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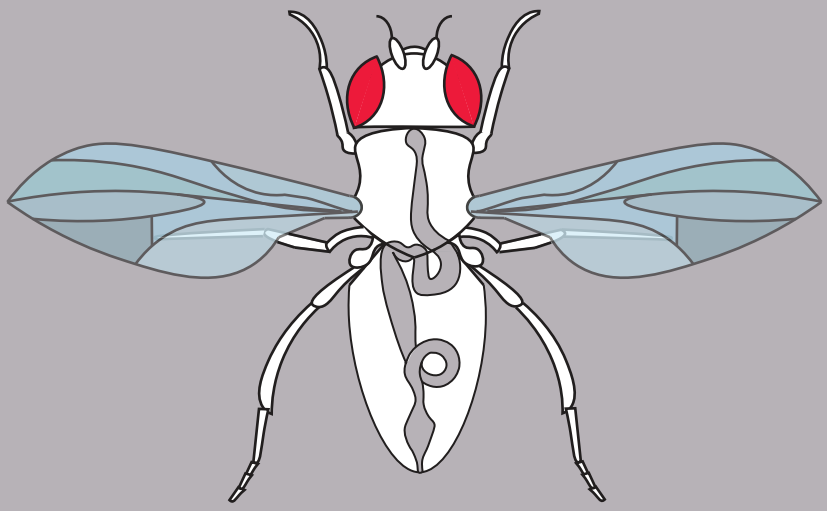
Regulation of Host-Microbe Interactions and Inflammatory Signalling in *Drosophila melanogaster*



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To my grandmother

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ABSTRACT

In order to eliminate harmful pathogens, while allowing beneficial microbes to persist, inflammatory signalling in the intestine needs to be carefully regulated. Overactive immune signalling can lead to chronic inflammation and an inflammatory environment is known to promote cancer development. The NF-κB family of transcription factors is a master regulator of inflammatory signalling and aberrant NF-κB signalling is characteristic for chronic inflammatory diseases, such as Crohn's disease and ulcerative colitis. As the inflammatory response constitutes a complex network in mammalian cells, we take advantage of using *Drosophila melanogaster*, with a far simpler immune system, as a model organism when studying inflammatory signalling. The aim of this thesis is to elucidate the regulation of intestinal inflammation during basal conditions and to advance the use of *Drosophila* as a platform for studying host-microbe interactions.

In order to investigate inflammatory regulation in the intestine, the fly microbiome needs to be manipulated. We, hence, started out by optimising a detailed protocol for rearing flies germ-free, or axenic. By carefully optimising the dechorionation of *Drosophila* embryos, sterile fly husbandry and validation of germ-free flies, we were able to successfully rear flies axenic in standard equipped laboratories. To further explore different avenues of modifying the microbiome, we, in a cross-disciplinary effort, designed antimicrobial mesoporous silica nanoparticles and characterised their antimicrobial properties. By using *Drosophila*, we were able to demonstrate *in vivo* antimicrobial activity of the designed particle against *Escherichia coli*, thereby, strengthening the use of *Drosophila* as a model in nanomedicine and drug development. Finally, to elucidate the regulation of inflammatory signalling in the intestine, we investigated the cellular regulation of *Drosophila* inhibitor of apoptosis 2 (Diap2), a potent inducer of NF-κB. We found a new role of the *Drosophila* caspase interleukin 1β-converting enzyme (Drice) as a regulator of inflammatory signalling in the fly gut. Drice acts by inducing the degradation of Diap2, thereby halting downstream NF-κB signalling. By studying the inflammatory phenotypes of the major immunological organs of the fly, we found that Drice acts specifically in the intestine, restraining inflammatory responses induced by commensal bacteria. In summary, the work in this thesis presents a new mode of inflammatory regulation in the *Drosophila* gut, and highlights the versatility of the fruit fly as a model organism. Due to well-conserved signalling pathways between mammals and *Drosophila*, research performed in the fly aids in understanding human inflammatory disease development and the interplay between human health, the microbiome and inflammatory signalling.

SAMMANFATTNING (ABSTRACT IN SWEDISH)

För att främja utvecklingen av symbiotiska förhållanden mellan mikrobiomet och värdorganismen men samtidigt skydda organismen mot sjukdomsframkallande bakterier, måste tarmens immunsignalering regleras noga. En överaktiv inflammationssignalering kan leda till kronisk inflammation och inflammationshärdar har visats gynna utvecklingen av cancerceller. NF-κB-transkriptionsfaktorer är nyckelkomponenter vid aktiveringen av inflammation och en rubbad NF-κB-signalering är kännetecknande för kroniska inflammationssjukdomar så som Crohns sjukdom och ulcerös kolit. Eftersom den inflammatoriska responsen utgör ett komplext nätverk i däggdjursceller, använder vi Drosophila melanogaster, eller bananflugan, med ett mycket enklare immunförsvar, som modellorganism vid inflammationsstudier. Målet med denna avhandling är att belysa de mekanismer som reglerar inflammationssignaleringen i tarmen under basala förhållanden och att främja användningen av Drosophila som en modell för att studera samverkan mellan värdorganismen och mikrobiomet.

För att studera hur inflammation regleras i tarmen måste flugans mikrobiom manipuleras. Vi började därmed med att optimera ett detaljerat protokoll som beskriver hur bananflugan kan odlas i sterila förhållanden, eller axeniskt. Genom att optimera dechorioneringen av flugembryon, upprätthållandet av flugor i sterila förhållanden, samt valideringen av axeniska flugor, lyckades vi erhålla sterila flugor i standardutrustade laboratorium. För att utforska andra sätt att manipulera flugans mikrobiom utvecklade vi i ett tvärvetenskapligt samarbete antimikrobiella mesoporösa kiseldioxid nanopartiklar och studerade deras antimikrobiella egenskaper. Med hjälp av Drosophila som modell kunde vi demonstrera antimikrobiell in vivo aktivitet hos nanopartiklarna mot Escherichia coli och därmed stärka rollen av Drosophila som modell vid utvecklingen av nanomedicin. Slutligen, för att utreda den cellulära regleringen av inflammation i flugans tarm på proteinnivå studerade vi hur proteinet Drosophila inhiberare av apoptos 2 (Diap2), en stark inducerare av NF-κB, regleras under basala förhållanden. Vi fann en ny roll för kaspaset Drosophila interleukin-1β-konverterande enzym (Drice) i moduleringen av inflammation och Diap2 i flugans tarm. Genom att inducera nedbrytningen av Diap2 hindrar Drice fortskridningen av den inflammatoriska signaleringen aktiverad av kommensaler. Vi har dessutom kunnat påvisa att Drice fungerar specifikt i tarmen och att avsaknad av Drice leder till kronisk tarminflammation, hyperproliferation och dysbios av tarmens mikroflora.

Sammanfattningsvis presenterar denna avhandling en ny kaspasmedierad mekanism vid regleringen av inflammation i bananflugans tarm och demonstrerar mångsidigheten hos Drosophila

som modellorganism vid studier beträffande mikrobiomet. Tack vare evolutionärt bevarade signaleringsräckor hos Drosophila och däggdjur bidrar forskning som erhållits i flugan till att förstå utvecklingen av inflammatoriska sjukdomar i människan samt samverkan mellan inflammation, mikrobiomet och människans välmående.

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. **Kietz**, **C**., Pollari, V., and Meinander, A. (2018) Generating germ-free *Drosophila* to study gut-microbe interactions: Protocol to rear *Drosophila* under axenic conditions. Current Protocols in Toxicology, 77, e52. doi: 10.1002/cptx.52

II. Karaman D.Ş., **Kietz**, **C***., Govardhanam, P*., Slita, A., Manea, A., Pamukçu, A., Meinander, A., Rosenholm, J.M. (2021) Core@shell structured ceria@mesoporous silica nanoantibiotics restrain bacterial growth *in vitro* and *in vivo*. *Manuscript.* *Equal contribution

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

PUBLICATIONS NOT INCLUDED IN THIS THESIS

Aalto, A.L*., Mohan, A.K*., Schwintzer, L., Kupka, S., **Kietz**, **C**., Walczak, H., Broemer, M., and Meinander, A. (2019). M1-linked ubiquitination by LUBEL is required for inflammatory responses to oral infection in *Drosophila.* Cell Death and Differentiation, 26, 860–876. doi: 10.1038/s41418-018-0164-x *Equal contribution

AUTHOR CONTRIBUTION

I. The author contributed to protocol design, experimental optimisation, analysing data and to writing the manuscript. The author and VP optimised how to rear flies axenic and validate axenity. VP optimised the antibiotic treatment of flies and performed qPCR. AM contributed to the design and the writing of the manuscript.

II. The author contributed to designing research, analysing data, to writing the manuscript, and performed the experimental research regarding the *in vivo* section of the manuscript, i.e., localisation of DiI-loaded nanoparticles in 3rd instar larvae and assessment of pathogen clearance upon nanoparticle and capsaicin treatment. DŞK and PG synthesised, characterized, loaded and imaged nanoparticles, measured capsaicin release and analysed morphology of bacteria. DŞK measured cerium release and assessed cytocompatibility of nanoparticles. AS and A. Manea measured the *in vitro* antibacterial activity of the nanoparticles. DŞK and AP analysed data. DŞK, JR and A. Meinander contributed to the design and writing of the manuscript.

III. The author contributed to designing research, analysing data, to writing the manuscript, and performed the following experimental procedures: Western blotting of S2 cell and fly lysates, qPCR of S2 cell and fly lysates, X-Gal staining of fly organs, immunofluorescence staining of fly midguts, 16S rRNA sequencing, caspase activity assay of fly midguts and S2 cells, transfections of S2 cells, ubiquitination assays of fly lysates and S2 cells, survival assays, pathogen clearance assays, assessment of cell viability and, reared flies under axenic conditions. AKM performed ubiquitin assays of S2 cells, VP reared flies under axenic conditions and performed qPCR, I-ET did survival assays and qPCR, and, PSR and AM performed survival assays. PSR, AM, PM designed experiments, analysed data and contributed to the writing of the manuscript.

ABBREVIATIONS

AFC	Axenic fly culture
AMP	Antimicrobial peptide
APAF-1	Adaptor protein apoptotic protease activating factor-1
ASC	Apoptosis-associated speck-like protein contain a caspase recruitment domain
BIR	Baculoviral IAP repeat
C2TA	Class II major histocompatibility complex transactivator
CA	Caspase activity assay
CARD	Caspase recruitment domain
CFU	Colony forming unit
c-FLIP	Cellular FLICE-like inhibitory protein
cIAP1/2	Cellular IAP1/2
COP-1	CARD-only protein-1
CrmA	Cowpox virus protein cytokine response modifier A
Cyld	Cylindromatosis
Damm	Death-associated molecule related to Mch2
DAMP	Danger-associated molecular patterns
DAP	Diaminopimelic acid
Dark	Death-associated APAF1-related killer
dBruce	*Drosophila* BIR repeat containing ubiquitin-conjugating enzyme
Dcp-1	*Drosophila* effector caspase-1
DD	Death domain
Decay	Death executioner caspase related to apopain/yama
DED	Death effector domain
DIABLO	Direct IAP-binding protein with low pI
Diap1/2	*Drosophila* iap1/2
Dif	Dorsal-related immunity factor
DISC	Death-induced signalling complex
Dredd	Death related ced-3/Nedd2-like caspase
Drice	*Drosophila* interleukin 1β-converting enzyme
Dronc	Death regulator Nedd2-like caspase
DSS	Dextran sulphate sodium
DUB	Deubiquitinating enzyme
Duox	*Drosophila* dual oxidase
dUsp36	*Drosophila* ubiquitin-specific protease 36
EC	Enterocyte
EB	Enteroblast
EEC	Enteroendocrine cell
FADD	Fas-associated death domain
GNBP1/3	Gram-negative binding protein 1/3
HET-E	Heterokaryon incompatibility gene E

Hid	Head involution defective
HOIL-1	Haem-oxidized IRP2 ubiquitin ligase-1
HOIP	HOIL-1-interacting protein
IAP	Inhibitor of apoptosis protein
IBD	Inflammatory bowel disease
IBM	IAP binding motif
IF	Immunofluorescence
IKK	IκB kinase
IκB	Inhibitor of κB
Imd	Immune deficiency
INCA	Inhibitory CARD
Ird5	Immune response deficient
ISC	Intestinal stem cell
JAK-STAT	Janus kinase signal transducer and activator of transcription
JNK	c-Jun terminal kinase
LRR	Leucine-rich repeat
LUBAC	Linear ubiquitin assembly complex
MAP	Mitogen-activated protein
MDP	Muramyl dipeptide
MSN	Mesoporous silica nanoparticles
NAIP	Neuronal apoptosis inhibitor protein
NEMO	NF-κB essential modifier
NF-κB	Nuclear factor κ-light-chain enhancer of activated B cells
NIK	NF-κB inducing kinase
NOD	Nucleotide-binding oligomerisation domain
NLR	NOD-like receptor
OTULIN	Ovarian tumour DUB with linear linkage specificity
PA	Pathogen clearance assay
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PGN	Peptidoglycan
PGRP	Peptidoglycan-recognition proteins
PRR	Pattern-recognition receptor
qPCR	Quantitative real-time polymerase chain reaction
RHD	Rel homology domain
RING	Really interesting new gene
RIPK1/2	Receptor interacting protein kinase 1/2
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
SA	Survival assay
SHARPIN	Shank-associated RH domain-interacting protein
SMAC	Second mitochondrial-derived activator of caspases
Strica	Serine/Threonine-rich caspase
TAB1/2/3	TAK1 binding protein 1/2/3

TAK1	Transformed growth factor β-activated kinase 1
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TP1	Telomerase-associated protein-1
TRAF	TNF receptor-associated factor
UA	Ubiquitin assay
UBA	Ubiquitin-associated domain
UBD	Ubiquitin binding domain
WB	Western blot
XIAP	X-linked IAP

INTRODUCTION

Vertebrate and invertebrate animals interact continuously with a diverse array of microbial communities hosted on their skin and mucosal surfaces (Qin et al., 2010). The interplay between host and microbiome affects several aspects of host physiology, ranging from proper immune system development and protection against pathogens, to efficient extraction of dietary energy and brain neurochemistry (Mohajeri et al., 2018). In humans, the intestinal epithelium is one of the largest interfaces for host-microbe interactions and the organ is being increasingly recognised for its role in human health and disease (Shreiner et al., 2015). To maintain intestinal homeostasis, harmful pathogens need to be eliminated, while simultaneously allowing for beneficial host-microbe interactions to be established. Furthermore, inflammation, apoptosis and regeneration need to be tightly controlled. Dysregulation of any of these cellular processes can lead to gastrointestinal infections, metabolic disorders, inflammatory bowel diseases (IBD) and to cancer (Garrett et al., 2010). A key player in the maintenance of intestinal immune homeostasis is the Nuclear factor κ-light-chain enhancer of activated B cells (NF-κB) family of transcription factors that regulates the expression of numerous inflammatory genes. Aberrant NF-κB signalling is characteristic for chronic inflammatory diseases, such as ulcerative colitis and Crohn's disease, which are both risk factors contributing to colon cancer (Kim and Chang, 2014). The activity of NF-κB is regulated at several steps, however, one of the most versatile modifiers of inflammatory NF-κB signalling is the highly conserved, posttranscriptional modification named ubiquitination (Wu et al., 2018). Ubiquitination entails the decoration of target proteins with single ubiquitin moieties or ubiquitin chains via a three-step enzymatic cascade (Komander and Rape, 2012). As ubiquitination is a highly versatile modification that can be rapidly induced or removed, ubiquitin and its regulators serve as interesting targets when tuning inflammatory signalling.

The fruit fly, *Drosophila melanogaster*, has been used as a model organism for over 100 years and has contributed greatly to advancements in the major fields of biology. As pathways regulating the inflammatory response in flies, such as the NF-κB signalling pathways, share a number of characteristics with those of mammals, and several anatomical features are conserved between vertebrate and invertebrate intestines, *Drosophila* serves as an attractive model when studying intestinal inflammation and its regulation. Furthermore, *Drosophila* harbours an easily manipulated microbiome with a far simpler composition than that of mammals, enabling detailed host-microbe studies to be carried out with relative ease in the fly.

This thesis aims to advance the knowledge of inflammatory regulation during host-microbe interactions and proposes a new immune-regulatory mechanism mediated by the caspase *Drosophila* interleukin 1β-converting enzyme (Drice). Drice maintains intestinal immune homeostasis by inducing the degradation of the NF-κB activating ubiquitin ligase *Drosophila* inhibitor of apoptosis 2 (Diap2), thereby halting harmful inflammatory signalling induced by commensal bacteria. This thesis aims, moreover, to further the use of *Drosophila* as a model for studying host-microbe interactions and as a platform for *in vivo* characterisation of antimicrobial nanoparticles.

REVIEW OF THE LITERATURE

1 *Drosophila melanogaster* as a model for studying intestinal host-microbe interactions

Progress in experimental biological sciences is driven by work in simpler systems, ranging from *in vitro* processes, studying purified biological material outside its natural context, to *in vivo* studies in model organisms (Matthews and Vossall, 2020). Concordantly, biological and medical research in the field of human pathology has relied heavily on the use of model organisms. In the case of intestinal biology and disease, mouse models have served as popular *in vivo* models due to their similarity to humans regarding anatomy and intestinal pathologies. However, as research involving mammals can be time-consuming and maintenance fees high, alternative invertebrate models are needed. The gastrointestinal tract of *Drosophila melanogaster* is reminiscent of the mammalian one, regarding both anatomical structures and epithelial cell composition. Furthermore, the fly and mammalian intestine display similar biological function, including food passage, digestion and nutrient absorption (Miguel-Aliaga et al., 2018, Lemaitre and Miguel-Aliaga, 2013). In combination with evolutionarily conserved signalling pathways, regulating intestinal development, regeneration and immunity, as well as an easily manipulated genome, *Drosophila* has emerged as an attractive model when studying gut physiology and host-microbe interactions (Apidianakis and Rahme, 2011).

1.1 *Drosophila* as a model organism

The first studies using *Drosophila* as a model organism dates back to the beginning of the 20th century, when the fly was used to study cross-breeding, sterility and genetics (Castle, 1906, Morgan, 1911, Sturtevant, 1959). Subsequent research performed in *Drosophila*, including the discovery of chromosomes and the function of genes, mutagenesis, and inheritance, enlightened our understanding of classical genetics, and laid down the foundations of genetics as a discipline and a tool for biological research (Kaufman, 2017). Later, with the advancement of molecular manipulations, studies using *Drosophila* have contributed greatly in elucidating the regulatory details of development, immunity and behavioural patterns. In 2000, the consortium of Berkeley *Drosophila* genome project and Celera Genomic *Drosophila melanogaster*, sequenced and assembled the *Drosophila melanogaster* genome (Adams et al., 2000, Myers et al., 2000). The genome encodes for approximately 14 000 genes on four chromosomes, of which three carry the majority of all genes (Adams et al., 2000, Myers et al., 2000). After the human genome project was finished a few years later, sequence analyses revealed the high

homologies between *Drosophila* and human genomes, thereby, strengthening the role of *Drosophila* as a model for understanding human biology and disease processes. Nearly 75% of human disease-related genes have been estimated to have functional orthologues in *Drosophila* (Reiter et al., 2001, Pandy and Nichols, 2011, Yamamoto et al., 2014), and several of these orthologues are expressed in the *Drosophila* tissues performing the same function as the mammalian equivalent (Chintapalli et al., 2007).

The *Drosophila* life cycle consists of four developmental stages: embryo, larva, pupa and adult, and lasts approximately nine days at 25°C (Figure 1). The embryo can be used in studies regarding pattern formation or cell fate determination, whereas the larva is commonly used for studying developmental and physiological processes, or behavioural patterns such as foraging (Yamaguchi and Yoshida, 2018). During the pupal stage, *Drosophila* undergoes metamorphosis. During this time the cells of the imaginal discs proliferate, differentiate and go through organogenesis to produce various adult tissues. Accordingly, the imaginal discs have been a valuable model when studying the genetics of tissue regeneration (Bergantiños et al., 2010). The adult *Drosophila* is a complex organism that is similar to mammals in many aspects. The adult fly brain consists of more than 100 000 neurons that mediate behavioural patterns such as circadian rhythm, learning, memory, feeding, aggression, courtship and grooming (Yamaguchi and Yoshida, 2018). Due to the advanced nervous system of *Drosophila*, the fly is used to study Alzheimer's and Parkinson's disease, and has emerged as a potent drug screening system for human neuropathologies (Pandy and Nichols, 2011). Furthermore, several of the adult fly's organs are functionally reminiscent to the mammalian ones, and the fly has served as a model in the study of the distinct pathologies of the heart, lung and kidney (Piazza and Wessells, 2011, Roeder et al., 2012, Helmstädter and Simons, 2017). *Drosophila* is additionally recognised as a powerful model for complex diseases such as diabetes and cancer (Graham and Pick, 2017, Mirzoyan et al., 2019). Finally, the high degree of conservation between the mammalian and *Drosophila* intestine, regarding biological function, such as food passage and digestion, cellular architecture and immune signalling, has made *Drosophila* a popular model for studying both intestinal health and disease progression (Miguel-Aliaga et al., 2018, Apidianakis and Rahme, 2011). Concordantly, research using *Drosophila* has contributed greatly to the characterisation of pathways regulating intestinal immunity, regeneration and homeostasis (Capo et al., 2019).

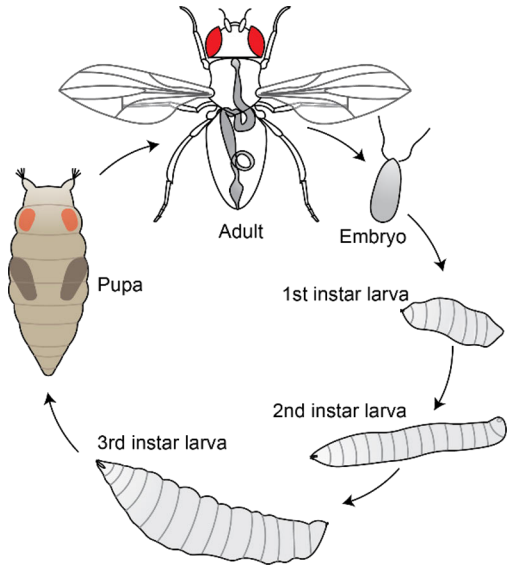

Figure 1. Life cycle and developmental stages of *Drosophila melanogaster*. *Drosophila* has four developmental stages: embryo, larva, pupa and adult. The larval stage is further subdivided into 1st, 2nd and 3rd instar larva. One life cycle, from fertilization to emergence of an adult fly, takes approximately nine days at 25°C. The gastrointestinal tract of the adult fly is visualised in grey.

1.2 The *Drosophila* digestive tract

The *Drosophila* digestive tract is a highly complex organ with its main functions in digestion, absorption and immunity. The fly gut impacts the activity of other organs by regulating numerous neuronal and endocrine signals modulating for instance, food uptake and nutrient storage. The intestinal epithelium serves, furthermore, as a key interface for host-microbe interactions that allows for symbiotic relationships to be established, while simultaneously acts as the first line of defence towards pathogens and protects the organism against external dangers (Miguel-Aliaga et al., 2018). In the following two sections, the anatomical structure of the *Drosophila* intestine and its cell types will be presented. Furthermore, key similarities and disparities between the mammalian and *Drosophila* intestine will be discussed.

1.2.1 Anatomical architecture of the *Drosophila* digestive tract

The *Drosophila* gut consists of a simple epithelium, surrounded by muscles, nerves and trachea, and is divided into foregut, midgut and hindgut (Figure 2) (Demerec, 1950, Shanbhag and Tripathi, 2009, Lemaitre and Miguel-Aliaga, 2013). The ectodermally derived foregut is further subdivided into oral cavity, oesophagus, crop and cardia and is

functionally reminiscent of the mammalian upper gastrointestinal tract. The *Drosophil*a foregut mediates physical and chemical processing of digested food, including degradation by enzymes released by the saliva (Miguel-Aliaga et al., 2018). The crop is a pouch-like structure connected to the foregut. A clear role of the *Drosophila* crop remains elusive, but studies conducted in other insects suggest a function in early digestion, microbial control and food storage (Stoffolano and Haselton, 2013). The cardia, also known as proventriculus, is a bulb-like structure shown to produce the protective intestinal peritrophic matrix and antimicrobial peptides (AMPs). The cardia may also act as a valve, regulating food-entry to the midgut (King, 1988, Tzou et al., 2000).

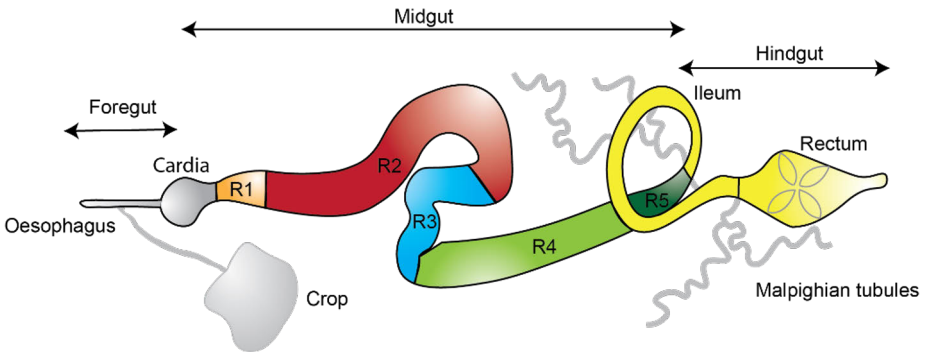

Figure 2. The *Drosophila* digestive tract. The *Drosophila* digestive tract is divided into foregut, midgut and hindgut. The foregut is subdivided into oesophagus, crop and cardia, and the midgut is subdivided into the five regions R1-R5, displaying distinct physiological properties. The hindgut is divided into ileum and rectum. On the border between midgut and hindgut the excretory Malpighian tubules discharge (figure adapted from Lemaitre and Miguel-Aliaga, 2013).

The endodermally derived midgut is located posterior to the cardia and is the principal site of enzymatic digestion and assimilation, with 349 putative digestive enzymes identified (Lemaitre and Miguel-Aliaga, 2013). The midgut occupies a large part of the *Drosophila* abdomen (Figure 1) and is subdivided into regions R1-R5, with specific digestive and metabolic functions (Figure 2) (Buchon et al., 2013, Marianes and Spradling, 2013). These five regions are separated by narrow epithelial boundaries and some of them are surrounded by a distinct set of muscles, suggesting a sphincter-like role in regulating the movement of food (Buchon et al., 2013). The posterior midgut is the most metabolically active and immune responsive region, and is analogous to the human small intestine. Whereas mammalian digestion occurs under acidic conditions, digestion in flies takes place mainly under neutral or basic conditions. Accordingly, the *Drosophila* gut is mainly neutral or mildly

alkaline, but some regions, for example the copper cell region (R3) and the hindgut are strongly acidic (Shanbhag and Tripathi, 2009). Genetic ablation of the copper cells or of the *Drosophila* V-ATPase, which mediates acidification of this region by H^+-pumping, leads to a higher abundance of gut microbiota, indicating an antibacterial role of the region (Overend et al., 2016, Li et al., 2016). In addition to the copper cell region, AMPs regulated by the Immune deficiency (Imd) signalling pathway and the *Drosophila* dual oxidase (Duox), generating reactive oxygen species (ROS), contribute greatly to intestinal immunity by shaping the abundance and composition of the microbiome (Broderick, 2016, Ha et al., 2005).

At the junction between midgut and the ectodermally derived hindgut, the Malpighian tubules, excretory organs equivalent of the vertebrate kidney, discharge (Figure 2) (Cohen et al., 2020). The hindgut corresponds functionally to the human colon (Micchelli and Perimon, 2006) and is subdivided into pylorus, a contractile sphincter connecting the midgut and hindgut, ileum and rectum (Figure 2) (Cohen et al., 2020, Miguel-Aliaga et al., 2018). The ileum and rectum, containing intricate epithelial infoldings called rectal papillae, mediate selective assimilation of water, ions and nutrients before excretion (Cohen et al., 2020).

The foregut and hindgut are lined with an impermeable cuticle, whereas the midgut is covered by a chitinous layer called the peritrophic membrane (Lehane, 1997, Hegedus et al., 2009). Underneath the peritrophic membrane, the intestinal epithelium is, furthermore, lined with an additional mucus layer (Figure 3) (Syed et al., 2008). The peritrophic membrane and the mucus layer serve a similar function as the human mucus layer, i.e., protecting the epithelial cells from harmful agents in the lumen (Hegedus et al., 2009). The epithelial cells are, furthermore, connected by septate junctions, a functional equivalent to tight junctions in the mammalian gut epithelium, separating the gut lumen from the body cavity and contributing to intestinal barrier function (Izumi et al., 2016, Izumi et al., 2019).

1.2.2 Cells of the *Drosophila* midgut

The adult *Drosophila* midgut epithelium contains three cell types: enterocytes (EC), which are large polyploid cells that secrete digestive enzymes and absorb nutrients, secretory enteroendocrine cells (EEC) and progenitor cells (Figure 3A, B). The progenitor cells can be further subdivided into intestinal stem cells (ISC) and undifferentiated ISC daughter cells, called enteroblasts (EB) (Miguel-Aliaga et al., 2018). EBs serve a similar function as the mammalian transit amplifying (TA) cells, giving rise to differentiated EECs and ECs (Figure 3A, B) (Miguel-Aliaga et al., 2018, Gehart and Clevers, 2019). The mammalian intestine contains, in addition to progenitor cells, absorptive ECs and secretory EECs, specialised absorptive M-cells and secretory Paneth, goblet and tuft cells,

however, no such specialised cells have been identified in the *Drosophila* gut (Van der Flier and Clevers, 2009, Ohno, 2016, Schneider et al., 2019). The *Drosophila* progenitor cells, scattered across the basal surface of the gut epithelium, are constantly replacing the cells of the midgut, renewing the midgut within one to two weeks under steady state conditions (Takashima et al., 2011, Micchelli and Perrimon, 2006, Ohlstein and Spradling, 2006). The activity of ISCs is influenced by the metabolic state of the fly, and by different external factors such as pathogenic bacteria or damage induced by corrosive agents (Amcheslavsky et al., 2009, Buchon et al., 2009a, O'Brien et al., 2011).

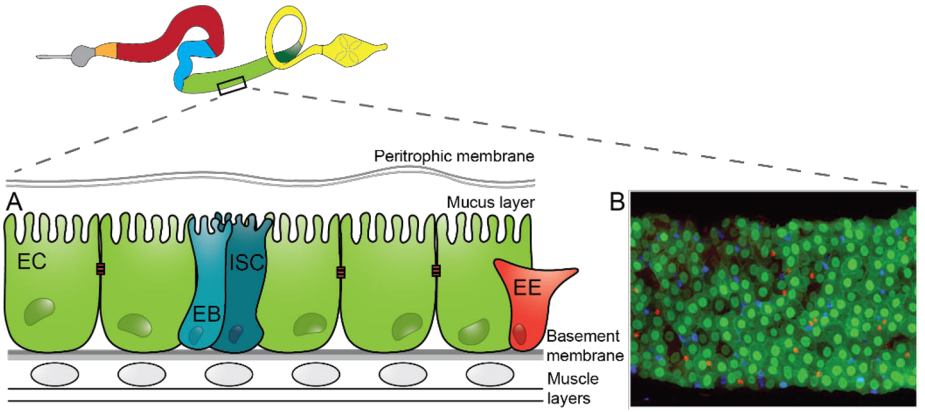

Figure 3. Cell types of the *Drosophila* gut. A) The intestinal epithelium consists of enterocytes (EC), progenitor cells (EB and ISC) and enteroendocrine cells (EEC) lined on the basement membrane. The epithelial cells are protected by a mucus layer and the peritrophic membrane. B) The different cell types of the *Drosophila* midgut can be visually distinguished using the cell markers escargot for ISCs and EBs (blue), prospero for EECs (red), and myo1A for ECs. (The fly line myo1A-GAL4 UAS-GFP/esg-lacZ was stained with anti-β-galactosidase and anti-prospero, Apidianakis and Rahme, 2011, figure adapted from Lemaitre and Miguel-Aliaga, 2013).

In contrast to the mammalian crypts of Lieberkühn, increasing the surface area of the intestine, extensive folding does not occur in the *Drosophila* gut (Allaire et al., 2018, Shanbhag and Tripathi, 2009). However, *Drosophila* ECs and ISCs do have cytoplasmic extensions, called microvilli, similar to the villi found in the human small intestine (Shanbhag and Tripathi, 2009). The epithelial layer, in both flies and humans, is aligned on the basal side on an extracellular collagenous matrix called the basement membrane (Figure 3A) (Sengupta and MacDonald, 2007). Underneath the basement membrane, circular muscles are present throughout the gut and an additional layer of longitudinal muscles surrounds the midgut (Figure 3A) (Hartenstein, 2005, Miguel-Aliaga et al.,

2018). These muscles are, contrary to the smooth muscles in the mammalian intestine, striated (Sandborn et al., 1967). A branched tracheal network overlies the musculature, supplying the gut with oxygen. The projections from the tracheal cells extrude through the visceral muscles allowing for close contact with the epithelial cells, and gas exchange (Li et al., 2013). Finally, the cells of the adult *Drosophila* gut are innervated by neurons known to regulate peristalsis, feeding behaviour and defecation (Cognigni et al., 2011, Olds and Xu, 2014, Zhang et al., 2014, Kuraishi et al., 2015). Little is, however, known regarding the effect of enteric neurons on absorption and digestion, and a possible neuronal modulation of the gut immune defence remains elusive.

1.3 The intestinal microbiome of *Drosophila*

In contrast to the human intestinal tract, estimated to host up to 100 trillion microbes from more than 1000 different taxa (Ley et al., 2006, Qin et al., 2010), a given laboratory fly population is usually associated with only four to eight aerobe or aerotolerant bacterial species. Probably due to the highly homogeneous laboratory fly food, containing antimicrobial preservatives, the regular transfer of flies to fresh food, and the limited influx of new bacterial species, flies sampled in the wild display a higher bacterial diversity than laboratory reared flies (Staubach et al., 2013). A robust feature of the gut microbiome of *Drosophila* is its variability and the bacterial composition of a single fly strain seems to vary over time. The growing consensus is that much of this variability is stochastic and is explained by the fact that the *Drosophila* gut is an open system where some microorganisms are lysed, passed through, or able to persist (Inamine et al., 2018, Obadia et al., 2017). During the different stages of the *Drosophila* life cycle, feeding behaviour and nutritional needs vary. This is reflected in the fluctuation of commensal bacterial load and changes in the dominant bacterial species during the life cycle (Bakula, 1969, Wong et al., 2011). As flies age, the overall load of resident bacteria increases. This is thought to be due to the age-related decrease in the efficiency of immune responses leading to the disturbance of gut homeostasis (Buchon et al., 2009b, Ren et al., 2007).

1.3.1 Composition of the *Drosophila* microbiome

Numerous studies have been conducted to identify the composition of the commensal bacteriome of the *Drosophila* gut (Erkosar et al., 2013, Brummel et al., 2004, Ren et al., 2007, Ridley et al., 2012, Ryu et al., 2008, Sharon et al., 2010, Storelli et al., 2011, Chandler et al., 2011, Wong et al., 2011). These studies have revealed that *Drosophila* is associated predominantly with bacterial species form the families *Lactobacillaceae* and *Enterococcaceae,* belonging to the phylum *Firmicutes*, and with

Acetobacteraceae and *Enterobacteriaceae* from the phylum *Proteobacteria*. On species level *Lactobacillus plantarum*, *Acetobacter pomorum*, *Lactobacillus brevis* and *Enterococcus faecalis* seem to associate frequently with flies (Erkosar et al., 2013). Largely due to intestinal barrier dysfunction and systemic immune responses, the bacterial composition changes as flies age, resulting in higher proportions of *Proteobacteria* (Clark et al., 2015). Interestingly, an increase in *Proteobacteria* has also been connected to inflammation in a genetic mouse model for colitis (Carvalho et al., 2012) and increased proportions of *Proteobacteria* versus *Firmicutes* is a signature of both IBD and aging in humans (Cheng et al., 2013, Clemente et al., 2012). The bacterial species *Wolbachia* (phylum: *Proteobacteria*) is an intracellular microbe harboured by a wide variety of arthropod, including *Drosophila*, hosts. *Wolbachia* is found in 40% of all insect species and a study done in 2005 revealed that approximately 30% of the *Drosophila* stocks housed at the Bloomington Stock Centre are infected with *Wolbachia* (Zug and Hammerstein, 2012, Clark et al., 2005). *Wolbachia* is found throughout somatic tissues and exists in the majority of hosts, including *Drosophila*, as an endosymbiont (Pietri et al., 2016).

Research on the composition of the *Drosophila* microbiome has focused predominantly on bacteria, however, yeasts, primarily from the phylum *Ascomycota*, are also commonly associated with the fly in nature (Chandler et al., 2012, Hamby et al., 2012). The relationship between yeast and *Drosophila* is considered to be mutualistic. Yeast can survive passage through the fly digestive tract, making *Drosophila* a vector for yeast dispersal (Coluccio et al., 2008) and can, besides serving as nutrition, affect both *Drosophila* physiology and behaviour (Anagnostou et al., 2010). A specific part of the fly immune system is devoted to recognition of fungal infection, indicating that flies are able to modulate and control yeast communities (Gottar et al., 2006, Lemaitre et al., 1996). In most studies investigating the role of yeast on *Drosophila* biology, baker's yeast, *Saccharomyces cerevisiae*, have been used. *S. cerevisiae* is, however, rarely found in wild populations of *Drosophila* species, and is, therefore, questioned as the best representative species when studying fly-yeast interactions (Hoang et al., 2015).

1.3.2 Role of specific bacteria on *Drosophila* physiology

By rearing flies germ-free, or axenic, the role of the fly microbiome has begun to be dissected. To investigate the role of a particular bacterial species on fly physiology researchers are using gnotobiotic flies, i.e., axenic flies re-introduced to specific bacteria. Studies have shown that lack of, or an altered microbiota impact larval development and energy homeostasis, as well as mating behaviour, locomotor behaviour, immune responses and lifespan of adult flies (Ridley et al., 2012, Shin et al., 2011,

Storelli et al., 2011, Sharon et al., 2010, Brummel et al., 2004, Schretter et al., 2018). Despite the inconsistency of the microbial composition in laboratory-reared *Drosophila*, the fly, similarly as higher organisms, display evolved symbiotic relationships with specific bacteria. For instance, Storelli and colleagues showed that *Lactobacillus plantarum* sufficiently on its own can promote larval development upon nutrient scarcity (Storelli et al., 2011) and Pais et al. revealed that stable colonisation of *Acetobacter thailandicus* in the *Drosophila* gut renders both host and bacteria with growth enhancing advantages (Pais et al., 2018).

The consequence of *Wolbachia* infections on *Drosophila* physiology remains largely uncharacterised, however, both beneficial and deleterious effects have been reported. The most well-studied effect of *Wolbachia* infections is a sperm-egg incompatibility during reproduction, referred to as cytoplasmic incompatibility (CI), which results in infected females maintaining a selective advantage over uninfected ones (Yen and Barr, 1973, Louis and Nigro, 1989). Apart from reproductive manipulation, *Wolbachia* has been shown to alter insulin signalling and, to some extent, be able to modify the commensal microbiome of *Drosophila*. *Wolbachia* infections have, furthermore, been shown to protect against RNA virus infections in the fruit fly (Teixeira et al., 2008, Ikeya et al., 2009, Simhadri et al., 2017).

The impact of the microbiome may result from direct effects of one bacterial species on its host or from indirect interactions between bacteria. Fast and colleagues have published a study, in which the interbacterial relationship between the pathogen *Vibrio cholerae* and the *Drosophila* symbiont *Acetobacter pasteurianus* was investigated. The study revealed that presence of *A. pasteurianus* intensifies the disease symptoms caused by *V. cholerae* and accelerates host death, whereas lack of *A. pasteurianus* relieves pathogenesis (Fast et al., 2018a). In another study, performed by Gould and colleagues, gnotobiotic flies were used in combinatorial experiments, where the effects of interactions between up to five bacterial species were studied. Interestingly, development and fecundity converged with higher bacterial diversity, whereas *Drosophila* lifespan and the composition of the microbiome seemed to be dependent on interactions between bacterial species (Gould et al., 2018).

To summarize, the generation of gnotobiotic flies has enabled researchers to dissect the effect of one, or a set of bacterial species on whole-organism physiology. As the *Drosophila* microbiome consists of aerobe and aerotolerant bacterial species (Cox and Gilmore, 2007), using the fly to study the effect of obligate anaerobes, which dominate the human intestine (Qin et al., 2010), is not suitable. The role of human aerobe, aerotolerant and facultative anaerobe bacteria and their metabolites on intestinal development, immunity and function can, on the

other hand, be explored (Liu et al., 2017). Furthermore, the *Drosophila* gut is well-suited for studying the effect of drugs on specific bacterial species, as well as elucidating the microbiota-induced interference of therapeutic drugs (Douglas, 2018).

2 Caspases and IAP proteins in cell signalling

Cell death and inflammation are fundamental cellular processes required for maintaining tissue homeostasis (Yang et al., 2015). Apoptotic activity often coincides with the inflammatory response, as apoptotic cells can stimulate inflammatory signalling and proinflammatory cytokines, such as Tumour necrosis factor α (TNFα), can activate cell death (Chen and Goeddel, 2002). Two families of proteins, namely caspases (cysteine-aspartic proteases) and Inhibitor of apoptosis proteins (IAPs), are central regulators of both inflammation and apoptosis (Van Opdenbosch and Lamkanfi, 2019, Budhidarmo and Day, 2015). In addition, these proteins regulate the activity of each other. Caspases are the key executors of apoptosis, although some have their main role in inflammatory signalling (Van Opdenbosch and Lamkanfi, 2019). IAPs are, on the other hand, and as their name dictates, potent inhibitors of apoptosis and act by modulating the activity of proapoptotic proteins, such as caspases. IAPs are, however, also important transduction intermediates in cellular signalling cascades, specifically during innate immune responses and NF-κB activation (Gyrd-Hansen and Meier, 2010, Kocab and Duckett, 2016). Due to the role of caspases and IAPs at the frontline of immunity and cell death, these proteins and their regulation have served as interesting targets when studying inflammatory disease and tissue homeostasis.

2.1 IAP proteins and their structure

IAP proteins were originally identified in the genomes of baculoviruses as proteins able to inhibit apoptosis in virus-infected insect cells (Clem et al., 1991). Since then, IAPs, characterised by the presence of one to three, approximately 70-amino acid long Baculoviral IAP repeat (BIR) domains (Crook et al., 1993, Birnbaum et al., 1994), have been identified in a wide range of organisms including yeast, worms, insects, fish and humans (Verhagen et al., 2001). The mammalian family of IAPs has eight members, of which X-linked IAP (XIAP), cellular IAP1 (cIAP1) and cellular IAP2 (cIAP2) are the most highly characterised ones. All three proteins harbour three BIR domains, a Ubiquitin-associated domain (UBA) and a C-terminal Really interesting new gene (RING) domain (Figure 4) (Budhidarmo and Day, 2015). Most BIR domains contain a surface groove that binds the N-terminus of specific sequences called IAP binding motifs (IBMs). IBMs are found in proapoptotic proteins such as in mammalian caspase-3, -7 and -9, in Second mitochondrial-derived activator of caspases (SMAC), also known as Direct IAP-binding protein with low pI (DIABLO) (Scott et al., 2005, Srinivasula et al., 2001), in the *Drosophila* caspases Drice and *Drosophila* effector caspase-1 (Dcp-1), as well as in the proapoptotic *Drosophila* proteins Head involution defective (Hid), Grim and Reaper (Zachariou et al., 2003, Tenev et al., 2005). By binding to the IBM domain

of these proteins, IAPs are able to modulate apoptotic signalling. The BIR-IBM interaction has, however, also been shown to play a role in immune signalling by functioning as a docking site promoting stability of signalling complexes (Paquette et al., 2010). Some BIR domains do not possess the IBM binding groove and convey other functions, such as dimerisation in the case of BIR1 of XIAP, and protein binding in the case of BIR1 of cIAP1 (Lu et al., 2007, Samuel et al., 2006).

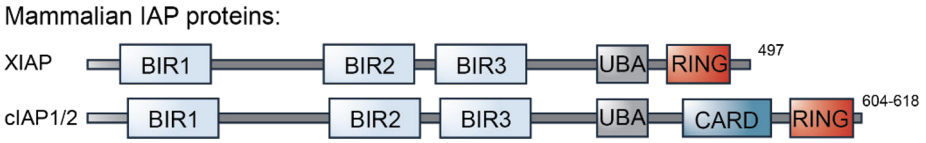

Figure 4. Protein domains of the most well-studied mammalian IAP-proteins. XIAP, cIAP1 and cIAP2 harbour three Baculoviral IAP repeat (BIR) domains, a Ubiquitin-associated (UBA) domain, and a C-terminal Really interesting new gene (RING) domain each. cIAP1 and cIAP2 carry in addition, a Caspase recruitment domain (CARD). The length of the proteins in amino acids is indicated to the right of the proteins (figure adapted from Budhidarmo and Day, 2015, Srinivasula and Ashwell, 2008).

The RING domain confers IAP proteins with E3 ubiquitin ligase activity (Deshaies and Joazeiro, 2009). E3 ligases constitute the final step, after E1 and E2, in the enzymatic cascade that is ubiquitination. The ubiquitin system is described in detail in section 2.2. IAP proteins can mediate their own, as well as the ubiquitination of their binding partners. Ubiquitination induced by IAPs has been shown to target substrates for proteasomal degradation, but also to activate, stabilise, and facilitate the recruitment of numerous proteins and protein complexes (Dumétier et al., 2020). The UBA domain is a member of the Ubiquitin binding domains (UBD) that recognise ubiquitin chains of different varieties in the cell, allowing binding to ubiquitinated proteins and, thereby, modulation of cell signalling (Gyrd-Hansen et al., 2008, Husnjak and Dikic, 2012). Interestingly, the UBA can also bind specific ubiquitin-charged E2s, implying a ubiquitination-promoting role of the domain (Budhidarmo and Day, 2014). In addition to their BIR, RING and UBA domains, cIAP1 and cIAP2 contain a Caspase recruitment domain (CARD) each (Figure 4) (Kocab and Duckett, 2016). The CARD domain regulates dimerisation needed for the E3 ligase activity of the cIAPs (Dueber et al., 2011, Lopez et al., 2011) and might allow for interaction with other CARD containing proteins, however, no such interaction partners have been found.

2.1.1 *Drosophila* IAP proteins

Drosophila encodes for four IAP proteins: *Drosophila* iap1 (Diap1), *Drosophila* iap2 (Diap2), Deterin and *Drosophila* BIR repeat containing ubiquitin-conjugating enzyme (dBruce) (Duckett et al., 1996, Jones et al., 2000, Vernooy et al., 2002). Diap1 carries two BIR domains and a C-terminal RING domain (Figure 5), and is the key inhibitor of apoptosis in *Drosophila*. Loss of Diap1 induces spontaneous caspase-mediated cell death, whereas gain-of-function mutants display abnormal growth phenotypes due to an excessive number of cells (Goyal et al., 2000, Lisi et al., 2000, Wang et al., 1999). Diap2 harbours three BIR domains, one UBA domain and a C-terminal RING domain (Figure 5) and is, thereby, on the basis of domain architecture, the closest homologue to mammalian IAPs. Although Diap2 is able to increase the apoptotic threshold of the cell, it has its main function in inflammatory NF-κB signalling (Ribeiro et al., 2007, Leulier et al., 2006a). Diap2 mutant flies succumb rapidly upon Gram-negative bacterial infection and are unable to induce expression of anti-inflammatory NF-κB target genes (Huh et al., 2007, Leulier 2006b, Gesellchen et al., 2005, Kleino et al., 2005). Deterin and dBruce harbour one BIR domain each, and have both been shown to suppress apoptosis when overexpressed (Jones et al., 2000, Vernooy et al., 2002). dBruce is vital during spermatid individualisation and absence of the IAP protein causes male sterility (Arama et al., 2003). Deterin and dBruce are, however, not as well characterised as Diap1 and Diap2, and their additional function as modulators of apoptosis or cell signalling remains obscure.

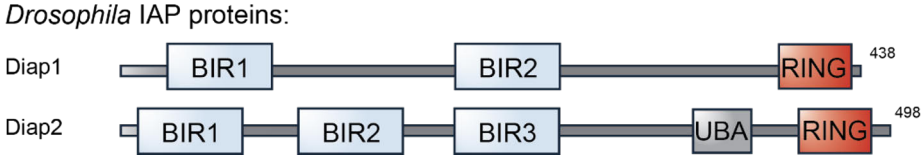

Figure 5. Protein domains of the most well-studied *Drosophila* IAPs. Diap2 harbours three Baculoviral IAP repeat (BIR) domains, a Ubiquitin-associated (UBA) domain, and a C-terminal Really interesting new gene (RING) domain. Diap1 harbours only two BIR domains and a RING domain. The length of the proteins in amino acids is indicated to the right of the proteins (figure adapted from Budhidarmo and Day, 2015, Srinivasula and Ashwell, 2008).

2.2 The ubiquitin system

Ubiquitination is a posttranslational modification catalysed by a three-step enzymatic process, in which proteins are decorated with single ubiquitin moieties or with polymeric chains made of several ubiquitin subunits (Komander and Rape, 2012). The ubiquitin moiety itself is a 76-

amino acid long protein, highly conserved throughout evolution. Ubiquitination is known to regulate a variety of cellular processes including protein degradation, endocytosis, autophagy, DNA repair, immunity and inflammation (Swatek and Komander, 2016). During ubiquitination, ubiquitin-activating enzymes, E1s, use ATP to generate a bond between their active site and the C-terminus of ubiquitin (Schulman and Harper, 2009), after which ubiquitin is transferred to the active site of a ubiquitin-conjugating enzyme, E2. The E2 enzyme, together with E3 ligases, add the ubiquitin moiety to the substrate, hence, generating a monoubiquitinated substrate (Figure 6A) (Ye and Rape, 2009, Deshaies and Joazeiro, 2009). Substrates monoubiquitinated on several sites are referred to as multi-monoubiquitinated. Additional ubiquitin moieties can be attached to the N-terminus (M1), or to one of seven lysine residues (K6, K11, K27, K29, K33, K48 and K63) on the first, substrate-attached ubiquitin, thereby, generating polyubiquitin chains. These chains can be of diverse length, and display different topology depending on the type of linkage between ubiquitin moieties (Figure 6B) (Komander and Rape, 2012). Adding to the complexity of ubiquitin modifications, ubiquitin chains can be homotypic or heterotypic. Ubiquitin moieties in homotypic chains are linked through the same residue, forming a chain of uniform topology, whereas heterotypic chains contain multiple linkage types, and are further subclassified as mixed or branched (Figure 6B) (French et al., 2021). The substrate specificity and site of ubiquitin modification are thought to be mediated by the E3 ligases, whereas the E2s play a role in determining the linkage type (Ye and Rape, 2009, Deshaies and Joazeiro, 2009). Differently ubiquitinated substrates are recognised by a variety of UBDs, binding specific types of ubiquitin chains, thereby, translating the ubiquitin modification into specific cellular outcomes (Figure 6A) (Husnjak and Dikic, 2012). Finally, ubiquitin chains and single moieties are continuously edited and removed by deubiquitinating enzymes, or DUBs (Komander et al., 2009a), making ubiquitination a highly dynamic cellular modification (Figure 6A).

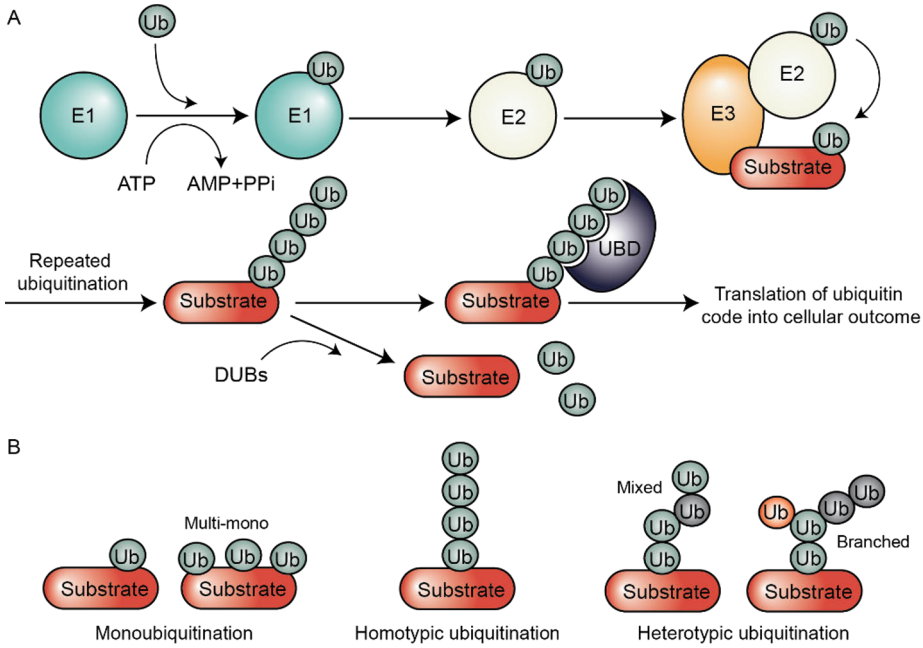

Figure 6. The ubiquitin system. A) Ubiquitin-activating enzymes, E1s, interact with ubiquitin in an ATP-dependent manner, whereafter the ubiquitin moiety is transferred to a ubiquitin-conjugating enzyme, E2. E2s together with E3 ligases add ubiquitin to the target substrate. Polyubiquitin chains are generated by repetition of the enzymatic cascade. The ubiquitin code is translated into cellular outcomes by proteins recognising ubiquitinated substrates via their Ubiquitin binding domains (UBDs). Deubiquitinating enzymes (DUBs) antagonise ubiquitination by cleaving off ubiquitin moieties attached to substrates. B) Substrates modified by a single ubiquitin moiety are referred to as monoubiquitinated, whereas multi-monoubiquitination entails the monoubiquitination on several sites of the substrate. Homotypic chains consist of ubiquitin moieties attached via the same linkage types, whereas heterotypic chains contain several different linkages. Heterotypic chains can be mixed or branched (figure modified from French et al., 2021, Swatek and Komander, 2016, Komander and Rape, 2012)

2.2.1 Translation of the ubiquitin code

Protein degradation was the first cellular function associated with ubiquitination (Chau et al., 1989, Hershko and Ciechanover, 1998). In eukaryotes, protein degradation is primarily mediated by the 26S proteasome, which recognises substrates ubiquitinated with K48-linked chains (Grice and Nathan, 2016). Interestingly, K48-linked ubiquitin is also the most abundant linkage type in the cell (Xu et al., 2009). The 26S proteasome is a barrel-like protein complex, consisting of the 20S core, executing proteolysis, and the 19S regulatory particle, capping one or both

ends of the core (Bard et al., 2018). The 19S particle, also known as the lid of the proteasome, harbours specific UBDs recognising ubiquitinated proteins and translocates appropriate substrates into the degradation core (Lander et al., 2012, Thrower et al., 2000, Bard et al., 2018). K48-linked ubiquitin chains, packed tightly against each other (Eddins et al., 2007), are thought to be the main cellular signal for proteasomal degradation (Thrower et al., 2000). However, the optimal length and number of K48-linked chains are under debate. Based on the study of Thrower and colleagues, chains of four or more ubiquitin moieties have been thought to be the signal for efficient degradation. This notion is, however, beginning to be challenged, as two diubiquitin K48-linked chains have been shown to be a more efficient degradation signal compared to a single tetraubiquitin chain (Lu et al., 2015). Furthermore, heterotypic K11/K48-linked chains have been shown to promote degradation of cell cycle substrates more efficiently than homotypic K48-linked chains (Meyer and Rape, 2014). These studies point towards an additional, unexplored, level of degradation kinetics modulating protein homeostasis.

K63-linked chains displaying an open topology are, in contrast to K48-linked chains, non-degradative and have been associated with several cellular processes, such as DNA damage responses, kinase activation and protein localisation (Chen and Sun, 2009, Komander et al., 2009b, Komander and Rape, 2012). However, one of the most well-studied roles of K63-linked chains lies within inflammatory signalling and NF-κB activation (Wu et al., 2018). K63-linked chains stimulate immune signalling by stabilising receptor complexes, recruiting downstream protein complexes and by activating kinases. Another NF-κB regulating chain type, adopting the similar extended conformation as K63-linked chains, are the M1-linked chains, catalysed by the Linear ubiquitin assembly complex (LUBAC) (Kirisako et al., 2006, Komander et al., 2009b), consisting of two regulatory subunits, Haem-oxidized IRP2 ubiquitin ligase-1 (HOIL-1) and Shank-associated RH domain-interacting protein (SHARPIN), and of the catalytic subunit HOIL-1-interacting protein (HOIP) (Tokunaga et al., 2011, Kirisako et al., 2006). The important immune-modifying role of M1-linked chains are reflected by severe, systemic, inflammatory phenotypes displayed by mice carrying mutations in components of the M1-linked ubiquitination machinery (Hrdinka and Gyrd-Hansen, 2017). Upon immune receptor activation, multiprotein complexes formed at the receptors are decorated with both K63- and M1-linked chains. Research indicates that K63-linked chains are induced first and act as recruiters of LUBAC. LUBAC subsequently catalyses the M1-ubiquitination of own substrates, or on pre-existing K63-linked chains, forming, hence, heterotypic K63/M1-linked chains (Emmerich et al., 2013). The homotypic and heterotypic chains recruit

ubiquitin-dependent kinase complexes such as the Transformed growth factor β-activated kinase 1 (TAK1) complex and the Inhibitor of κB (IκB) kinase (IKK) complex, which are both crucial for downstream signalling and subsequent NF-κB activation (Kanayama et al., 2004, Wu et al., 2006).

Monoubiquitination and homotypic chains of "atypical" ubiquitin linkages (K6, K11, K27, K29, K33) have not received as much attention as K48-, K63- and M1-linked ubiquitination and the regulation of these chain types and their biological function remain relatively unexplored. Some cellular functions have, however, already been associated with atypical ubiquitin chains, such as removal of damaged mitochondria with K6-linked chains (Cunningham et al., 2015), cell cycle regulation with K11-linked chains (Wickliffe et al., 2011), DNA damage and autoimmunity with K27-linked chains (Gatti et al., 2015, Liu et al., 2014), proteasomal degradation with K29-linked chains (Johnson et al., 1995), trafficking with K33-linked chains (Yuan et al., 2014), and, finally, endocytosis with monoubiquitination (Haglund et al., 2003). Furthermore, the regulation and function of heterotypic mixed and branched chains are only beginning to be elucidated (French et al., 2021).

2.3 Caspases and their activation

Caspases are a family of cysteine proteases, cleaving their substrates on the C-terminal side of aspartate residues. Caspases have been identified in all metazoans, ranging from *Caenorhabditis elegans* and *Drosophila*, to mouse and human (Lamkanfi et al., 2002). Caspases consist of an amino-terminal prodomain of variable size, followed by one large, ∼20 kDa, and one small, ∼10 kDa, subunit that together form the catalytically active protease domain. The murine and human caspases are, based on their described function and domain architecture, typically divided into inflammatory caspases (caspase-1, -4, -5, -11) and apoptotic caspases (caspase-3, -6, -7 -8, -9, -10) (Figure 7) (Van Opdenbosh and Lamkanfi, 2019). The functions of caspase-2, -12 and -14 are not completely understood, but they seem to work beyond cell death and inflammation, and are, hence, not classified as inflammatory nor apoptotic (Fava et al., 2012, Vande Walle et al., 2016, Denecker et al., 2008). The apoptotic caspases can be further subdivided into initiator, or apical caspases (caspase-8, -9, -10), and effector caspases (caspase-3, -6, -7) depending on their position in the apoptotic signalling cascade. The apoptotic initiator caspases and inflammatory caspases contain specific recruitment domains, i.e., Death effector domains (DEDs) or CARD domains, in the N-terminus, enabling protein-protein interactions. The effector caspases, on the other hand, have short prodomains lacking specific domains (Figure 7) (Van Opdenbosh and Lamkanfi, 2019).

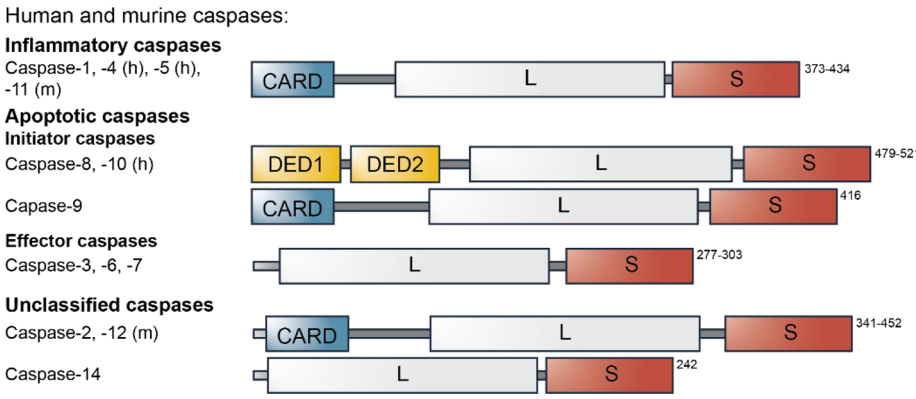

Figure 7. Protein domains of human and murine caspases. Caspases contain a small (S) (~10 kDa) subunit and a large (L) (~20 kDa) subunit that together form the protease domain. In addition to the L and S subunits, inflammatory caspases (caspase-1, -4, -5, -11) and apoptotic initiator caspases (caspase-8, -9, -10) contain long N-terminal prodomains harbouring DED or CARD domains. Effector caspases (caspase-3, -6, -7) have short prodomains lacking specific protein domains. The unclassified caspases caspase-2 and -12 contain a long prodomain with a CARD domain, whereas unclassified caspase-14 has a structure resembling that of an effector caspase. The length of the caspases in amino acids is indicated to the right of the proteins, m stands for murine, h for human (figure adapted from Van Opdenbosh and Lamkanfi, 2019, Lamkanfi et al., 2002).

Caspases are synthesised as zymogens, requiring dimerisation and excision of the prodomain to be activated. Activation of initiator caspases are described by the "induced proximity" model, wherein inactive monomers of the caspases are recruited to oligomeric signalling platforms to dimerise, undergo proteolytic cleavage and gain activity (Muzio et al., 1998, Boatright et al., 2003). Two classical examples of caspase activation by induced proximity are found in the intrinsic and extrinsic apoptosis signalling pathways. Upon activation of the mammalian intrinsic apoptotic pathway, the adaptor protein Apoptotic protease activating factor-1 (APAF-1), together with cytochrome c released from the mitochondria, form a protein complex called apoptosome. The apoptosome recruits caspase-9 via its CARD domains, and activates the caspase through proximity-induced dimerisation (Acehan et al., 2002, Boatright et al., 2003). In the extrinsic pathway, activation of the Fas receptor promotes clustering of the adaptor protein Fas-associated death domain (FADD), leading to the recruitment of caspase-8 or -10 via their DED domains (Medema et al., 1997, Scott et al., 2009, Wachmann et al., 2010). These protein clusters are called Death-induced signalling complexes (DISCs) and promote caspase activation through dimerisation (Boatright et al., 2003). In the case of the inflammatory caspase-1 and its

activation, the inflammasome has been described (Martinon et al., 2002). Inflammasomes consist of Nucleotide oligomerisation domain (NOD)-like receptors (NLRs), Apoptosis-associated speck-like protein contain a caspase recruitment domain (ASC) and caspase-1. Upon activation of NLRs, ASC is recruited to the receptor and the inflammasome is assembled (Masumoto et al., 2001). After assembly, caspase-1 binds to the complex via a CARD-CARD interaction, leading to the proximity-induced activation of the caspase (Srinivasula et al., 2002, Stehlik et al., 2003). Whereas zymogens of initiator and inflammatory caspases are monomeric, the inactive precursors of effector caspases exist in the cytosol as dimers, typically activated by initiator caspase-mediated cleavage (Ramirez and Salvesen, 2018). The dimers of caspase-3 and caspase-7 are activated by proteolysis of a linker region located between the large and small subunit (Li et al., 1997, Riedl, 2001), whereas, caspase-6, interestingly, is often activated by caspase-3 instead of initiator caspases, and seems to be able to undergo autoactivation both *in vitro* and *in vivo* (Hirata et al., 1998, Wang et al., 2010).

2.3.1 *Drosophila* caspases

Seven caspases have been identified in *Drosophila*, three of which have similar structure as mammalian initiator caspases, namely Death related ced-3/Nedd2-like caspase (Dredd), Death regulator Nedd2-like caspase (Dronc) and Ser/Thr-rich caspase (Strica) (Chen et al., 1998, Dorstyn et al., 1999a, Vernooy et al., 2000), and four with structural similarities to effector caspases, called Drice, Death-associated molecule related to Mch2 (Damm), Death executioner caspase related to apopain/yama (Decay) and Dcp-1 (Figure 8) (Fraser et al., 1997, Vernooy et al., 2000, Dorstyn et al., 1999b, Song et al., 1997). The caspase-9 homologue Dronc, which is one of the three *Drosophila* initiator caspases, is considered to be the main apoptosis-inducer in the fly (Hay and Guo, 2006). Dronc is activated by binding via CARD-CARD interactions to the *Drosophila* apoptosome, formed by the adaptor protein Death-associated Apaf1-related killer (Dark) (Yu et al., 2006, Yuan et al., 2011). Upon activation, Dronc cleaves and activates effector caspases Drice and Dcp-1, which in turn cleave downstream substrates, thereby, executing apoptosis (Hawkins et al., 2000, Meier et al., 2000). Drice and Dcp-1 are homologous to mammalian effector caspase-3 and seem to have partially overlapping functions during apoptosis. Dcp-1 mutants display milder defects in apoptotic signalling compared to Drice mutants, however, the phenotype of double Drice/Dcp-1 mutant is stronger than that of either mutant alone (Kondo et al., 2006, Xu et al., 2006, Muro et al., 2006, Lee et al., 2011). The initiator caspase Dredd was initially thought to function in apoptotic signalling (Chen et al., 1998), but has since then been established as key inducer of the inflammatory response triggered by Gram-negative bacteria (Leulier

et al., 2000). Dredd contains two DEDs in its prodomain, which are important for interaction with the adaptor protein *Drosophila* Fadd (dFadd), recruiting Dredd to the bacteria-activated receptor complex (Hu and Yang, 2000). The caspases Decay, Strica and Damm have not received as much attention as the other *Drosophila* caspases, however, Decay was found recently to regulate wing size, independently of Dronc-induced apoptosis (Shinoda et al., 2019). Strica is known to contain a unique serine and threonine rich prodomain, however the function of Strica, or Damm, remains largely unknown (Doumanis et al., 2001).

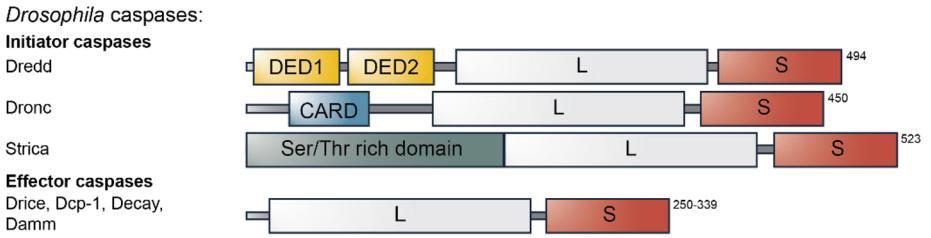

Figure 8. Protein domains of *Drosophila* caspases. In addition to the long (L) and short (S) subunits of the protease domain, the initiator caspases Dronc and Dredd contain long N-terminal prodomains harbouring two DED or a CARD domain, respectively. The atypical *Drosophila* initiator caspase Strica does not contain DED or CARD domains, but harbours instead a Ser/Thr rich prodomain. Effector caspases (Drice, Dcp-1, Decay and Damm) have short prodomains lacking specific protein domains. The length of the caspases in amino acids is indicated to the right of the proteins (figure adapted from Lamkanfi et al., 2002).

2.3.2 Regulation and inhibition of caspases

As proteolysis of caspases is irreversible, the activity of caspases needs to be carefully regulated. Some of the first caspase inhibitors were found in viruses, used to block the host defence upon viral infection. The two best characterised viral caspase inhibitors are the Cowpox virus protein cytokine response modifier A (CrmA) and the baculovirus protein p35 (Bump et al., 1995, Zhou et al., 1997). Both proteins bind to caspases in a relatively non-selective manner, and function as so called "suicide substrates" that bind to the active site of the caspase and stay bound after being cleaved, thereby, physically blocking the catalytic pocket (Swanson et al., 2007, Lu et al., 2006). Ectopic expression of CrmA or p35 in mammalian and *Drosophila* cells have been extensively used in order to study the role of caspases in cell signalling.

In addition to synthesising caspases as inactive zymogens requiring specific modes of activation, the cell employs several strategies, such as decoy proteins, posttranscriptional modifications and caspase inhibitors, to regulate caspase activity (Parrish et al., 2013, Pop and Salvesen, 2009). The main group of caspase regulators found in metazoans is the IAP family

of proteins, whose members interact via their BIR domain with the IBM domain of caspases. In mammals, XIAP is the only known IAP able to inhibit the enzymatic activity of caspases directly by binding. XIAP has been shown to inhibit caspase-3 and caspase-7 via its BIR2 domain, whereas caspase-9 is inhibited via the BIR3 domain (Scott et al., 2005, Shiozaki et al., 2003).

In *Drosophila*, Diap1 inhibits the initiator caspase Dronc and the effector caspases Drice and Dcp-1 by binding to, and ubiquitinating the caspases (Wilson et al., 2002, Zachariou et al., 2003, Ditzel et al., 2008). In the case of Drice, Diap1 inhibits the caspase by binding via its BIR1 domain to the active form of Drice. This binding is antagonized by the proapoptotic protein Reaper, increasing the pool of free, active Drice during apoptosis (Zachariou et al., 2003). Diap2 has also been shown to lower the apoptotic threshold of the cell by inhibiting Drice (Ribeiro et al., 2007). The Diap2-mediated inhibition of Drice is mechanism-based and resembles caspase inhibition mediated by p35. Diap2 binds to active Drice in a dual manner via a covalent adduct formed between the catalytic cysteine 211 of Drice and aspartic acid 100 of Diap2, and through a binding between the IBM domain of Drice and the BIR3 domain of Diap2 (Figure 9) (Ribeiro et al., 2007). As a consequence of the Drice-Diap2 interaction, Diap2 is cleaved and Drice ubiquitinated. The details of this event, such as ubiquitination site or chain type, and how complex formation affects Diap2-activity remains, however, elusive (Ribeiro et al., 2007).

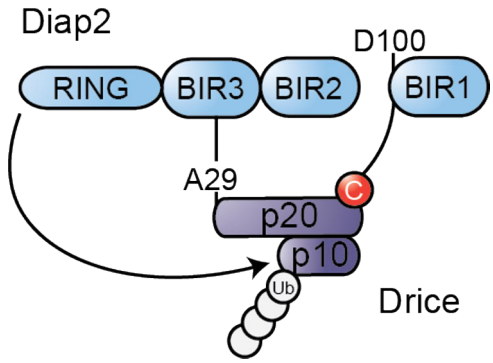

Figure 9. The Diap2-Drice complex. Diap2 and Drice interacts via alanine 29 (A29), part of the IBM domain of Drice, and the BIR3 domain, and, additionally, through a covalent binding formed between the catalytic cysteine 211 (C211) of Drice and aspartic acid 100 (D100) of Diap2. Cleavage of the BIR1 domain and ubiquitination of Drice are known consequences of the interaction. The proteins are depicted as monomeric for clarity, however, both proteins dimerise prior to activation (figure modified from Ribeiro et al., 2007).

Activated caspases are short-lived species with a more dynamic turnover compared to the one of inactive zymogens (Tawa et al., 2004), suggesting an involvement of the proteasomal pathway in caspase regulation. As IAPs possess E3 ligase activity, the proteins might be able to target caspases for proteasomal degradation. Indeed, both cIAP1 and XIAP, have been shown to ubiquitinate a processed form of caspase-3 in different settings, leading to its subsequent proteasomal degradation (Choi et al., 2009, Suzuki et al., 2001). XIAP has, furthermore, been shown to polyubiquitinate active caspase-9 *in vitro*, and inhibition of the proteasome together with overexpression of XIAP promotes accumulation of polyubiquitinated caspase-9 (Morizane et al., 2005). Similarly in *Drosophila* S2 cells, Diap1 appears to target the apoptosome-bound Dronc for degradation (Muro et al., 2002). However, an *in vivo* occurrence or the details of Diap1 or Diap2-mediated proteasomal degradation of caspases, remain elusive.

Decoy proteins are structurally related to caspase prodomains and compete for caspase binding sites in activation platforms, thus, preventing activation of initiator and inflammatory caspases. Cellular FLICE-like inhibitory protein (c-FLIP) is a catalytically inactive pseudo-caspase-8 that binds to the DISC, inhibiting any subsequent recruitment of caspase-8 to the site (Irmler et al., 1997). However, interestingly, a long isoform of c-FLIP has in contrast been shown to facilitate caspase activation, thereby exemplifying the complexity of caspase regulation (Micheau et al., 2002, Chang et al., 2002). Finally, in the case of inflammasome-mediated activation of caspase-1, decoy proteins harbouring CARD domains, such as CARD-only protein-1 (COP-1), ICEBERG and Inhibitory CARD (INCA), inhibit caspase-1 recruitment to the inflammasome, attenuating, thereby, the inflammatory signal (Lee et al., 2001, Druilhe et al., 2001, Lamkanfi et al., 2004).

3 The host defence system

The host defence system includes all mechanisms, both constitutive and inducible, used by an organism to defend itself against harmful organic or inorganic substances. In humans, the defence system consists of three layers: anatomical and physiological barriers, the adaptive immune system and the innate immune system. Anatomical and physiological barriers, such as the skin, low stomach pH and bacteriolytic lysozyme in tears and saliva, provide an important first line of defence against pathogens (Turvey and Broide, 2010). The adaptive immune system consists of T cells and B cells, which are lymphocytes displaying a wide repertoire of unique receptors, recognising specific antigens. After the initial antigen encounter, the clonal expansion of lymphocytes required to clear a pathogen takes three to five days, giving pathogens ample time to cause damage (Chaplin, 2010). The innate immune system augments the anatomical and physiological barriers and can generate an inflammatory response within minutes, closing the gap between pathogen exposure and proper immune response. The innate immune system is activated by pattern-recognition receptors (PRRs) recognising pathogen-associated molecular patterns (PAMPs) or host-derived danger-associated molecular patterns (DAMPs) (Takeuchi and Akira, 2010). Activation of PRRs induces downstream innate immune responses, which can be mediated by cell-dependent mechanisms, such as phagocytosis, or by secreted factors, such as antimicrobial proteins and proinflammatory cytokines (Gasteiger et al., 2017). The cytokines trigger, furthermore, the maturation of the adaptive immune response (Iwasaki and Medzhitov, 2015). In addition to being indispensable for the immune defence, the innate immune system has emerged as a crucial regulator of human inflammatory diseases. Dysregulated innate immune responses have been connected to the pathogenesis of asthma, and to development of autoimmune diseases such as type 1 diabetes and IBD (Pivniouk et al., 2020, Cabrera et al., 2016, Segal, 2019).

In contrast to mammals, *Drosophila* does not carry an adaptive immune system, but relies solely on an innate immune response when combating infections. The innate immune system is aided, similarly as in humans, by physical barriers, such as the epithelial lining beneath the cuticle in the digestive tract and trachea (Lemaitre and Hoffmann, 2007). The *Drosophila* innate immune system can be divided into a cellular and a humoral response. The cell-mediated response entails phagocytosis and the encapsulation of parasites, and is carried out by differentiated haemocytes located in the *Drosophila* haemolymph (Vlisidou and Wood, 2015). Phagocytosis is mediated by the macrophage-like plasmatocytes, leading to the disposal of both apoptotic bodies and invading pathogens (Melcarne et al., 2019), whereas encapsulation is an elaborate defence

mechanism of *Drosophila* larvae, mediated by lamellocytes, protecting the host from larger intruders, such as parasitoid eggs (Kim-Jo et al., 2019). The humoral arm of the innate immune system is the most well-studied part of the *Drosophila* immune response and is characterised by the infection-induced activation of NF-κB signalling, culminating in production and secretion of antimicrobial peptides. As several features of the *Drosophila* humoral immune response, such as microbial sensing by PRRs, as well as the components and regulatory mechanisms of NF-κB-mediated inflammatory signalling, are conserved in higher organisms, research done in *Drosophila* has aided greatly in elucidating the details of the mammalian innate immune system (Lemaitre and Hoffman, 2007). The following sections will present both mammalian and *Drosophila* NF-κB signalling and discuss their regulation during immune responses, with emphasis on intestinal immunity.

3.1 The mammalian NF-κB signalling pathway

The NF-κB family of transcription factors is a master regulator of host defence and controls the expression of numerous proinflammatory genes. In addition to being essential for both innate and adaptive immune responses, NF-κB also regulates genes involved in differentiation, proliferation and survival. The NF-κB transcription factors are activated by a variety of receptors, ranging from PRRs recognising PAMPs and DAMPs (Carmody and Chen, 2007, Feldman et al., 2015), to cytokine, antigen and growth factor receptors (Hayden and Ghosh, 2014, Schulze-Luehrmann and Ghosh, 2006, Shostak and Chariot, 2015). NF-κB can also be activated by environmental stresses, such as reactive oxygen species, ultraviolet light and irradiation (Lingappan, 2018, László and Wu, 2008, Singh et al., 2015). Given its influence on the vast array of different biological processes, dysregulation of NF-κB can have severe consequences, leading to the development of chronic inflammatory diseases, autoimmune diseases, neurodegenerative disorders, cardiovascular disorders, and cancer (Zhang et al., 2017).

The first NF-κB transcription factor was discovered in 1986 as a sequence-specific DNA binding protein in activated B lymphocytes (Sen and Baltimore, 1986). To date, five mammalian NF-κB transcription factors, p65 or RelA, RelB, c-Rel and, p105 and p100, precursors of p50 and p52, respectively, have been described. All of the transcription factors share a conserved Rel homology domain (RHD), mediating sequence-specific DNA binding, dimerisation, and binding of inhibitory proteins (Smale, 2012). The transcription factors hetero- or homodimerise to form 15 possible cytosolic dimers with specific transcriptional properties (Smale, 2012). In their resting state, the NF-κB dimers are sequestered in the cytoplasm by proteins from the IκB family. The IκB proteins contain an ankyrin-repeat domain, which blocks the dimer's nuclear localisation

signal (Mitchell et al., 2016). The precursor proteins p105 and p100 contain a C-terminal ankyrin-repeat domain, functioning, thereby, as inhibitory proteins themselves. Partial degradation of p105 and p100 interrupts their inhibitory function, yielding free p50 and p52 NF-κB proteins (Smale, 2012).

NF-κB activation is mediated through two major signalling pathways: the canonical and the non-canonical pathway (Figure 10) (Shih et al., 2011). The canonical pathway induces rapid activation of NF-κB downstream of PRRs, T cell and B cell receptors, and proinflammatory cytokine receptors, such as members of the TNF-receptor (TNFR) superfamily. Proteins, i.e., adaptor molecules, kinases and ubiquitin ligases, recruited to the different types of receptors vary, however, they all aim at recruiting TAK1 to the complex. TAK1 in turn, activates the IKK complex, consisting of the kinases IKKα and IKKβ, as well as the regulatory subunit NF-κB essential modifier (NEMO), by phosphorylation. The activated IKK complex phosphorylates IκB, thereby, targeting IκB for proteasomal degradation, resulting in the release of the NF-κB dimer and subsequent nuclear translocation (Figure 10) (Wertz and Dixit, 2010). The liberated dimers in the canonical pathway are largely composed of the p65, p50 and c-Rel subunits (Hoffmann and Baltimore, 2006).

The activity of the noncanonical pathway is mediated foremost by members of the TNFR superfamily, but can also be stimulated by RNA viruses and by pathogenic bacteria (Sun, 2017, Struzik and Szulc-Dąbrowska, 2019). Activation of the non-canonical NF-κB pathway relies on *de novo* protein synthesis, and is, in contrast to the canonical pathway, slow and persistent. Receptor activation leads to the stabilisation of NF-κB inducing kinase (NIK), which is subjected to continuous proteasomal degradation in unstimulated cells (Liao et al., 2004). Stabilisation of NIK allows for the kinase to activate IKKα by phosphorylation (Xiao et al., 2001). IKKα phosphorylates in turn p100, targeting the NF-κB precursor for partial proteasomal degradation, generating, thus, the p52 protein and freeing, predominantly, RelB/p52 dimers to enter the nucleus (Figure 10) (Senftleben et al., 2001, Sun, 2017).

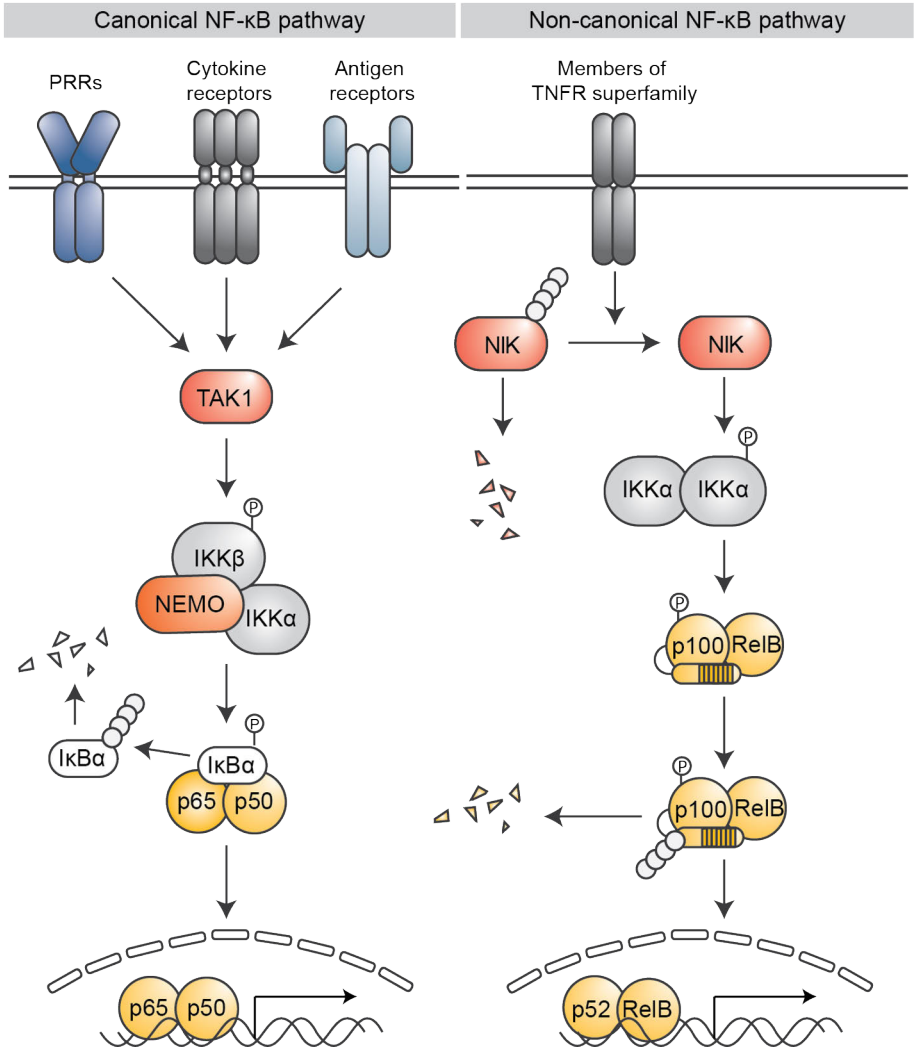

Figure 10. The NF-κB signalling pathway. The canonical NF-κB pathway occurs downstream of Pattern-recognition receptors (PRRs), cytokine receptors and antigen receptors. Each receptor activates a distinct set of signalling mediators that aim at activating TAK1. TAK1 activates the IKK complex consisting of NEMO, IKKβ and IKKα, by phosphorylation. The activated complex targets IκBα for proteasomal degradation by phosphorylation. The released NF-κB transcription factor dimer translocates to the nucleus and activates target gene expression. The non-canonical NF-κB pathway is activated mainly by receptors that are members of the Tumour necrosis factor receptor (TNFR) superfamily. Receptor activation disrupts the continuous degradation of NIK. Stabilised NIK activates IKKα by phosphorylation, which in turn phosphorylates p100, the precursor for p52. Through partial degradation of p100 the p52/RelB dimer is free to translocate to the nucleus and induce gene expression (figure adapted from Sun, 2017).

3.2 NF-κB-mediated immune signalling in *Drosophila melanogaster*

A hallmark of the *Drosophila* innate immune response is the PRR-mediated activation of NF-κB signalling that culminates in the production and secretion of AMPs. In *Drosophila*, PGN-recognition proteins (PGRPs) are the main PRRs and sensors of microbial non-self. *Drosophila* encodes for 13 PGRPs that all share a common 160-amino acid long PGRP domain that mediates recognition of PGN (Kurata, 2014). In addition to the PGRP domain, some PGRPs harbour amidase activity and are able to modulate immune signalling directly by inducing degradation of PGN (Zaidman-Rémy et al., 2006, Bischoff et al., 2006, Zaidman-Rémy et al., 2011). The non-catalytic PGRPs function as bacterial receptors inducing immune signalling. Some of these receptors are extracellular and function as circulating bacterial sensors (Michel et al., 2001, Bischoff et al., 2004), whereas others are located in the cell membrane or intracellularly (Choe et al., 2002, Gottar et al., 2002, Rämet et al., 2002, Takehana et al., 2002).

The production of AMPs is controlled by two *Drosophila* NF-κB pathways, namely the Imd and Toll signalling pathways (Hetru and Hoffmann, 2009, Lemaitre and Hoffmann, 2007, De Gregorio et al., 2002). The Toll pathway is a well-conserved signalling cascade first identified for its role in determining the dorso-ventral axis of *Drosophila* embryos (Anderson et al., 1985). In the search of Toll homologues, TLRs were identified in higher organisms, and now Toll and TLRs are best known for their role in innate immunity (Anthoney et al., 2018, Valanne et al., 2011). Toll signalling in *Drosophila* is initiated by extracellular circulating PRRs that recognise cell wall components from fungi or Gram-positive bacteria. Fungal β-glucan is identified by Gram-negative binding protein 3 (GNBP3) (Gottar et al., 2006), whereas Lys-type PGN from the cell wall of Gram-positive bacteria is reportedly identified by PGRP-SD, or by a complex consisting of PGRP-SA and GNBP1 (Figure 11) (Steiner, 2004, Bischoff et al., 2004, Wang et al., 2006). These PPRs induce a serine-cascade leading to the cleavage of the extracellular cytokine Spätzle (Jang et al., 2006). Cleaved Spätzle binds to the Toll receptor, activating an intracellular signalling cascade, culminating in the degradation of the inhibitory factor Cactus, inhibiting the NF-κB transcription factors Dorsal-related immunity factor (Dif) and Dorsal in resting cells (Figure 11) (Weber et al., 2003, Wu and Anderson, 1998). Upon dissociation from Cactus, Dif and Dorsal translocate to the nucleus and activate the transcription of AMP genes (Meng et al., 1999, Reichhart et al., 1993).

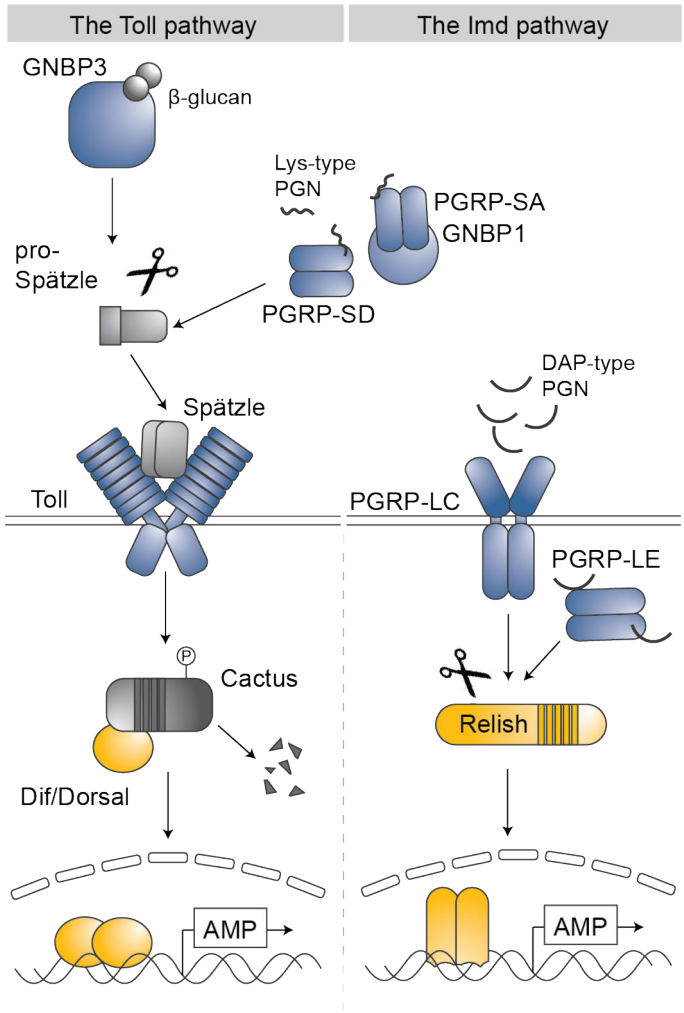

Figure 11. The *Drosophila* Toll and Imd pathway. Circulating pattern-recognition proteins GNBP3 and PGRP-SD or PGRP-SA and GNBP1, recognise β-glucan from fungi and Lys-type peptidoglycan (PGN) from Gram-positive bacteria, respectively, thereby initiating an extracellular protein cascade, culminating in the cleavage of pro-Spätzle. Cleaved Spätzle activates the Toll receptor, leading to the phosphorylation-dependent degradation of the inhibitory protein Cactus and nuclear translocation of transcription factors Dif and/or Dorsal. Diaminopimelic acid (DAP)-type PGN, originating from Gram-negative bacteria, activate the transmembrane receptor PGRP-LC or the intracellular receptor PGRP-LE. The extracellular PGRP-SD has also been reported to induce Imd signalling by bringing PGN to the proximity of PGRP-LC. Receptor activation leads to the cleavage of transcription factor Relish and its subsequent translocation to the nucleus. The Toll and Imd pathways activate a distinct set of AMPs depending on the type of microbial stimuli (figure modified from Kurata, 2014, Lemaitre and Hoffmann, 2007, Iatsenko et al., 2016).

Whereas the Toll pathway is activated by circulating proteins recognising microbial structures, the Imd pathway is initiated by direct interaction between receptor and pathogen. Diaminopimelic acid (DAP)-type PGN, present in the cell wall of Gram-negative bacteria, is recognised by the transmembrane receptor PGRP-LC, or the intracellular receptor, PGRP-LE (Choe et al., 2002, Gottar et al., 2002, Rämet et al., 2002, Takehana et al., 2002). Interestingly, in contrast to its assumed role as an inducer of Toll signalling, PGRP-SD has also been reported to function as an Imd specific PRR (Iatsenko et al., 2016). PGRP-SD was shown to enhance the activation of PGRP-LC by promoting relocalisation of extracellular PGN to the cell surface (Iatsenko et al., 2016). Receptor activation leads to the cleavage-dependent activation and nuclear translocation of the NF-κB transcription factor Relish, and, finally, to production and secretion of AMPs (Figure 11) (Stöven et al., 2003, Hedengren et al., 1999). The Imd pathway mediates a rapid response to pathogens and Relish has been shown to translocate to the nucleus within minutes, with AMP levels peaking a few hours post infection. Activation of the Toll transcription factors is on the other hand slower, and the AMP production can be sustained for days (Lemaitre et al., 1997).

AMPs can be produced by the epithelial cells of the digestive, respiratory and reproductive tracts, thereby modulating local immune responses, or by the fat body, an organ homologous to the mammalian liver. The fat body produces AMPs in response to pathogens entering the body cavity, also known as the haemocoel, and are secreted systemically into the haemolymph (Tzou et al., 2000, Lemaitre and Hoffmann, 2007). AMPs are small, <10 kDa, with the exception of the 25 kDa attacin, cationic peptides displaying distinct antibacterial and, or, antifungal activities (Imler and Bulet, 2005). The characterised AMPs of *Drosophila* are currently divided into seven gene families: *Drosocin*, *Attacins* (four genes), *Diptericins* (two genes), *Drosomycin*, *Metchnikowin*, *Cecropins* (four genes) and *Defensin* (Bulet et al., 1996, Wicker et al., 1990, Levashina et al., 1995, Ekengren and Hultmark 1999, Cociancich et al., 1993, Tzou et al., 2002, Hedengren et al., 2000). However, as many additional genes encoding small peptides are known to be upregulated during infection, the number of AMP families might increase in the future (De Gregorio et al., 2002).

Upon a septic bacterial infection, the production and release of AMPs in the fat body are regulated by both the Imd and Toll signalling pathways (Hetru and Hoffmann, 2009, Lemaitre and Hoffmann, 2007, De Gregorio et al., 2002). However, interestingly, local immune responses mediated by epithelial cells are believed to be solely controlled by the Imd pathway, as no role for Toll signalling has been identified in the host defence of the gut (Buchon et al., 2009a, Broderick, 2016), and several of the key members of the Toll pathway do not seem to be expressed in the epithelial cells of the trachea (Wagner et al., 2008, Akhouayri et al., 2011). Furthermore,

AMP expression in the Malpighian tubules and salivary glands have been shown to be regulated by Imd signalling (Tzou et al., 2000, Verma and Tapadia, 2012, Abdelsadik and Roeder, 2010). As the Toll signalling pathway is the only known mediator of a fungal or Gram-positive bacterial infection, the regulation of these microbes during local immune-responses or in the maintenance of beneficial host-microbe interactions, remain elusive.

3.2.1 The *Drosophila* Imd pathway

The Imd pathway is activated upon bacterial stimulation when PGN from the cell wall of Gram-negative bacteria is recognised by the transmembrane receptor PGRP-LC or by the intracellular receptor PGRP-LE (Choe et al., 2002, Gottar et al., 2002, Rämet et al., 2002, Takehana et al., 2002). Whereas the systemic activation of Imd is dependent on PGRP-LC, the intestinal immune response towards commensal and pathogenic bacteria is regulated by both PGRP-LC and PGRP-LE, expressed at varying levels in different regions of the gut (Bosco-Drayon et al., 2012, Neyen et al., 2012). The receptors are thought to homodimerise or -oligomerise upon ligand binding, whereafter the adaptor protein Imd is recruited to the complex (Figure 12) (Mellroth et al., 2005, Georgel et al., 2001, Choe et al., 2005). The details of the formation of the signalling complex formed immediately downstream of PGRP receptors remains largely elusive, however, the receptors and Imd have been shown to form functional amyloids, required for Imd signalling to proceed (Kleino et al., 2017). The Imd protein has no homologue in mammals, but contains a death domain (DD) sharing sequence homology with the DD of mammalian Receptor interacting protein kinase 1 (RIPK1) (Georgel et al., 2001), involved in NF-κB signalling in mammals. Imd binds via its DD domain to dFadd, which in turn recruits the caspase-8 homologue Dredd through its DED domain (Figure 12) (Naitza et al., 2002, Hu and Yang, 2000). Upon recruitment to the signalling complex, activated Dredd cleaves Imd, exposing an IBM domain of Imd, to which the E3 ligase Diap2 binds via its BIR domains (Kim et al., 2014, Paquette et al., 2010). Cytoplasmic Relish is in an autoinhibitory state, wherein C-terminal ankyrin repeats mask its nuclearisation signal (Stöven et al., 2000). Upon activation, Dredd cleaves off the inhibitory C-terminal part, freeing the N-terminal fragment of Relish to enter the nucleus (Figure 12) (Stöven et al., 2003).

Diap2 K63-ubiquitinates both Dredd and Imd (Paquette et al., 2010, Meinander et al., 2012). Ubiquitination of Dredd is required for activation of the caspase and flies carrying a point mutation in the ubiquitination site of Dredd, do not cleave Imd nor Relish, and succumb to Gram-negative bacterial infections (Meinander et al., 2012). The ubiquitin chains on Imd are thought to recruit *Drosophila* Tak1 (dTak1), via *Drosophila* Tak1-binding protein (dTab2), as the mammalian Tab2 homologue has been

shown to interact specifically with K63-linked ubiquitin chains (Figure 12) (Kulathu et al., 2009, Kleino and Silverman, 2014). In addition to driving Imd signalling, dTak1 activates the c-Jun terminal kinase (JNK) pathway, a conserved stress sensing pathway in eukaryotic cells. *Drosophila* JNK signalling is needed for normal AMP release and required for Imd-induced epithelial shedding (Kallio et al., 2005, Zhai et al., 2018, Tafesh-Edwards and Eleftherianos, 2020). Interestingly, activation of Relish has been shown to attenuate JNK activity, indicating a dual role of the Imd pathway in shaping JNK-induced immune activation (Park et al., 2004).

Recruited dTak1 and dTab2 activate the IKK complex, consisting of a regulatory subunit, Kenny, homologous to mammalian NEMO or IKKγ, and of a catalytic subunit called Immune response deficient 5 (Ird5), homologous to mammalian IKKβ (Figure 12) (Rutschmann et al., 2000, Silverman et al., 2000). Similarly, as mammalian NEMO, Kenny has been shown to be M1-ubiquitinated by the LUBAC orthologue Lubel, in flies (Aalto et al., 2019), leading to the stabilisation of the IKK complex. An activated IKK complex is required for subsequent activation of Relish, however, the details of this interaction remain largely elusive (Rutschmann et al., 2000, Silverman et al., 2000). Ird5 has been shown to phosphorylate residues in the N-terminal part of Relish, facilitating transcription and recruitment of RNA polymerase II. This event is, however, not needed for Relish cleavage nor for nuclear translocation (Ertürk-Hasdemir, et al., 2009). Although the Toll and Imd pathway have been considered to serve independent functions of the *Drosophila* immune system, crosstalk at the level of the NF-κB transcription factors have been demonstrated. All forms of homo- and heterodimers of Relish, Dorsal and Dif have been identified in overexpression systems, and the Relish-Dif dimer is known to regulate the expression of certain AMPs (Tanji et al., 2010). Although no such interaction has been described to date, upstream Toll pathway signalling molecules might also affect the activity of Imd pathway members.

Traditionally, the *Drosophila* Imd pathway has been compared to the mammalian canonical NF-κB pathway activated by the cytokine receptor TNFR1. The Imd and TNFR1 pathways both activate inflammatory NF-κB and share a number of homologous protein components, regulated in a conserved manner (Falschlehner and Boutros, 2012, Myllymäki et al., 2014). However, in contrast to the PGRP receptors of Imd signalling, TNFR1 does not function as a sensor of infection, but as an amplifier of the immune response. In the case of intestinal immunity, the NOD2 pathway might serve as a better mammalian analogue for the Imd pathway. Similarly, as the TNFR and Imd pathway, the NOD2 and Imd pathway contain several conserved signalling proteins and rely on the same modes of regulation when activating NF-κB. Additionally, both the NOD2 and Imd

pathway are stimulated directly by bacteria, are key regulators of NF-κB activity in barrier epithelia, and crucial for maintaining intestinal homeostasis (Buchon et al., 2014, Al Nabhani et al., 2017). Next, the details of both inflammatory TNFR1 and NOD2 signalling are discussed.

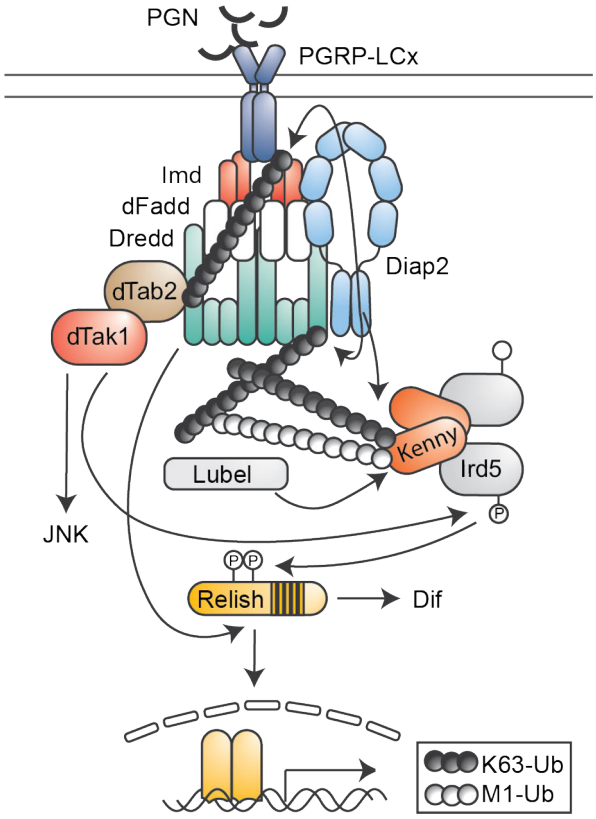

Figure 12. Components of the Imd pathway. The Imd pathway is activated when peptidoglycan (PGN), derived from Gram-negative bacteria, binds to the membrane receptor PGRP-LC. Upon activation, the adaptor proteins Imd and dFadd, as well as the caspase Dredd, are recruited to the receptor complex. Dredd cleaves Imd, whereafter Diap2 ubiquitinates both Dredd and Imd. Both Lubel and Diap2 are known to ubiquitinate Kenny. The dTak1-dTab2 complex and the IKK complex are thought to be recruited to the receptor complex via the ubiquitin chains attached to Imd and Dredd. dTak1 activates also the JNK pathway in response to bacterial infection. To activate Relish, Ird5 of the IKK complex phosphorylates Relish, whereas Dredd cleaves off the inhibitory ankyrin repeats. After activation, Relish translocates to the nucleus and induces gene transcription. By forming Relish-Dif dimers, the transcriptional properties of Relish can be modified (figure modified from Myllymäki et al., 2014, Falschlehner and Boutros, 2012).

3.3 The TNFR1 signalling pathway

TNFα is a master cytokine, belonging to the TNF superfamily of cytokines that affects a variety of cellular responses, ranging from inflammatory gene expression and proliferation, to apoptosis and necroptosis. TNFα is secreted primarily by macrophages in response to PRR activation and amplifies the inflammatory response by binding to TNFR1 and TNFR2 (Grivennikov et al., 2005, Wajant and Siegmun, 2019). Activation of TNFR1 drives the immune responses and cell survival through NF-κB, but can also, depending on the physiological circumstances, induce programmed cell death by apoptosis or necroptosis (Holbrook et al., 2019). TNFR1-induced activation of NF-κB is mediated by a protein complex referred to as Complex I, whereas the protein complexes inducing cell death are known as Complex IIa, IIb and IIc (Gough and Myles, 2020). Here, the assembly of Complex 1 and its subsequent activation of NF-κB is discussed (Figure 13).

TNFR1 belongs to the death receptor subgroup of the TNFR super family and is expressed in most cell types (Park et al., 2007). TNFR1, harbouring a DD in its cytoplasmic part, undergoes a conformational shift upon ligand binding, enabling the recruitment of TNFR1-associated death domain (TRADD) and RIPK1 (Figure 13) (Hsu et al., 1995, Hsu et al., 1996a). TRADD further recruits the adaptor protein TNF receptor-associated factor 2 (TRAF2) via its N-terminal TRAF-binding domain (Hsu et al., 1996b). TRAF5 has also been shown to modulate TNFR1-induced NF-κB activity. The details of the TRAF2 versus TRAF5-mediated regulation remains, however, obscure (Tada et al., 2001). TRAF2 recruits the E3 ligases cIAP1 and cIAP2, which modify RIPK1 and themselves with K63-linked ubiquitin chains (Bertrand et al., 2008, Mahoney et al., 2008, Varfolomeev et al., 2008). The K63-linked chains allow for recruitment of LUBAC, which in turn modifies RIPK1 and NEMO with M1-linked ubiquitin chains (Haas et al., 2009, Ikeda et al., 2011, Tokunaga et al., 2009). The ubiquitin chains function as scaffolds for efficient recruitment and activation of the TAK1-TAB2/TAB3 and the IKK complex. Activated IKK induces the degradation of IκB, thereby freeing the NF-κB dimer to enter the nucleus (Figure 13).

In *Drosophila*, the sole homologue of TNF identified is Eiger (Igaki et al., 2002, Moreno et al., 2002). Eiger regulates cell death, host defence, tissue growth and regeneration via its receptors Wengen and Grindelwald (Igaki and Miura, 2014, Kanda et al., 2002, Andersen et al., 2015). However, in contrast to mammalian TNFR1 signalling, Eiger exerts its function mainly through JNK, and not NF-κB.

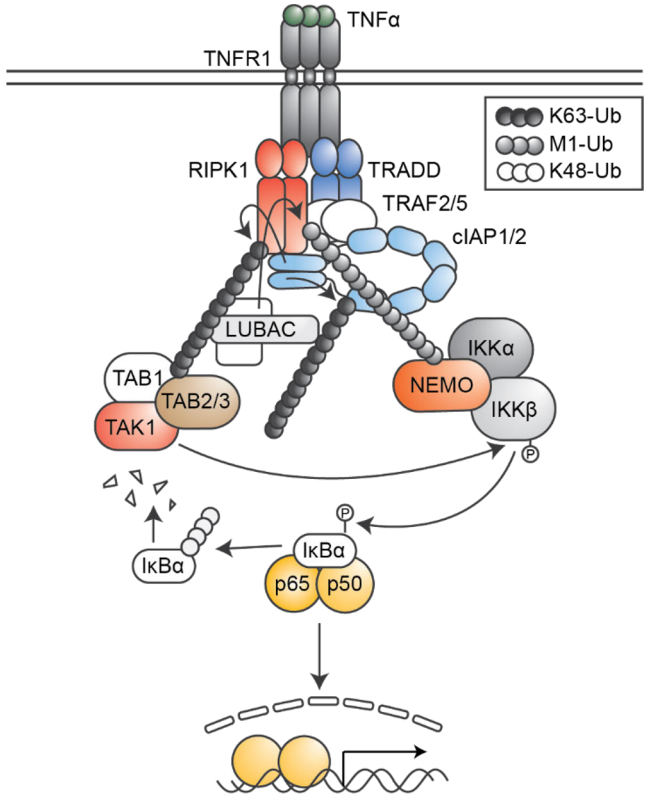

Figure 13. The TNFR1 signalling pathway. The signalling pathway is induced upon activation of TNFR1 by the cytokine TNFα. Receptor activation leads to the recruitment of TRADD, RIPK1 and TRAF2 or 5. TRAF2/5 recruits cellular cIAP1 and 2, which ubiquitinate RIPK1 and themselves with K63-linked chains. The complex consisting of TAK1, TAB1 and TAB2 or TAB3, and LUBAC are recruited to the receptor complex via the K63-linked ubiquitin chains. LUBAC synthesises M1-linked ubiquitin chains on RIPK1 and on NEMO, part of the IKK complex. Upon TAK1-mediated activation of the IKK complex, the complex phosphorylates IκB, thereby, targeting the protein for degradation. Degradation of IκB releases the NF-κB dimer for nuclear translocation and subsequent target gene activation. Conserved components between the Imd and TNFR1 signalling pathways are indicated by similar colours, see Figure 12 (figure adapted from Wajant and Siegmun, 2019, Falschlehner and Boutros, 2012).

3.4 The NOD2 signalling pathway

The mammalian NOD2 is a member of the NLR family of PRRs and is considered to be a key regulator of intestinal health and host-microbe interactions (Al Nabhani et al., 2017). NOD2 modulates both innate and adaptive immune responses by regulating NF-κB and the Mitogen-activated protein (MAP) kinase pathways. *NOD2* mutations have been associated with several inflammatory diseases, including Crohn's disease,

Graft-versus-host disease and Blau syndrome (Sidiq et al., 2016, Landfried et al., 2010, Miceli-Richard et al., 2001).

Intestinal NF-κB signalling is induced when NOD2, expressed by both haematopoietic and non-haematopoietic cells in the intestinal epithelium, recognises muramyl dipeptide (MDP) derived from PGN of Gram-negative and Gram-positive bacteria (Figure 14) (Girardin et al., 2003, Al Nabhani et al., 2017, Ferrand et al., 2019). The NOD2 protein contains two N-terminal CARD-domains, a C-terminal leucine-rich repeat (LRR) and a central NACHT domain. NACHT is an acronym standing for NAIP (Neuronal apoptosis inhibitor protein), C2TA (Class II major histocompatibility complex transactivator), HET-E (Heterokaryon incompatibility gene E) and TP1 (Telomerase-associated protein-1). The CARD domains interact with downstream adaptor proteins (Ogura et al., 2001), the NACHT domain mediates oligomerisation (Proell et al., 2008) and the LRR domain is involved in the recognition of MDP (Laroui et al., 2011). During steady-state conditions, NOD2 exists most probably, in an autoinhibitory state in the cytosol (Lechtenberg et al., 2014). Upon ligand recognition, NOD2 oligomerises via the NACHT domain and undergoes a conformational change, whereafter NOD2 recruits the serine-threonine kinase RIPK2 via a CARD-CARD interaction (Park et al., 2007). After binding to the receptor, RIPK2 undergoes autophosphorylation (Pellegrini et al., 2017) and ubiquitination (Hasegawa et al., 2008). RIPK2 has been associated with a number of ubiquitin E3 ligases, including XIAP (Krieg et al., 2009), cIAP1 and cIAP2 (Bertrand et al., 2009), the TRAF ligases -2, -5, and -6 (McCarthy et al., 1998, Yang et al., 2007), and Pellino3 (Yang et al., 2013). Whereas the role of TRAF- and cIAP1/2-mediated ubiquitination of RIPK2 in NOD2 signalling is not completely clear, XIAP, adding K63-linked chains on RIPK2, is an indispensable component of the pathway (Bertrand et al., 2009, Damgaard et al., 2012, Stafford et al., 2018). XIAP-mediated ubiquitination of RIPK2 is essential for the recruitment of LUBAC, which conjugates M1-linked linear ubiquitin chains on RIPK2 (Damgaard et al., 2012). It is thought that the ubiquitin chains on RIPK2 function as a scaffold, or binding platform, recruiting TAK1, via TAB1, TAB2 or TAB3, and the IKK complex to the receptor complex. The activated IKK complex targets IκB for degradation, thereby, activating NF-κB and subsequent gene transcription (Figure 14) (Kanayama et al., 2004, Wang et al., 2001, Chen et al., 2006, Yang et al., 2007, Hasegawa et al., 2008).

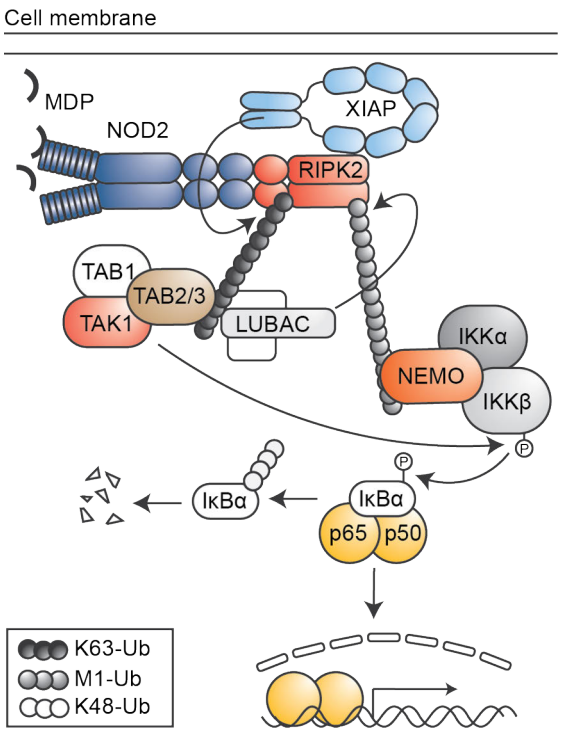

Figure 14. The mammalian NOD2 pathway. The intracellular NOD2 receptor is activated by muramyl dipeptide (MDP) derived from Gram-positive and Gram-negative bacteria. Activated NOD2 binds to RIPK2 that is thereafter K63-ubiquitinated by XIAP. Other E3 ligases omitted from the figure have also been shown to ubiquitinate RIPK2. LUBAC is recruited to the complex in an XIAP-dependent manner, and adds M1-linked chains on RIPK2. It is believed that the TAK1-TAB1-TAB2/3 complex and the IKK complex are recruited to the receptor complex via the ubiquitin chains attached to RIPK2. The catalytic subunit of the IKK complex, IKKβ, phosphorylates the inhibitory IκB protein, hence, targeting it for K48-linked ubiquitination and proteasomal degradation, thereby, freeing the NF-κB dimer to enter the nucleus and activate target gene transcription. Conserved components between the Imd and NOD2 signalling pathways are indicated by similar colours, see Figure 12 (figure based on Al Nabhani et al., 2017, Martínez-Torres and Chamaillard, 2019, and Falschlehner and Boutros, 2012).

3.5 Ubiquitin-mediated regulation of NF-κB signalling

Due to its strong proinflammatory function, increased activity of NF-κB contributes to the development of both acute and chronic inflammatory diseases (Zhang et al., 2017). Concordantly, inhibition of NF-κB has been shown to have anti-inflammatory effects *in vivo* (Greten et al., 2004, Acharyya et al., 2007, Arkan et al., 2005). However, mouse models

wherein NF-κB was inhibited in non-immune intestinal epithelial cells, developed severe chronic inflammatory conditions (Nenci et al., 2007, Zaph et al., 2007), indicating, hence, a dual immune regulatory role of NF-κB in certain tissues (Pasparakis, 2009, Wullaert et al., 2011). Maintaining immune homeostasis in the intestinal epithelia is particularly challenging, as the vast array of microbes call for a highly specialised immune response that is effective in eliminating pathogens, while allowing for beneficial host-microbe interactions to be established. Due to the properties of ubiquitination, functioning as a potent inducer of NF-κB, while simultaneously being highly dynamic and editable, the cell takes advantage of modifying inflammatory signalling by regulating ubiquitination and its inducers.

3.5.1 Regulation of intestinal NOD2 signalling by DUBs

As discussed earlier, E3 ligases are key induces of NF-κB signalling. In the case of mammalian NOD2 signalling, XIAP is one of the main E3 ligases driving NF-κB activity, and cells lacking XIAP are unable to induce NF-κB upon receptor stimulation (Krieg et al., 2009). Concordantly, *XIAP*$^{-/-}$ mice cannot clear intracellular bacterial infections and the absence or defective function of XIAP is connected to the development of IBD in humans (Bauler et al., 2008, Worthey et al., 2011, Zeissig et al., 2015, Parackova et al., 2020). Recently developed selective XIAP antagonists interfering with the XIAP-RIPK2 binding, was showed to block NOD2-induced ubiquitination of RIPK2, activation of NF-κB, and to decrease cytokine and chemokine production (Goncharov et al., 2018). The antagonists hold thus promise, unlike pan-IAP antagonists, activating cell death, NF-κB signalling, or cIAP1/2 autoubiquitination and proteasomal degradation (Varfolomeev et al., 2007, Vince et al., 2007), as possible therapeutic agents for NOD2-mediated inflammatory disease (Goncharov et al., 2018). Similarly to XIAP, the LUBAC complex seems to be indispensable in NOD2 signalling, and the transcription of NOD2 target genes is severely decreased in the absence of the LUBAC component SHARPIN (Damgaard et al., 2012).

The cell counteracts the activity of XIAP and LUBAC by expressing specialised DUBs, such as Cylindromatosis (CYLD), Ovarian tumour DUB with linear linkage specificity (OUTLIN) and A20 (Lork et al., 2017). CYLD mediates the cleavage of various polyubiquitin linkages, with a preference for K63- and M1-linked chains (Sato et al., 2015, Ritorto et al., 2014), whereas OTULIN exclusively hydrolyses M1-linked ubiquitin chains (Keusekotten et al., 2013). A20 exhibits activity towards K63-linked chains *in vivo*, however, interestingly, A20 also holds E3 ligase activity, enabling a dual ubiquitin-editing role (Wertz et al., 2004). CYLD has been shown to regulate NF-κB signalling downstream from a number of receptors, among them the NOD2 receptor (Sun, 2010). The DUB acts by

limiting K63- and M1-linked chains on RIPK2, thereby, decreasing cytokine production (Lork et al., 2017, Wex et al., 2016, Hrdinka et al., 2016). CYLD knockout mice, accordingly, display intestinal inflammation and are more susceptible to dextran sulphate sodium (DSS)-induced colon inflammation and tumour development (Zhang et al., 2006, Reiley et al., 2007). Furthermore, a genome-wide association study revealed CYLD polymorphisms to be strongly associated with Crohn's disease in humans (Cleynen et al., 2014). OTULIN controls NOD2 signalling by limiting the accumulation of M1-chains associated with RIPK2 (Fiil et al., 2013). Loss of OTULIN results in severe inflammatory phenotypes (Damgaard et al., 2016), however, how intestinal immune homeostasis is affected in OTULIN mutants, remains elusive. Finally, genome-wide association studies have identified, similarly as CYLD, A20 as a susceptibility gene for inflammatory diseases, such as psoriasis and Crohn's disease (Vereecke et al., 2011, Hammer et al., 2011, Barmada et al., 2004). A20 deficient mice die prematurely due to severe multiorgan inflammation, hence, reflecting the important role of A20 in inflammatory regulation (Lee et al., 2000). Mice with an enterocyte-specific A20 deletion are hypersensitive to DSS-induced colitis (Vereecke et al., 2010) and when A20 is deleted in both enterocytes and myeloid cells, mice develop spontaneous intestinal inflammation (Vereecke et al., 2014). Mechanistically, A20 is required for the termination of MDP-induced NF-κB signalling and counteracts NOD2 signalling by removing ubiquitin chains attached on RIPK2 (Hitotsumatsu et al., 2008).

3.5.2 Ubiquitin-mediated regulation of intestinal Imd signalling

Several regulators of intestinal Imd signalling have been identified in *Drosophila*. Among these are secreted amidase PGRPs that degrade PGN in the gut, preventing excessive immune activation (Paredes et al., 2011, Zaidman-Rémy et al., 2006), Poor Imd response upon knock-in (Pirk) that regulates intestinal immune tolerance by interacting with Imd (Kleino et al., 2008, Lhocine et al., 2008), and the transcription factor Caudal, regulating AMP expression in specific compartments of the gut (Ryu et al., 2008). The last section of this thesis will, however, only focus on the E3 ligases known to drive intestinal Imd signalling and the proteins shown to directly counteract their activity.

Similarly, as the E3 ligase activity of XIAP is needed for NOD2 signalling to proceed (Krieg et al., 2009, Damgaard et al., 2012), the IAP protein Diap2 is essential for the Relish-mediated antibacterial immune response (Kleino et al., 2005, Leulier et al., 2006b, Valanne et al., 2007). Diap2 mutants cannot mount septic nor local immune responses against Gram-negative bacteria and succumb rapidly upon pathogen exposure (Huh et al., 2007, Leulier 2006b, Gesellchen et al., 2005, Kleino et al., 2005). Diap2 acts by mediating the K63-linked ubiquitination of pathway members

Imd, Dredd and Kenny, hence, stimulating downstream signalling (Paquette et al., 2010, Meinander et al., 2012, Aalto et al., 2019).

The *Drosophila* LUBAC orthologue, Lubel, is shown to catalyse the formation of M1-linked linear chains on Kenny upon bacterial infection (Aalto et al., 2019). The E3 ligase seems to function specifically in the intestinal epithelia, as Lubel mutants are unable to mount an immune-response upon oral infection with Gram-negative bacteria, but do survive a septic infection. Furthermore, overexpression of the catalytic domain of Lubel induces intestinal inflammation in the absence of infection (Aalto et al., 2019).

Similarly as the mammalian counterpart, the *Drosophila* Cyld counteracts Diap2 and Lubel by removing both K63- and M1-linked ubiquitin chains of target proteins (Tsichritzis et al., 2007, Aalto et al., 2019). Cyld mutants display an increased expression of Imd target genes, but are, interestingly, more sensitive to septic infections compared to wild type flies. As Cyld is also involved in regulating metabolic signalling and fat storage, the impaired immune response might be explained by a dysfunction of the fat body (Tsichritzis et al., 2007). Finally, another DUB known as *Drosophila* ubiquitin-specific protease 36 (dUsp36), or Scrawny, has been shown to prevent the accumulation of K63-polyubiquitinated Imd, while promoting its degradation. Silencing of dUsp36 stimulates activity of the Imd pathway, which is lost when flies are reared axenic, indicating a role of dUsp36 in regulating immune responses induced by commensal bacteria (Thevenon et al., 2009).

3.6 *Drosophila* as a model for studying intestinal host defence

The well-characterised immune system of *Drosophila*, displaying similar stimulatory and regulatory mechanisms with those of mammals, strengthens the role of the fruit fly as a model when studying intestinal immunity and host defence. However, due to physiological disparities between the *Drosophila* and the mammalian intestine, all manifestations of human intestinal host defence cannot be studied in the fly. For instance, the fly gut does not have a lamina propria, i.e., a layer of connective tissue containing resident immune cell population that regulate intestinal immunity and bacterial populations (Mowat and Agace, 2014), nor does the fly gut contain highly specialised immune active epithelial cells, such as the AMP producing Paneth cells or absorptive M cells (Bevins and Salzman, 2011, Ohno, 2016). Furthermore, as *Drosophila* lacks an adaptive immune system, the role of immune cells in intestinal diseases, such as the role of T cells in IBD, cannot be investigated in the fly.

When elucidating the regulatory mechanisms of host defence, *Drosophila* is best suited for research regarding well-conserved pathways and specific effects. Among these are the Janus kinase protein and signal

transducer and activator of transcription (JAK-STAT) signalling pathway inducing immune responses and tissue growth (Panayidou and Apidianakis, 2013), the JNK pathway activating intestinal stress responses (Tafesh-Edwards and Eleftherianos, 2020), ROS signalling maintaining immue homeostasis (Ha et al., 2005, Jones et al., 2013), and finally, as outlined in the sections above, the NF-κB signalling pathways regulating immune responses, tissue homeostasis and host-microbe interactions (Lemaitre and Hoffmann, 2007). The devoted characterisation of the molecules involved in sensing and signalling in these immune-regulatory pathways, are gradually enabling more complex studies regarding host-microbe interations and immune signalling, to be carried out the fly.

OUTLINE AND KEY AIMS OF THESIS

Intestinal immune homeostasis is crucial for human health and aberrant regulation of host-microbe interactions is connected to chronic inflammation, metabolic diseases and cancer development. The evolutionarily conserved NF-κB signalling pathways are master mediators of immune responses in the intestinal epithelia and their regulation is, thereby, of specific interest when tuning inflammatory signalling. The key aims of this thesis are to advance the use of *Drosophila melanogaster* as a model for studying host-microbe interactions and to elucidate the molecular mechanisms regulating ubiquitin-induced inflammatory NF-κB signalling in the intestine of *Drosophila*.

Key aims of this thesis:

- To study host-microbe interactions in the *Drosophila* gut by rearing flies under axenic conditions.
- To externally manipulate the fly microbiome with developed combinatory antimicrobial nanoparticles.
- To investigate the specific regulation of inflammatory NF-κB signalling occurring in microbiotic environments.
- To elucidate caspase-mediated regulation of ubiquitin E3 ligases during inflammatory signalling.
- To determine the role of the *Drosophila* caspase Drice as a regulator of NF-κB signalling and intestinal homeostasis.

EXPERIMENTAL PROCEDURES

In this section, the experimental procedures (Table 1) used during the thesis work are briefly presented. More detailed information on material and methods can be found in the original articles.

Table 1. Experimental procedures used in this thesis.

Experimental procedures	**Study**
16S sequencing	III
Axenic fly culture (AFC)	I, III
Caspase activity assay (CA)	III
Cell culture	III
Cell viability assay	III
Drosophila dissections	III
Drosophila crossings	III
Image analysis	I, III
Immunofluorescence (IF)	III
Fluorescence microscopy	III
Light microscopy	II, III
Pathogen clearance assay (PA)	II, III
Polymerase chain reaction (PCR)	I, III
Quantitative reverse transcription PCR (qPCR)	I, III
SDS-PAGE and Western blotting (WB)	III
Statistical analysis	I, II, III
Survival assay (SA)	III
Transfection	III
Ubiquitination assay (UA)	III
X-gal staining	III

1 Fly husbandry

The flies used were maintained at 20°C or 25°C with a 12-h light-dark cycle on Nutri-fly BF (Dutscher Scientific, Essex, UK) food. Adult flies were used for all experiments, except for those regarding nanoparticles, where 3rd instar larvae were used. The *Canton*^{S} or *yellow white* (*yw*) strains were used as wild type flies and, when studying the activity of the Imd pathway, the *Diap2*^{7c} mutant was used as negative control. In experiments regarding flies expressing genes under the UAS-Gal4 system, the Gal4 driver line was additionally used as internal control. All fly lines and their applications are listed in Table 2. The *Wolbachia* positive or negative state of all fly lines was assessed by PCR with *Wolbachia* specific primers (Table 2). To rear flies under axenic conditions, embryos were dechorionated

with bleach whereafter they were transferred onto sterile food in a sterile environment. After hatching, flies were confirmed to be germ-free by PCR of the 16S ribosomal RNA (rRNA) gene and by growing fly homogenates on Luria Bertani (LB, Sigma, Saint Louis, Missouri, US) agar plates and counting colonies.

Table 2. *Drosophila* lines used during thesis work, their applications and their *Wolbachia* positive or negative state.

Fly lines	**Application**	***Wolbachia***
*Canton^{S}**	WB, qPCR, IF, 16S seq., SA, UA	+
Canton^{S} larvae	Nanoparticle and capsaicin treatment	+
*Yellow white** (*yw*)	AFC, FA	+
*DaughterlessGal4** (*DaGal4*)	qPCR, PA	+
*UbiquitousGal4** (*UbiGal4*)	WB, qPCR, FA, AFC, 16S seq., PA	-
*NP1Gal4**	qPCR	-
*Diptericin-LacZ** (*Dipt-LacZ*)	X-Gal staining	-
Diap2^{7c} #	WB, qPCR, SA, PA, UA	+
UAS-p35	qPCR	-
Drice^{17}	WB, qPCR, IF, SA	-
UbiGal4; UAS-Diap2^{WT}	WB, qPCR, IF, AFC, 16S seq.	+
UbiGal4; UAS-Diap2^{WT}/Dipt-LacZ	X-Gal staining	+
UbiGal4; Drice-RNAi	WB, qPCR, IF, AFC, 16S seq., PA, UA	-
UbiGal4; UAS-Drice-RNAi/Dipt-LacZ	X-Gal staining	-
UAS-Drice^{WT}; DaGal4	qPCR, SA, PA, UA	-
UAS-Drice^{WT}/UbiGal4; UAS-Drice-RNAi	WB, qPCR	-
UAS-Drice^{C211A}/UbiGal4; UAS-Drice-RNAi	WB, qPCR	-
Diap2^{7c}; UAS-Diap2^{WT}/DaGal4	qPCR, SA, PA	+
Diap2^{7c}; UAS-Diap2$^{\Delta 100}$/DaGal4	qPCR, SA, PA	+
NP1Gal4; UAS-p35	qPCR, FA	-
NP1Gal4; UAS-p35/Dipt-LacZ	X-Gal staining	-
PGRP-LC$^{\Delta 5}$	FA	-

* Fly lines used as positive controls
# Fly line used as negative control

2 16S rRNA sequencing

To identify the bacterial species residing in the *Drosophila* gut, we performed 16S rRNA sequencing on DNA extracted from adult flies. Amplification and Illumina MiSeq sequencing of the V1-V3 region of the bacterial 16S rRNA gene, as well as selection of operational taxonomic units (OTUs) and taxonomy assignment of OTUs were done using Eurofins Genomics InView Microbiome Profiling 3.0 service.

3 Survival assays (SA) and pathogen clearance assays (PA)

The ability of flies to fend off septic pathogenic infections was assessed by infecting flies with the Gram-negative bacteria *Erwinia carotovora carotovora 15* (*Ecc15*) and monitoring the survival of flies for four days. The septic infection was induced by pricking adult flies in the lateral thorax with a needle previously dipped in concentrated *Ecc15* solution. To study the ability of *Drosophila* to clear ingested pathogens, the number of colonies was assessed in flies infected orally with *Escherichia coli* (*E. coli*). To distinguish between commensal and pathogenic bacteria we used ampicillin resistant *E. coli*, and plated the fly lysates on LB plates containing ampicillin. The same assay was employed to investigate the effect of capsaicin and capsaicin-loaded nanoparticles on ingested *E. coli* in 3rd instar larvae.

4 Cell culture

To study protein expression, protein-protein interactions and the ubiquitination patterns of specific proteins in the Imd pathway, *Drosophila* Schneider S2 cells (Invitrogen, Waltham, Massachusetts, US) were used. The S2 cells were grown at 25°C using Schneider's cell medium supplemented with 10% fetal bovine serum (Biowest, Nuaillé, France), L-glutamine (Sigma) and penicillin/streptomycin (Sigma). The cells were transfected using the Effectene transfection reagent (QIAGEN, Hilden, Germany).

5 Protein expression and protein-protein interaction studies

To study cell proliferation, the number of phospho-histone H3 (PHH3) (Ser10, #9701, Cell Signaling Technology, Danvers, Massachusetts, US) expressing cells in dissected fly guts was counted by immunofluorescence staining (IF). Protein-protein interactions were assessed in transfected S2 cells by immunoprecipitation (IP) using HA-tagged beads (Sigma).

Ubiquitin assays (UA), investigating the ubiquitin patterns in adult flies or in transfected S2 cells were done by performing pulldowns with the recombinant GST-Tandem ubiquitin binding entity (TUBE) protein during denaturing conditions. The protein expression in samples from IP experiments, UAs, transfected S2 cells, whole flies and fly organs were investigated by SDS-PAGE and Western blotting (WB) using the antibodies listed in Table 3.

Table 3. Primary antibodies used, their source and their application.

Antibody	Company or reference	Application
Diap2	Tenev et al., 2005	WB, UA, IP
Drice	Leulier et al., 2006a	WB, UA, IP
Actin (C-11)	Santa Cruz Biotechnology	WB, UA, IP
V5 (clone SV5-Pk1)	Bio-Rad	UA, IP
HA (clone 3F10)	Roche	UA, IP
K63 (clone Apu3)	Millipore	WB, UA
K48 (clone Apu2)	Millipore	WB
Phospho-histone H3	Cell Signalling	IF

6 Measurement of NF-κB target gene activity

Quantitative reverse transcription PCR (qPCR) was employed to study the expression of NF-κB target genes *Drosocin* and *Diptericin* during basal conditions and after septic infection in adult flies, in conventionally reared flies, in axenic flies, and in transfected *Drosophila* S2 cells. The expression of the housekeeping gene *Ribosomal protein 49* (*rp49*) was used for normalization. To study local NF-κB activity, dissected guts and fat bodies of *Diptericin-LacZ* reporter fly lines, combined with mutants of interest, were stained with X-gal.

7 Measurement of caspase activity (CA) and cell viability

Caspase activity was assessed by adding the profluorescent substrate Z-DEVD-R110 (Apo-ONE ® Homogeneous Caspase-3/7 Assay, Promega, Madison, Wisconsin, US), recognised specifically by caspase-3 and -7, onto lysed fly guts or transfected S2 cells, and thereafter measuring emitted fluorescence. Cell viability of transfected cells was investigated by spectrophotometry after addition of the WST-1 reagent (Roche, Basel, Switzerland).

RESULTS AND DISCUSSION

1 Manipulation of the *Drosophila* microbiome (I, II)

Largely due to the complexity of the human intestinal microbiome, with a composition that varies greatly between individuals (Ley et al., 2006), studying specific bacterial species and their effect on host physiology is challenging. Adding to the complexity of host-microbe interactions, external factors, such as nutrition, invading pathogens and drug exposure, continuously modify the bacterial composition and challenge intestinal homeostasis. To unravel the network of host-microbe interactions, animal models in which the microbiome and its composition can be easily manipulated need to be established. *Drosophila* serves as an excellent candidate for this purpose, as the fly, while carrying a microbiome with a relatively simple bacterial composition, maintains advanced host-microbe interactions affecting organism health. Furthermore, the fly shares several similarities with the mammalian intestine regarding both anatomical and cellular architecture as well as biological function (Apidianakis and Rahme, 2011, Liu et al., 2017, Capo et al., 2019). Finally, the *Drosophila* gut provides a far more complex platform to study microbe-drug interactions than methods based on biochemical assays or cell cultures. To further the use of *Drosophila* as a model for studying host-microbe interactions, we optimised a protocol for rearing flies under axenic conditions and took advantage of the foraging behaviour of *Drosophila* larvae to externally target intestinal pathogens with antimicrobial nanoparticles *in vivo*.

1.1 Rearing *Drosophila* under axenic conditions

To ease the use of *Drosophila* in host-microbe studies, we developed a step-by-step protocol of how to rear flies under axenic conditions in standard equipped laboratories. We optimised the two most commonly used methods of rearing axenic flies, i.e., supplementing dietary media with antibiotics or dechorionation of *Drosophila* eggs. The method of dechorionating eggs was first described by Marion Bakula (Bakula, 1969), and entails the removal of the outermost layer of the egg, the chorion, using bleach (I, Fig 1). Normally, the chorion is coated with bacteria originating from the mother and is the main bacterial transmission route between adult females and progeny. Emerging larvae ingest the chorion, forming, hence, the basis of their own microbiota (Bakula, 1967). Removal of the chorion leads to a sterile egg, and when transferred onto sterile food in a sterile environment, adult axenic flies can be reared. This method has been implemented by several groups, although with varying protocols regarding concentration of bleach, time points and washing steps (Brummel et al., 2004, Ryu et al., 2008, Shin et al., 2011, Storelli et al., 2011, Ridley et al., 2012, Blum et al., 2013, Schretter et al., 2018, Sharon et al.,

2012). The studies performed by these groups showed that removal of all bacteria affects immune regulation (Ryu et al., 2008), development (Shin et al., 2011, Storelli et al., 2011), energy homeostasis (Ridley et al., 2012), interferes with mating preference (Sharon et al., 2010), leads to hyperactive locomotor behaviour (Schretter et al., 2018) and shortens lifespan (Brummel et al., 2004). We reported a significant decrease in the basal AMP expression of axenic flies compared to conventionally reared flies (I, Fig 5).

Supplementing dietary media with antibiotics is considered a far simpler method than dechorionation when rearing flies axenic. Both broad-spectrum antibiotics such as streptomycin or tetracycline, or different combinations of antibiotics have been used for this purpose (Ridley et al., 2013, Sharon et al., 2010, Brummel et al., 2004, Fast et al., 2018b, Heys et al., 2018). We described how by allowing adult flies to lay eggs onto food supplemented with tetracycline, removing the adults, and letting the eggs develop in the antibiotic-supplemented food, adult germ-free flies can be obtained.

Regardless of the sterilisation process, the axenic flies need to be confirmed to be germ-free. A simple way to confirm axenity is to plate fly homogenates on agar plates and check for colony formation. We describe how to use LB plates for this purpose (I, Fig 2). However, as all bacterial species do not grow on LB, other growth media, such as nutrient agar or De Man, Rogosa and Sharpe (MRS) agar should be used in addition to confirm the absence of a broader variety of bacterial species. The small subunit of the prokaryotic ribosome is called 16S and is conserved between bacterial species (Weisburg et al., 1991). The 16S gene can, hence, be used to identify bacteria in a sample. We optimised a protocol for performing PCR on 16S ribosomal RNA from fly lysates to identify, in a highly sensitive manner, any bacterial contaminations in our axenic flies (I, Fig 3).

1.1.1 Considerations when rearing flies axenic

Disturbing the resident microbiota by supplementing diet with antibiotics or by dechorionation may lead to unspecific effects. Antibiotics treatment seems to, however, be the harsher option as it can, besides acting on bacteria, also act on host proteins (Brodersen et al., 2000). Diets supplemented with tetracycline has, furthermore, been shown to have a transgenerational effect on mitochondria, affecting insect embryo development and sperm viability (Ballard and Melvin, 2007, Zeh et al., 2012, O'Neill et al., 1997). We, furthermore, found that the developmental time of flies fed antibiotic-supplemented food was markedly prolonged compared to that of control flies.

The effect of dechorionation on fly physiology remains unclear. Ridley and colleagues reported no effect of chorion removal on survivorship

form egg to adult, whereas a study conducted by Heys and colleagues showed an increased mortality rate during the development of flies hatched from dechorionated eggs compared to conventionally reared flies (Ridley et al., 2013, Heys et al., 2018). As the chorion acts as an outer barrier, protecting dipterans from external changes, dechorionation might lead to an egg more sensitive to environmental shifts, which in turn could explain the higher mortality rate reported by Heys et al (Chapman et al., 2013).

1.2 Capsaicin-loaded silica nanoparticles (NAB) target *Escherichia coli* in the *Drosophila* intestine

Traditionally, antibiotics, probiotics and microbial transplants have been used to manipulate the microbiota, acting by eliminating pathogens or restoring a dysbiotic microflora (Konstantinidis et al., 2020, Hemarajata and Versalovic, 2013, Young, 2016). However, especially in the case of antibiotics, the adverse effects, including decreased microbial diversity, increased disease susceptibility, development of allergic conditions, and, the most concerning, the emergence of multidrug resistant bacterial strains, call for alternative methods when targeting bacteria (Konstantinidis et al., 2020). Tuneable nanoparticles, acting as carriers of therapeutic agents, are used to manipulate the microbiome in a more controlled manner and serve as attractive alternatives to antibiotics. Furthermore, combinatory nanoparticle designs, exhibiting multiple antibacterial properties, hold promise when eliminating antibiotic-resistant bacteria (Wang et al., 2017).

Nanoparticles are submicron (10-1000 nm) colloidal particles, used as drug carrier systems, where the drug can be dissolved, entrapped, encapsulated or attached to a nanoparticle matrix (Mohanraj and Chen, 2006). By encapsulating proteins in a nanocarrier, unwanted properties of the protein, such as poor solubility and stability, difficulty in crossing cell membranes or lack of specificity, can be modified (Xu et al., 2019). Clinically available nanoparticles are usually constructed of organic materials, such as lipids and polymers, however, the intrinsic instability and limited drug-loading capacity of these materials are restricting their use as drug delivery systems (Elsabahy and Wooley, 2012, Puri et al., 2009). In order to improve the features of nanoparticles, particles made of inorganic materials, such as tuneable mesoporous silica nanoparticles (MSNs), with a high chemical stability, have been developed (Xu et al., 2019). We designed a combinatorial nanoparticle (NAB), employing MSN as a mesoporous shell, cerium oxide as core, capsaicin as loaded drug and chitosan as final coat (II, Scheme 1). Upon synthesis, the cytocompatibility of the designed particle and its constructs, as well as the antibacterial properties of the particle, were evaluated *in vitro*. The *in vivo*

characterisation was performed by orally administering nanoparticles to *E. coli* infected *Drosophila* larvae.

When studying the effect of nanoparticles in *Drosophila*, the particles have generally been administered through ingestion, however, injection and inhalation are also possible routes of delivery (Chifiriuc et al., 2016, Posgai et al., 2009). Depending on the chosen route of administration and monitored *Drosophila* life stage, the effect of nanoparticles on behaviour, development, mutagenesis, or even on modulating the activity of specific signalling pathways, can be assessed. To study the antimicrobial activity of NAB, we orally infected *Drosophila Canton*^{S} larvae with *E. coli*, whereafter the nanoparticles were administered through ingestion. When we, after treatment, plated larval homogenates and counted the number of colony forming units (CFU), we found a significant decrease in number of CFU in larvae treated with nanoparticles, hence, indicating that the constructed nanoparticle is, indeed, suitable for targeting bacteria residing in the *Drosophila* gut (II, Fig 6B). Capsaicin is a component of *Capsicum* plants (chili peppers), shown to exhibit antimicrobial activity against a variety of pathogens, among these *Staphylococcus aureus* and *E. coli* (Wang et al., 2019, Füchtbauer et al., 2021). When we assessed the antimicrobial activity of capsaicin alone, we found that the concentration of free capsaicin needed to eliminate *E. coli* was significantly higher than that needed when loaded to the particle (II, Fig 6C). These results can be explained by the intrinsic antimicrobial activity of the unloaded nanoparticle, but might also point towards a more efficient, nanoparticle-mediated drug delivery.

Although future characterisation, especially regarding toxicity of the developed nanoparticles is needed, the results from our *in vivo* experiments are encouraging in regard of using nanoparticles for manipulating commensal microbes or targeting pathogenic bacteria residing in the gut. Our study further strengthens the use of *Drosophila* as a platform for drug testing and nanomedicine studies.

2 Caspase-mediated regulation of inflammatory signalling and host-microbe interactions (III)

In order for symbiotic host-microbe relationships to be established, while simultaneously eliminating harmful pathogens, intestinal immune responses need to be tightly regulated (Miguel-Aliaga et al., 2018, Mohajeri et al., 2018). Dysregulation of these responses can lead to chronic inflammation, connected to the development of several diseases such as IBDs and gastrointestinal cancer (Garrett et al., 2010). IAP proteins are key regulators of inflammation and able to induce NF-κB signalling by ubiquitination (Gyrd-Hansen and Meier, 2010). The proteins,

furthermore, modify the activity of caspases and, thereby, regulate apoptosis (Budhidarmo and Day, 2015). As inflammation and apoptosis both impact intestinal homeostasis, the interplay between IAPs and caspases are particularly interesting when studying the regulation of intestinal inflammation. Hence, to elucidate inflammatory regulation in *Drosophila*, we set out to investigate how the *Drosophila* iap2 protein is regulated in the intestine.

2.1 The Diap2-Drice complex regulates Imd signalling in the intestine

The fat body is the major immune responsive organ in the fly, activated upon septic infections. When a pathogen enters the haemocoel and the haemolymph, inflammatory NF-κB signalling is induced in order to eliminate the intruder (Lemaitre and Hoffmann, 2007). An equally important part of the immune system are the local immune responses taking place in the epithelial layers of gut and trachea, shaping the abundance and composition of luminal microorganisms. Activation of inflammatory signalling at the epithelial interfaces needs to be tightly regulated in order to avoid excess immune responses, harming commensal bacteria and disrupting gut homeostasis. As Diap2 is the key pathway inducing ligase, shown to be required for Imd signalling to proceed, we set out to investigate whether Diap2 is differently regulated in the *Drosophila* gut versus in the fat body. In order to detect inflammation, we used qPCR to measure expression of Relish-dependent AMP genes in whole fly lysates. By further staining the gut and fat body of *Diptericin-LacZ* reporter flies combined with our mutants of interest, we were able to identify the organ responsible for a possible AMP expression. When studying NF-κB signalling in transgenic flies ectopically expressing Diap2, we found inflammatory genes to be upregulated in the fly gut but not in the fat body (III, Fig 1A-D). Furthermore, we detected full length Diap2 in lysates from the fat body of wild type flies, but found Diap2 to be absent in gut lysates (III, Fig 1G). In the transgenic Diap2 flies, however, Diap2 was stabilised in both fat body and gut, indicating that increased expression of Diap2 induces inflammation only in the intestine (III, Fig 1H).

As Drice and Diap2 are known to interact by forming a stable complex (Ribeiro et al., 2007), we hypothesised that Drice is regulating Diap2 in the intestine. When we investigated the inflammatory phenotype of Drice mutant flies, it indeed correlated with that of flies ectopically expressing Diap2, displaying upregulated inflammatory signalling in the gut, but not in the fat body (III, Fig 2A, B, D, E). Accordingly, expressing $Drice^{WT}$ ubiquitously in the fly attenuated pre-existing basal expression of AMPs (III, Fig 2C). In addition to being essential during host defence, inflammation is important for regeneration and tissue repair.

Concordantly, an aberrant or a prolonged inflammatory response is connected to hyperplasia and cancer development (Medzhitov, 2008, Greten and Grivennikov, 2019). When we stained the guts of Diap2 transgenic or Drice mutant flies for dividing cells, we, indeed, found both fly lines to have an increased number of proliferating cells (III, Fig 1E, 2G).

The inflammatory signalling in the intestine seemed to be driven by excessive levels of Diap2, as the protein was stabilised in the guts of Drice mutant flies (III, Fig 2I). In mammals, XIAP has been shown to be degraded via the proteasome (Yang et al., 2000). The levels of XIAP seem to be regulated by its own E3 ligase activity, as removal of the RING domain stabilises XIAP in apoptotic thymocytes (Schile et al., 2008). Similarly, we found that inhibition of the proteasome lead to a stabilisation of cleaved Diap2 in the fly intestine but also of Drice, indicating that formation of the Drice-Diap2 complex induces the degradation of both proteins in a manner that is preceded by cleavage of Diap2 (III, Fig 2J). As Diap2-Drice complex formation requires ubiquitination of Drice (Figure 9) (Ribeiro et al., 2007), the E3 ligase activity of Diap2, as in the case of XIAP, seems to be needed for degradation.

2.2 The catalytic activity of Drice is needed to restrain Imd signalling

As formation of the Diap2-Drice complex is dependent on Drice-mediated cleavage of Diap2 (Figure 9) (Ribeiro et al., 2007), we wanted to investigate whether the catalytic activity of Drice is needed for Drice-mediated regulation of Imd signalling. We generated flies expressing a catalytic mutant of Drice, and found the flies unable to rescue the Drice mutant phenotype (III, Fig 3A). The viral caspase inhibitor p35 is known to inhibit Drice by trapping the catalytic machinery of the caspase via a covalent thioacyl linkage (Kim et al., 2014). The need of Drice's catalytic activity for regulating Diap2 was further strengthened by the fact that flies expressing p35 mimicked the inflammatory phenotype of Drice mutants (III, Figure 3C, D). p35 has been shown to also inhibit Dcp-1 (Kim et al., 2014), however, Drice is the only effector caspase known to interact with Diap2 (Leulier et al., 2006a), indicating that Drice alone, inhibits Diap2-mediated activation of Relish target genes.

Imd pathway members Imd, Dredd and Kenny are targets of pathogen-induced Diap2-mediated K63-linked ubiquitination (Paquette et al., 2010, Meinander et al., 2012, Aalto et al., 2019). To investigate if loss of Drice activity affects Diap2-mediated ubiquitination *in vivo*, we fed flies with the cell-permeable caspase-3 inhibitor Z-DEVD-FMK and pulled down ubiquitin chains with GST-TUBE from whole fly lysates. Indeed, inhibition of Drice led to an increase in Diap2-mediated K63-linked ubiquitination (III, Fig 4B). To further assess if Drice interferes with the ubiquitination of the known Diap2 targets Dredd and Kenny, we performed GST-TUBE pulldowns in S2 cells transfected with Diap2, Dredd or Kenny. Drice

activity was induced by overexpressing Drice, and inhibited by overexpressing a catalytic mutant form of Drice, Drice^{C211A}, or by treating cells with Z-DEVD-FMK. The presence of Drice decreased the amount of Diap2 and ubiquitinated Dredd and Kenny, whereas overexpression of Drice^{C211A} or treatment with Z-DEVD-FMK did no longer decrease Diap2 levels and increased the amount of ubiquitinated proteins (III, Fig 4C, D). The ubiquitin assays indicate that Drice, by regulating the levels of Diap2, also restrains the ability of Diap2 to ubiquitinate its targets. While our overexpression system does not dictate an *in vivo* occurrence, it seems likely that Drice does not interfere with the binding of Diap2 to Dredd and Kenny, but merely decreases their interaction by inducing the degradation of Diap2. As ubiquitination of Dredd and Kenny are crucial steps needed to further the Imd pathway (Meinander et al., 2012, Aalto et al., 2019), a decrease in the ubiquitination of these targets would, concordantly, restrain inflammatory signalling.

2.3 The microbiome in Diap2 and Drice mutant flies

Chronic inflammatory conditions, such as IBDs, disrupt the balance of the intestinal microbiome and are frequently associated with bacterial community dysbiosis (Clemente et al., 2012). To investigate the bacterial composition of Diap2 and Drice mutant flies we performed 16S sequencing on lysates from adult flies. The 16S gene contains highly conserved regions as well as variable regions, specific for different bacterial species. By taking advantage of the conserved regions for primer binding, the variable region can be amplified and sequenced in order to identify the different bacterial species within a sample. The effect of a *Wolbachia* infection on the composition of the commensal microbiome is not completely clear, as results seem to vary with the genotypes studied (Simhadri et al., 2017). However, as *Wolbachia* has been shown to impact host physiology (Ikeya et al., 2009), we decided to choose controls with a similar *Wolbachia* status for the 16S sequencing. Our *Canton^{S}* and the transgenic Diap2 flies are *Wolbachia* positive, whereas the driver line *UbiGal4* and the Drice mutants are *Wolbachia* negative. These lines were, hence, compared in the 16S sequencing. We found both the Diap2 transgenic and Drice mutant flies to, compared to the control lines, have an increased proportion of bacteria from the phylum *Proteobacteria* versus *Firmicutes*, (III, Fig 1F, 2H), a notion associated with IBD and aging in both humans and flies (Cheng et al., 2013, Clemente et al., 2012, Clark et al., 2015).

We found all sequenced fly lines to harbour bacterial species predominantly from the families *Acetobacteraceae* and *Lactobacillaceae*. *Canton^{S}*, the Diap2 transgenic flies and the Drice mutant flies harboured, in addition, bacterial species from the family *Enterococcaceae* (Figure 15).

These three bacterial families are consistently reported to associate with *Drosophila* (Erkosar et al., 2013, Brummel et al., 2004, Ren et al., 2007, Ridley et al., 2012, Ryu et al., 2008, Sharon et al., 2010, Storelli et al., 2011, Chandler et al., 2011, Wong et al., 2011). To investigate the effect of a pathogenic disturbance on the bacterial composition, we infected flies orally with the Gram-negative pathogen *Ecc15* and performed 16S sequencing after a 24-h recovery period (Figure 15). The proportion of *Lactobacillaceae* increased, while the proportion of *Acetobacteraceae* decreased, in *UbiGal4* and in the Diap2 and Drice mutant fly lines post infection. Interestingly, *Canton^S* displayed, on the contrary, an increase in the relative abundance of *Acetobacteraceae* coupled with a decrease in the proportion of *Lactobacillaceae* (Figure 15). Although a clear pathogen-induced shift in the bacterial composition can be detected, conclusions regarding the role of specific bacterial species during infection cannot be drawn. However, species that exhibit clear changes in proportion, such as *L. pseudomesenteroides* in the case of *UbiGal4*, and *L. plantarum* in the case of *Canton^S,* might serve as interesting candidates when elucidating the effect of internal microbial interactions on host health and in the protection against pathogens.

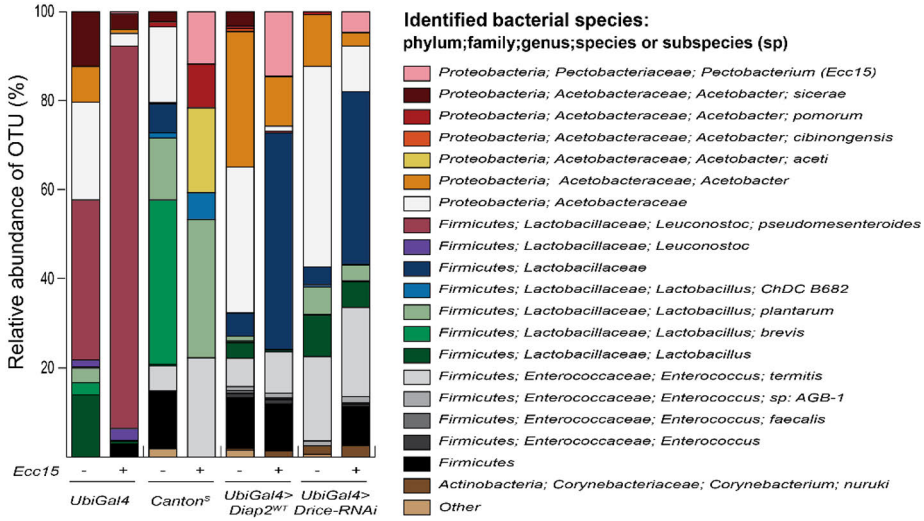

Figure 15. *Drosophila* bacterial composition post oral infection. Bacterial 16S rRNA metagenomics analysis of the 1V-3V region in *UbiGal4*, *Canton^S*, *UbiGal4;Diap2^{WT}* and *UbiGal4;Drice-RNAi* adult flies before and after oral infection with *Ecc15*. Colours indicate identified operational taxonomic units (OTUs), n = 1. The class and order of identified bacterial species are not specified and the proportion of *Wolbachia* species have been omitted from the *Canton^S* and *UbiGal4;Diap2^{WT}* samples for easier comparison of bacterial species residing in the gut lumen.

2.4 Drice restrains inflammatory signalling induced by the resident microbiome

As Diap2 is required for Imd signalling to proceed, we next studied the immune response upon pathogenic Gram-negative infection in flies ectopically expressing Drice and in Drice mutants. We found that both fly lines were able to fend off both septic and oral pathogenic infections, indicating that Drice only has negative regulatory effect during basal conditions (III, Fig 5A-F). This notion was further strengthened by the fact that ingestion of pathogenic bacteria leads to a rapid accumulation of both Diap2 and Drice, suggesting a disruption of the Diap2-Drice complex during infection (III, Fig 5H). Complex disruption seems to allow Diap2 to be redirected for ubiquitination of other target proteins, thereby, driving downstream Imd signalling. A similar IAP-induced shift in target ubiquitination upon receptor activation has been shown in non-canonical NF-κB signalling, where cIAPs switch from degradation-inducing ubiquitination of NIK to ubiquitination of TRAF2/3, releasing NIK to activate downstream signalling (Zarnegar et al., 2008).

The Drice-cleaved form of Diap2, $Diap2^{\Delta 100}$, is a functional E3 ligase able to interact with both Imd and Dredd (Figure 16A, B) and to ubiquitinate Dredd (Figure 16C). However, as Diap2 cleavage is a consequence of interaction with Drice, we wanted to examine if cleavage reduces the activity of Diap2 in Imd signalling. We, hence, performed oral and septic survival assays and measured AMP expression after septic infection in flies expressing only $Diap2^{\Delta 100}$. As expected, these flies were able to fend off pathogens equally well as control flies and flies expressing $Diap2^{WT}$ (III, Fig S4C-E), indicating, hence that cleavage of Diap2 is not enough to restrain inflammatory signalling. Another consequence of Diap2-Drice interaction is the separation of the BIR1 domain from Diap2 (Ribeiro et al., 2007). Interestingly, a nonsense mutation E99X in XIAP that introduces a stop codon after the BIR1 domain was found in an early onset Crohn's disease patient (Zeissig et al., 2015). This mutation led to a defect in intestinal NOD2 signalling, without affecting immune signalling in T cells or peripheral blood mononuclear cells (Zeissig et al., 2015). The BIR1 domain has, furthermore, been shown to mediate XIAP-induced NF-κB signalling by bringing TAB1 and XIAP together, leading to the activation of TAK1 (Lu et al., 2007). As separation of BIR1 in *Drosophila* seems to take place only in the intestine, the role of the yet unidentified *Drosophila* TAB1 homologue, could unravel yet another level of epithelial inflammatory regulation.

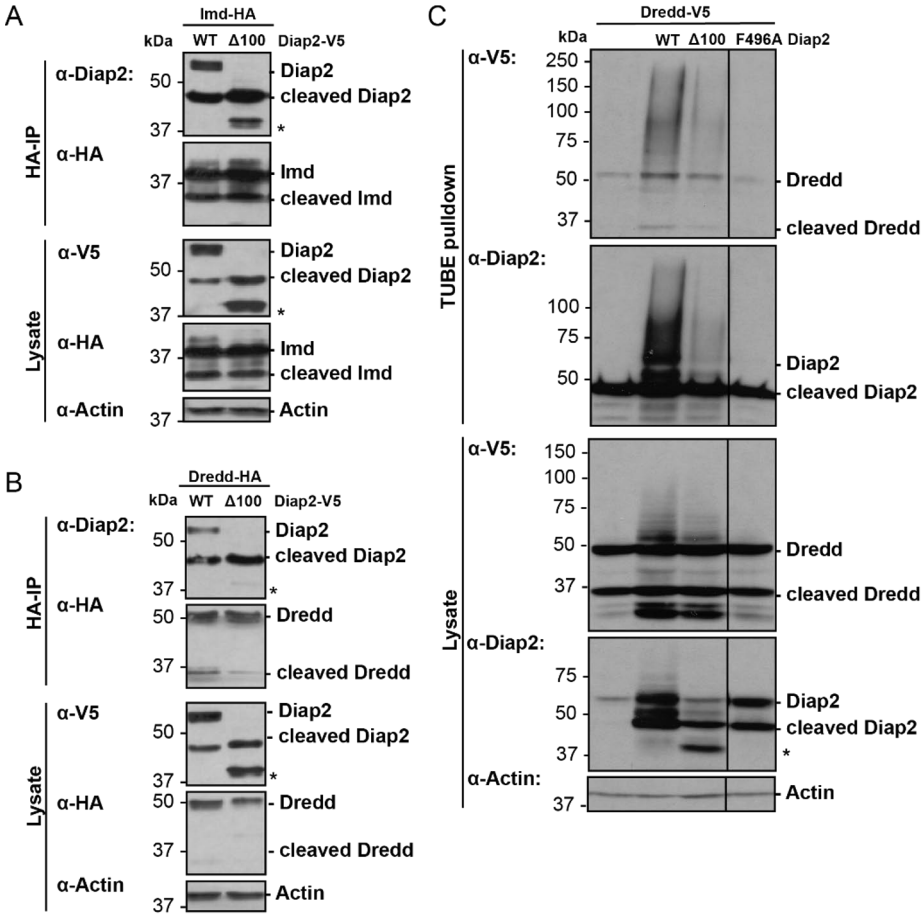

Figure 16. Diap2 $^{\Delta 100}$ interacts with both Imd and Dredd and is a functional E3 ligase. A, B) *Drosophila* S2 cells were transfected with HA-tagged Imd (A) or HA-tagged Dredd (B) together with V5-tagged Diap2^{WT} or Diap2 $^{\Delta 100}$, whereafter a HA-IP was performed, and the samples were analysed by Western blotting with α-V5, α-HA, α-Diap2 and α-Actin antibodies, n = 3. C) *Drosophila* S2 cells were transfected with V5-tagged Dredd and Diap2^{WT}, Diap2 $^{\Delta 100}$ or the catalytically dead Diap2^{F496A}, whereafter ubiquitin chains were isolated with GST-TUBE at denaturing conditions, and the samples were analysed by Western blotting with α-V5, α-Diap2 and α-Actin antibodies, n = 3.

As the bacterial presence is constant in the gut, and the fat body only encounters bacteria during systemic infections, we hypothesised that the commensal microbiome activates Diap2-mediated inflammatory signalling, which in the absence of Drice leads to excessive activation of NF-κB. When eliminating all bacteria by rearing flies axenic, we found as expected, the inflammatory phenotypes of Drice mutant and Diap2

transgenic flies to be rescued (III, Fig 6A, C). Interestingly, the levels of Diap2 remained low in the intestine of axenic flies, pointing towards a continuous degradation of the Drice-Diap2 complex also in the absence of bacteria (III, Fig S5). The results from Study III lead us to propose the following model of Drice-mediated regulation of Diap2 and inflammatory signalling (Figure 17): Commensal bacteria trigger the formation of an initial PGRP-LC or PGRP-LE receptor complex consisting of the receptor, Imd, dFadd and Dredd. The complex competes for Diap2-recruitment to further activate Imd signalling. To avoid unnecessary activation of NF-κB, active Drice binds to Diap2, forming the inhibitory Drice-Diap2 complex, hence, triggering its subsequent degradation. Drice restrains, thereby, Diap2 from interacting with pathway inducers Dredd and Kenny, bringing the pathway to a stop. In the absence of Drice, Diap2 is free to interact with and ubiquitinate its substrates, stimulating activation of Relish target genes. The excessive amounts of AMPs, targeting commensal bacteria, are secreted to the gut lumen, leading to a disturbed gut homeostasis. Interestingly, Drice induces the degradation of Diap2 also during axenic conditions. In contrast to conventionally reared flies, removal of Drice in germ-free flies does not induce Relish target gene expression, indicating that accumulated levels of Diap2 without receptor triggering is not enough to induce NF-κB.

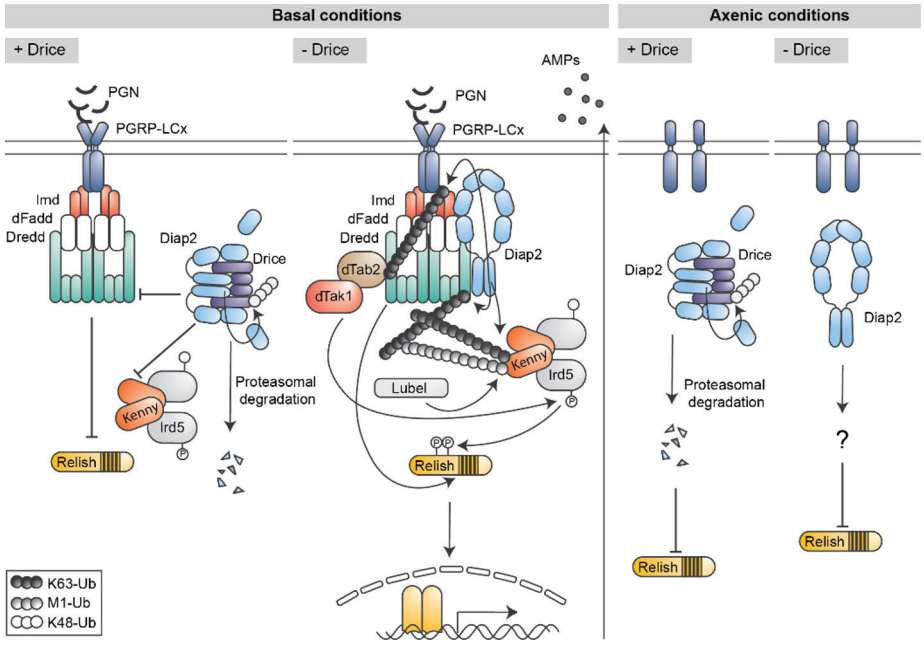

Figure 17. Proposed model for Drice-mediated regulation of the Imd pathway. During basal conditions, peptidoglycan (PGN) from the cell wall of commensal bacteria activate the PGRP-LC receptor, leading to the recruitment of Imd, dFadd and Dredd. The receptor complex competes for Diap2 recruitment to activate downstream signalling. Drice halts Imd signalling by forming an inhibitory complex with Diap2, targeted for proteasomal degradation. In the absence of Drice, excessive amounts of antimicrobial peptides (AMPs) are secreted into the intestinal lumen, disturbing gut homeostasis as unrestrained Diap2 is able to ubiquitinate Imd, Dredd and Kenny. Ubiquitin chains on Imd recruit the dTak1-dTab2 complex, leading to dTak1-mediated activation of Ird5. Ird5 in turn phosphorylates Relish. Ubiquitin-dependent activation of Dredd and Dredd-mediated cleavage of Relish precedes translocation of the Relish dimer to the nucleus and activation of NF-κB target genes. During axenic conditions Drice still targets Diap2 for degradation. The regulation of Diap2 during axenic conditions in the absence of Drice remains elusive, however, Diap2 alone is unable to drive Relish target genes expression without receptor stimulation.

CONCLUDING REMARKS

The role of the intestinal microbiome on human health is being increasingly recognised and unbalanced immune responses towards bacteria are connected to dysbiosis, IBDs and cancer development (Clemente et al., 2012, Shreiner et al., 2015). However, largely due to the complexity of the human microbiota, the details of host-microbe interactions remain largely elusive. As *Drosophila melanogaster* and mammals display a high degree of conservation regarding biological function, cellular structure and inflammatory signalling of the intestine, *Drosophila* serves as an excellent model when studying intestinal immune homeostasis (Apidianakis and Rahme, 2011, Capo et al., 2019). The aim of this thesis has been to advance the knowledge of inflammatory regulation in the intestine by using the fruit fly as a model, and to further the use of *Drosophila* as a platform for investigating host-microbe interactions.

The rich bacterial community associated with *Drosophila*, combined with standardised protocols of rearing flies with a defined microbiota, provide scientists with a versatile model in which to study host-microbe interactions. In deciphering the specific contributions of the microbiome on a particular host phenotype, the use of gnotobiotic flies, monoassociated with a single bacterial species, has emerged as an imperative approach. However, to more accurately model the complexity of the microbiome, combinatory studies, using gnotobiotic flies polyassociated with several bacterial species, are required. These studies focusing on microbe-microbe, as well as host-microbe interactions, reveal a fascinating new level of microbial influence that has only begun to be elucidated.

Adding to the complexity of host-microbe interactions, the microbiome is continuously modified by external factors such as the environment, nutrition and drugs. When it comes to antimicrobial drugs, the adverse effects of antibiotics on the commensal microbiome and, importantly, the development of antibiotic resistance, call for alternative methods when targeting bacteria. Nanoparticles, displaying different, combinatory modes of antimicrobial action compared to traditional antibiotics, provide a promising strategy when managing infections caused by resistant bacteria. In the development of nanoparticles and antimicrobial agents, *Drosophila* provides a platform, not only for initial *in vivo* drug-screenings, but also for assessing microbe-drug interactions affecting host physiology. With the increasing evidence indicating that the microbiome greatly influences the efficacy of drugs (Wilkinson et al., 2018), investigating microbial drug metabolism in gnotobiotic flies might reveal key species interfering with drug therapies in patients.

Finally, by taking advantage of the sophisticated genetic tool-box of *Drosophila*, novel regulators of intestinal homeostasis can be identified.

We have shown that the effector caspase Drice acts, in addition to its known role as an apoptosis inducer, as a negative regulator of the ubiquitin E3 ligase Diap2 and intestinal NF-κB signalling. Interestingly, this caspase-mediated regulatory step does not affect pathogen-induced inflammatory responses, and might hence, serve as a target for specific regulation of epithelial immune responses during chronic inflammatory diseases. Taken together, this thesis has described the generation and use of axenic *Drosophila*, *Drosophila* as an *in vivo* model to assess the antimicrobial effects of nanoparticles, as well as a novel caspase-mediated regulatory step of inflammatory signalling in the *Drosophila* gut. In combination, these three studies reveal the versatility and true potential of *Drosophila* as a model for host-microbe interactions.

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Christa Kietz

Regulation of Host-Microbe Interactions and Inflammatory Signalling in *Drosophila melanogaster*

Vertebrate and invertebrate animals interact continuously with a diverse array of microbial communities hosted on their skin and mucosal surfaces. In humans, the intestinal epithelium is one of the largest interfaces for host-microbe interactions and the organ is being increasingly recognised for its role in human health and disease. Due to evolutionarily conserved signalling pathways, regulating intestinal development, regeneration and immunity, the fruit fly, *Drosophila melanogaster*, has emerged as an attractive model when studying gut physiology. By using *Drosophila* as a model, this thesis aims to advance the knowledge of inflammatory regulation in the intestine and proposes a new immune-regulatory mechanism mediated by the caspase *Drosophila* interleukin 1β-converting enzyme (Drice) in the fly gut. This thesis describes, moreover, the use of *Drosophila* as a model for studying intestinal host-microbe interactions and as a platform for *in vivo* characterisation of antimicrobial nanoparticles.

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