eLife Sciences Publications Ltd, 2014(3). doi: 10.7554/eLife.03464.

Rider, P. *et al.* (2017) 'Alarmins: Feel the Stress', *The Journal of Immunology*, 198(4), pp. 1395–1402. doi: 10.4049/jimmunol.1601342.

Ritorto, M. S. *et al.* (2014) 'Screening of DUB activity and specificity by MALDI-TOF mass spectrometry', *Nature Communications*. Nature Publishing Group, 5(1), pp. 1–11. doi: 10.1038/ncomms5763.

Rittinger, K. and Ikeda, F. (2017) 'Linear ubiquitin chains: enzymes, mechanisms and biology.', *Open biology*. The Royal Society, 7(4). doi: 10.1098/rsob.170026.

Rivière, C., Pawlus, A. D. and Mérillon, J. M. (2012) 'Natural stilbenoids: Distribution in the plant kingdom and chemotaxonomic interest in Vitaceae', *Natural Product Reports*, pp. 1317–1333. doi: 10.1039/c2np20049j.

Rivkin, E. *et al.* (2013) 'The linear ubiquitin-specific deubiquitinase gumby regulates angiogenesis', *Nature*, 498(7454), pp. 318–324. doi: 10.1038/nature12296.

Ronau, J. A., Beckmann, J. F. and Hochstrasser, M. (2016) 'Substrate specificity of the ubiquitin and Ubl proteases', *Cell Research*. Nature Publishing Group, pp. 441–456. doi: 10.1038/cr.2016.38.

Rothe, M. *et al.* (1995) 'The TNFR2-TRAF Signaling Complex Contains Two Novel Proteins Related to Baculoviral Inhibitor of Apoptosis Proteins', *Cell*, 83, pp. 1243–1252.

Routa, J. *et al.* (2017) 'Wood extractives of Finnish pine, spruce and birch-availability and optimal sources of compounds A literature review', *Natural resources and bioeconomy studie*, (73). Available at: http://urn.fi/URN:ISBN:978-952-326-495-3 (Accessed: 25 January 2022).

Rubartelli, A. and Sitia, R. (2009) 'Stress as an intercellular signal: the emergence of stress-associated molecular patterns (SAMP).', *Antioxidants & redox signaling*, 11(10), pp. 2621–2629. doi: 10.1089/ars.2009.2377.

Rubin, G. M. *et al.* (2000) 'Comparative Genomics of the Eukaryotes', *Science*, 287(5461), pp. 2204–2215.

Rutschmann, S. *et al.* (2000) 'Role of Drosophila IKKγ in a Toll-independent antibacterial immune response', *Nature Immunology*, 1(4), pp. 342–347. doi: 10.1038/79801.

Ryu, J.-H. *et al.* (2004) 'The homeobox gene Caudal regulates constitutive local expression of antimicrobial peptide genes in Drosophila epithelia', *Molecular and cellular biology*. Mol Cell Biol, 24(1), pp. 172–185. doi: 10.1128/MCB.24.1.172-185.2004.

Ryu, J. H. *et al.* (2006) 'An essential complementary role of NF-κB pathway to microbicidal oxidants in Drosophila gut immunity', *EMBO Journal*. European Molecular Biology Organization, 25(15), pp. 3693–3701. doi: 10.1038/sj.emboj.7601233.

Ryu, J. H. *et al.* (2008) 'Innate immune homeostasis by the homeobox gene caudal and commensal-gut mutualism in Drosophila', *Science (New York, N.Y.)*. Science, 319(5864), pp. 777–782. doi: 10.1126/SCIENCE.1149357.

Sasaki, K. and Iwai, K. (2015) 'Roles of linear ubiquitinylation, a crucial regulator of NF-κB and cell death, in the immune system', *Immunological Reviews*, 266(1), pp. 175–189. doi: 10.1111/imr.12308.

Sasaki, Y. *et al.* (2013) 'Defective immune responses in mice lacking LUBAC-mediated linear ubiquitination in B cells', *The EMBO Journal*. John Wiley & Sons, Ltd, 32(18), pp. 2463–2476. doi: 10.1038/EMBOJ.2013.184.

Sato, Y. *et al.* (2008) 'Structural basis for specific cleavage of Lys 63-linked polyubiquitin chains', *Nature*, 455(7211), pp. 358–362. doi: 10.1038/nature07254.

Sato, Y. *et al.* (2009) 'Structural basis for specific recognition of Lys 63-linked polyubiquitin chains by NZF domains of TAB2 and TAB3', *The EMBO Journal*, 28, pp. 3903–3909. doi: 10.1038/emboj.2009.345.

Sato, Y. *et al.* (2011) 'Specific recognition of linear ubiquitin chains by the Npl4 zinc finger (NZF) domain of the HOIL-1L subunit of the linear ubiquitin chain assembly complex', *Proceedings of the National Academy of Sciences of the United States of America*, 108(51), pp. 20520–20525. doi: 10.1073/pnas.1109088108.

Sato, Y. *et al.* (2015) 'Structures of CYLD USP with Met1-or Lys63-linked diubiquitin reveal mechanisms for dual specificity', *Nature Structural and Molecular Biology*. Nature Publishing Group, 22(3), pp. 222–229. doi: 10.1038/nsmb.2970.

Schaeffer, V. *et al.* (2014) 'Binding of OTULIN to the PUB Domain of HOIP Controls NF-κB Signaling', *Molecular Cell*, 54(3), pp. 349–361. doi: 10.1016/j.molcel.2014.03.016.

Schlesinger, D. H., Goldstein, G. and Niall, H. D. (1975) 'The Complete Amino Acid Sequence of Ubiquitin, an Adenylate Cyclase Stimulating Polypeptide Probably Universal in Living Cells', *Biochemistry*, 14(10), pp. 2214–2218. doi: 10.1021/bi00681a026.

Schlicher, L. *et al.* (2016) 'SPATA2 promotes CYLD activity and regulates TNF-induced NF-kappaB signaling and cell death', *EMBO reports*, 17(10), pp. 1485–1497. doi: 10.15252/embr.201642592.

Schneider, I. (1972) 'Cell lines derived from late embryonic stages of Drosophila melanogaster.', *Journal of Embryology and Experimental Morphology*. J Embryol Exp Morphol, 27(2), pp. 353–365. doi: 10.1242/dev.27.2.353.

Schreiber, S., Nikolaus, S. and Hampe, J. (1998) 'Activation of nuclear factor kappa B inflammatory bowel disease', *Gut*. Gut, 42(4), pp. 477–484. doi: 10.1136/GUT.42.4.477.

Scott, D. C. *et al.* (2016) 'Two Distinct Types of E3 Ligases Work in Unison to Regulate Substrate Ubiquitylation.', *Cell*. Cell Press, 166(5), pp. 1198-1214.e24. doi: 10.1016/j.cell.2016.07.027.

Sen, R. and Baltimore, D. (1986) 'Multiple nuclear factors interact with the immunoglobulin enhancer sequences', *Cell*. Cell, 46(5), pp. 705–716. doi: 10.1016/0092-8674(86)90346-6.

Seymour, R. E. *et al.* (2007) 'Spontaneous mutations in the mouse Sharpin gene result in multiorgan inflammation, immune system dysregulation and dermatitis', *Genes & Immunity 2007 8:5*. Nature Publishing Group, 8(5), pp. 416–421. doi: 10.1038/sj.gene.6364403.

Shih, Susan C *et al.* (2000) *Monoubiquitin carries a novel internalization signal that is appended to activated receptors*, *The EMBO Journal*.

Shimizu, Y., Taraborrelli, L. and Walczak, H. (2015) 'Linear ubiquitination in immunity', *Immunological Reviews*, 266(1), pp. 190–207. doi: 10.1111/imr.12309.

Shin, N. R., Whon, T. W. and Bae, J. W. (2015) 'Proteobacteria: Microbial signature of dysbiosis in gut microbiota', *Trends in Biotechnology*. Elsevier Ltd, pp. 496–503. doi: 10.1016/j.tibtech.2015.06.011.

Shu, H. D., Takeuchi, M. and Goeddel, D. V. (1996) 'The tumor necrosis factor receptor 2 signal transducers TRAF2 and c-IAP1 are components of the tumor necrosis factor receptor 1 signaling complex', *Proceedings of the National Academy of Sciences*. National Academy of Sciences, 93(24), pp. 13973–13978. doi: 10.1073/PNAS.93.24.13973.

Silverman, N. *et al.* (2000) 'A Drosophila IκB kinase complex required for relish cleavage and antibacterial immunity', *Genes and Development*, 14(19), pp. 2461–2471. doi: 10.1101/gad.817800.

Silverman, N. *et al.* (2003) 'Immune activation of NF-kappaB and JNK requires Drosophila TAK1.', *The Journal of biological chemistry*. Elsevier, 278(49), pp. 48928–48934. doi: 10.1074/JBC.M304802200/ATTACHMENT/005F6512-779F-463F-BC66-0E95A2A84234/MMC1.PDF.

Smit, J. J. *et al.* (2012) 'The E3 ligase HOIP specifies linear ubiquitin chain assembly through its RING-IBR-RING domain and the unique LDD extension', *The EMBO Journal*, 31. doi: 10.1038/emboj.2012.217.

Souza Monteiro de Araujo, D. *et al.* (2020) 'TRPA1 as a therapeutic target for nociceptive pain', *Expert Opinion on Therapeutic Targets*, pp. 997–1008. doi: 10.1080/14728222.2020.1815191.

Stafford, C. A. *et al.* (2018) 'IAPs Regulate Distinct Innate Immune Pathways to Co-ordinate the Response to Bacterial Peptidoglycans', *Cell Reports*, 22(6), pp. 1496–1508. doi: 10.1016/j.celrep.2018.01.024.

Stewart, M. D. *et al.* (2016) 'E2 enzymes: More than just middle men', *Cell Research*. Nature Publishing Group, pp. 423–440. doi: 10.1038/cr.2016.35.

Stieglitz, B. *et al.* (2012) 'LUBAC synthesizes linear ubiquitin chains via a thioester intermediate', *Nature Publishing Group*, 13. doi: 10.1038/embor.2012.105.

Stieglitz, B. *et al.* (2013) 'Structural basis for ligase-specific conjugation of linear ubiquitin chains by HOIP', *Nature*, 503(7476), pp. 422–426. doi: 10.1038/nature12638.

Storer, A. and Ménard, R. (1994) 'Catalytic mechanism in papain family of cysteine peptidases', *Methods in Enzymology*. Academic Press, 244(C), pp. 486–500. doi: 10.1016/0076-6879(94)44035-2.

Stöven, S. *et al.* (2003) 'Caspase-mediated processing of the drosophila NF-κB factor relish', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 100(10), pp. 5991–5996. doi: 10.1073/pnas.1035902100.

Sun, S. C. (2017) 'The non-canonical NF-κB pathway in immunity and inflammation', *Nature reviews. Immunology*. Nat Rev Immunol, 17(9), pp. 545–558. doi: 10.1038/NRI.2017.52.

Swatek, K. N. and Komander, D. (2016) 'Ubiquitin modifications', *Cell Research*. Nature Publishing Group, pp. 399–422. doi: 10.1038/cr.2016.39.

Taillebourg, E. *et al.* (2012) 'The deubiquitinating enzyme USP36 controls selective autophagy activation by ubiquitinated proteins', *Autophagy*. doi: 10.4161/auto.19381.

Takehana, A. *et al.* (2004) 'Peptidoglycan recognition protein (PGRP)-LE and PGRP-LC act synergistically in Drosophila immunity', *EMBO Journal*, 23(23), pp. 4690–4700. doi: 10.1038/sj.emboj.7600466.

Takiuchi, T. *et al.* (2014) 'Suppression of LUBAC-mediated linear ubiquitination by a specific interaction between LUBAC and the deubiquitinases CYLD and OTULIN', *Genes to Cells*, 19(3), pp. 254–272. doi: 10.1111/gtc.12128.

Taniguchi, K. and Karin, M. (2018) 'NF-κB, inflammation, immunity and cancer: coming of age', *Nature Reviews Immunology 2018 18:5*. Nature Publishing Group, 18(5), pp. 309–324. doi: 10.1038/nri.2017.142.

Tauszig, S. *et al.* (2000) 'Toll-related receptors and the control of antimicrobial peptide expression in Drosophila', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 97(19), pp. 10520–10525. doi: 10.1073/pnas.180130797.

Tenno, T. *et al.* (2004) 'Structural basis for distinct roles of Lys63- and Lys48-linked polyubiquitin chains', *Genes to Cells*. John Wiley & Sons, Ltd, 9(10), pp. 865–875. doi: 10.1111/j.1365-2443.2004.00780.x.

Thevenon, D. *et al.* (2009) 'The Drosophila Ubiquitin-Specific Protease dUSP36/Scny Targets IMD to Prevent Constitutive Immune Signaling', *Cell Host and Microbe*. doi:

10.1016/j.chom.2009.09.007.
Tigno-Aranjuez, J. T. *et al.* (2014) 'In Vivo Inhibition of RIPK2 Kinase Alleviates Inflammatory Disease', *Journal of Biological Chemistry*. Elsevier, 289(43), pp. 29651–29664. doi: 10.1074/JBC.M114.591388.
Tokunaga, F. *et al.* (2009) 'Involvement of linear polyubiquitylation of NEMO in NF-κB activation', *Nature Cell Biology*. Nature Publishing Group, 11(2), pp. 123–132. doi: 10.1038/ncb1821.
Tokunaga, F. *et al.* (2011) 'SHARPIN is a component of the NF-κB-activating linear ubiquitin chain assembly complex', *Nature*, 471(7340), pp. 633–636. doi: 10.1038/nature09815.
Tokunaga, F. *et al.* (2012) 'Specific recognition of linear polyubiquitin by A20 zinc finger 7 is involved in NF-κB regulation', *EMBO Journal*, 31(19), pp. 3856–3870. doi: 10.1038/emboj.2012.241.
Tokunaga, F. and Iwai, K. (2009) 'Involvement of LUBAC-mediated linear polyubiquitination of NEMO in NF-kappaB activation', *Tanpakushitsu kakusan koso. Protein, nucleic acid, enzyme*, pp. 635–642.
Tseng, P. H. *et al.* (2010) 'Different modes of ubiquitination of the adaptor TRAF3 selectively activate the expression of type I interferons and proinflammatory cytokines', *Nature immunology*. Nat Immunol, 11(1), pp. 70–75. doi: 10.1038/NI.1819.
Tsichritzis, T. *et al.* (2007) 'A Drosophila ortholog of the human cylindromatosis tumor suppressor gene regulates triglyceride content and antibacterial defense', *Development*, 134(14), pp. 2605–2614. doi: 10.1242/dev.02859.
Tzou, P. *et al.* (2000) 'Tissue-Specific Inducible Expression of Antimicrobial Peptide Genes in Drosophila Surface Epithelia', *Immunity*. Cell Press, 13(5), pp. 737–748. doi: 10.1016/S1074-7613(00)00072-8.
Ugur, B., Chen, K. and Bellen, H. J. (2016) 'Drosophila tools and assays for the study of human diseases', *Disease Models & Mechanisms*. The Company of Biologists, 9(3), pp. 235–244. doi: 10.1242/DMM.023762.
Valanne, S., Wang, J.-H. and Rämet, M. (2011) 'The Drosophila Toll Signaling Pathway', *The Journal of Immunology*. The American Association of Immunologists, 186(2), pp. 649–656. doi: 10.4049/jimmunol.1002302.
Välimaa, A. L. *et al.* (2007) 'Antimicrobial and cytotoxic knotwood extracts and related pure compounds and their effects on food-associated microorganisms', *International Journal of Food Microbiology*. Elsevier, 115(2), pp. 235–243. doi: 10.1016/j.ijfoodmicro.2006.10.031.
Vandenabeele, P. *et al.* (2010) 'Molecular mechanisms of necroptosis: An ordered cellular explosion', *Nature Reviews Molecular Cell Biology*. Nature Publishing Group, 11(10), pp. 700–714. doi: 10.1038/nrm2970.
Varfolomeev, E. *et al.* (2008) 'c-IAP1 and c-IAP2 Are Critical Mediators of Tumor Necrosis Factor α (TNFα)-induced NF-κB Activation', *The Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology, 283(36), p. 24295. doi: 10.1074/JBC.C800128200.
Venken, K. J. T. and Bellen, H. J. (2007) 'Transgenesis upgrades for Drosophila melanogaster', *Development*. The Company of Biologists, 134(20), pp. 3571–3584. doi: 10.1242/DEV.005686.
Verdecia, M. A. *et al.* (2003) 'Conformational flexibility underlies ubiquitin ligation mediated by the WWP1 HECT domain E3 ligase', *Molecular Cell*. Cell Press, 11(1), pp. 249–259. doi: 10.1016/S1097-2765(02)00774-8.
Verhelst, K. *et al.* (2012) 'A20 inhibits LUBAC-mediated NF-κB activation by binding linear polyubiquitin chains via its zinc finger 7', *EMBO Journal*, 31(19), pp. 3845–3855. doi: 10.1038/EMBOJ.2012.240.
Vidai, S. *et al.* (2001) 'Mutations in the Drosophila dTAK1 gene reveal a conserved function for MAPKKKs in the control of rel/NF-κB-dependent innate immune responses', *Genes & Development*. Cold Spring Harbor Laboratory Press, 15(15), pp. 1900–1912. doi: 10.1101/GAD.203301.
Vijay-Kumar, S. *et al.* (1985) 'Three-dimensional structure of ubiquitin at 2.8 A resolution.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 82(11), pp. 3582–3585. doi: 10.1073/pnas.82.11.3582.

Vijay-kumar, S., Bugg, C. E. and Cook, W. J. (1987) 'Structure of ubiquitin refined at 1.8 Å resolution', *Journal of Molecular Biology*. Academic Press, 194(3), pp. 531–544. doi: 10.1016/0022-2836(87)90679-6.

Vlisidou, I. and Wood, W. (2015) '*Drosophila* blood cells and their role in immune responses', *FEBS Journal*. Blackwell Publishing Ltd, 282(8), pp. 1368–1382. doi: 10.1111/febs.13235.

Wagner, S. *et al.* (2008) 'Ubiquitin binding mediates the NF-κB inhibitory potential of ABIN proteins', *Oncogene 2008 27:26*. Nature Publishing Group, 27(26), pp. 3739–3745. doi: 10.1038/sj.onc.1211042.

Wagner, S. A. *et al.* (2016) 'SPATA2 links CYLD to the TNF-α receptor signaling complex and modulates the receptor signaling outcomes.', *The EMBO journal*. European Molecular Biology Organization, 35(17), pp. 1868–84. doi: 10.15252/embj.201694300.

Walczak, H. (2011) 'TNF and ubiquitin at the crossroads of gene activation, cell death, inflammation, and cancer', *Immunological Reviews*. doi: 10.1111/j.1600-065X.2011.01066.x.

Walden, H. and Rittinger, K. (2018) 'RBR ligase-mediated ubiquitin transfer: A tale with many twists and turns', *Nature Structural and Molecular Biology*. Nature Publishing Group, 25(6), pp. 440–445. doi: 10.1038/s41594-018-0063-3.

Wang, K. *et al.* (2013) 'Whole-genome DNA/RNA sequencing identifies truncating mutations in RBCK1 in a novel Mendelian disease with neuromuscular and cardiac involvement', *Genome Medicine*. BioMed Central, 5(7), pp. 1–8. doi: 10.1186/GM471/FIGURES/3.

Wang, L., Du, F. and Wang, X. (2008) 'TNF-α Induces Two Distinct Caspase-8 Activation Pathways', *Cell*. Cell Press, 133(4), pp. 693–703. doi: 10.1016/J.CELL.2008.03.036.

Wang, X., Herr, R. A. and Hansen, T. H. (2012) 'Ubiquitination of Substrates by Esterification', *Traffic*. John Wiley & Sons, Ltd, 13(1), pp. 19–24. doi: 10.1111/J.1600-0854.2011.01269.X.

van Well, E. M. *et al.* (2019) 'A protein quality control pathway regulated by linear ubiquitination', *The EMBO Journal*. EMBO, 38(9). doi: 10.15252/embj.2018100730.

Wenzel, D. M. *et al.* (2011) 'UBCH7 reactivity profile reveals parkin and HHARI to be RING/HECT hybrids', *Nature*. Nature Publishing Group, 474(7349), pp. 105–108. doi: 10.1038/nature09966.

Werner, T. *et al.* (2000) 'A family of peptidoglycan recognition proteins in the fruit fly Drosophila melanogaster', *Proceedings of the National Academy of Sciences*. National Academy of Sciences, 97(25), pp. 13772–13777. doi: 10.1073/PNAS.97.25.13772.

Wiborg, O. *et al.* (1985) 'The human ubiquitin multigene family: some genes contain multiple directly repeated ubiquitin coding sequences.', *The EMBO Journal*. Wiley, 4(3), pp. 755–759. doi: 10.1002/j.1460-2075.1985.tb03693.x.

Van Wijk, S. J. L. *et al.* (2012) 'Molecular Cell Resource Fluorescence-Based Sensors to Monitor Localization and Functions of Linear and K63-Linked Ubiquitin Chains in Cells'. doi: 10.1016/j.molcel.2012.06.017.

Van Wijk, S. J. L. *et al.* (2017) 'Linear ubiquitination of cytosolic Salmonella Typhimurium activates NF-κB and restricts bacterial proliferation', *Nature Microbiology*. Nature Publishing Group, 2. doi: 10.1038/nmicrobiol.2017.66.

Winborn, B. J. *et al.* (2008) 'The deubiquitinating enzyme ataxin-3, a polyglutamine disease protein, edits Lys63 linkages in mixed linkage ubiquitin chains', *Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology, 283(39), pp. 26436–26443. doi: 10.1074/jbc.M803692200.

Wu, C. J. *et al.* (2006) 'NEMO is a sensor of lys 63-linked polyubiquitination and functions in NF-κB activation', *Nature Cell Biology*. Nature Publishing Group, 8(4), pp. 398–406. doi: 10.1038/ncb1384.

Wu, S. C. *et al.* (2012) 'Infection-induced intestinal oxidative stress triggers organ-to-organ immunological communication in Drosophila', *Cell Host and Microbe*. Cell Press, 11(4), pp. 410–417. doi: 10.1016/j.chom.2012.03.004.

Xu, P. *et al.* (2009) 'Quantitative Proteomics Reveals the Function of Unconventional Ubiquitin Chains in Proteasomal Degradation', *Cell*, 137(1), pp. 133–145. doi: 10.1016/j.cell.2009.01.041.

Yagi, H. *et al.* (2012) 'A non-canonical UBA-UBL interaction forms the linear-ubiquitin-chain assembly complex', *EMBO reports*, 13, pp. 462–468. doi: 10.1038/embor.2012.24.

Yagi, Y. *et al.* (2013) 'fat facets induces polyubiquitination of Imd and inhibits the innate immune response in Drosophila', *Genes to cells : devoted to molecular & cellular mechanisms*. Genes Cells, 18(11), pp. 934–945. doi: 10.1111/GTC.12085.

Yamaguchi, M. and Yoshida, H. (2018) 'Drosophila as a model organism', *Advances in Experimental Medicine and Biology*, 1076, pp. 1–10. doi: 10.1007/978-981-13-0529-0_1.

Yamanaka, K. *et al.* (2003) 'Identification of the ubiquitin–protein ligase that recognizes oxidized IRP2', *Nature Cell Biology 2003 5:4*. Nature Publishing Group, 5(4), pp. 336–340. doi: 10.1038/ncb952.

Yang, Y. *et al.* (2007) 'NOD2 pathway activation by MDP or Mycobacterium tuberculosis infection involves the stable polyubiquitination of Rip2', *The Journal of biological chemistry*. J Biol Chem, 282(50), pp. 36223–36229. doi: 10.1074/JBC.M703079200.

Ye, Y. and Rape, M. (2009) 'Building ubiquitin chains: E2 enzymes at work', *Nat Rev Mol Cell Biol*, 10(11), pp. 755–764. doi: 10.1038/nrm2780.

Yu, L. *et al.* (2013) 'Modulation of TRP channels by resveratrol and other stilbenoids.', *Molecular pain*. SAGE Publications, 9, p. 3. doi: 10.1186/1744-8069-9-3.

Zaidman-Rémy, A. *et al.* (2011) 'Drosophila Immunity: Analysis of PGRP-SB1 Expression, Enzymatic Activity and Function', *PLOS ONE*. Public Library of Science, 6(2), p. e17231. doi: 10.1371/JOURNAL.PONE.0017231.

Zhang, Q., Lenardo, M. J. and Baltimore, D. (2017) '30 Years of NF-κB: A Blossoming of Relevance to Human Pathobiology', *Cell*. NIH Public Access, pp. 37–57. doi: 10.1016/j.cell.2016.12.012.

Zheng, N. *et al.* (2000) 'Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin-protein ligases', *Cell*. Cell Press, 102(4), pp. 533–539. doi: 10.1016/S0092-8674(00)00057-X.

Zhou, Q. *et al.* (2016) 'Biallelic hypomorphic mutations in a linear deubiquitinase define otulipenia, an early-onset autoinflammatory disease', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 113(36), pp. 10127–10132. doi: 10.1073/pnas.1612594113.

Zhou, R. *et al.* (2005) 'The role of ubiquitination in Drosophila innate immunity', *Journal of Biological Chemistry*, 280(40), pp. 34048–34055. doi: 10.1074/jbc.M506655200.

Zhuang, Z. H. *et al.* (2006) 'Drosophila TAB2 is required for the immune activation of JNK and NF-kappaB', *Cellular signalling*. Cell Signal, 18(7), pp. 964–970. doi: 10.1016/J.CELLSIG.2005.08.020.

Zinngrebe, J. *et al.* (2014) 'Ubiquitin in the immune system', *EMBO Reports*, 15, pp. 28–45. doi: 10.1002/embr.201338025.

Zinngrebe, J. *et al.* (2016) '--LUBAC deficiency perturbs TLR3 signaling to cause immunodeficiency and autoinflammation', *The Journal of Experimental Medicine*, 213(12), pp. 2671–2689. doi: 10.1084/jem.20160041.

Anna Aalto

Met1-ubiquitination determines inflammatory signalling in *Drosophila melanogaster*

The innate immune response is a first line of defence against external and internal insults, such as pathogens, damaged cells and environmental irritants. One of the main inflammatory pathways activated in this response is the nuclear factor κ-light-chain enhancer of activated B cells (NF-κB) signalling pathway leading to activation of the transcription factor NF-κB. During basal conditions, this pathway is tightly regulated, as unwanted activation of NF-κB is associated with chronic inflammation and cancer progression. Post-translational modifications (PTMs), such as ubiquitination, play a key role in the regulation of the inflammatory NF-κB signalling. This thesis aims to bring more light into the ubiquitin-mediated regulation of the NF-κB signalling, with a particular focus on Met1-linked ubiquitination. By using the biological model organism fruit fly, *Drosophila melanogaster*, this thesis demonstrates that Met1-ubiquitination has a key role in activating the NF-κB upon pathogen infection and that it is involved in sterile inflammation. Additionally, this thesis highlights the benefits of investigating the innate immune responses in the fly and describes an optimised method/platform for inducing and detecting intestinal inflammation and for screening anti-inflammatory compounds, such as stilbenoid compounds.

ISBN 978-952-12-4207-6