

**Understanding the role of Toll signalling in *Drosophila*
midgut stem cell proliferation**



**A thesis submitted for the degree of Master of Science (by research) in the University of
Oxford, Trinity Term 2023**

Aiswarya Udayakumar

St. Catherine's College

Department of Biochemistry

Abstract

The intestinal epithelium is a site of complex interaction between microbiome, epithelial cells, and the immune system through a multidirectional crosstalk achieved using signalling molecules and metabolites secreted by the microbiome and host cells. This cross talk maintains epithelial turnover and the gut microbial population while integrating nutrient absorption and host defence. In this context, innate immune pathways have been extensively studied to understand their role in the maintenance of microbial and epithelial homeostasis and to study the response against infections and stress. In mammals, Toll-Like Receptors (TLRs) have been widely researched for their role in maintaining the commensals and influencing the epithelial regeneration. However, the complexity of mammalian systems and inability to standardize the models makes it challenging to study interactions of TLRs with different pathways in the gut. The immune and developmental pathways involved in epithelial plasticity are evolutionarily conserved. Therefore, using *Drosophila melanogaster* as a model organism, we aim to understand the role of Toll signalling in regulating epithelial turnover during infection. To this end, we used a constitutively active form of Toll receptor to mimic Toll activation. Our results reveal that, the canonical Toll pathway was involved in the intestinal stem cell (ISC) proliferation. It also led to the enteroblasts getting blocked from differentiating and/or (mis)differentiating that caused accumulation of esg+ cells in the gut resulting in dysplasia. We found that the Jun N-terminal kinase (JNK) pathway was acting downstream of Toll in mediating Toll-induced dysplasia. Moreover, the developmental pathway Notch, had a potential antagonistic role upstream or in parallel to the Toll pathway. Taken together our results place Toll as the signalling element to integrate epithelial renewal and immune response.

Acknowledgements

I would like to express my deepest gratitude and appreciation to all those who have supported me throughout the journey of completing my master's thesis.

First and foremost, I am immensely thankful to my supervisor, Prof. Petros Ligoxygakis, for his unwavering guidance, patience, and invaluable insights. His expertise and commitment to excellence have been instrumental in shaping my project research. His kindness and patience throughout my failures gave me the confidence to learn from my mistakes. I will always be indebted to him for this wonderful learning opportunity.

I extend my heartfelt appreciation to Dr. Jeff Lee, a postdoctoral scholar from the Davis lab, whose expertise in code development for image analysis and patience in addressing my queries have been invaluable. My gratitude also extends to Prof. Tim Nott for imparting the essential skills required for operating the Leica SP5 confocal microscope, without which my data acquisition would have remained unattainable.

To our previous lab member and collaborator, Shivohum Bahuguna, a PhD scholar at the German Cancer Research Center (DKFZ), I owe a debt of gratitude for his constant guidance and unwavering patience during my research. Despite the geographical distance, he generously shared his knowledge, techniques, and valuable writing insights. His attention to detail and teaching prowess are truly remarkable, and I foresee a bright future in his academic pursuits.

I am deeply grateful to our lab member and my close friend, Grace Critchley, for her unwavering support and motivational encouragement throughout this project. My thanks also extend to Kayo Akpobaro for his invaluable assistance with procurement.

I offer heartfelt thanks to my thesis committee members, Prof. Andre Furger and Prof. Ilan Davis, along with my transfer committee member, Prof. Alberto Baena. Their constructive feedback and invaluable suggestions have elevated the quality of this work. Gratitude is also extended to my graduate advisors, Prof. Paul Elliot and Prof. David Staunton, who provided invaluable advice on academia and research.

The supportive environment provided by the faculty and staff, especially the ever-helpful graduate administrator Dawn Gibbons, was indispensable to the success of my research.

A special mention goes to my friend from the Davis lab, Jana Joha, who not only offered friendship but also served as a pillar of support throughout my project. My sincere appreciation goes to the cherished friends who made my time at Oxford truly memorable, including Shreyoshee Bandyopadhyay, Nora Rebaa, Ritapriya Bandyopadhyay, Dr. Eboni Bucknor, Devi Satarkar, Nikita Manoharan, Setareh Rezazad, Qiaoyu Tian, Anna Schwarzenbach, Yimo Zhang, Sophia Gotzig, and Julia Olivares.

My deepest gratitude is reserved for my best friend, Rohan Rout, whose unwavering support, despite the distance, has been my constant. I also acknowledge the support of my friends back home, Aastha Banerjee and Simran Hora, whose care and encouragement have been my guiding lights.

Lastly and most importantly, I owe an immeasurable debt of gratitude to my mother, Bindu Udayakumar, for instilling in me the aspiration to reach for the stars and for shaping me into the person I am today. To my father, Udayakumar Vasudevan, whose guiding presence has been constant in life. And to my sister, Divyasri Udayakumar, whose attentive ear, and boundless cheerfulness has inspired me to come this far.

This thesis is the culmination of the unwavering support and encouragement of these extraordinary individuals. I am deeply thankful for your pivotal roles in this significant milestone of my academic journey.

- Aiswarya Udayakumar, October 2023

Contents

Abstract.....	ii
Acknowledgements	iii
Figures.....	vii
Tables	x
Abbreviations.....	xi
1 INTRODUCTION	1
1.1 Mammalian innate immunity: Toll-like receptor signalling	1
1.2 Drosophila innate immunity.....	4
1.2.1 IMD pathway.....	5
1.2.2 The Toll pathway.....	6
1.3 Epithelial and microbial homeostasis in the mammalian gut	8
1.3.1 Anatomy of the mammalian gut.....	8
1.3.2 Cellular composition of the mammalian intestine	10
1.3.3 Role of TLR in intestinal epithelial homeostasis	11
1.4 Epithelial and microbial homeostasis in the Drosophila gut.....	13
1.4.1 Anatomy of the Drosophila gut.....	13
1.4.2 Cellular composition of the Drosophila midgut	15
1.4.3 Drosophila gut microbiome.....	18
1.5 Multiple pathways regulate epithelial homeostasis in the Drosophila gut.	19
1.5.1 Innate immunity in epithelial homeostasis	19
1.5.2 JNK pathway maintains gut epithelial composition.....	20
1.5.3 Notch signalling in Drosophila gut and its interaction with JNK.....	23
1.6 Context for the development of the project.....	25
1.7 The project aims to answer the following questions.	26
1) Does canonical Toll signalling regulate epithelial renewal in Drosophila midgut? .	26
2) Does JNK interact with Toll pathway in maintaining epithelial renewal?.....	26
2 MATERIALS AND METHODS	26
2.1 Drosophila genetics: Spatio-temporal control of gene expression	26
2.2 Drosophila husbandry	27
2.3 Infection models.....	29
2.4 Polylysine slide preparation	29
2.5 Gut dissection and immunostaining	30
2.6 Imaging	31
2.7 Image processing and analysis	32
2.8 Quantification of pH3+ cell numbers.	34
2.9 Culture dependent quantification of gut microbiota (CFU)	34

3	RESULTS	34
3.1	<i>Toll is required for infection-induced stem cell mitosis.</i>	34
3.2	<i>Activating Toll pathway increases the number of intestinal progenitors in the Drosophila midgut.....</i>	36
3.3	<i>Activating Toll pathway increases mitotic cells in the gut.</i>	41
3.4	<i>Toll signalling influences cell fates in the posterior midgut.</i>	43
3.5	<i>Toll activation increases the number pH3+ cells in young flies</i>	47
3.6	<i>Toll activation increases the ISC population in midguts.</i>	48
3.7	<i>Activation of Toll pathway does not significantly influence EE population in the Drosophila midgut.....</i>	51
3.8	<i>Toll activation in stem cells increases the number of mitotic cells.....</i>	54
3.9	<i>Toll activation in progenitors influence intracellular localization of Dif protein.</i>	56
3.10	<i>Dif is required for Toll^{10b} induced expansion of esg+ cells.....</i>	57
3.11	<i>Cactus^{RNAi} increases mitotic cells in the gut.</i>	60
3.12	<i>Spaetzle expression in progenitors induces stem cell mitosis.</i>	61
3.13	<i>Increased expression of Puckered on Toll activation highlights the involvement of JNK signalling.</i>	62
3.14	<i>JNK inhibition reduced Toll-mediated expansion of esg+ cells in the gut.</i>	63
3.15	<i>JNK signalling does not contribute to epithelial renewal in homeostatic condition.....</i>	66
3.16	<i>Activation of Toll pathway increases cultivable bacterial density in the gut.</i>	68
4	DISCUSSION.....	69
4.1	<i>Activation of Toll signalling in progenitors results in higher rates of stem cell mitosis combined with EBs getting blocked and/or (mis) differentiating.</i>	71
4.2	<i>Canonical Toll pathway in ISCs and EBs is involved in promoting activated Toll-induced dysplasia.</i>	72
4.3	<i>Toll interacts with JNK to maintain epithelial barrier.</i>	74
4.4	<i>Conclusions.....</i>	75
4.5	<i>Future directions</i>	76
4.5.1	<i>Does Toll pathway interact with Notch to cause dysplasia in the activated form?</i>	76
4.5.2	<i>Does Spaetzle act as a mitogen?</i>	78
4.5.3	<i>Mechanism of JNK and Toll interaction.....</i>	78
4.5.4	<i>What role does the bacteriome play in dysplasia caused by Toll activation?.....</i>	78
5	APPENDIX.....	79
5.1	<i>Fly food recipe</i>	79
5.2	<i>De Man, Rogosa and Sharpe (MRS) media composition</i>	80

<i>Bibliography</i>	81
---------------------------	-----------

Figures

INTRODUCTION

Figure 1.1: Representation of TLR signalling in mammals (humans) involving MyD88-dependent and MyD88-independent signalling. Figure taken from Nouri et al., 2021[36].	4
Figure 1.2: Representation of Drosophila IMD pathway. Figure taken from (Kuraishi et al., 2013)[46].	5
Figure 1.3: Representation of Toll signalling in Drosophila melanogaster. [59].	7
Figure 1.4: Simplified diagrammatic comparison between Toll signalling in Drosophila and Toll-like receptor 4 signalling in mammals. Similar color coding has been used to represent protein orthologs.	7
Figure 1.5: Comparison between Human and Drosophila gut. Organs with similar functions have been coded with similar colors. Figure taken from (Sadaqat et al., 2022)[138].	14
Figure 1.6: Anatomical divisions of the Drosophila gut. Different regions have varied morphology and function. The posterior midgut is functionally equivalent to human small intestine. This project focuses on the R4 region of the midgut. Figure taken from (Capo et al., 2019)[132].	15
Figure 1.7: Representation of various stem cell fates in Drosophila midgut. These cells express unique genetic markers that can be exploited to label them using immunostaining.	16
Figure 1.8: Comparison between mammalian (A) and Drosophila (B) cellular composition in the gut.	17
Figure 1.9: Representation of conventional Drosophila JNK pathway. [178]	21
Figure 1.10: Representation of interaction between JNK and IMD pathways. Figure adapted from (Tafesh-Edwards & Eleftherianos, 2020)[177].	22
Figure 1.11: The role of Notch signalling in cell fate decisions of the Drosophila midgut epithelial cells.	24

MATERIALS AND METHODS

Figure 2.1: Representation of z-stacks taken for the gut samples.	32
Figure 2.2: Histograms for density of nucleus and major axis length.	33

RESULTS

Figure 3.1: Toll pathway is involved in cell mitosis induced by Ecc15 infection.	35
Figure 3.2: Figure representing genetic manipulation performed in experimental flies.	36
Figure 3.3: Activation of Toll signalling in progenitor cells markedly increases the number of progenitors.	37
Figure 3.4: Toll activation leads to a significant expansion of cells in the midgut.	37
Figure 3.5: Toll activation increases the proportion of esg+ cells in the midgut.	38
Figure 3.6: Toll activation increases the proportion of cells ISCs, EBs and EEs in the midgut.	38
Figure 3.7: Toll activation decreases the proportion of large nuclei cells in the midgut.	39
Figure 3.8: Toll activation increases the proportion of small nuclei cells that are GFP+ (esg+).	39
Figure 3.9: Toll activation increases the proportion of large nuclei cells that are GFP+ (esg+).	40
Figure 3.10: Activation of Toll signalling in progenitors increases cell mitosis.	42
Figure 3.11: Toll activation in progenitors increases cell mitosis.	43
Figure 3.12: Blocking the Toll pathway using Dif ^{RNAi} in progenitors does not block stem cell proliferation.	44
Figure 3.13: Blocking Toll pathway using Dif ^{RNAi} does not block stem cell mitosis.	45
Figure 3.14: Suppressing levels of dif does not influence total cells in the midgut.	45
Figure 3.15: Suppressing levels of dif does not influence the proportion of esg+ cells in the midgut.	46
Figure 3.16: Suppressing levels of dif reduces the proportion of small nuclei cells in the midgut.	46
Figure 3.17: Suppressing levels of dif increases the proportion of large nuclei cells in the midgut.	47
Figure 3.18: Toll activation results in increased number of pH3+ cells in young fly guts.	48
Figure 3.19: Activation of Toll signalling increases the number of ISCs in the midgut.	49
Figure 3.20: Toll activation does not significantly alter the proportion of stem cells in the midgut.	50
Figure 3.21: Toll activation does not significantly alter the proportion of stem cells in the midgut.	50
Figure 3.22: Toll activation alters the number of small nuclei cells in the midgut.	51
Figure 3.23: Activation of Toll signalling in progenitors influences cell fates in the posterior midgut.	53
Figure 3.24: Toll activation does not significantly alter the proportion of Ees.	53
Figure 3.25: Toll activation increases the proportion of Ees that are GFP+ (esg+).	54
Figure 3.26: Activating Toll pathway only in ISCs does not contribute to dysplasia as seen with Toll activation in esg+ cells (ISCs and EBs).	55
Figure 3.27: Toll activation in stem cells increases the pH3+ cell counts.	56

Figure 3.28: Activating Toll pathway influences intracellular localization of Dif protein.....	57
Figure 3.29 : Blocking dif suppresses Toll activation-induced esg+ cell expansion.	58
Figure 3.30: Suppressing levels of dif reduces stem cell mitosis induced by Toll activation.	58
Figure 3.31: Suppressing levels of dif reduces the expansion of total cell population in flies with activated Toll signalling.	59
Figure 3.32: Suppressing levels of dif reduces the expansion of esg+ cells in flies with activated Toll signalling..	59
Figure 3.33: Cactus ^{RNAi} expressed in progenitor cells potentially influences esg+ cell counts.	60
Figure 3.34: Suppressing levels of cactus increases stem cell mitosis.....	61
Figure 3.35: Activated form of Spaetzle induces stem cell proliferation.....	62
Figure 3.36: Activation of the Toll pathway leads to JNK signalling activation.	63
Figure 3.37: Blocking JNK pathway rescues Toll-mediated expansion of GFP+ cell population.....	64
Figure 3.38: Blocking JNK pathway suppresses Toll-mediated increase in stem cell mitosis.....	64
Figure 3.39: Blocking JNK pathway reduces the expansion of total cell population in flies with activated Toll signalling.	65
Figure 3.40: Blocking JNK pathway reduces the expansion of esg+(GFP+) cells in flies with activated Toll signalling.	65
Figure 3.41: Blocking JNK pathway does not influence stem cell proliferation in homeostasis.....	66
Figure 3.42: Blocking JNK pathway using Bsk ^{DN} does not block stem cell mitosis.	67
Figure 3.43: Blocking JNK pathway does not influence total cell counts in homeostasis.	67
Figure 3.44: Blocking JNK pathway does not influence the number of progenitors in homeostasis.	68
Figure 3.45: Toll activation in progenitors increases cultivable bacterial density in the gut.	69

DISCUSSION

Figure 4.1: Blocking Dif rescues Notch ^{RNAi} induced tumors.....	77
Figure 4.2: Diagrammatic representation of the main findings from the project.	77

Tables

Table 2.1: <i>Drosophila melanogaster</i> strains used in this study.	29
Table 5.1: Composition of the conventional fly food used in this project.	79
Table 5.2: Composition of de Man, Rogosa and Sharpe (MRS) agar for analyzing the cultivable gut microbial load of flies.	80

Abbreviations

AMPs	Antimicrobial peptides
AP-1	Activator protein 1
ASK1	Apoptosis signal-regulating kinase 1
BA	Bile acid
BMP	Bone metalloprotease
BSA	Bovine serum albumin
Bsk	Basket
CFUs	Colony forming units.
CRCs	Colorectal cancers
DAMPs	Damage associated molecular patterns.
DAP	Diaminopimelic acid
DAP	γ -D-glutamyl-meso-diaminopimelic acid
DCs	Dendritic cells
DI	Delta
EBs	Enteroblasts
Ecc15	<i>Erwinia carotovora carotovora 15</i>
ECs	Enterocytes
EE	Enteroendocrine
EEPs	Enteroendocrine progenitors
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
Esg	Escargot
GF	Germ-free
GI	Gastrointestinal
GNBP	β -glucan recognition proteins
Hep	Hemipterous
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IECs	Intestinal epithelial cells
IFNs	Interferons
IKK	I κ B kinase (IKK)
IMD	Immunodeficiency
IRAK4	Interleukin-1 receptor-associated kinase 4

IRF3	Interferons regulatory factor 3
ISCs	Intestinal stem cells
JAK/STAT	Janus kinase/Signal transducers and activators of transcription
JNK	c-Jun NH ₂ -terminal kinase
JNKKs	JNK kinase kinases
JNKs	JNK kinases
LP	Lamina propria
LPS	Lipopolysaccharides
M cells	Microfold cells
MAPKs	Mitogen activated protein kinases
Mekk1	MAPK kinase kinase 1
miRNA	microRNAs
Mkk4	MAPK kinase 4
MRS	De Man, Rogosa and Sharpe
MyD88	Myeloid differentiation primary response 88
NA	Numerical aperture
NK	Natural killer
NLRs	NOD-like receptors
PAMPs	Pathogen- associated molecular patterns.
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with Triton
PCNA	Proliferating cell nuclear antigen.
PCs	Paneth cells
PD	Parkinson's disease
PGN	Peptidoglycan
PGRP	Peptidoglycan recognition protein
pH3	phospho-Histone 3
Pros	Prospero
PRRs	Pattern recognition receptors
Puc	Puckered
Pvr	PDGF/VEGF receptor
RA	Rheumatoid arthritis
RIP-1	Receptor-interacting protein 1
RLR	RIG-I-like receptor
ROS	Reactive oxygen species
SCFAs	Short chain fatty acids
SLE	Systemic lupus erythematosus
Slpr	Slipper
SPE	Spätzle processing enzyme
Spz	Spätzle

sTLR	soluble Toll-like receptor
Su(H)	Suppressor of Hairless
T2D	Type-2 diabetes
TAK1	Transforming growth factor beta activated kinase 1
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TNFR	Tumor necrosis factor α receptor
TRAF6	Tumor necrosis factor receptor (TNFR)-associated factor 6
TRIF	TIR domain-containing adaptor protein inducing interferon beta
UAS	Upstream activation sequence
Upd3	Unpaired 3
Wg	Wingless

1 INTRODUCTION

The role of the Toll pathway in immunity was first described in *Drosophila* by the Hoffman lab, who found that Toll was driving immune responses (including secretion of antimicrobial peptides (AMPs)) following fungal infection[1]. Beutler *et al.*, later discovered the function of Toll-like receptor 4 (TLR4), against Gram-negative bacteria[2]. We now know that TLR4 is one of the members of the TLR family, which shares a great extent of similarity with Toll. These two discoveries became central in the notion of the evolutionary conservation of innate immune responses in vertebrates and invertebrates.

1.1 Mammalian innate immunity: Toll-like receptor signalling

Mammalian Toll-like receptors (TLRs) are pattern recognition receptors (PRRs) that recognize conserved molecules on microbes which are necessary for their survival. These are collectively called pathogen associated molecular patterns (PAMPs). TLRs also recognise signatures of cellular stress during inflammation and cell death, called damage associated molecular patterns (DAMPs), like reactive oxygen species (ROS)[3]. Due to its primary role in innate immune signalling and association with wide range of diseases, mammalian TLR signalling has been extensively studied. There are 10 TLRs in humans and 12 in mice, each one responsible for recognition of specific microbial components like lipopolysaccharides (LPS), bacterial DNA, peptidoglycans (PGN) from bacteria, zymosan and beta-glucan from fungi, single stranded RNA (ssRNA) and double stranded RNA (dsRNA) from viruses, to name a few[4]. The activation of the TLR pathway leads to secretion of proinflammatory cytokines, chemokines, cell adhesion molecules, co-stimulatory molecules, and type 1 interferon genes[5], [6]. TLRs are present on immune cells like macrophages, mast cells, basophil, eosinophil, dendritic cell (DC), neutrophils, Natural killer (NK) cells, B and T-lymphocytes. Smooth muscle cells, epithelial cells, endothelial cells, and fibroblasts are the non-immune cells that have TLR expression[7]–[10]. The selective expression of TLR2 and TLR4 on mucosal epithelial cells have been researched in sites like the gut, reproductive tract, upper and lower respiratory tract, and oral cavity[11]. In the oral mucosa, mRNA for TLRs can be found in the epithelial cells, while the expression of TLR protein is variable and inducible. Invasion by pathogen and subsequent inflammation induces the expression of

various TLR proteins based on the severity of infection and the nature of the pathogen[12], [13]. Activation of Toll signalling mediates expression of anti-microbial protein like defensins. β -defensins thus secreted further induce TLR signalling and recruit immune cells to the site of pathogen invasion[14], [15]. Similarly in the airway epithelium, despite the presence of TLR mRNA in epithelial cells, the protein expression depends on the state of the tissue (normal, inflammation or allergy)[16], [17]. TLR2 and TLR4 are the most relevant receptors in the airway epithelium and an increase in expression of these receptors have been reported on pathogen invasion[18], [19]. TLR activation in this tissue site induces different responses based on the location along the respiratory tract. TLRs in the upper respiratory tract induce secretion of mucins and therefore increase the mucus secretion by cells directly. IL-8 and TNF- α secreted because of TLR activation, further induces mucin secretion[20], [21]. The lower respiratory tract is exposed to pathogens that descends from the upper tract leading to TLR expression on epithelial cells and production of anti-microbial components like defensins, lysozymes, nitric oxide (NO) and cytokines[11], [22]. The cytokines in the upper and lower respiratory tract mediate recruitment of phagocytes into the tissue and enable infection clearance[23]. In the gut mucosal epithelium, mRNA for TLR1 to TLR9 has been reported but low levels of constitutively expressed TLR2, TLR4 and TLR5 is seen on the epithelial cells. The intestines are constantly exposed to high microbial densities and therefore, tightly regulate expression of TLRs and related signalling pathways. High local levels of factors like IFN- γ or TNF- α induces expression of TLR4 and is seen to contribute to chronic inflammation resulting in conditions like Crohn's disease[24]. TLR5 expression is seen on the basolateral side of cells and gets activated when microorganisms cross the epithelial barrier during invasion[25].

TLRs operate through myeloid differentiation primary response 88 (MyD88)-dependent or Toll/interleukin-1 receptor domain-containing adaptor protein inducing interferon beta (TRIF)-dependent signalling pathways[9], [26]. Dimerization of the Toll receptor on microbial sensing, leads to activation and signal transduction through the intracellular (Toll/Interleukin-1 receptor) TIR domain[27]. In the context of MyD88-dependent signalling, the activated TIR domain recruits an adaptor protein called MyD88, which contains a TIR domain on its C-terminal leading to an interaction between the TIR domain of the two proteins, hence activating MyD88. MyD88 then interacts with a kinase called Interleukin-1 receptor-associated kinase 4 (IRAK4) through the death domain of both the molecules. IRAK4 transmits the signal by phosphorylating IRAK1, which then recruits tumor necrosis factor receptor (TNFR)-associated factor 6

(TRAF6)[28], [29]. The signal can be transmitted in two separate ways after TRAF6. One is the NF- κ B pathway where TRAF6 aids the activity of I κ B kinase (IKK) complex responsible for phosphorylating I κ B, which is the inhibitor of the transcription factor NF- κ B. Once phosphorylated, I κ B dissociates from NF- κ B and the transcription factor is then freed to translocate into the nucleus and mediate gene expression[30]. The second pathway is TRAF6-mediated activation of mitogen activated protein kinases (MAPKs), terminating with the activation of AP-1 transcription factor[26](**Fig 1.1**). Alternatively, under specific conditions, some TLRs interact with TRIF, which activates NF- κ B through receptor-interacting protein (RIP-1), in a MyD88-independent manner. TRIF also enhances the activity of interferon regulatory factor 3 (IRF3) via TRAF3 and kinases to drive the expression of type 1 interferons (IFNs)[31]–[34] (**Fig 1.1**).

The expression of TLRs is compartmentalized within cells. TLRs are expressed on plasma membrane, and they facilitate the recognition of surface molecules derived from various pathogens. TLRs are also expressed on organelle membranes like endosomes, lysosomes, and endoplasmic reticulum (ER) enabling them to sense intracellular bacteria and viruses through their nucleic acid signatures. TLR1, 2, 4, 5, 6, and 10 are expressed on the cell membrane while TLR3, 7, 8, and 9 are localized on organelle membranes (reviewed in [35])(**Fig 1.1**).

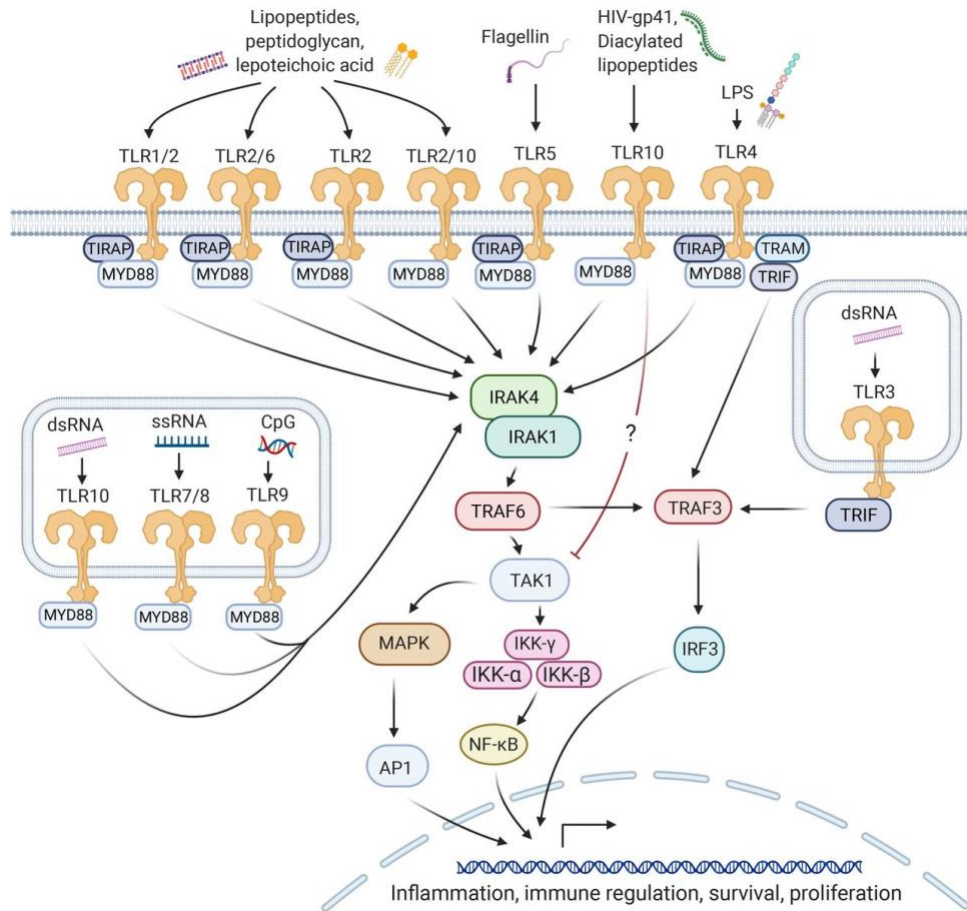


Figure 1.1: Representation of TLR signalling in mammals (humans) involving MyD88-dependent and MyD88-independent signalling. Figure taken from Nouri *et al.*, 2021[36].

1.2 *Drosophila* innate immunity

Drosophila employs multiple routes to safeguard itself from pathogens, through humoral and cell-mediated responses. First is the physical impediment, through a layer of epithelium[37]. Apart from this, flies contain plasmatocytes, which are macrophage-like cells involved in the phagocytosis of invaders[38], [39]. The lamellocytes are specialized cells that encapsulate larger foreign bodies, like wasp eggs, as they cannot be phagocytosed[40]. The humoral component of the response is carried out by antimicrobial peptides (AMPs) released into the hemolymph (blood equivalent for flies), from the fat body, which is the functional equivalent of human liver[41].

Drosophila has two innate immune signalling pathways: The Toll and the Immunodeficiency pathway(IMD).

1.2.1 IMD pathway

Imd pathway is one of the innate immunity signalling pathways in *Drosophila* and is homologous to the mammalian tumor necrosis factor α receptor (TNFR) signalling pathway. Membrane-associated peptidoglycan recognition protein–LC (PGRP-LC) recognizes γ -D-glutamyl-meso-diaminopimelic acid (DAP) type peptidoglycan, and cytosolic PGRP-LE recognizes monomeric DAP-type peptidoglycan present on gram negative bacteria. Upon peptidoglycan (PGN) recognition, a signalling cascade similar to vertebrate TNF α receptor and TLR signalling is activated. After recruiting various adaptors, caspases and kinases, the signal terminates at the phosphorylation of the NF- κ B homolog Relish, and it is cleaved (**Fig 1.2**). The free fragment of Relish then translocates into the nucleus to mediate the gene expression of effectors (AMPs), like *attacin* and *dipthericin*[42]–[45]. The IMD pathway has been widely studied in the context of the gut [46], [47]. This project focuses on the less explored function of Toll signalling in the gut and this pathway is reviewed in the following section.

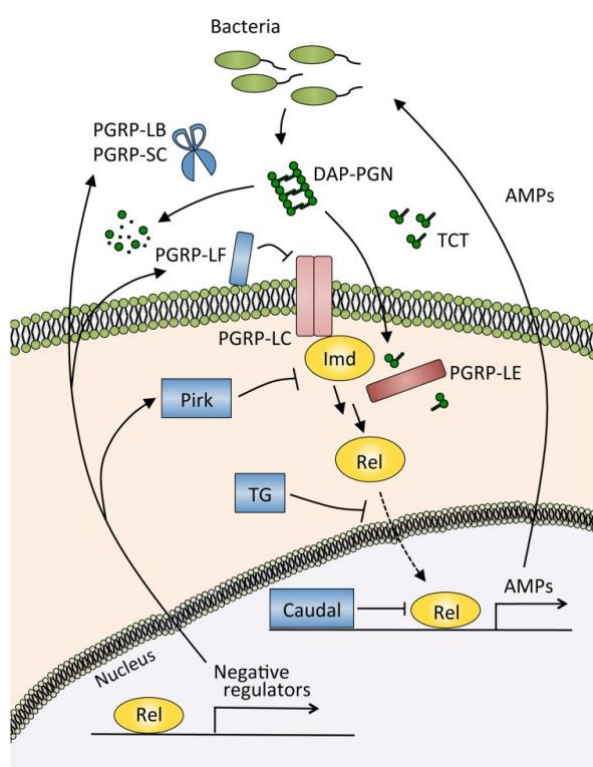


Figure 1.2: Representation of *Drosophila* IMD pathway. Figure taken from (Kuraishi *et al.*, 2013)[46].

1.2.2 The Toll pathway

The *Drosophila* Toll receptor, unlike mammalian TLRs, does not recognize pathogens by direct binding. The pathogens are recognized in the haemolymph, which is the insect equivalent of blood. This is done by peptidoglycan recognition proteins (PGRPs) and β -glucan recognition proteins (GNBPs), which are secreted into the haemolymph. On PAMP binding, they trigger activation of the Toll ligand, Spätzle (Spz) and thereby mediate its proteolytic cleavage through a distinct proteolytic cascade, that ends at Spätzle processing enzyme (SPE) cleaving Spz[48], [49]. In the inactive form, the prodomain of Spz is associated to the C-terminal region, thus masking the hydrophobic Spz region. On activation induced by proteolysis, conformational changes within the protein cause binding to the Toll receptor. Until Spz binds to the receptor, the prodomain remains associated with the Spz region and gets released once receptor binding occurs[50], [51]. There are two potential models of Spz and Toll binding. The older model suggests that two Toll receptors are bound by one Spz dimer. The newer model predicts that the Toll receptors, that are also dimers, bind at each N-terminus to a Spz dimer. The proteolytic cascades leading to Spz activation also vary depending on the microorganism that initiated the response[52], [53].

On Spz binding, the activated dimeric Toll receptor binds to the adaptor protein MyD88 located intracellularly via its TIR domain, as already discussed in the context of mammalian TLRs [54]. MyD88 recruits another adaptor protein, Tube (ortholog of human IRAK4) and a kinase called Pelle (ortholog of human IRAK1) to form a complex called MyD88-Tube -Pelle complex and they interact through their Death domains (DD). MyD88 and Pelle bind to each other through separate death domains on Tube[55]–[57]. The complex is then responsible for phosphorylation and eventual degradation of the I κ B factor, Cactus. Under non-signalling conditions, Cactus is bound to either of the NF- κ B factors, Dorsal and/or Dif, in a context-dependent manner. After dissociation of Cactus, Dorsal/Dif is free to undergo nuclear translocation and mediate the expression of a set of target genes aiding host defence mechanism, like AMPs[58]. For a schematic, see **Fig 1.3**. The similarities between Toll signalling and TLR signalling is depicted in **Fig 1.4**.

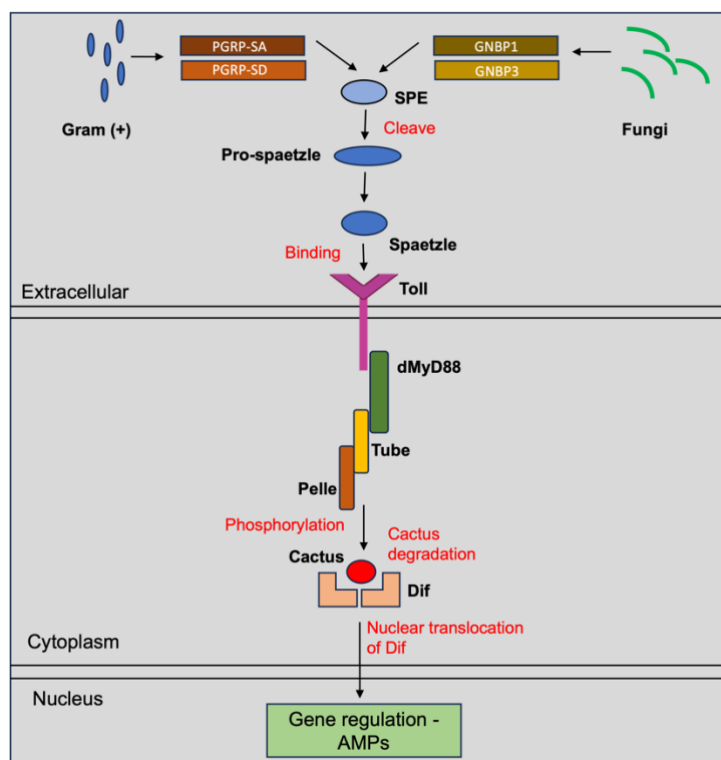


Figure 1.3: Representation of Toll signalling in *Drosophila melanogaster*. [59]

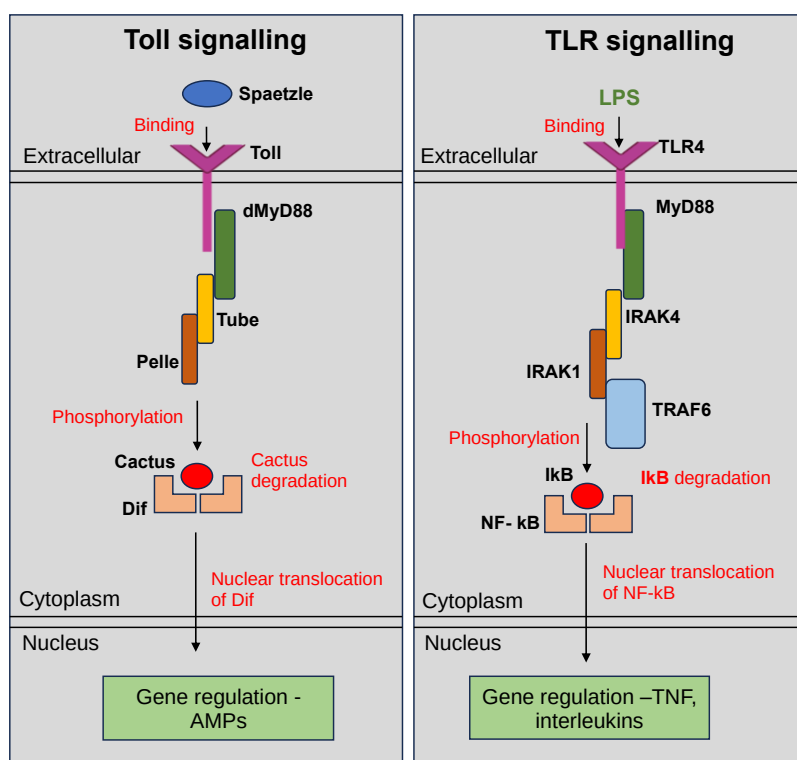


Figure 1.4: Simplified diagrammatic comparison between Toll signalling in *Drosophila* and Toll-like receptor 4 signalling in mammals. Similar color coding has been used to represent protein orthologs.

As TLR signalling is one of the primary pathways involved in the innate immunity response, with very potent effectors, the signalling is strictly regulated. For example, auto-immune diseases like systemic lupus erythematosus (SLE), Crohn's disease, multiple sclerosis, and rheumatoid arthritis (RA), can arise due to aberrant activation of TLR pathway by self-antigens[60]. They are also implicated in type 2 diabetes and Alzheimer's[61]–[63]. In cancers, TLR signalling effectors can have contrasting roles. Even though immune activation through TLRs have been associated with immune detection of tumor antigens and an anti-tumoral response, constant inflammation by TLR stimulation has been linked with exacerbation of many cancer types[64]. Therefore, in a homeostatic state, numerous negative regulators are responsible for the inhibition of the TLR signalling pathway at various levels. These include soluble receptors like soluble TLR (sTLR), co-stimulatory molecules, intracellular regulators, or transmembrane proteins[65], [66]. Several microRNAs (miRNAs) have also been identified in the regulation of TLR pathway and have been involved in autoimmune diseases[67], [68]. These regulators maintain a balance between activation of TLRs on exposure to PAMPs and inhibit over-activation of the signalling cascade. Fine tuning TLR signalling and hence, immune activation is of great significance in context of the gut.

1.3 Epithelial and microbial homeostasis in the mammalian gut

1.3.1 Anatomy of the mammalian gut

The human gastrointestinal (GI) tract houses trillions of microorganisms. The small and large intestines form the major portions of the human GI tract and is also a site of the highest microbial load in the human body[69]. They play a crucial part in digesting a complex range of dietary components and in nutrient absorption. The small intestine is divided into three subsections namely: duodenum, jejunum, and ileum, based on their structural characteristics and functions. The duodenum is a site of digestion, where food entering from the stomach is broken down using enzymes from pancreatic secretions. With its high surface area attained through circular folding and villi, the jejunum is the site of absorption of most small nutrient particles from the digested food. The ileum also contains villi and absorbs the components not taken up by the jejunum like bile acids (BA), vitamins and other nutrients (reviewed in [70])

In the small intestine, a monolayer of intestinal epithelial cells (IECs) along with the secreted mucus layer, forms the barrier that physically impedes the entry of these microbes into tissues. It also forms a barrier between tissue resident immune cells present in the lamina propria (LP) and the microbes[71]. The gut microbiota is in constant bidirectional communication with the immune and epithelial cells of the gut through metabolites and peptides derived both from the host and the microbiome. This cross talk helps to maintain the microbial homeostasis in terms of population density and diversity[72], [73].

The human microbiome is comprised of hundreds of species-level phylotypes but is dominated by the anaerobic bacteria of the phyla *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Verrucomicrobia* and *Actinobacteria*[74], [75]. The members of this diverse microbiome composition play their unique role in metabolizing complex dietary components in the gut, thus aiding digestion and nutrient absorption by the epithelium. The intestinal interface is also a site of fine balance in the metabolites of both microbial origin and transformation of host or dietary derived substrates[76]. The anaerobes process complex carbohydrates and generate short chain fatty acids (SCFAs) which serve as energy sources for the maintenance of the microbiome as well as for IECs. SCFAs have also been extensively studied for their anti-inflammatory role in the intestinal tract[72], [77]. In addition, the microbes also modify endogenous compounds secreted by other members of the microbiome and host. The secondary metabolites generated by the microbiota through modification of bile acids(BAs) from the liver are also responsible for modulating immune response in the gut[78].

A lot of factors like diet, lifestyle and the genetic disposition are responsible for determining the microbiome composition in every individual and this composition remains relatively constant. However, due to perturbation in the crosstalk between the host and the microbiota, the microbial composition can be altered, favoring pathogens, and making the intestinal interface vulnerable to pathogenic insult. This is known as dysbiosis. Loss of commensals further disrupts the immunosuppressive environment enabled by them and hence predisposes the host to inflammatory and tumorigenic pathologies[73], [79]. Constant or prolonged inflammation in the gut has been linked to various diseases of the intestine like inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), coeliac disease and even colorectal cancers (CRCs)[80], [81]. Common factors in the modern lifestyle, such as excess calorie intake, lack of physical activity and factors like stress have an impact on gut brain axis and change the microbiome profile. This has been linked to many metabolic

disorders [82]–[84]. It's also a major contributing factor to extra-intestinal diseases like asthma, cardiovascular diseases, allergies, obesity, type-2 diabetes(T2D) and Parkinson's disease (PD)[85], [86].

1.3.2 Cellular composition of the mammalian intestine

As the intestinal interface is a site for constant stresses like toxins secreted by microbes, dietary toxins and pathogen insults, maintenance of the organ structure and function requires quick turnover and regeneration. The human intestinal epithelium has a turnover rate of 5-7 days[87]. This self-renewal is possible due to the presence of specialized stem cells that proliferate and regenerate the entire intestine. In humans, the intestinal tract consists of pluripotent intestinal epithelial stem cells (pluripotent IECs) which are involved in forming most IEC types[88]. The stem cells are restricted to the base of the crypts in the villi and while moving upward in the crypt, proliferate to form a transient cell which then differentiates into the various cell types. As the differentiated cells reach the apex of the villi, they undergo shedding into the lumen. This process regulates the maintenance of epithelial homeostasis in the intestine. The different IEC types in the intestine are enterocytes, Paneth cells (PCs), goblet cells, exocrine cells, endocrine cells, neuroendocrine cells, tuft cells and microfold cells (M cells)[89]. The IECs also maintain the barrier integrity through intracellular junctions that aid cell-to-cell interaction and also regulate paracellular permeability and transepithelial exchange of substances. Loss of intracellular junctions leads to leaky gut, which occurs when the intestinal barrier loses its integrity and is unable to regulate the passage of compounds and microbes into subepithelial sites and LP. This is seen in many gut-related disorders, especially IBD[90], [91].

The majority of cells present in the intestine are the absorptive enterocytes. They cater to the absorption of water, peptides, sugar, ions and many other compounds. They are also involved in secretion of immunoglobins[92]. 10% of IECs are composed of mucus-secreting goblet cells. The mucus layer is an integral part of the intestine, shielding the epithelial cells from the pathogens and the digestive enzymes in the lumen, in addition to ensuring the smooth passage of food through the gut[93]. Paneth cells are integral in the secretion of antimicrobial peptides and proteins. They are involved in bacterial sensing through TLR activation, thereby producing antimicrobial factors. They also help maintain the stemness of ISCs by providing them with growth factors. Their presence is restricted to the small intestine[94]–[96]. The neuroendocrine cells

play their role as chemoreceptors by detecting harmful substances, initiating digestion and protective responses. They achieve this by releasing peptides or intestinal hormones into the blood stream upon stimulation, thereby activating the nervous system[97]. Enteroendocrine cells secrete hormones[98]. There is also a constant sampling of the luminal content by the M cells, leading to presentation of antigens to underlying immune cells in the LP[99], contributing to the decision of when inflammation needs to be triggered.

As the turnover rate of the intestinal cells is high and insults on them frequent, it is extremely critical to achieve a proper balance between stem cell proliferation and IEC death, during both homeostasis and during conditions of tissue damage, as observed in infections. Maintenance of homeostasis is multifaceted and requires an interplay of various systems working in harmony: mainly, immune signalling pathways, microbiome, and metabolism of the host. The intestinal stem cell (ISC) function is also maintained by neighboring PCs, intestinal stromal cells and intestinal myofibroblasts[100], [101]. There are multiple pathways that form a complex network in regulating the ISC proliferation. Some of the prominent ones are Wnt, bone metalloprotease (BMP) and Notch signalling[102].

1.3.3 Role of TLR in intestinal epithelial homeostasis

Various studies highlight the importance of microbiota in maintaining ISC proliferation rate using germ-free models (GF) and intestinal organoids[103]–[105]. IECs also express PRRs, like TLR, NOD-like receptors (NLR), RIG-I-like receptor (RLR), that sense the microbial composition in the lumen[106]–[108]. In addition to being involved in the pathogen-induced pro-inflammatory response, these signalling pathways are also necessary in the maintenance of immune tolerance and repair of damage in homeostatic conditions[109]. GF mice are shown to have a compromised intestinal barrier as indicated by higher mucus penetrability, reduced permeability through paracellular transport, reduced production of AMPs, diminished IEC proliferation, slower motility and reduced number of lymphocytes present in the intraepithelial regions[73], which indicates that the microbiome through immune pathways, contribute to maintaining the intestinal integrity.

As diseases like IBD and CRCs are a result of chronic inflammation, understanding the role of TLRs signalling and its interaction with the commensals and also other developmental pathways, in maintaining the integrity of the intestinal epithelium is crucial. In the gut, TLR1-9 are expressed on various cell-types like

the IECs, immune cells, stromal cells and some other non-immune cells present in the microenvironment[7], [8].

The role of TLR signalling in the mammalian model is complex and sometimes contrasting. For example, one of the first studies done in the field by Rakoff-Nahoum *et al.*, depicted that MyD88-deficient mice had increased epithelial cell proliferation due to TLR signalling disruption. Similar results were later observed in TLR1 and TLR9-deficient mice[110]–[112]. Further, activating TLR4 using external lipopolysaccharide (LPS) administration, suppressed ISC proliferation and induced cell death in organoids[113], [114]. Parallely, *Tlr4*–/– organoids are protected from apoptosis induced by LPS exposure. Similar findings are also observed in *in vivo* studies conducted on mono-colonized gnotobiotic mice[113], [114]. These results show that on activating TLR signalling, there is reduced cell proliferation and higher apoptosis, thus inhibiting epithelial cycling and renewal. Contradictory evidences indicate an increased IEC proliferation in small intestines and colon on TLR4 activation. Further TLR4 deficiency has been shown to downregulate proliferating cell nuclear antigen (PCNA) in small intestinal IECs, which is a marker to reflect cell turnover rates[115], [116]. These differences could potentially be due to the variability of microbial composition in the transgenic mice, and is also an indication of how the microbial variations could completely modulate signalling pathways. It is also more challenging to standardize mammalian systems to carry out these studies.

TLR signalling also impacts pathways that determine stem cell lineages and their proliferation. One such pathway is Notch. Activating Notch signalling in mice directs the stem cells to an absorptive lineage[117], [118]. Notch inhibition on the other hand supports secretory lineage. However, the involvement of Toll signalling with the Notch pathway is not well understood. While some studies show that TLR signalling has a stimulatory effect on Notch signalling in IECs, there are studies that demonstrate a completely reverse phenomenon[113], [116], [119], [120]. Therefore, understanding the interplay between these pathways is of significance.

Apart from the role in epithelial homeostasis and immune sensing, TLRs also regulate barrier permeability during infection. MyD88-deficiency increased the permeability of gut in mice with induced colitis. Ablation of TLR signalling using *Myd88*–/– and *Tlr2*–/– mice displayed early loss of tight junction and onset of colitis on DSS treatment[121], [122]. Similar results have been observed in *Myd88*–/– and *Tlr1*–/– mice[111], [123]. On the contrary, TLR4 signal induction has demonstrated leaky gut phenotypes in mouse models and in human IEC

lines[119], [124], [125]. Therefore, different TLRs could have varied impacts on the cell-junctions.

TLR signalling in IECs further shapes immune responses by releasing factors that induce chemoattraction of immune cells to lamina propria during infections[126], [127]. It's also involved in modulating T-cell differentiation and priming properties of dendritic cells (DCs)[128], [129]. In addition to this, it also aids class switching in B-cells to increase the number of IgA producing B-cells in the lamina propria, that are then released into the lumen to bind antigens[130].

These studies highlight the importance of TLR signalling in the gut but also represent the complexity of working with mammalian models. Mammalian systems have a very diverse microbial population that makes understanding the interaction of pathways very challenging. *Drosophila melanogaster* provides for an excellent model to study this complex system. Full sequencing of *Drosophila* genome has shown that 75% of genes correlated to human diseases have functional orthologs in flies[131]. They would be ideal to study the role of TLR signalling in the maintenance of epithelial homeostasis in both normal as well as diseased states, given the conserved nature of TLR signalling pathway and the similarity between the GI tract and *Drosophila* gut, in terms of function and stem cell lineages. *Drosophila* also has a microbiome that is less complex as compared to mammals. It is genetically amenable and yet a functionally analogous model for studying diseases like IBD and identifying therapeutic targets and disease markers[132].

1.4 Epithelial and microbial homeostasis in the *Drosophila* gut

1.4.1 Anatomy of the *Drosophila* gut

The *Drosophila* gut shares structural and functional similarities with human GI tract. It also shares similar stem cell lineages. *Drosophila* gut is mainly sectioned into the foregut, midgut and hindgut along the anteroposterior axis. The crop, pharynx and esophagus form the foregut. The crop is used for food storage prior to digestion. The cardia (proventriculus) is located at the junction between the foregut and the midgut. It regulates the entry of food into the midgut and is involved in AMP synthesis[133]. The midgut has an endodermal origin while the other two have ectodermal origin. The midgut is further divided into six regions (R0-R5), based on distinct digestive and metabolic functions[134]. The midgut is functionally equivalent to human stomach and small intestines. The posterior

midgut (R4-R5) region is responsible for active immune responses as well as metabolism and therefore can be compared to the small intestine(**Fig 1.6**). This region also contains the stem cells that are more active in maintenance of the epithelium in homeostasis and damage-associated tissue repair[132], [135]. The hindgut shares similarity with the colon. The malpighian tubules are connected to the gut at the junction between posterior midgut and hindgut. Together the tubules and hindgut regulate water and ion homeostasis and are functionally equivalent to human kidneys[135], [136]. A distinguishing factor between human and *Drosophila* gut is the absence of intestinal villi and crypts in flies[137]. *Drosophila* gut consists of a monolayer of columnar epithelium surrounded by visceral muscles, trachea, nerves and Malpighian tubules[134]. A comparison between the *Drosophila* and human gut is shown in **Fig 1.5**.

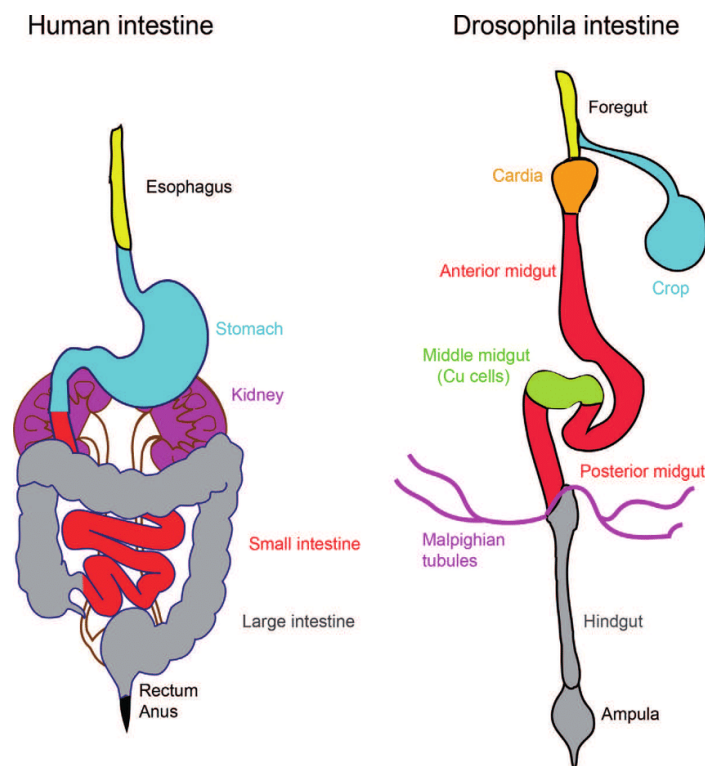


Figure 1.5: Comparison between Human and *Drosophila* gut. Organs with similar functions have been coded with similar colors. Figure taken from (Sadaqat *et al.*, 2022)[138].

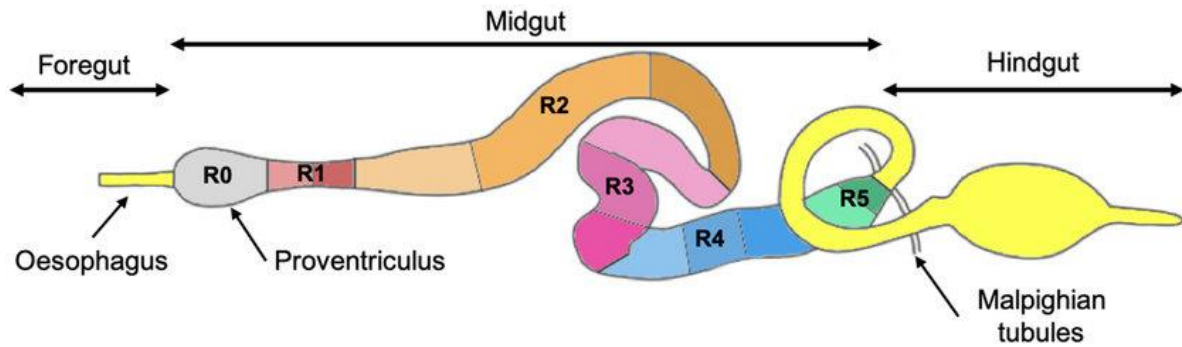


Figure 1.6: Anatomical divisions of the *Drosophila* gut. Different regions have varied morphology and function. The posterior midgut is functionally equivalent to human small intestine. This project focuses on the R4 region of the midgut. Figure taken from (Capo *et al.*, 2019)[132].

1.4.2 Cellular composition of the *Drosophila* midgut

There are different cell-types in the *Drosophila* adult midgut epithelium. The intestinal stem cells (ISCs) give rise to all other cell-types in the midgut. The *Drosophila* posterior midgut is maintained by approximately 1,000 ISCs that are scattered amongst 10,000 posterior midgut cells[139]. They express the Notch ligand *Delta* (DI) which is generally used to identify the ISC population. They divide symmetrically to form ISC daughter cells. Asymmetric ISC divisions form resident niche daughter stem cells and transient enteroblasts (EBs) or enteroendocrine progenitors (EEPs), which give rise to enterocytes(ECs) or enteroendocrine cells(EEs) respectively (**Fig 1.7**). The differentiating ISC that expresses *prospero* (*pros*) form the EEPs and EEs, while the ones that do not express *pros* form the ECs via the transient EB state, with high levels of Notch signalling[135], [140]–[143]. EBs are diploid and express Suppressor of Hairless (Su(H)), which is a transcriptional reporter of Notch pathway. ISCs are also located more basally and move towards the apical region on differentiation. The ISCs, EBs and EEPs form the progenitor cell population, while the ECs and EEs form the terminally differentiated cell types. The progenitor cells express a transcription factor called *escargot* (*esg*) that aids identification and labelling of this cell population[143], [144]. In a manner analogous to mammalian gut, the subregions of *Drosophila* gut show variation in stem cell cycling rates and expression of markers like DI[145], justifying the differences in regional physiology with variation in stem cell characteristics. Studies also show that these subregions are also re-established following tissue injury[146]. The stem cells that originate in a region remain restricted to that region due to the presence of regional boundaries that can be observed using stem cell clones.

This is also observed in tumours derived from stem cells. Therefore, daughter stem cells carry region-specific information that maintains the distinct regions of the gut[147]. In this study we focus on the stem cells of the R4 region as the posterior midgut is more immunologically active and the stem cells of R4 are known to actively participate in tissue regeneration apart from having high turnover rate[135], [148].

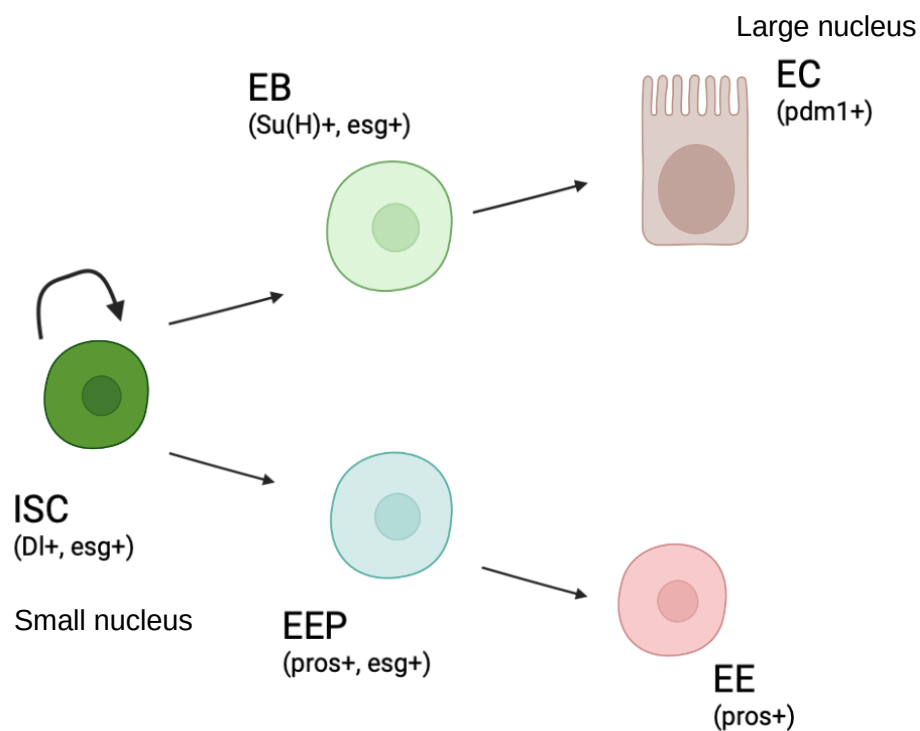


Figure 1.7: Representation of various stem cell fates in *Drosophila* midgut. These cells express unique genetic markers that can be exploited to label them using immunostaining.

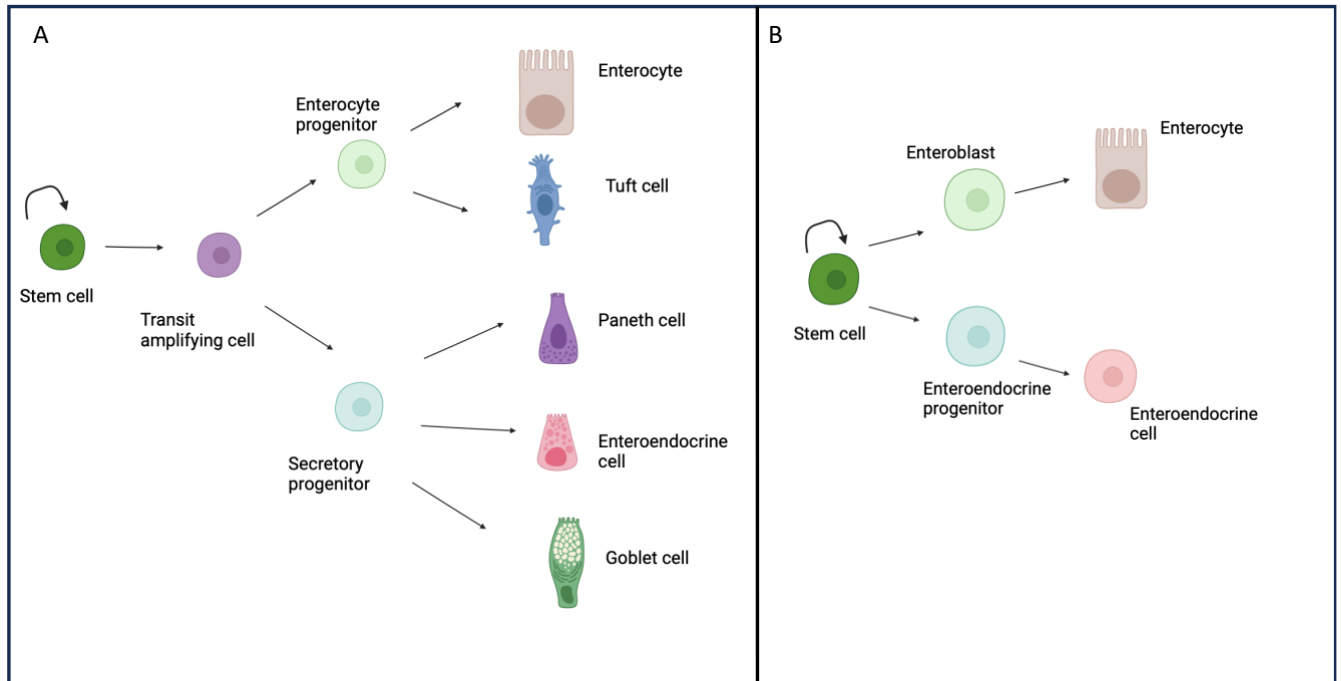


Figure 1.8: Comparison between mammalian (A) and *Drosophila* (B) cellular composition in the gut.

The enterocytes (ECs) are the absorptive cells of the gut and make up 90% of the differentiated cells in the gut[140]. They are involved in metabolism by secretion of digestive compounds, nutrient absorption, transport and immune responses. They are morphologically distinct from other cell populations due to the presence of large polyploid nuclei. They vary in function and morphology in different gut regions but mostly express the gene *Myosin31DF*, used for EC identification. The midgut ECs have also been found to express *pdm1*. The EEs comprise 10% of cell population of the gut[140], [149]–[152]. They are involved in secreting hormones and peptides that are involved in various functions like gut mobility, and inter-organ communication involved in the gut-brain axis to modulate metabolism and behaviour in response to nutrient availability. They are also pivotal in innate immune response, as they are the first to provide antimicrobial defence. *Prospero (pros)* is the genetic marker for EEs. The ECs and EEs thereby orchestrate antimicrobial responses as performed by PCs and goblet cells in the mammalian counterpart[149], [150], [153].

Digestion in fly guts also take place under neutral or slightly basic pH conditions, except for the copper cell region (R3) and hindgut which are strongly acidic[133], [154], [155]. The mammalian intestine on the other hand has very low pH in the upper region and decreases along the length and this shapes the density and diversity of microbes found in different regions[133], [156]. The bacterial species

of *Drosophila* gut are more aero-tolerant. The physiochemical properties of various sections of gut determines the bacterial colonization in that region. As discussed in the context of mammals, *Drosophila* gut epithelial cells are in constant cross talk with the microbiota through microbiota or host-derived metabolites. This maintains the microbial population as well as epithelial barrier integrity and cell composition[157], [158].

The presence of intestinal stem cells in *Drosophila* was discovered in 2006[135], and the finding that these stem cells were responsible for epithelial regeneration, even in homeostatic conditions opened up new venues to study stem cell maintenance and organ plasticity. The midgut is a site of constant insults from external and internal stressors like host metabolic and nutritional changes or physical, chemical and biological damages like pathogens and toxins. This leads to the loss of epithelial cells, mainly ECs and therefore the ISCs need to constantly replace the lost cells[159]. Various signals from the neighbouring cells like visceral muscles, and differentiated EEs and ECs control ISC proliferation and differentiation. The signalling pathways that predominantly control the ISC self-renewal and differentiation like the Wnt/ β -catenin pathway (Wingless (Wg) in *Drosophila*) or Notch pathway, discussed in the context of mammals, are conserved across humans and *Drosophila*[159]. Aberrant signalling or inflammation due to presence of pathogens can lead to over-proliferation of the epithelial cells, giving rise to tumour-like conditions, and dysplasia[160], [161].

Studies also show the involvement of immune pathways (IMD) and intestinal bacteria in regulating epithelial homeostasis. However, the role of Toll pathway and its interaction with other signalling mechanisms in maintenance of the intestinal barrier homeostasis has not been fully understood. The next section discusses the work already done in the field of *Drosophila* gut.

1.4.3 *Drosophila* gut microbiome

The midgut is a site of constant pathogen and toxin threat. Apart from that it also maintains commensals, which are transmitted maternally and via external environment [162], [163]. Microbiome composition does vary across species and population of *Drosophila*. However, 20 species make up about 90% of the microbial population in flies. Bacteria like *Firmicutes* (*Lactobacillus* and *Enterococcus*) and *Proteobacteria* (*Acetobacteraceae* and *Enterobacteriaceae*) dominate *Drosophila* gut and reach

densities of about 10^6 per fly gut[164], [165]. *Lactobacillaceae* and *Acetobacteraceae*, dominate the microbial populations. Microbial compositions and densities get altered throughout the lifespan of the fly. Newly eclosed flies have low densities of gut microbiome due to expression of several AMPs in the pupal stage. As the flies age, the microbial population becomes more abundant and the variability increases[166].

These resident microbes are extremely beneficial to the host and are involved in various biological processes such as energy harvest, maintaining the integrity of gut barrier, protecting against pathogens and modulating host immunity[167]. Several studies highlight the importance of the commensals in improving lifespan of flies when infected with *Serratia marcescens* or *Pseudomonas aeruginosa*[168], [169]. The exact advantages that the microbiota provides in infection resistance and tissue repair after pathogenic insult however, has not been well understood.

1.5 Multiple pathways regulate epithelial homeostasis in the *Drosophila* gut.

The resident microbes are known to increase the basal levels of c-Jun NH₂-terminal kinase (JNK), Janus kinase/Signal transducers and activators of transcription (JAK/STAT) and Immune deficiency (IMD) signalling. These are known for their roles in ISC renewal and differentiation during infections as well as in the maintenance of epithelial composition in the homeostatic conditions. JAK/STAT activation occurs in ISCs while JNK and IMD is triggered in ECs[170], [171]. Microbe-associated JAK/STAT pathway activation has shown to increase mitotic cells, labelled using phosphor-Histone 3(pH3) and escargot positive cells, which comprise ISC and EB population [172], [173].

1.5.1 Innate immunity in epithelial homeostasis

All cell types within the midgut express IMD pathway components and the ECs, EBs and EEs are involved in infection-induced AMP expression. In the gut, the PGRP-LE receptor present in the cytoplasm is responsible for bacterial recognition. Imd activity is modulated by negative regulators like amidases PGRP-SC and PGRP-LB that are involved in ubiquitination/de-ubiquitination of downstream molecules of the IMD pathway. This helps in the maintenance of immune tolerance toward commensals in the gut and also prevents overactivation of immune signalling[174]. Studies on GF mice with mutations on

Relish or PGRP-LC show diminished ISC division and renewal of the epithelial barrier. This is an indication of the importance of gut microbes and their recognition through immune signalling, in maintaining the epithelial barrier through ISC proliferation in homeostatic conditions[170]. Tak1, which is a protein activated downstream of the IMD pathway, also activates JNK signalling and AP-1 (downstream to JNK) nuclear localization resulting in expression of genes involved in response to stress[175].

In homeostatic conditions, the role of IMD is central in microbial recognition through Diaminopimelic acid (DAP)-type PGN. This helps maintain the epithelial composition[47]. GF flies show about 50% increase in EE cell proportion as compared to conventionally reared flies. Similar increase in EEs is seen in *Imd/Relish* mutants compared to isogenic controls. This indicates that basal *Imd* activation is required to maintain epithelial composition. It was further confirmed that cell-autonomous activation of IMD pathway in ISCs was sufficient to maintain the cell composition of the gut. This is also mediated by Notch acting downstream or parallel to IMD. In the same study, when flies were infected with *Erwinia carotovora carotovora* (Ecc15), which is a *Drosophila* pathogen, the EE population increased in the midgut due to an increased ISC differentiation towards EE phenotype. However, the IMD-Relish pathway has been established to be strongly stimulated during infections with Ecc15. It was observed that during infections, virulence associated factors activate JAK/STAT and it overrides the IMD in inducing ISC differentiation toward EEs. Regulation of epithelial homeostasis and ISC behavior occurs with various levels of interaction between immune and developmental pathways[176].

1.5.2 JNK pathway maintains gut epithelial composition.

c-Jun N-terminal kinase (JNK) is a mitogen activated protein kinase (MAPK)- type kinase cascade. Conventional JNK pathway involves *Drosophila* TNFR ligand Egr binding to the two TNF receptors Grindelwald (Grnd) and Wengen (Wgn), resulting in the activation of the JNK kinase cascade. Mammals express three JNK proteins while the *Drosophila bsk* gene encodes a unique JNK protein, making it easier to analyse its role. In *Drosophila*, stress activates the pathway and the signal is transduced through JNK kinase kinases (JNKKs), like Slipper (Slpr) or Msn, apoptosis signal-regulating kinase 1 (ASK1), transforming growth factor beta activated kinase 1 (TAK1), and MAPK kinase kinase 1 (Mekk1). These

phosphorylate the JNK kinases (JNKKs): Hemipterous (Hep) or MAPK kinase 4 (Mkk4). The JNKKs then phosphorylate JNK(Bsk-Basket). Conventionally, activated Bsk phosphorylates activator protein 1 (AP-1) transcription factor, which is a heterodimer of c-Jun and Fos (Jra and Kay, respectively in *Drosophila*). AP-1 mediates the expression of target genes involved in multiple cellular process like cell proliferation and apoptosis (**Fig 1.8**). One particular transcriptional target of JNK is *puckered* (*puc*) and it inhibits Bsk in a feedback loop by dephosphorylating Bsk, thus acting as a negative regulator of the pathway[177], [178]. IMD pathway activates JNK pathway through activated TAK1 [172]. Imd and JNK also share common negative regulators which are PDGF and VEGF-related receptors Pvr and Erk. Activation of JNK and Imd promotes expression of PDGF- and VEGF-related ligands Pvf2 and Pvf3. These two ligands activate Pvr, that inhibits the phosphorylation of Bsk through Erk. Relish phosphorylation is also inhibited by Pvr. Further, Relish (transcription factor downstream of IMD pathway) is involved in the proteasomal degradation of TAK1, adding another layer of regulation to JNK-mediated response. Interaction of IMD and JNK pathways is extensively discussed in the review Tafesh-Edwards & Eleftherianos, 2020[177]

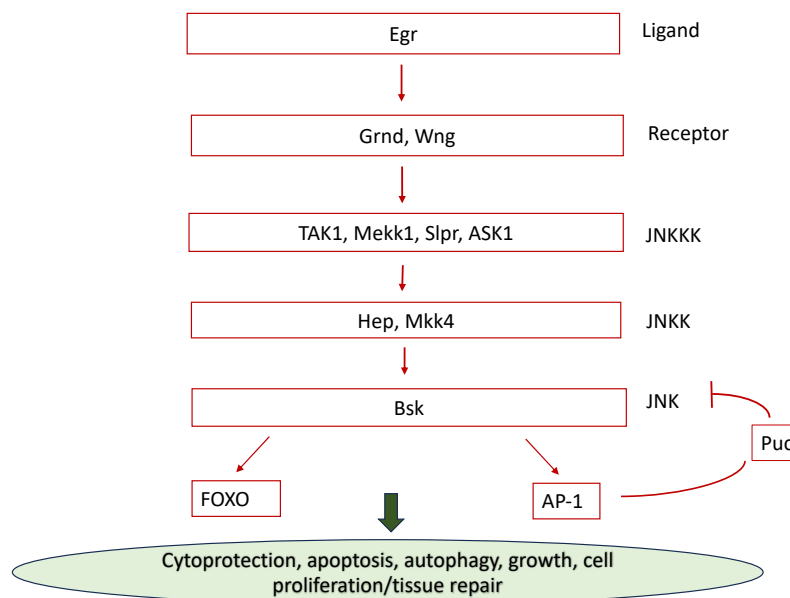


Figure 1.9: Representation of conventional *Drosophila* JNK pathway. [178]

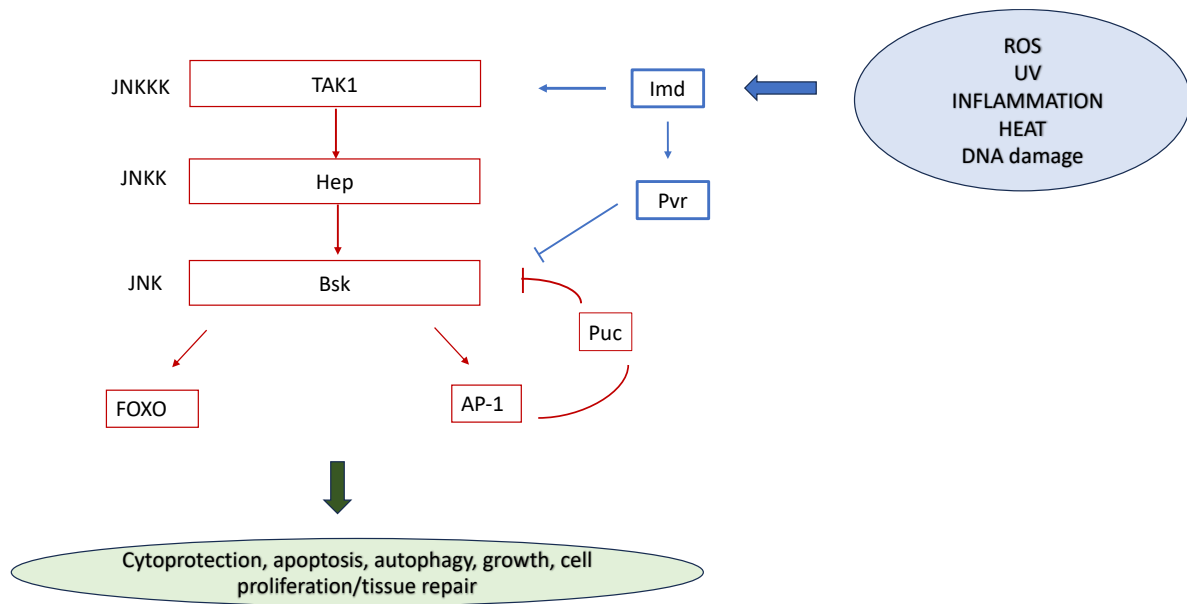


Figure 1.10: Representation of interaction between JNK and IMD pathways. Figure adapted from (Tafesh-Edwards & Eleftherianos, 2020)[177].

Various external and internal stimuli can activate JNK signalling such as growth factors, DNA damage, oxidative stress, pathogens, UV radiation and cytokines. Activation of JNK is seen to increase mitosis and the stem cell population in fly midguts[179]. Therefore, JNK activation is crucial in order to maintain the cytoprotective and regenerative responses during homeostasis, chronic stress, infections and even in age-related stress conditions and to promote longevity [179], [180]. Even though organism-wide upregulation of the JNK pathway increases longevity by mediating higher stress tolerance, JNK activation in the gut of *Drosophila* is seen to have opposing effects. In homeostasis, JNK signalling ensures protection from stress damage by inducing ISC proliferation and EC apoptosis and thus maintain the epithelial turnover rate. Aging sets this balance askew and progressively promotes symmetric divisions of ISC leading to an increase of ISC population in aged guts. Apart from this, aged guts also have an accumulation of mis-differentiated cells[181]. This results in the loss of epithelial homeostasis and eventually terminates at dysplasia and loss of epithelial barrier integrity. Despite the general reputation of JNK being the cytoprotective signalling pathway, aged guts are seen to have a heightened JNK activity that contributes to the deterioration of epithelial integrity[179], [181], [182]. Age associated loss of morphology and intestinal structure can be reduced using *hep*¹ mutants or using flies with diminished JNK activity in the ISCs and EBs. Further, JNK activation in progenitors induces age-associated ISC accumulation. JNK is

also involved in mis-differentiation of ISCs[181]. This age-related upregulation of JNK can be due to the accumulation of reactive oxygen species (ROS), as the presence of ROS has been linked to JNK activation in many tissues. ROS is generated as a product of age-induced damage to mitochondria[183]–[185]. Activation of JNK in ECs leads to EC death and ISC proliferation to replace the cell loss. This further reduces the lifespan of flies[186], [187].

Ageing also induces tumorigenic phenotypes due to ISC over-proliferation and mis-differentiation[181]. JNK is known to have both anti and pro-tumorigenic effects during tumour progression[188]. JNK signalling in targeted cells induces cell-death, although through other mechanisms if JNK-induced apoptosis is suppressed, JNK signalling causes tumour progression and invasiveness. The pro-tumorigenic effects of JNK is evident in *Drosophila* midgut tumours induced using loss of bone morphogenetic protein (BMP) signalling. These tumours had aberrant JNK activation that led to the loss of barrier function and integrity. Blocking JNK rescued this phenotype[189]. Similar findings have been replicated in mouse models using β -catenin-mediated tumour induction. Inhibiting JNK signalling in these tumours resulted in reduced tumour size, number and improved lifespan[190].

There is also differential impact on the activation of JNK in diverse cell populations in the gut. For instance, overexpression of JNK induces ISC over-proliferation. JNK regulates spindle orientation in ISCs, leading to more symmetric divisions[191]. However, in the case of ECs, JNK activation has been linked to apoptosis due to the expression of Ets21c transcription factor, that expresses genes involved in programmed cell death. Contrary to this, Ets21c has been known to promote ISC proliferation through upregulation of JAK/STAT pathway or PDGF/VEGF receptor (Pvr) signalling[192]. The result of JNK activation is context dependent as it promotes the expression of distinct sets of genes in different cell-types. Closing the gap in the knowledge of other factors interacting with JNK, leading to its varied impacts could widen the understanding of the role it plays in maintaining epithelial homeostasis in the gut.

1.5.3 Notch signalling in *Drosophila* gut and its interaction with JNK.

ISC differentiation into ECs is mediated by multiple signals from diverse developmental pathways like Notch, Dpp/TGF β and the transcription factor Sox21a[143], [193], [194]. Notch is central in regulating proliferation and cell-

fate in ISCs. Differential Notch activation regulates ISC differentiation into ECs or EEs. High levels of Notch ligand Delta (DI) in ISCs activates high Notch signalling in daughter cells and promotes differentiation into ECs. By contrast, when there is an asymmetric localisation of Pros leading to the formation of EEs, ISCs remain multipotent through weak Notch signal from neighbouring EEs. Thus Notch acts in a bidirectional and context-dependent manner[142], [143](**Fig 1.9**).

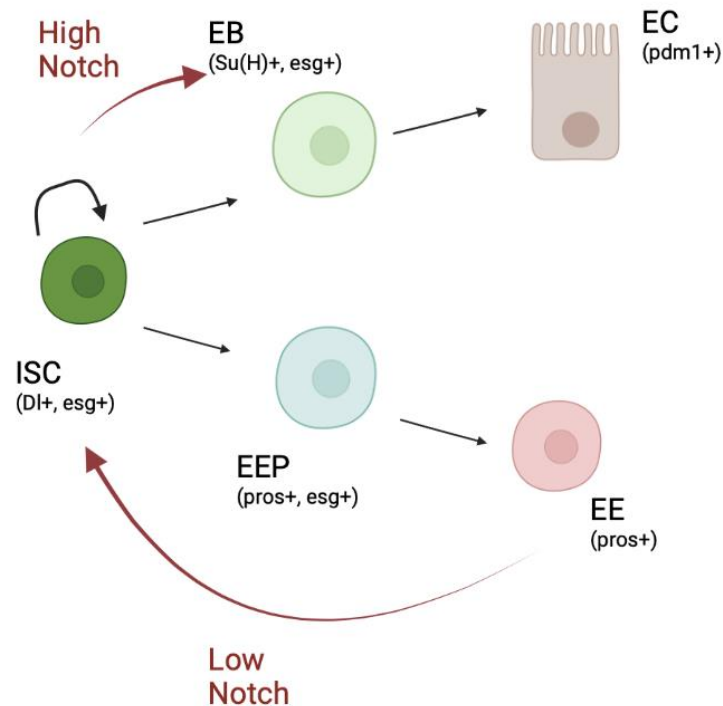


Figure 1.11: The role of Notch signalling in cell fate decisions of the *Drosophila* midgut epithelial cells.

As discussed, aged midguts contain over-proliferating ISCs along with the presence of mis-differentiated cells. These cells also have elevated JNK levels. Ectopic Delta/Notch signalling in these cells can block the over-proliferative phenotype but lead to their abnormal differentiation. There was also widespread activation of Notch in JNK-upregulated conditions. JNK and Notch signalling therefore interact, with DI being potentially downstream of JNK pathway to maintain a balance between cell proliferation and differentiation. This balance is generally lost in aged guts[181].

Therefore discerning the interaction between immune pathways and the pathways that determine ISC cell fate and proliferation is critical in understanding how the microbiome and the immune system interact to maintain

epithelial turnover in normal conditions and mediate tissue repair during infection and stress-induced damage.

1.6 Context for the development of the project

The significant role played by the Toll pathway in host defense has been extensively researched. As the gut is the site of constant pathogen insults and is the hub of commensals, innate immunity needs to be active to maintain a balance between the pathogens and the beneficial microbes. IMD has been explored widely in this regard and Toll is considered to not be involved in the gut immunity[47]. However, research on the role of Toll in mediating immune response and epithelial turnover in the gut is lacking.

Previous work done in the Ligoxygakis lab places Toll as a central element linking immune sensing, metabolism, and microbial populations, in the gut of *Drosophila*. Shivohum Bahuguna *et al.*, showed that the flies that lacked Toll signalling through loss of PGRP-SA, Dif or Spz in whole flies or even in enterocytes, had a significantly lower bacterial density in comparison to their genetic backgrounds[195]. PGRP-SA loss also impacts the diversity of microbial composition with a major drop in the density of *Lactobacillaceae*. Loss of Toll signalling in ECs also negatively impacted lifespan of flies and reduced ISC proliferations and formation of EBs, indicating that basal level Toll activation was required for maintenance of bacteriome and epithelial composition. Inhibiting TOR using Rapamycin or using RNAi of mTOR restored the bacterial densities but could not restore bacterial diversity. mTOR is involved in responses such as protein synthesis, growth, lipid and glucose metabolism, and autophagy. It carries out these functions in response to upstream cues including growth factors, insulin, amino acids, oxygen, and energy levels, through complex interactions. 4E-BP was found to act downstream to mTOR and enable fat catabolism that provided for the energy demands of the commensals and maintained their densities. mTOR acted as an inhibitor of 4E-BP in the absence of PGRP-SA- Toll-Dif signalling. 4E-BP was found to act downstream of Toll. It was also confirmed that 4E-BP had NF- κ P binding sites. Brummer, which is a lipase was also identified as a necessary protein in maintaining the intestinal environment through lipid breakdown, downstream of 4E-BP. Peptidoglycan (PG) binding to PGRP-SA was essential for maintaining the commensals as mutant

forms of PGRP-SA with lower PG binding, was unable to maintain the microbial densities. Therefore, this study proved the hypothesis that microbial sensing by Toll pathway was responsible for regulating lipid metabolism and enabling the retainment of commensal population in homeostatic conditions[195]. This study hinted towards the potential role of Toll signalling in regulating epithelial turnover and my project aims to understand the role of Toll signalling in linking immune sensing and epithelial homeostasis in the *Drosophila* midgut.

1.7 The project aims to answer the following questions.

- 1) Does canonical Toll signalling regulate epithelial renewal in *Drosophila* midgut?
- 2) Does JNK interact with Toll pathway in maintaining epithelial renewal?

2 MATERIALS AND METHODS

2.1 *Drosophila* genetics: Spatio-temporal control of gene expression

The GAL4/UAS system is an established tool used in the *Drosophila* genetic manipulation. The system was adapted from yeast, where GAL4 regulates the galactose metabolism. It functions on the principle that transcriptional activation of genes required for galactose metabolism occurs when GAL4 binds to the upstream activation sequence (UAS) and mediates gene transcription. It is a bipartite system containing the first stable transgenic GAL4 expressing driver line, where GAL4 is coded downstream to specific promoters (cell, tissue, organ specific promoters). The second stable transgenic line carries a UAS-dependent transgene, which could be an RNAi, a gene coding for a protein or various markers. When these two lines are crossed, the progeny expresses the transgene wherever GAL4 is expressed. This enables spatial control of gene expression[196] .

To refine gene expression further, another layer of regulation is added using GAL80. GAL80 binds to GAL4 and inhibits its activity in promoting gene transcription. In this project we used a temperature sensitive variant of GAL80 called GAL80^{ts}, that can be inactivated at 29°C. At 18°C, GAL4 activity is inhibited due to the active GAL80 [197]. Therefore, the flies containing GAL4/UAS/GAL80^{ts} are maintained at 18°C, to prevent expression of transgenes during

development. The control and experimental flies (adults) are then moved to 29°C for inhibition of GAL80, enabling GAL4 binding to UAS and mediating transgene expression. This allows for temporal control of gene expression.

2.2 *Drosophila* husbandry

Flies were reared at 18°C or 29°C, 70% humidity, on standard cornmeal-molasses medium (Appendix 5.1: *Drosophila* media recipe) under a 12 hour light and 12 hour dark cycle. Backup stocks for each line were maintained at 18°C. The crosses for experiments with temperature sensitive lines were maintained at 18°C. 0-2 day old progeny from the crosses (experimental flies) were collected and shifted to 29°C. Approximately 15 to 20 female flies and 3-4 male flies were kept in each vial for every experiment, and tipped into fresh food every two days till the time point of the experiment. Only females were used in experiments to negate sex-specific differences in gut physiology between male and female flies. *Drosophila* strain w^{1118} was used as genetic background for preparing genetic immune mutants. In this project we used *esg-Gal4*, *tub-GAL80^{ts}*, *UAS-GFP (esg^{ts})* flies crossed with their genetic background w^{1118} , as controls in most experiments.

Fly lines used in this study, their source and description is given below (**Table 2.1**)

Genotype	Source	Description
w^{1118}	Bloomington (RRID:BDSC 3605)	Flies have a loss of function mutation of <i>w</i> in gene CG2759 resulting in white eyes.
<i>esg^{ts}</i>	Buchon <i>et al.</i> , 2013[198]	Esg+ cells (ISCs and EBs) have GAL4 expression and are labelled with GFP. GAL80 ^{ts} is also included to regulate gene expression and is controlled via a tubulin enhancer.

<i>ISC^{ts}</i>	Jiang and Edgar 2009[199]	This is an ISC-specific expression system. Esg ⁺ cells have GAL4 expression and are labelled with GFP. GAL80 ^{ts} is also included to regulate gene expression via <i>Su(H)</i> enhancer, thus precluding expression in EBs.
<i>UAS-Toll^{10b}</i> (II)	Shia <i>et al.</i> ,2009[200]	Codes for a constitutively active form of Toll receptor under UAS control.
<i>UAS-Toll^{10b}</i> (X)	Bloomington (RRID:BDSC 58987)	Codes for a constitutively active form of Toll receptor under UAS control.
<i>UAS-Toll^{RNAi KK}</i>	VDRC ID : 100078(library KK)	UAS regulatory sequences drive expression of an inverted repeat(Toll).
<i>UAS-Cactus^{RNAi}</i>	Bloomington (RRID:BDSC 31713)	Expresses dsRNA for RNAi of <i>cactus</i> under UAS control
<i>UAS-Dif^{RNAi 1}</i>	Bloomington (RRID:BDSC 30513)	Expresses dsRNA for RNAi of <i>dif</i> under UAS control
<i>UAS-Dif^{RNAi 2}</i>	Bloomington (RRID:BDSC 29514)	Expresses dsRNA for RNAi of <i>dif</i> under UAS control
<i>UAS-Dif^{RNAi 3}</i>	VDRC ID : 100537(library KK)	UAS regulatory sequences drive expression of an inverted repeat (Dif)
<i>UAS-Bsk^{DN}</i>	Bloomington (RRID:BDSC 6409)	A non-activatable mutant form of Bsk (Bsk ^{DN}) is expressed under the control of UAS regulatory sequences.
<i>UAS-Spz^{GFP}</i>	Cho <i>et al.</i> ,2010[201]	The construct uses cDNA sequence of a processed form of Spz tagged to GFP protein under UAS regulatory sequence.

<i>UAS-Notch^{RNAi}</i>	VDRC ID : 27228(library GD)	UAS regulatory sequences drive expression of an inverted repeat(Notch).
<i>puc-lacZ</i>	Zhou <i>et al.</i> , 2017[202]	Reporter enzyme lacZ is expressed in cells expressing puckered.
<i>esg^{ts}; Dl-lacZ</i>	Kind gift of Bruce Edgar, University of Utah, Salt Lake City, UT	Reporter enzyme lacZ is expressed in cells expressing Delta. Esg ⁺ cells have GAL4 expression and are labelled with GFP. GAL80 ^{ts} is also included to regulate gene expression.

Table 2.1: *Drosophila melanogaster* strains used in this study.

2.3 Infection models

To prepare the bacterial culture, bacteria culture was grown in a flask containing Rifampicin in 1X LB broth. OD at 600nm was measured using a nanodrop. Literature review suggested an OD₆₀₀ = 200 value as an optimum for infection models using *Erwinia carotovora carotovora* (Ecc15) strain. The volume required for OD=200 was calculated and that volume of bacterial culture was pelleted by spinning down the culture using a centrifuge. This was then resuspended in 1ml of 5% sucrose in PBS and used for infection.

7d old flies kept at 18°C, were shifted to 29°C and starved for 6 hours. The flies were then transferred to cages containing filter paper with 1ml high-dose strain of Ecc15 bacteria (OD₆₀₀=200). The flies were then left overnight at 29°C. These fly guts were then dissected the following day and immuno-stained.

2.4 Polylysine slide preparation

For preparation of the polylysine coated slides, the contents of 1g bottle of polylysine(Sigma P1524) were dissolved in 170ul ddH₂O, in a small beaker using a magnetic flea stirrer. After the solution appeared clear, 10ml of this solution

was aliquoted and stored in -20°C as 10X polylysine stocks. When the slides needed coating, 3 aliquots of the stock solution were defrosted and used. Working solution was made by mixing 30ml of 10X polylysine solution with 170ul ddH₂O and 1.5ml of Kodak Photo-Flo. The glass slides were loaded onto a staining jar and the working solution was poured into the jar. The slides were left in polylysine solution on the rocker for 10 mins. The solution was then poured out into a falcon tube and the slides were dried at 60°C for 10 mins in the oven. The slides underwent a total of 5 polylysine coating and drying cycles. The slides were then air-dried. After drying, the surface of the slide that had the polylysine coat was bordered with silicone to create a well to perform immunostaining steps and hold antibody solutions. When the silicone ring was dry, the slides were stored at 4°C.

2.5 Gut dissection and immunostaining

The polylysine coated slides make the *Drosophila* gut stick to its surface and have been used for all the steps of immunostaining to preserve the integrity of the whole length of the tissue.

For gut immunostaining, individual guts from anesthetized flies were dissected at selected time points (10d adult flies)(n=10-15) into cold 1X phosphate buffered saline (PBS). The guts were then arranged onto the prepared polylysine slides containing cold 1X PBS in the well, made of silicone. After arranging the samples on the slide, the PBS is replaced with 4% formaldehyde fixative diluted in 1XPBS, for 40 minutes. The slide was always covered so the samples remain in dark in all the steps moving forward. After fixing, guts were washed twice in 0.1% Phosphate-buffered saline with Triton (PBST) containing 1X PBS and 0.1% Triton X-100, for 5 minutes per wash cycle. The sample was then incubated in 1% Bovine serum albumin (BSA) diluted in 0.1% PBST for 50 minutes (blocking). Guts were then incubated in the primary antibody of desired dilution overnight at 4°C. The primary antibody was then removed and the guts underwent three washing cycles with 0.1% PBST for 5, 20 and 10 mins per cycle in that respective order. The guts were then incubated in secondary antibody of desired concentration for 1.5 hours at room temperature. The guts were then washed with 0.1% PBST for 10, 20 and 15 mins per cycle. After removing the PBST, the guts were incubated in 1:1000 concentration (diluted in PBS) of nuclear stain To-Pro-3 (Invitrogen-T3605) for 25 mins. After two more rounds of wash cycles for 5 mins each, VECTASHIELD (anti-fade mounting medium H-1000-without DAPI

from Vector Laboratories) was added to the sample and the slide was covered with a coverslip. The coverslip was then sealed using nail varnish. The slides were stored at 4°C and imaged within 3-4 days. The control guts were placed on the same slide as experimental guts for direct comparison.

The following primary antibodies were used at specified concentrations and diluted using 0.1% PBST in 1% Bovine serum albumin (BSA).

1. Rabbit Phospho-Histone H3 (Ser10) Polyclonal Antibody(Cat# PA5-17869 from Invitrogen) -1:200
2. Mouse Prospero (MR1A) (AB_528440 from DSHB)- 1:50
3. Rabbit anti-Dif antibody (Ligoxygakis *et al.*, 2002[203]) 1:200
4. Anti-beta galactosidase mAb (Cat # Z3781 from Promega) -1:500

The secondary antibodies used were Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 568 (Cat #A-11036 from Invitrogen) and Alexa fluor 546 anti mouse(Cat # A-21123 from Invitrogen).

2.6 Imaging

The samples were imaged using Leica TCS SP5 confocal laser scanning microscope and all images were captured using 63X magnification objective with a numerical aperture (NA) of 1.4. The oil used for microscope was Leica Type F Immersion liquid ($n_e^{23} = 1,5180$ and $v_e = 46$). The Leica SP detector was used for capturing images. The voxel size of the images were (x;y;z) = 481.47nm; 481.47nm; 2um.

Z-stacks of the each gut was taken at the R4 region of the posterior midgut. Every sample varied in thickness, due to variation in gut physiology or due to slight variations in sample preparation. To have uniform images and consistent sampling of the guts, z-stacks were taken from the start of the gut to the middle of the gut, ending at the lumen. The midpoint was calculated by dividing the start and the end by 2. Imaging was only done till the mid-point to avoid deteriorated resolution and quality of images with increased penetration into the sample depth. 2um thick slices were taken for the z-stack.

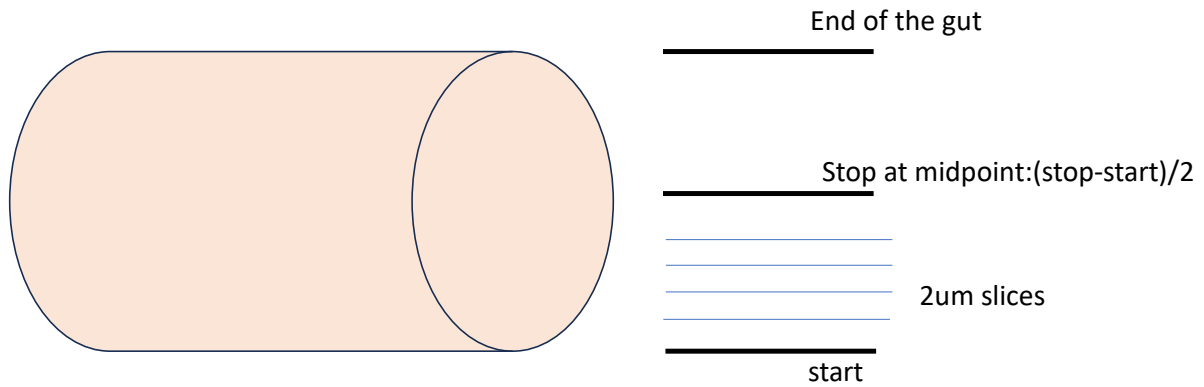


Figure 2.1: Representation of z-stacks taken for the gut samples.

The start and stop points were taken using the objective. The midpoint of the gut was calculated using: $(\text{stop} - \text{start})/2$. 2um thick z-stacks were taken from start to the midpoint (ending at the lumen).

The samples used in this project had progenitor cells labelled with green fluorescent protein (GFP). Therefore, all samples had a channel for the detection of GFP which was kept consistent through all the samples. For imaging GFP in guts, Argon laser was powered up to 30% of its maximum capacity. The excitation wavelength used was 488 operating at 20% of the maximum intensity. The gain was set to 910. The emission wavelength was 502-568 nm. To-pro-3 used to label the nucleus was a far red stain. The excitation wavelength used for its detection was 633 nm operated at 10% of the maximum intensity with the gain in the range of 840-850. For detection of Alexa fluor 568 secondary antibody the excitation laser used was 543 nm and emission was set at 565-635nm. For detection of the proteins probed with Alexa fluor 546 antibody, the excitation wavelength used was 543 nm and the detection wavelength used was 554-635nm. The excitation laser intensity and gains were varied to account for the abundance and localisation of the protein of interest targeted with the antibody. The images were visualised using Fiji-2 (image J).

2.7 Image processing and analysis

To measure nuclear size, nuclei channels were first median filtered using the Scipy `ndimage` module[204] at kernel radius size of 3px. Filtered images were then segmented using Cellpose v2.0.1[205] with 3D mode enabled. Cell size assessment was made with major axis length of each nuclei quantified using Scikit-Image `regionprops` function[206]. The large and small nuclei cells were filtered based on threshold set from histograms plotted for density of cells

against its major axis length for control fly guts and this threshold was used for all other samples (**Fig 2.2**).

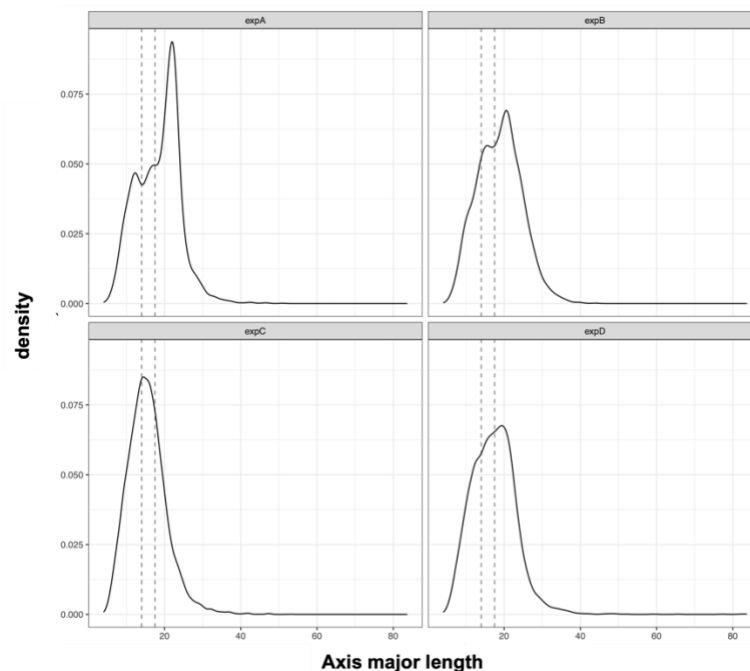


Figure 2.2: Histograms for density of nucleus and major axis length.

The figure represents different histograms obtained using different samples from control (*esg^{ts}* crossed with *w¹¹¹⁸*) fly midguts. 10d old flies with stained nuclei were imaged using confocal microscopy and 2um thick z-stacks were obtained. The 3D volumes of the nuclei were quantified, and the major axis length was plotted against density (number of nucleus with that axis length).

For quantification of fluorescence intensity levels, segmented nuclei masks were expanded by 2px in 3D and the original nuclei masks were subtracted in order to obtain a 3D ring mask around the nucleus, representing a sample of cytoplasmic volume. Fluorescence channels to be quantified were background subtracted (rolling ball, 20px radius) and were quantified within 3D ring masks using Scikit-Image `regionprops` function. Intensity thresholds were set by plotting a distribution of integrated fluorescence intensities and by setting 1.5x normalised fluorescence cut-off from internal control groups. Statistical comparisons were performed in R (4.0.4) environment. All the statistical tests used have been mentioned in the figure legend. All analysis scripts are available in: https://github.com/jefflee1103/Image-analysis/tree/main/people/Ash_U.

2.8 Quantification of pH3+ cell numbers.

For quantifying the pH3+ cell counts for the whole gut, argon laser arc lamp was used. This allowed for visualising pH3+ cells through the eyepiece of the microscope using excitation filter of 515-560nm and emission filter of 590nm. pH3+ cells were counted manually, going through the anteroposterior axis of the gut stack by stack using the eyepiece. Approximately 10-15 guts from 3 different crosses were used to quantify pH3 + cell counts.

2.9 Culture dependent quantification of gut microbiota (CFU)

Five *Drosophila* guts were dissected in 1X Phosphate-buffered saline (PBS) solution and then homogenized using a sterile needle homogeniser until the guts appear completely broken. The resulting suspension was then diluted to form 1:100 dilution and 200ul was plated onto three plates containing De Man, Rogosa and Sharpe (MRS) agar (Appendix 5.2: MRS media recipe) and incubated at 30°C for 48 hours. The colonies in each plate were counted, and log₁₀ values of the colony forming units (CFU's) were calculated. These three plates form the technical replicates for the experiment. This entire procedure was repeated thrice using flies from different generations to account for biological variations in samples. The colonies were counted using ImageJ by setting thresholds to form masks containing the colonies and then using the particle counter function. The log₁₀ CFU values of mutants and controls were statistically compared using Student's t-test, and graphs were plotted in GraphPad Prism.

3 RESULTS

3.1 Toll is required for infection-induced stem cell mitosis.

Previous work by Bahuguna *et al.*, had hinted towards the potential role of Toll signalling in regulating epithelial turnover during infections[195]. Our collaborator Shivohum Bahuguna, Ph.D. student at German Cancer Research Center (DKFZ), further analyzed the possible role of Toll signalling in infection related epithelial regeneration, using Ecc15 oral infection models. Oral infection using Ecc15 is a well-established infection model in *Drosophila*[207]. Ecc15 is a

Gram-negative bacterium and has been found to trigger both systemic and gut immune response[47]. To study the impact of Toll signalling during infections, different *UAS-RNAi* lines were used to reduce the expression of *toll* and its downstream effector *dif*, using *esg^{ts}* drivers. *Esg^{ts}* flies that expressed *UAS-Toll^{RNAiKK}*, or one of three *UAS-Dif^{RNAi}* lines were used to quantify stem cell mitosis. Control flies were *esg^{ts}* crossed with the *w¹¹¹⁸* genetic background. An antibody against the phosphorylated form of Histone-3 (pH3) labels mitotic ISCs, is an indicator of cell proliferation rates and has been used to quantify mitotic cells in infected guts. On measuring the pH3+ cells in whole guts of 7d old flies with an Ecc15 oral infection, we observed that in controls, the pH3+ cell counts were significantly higher as compared to flies that had RNAi to silence *toll* or *dif* in progenitors (**Fig. 3.1**). *Toll^{RNAi}* showed maximum suppression in the levels of pH3. These results indicated the potential role of the canonical Toll pathway (Toll-Dif pathway) in promoting stem cell proliferation and epithelial renewal that helps restore healthy epithelial barrier function during infection.

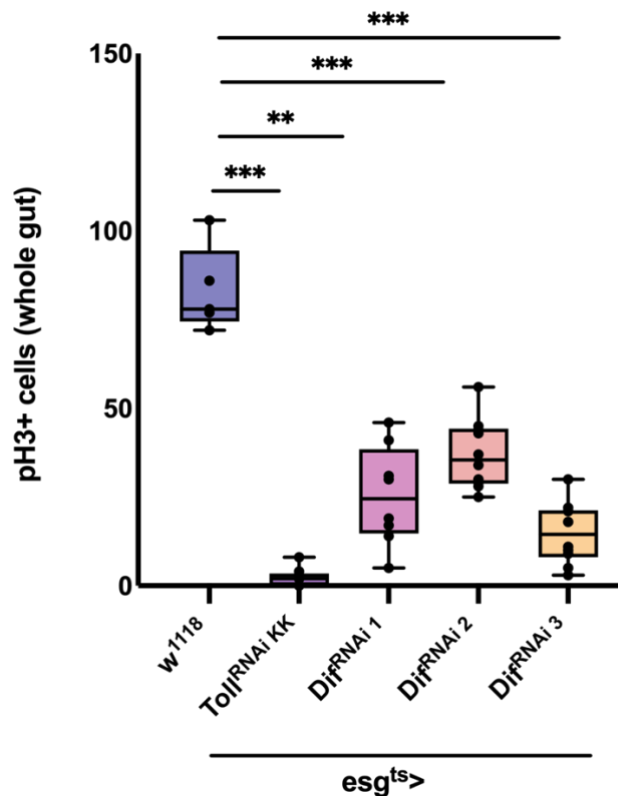


Figure 3.1: Toll pathway is involved in cell mitosis induced by Ecc15 infection.

The graph represents pH3+ cell counts from whole guts of 7d old female controls (*w¹¹¹⁸* crossed with *esg^{ts}*) (*n*=5), *UAS-Toll^{RNAiKK}* (*n*=9), *UAS-Dif^{RNAi1}* (*n*=8), *UAS-Dif^{RNAi2}* (*n*=10), and *UAS-Dif^{RNAi3}* (*n*=10) under an *esg^{ts}* driver infected with Ecc15. The data was obtained by performing the experiment thrice. The x-axis represents different genotypes.. The y-axis represents the number of pH3+ cells present in the whole gut. The pH3+ counts of control flies and flies with RNAi for *toll* and *dif* were statistically compared using Mann-Whitney test (ns = not significant, **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001).

3.2 Activating Toll pathway increases the number of intestinal progenitors in the *Drosophila* midgut.

The previous result highlighted the importance of Toll signalling in epithelial renewal during conditions of infection. To mimic Toll activation during infection, we used a constitutively active form of the Toll receptor under UAS (*UAS-Toll^{10b}*). It is to be noted that the following sections do not have infection experiments. Another thing to keep in mind is that this report constantly mentions GFP+ cells. When *esg^{ts}* drivers are used (as seen in majority of experiments), the GFP+ cells are equivalent to *esg*+ cells or progenitors. Also, unless mentioned otherwise, *Toll^{10b}* is used throughout this report to denote *UAS-Toll^{10b}*. *Toll^{10b}* is a gain of function allele, namely an amino acid replacement (C781Y) on the external surface of the extracellular part of the protein near the start of the transmembrane domain[208]. *Esg^{ts}* (*esg-GAL4, tub-GAL80^{ts}, UAS-GFP*) was used to drive *Toll^{10b}* expression in progenitor cells. Despite increasing evidence to support the presence of enteroendocrine progenitors (EEPs) in drosophila midguts, their numbers in the gut are very limited[209]. The controls used were *esg^{ts}* flies crossed with the *w¹¹¹⁸* genetic background. Both the control and mutant flies had progenitor cells marked through *UAS-GFP*. On Toll activation, we observed a significant increase in GFP+ cells in the R4 region of 10d old female *Drosophila* midguts (**Fig 3.3**).

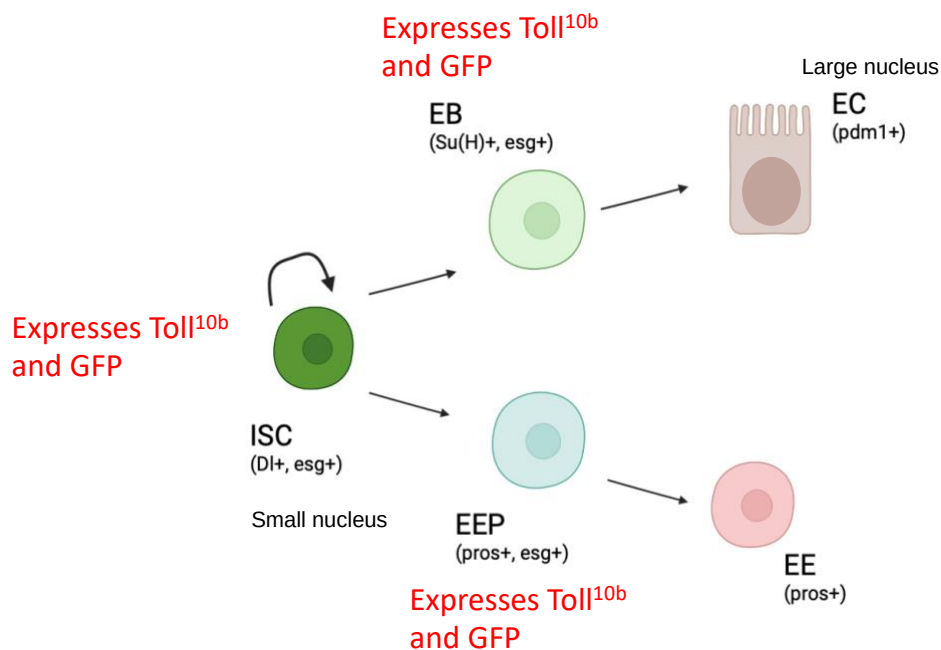


Figure 3.2: Figure representing genetic manipulation performed in experimental flies.

The progenitors (ISC, EBs and EEPs) are labelled with GFP and express Toll^{10b} for experimental flies. It is to be noted that the controls have their progenitors labelled with GFP but do not express Toll^{10b}.

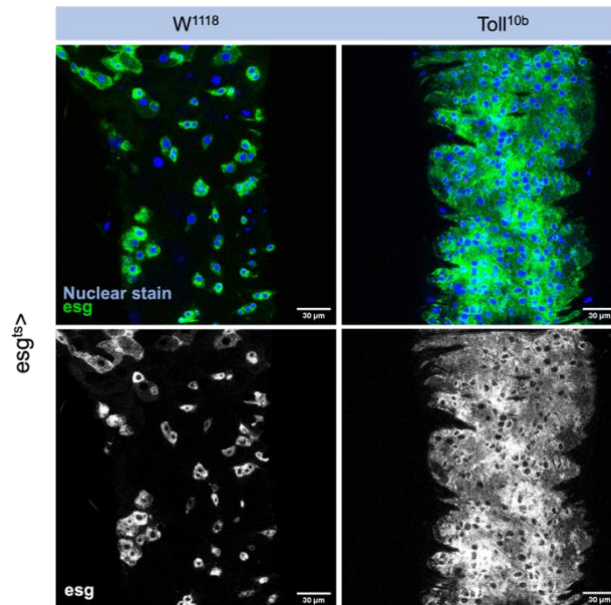


Figure 3.3: Activation of Toll signalling in progenitor cells markedly increases the number of progenitors.

The figure represents a single frame from z-stacks obtained using confocal imaging. The genotypes are control (*w¹¹¹⁸* crossed with *esg^{ts}*) and *esg^{ts}, UAS-Toll^{10b}*. 2µm thick z-stacks were obtained from the posterior midgut region (R4) of 10d old female flies and a single slice has been represented here. The progenitors (ISCs and EBs) are GFP+ (green) and the nucleus is shown in blue (upper panel). The lower panel represents GFP+ cells in white. Scale bar = 30µm.

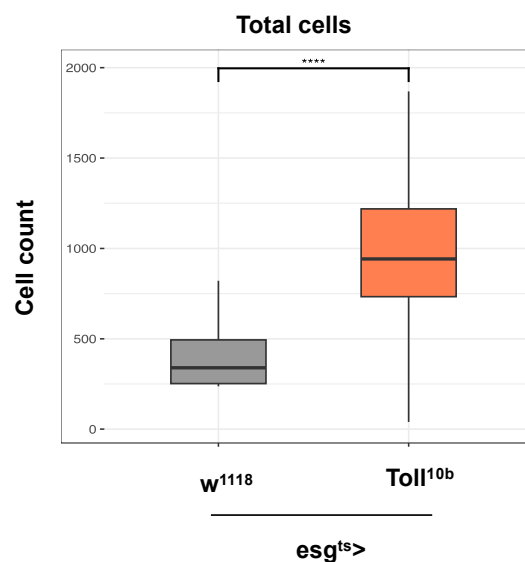


Figure 3.4: Toll activation leads to a significant expansion of cells in the midgut.

The graph represents the total cell counts in the midgut region of 10d old female control (*esg^{ts}* crossed with *w¹¹¹⁸*) (*n*= 18) and *esg^{ts}, UAS-Toll^{10b}* (*n*=19) flies. The results were obtained by performing the experiment thrice. The x-axis represents different genotypes. The y-axis represents the total number of cells in a section of the R4

midgut region. The cell counts in control and *esg^{ts}, UAS-Toll^{10b}* were statistically compared using Student's t-test (ns = not significant, *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001).

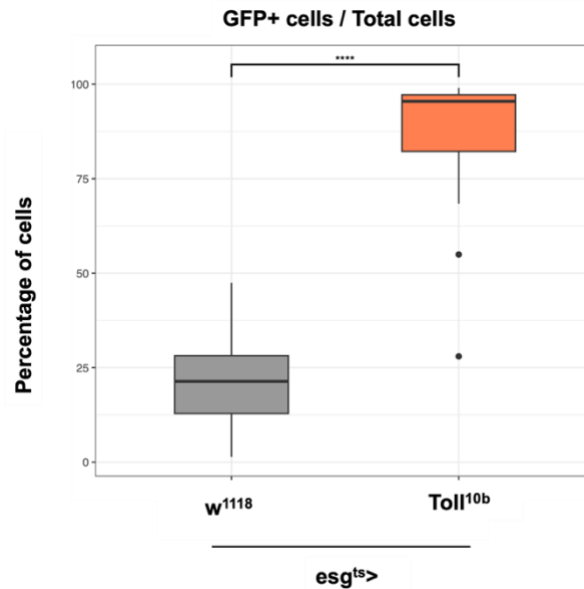


Figure 3.5: Toll activation increases the proportion of *esg*⁺ cells in the midgut.

The graph represents percentage of cells that are GFP+ in the midgut region of 10d old female control (*esg^{ts}* crossed with *w¹¹¹⁸*) (n= 18) and *esg^{ts}, UAS-Toll^{10b}* (n=19) flies. The results were obtained by performing the experiment thrice. The x-axis represents different genotypes. The y-axis represents the percentage of GFP+ cells present in proportion to the total number of cells in a section of the R4 midgut region. The percentages in control and *esg^{ts}, UAS-Toll^{10b}* were statistically compared using Wilcoxon test (ns = not significant, *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001).

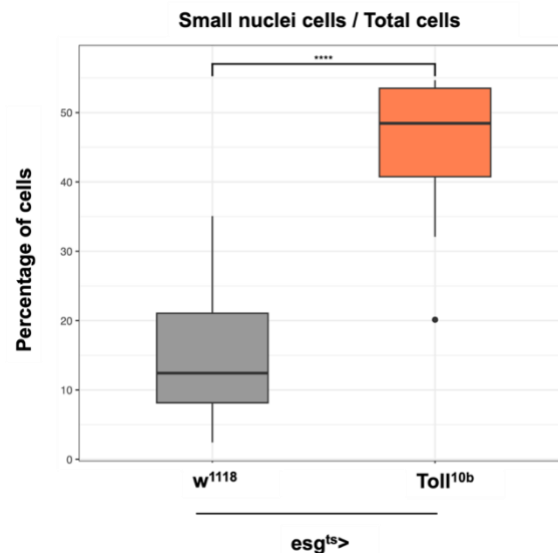


Figure 3.6: Toll activation increases the proportion of cells ISC, EBs and EEs in the midgut.

The graph represents percentage of cells that have a small nucleus (ISCs, EBs, EEs) in the midgut region of 10d old female control (*esg^{ts}* crossed with *w¹¹¹⁸*) (n=18) and *esg^{ts}, UAS-Toll^{10b}* (n=19) flies. The results were obtained by performing the experiment thrice. The x-axis represents different genotypes. The y-axis represents the percentage of small nuclei cells present in proportion to the total number of cells in a section of the R4 midgut

region. The percentages in control and *esg^{ts}*, *UAS-Toll^{10b}* were statistically compared using Wilcoxon test (ns = not significant, *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001).

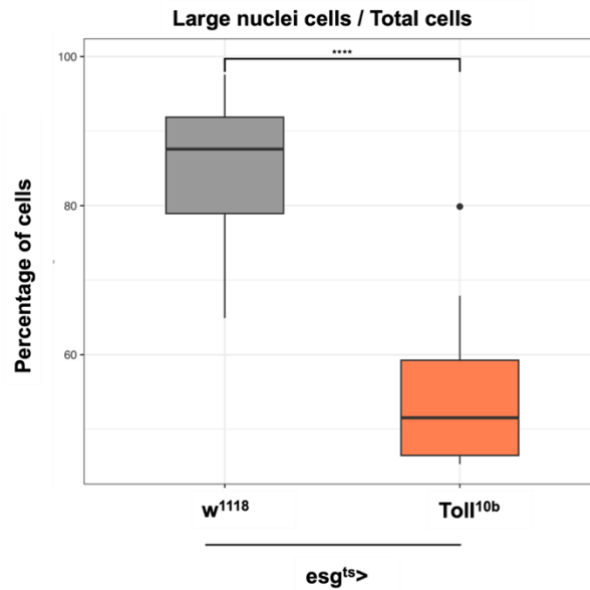


Figure 3.7: Toll activation decreases the proportion of large nuclei cells in the midgut.

The graph represents percentage of cells that have a large nucleus (ECs) in the midgut region of 10d old female control (*esg^{ts}* crossed with *w¹¹¹⁸*) (n=18) and *esg^{ts}*, *UAS-Toll^{10b}* (n=19) flies. The results were obtained by performing the experiment thrice. The x-axis represents different genotypes. The y-axis represents the percentage of large nuclei cells present in proportion to the total number of cells in a section of the R4 midgut region. The percentages in control and *esg^{ts}*, *UAS-Toll^{10b}* were statistically compared using Wilcoxon test (ns = not significant, *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001).

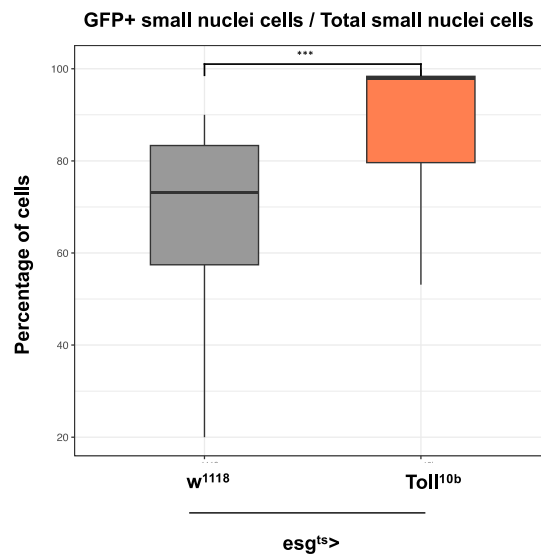


Figure 3.8: Toll activation increases the proportion of small nuclei cells that are GFP+ (*esg+*).

The graph represents percentage of small nuclei cells (ISCs, EBs, EEs) that are GFP+ in the midgut region of 10d old female control (*esg^{ts}* crossed with *w¹¹¹⁸*) (n=18) and *esg^{ts}*, *UAS-Toll^{10b}* (n=19) flies. The results were obtained

by performing the experiment thrice. The x-axis represents different genotypes. The y-axis represents the percentage of GFP+ cells with a small nucleus in proportion to the total number of cells with a small nucleus in a section of the R4 midgut region. The percentages in control and *esg^{ts}, UAS-Toll^{10b}* were statistically compared using Wilcoxon test (ns = not significant, *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001).

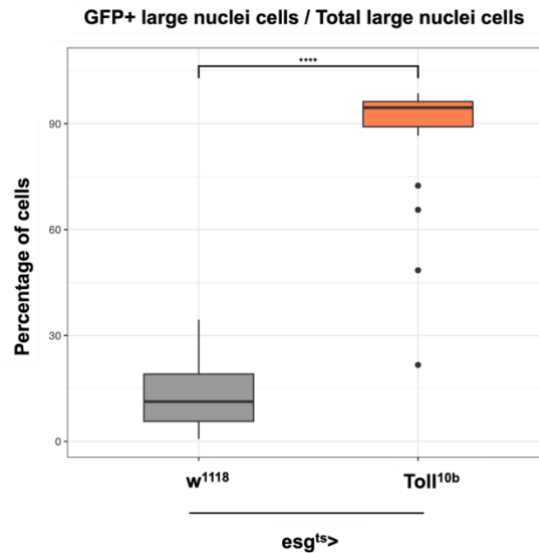


Figure 3.9: Toll activation increases the proportion of large nuclei cells that are GFP+ (*esg*+).

The graph represents percentage of large nuclei cells (ECs) that are GFP+ in the midgut region of 10d old female control (*esg^{ts}* crossed with *w¹¹¹⁸*) (n=18) and *esg^{ts}, UAS-Toll^{10b}* (n=19) flies. The results were obtained by performing the experiment thrice. The x-axis represents different genotypes. The y-axis represents the percentage of GFP+ cells with a large nucleus in proportion to the total number of cells with a large nucleus in a section of the R4 midgut region. The percentages in control and *esg^{ts}, UAS-Toll^{10b}* were statistically compared using Wilcoxon test (ns = not significant, *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001).

It was clearly visible that even some of the cells with large nuclei, a characteristic of ECs, were GFP+. We quantified the proportions of *esg*+ , small nuclei and large nuclei cells in the gut. The following conclusions were drawn from the data obtained through image analysis (depicted in **Fig 3.4- Fig 3.9**).

1. On Toll activation, there is a massive expansion in the total cell population, which results in a significantly high number of cells observed per frame in flies with activated Toll as compared to controls (**Fig 3.4**).
2. The proportion of *esg*+ cells (progenitors) is almost always constant in conditions of homeostasis (the proportion of progenitors is generally constant to maintain constant epithelial turnover rates), as observed in control flies. However, on Toll activation, this proportion is increased with more than 90% of the cells in the region being GFP+ (*esg*+) (**Fig 3.5**).
3. The proportion of small nuclei cells increases (ISCs, EBs, EEs) (**Fig 3.6**)

4. The proportion of cells with large nuclei (potentially ECs) decreases (**Fig 3.7**).
5. The ratio of both small and large nuclei cells that are GFP+ increases in *Toll*^{10b} with more than 90% of the large nuclei cells being GFP+ (**Fig 3.8, Fig 3.9**).

These results potentially mean that the rate of ISC proliferation was increased in *Toll*^{10b} flies (explained by the massive increase in the total number of cells) and/or *Toll*^{10b} expression in EBs blocked them from going further (thus accumulating GFP+ (*esg*+) cells and also explained by the decrease in proportion of large nuclei cells and an increase in the proportion of cells with small nuclei) and/or causes (mis) differentiation of EBs at a higher rate that the GFP perdurance was observed. To distinguish between these non-mutually exclusive scenarios, the following experiments were performed. A limitation in this report is the lack of *pdm1* staining to confirm the identity of cells with a large nucleus as ECs. However, *ph3* staining, *prospero* staining and the use of *DI-lacZ* has been used to label mitotic cells, EEs and ISCs respectively in the experiments that follow. Another limitation is the use of *esg*^{ts} drivers that induce transgene expression in both ISCs and EBs, hence making it difficult to understand which cell population contributes more to the dysplasia observed on Toll activation.

3.3 Activating Toll pathway increases mitotic cells in the gut.

To further understand the mechanism that led to such a significant expansion of the *esg*+ cell population in the midgut, *ph3* staining was used. *ph3* stains for mitotic cells or the cells arrested in the G2/M phase of the cell cycle. Therefore, the number of *ph3*+ cells in the whole gut of 10d *esg*^{ts} and *esg*^{ts} > *UAS-Toll*^{10b} were compared. *Toll*^{10b} flies showed a significant increase in the number of *ph3*+ cells as compared to *esg*^{ts} controls. These cells were also GFP+ and had small nuclei. In homeostatic conditions, ISCs are the only cells capable of undergoing mitosis in the gut[210] and a *ph3*+ cell could be a dividing ISC. Additionally, as discussed in section 3.2, the increase in *ph3* levels also corroborated with an increase in the number of *esg*+ cells and an increase in the total number of cells in the gut, indicating a potential expansion in the number of ISCs or increased transition of ISCs to EBs. The significant expansion of the total number of cells in the gut rules out the possibility of cell cycle arrest at G2/M phase. Further, as the experiment involves overexpressing an activated Toll receptor in both the progenitors (ISCs and EBs), the increase in *ph3*+ cells could also be due to mis-differentiation or non-differentiation of EBs as a result of an activated Toll signalling causing EB accumulation and depletion of ECs (large nuclei) (as shown

in the section 3.2). This could further stimulate ISC divisions to account for the depleted EC pool causing dysplasia and the observed increase in pH3+ cells, *esg*+ cells and total number of cells in the gut. To completely understand the impact of activation of Toll signalling on stem cells and EBs, it is important to use cell-specific drivers like *DI-GAL4* and *Su(H)-GAL4* to drive the expression of *Toll*^{10b} in stem cells and EBs separately. The use of *esg-GAL4* drivers activate Toll signalling in two different kinds of cells in the Drosophila gut and therefore any phenotypes observed cannot be directly linked to the impact of activated Toll signalling in just one cell population. This represents another limitation of this study.

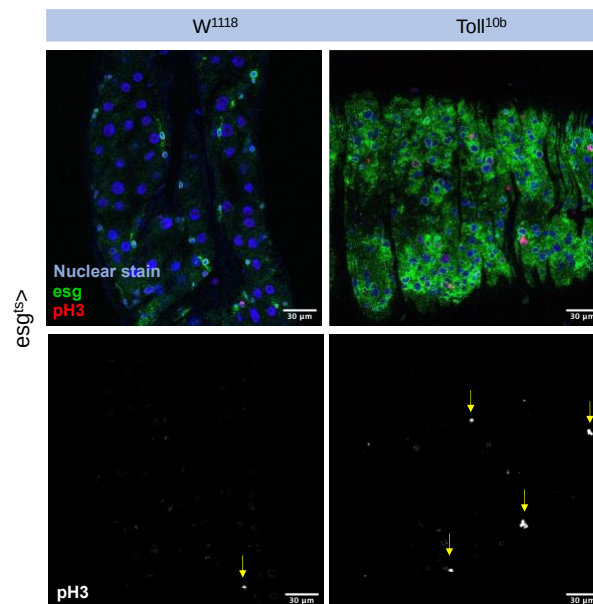


Figure 3.10: Activation of Toll signalling in progenitors increases cell mitosis.

The figure represents a single frame from z-stacks obtained using confocal imaging. The genotypes are control (*w*¹¹¹⁸ crossed with *esg*^{ts}) and *esg*^{ts}, *UAS-Toll*^{10b}. The yellow arrows point toward the pH3+ cells. 2μm thick z-stacks were obtained from the posterior midgut region (R4) of 10d old female flies and a single slice has been represented here. The progenitors (ISCs and EBs) are GFP+ (green), mitotic cells are stained with pH3 antibody (red), and the nucleus is shown in blue (upper panel). The lower panel represents pH3+ cells in white. Scale bar = 30μm.

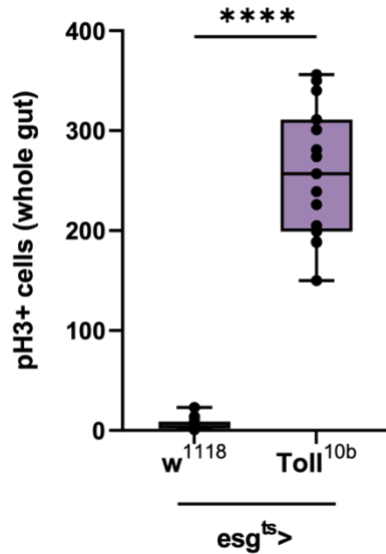


Figure 3.11: Toll activation in progenitors increases cell mitosis.

The graph represents pH3+ cell counts from whole guts of 10d old female control (*esg^{ts}* crossed with *w¹¹¹⁸*) (n=15) and *esg^{ts}, UAS-Toll^{10b}* (n=15) flies. The x-axis represents different genotypes. The y-axis represents the number of pH3+ cells present in the whole gut. This experiment had three biological replicates. The pH3+ counts of control and *esg^{ts}, UAS-Toll^{10b}* were statistically compared using Mann-Whitney test (ns = not significant, *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001).

3.4 Toll signalling influences cell fates in the posterior midgut.

Dif is the transcription factor that acts as the effector of Toll signalling pathway. On Toll activation, it translocates into the nucleus and mediates gene expression[58]. We further wanted to see if blocking Dif could give opposing results as observed in *Toll^{10b}* flies. For this, *Dif^{RNAi}* was expressed in progenitors using *esg^{ts}* driver. pH3 staining was performed to quantify mitotic cells in the gut. Confocal images were also quantified and analyzed. **Fig 3.12** and **Fig 3.15** clearly indicate that the proportion of GFP+ cells (progenitors) remain constant in control and *Dif^{RNAi}* fly guts. The total number of cells remain relatively constant (**Fig 3.14**), which meant that depletion of Toll signalling did not adversely impact the maintenance of epithelium. An interesting observation was that, in contrast to guts with activated Toll signalling, the proportion of cells with small nuclei decreased and consequently, the proportion of cells with large nuclei was higher (**Fig 3.16** and **Fig 3.17**). The number of pH3+ cells in the whole guts was also higher than control flies (**Fig 3.13**). This is an indication that the dividing stem cells differentiated into an EB and subsequently an EC at a higher rate. As the driver used was *esg-GAL4*, this could also mean that on depleting Toll in EBs, they differentiated into ECs at a higher rate and to maintain the proportions of EBs, the ISCs divided more, thereby exhibiting higher pH3+ cells in *Dif^{RNAi}* fly guts. Toll signalling could potentially be inhibiting differentiation of EBs to ECs, which

explains why flies with activated Toll signalling (*Toll*^{10b} flies) had a lower proportion of large nuclei cells (**Fig 3.7**). This experiment elucidates the potential role of Toll signalling in cell fate decision by determining the rates of differentiation to an EC. To further understand the effects of cell-specific depletion of the Toll pathway, it is necessary to express RNAi for molecules in the Toll pathway using cell-specific drivers. It is also important to label ECs to confirm that the large nuclei cells are truly ECs.

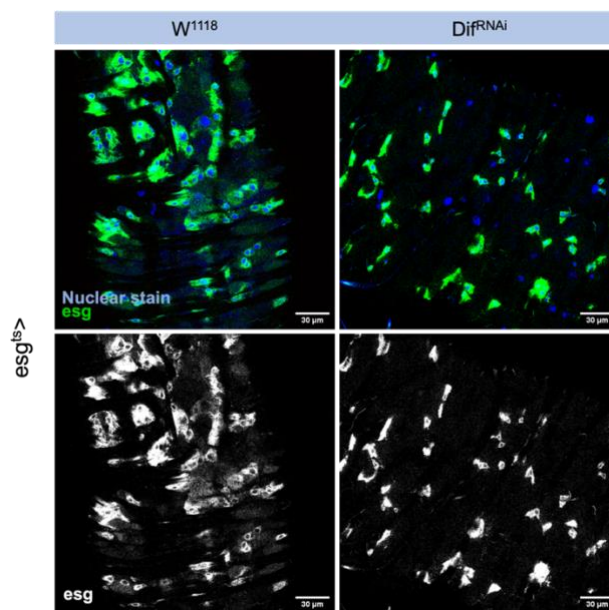


Figure 3.12: Blocking the Toll pathway using *Dif*^{RNAi} in progenitors does not block stem cell proliferation.

The figure represents a single frame from z-stacks obtained using confocal imaging. The genotypes are control (*w*¹¹¹⁸ crossed with *esg*^{ts}) and *esg*^{ts}; *UAS-Dif*^{RNAi}. 2μm thick z-stacks were obtained from the posterior midgut region (R4) of 10d old female flies and a single slice has been represented here. The progenitors (ISCs and EBs) are GFP+ (green) and the nucleus is shown in blue (upper panel). The lower panel represents GFP+ cells in white. Scale bar = 30μm.

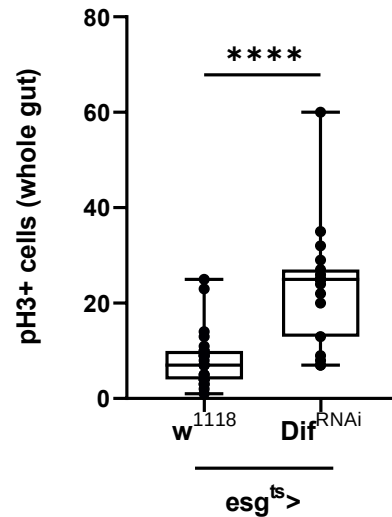


Figure 3.13: Blocking Toll pathway using *Dif*^{RNAi} does not block stem cell mitosis.

The graph represents pH3+ cell counts from whole guts of 10d old female control (*esg*^{ts} crossed with *w*¹¹¹⁸) (n=23) and *esg*^{ts}; *UAS-Dif*^{RNAi} (n=19) flies. This experiment had three biological replicates. The x-axis represents different genotypes. The y-axis represents the number of pH3+ cells present in the whole gut. The pH3+ counts of control and *esg*^{ts}; *UAS-Dif*^{RNAi} were statistically compared using Mann-Whitney test (ns = not significant, *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001).

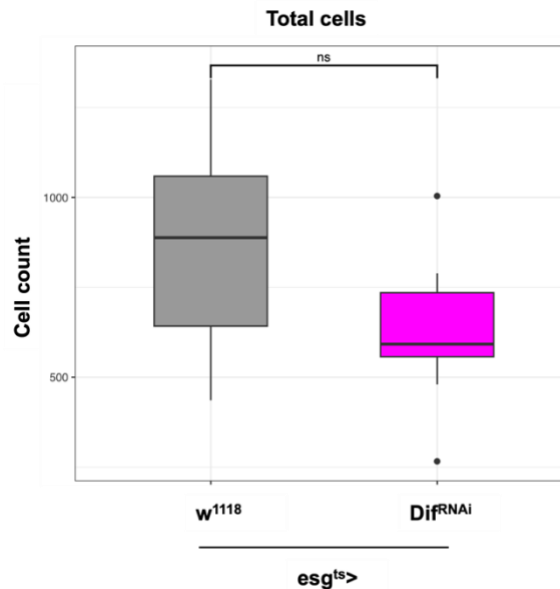


Figure 3.14: Suppressing levels of *dif* does not influence total cells in the midgut.

The graph represents total cells present in a midgut region of 10d old female control (*esg*^{ts} crossed with *w*¹¹¹⁸) (n=7) and *esg*^{ts}; *UAS-Dif*^{RNAi} (n=13) flies. This experiment had three biological replicates. The x-axis represents different genotypes. The y-axis represents total cell counts in a section of the R4 midgut region. The cell counts in control and *esg*^{ts}; *Dif*^{RNAi} were statistically compared using Wilcoxon test (ns = not significant, *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001).

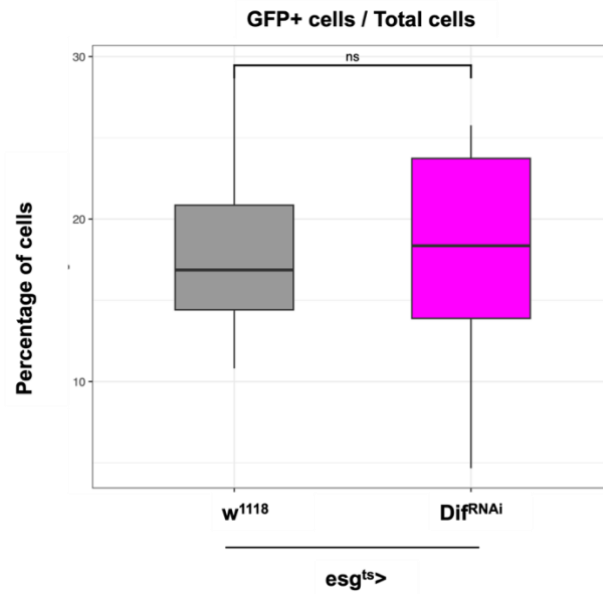


Figure 3.15: Suppressing levels of *dif* does not influence the proportion of *esg*^{ts} cells in the midgut.

The graph represents the proportion of GFP+ cells present in a midgut region of 10d old female control (*esg*^{ts} crossed with *w*¹¹¹⁸) (n=7) and *esg*^{ts}; *UAS-Dif*^{RNAi} (n=13) flies. The x-axis represents different genotypes. The y-axis represents the percentage of GFP+ cells in proportion to the total number of cells in a section of the R4 midgut region. The percentages in control and *esg*^{ts}; *UAS-Dif*^{RNAi} were statistically compared using Wilcoxon test (ns = not significant, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

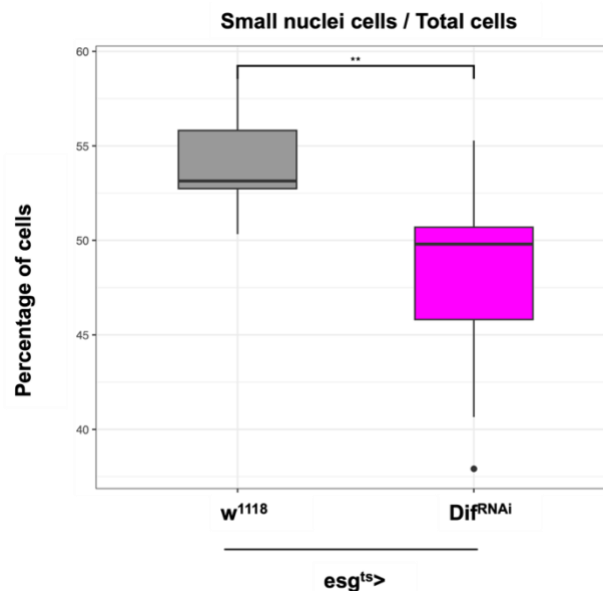


Figure 3.16: Suppressing levels of *dif* reduces the proportion of small nuclei cells in the midgut.

The graph represents percentage of cells that have a small nucleus (ISCs, EBs, EEs) in the midgut region of 10d old female control (*esg*^{ts} crossed with *w*¹¹¹⁸) (n=7) and *esg*^{ts}; *UAS-Dif*^{RNAi} (n=13) flies. This experiment had three biological replicates. The x-axis represents different genotypes. The y-axis represents the percentage of small nuclei cells present in proportion to the total number of cells in a section of the R4 midgut region. The percentages in control and *esg*^{ts}; *UAS-Dif*^{RNAi} were statistically compared using Wilcoxon test (ns = not significant, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

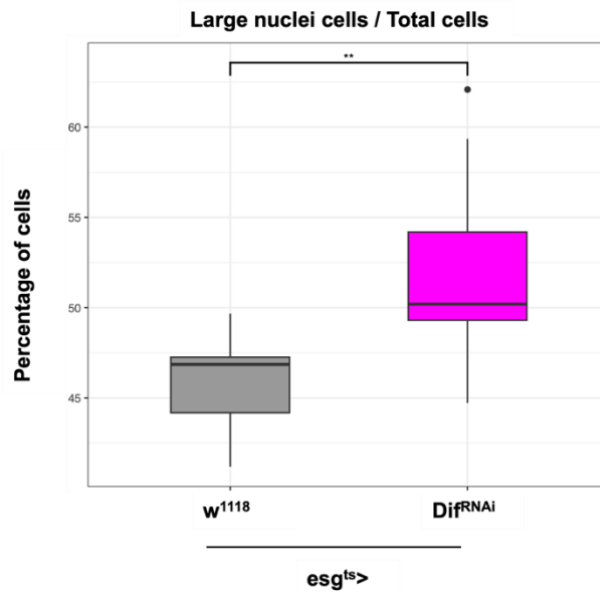


Figure 3.17: Suppressing levels of *dif* increases the proportion of large nuclei cells in the midgut.

The graph represents percentage of cells that have a large nucleus (ECs) in the midgut region of 10d old female control (*esg^{ts}* crossed with *w¹¹¹⁸*) ($n=7$) and *esg^{ts}; UAS-Dif^{RNAi}* ($n=13$) flies. This experiment had three biological replicates. The x-axis represents different genotypes. The y-axis represents the percentage of large nuclei cells present in proportion to the total number of cells in a section of the R4 midgut region. The percentages in control and *esg^{ts}; UAS-Dif^{RNAi}* were statistically compared using Wilcoxon test (ns = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

3.5 Toll activation increases the number pH3+ cells in young flies

Activating Toll pathway resulted in a significant increase in the *esg*⁺ cell population in guts. Higher number of mitotic cells (pH3+ cells) and an increased total cell count were also found in the guts with Toll activation. It was however imperative to ensure that this phenotype observed was true and not an artefact of higher amounts of activated Toll^{10b} proteins present in the progenitors. Therefore we also measured the pH3+ cell counts in 1d and 3d female fly guts to look at the impact of short term Toll activation through *esg^{ts}* driver. As observed from the pH3+ cell counts of whole guts, it became clear that inducing Toll, increased the number of mitotic cells even in very young guts (**Fig 3.18**). It was also interesting to see the increase in pH3+ cell numbers with Toll activation in progenitor cells even for a day. Quantifying the total cell counts in young guts would be necessary to confirm that the increase in pH3 levels indicate higher mitosis rates.

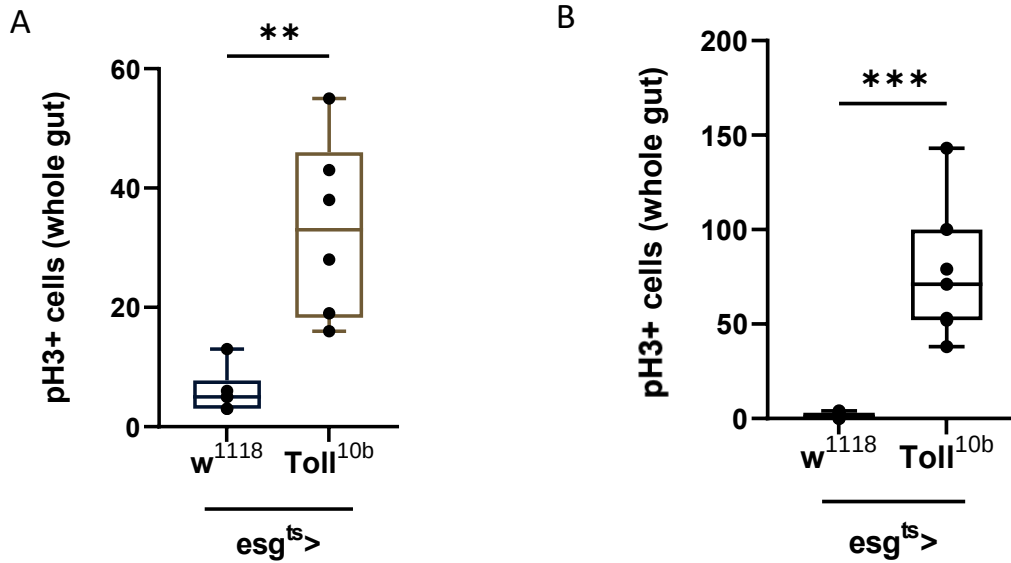


Figure 3.18: Toll activation results in increased number of pH3+ cells in young fly guts.

The graph represents pH3+ cell counts from whole guts of 1d old (A) and 3d old (B) female control (*esg^{ts}* crossed with *w¹¹¹⁸*) and *esg^{ts}, UAS-Toll^{10b}* flies. The sample size for 1d old guts were n=6, for both control and *esg^{ts}, UAS-Toll^{10b}*. The sample size for 3d old guts was n=7 for both genotypes. The results were obtained by performing the experiments thrice. The x-axis represents different genotypes. The y-axis represents the number of pH3+ cells present in the whole gut. The pH3+ counts of control and *esg^{ts}, UAS-Toll^{10b}* were statistically compared using Mann-Whitney test (ns = not significant, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

3.6 Toll activation increases the ISC population in midguts.

On activating Toll in the progenitors, there was an increase in the number of pH3+ cells observed (explained in section 3.3). A potential explanation for this phenotype was that, activating Toll in ISCs lead to higher rates of ISC divisions. Alternatively, as discussed in section 3.3, the increase in pH3+ cells could be due to EB mis-differentiation or non-differentiation due to increased Toll signalling. This could be causing accumulation of EBs (corroborated with an increase in the proportion of small nuclei cells) in the gut, and EC depletion, resulting in the ISCs to divide further to replenish the EC population in the gut. However, the question of whether these ISCs divided symmetrically to form more ISCs, or asymmetrically to form EBs that then accumulated, had to be determined. To quantify the proportion of cells that were ISCs when *Toll^{10b}*, we used *esg^{ts}; Dl-lacZ* flies in combination with *UAS-Toll^{10b}*. Delta (DI), the Notch ligand, is a stem cell marker[143] and we used flies that have the last 10 amino acids of DI replaced by the coding region of lacZ[211]. LacZ was labelled with anti-β galactosidase antibody. Therefore *esg^{ts}; Dl-lacZ* (crossed with *w¹¹¹⁸*) flies were used as controls and *esg^{ts}; Dl-lacZ* were crossed with *Toll^{10b}* flies to have activated Toll signalling in *esg+* cells in mutants. Immunostaining of 10d old fly midguts

revealed an increase in the number of lacZ+ cells in Toll^{10b} fly midgut as compared to controls (**Fig 3.19**). However, the proportion of lacZ+ cells remain relatively the same, because of massive increase in total cell counts observed in Toll^{10b} guts (**Fig 3.20** and **Fig 3.21**). A notable result was that, among the population of small nuclei cells, the proportion of lacZ+ cells was lower in flies with activated Toll signalling. In conditions of homeostasis, the proportion of progenitors are constantly maintained. On Toll activation, there is a marked increase in the number of small nuclei cells, and from **Fig 3.22**, it is evident that only a small proportion of these are stem cells. The other small nuclei cells could either be blocked EBs, differentiated EEs or mis-differentiated cells. Therefore, we wanted to determine the proportion of EEs on Toll activation, explained in the following section.

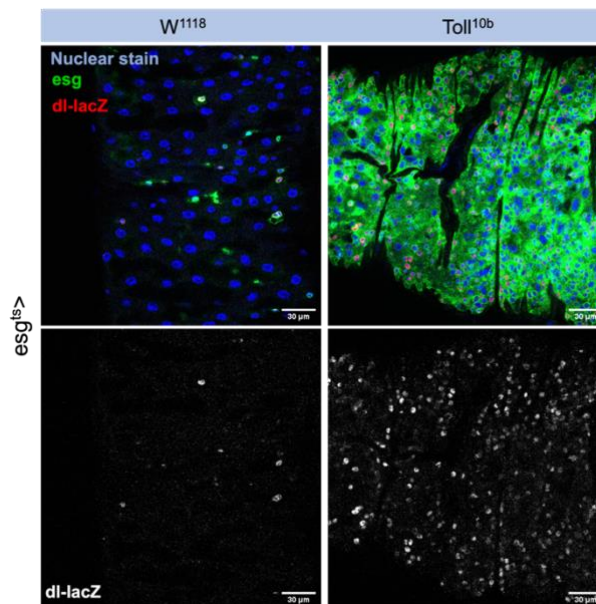


Figure 3.19: Activation of Toll signalling increases the number of ISCs in the midgut.

The figure represents a single frame from z-stacks obtained using confocal imaging. The genotypes are control (*w¹¹¹⁸* crossed with *esg^{ts}*) and *esg^{ts}, UAS-Toll^{10b}*. 2μm thick z-stacks were obtained from the posterior midgut region (R4) of 10d old female flies and a single slice has been represented here. The progenitors (ISCs and EBs) are GFP+ (green) and the nucleus is shown in blue (upper panel). The ISCs expressed lacZ which was labelled using anti-β-galactosidase antibody (red). The lower panel represents lacZ+ cells in white. Scale bar = 30μm.

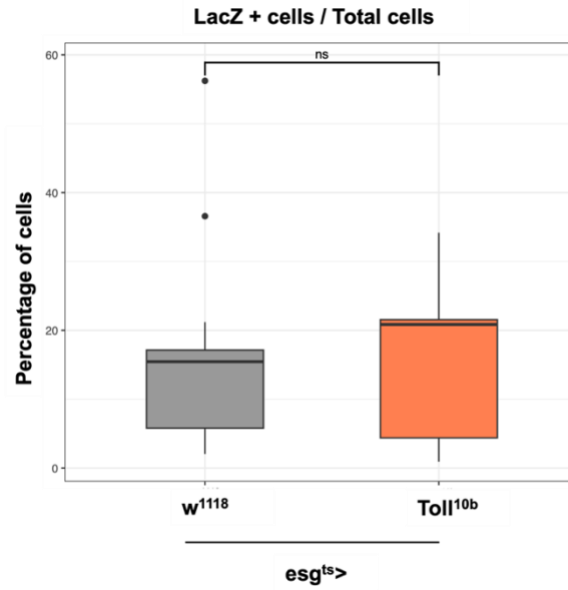


Figure 3.20: Toll activation does not significantly alter the proportion of stem cells in the midgut.

The graph represents percentage of lacZ+ cells (ISCs) in the midgut region of 10d old female control (*esg^{ts}* crossed with *w¹¹¹⁸*) (n=13) and *esg^{ts}, UAS-Toll^{10b}* (n=14) flies. This experiment had three biological replicates. The x-axis represents different genotypes. The y-axis represents the percentage of lacZ+ cells (ISCs) in proportion to the total number of cells in a section of the R4 midgut region. The percentages in control and *esg^{ts}, UAS-Toll^{10b}* were statistically compared using Wilcoxon test (ns = not significant, *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001).

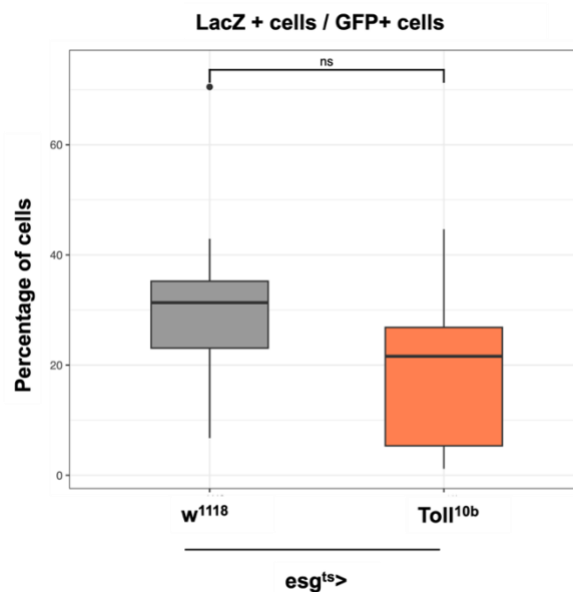


Figure 3.21: Toll activation does not significantly alter the proportion of stem cells in the midgut.

The graph represents the proportion of GFP+ cells that are lacZ+ (ISCs) in the midgut region of 10d old female control (*esg^{ts}* crossed with *w¹¹¹⁸*) (n=13) and *esg^{ts}, UAS-Toll^{10b}* (n=14) flies. This experiment had three biological replicates. The x-axis represents different genotypes. The y-axis represents the percentage of lacZ+ cells (ISCs) in proportion to the total number of GFP+ cells in a section of the R4 midgut region. The percentages in control and *esg^{ts}, UAS-Toll^{10b}* were statistically compared using Wilcoxon test (ns = not significant, *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001).

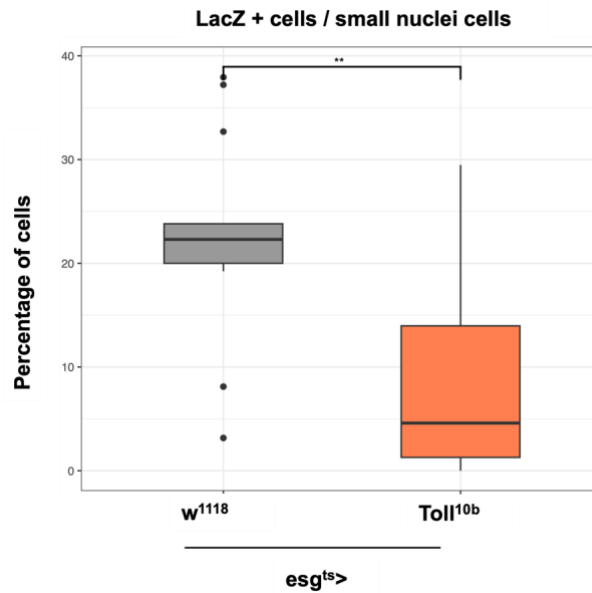


Figure 3.22: Toll activation alters the number of small nuclei cells in the midgut.

The graph represents percentage of lacZ+ cells (ISCs) in proportion to the small nuclei cells in the midgut region of 10d old female control (*esg^{ts}* crossed with *w¹¹¹⁸*) (n=13) and *esg^{ts}, UAS-Toll^{10b}* (n=14) flies. This experiment had three biological replicates. The x-axis represents different genotypes. The y-axis represents the percentage of lacZ+ cells (ISCs) in proportion to the total number of cells with a small nucleus in a section of the R4 midgut region. The percentages in control and *esg^{ts}, UAS-Toll^{10b}* were statistically compared using Wilcoxon test (ns = not significant, *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001).

3.7 Activation of Toll pathway does not significantly influence EE population in the *Drosophila* midgut.

To further understand if the proliferating ISCs differentiate or get blocked at the EB stage, it is important to label the differentiated cell populations of the midgut, which are the EEs and ECs. Prospero(pros) is a genetic marker for EE cells in the midgut[135], [144] and the number of pros+ cells were compared in *esg^{ts}* (crossed with *w¹¹¹⁸*) and *esg^{ts}>UAS-Toll^{10b}* flies. As seen in **Fig 3.23**, there was an increase in the number of pros+ cells observed in *Toll^{10b}* flies. However, the proportion of pros+ cells in comparison to total cells was insignificant (**Fig 3.24**). This indicated that the increase in small nuclei cells observed on Toll activation is not due to a very high increase in EE numbers. However, an interesting result from **Fig 3.25** was that a huge proportion of EEs were also GFP+ on Toll activation. This could be enteroendocrine progenitors (EEPs). Toll activation therefore potentially increases EEP numbers in the gut. This could be a result of the fast rates of ISC proliferation, thereby generating a lot of EEPs. It could be possible that these enteroendocrine progenitors also get blocked, which is why they still retain GFP. From all the experiments discussed until this section, we can make the following conclusions:

- The expansion of the total number of cells in the guts with activated Toll signalling in the progenitors and a significant increase in the number of pH3+ cells in these guts point towards higher rate of cell mitosis in the gut.
- Further, on quantifying the proportions of ISCs (using *DI-lacZ*), and EEs (using prospero antibody), in the guts with activated Toll signalling, it was observed that despite an increase in the number of ISCs and EEs in these guts, the proportions of these cells with respect to total number of cells remained similar to that of control guts.
- This result combined with the data from section 3.2, where it was found that activated Toll signalling in progenitors greatly increased the proportion of small nuclei cells points out that this expansion of small nuclei GFP+ (*esg*+) cells mostly comprised of EBs that were blocked at the stage as they were non-differentiation or mis-differentiating EBs that retained *esg* marker and therefore expressed GFP. As all experiments performed till now use *esg-GAL4* drivers to express *Toll^{10b}*, the EBS also have an active Toll signalling that could potentially be blocking its proper differentiation.
- To validate these findings, it is necessary to label EBs and justify that the expansion of small nuclei GFP+ cells are largely comprised of EBs. Further it is also necessary to use ISC and EB specific drivers to clearly define the impact of activated Toll signalling in these cells. This is one of the limitations of this study.

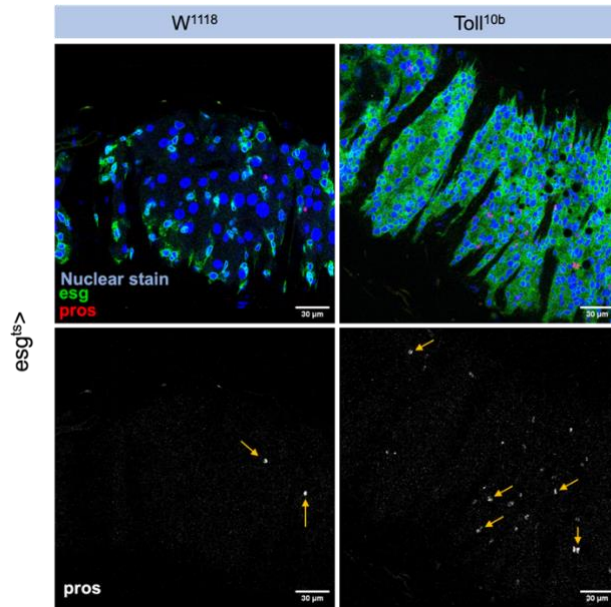


Figure 3.23: Activation of Toll signalling in progenitors influences cell fates in the posterior midgut.

The figure represents a single frame from z-stacks obtained using confocal imaging. The genotypes are control (w^{1118} crossed with esg^{ts}) and esg^{ts} , $UAS-Toll^{10b}$. 2μm thick z-stacks were obtained from the posterior midgut region (R4) of 10d old female flies and a single slice has been represented here. The progenitors (ISCs and Ebs) are GFP+ (green), Ees are stained with prospero antibody (red), and the nucleus is shown in blue (upper panel). The lower panel represents pros+ cells in white. The arrows point toward pros+ cells. Scale bar = 30μm.

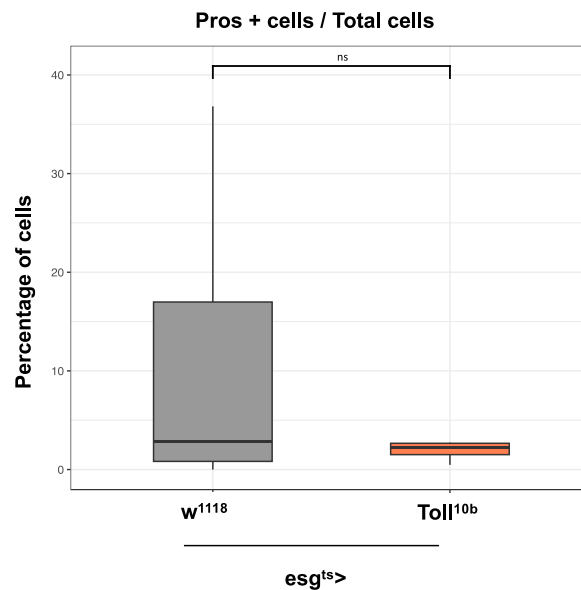


Figure 3.24: Toll activation does not significantly alter the proportion of Ees.

The graph represents percentage of pros+ cells (Ees) in the midgut region of 10d old female control (esg^{ts} crossed with w^{1118}) (n=15) and esg^{ts} , $UAS-Toll^{10b}$ (n=15) flies. This experiment had three biological replicates. The x-axis represents different genotypes. The y-axis represents the percentage of pros+ cells (Ees) in proportion to the total number of cells in a section of the R4 midgut region. The percentages in control and esg^{ts} , $UAS-Toll^{10b}$ were statistically compared using Wilcoxon test (ns = not significant, *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001).

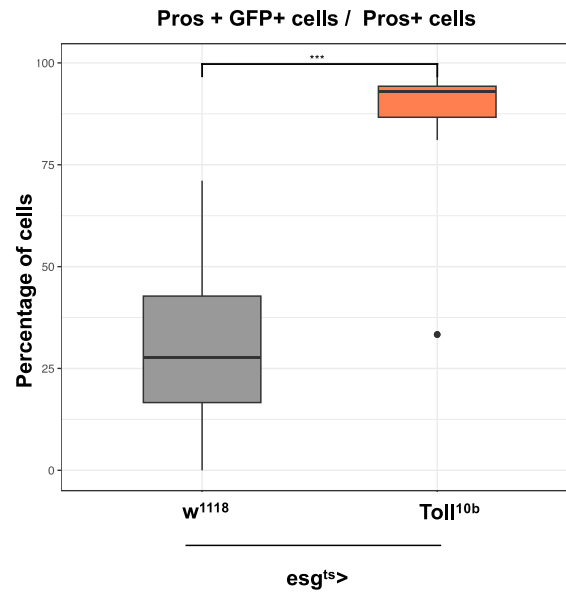


Figure 3.25: Toll activation increases the proportion of Ees that are GFP+ (*esg*+).

The graph represents percentage of *pros*+ cells (EEs) that are also GFP+ in the midgut region of 10d old female control (*esg^{ts}* crossed with *w¹¹¹⁸*) (n=15) and *esg^{ts}, UAS-Toll^{10b}* (n=15) flies. This experiment had three biological replicates. The x-axis represents different genotypes. The y-axis represents the percentage of *pros*+ GFP+ cells in proportion to the total number of *pros*+ cells in a section of the R4 midgut region. The percentages in control and *esg^{ts}, UAS-Toll^{10b}* were statistically compared using Wilcoxon test (ns = not significant, *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001).

3.8 Toll activation in stem cells increases the number of mitotic cells.

All the above results focused on Toll activation in *esg*+ cells, which are ISCs and EBs. We further wanted to determine if Toll activation in stem cells alone was sufficient to induce accelerated stem cell proliferation and dysplasia. To study this, we used *ISC^{ts}* (*esg-GAL4, Su(H)-GAL80^{ts}, UAS-GFP*) flies to drive *UAS-Toll^{10b}* expression. These flies have *Toll^{10b}* expression only in the stem cells. Hence, the stem cells have activated Toll signalling and are also labelled with GFP. One limitation of this driver is that, to repress transgene expression in EBs, the flies are grown at 18°C so the temperature sensitive GAL80 blocks GAL4 activity in EBs and therefore, the transgenes, in this case GFP and *Toll^{10b}* are expressed only in ISCs. The limitation of this driver is the expression of transgenes even during development. It is also the reason why the use of this driver was limited to this experiment. The controls used were the progeny of *ISC^{ts}* flies crossed with *w¹¹¹⁸* genetic background. pH3+ cell counts were measured to quantify mitotic divisions and it was seen that Toll activation in stem cells alone led to a marked increase in pH3+ cells in the whole gut of flies (**Fig 3.27**). However, the increase in pH3+ cells was lower than *esg^{ts}> UAS-Toll^{10b}* fly guts. Also from Fig 3.26, the increase in GFP on Toll activation in stem cells is not close to the GFP+ cell

expansion observed in flies with activated Toll signalling in both ISCs and EBs. This could indicate that Toll activation in both ISCs and EBs contribute to the Toll-mediated dysplasia in the posterior midgut, with Toll activation in EBs potentially contributing more to the dysplasia seen in the gut. Further quantification of the proportion of *esg*⁺ cells is required to validate this. Also, cell-specific drivers for EB to express *Toll*^{10b} in EBs only, would be necessary to support this hypothesis. Further quantification of total number of cells is necessary to conclude that the increase in pH3⁺ cells with activated Toll signalling represents ISC proliferation. Experiments on these lines form the future prospective for this project.

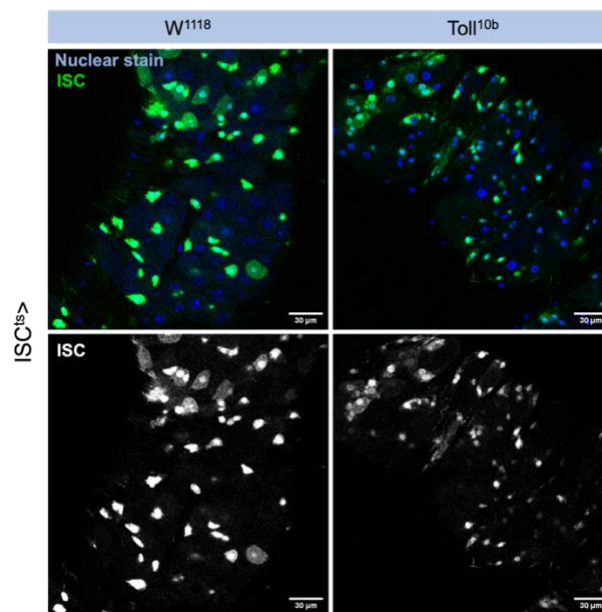


Figure 3.26: Activating Toll pathway only in ISCs does not contribute to dysplasia as seen with Toll activation in *esg*⁺ cells (ISCs and EBs).

The figure represents a single frame from z-stacks obtained using confocal imaging. The genotypes are control (*w*¹¹¹⁸ crossed with *ISC*^{ts}) and *ISC*^{ts}, *UAS-Toll*^{10b}. 2um thick z-stacks were obtained from the posterior midgut region (R4) of 10d old female flies and a single slice has been represented here. ISCs are GFP⁺ (green) and the nucleus is shown in blue (upper panel). The lower panel represents GFP⁺ cells in white. Scale bar = 30um.

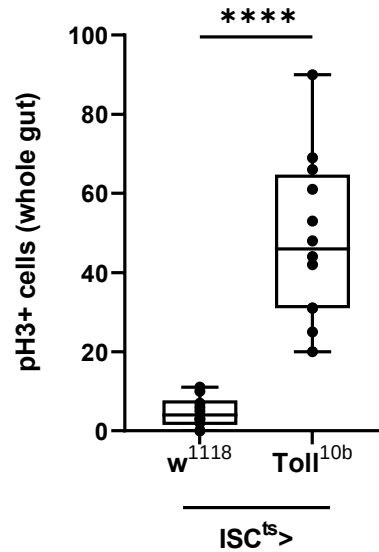


Figure 3.27: Toll activation in stem cells increases the pH3+ cell counts.

The graph represents pH3+ cell counts from whole guts of 10d old female control (*ISC^{ts}* crossed with *w¹¹¹⁸*) (n=10) and *ISC^{ts}, UAS-Toll^{10b}* (n=12) flies. This experiment had three biological replicates. The x-axis represents different genotypes. The y-axis represents the number of pH3+ cells present in the whole gut. The pH3+ counts of control and *ISC^{ts}, UAS-Toll^{10b}* were statistically compared using Mann-Whitney test (ns = not significant, *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001).

3.9 Toll activation in progenitors influence intracellular localization of Dif protein.

Dif is the transcription factor operating downstream of the Toll pathway in *Drosophila*. Signal transduction into the cell after Toll activation terminates with Dif translocating into the nucleus to mediate transcription of genes involved in host defense, like AMPs[58]. To analyse if there was nuclear translocation of Dif protein in *esg^{ts}> UAS-Toll^{10b}* flies, we stained Dif using an antibody in control (*esg^{ts}* crossed with *w¹¹¹⁸*) and *Toll^{10b}* fly guts. **Fig 3.28** is a representative image of Dif staining. It was observed that in control flies, there was higher Dif staining, observed in the cytoplasm of cells. In *Toll^{10b}* flies, the cytoplasmic levels of Dif seemed to deplete and staining of Dif was found closer to the nucleus. The inability to stain Dif inside the nucleus could be a limitation of the antibody to penetrate the nucleus. This result supported our hypothesis that canonical Toll pathway was causing dysplasia in Toll activated midguts. However, as Dif nuclear localization cannot be seen, we used other experiments to determine if the canonical pathway is involved.

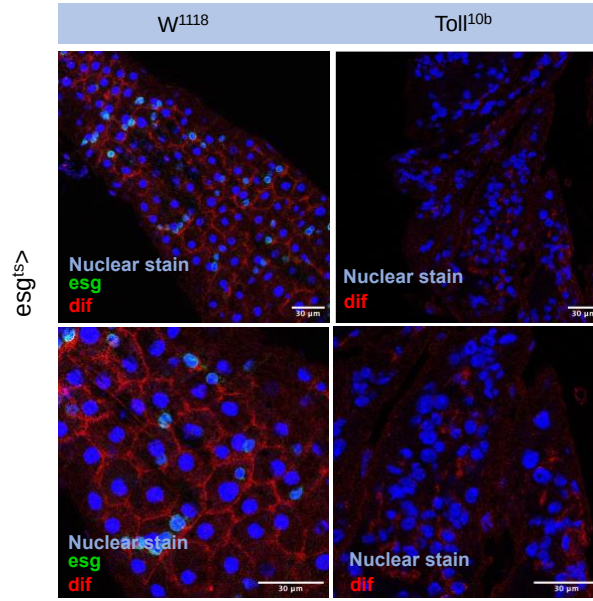


Figure 3.28: Activating Toll pathway influences intracellular localization of Dif protein.

The figure represents a single frame obtained using confocal imaging. The genotypes are control (w^{1118} crossed with esg^{ts}) and esg^{ts} , $UAS-Toll^{10b}$. Images were obtained from the posterior midgut region (R4) of 10d old female flies, and a single image has been represented here. The progenitors (ISCs and EBs) are GFP+ (green) and the nucleus is shown in blue. Dif is marked with red. 63X magnification with no zoom (upper panel). 63X magnification with 3X zoom (lower panel). Scale bar = 30μm.

3.10 Dif is required for $Toll^{10b}$ induced expansion of esg^+ cells.

Dif is the NF-κB factor acting downstream of Toll in *Drosophila*[58] . We wanted to confirm that the $Toll^{10b}$ -induced expansion of esg^+ cells was mediated by the canonical Toll pathway and was not due to an artefact produced by $Toll^{10b}$ expression. For this, it was important to analyse the effect of depleting Dif protein in the cells using Dif^{RNAi} in $Toll^{10b}$ flies. Therefore, esg^{ts} was used to drive expression of both $UAS-Toll^{10b}$ and $UAS-Dif^{RNAi}$. The **Fig 3.29** shows that the number of GFP+ (esg^+) cells on Dif^{RNAi} expression in guts with activated Toll signalling was significantly lower than the guts without Dif^{RNAi} . The levels of total cells were lower in the R4 region and the proportion of GFP+ (esg^+) cells in $Toll^{10b}; Dif^{RNAi}$ guts was comparable to controls (esg^{ts} flies crossed with w^{1118}). The graph (**Fig 3.30**) shows suppression of pH3+ cell counts when Dif^{RNAi} is co-expressed in $Toll^{10b}$ flies. Therefore, our hypothesis that the canonical Toll pathway is responsible for Toll mediated dysplasia was confirmed.

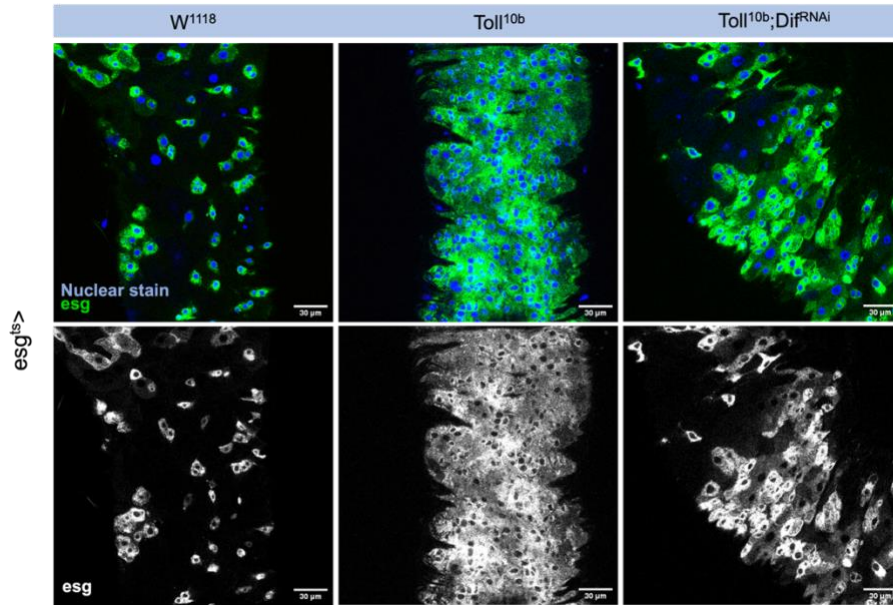


Figure 3.29 : Blocking *dif* suppresses Toll activation-induced *esg*⁺ cell expansion.

The figure represents a single frame from z-stacks obtained using confocal imaging. The genotypes are control (*w*¹¹¹⁸ crossed with *esg*^{ts}) and *esg*^{ts}, *UAS-Toll*^{10b} and *esg*^{ts}, *UAS-Toll*^{10b}; *UAS-Dif*^{RNAi}. 2μm thick z-stacks were obtained from the posterior midgut region (R4) of 10d old female flies and a single slice has been represented here. The progenitors (ISCs and EBs) are GFP⁺ (green) and the nucleus is shown in blue (upper panel). The lower panel represents GFP⁺ cells in white. Scale bar= 30μm.

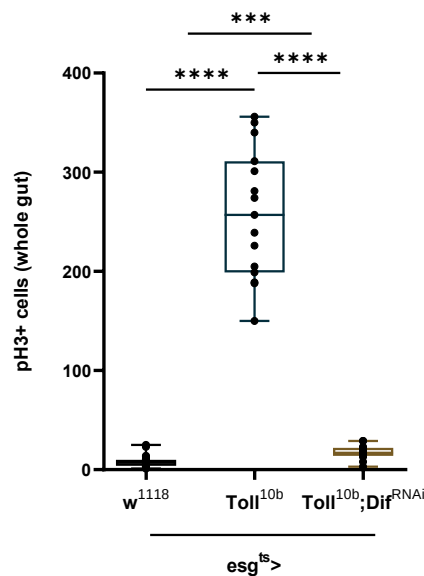


Figure 3.30: Suppressing levels of *dif* reduces stem cell mitosis induced by Toll activation.

The graph represents pH3⁺ cell counts from whole guts of 10d old female control (*esg*^{ts} crossed with *w*¹¹¹⁸) (n=23) and *esg*^{ts}, *UAS-Toll*^{10b} (n=15) and *esg*^{ts}, *UAS-Toll*^{10b}; *UAS-Dif*^{RNAi} (n=15) flies. This experiment had three biological replicates. The x-axis represents different genotypes. The y-axis represents the number of pH3⁺ cells present in the whole gut. The pH3⁺ counts of control and *esg*^{ts}, *UAS-Toll*^{10b} and *esg*^{ts}, *UAS-Toll*^{10b}; *UAS-Dif*^{RNAi} were statistically compared using Mann-Whitney test (ns = not significant, *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001).

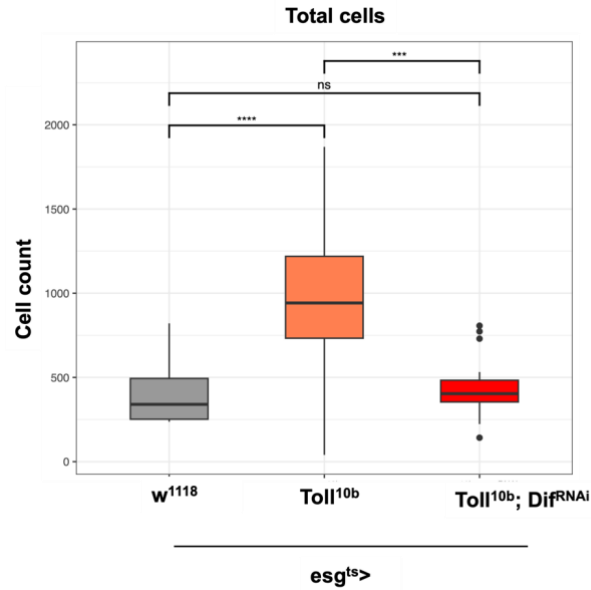


Figure 3.31: Suppressing levels of *dif* reduces the expansion of total cell population in flies with activated Toll signalling.

The graph represents total cells present in a midgut region of 10d old female control (*esg^{ts}* crossed with *w¹¹¹⁸*) (n=17), *esg^{ts}, UAS-Toll^{10b}* (n=19) and *esg^{ts}, UAS-Toll^{10b}; UAS-Dif^{RNAi}* (n=16) flies. This experiment had three replicates. The x-axis represents different genotypes. The y-axis represents total cell counts in a section of the R4 midgut region. The cell counts in control, *esg^{ts}, UAS-Toll^{10b}* and *esg^{ts}, UAS-Toll^{10b}; UAS-Dif^{RNAi}* were statistically compared using Kruskal-Wallis with post hoc rank sum Dunn test (ns = not significant, *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001).

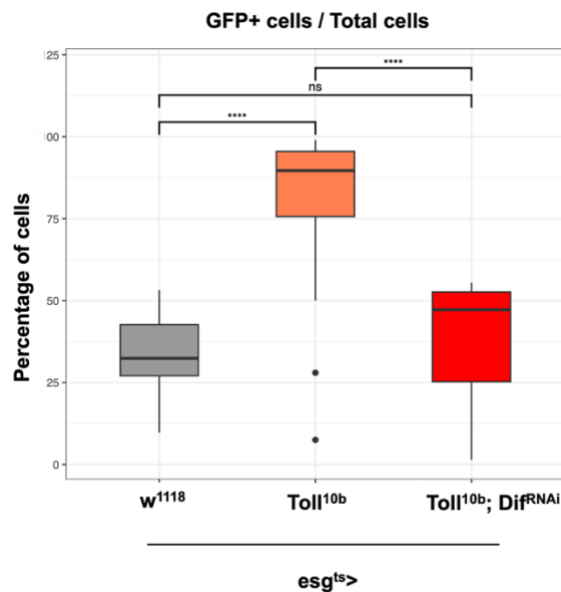


Figure 3.32: Suppressing levels of *dif* reduces the expansion of *esg*⁺ cells in flies with activated Toll signalling.

The graph represents the proportion of GFP+ cells present in a midgut region of 10d old female control (*esg^{ts}* crossed with *w¹¹¹⁸*) (n=17), *esg^{ts}, UAS-Toll^{10b}* (n=19) and *esg^{ts}, UAS-Toll^{10b}; UAS-Dif^{RNAi}* (n=16) flies. This experiment had three replicates. The x-axis represents different genotypes. The y-axis represents the percentage of GFP+ cells in proportion to the total number of cells in a section of the R4 midgut region. The percentages in control, *esg^{ts}, UAS-Toll^{10b}* and *esg^{ts}, UAS-Toll^{10b}; UAS-Dif^{RNAi}* were statistically compared using Kruskal-Wallis with post hoc rank sum Dunn test (ns = not significant, *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001).

3.11 *Cactus*^{RNAi} increases mitotic cells in the gut.

Cactus is the *Drosophila* homologue of I κ -B in mammals. It represses the function of Dif by binding to it and preventing its translocation into the nucleus[58]. We hypothesized that, depleting the levels of Cactus in progenitor cells would result in a phenotype comparable to that observed on *Toll*^{10b} expression in progenitor cells. To analyse this hypothesis, we expressed *cactus*^{RNAi} in *esg*^{ts} flies. *Esg*^{ts} flies crossed with *w*¹¹¹⁸ acted as controls. There was a slight increase in GFP+ cells observed in *cactus*^{RNAi} flies (**Fig 3.33**). However, further quantification of the number of GFP+ cells or total number of cells is necessary to confirm this. We quantified stem cell mitosis rate using pH3 staining and it became clear that the levels of pH3+ cells were increased in *cactus*^{RNAi} guts as compared to its genetic background (**Fig 3.34**). Hence, depleting Cactus induced stem cell divisions in fly guts. The *esg*+ cell counts and pH3 levels were not as high as the *Toll*^{10b} expression phenotype. This is possibly due to the fact that on Toll activation, the entire signalling cascade, beginning from the receptor, the adaptors and kinases, are all activated. Cactus degradation is near the end of the signalling pathway and hence only depleting Cactus does not produce the substantial impact seen on Toll activation. However, depleting Cactus alone also increases the number of pH3+ cells, and this further confirms the role of canonical Toll-Cactus-Dif pathway in inducing dysplasia in the *Drosophila* gut.

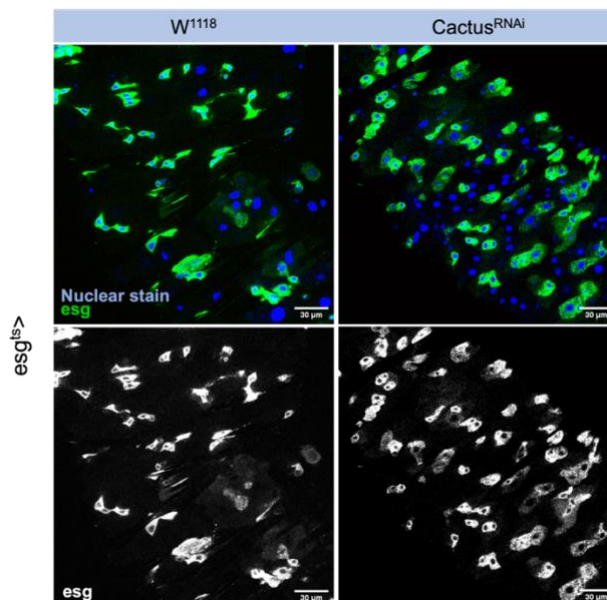


Figure 3.33: *Cactus*^{RNAi} expressed in progenitor cells potentially influences *esg*+ cell counts.

The figure represents a single frame from z-stacks obtained using confocal imaging. The genotypes are control (*w*¹¹¹⁸ crossed with *esg*^{ts}) and *esg*^{ts}; *UAS-Cactus*^{RNAi}. 2μm thick z-stacks were obtained from the posterior midgut region (R4) of 10d old female flies and a single slice has been represented here. The progenitors (ISCs and EBs)

are GFP+ (green) and the nucleus is shown in blue (upper panel). The lower panel represents GFP+ cells in white. Scale bar = 30um.

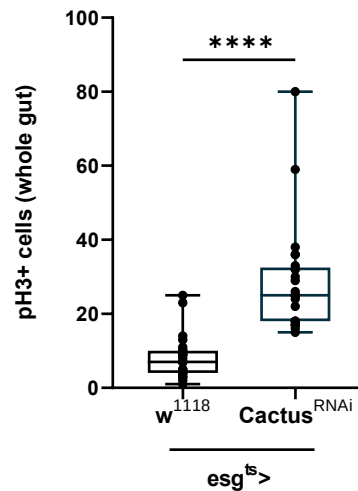


Figure 3.34: Suppressing levels of *cactus* increases stem cell mitosis.

The graph represents pH3+ cell counts from whole guts of 10d old female control (*esg^{ts}* crossed with *w¹¹¹⁸*) (n=23) and *esg^{ts}; UAS-Cactus^{RNAi}* (n=25) flies. This experiment had three replicates. The x-axis represents different genotypes. The y-axis represents the number of pH3+ cells present in the whole gut. The pH3+ counts of control and *esg^{ts}; UAS-Cactus^{RNAi}* were statistically compared using Mann-Whitney test (ns = not significant, *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001).

3.12 Spaetzle expression in progenitors induces stem cell mitosis.

Spaetzle is the Toll ligand in *Drosophila*. It is cleaved by a serine protease (Spaetzle processing enzyme) before binding to Toll receptor, causing the receptor to dimerize, thus activating the signalling pathway[212]. All the previous experiments showed that activating Toll through a constitutively active form of the receptor could generate a response that caused dysplasia. We wanted to see if expressing an active form of Spz by using *UAS-Spz* in progenitors could induce stem cell proliferation and if Spaetzle could potentially be acting as a mitogen to activate immune pathway and mediate cell proliferation. We therefore used *UAS-Spz* under *esg^{ts}* driver line. pH3 staining was done to measure the stem cell mitosis. From the pH3+ cell counts (**Fig 3.35**), there is evidence to say that an active form of Spz in progenitors does increase stem cell mitosis. Experiments to quantify increase in total cell counts are paramount to confirm that the activated Spz in progenitors increases cell proliferation.

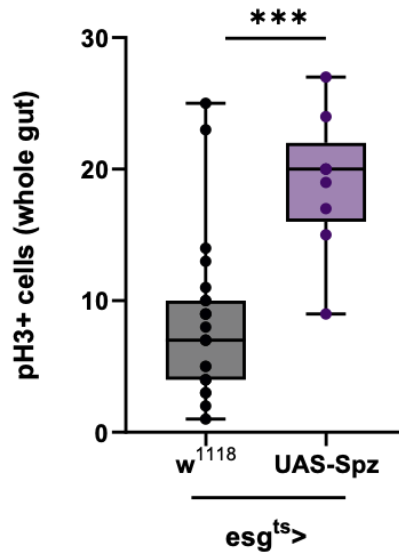


Figure 3.35: Activated form of Spaetzle induces stem cell proliferation.

The graph represents pH3+ cell counts from whole guts of 10d old female control (*esg^{ts}* crossed with *w¹¹¹⁸*) (n=23) and *esg^{ts}; UAS-Spz^{GFP}* (n=9) flies. The x-axis represents different genotypes. This experiment had three replicates. The y-axis represents the number of pH3+ cells present in the whole gut. The pH3+ counts of control and *esg^{ts}; UAS-Spz^{GFP}* were statistically compared using Mann-Whitney test (ns = not significant, *p < 0.05, ***p<0.01, ****p<0.001, *****p<0.0001).

3.13 Increased expression of Puckered on Toll activation highlights the involvement of JNK signalling.

JNK pathway is known to be involved in its cytoprotective role by mediating response against stress in the *Drosophila* gut. In aged guts however, JNK is seen to exacerbate age-induced dysplasia by causing ISC over-proliferation and mis-differentiation[181], [191]. It is also seen to worsen BMP loss-induced tumors[189]. To determine if JNK had a role in the accelerated ISC proliferation observed on activating Toll pathway, we measured puckered (*puc*) expression in cells of the midgut. Puckered is a transcriptional target acting downstream of JNK pathway[177], [178]. Antibody (β -galactosidase) were used to label *puc* (tagged to lacZ) in 10d *esg^{ts}* and *esg^{ts}; Toll^{10b}* midguts. The staining evidently shows an increase in the expression of cells labelled with *puc* antibody in *Toll^{10b}* flies, showing that JNK signalling could be upregulated on Toll signalling activation (**Fig 3.36**). This experiment was performed by Shivohum Bahuguna, Ph.D. student at German Cancer Research Center (DKFZ). This led to the

hypothesis that JNK could potentially be acting downstream of Toll and thereby mediating dysplasia in midguts.

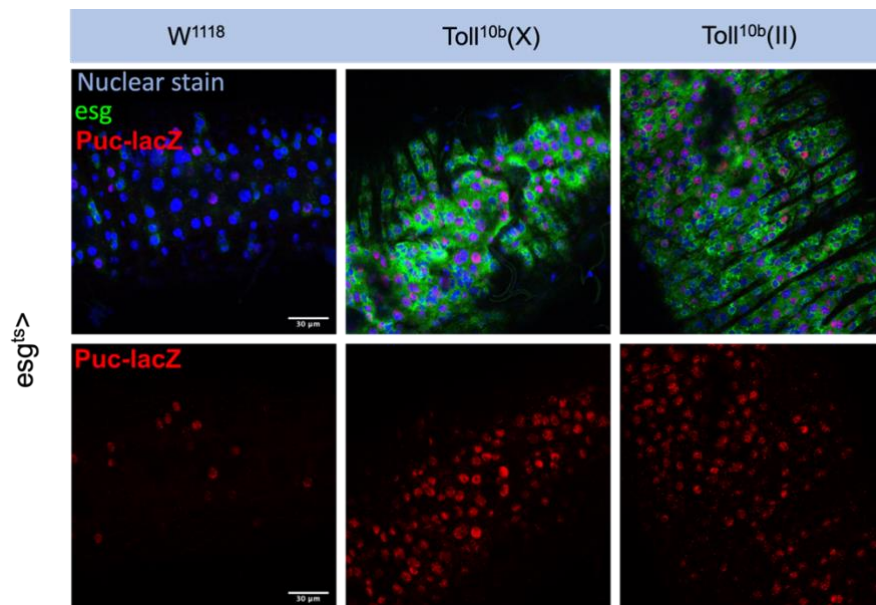


Figure 3.36: Activation of the Toll pathway leads to JNK signalling activation.

The figure represents a single slice from a section of posterior midgut R4 region of 10d old female flies obtained using confocal imaging. The genotypes are control (*esg^{ts}* crossed with *w¹¹¹⁸*), *esg^{ts}; UAS-Toll^{10b}* (*Toll^{10b}* on the X chromosome) and *esg^{ts}; UAS-Toll^{10b}* (*Toll^{10b}* on the 2nd chromosome). The upper panel consists of the progenitors that are GFP+ (green). Puc+ cells also express lacZ which is stained using anti-β-galactosidase antibody (red) and the nucleus is shown in blue. The lower panel is a representative of lacZ+ cells (red). Scale bar = 30μm.

3.14 JNK inhibition reduced Toll-mediated expansion of *esg*+ cells in the gut.

The previous result showed that Toll activation induced JNK signalling and therefore increased levels of transcriptional target of JNK (puckered) in cells. However, we wanted to determine if JNK was necessary for the phenotype observed on Toll activation. We used *Bsk^{DN}* mutants, which is the non-activable mutant form of Bsk (*Drosophila* homologue of JNK) expressed under UAS. The control flies used were *esg^{ts}* and the mutants analysed were *esg^{ts}> UAS-Toll^{10b}* and *esg^{ts}> UAS-Toll^{10b}; UAS-Bsk^{DN}*. The latter had both activation of Toll and loss of JNK signalling in their ISCs and EBs. The images shown (**Fig 3.37**), point evidently to the fact that loss of JNK signalling inhibited the Toll-mediated dysplasia in the midgut. **Fig 3.39** and **3.40**, clearly point out the role of JNK signalling. On blocking JNK, the total cells and the proportion of *esg*+ cells were reduced and were comparable to controls, in flies with activated Toll pathway.

pH3 staining also confirmed that JNK was necessary to mediate Toll-induced stem cell mitosis (**Fig 3.37**). This provided evidence that JNK was acting downstream of Toll signalling in maintaining the epithelial barrier.

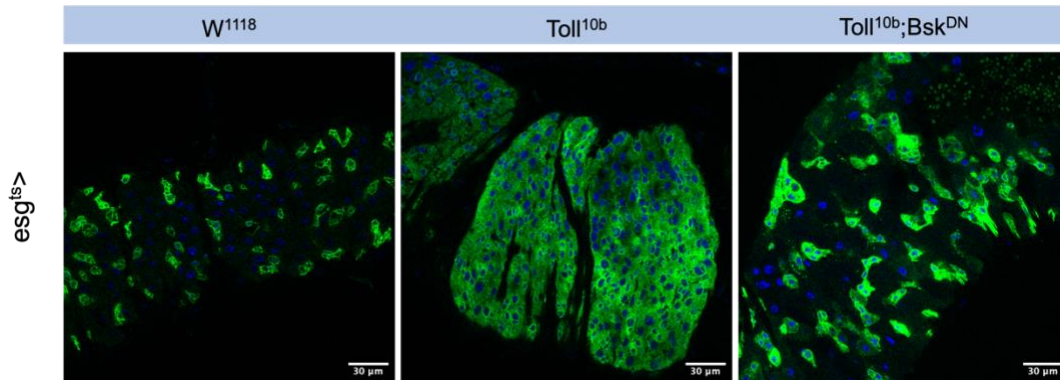


Figure 3.37: Blocking JNK pathway rescues Toll-mediated expansion of GFP+ cell population.

The figure represents a single slice from a section of posterior midgut R4 region of 10d old female flies obtained using confocal imaging. The genotypes are control (*esg^{ts}* crossed with *w¹¹¹⁸*), *esg^{ts}, UAS-Toll^{10b}* and *esg^{ts}, UAS-Toll^{10b}; UAS-Bsk^{DN}*. The progenitors are GFP+ (green) and the nucleus is shown in blue. Scale bar = 30μm.

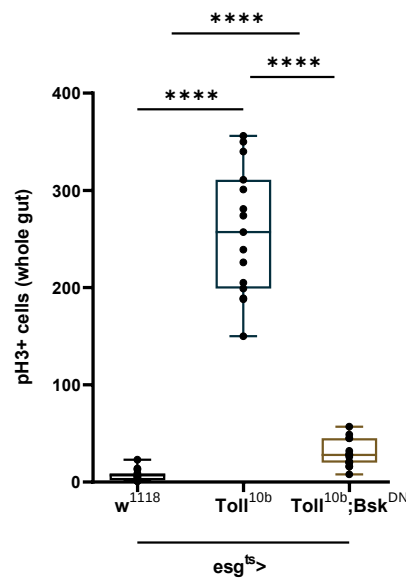


Figure 3.38: Blocking JNK pathway suppresses Toll-mediated increase in stem cell mitosis.

The graph represents pH3+ cell counts from whole guts of 10d old female control (*esg^{ts}* crossed with *w¹¹¹⁸*) (n=15) and *esg^{ts}, UAS-Toll^{10b}* (n=15) and *esg^{ts}, UAS-Toll^{10b}; UAS-Bsk^{DN}* (n=15) flies. This experiment had three replicates. The x-axis represents different genotypes. The y-axis represents the number of pH3+ cells present in the whole gut. The pH3+ counts of control and *esg^{ts}, UAS-Toll^{10b}* and *esg^{ts}, UAS-Toll^{10b}; UAS-Bsk^{DN}* were statistically compared using Mann-Whitney test (ns = not significant, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

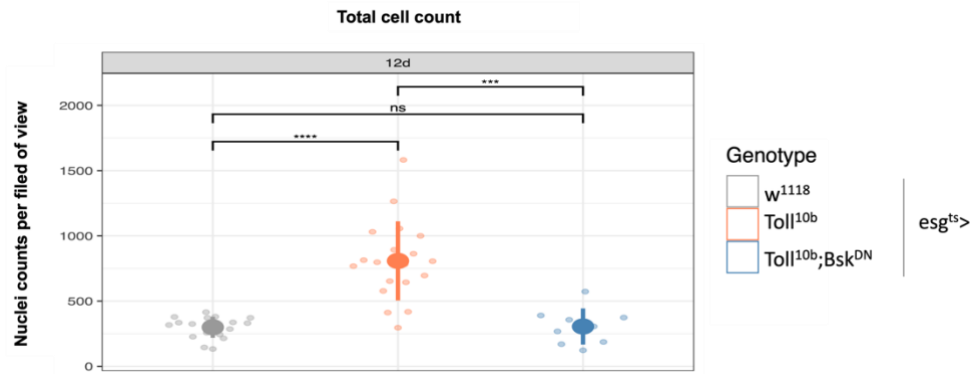


Figure 3.39: Blocking JNK pathway reduces the expansion of total cell population in flies with activated Toll signalling.

The graph represents total number of cells present in a midgut region of 10d old female control (*esg^{ts}* crossed with *w¹¹¹⁸*) (n=18), *esg^{ts}, UAS-Toll^{10b}* (n=19) and *esg^{ts}, UAS-Toll^{10b}; UAS-Bsk^{DN}* (n=9) flies. This experiment had three replicates. The x-axis represents different genotypes. The y-axis represents the nuclei counts per field of view (total number of cells) in a section of the R4 midgut region. The cell counts in control, *esg^{ts}, UAS-Toll^{10b}* and *esg^{ts}, UAS-Toll^{10b}; UAS-Bsk^{DN}* were statistically compared using Dunn's test (ns = not significant, *p < 0.05, ***p<0.01, ****p<0.0001).

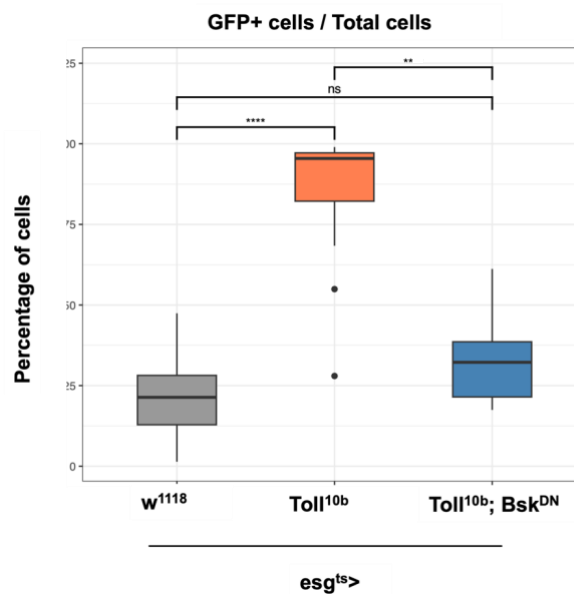


Figure 3.40: Blocking JNK pathway reduces the expansion of *esg*+(GFP+) cells in flies with activated Toll signalling.

The graph represents the proportion of GFP+ cells present in a midgut region of 10d old female control (*esg^{ts}* crossed with *w¹¹¹⁸*) (n=18), *esg^{ts}, UAS-Toll^{10b}* (n=19) and *esg^{ts}, UAS-Toll^{10b}; UAS-Bsk^{DN}* (n=9) flies. This experiment had three replicates. The x-axis represents different genotypes. The y-axis represents the percentage of GFP+ cells in proportion to the total number of cells in a section of the R4 midgut region. The percentages in control, *esg^{ts}, UAS-Toll^{10b}* and *esg^{ts}, UAS-Toll^{10b}; UAS-Bsk^{DN}* were statistically compared using Kruskal-Wallis with post hoc rank sum Dunn test (ns = not significant, *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001).

3.15 JNK signalling does not contribute to epithelial renewal in homeostatic condition.

The previous results depict that JNK was involved in epithelial renewal and stem cell proliferation under Toll activation. To further confirm whether JNK signalling was required even without Toll activation, as in the case of homeostasis, *esg^{ts}* was used to drive *UAS-Bsk^{DN}* expression in progenitors. The results (**Fig 3.41, Fig 3.43, Fig 3.44**) showed that the number of total cells and the proportion of *esg*+ cells appeared similar to that of control (*esg^{ts}* crossed with *w¹¹¹⁸*) midguts. On comparing the pH3+ cells in controls and *Bsk^{DN}* fly guts, there was only a small reduction in pH3+ cell numbers and the difference was not very significant (**Fig 3.42**). This is an indication that JNK signalling is not necessary for maintenance of epithelial turnover in homeostatic conditions.

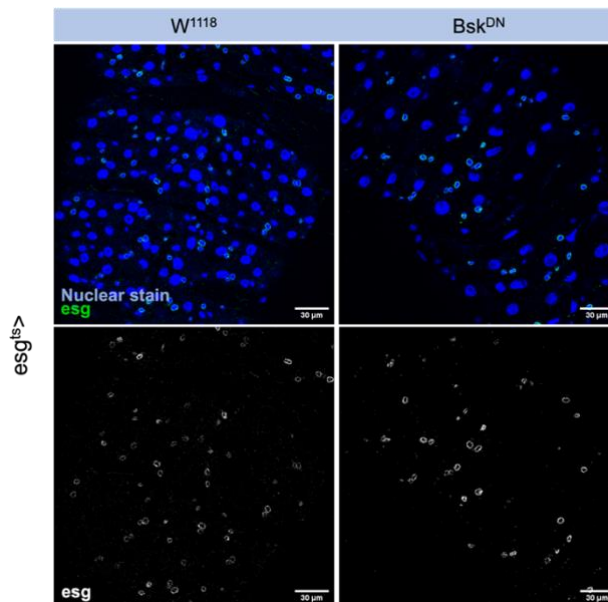


Figure 3.41: Blocking JNK pathway does not influence stem cell proliferation in homeostasis.

The figure represents a single frame from z-stacks obtained using confocal imaging. The genotypes are control (*w¹¹¹⁸* crossed with *esg^{ts}*) and *esg^{ts}; UAS-Bsk^{DN}*. 2μm thick z-stacks were obtained from the posterior midgut region (R4) of 10d old female flies and a single slice has been represented here. The progenitors (ISCs and EBs) are GFP+ (green) and the nucleus is shown in blue (upper panel). The lower panel represents GFP+ cells in white. Scale bar = 30μm.

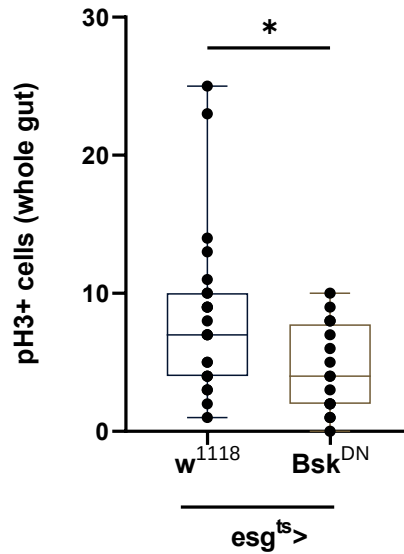


Figure 3.42: Blocking JNK pathway using *Bsk^{DN}* does not block stem cell mitosis.

The graph represents pH3+ cell counts from whole guts of 10d old female control (*esg^{ts}* crossed with *w¹¹¹⁸*) (n=23) and *esg^{ts}; UAS-Bsk^{DN}* (n=16) flies. This experiment had three replicates. The x-axis represents different genotypes. The y-axis represents the number of pH3+ cells present in the whole gut. The pH3+ counts of control and *esg^{ts}; UAS-Bsk^{DN}* were statistically compared using Mann-Whitney test (ns = not significant, *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001).

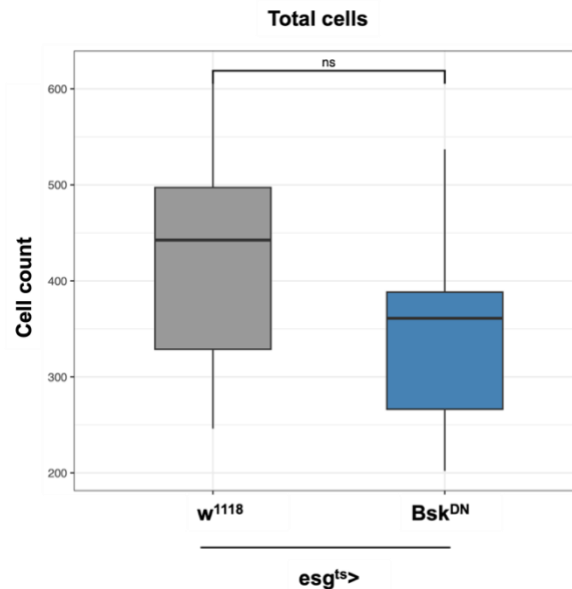


Figure 3.43: Blocking JNK pathway does not influence total cell counts in homeostasis.

The graph represents total number of cells present in a midgut region of 10d old female control (*esg^{ts}* crossed with *w¹¹¹⁸*) (n=10) and *esg^{ts}; UAS-Bsk^{DN}* (n=12) flies. This experiment had three replicates. The x-axis represents different genotypes. The y-axis represents the total number of cells in a section of the R4 midgut region. The cell counts in control and *esg^{ts}; UAS-Bsk^{DN}* were statistically compared using Wilcoxon test (ns = not significant, *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001).

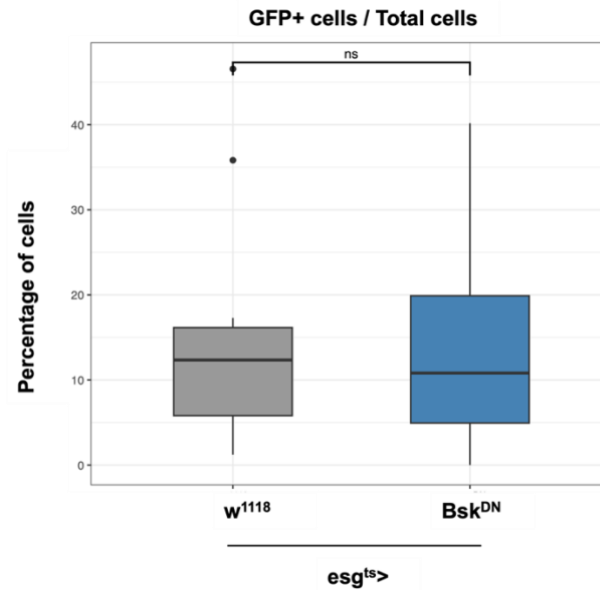


Figure 3.44: Blocking JNK pathway does not influence the number of progenitors in homeostasis.

The graph represents the proportion of GFP+ cells present in a midgut region of 10d old female control (*esg^{ts}* crossed with *w¹¹¹⁸*) (n=10) and *esg^{ts}; UAS-Bsk^{DN}* (n=12) flies. This experiment had three replicates. The x-axis represents different genotypes. The y-axis represents the percentage of GFP+ cells in proportion to the total number of cells in a section of the R4 midgut region. The percentages in control and *esg^{ts}; UAS-Bsk^{DN}* were statistically compared using Wilcoxon test (ns = not significant, *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001).

3.16 Activation of Toll pathway increases cultivable bacterial density in the gut.

Bahuguna *et al.*, had found that Toll pathway was required for maintaining lipid metabolism that aided retention of microbial density and diversity in *Drosophila* gut[195]. We wanted to establish if *Toll^{10b}* could impact microbial density. Further, we wanted to analyse if JNK also had a role in maintaining the bacteriome. We therefore used *esg^{ts}* flies to drive *Toll^{10b}*. In addition to that we also looked at a combination of *UAS-Toll^{10b}; UAS-Bsk^{DN}* to study the interaction of pathways and its impact on bacterial density. We dissected guts from different genotypes and plated them on MRS agar to determine microbial densities. The graph (Fig 3.45) showed that on activation of Toll, there was a significant increase in the cultivable bacterial density. In flies with depletion of Bsk in *Toll^{10b}* flies, there wasn't a significant reduction of microbial density as compared to *Toll^{10b}* fly guts. Therefore blocking JNK is only able to rescue Toll-mediated dysplasia and not the increase in microbial densities. This result was very interesting as activating an immune pathway led to an increase in microbial density in the gut. More research needs to be done in these lines to understand the mechanism behind this phenotype. Future scope of this work is discussed in section 4.5. A limitation of this study is the use of only one kind of agar as not all bacteria in the gut grows on MRS agar.

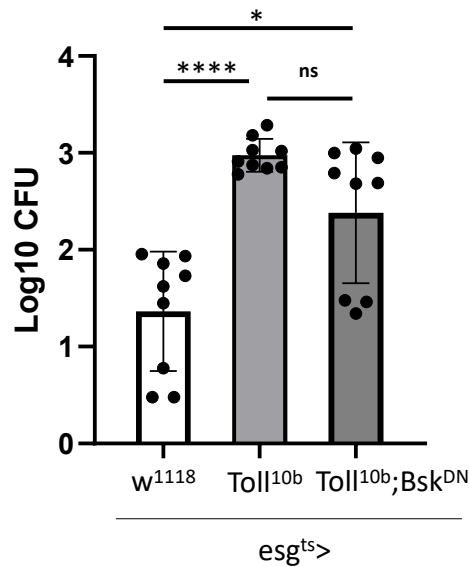


Figure 3.45: Toll activation in progenitors increases cultivable bacterial density in the gut.

The figure represents microbial densities obtained on plating 10d old female fly guts from control (*esg^{ts}* crossed with *w¹¹¹⁸*), *esg^{ts} > UAS-Toll^{10b}* and *esg^{ts} > UAS-Toll^{10b}; UAS-Bsk^{DN}* (n=15 for all genotypes). Five guts per genotype were plated into three plates to form technical replicates, and this was performed thrice to obtain three biological replicates. The x-axis represents different genotypes, and the y-axis represents the log₁₀ CFU for the different genotypes. Mann-Whitney test was used to perform statistical analysis (ns = not significant, *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001).

4 DISCUSSION

The human intestine has high rates of tissue renewal and has always attracted research to comprehend the maintenance of the epithelial and microbial homeostasis. Being a site of constant toxin and pathogenic insult, the epithelial stem cells need to constantly replace the damaged cells with healthy ones. In addition to that, they also need to maintain the proportion of various cells present in the intestines, such as enterocytes, enteroblasts, tuft cells, goblet cells, enteroendocrine, Paneth cells etc. The intestines also contain the highest microbial load in the human body and the gut microbiome exerts a significant impact on the metabolism of complex carbohydrates, thereby aiding absorption. Hence, the intestines also form the site of complex immune signalling homeostasis that tolerates the commensals but is responsive towards pathogens

that enter the tract through food. This high degree of regulation is maintained by constant cross talk between the host and the microbes achieved through metabolites secreted by the host cells and microbes. To understand this multifaceted and complex microenvironment present in the gut, scientists have delved deep into decoding the signalling pathways, immune and metabolic signatures that comprise a healthy epithelial barrier (reviewed in [71]).

As innate immune signalling is of prime importance in recognizing the microbial patterns and orchestrating either anti-inflammatory or pro-inflammatory responses, pathways like TLR, have been extensively researched. TLR signalling is one of the primary innate immune signalling pathways responsible for sensing unique microbial patterns on the microbes. The role of TLR signalling in maintaining the gut microbiome and impacting epithelial renewal is highly sought after. However, the complexity of mammalian microbiome with respect to the variability in diversity and composition of microbes, and the complex immune signalling pathways involved, makes it extremely challenging (reviewed in [73]).

Discovery of constant renewal of the *Drosophila* gut epithelial barrier by stem cells, paved the way to a whole new area of research in understanding stem cell proliferation and maintenance of adult tissue homeostasis. Research on the simpler yet, evolutionarily and functionally conserved mechanisms of *Drosophila* intestinal epithelial and microbial homeostasis, provided a platform to better understand the mammalian gut. The aim is to answer questions related to what developmental pathways could contribute to maintaining ISC proliferation rate in homeostasis and conditions of infection. As *Drosophila* also has a microbiome, it is also a well-suited model to study the role of microbes in determining epithelial turnover rates (reviewed in [132]). As immune signalling bridges the gap between microbes and epithelial cells, it is also important to understand the mechanism by which immune signalling pathways interact with other pathways that maintain epithelial homeostasis. *Drosophila* has two innate immune pathways namely: IMD and Toll. IMD signalling has been researched to understand its role in epithelial renewal[47]. In this study we look at the functions of Toll signalling in maintaining the epithelial barrier.

4.1 Activation of Toll signalling in progenitors results in higher rates of stem cell mitosis combined with EBs getting blocked and/or (mis) differentiating.

The mechanism involved in the epithelial renewal of mammalian and *Drosophila* gut is quite similar[213], [214]. Upon damage induced by stress, infection, or toxins, it is also expected that the epithelial lining would undergo faster stem cell proliferation and epithelial regeneration, to replace the damaged epithelial cells in both models[215][47], [216]. However, the mammalian models give contrasting results with some data suggesting that activation of the TLR4 signalling using LPS suppresses stem cell renewal and epithelial cycling, while other studies indicate that inducing TLR4 signalling also increases stem cell proliferation (reviewed in [73]). These results highlight the variability and complexity of mammalian systems. Toll pathway in *Drosophila* has been known to contribute to responses against infection by upregulating genes that code for antimicrobial peptides that aid in the infection clearance[58], but there has been no research investigating its role in epithelial regeneration.

To investigate if in addition to immune response, activation of Toll signalling could also be linked to an increased stem cell proliferation in the gut, we used RNAi-mediated suppression of Toll signalling in Ecc15 infection models. Infection induced an increase in mitotic ISCs as shown by the increase in the number of pH3+ cells in the whole fly guts as compared to uninfected controls. It was observed that on suppression of Toll signalling using *Toll^{RNAi}* or *Dif^{RNAi}*, the number of mitotic ISCs in the gut was reduced as compared to infected guts with a functional Toll signalling. This result indicated that the Toll signalling, induced by infection could also contribute to an increased stem cell proliferation. Further we wanted to investigate the impact of constant activation of the Toll signalling in midguts that mimicked Toll activation during infection.

We therefore used *UAS-Toll^{10b}* in combination with *esg^{ts}* drivers to activate Toll signalling in progenitors. Toll activation led to an expansion in cell population (total cells) and more than 90% of the cells in the gut were GFP+ (*esg*+) (**Fig 3.4, Fig 3.5**). This was also verified by pH3 staining that confirmed a significant increase in stem cell divisions on Toll activation (**Fig 3.11**). An increase in stem cell mitosis was observed even in young fly guts (1-3d old) (**Fig 3.18**). There was also a marked increase in small nuclei (ISCs, EBs, EEPs, EEs) cell population and a reduction in the proportion of cells with large nuclei (conventional ECs) (**Fig 3.6, Fig 3.7**). Upon analysing the proportions of different cell populations using *DI-lacZ* flies to quantify the ISCs and prospero staining to quantify EEs, we

observed that even though the individual population of these cells increased, their proportions (in comparison to the total number of cells per field of view) remained relatively comparable to control fly guts (**Fig 3.20, Fig 3.24**). Therefore, the large increase in the proportion of cells with small nuclei could be attributed to two mutually exclusive scenarios: the stem cells divide rapidly on Toll activation, and as EBs also have activated Toll, they get blocked at the EB stage and their differentiation is inhibited due to increased Toll signalling. This could also explain the increase in pH3+ cell counts as they could be ISCs dividing at a higher rate to account for the depleting EC population. Another explanation for this phenotype could be that the EBs undergo (mis)differentiation to form a cell type that does not have a nucleus as large as ECs and also expresses GFP. To differentiate between these cell types, it is important to label ECs and EBs using cell-specific markers. In case the guts with activated Toll signalling show an increase in cells labelled with EB marker and are GFP+, this would mean that there was an accumulation of EBs and that EBs are unable to differentiate. However, if majority of cells (GFP+ cells) in the gut get labelled with EC specific markers and are GFP+, this would mean that there are differentiation defects/mis-differentiation of EBs. The possibility that an activated Toll signalling blocks differentiation into ECs was further supported by depleting levels of *dif* (using *Dif^{RNAi}*) in *esg^{ts}* flies. These flies had higher proportions of large nuclei cells and a smaller proportion of cells with a small nucleus (**Fig 3.16, Fig 3.17**). Therefore, blocking Toll signalling, led to the formation of more ECs from EBs and hence depleting Toll is seen to promote differentiation into ECs. Interestingly, *Dif^{RNAi}* flies had slightly higher stem cell mitosis than controls without the RNAi. This could be the ISCs dividing to replenish the pool of EBs that differentiate at a higher rate to form ECs. This signifies the role of Toll signalling in cell fate decisions. However, confirmation of cell identifies as EBs or ECs using markers are fundamental to draw strong conclusions. It is also important to use cell-specific drivers to induce Toll expression in ISCs and EBs separately to verify that the activation of Toll in EBs blocks their differentiation.

4.2 Canonical Toll pathway in ISCs and EBs is involved in promoting activated Toll-induced dysplasia.

We confirmed that the expansion of total cell population and increase in the proportion of *esg*+ cells was achieved through canonical Toll signalling, as we observed that the cytoplasmic levels of Dif found in control flies was depleted in flies with active Toll signalling, which could potentially be due to Dif translocating

into the nucleus (**Fig 3.28**). Additionally, suppression of *dif* expression using *Dif^{RNAi}* in combination with *Toll^{10b}* reduced stem cell proliferation observed in *Toll^{10b}* fly midguts (observed as suppression of pH3+ cell counts) (**Fig 3.30**). The total cell counts and the proportion of *esg*+ cells in the R4 region also reverted to the levels observed in control fly guts on co-expression of *Toll^{10b}* and *Dif^{RNAi}* (**Fig 3.31, Fig 3.32**). These flies had slightly increased levels of pH3+ cells as compared to controls (**Fig 3.30**). This could be attributed to the fact that RNAi mediated depletion only leads to lowered amounts of the protein and does not completely remove the protein in the cells. Therefore, there would still be some molecules of Dif to carry out gene expression downstream to Toll. Parallely, *Cactus^{RNAi}* also increased the number of pH3+ cells, indicating an increase in stem cell mitosis (**Fig 3.34**). The increase in stem cell mitosis on expressing *Cactus^{RNAi}* was not as impactful as *Toll^{10b}* because in the classical Toll pathway, Cactus degradation is almost at the end of the signalling cascade and only depleting Cactus levels therefore does not give the full-fledged effect seen on activating the Toll receptor (using *Toll^{10b}*). On activating Toll receptor, the whole signalling cascade is activated and as it includes various adaptor proteins and kinases, they could have their separate roles in interacting with other molecules and contributing to dysplasia. We explored the phenotype further by using an activated form of Spz (*UAS-Spz^{GFP}*) and expressing it in progenitors. These flies also had higher levels of stem cell mitosis (**Fig 3.35**). Studies show that Spz could potentially be a transcriptional target of the Toll pathway [217] and we hypothesise that Toll activation could potentially also induce Spz expression, that could be acting as a mitogen in further inducing cell proliferation, worsening dysplasia in the gut. Experiments on these lines are necessary to substantiate this hypothesis.

Apart from this, we found that Toll activation in both ISCs and EBs was necessary to induce high rates of stem cell proliferation as observed in *esg^{ts}>UAS-Toll^{10b}* guts. Even though the pH3+ cell counts in *ISC^{ts}> UAS-Toll^{10b}* was much higher than controls, it was not as high as the pH3+ cell counts observed in *esg^{ts}>UAS-Toll^{10b}*. Therefore, it can be concluded that Toll activation in EBs was necessary to induce the dysplasia phenotype and activation of Toll in EBs could also be inducing the stem cells to divide more, due to potential depletion of EC population, further increasing the levels of pH3+ cell counts in flies with activated Toll signalling in *esg*+ cells. Toll activation only in EBs would be necessary to confirm our hypothesis that EBs contribute more to dysplasia.

4.3 Toll interacts with JNK to maintain epithelial barrier.

In *Drosophila*, a complex interaction of pathways like Notch, Wnt, JAK/STAT, epidermal growth factor receptor (EGFR), JNK signalling, etc. are responsible for maintaining the stem cell population and determining cell fate in the gut (reviewed in [218]). In the mammalian system, the stem cell niche is maintained by Notch, Bone Morphogenetic Protein (BMP), Epidermal growth factor (EGF), Wnt, Hippo signalling etc. (reviewed in [219]). The pathways that regulate epithelial renewal is highly conserved across these species, which makes *Drosophila* an ideal model to study the interaction of pathways that regulate epithelial homeostasis. Therefore, we sought to explore which pathways could be responsible for interacting with Toll and aid dysplasia in the gut. In mammals, JNK interacts with Wnt pathway to regulate epithelial homeostasis and tumorigenesis, where JNK activation was seen to increase stem cell proliferation. JNK signalling activation also made mice more susceptible to colon cancer induced by inflammation[220]. However, the exact role of JNK signalling in tumors is ambiguous[188], [220]. JNK has also been implicated in cases of IBS[221]. The mechanism and interaction of JNK with other pathways is not well understood. There is also a potential for JNK-specific inhibitors in treatment of Inflammatory Bowel Disease (IBD)[221]. However, the complexity of mammalian models makes signalling pathways very context dependent and specific. In *Drosophila*, JNK is known to be upregulated in fly guts with age and when exposed to oxidative stress[222]. In the context of development and infections with Gram-positive bacteria, fungi and viruses, Toll is found to activate JNK and mediate cell death through ROS[223]. However, in the context of gut, the role of JNK is quite contrary. It is involved in promoting the proliferation of ISCs and mis-differentiated daughter cells. These mis-differentiated cells expressed esg but were polyploid (characteristic of an EC) and did not express EC-specific markers[224]. In my work, as activation of Toll in progenitors also leads to accumulation of cells, that had a slightly larger nuclei size as compared to ISC/EB but also were GFP+ (esg+), therefore we explored the interaction of JNK and Toll pathways.

In fly guts with activated Toll signalling, we found an increased level of transcription target of JNK (Puc) (**Fig 3.36**). Further, when we expressed Bsk^{DN} (a non-activable form of JNK) in progenitors with activated Toll signalling, we observed that blocking JNK in Toll activated fly guts, rescued the dysplasia phenotype, and brought down the levels of total cell numbers, GFP+ (esg+) cell proportions and pH3+ cells count to the levels of controls (**Fig 3.38- Fig 3.40**). As blocking JNK rescued *Toll*^{10b} phenotype, it clearly implied that JNK acted

downstream of Toll in inducing stem cell mitosis and the expansion of *esg*⁺ cell numbers. On measuring cultivable bacterial colonies in the gut of control and Toll-activated flies, it was found that the increase in bacterial density on Toll activation could further activate Toll and worsen the condition of the gut. It is also unclear as to how activating an immune pathway could lead to a substantial increase in bacterial densities observed in *Toll*^{10b} flies. There definitely could be metabolic aspects involved, as Toll is involved in lipid metabolism and microbial retention in the gut (as Bahuguna *et al.*, showed in ECs[195]). However, we are yet to find if this increase in bacterial density leading to dysbiosis is responsible for inducing dysplasia or if Toll-mediated dysplasia contributes the shift in gut bacterial densities.

We then analysed if basal levels (without Toll activation) of JNK signalling was necessary for epithelial renewal and for this we used *esg*^{ts}>*UAS-Bsk*^{DN} flies and compared them to controls (*esg*^{ts} crossed with *w*¹¹¹⁸). We found that basal levels of JNK was not necessary to maintain stem cell proliferation as the total number of cells in the gut, pH3⁺ cell counts and *esg*⁺ cell proportions remained comparable to controls. This indicates that in conditions of Toll activation, as that on infection, JNK aids Toll-mediated stem cell renewal by functioning downstream to Toll but does not play a role in maintaining the epithelium during homeostasis.

4.4 Conclusions

Taken together, the results from this project strongly support the hypothesis that Toll signalling is involved in regulating epithelial renewal. Activated Toll signalling increases stem cell mitosis. It also results in EBs being blocked and/or (mis) differentiating. Toll-induced stem cell mitosis and dysplasia occurs through canonical Toll pathway. In uninfected conditions, Toll pathway is not involved in stem cell mitosis but is involved in cell-fate decisions. Further, Toll signalling increases cultivable bacterial densities. This increase in bacterial load could either be the result of dysplasia or be the causative agent for it. Thus, the more gut bacteria, the more induction of ISCs. Exploring this result in axenic flies would be one of the future directions of the work. JNK pathway acts downstream to Toll in mediating Toll-induced dysplasia in the R4 region of the posterior midgut. However, basal levels of JNK signalling in non-infected conditions, is not necessary for the maintenance of epithelial turnover.

4.5 Future directions

4.5.1 Does Toll pathway interact with Notch to cause dysplasia in the activated form?

As Notch is the central pathway for stem cell divisions and is highly conserved across large evolutionary distances[159], we hypothesize that Notch could be involved in Toll-mediated dysplasia. High Notch activity is known for the following:

- 1- Suppression of ISC proliferation[225].
- 2- Blocking EB to EE differentiation[225].
- 3- Daughters of ISCs with high DI protein receive strong N signal and therefore differentiate to form ECs[143].

In our dysplasia phenotype observed on Toll activation, we observe the opposite effects. There is higher ISC proliferation and there is a constant proportion of EEs as compared to controls (**Fig 3.23**). Also, the proportion of large nuclei cells (ECs) is lower on Toll activation (**Fig 3.6**). These are all indicative of low N signalling phenotype. Therefore, we believed Notch and Toll pathways had opposing effects.

Depletion of Notch in the *Drosophila* gut has been known to induce tumors rich in EEs[160], [226]. To study the interaction of Notch pathway with Toll, Shivohum Bahuguna, former master's student in the lab, used depletion of Dif in *Notch^{RNAi}* - induced tumor fly guts. *Dif^{RNAi}* caused rescue of tumor phenotype (**Fig 4.1**). We therefore believe that Notch could be either upstream to Toll or acting parallel to it, but the pathways act in opposing manner. This means that activating Toll could lead to inhibition of Notch pathway that leads to dysplasia or Toll-mediated dysplasia could influence Notch signalling and inhibit its activity, and this worsens the epithelial homeostasis. Experiments to further validate the interaction of Toll and Notch signalling is necessary. This could be done using *UAS-Notch^{intra}* (UAS regulatory sequences that drives the expression of the intracellular domain of N[227]), to see if expression of N can reverse dysplasia caused by Toll activation.

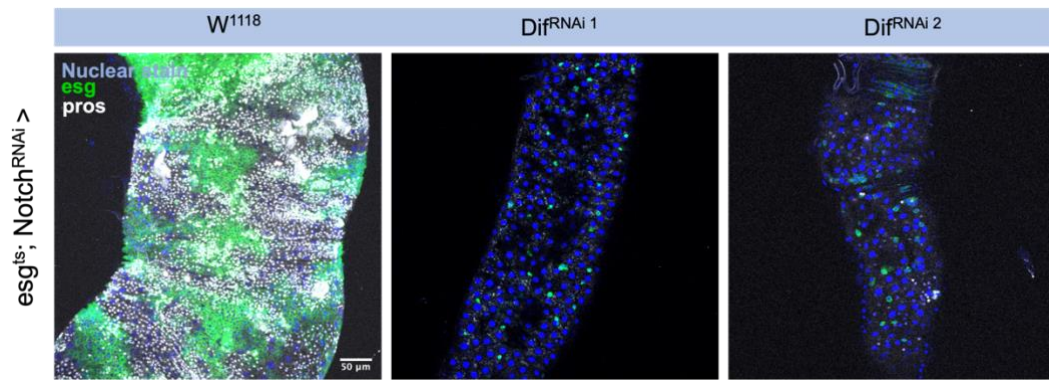


Figure 4.1: Blocking Dif rescues *Notch*^{RNAi} induced tumors.

The figure represents a single section of posterior midgut R4 region of 10d old female flies obtained using confocal imaging. The genotypes are *esg*^{ts}; *UAS-Notch*^{RNAi}, *esg*^{ts}; *UAS-Notch*^{RNAi}, *UAS-Dif*^{RNAi 1} and *esg*^{ts}; *UAS-Notch*^{RNAi}, *UAS-Dif*^{RNAi 2}. The progenitors (ISCs and EBs) are GFP+ (green), EEs are stained with prospero (white), and the nucleus is shown in blue. Scale bar = 50μm. (Experiment performed by Shivohum Bahuguna, German Cancer Research Center (DKFZ)).

The following representation sums up the work done in this project (**Fig 4.2**).

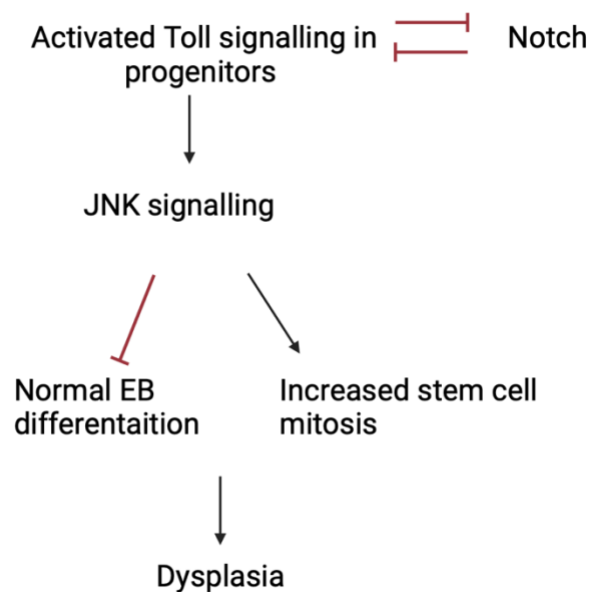


Figure 4.2: Diagrammatic representation of the main findings from the project.

The figure represents our research model. Toll activation in progenitors increased stem cell proliferation and blocked the normal differentiation of EBs, causing its accumulation. JNK was found to be downstream of Toll in inducing dysplasia. Notch has potential antagonistic role either upstream or parallel to Toll pathway. Figure created using BioRender.

4.5.2 Does Spaetzle act as a mitogen?

In this project we saw that expressing a processed form of Spaetzle that can directly bind to Toll in progenitor cells, led to an increase in pH3⁺ cells in the gut. We believe that Spz could be acting as a mitogen. Studies show that Spz could potentially be a transcriptional target of the Toll pathway [217] we assume that Toll activation could potentially also induce Spz expression, that could be acting as a mitogen in further inducing stem cell proliferation, hence worsening dysplasia in the gut. To analyse this, we would have to use *UAS-Spz^{RNAi}* expression in progenitors with activated Toll to see if the condition of dysplasia gets reversed on Spz depletion.

4.5.3 Mechanism of JNK and Toll interaction

In this project, we found that JNK acted downstream to Toll pathway in inducing stem cell proliferation and expansion of esg⁺ cells. To explore the process further, we can study at which point JNK and Toll interact. For this, we plan on using RNAi for *eiger* or *wengen*, which is the ligand and receptor for JNK pathway and observe if RNAi for these molecules could rescue *Toll^{10b}* phenotype. This would help understand if Toll-induced stress activates JNK or whether the pathways have a more direct interaction.

4.5.4 What role does the bacteriome play in dysplasia caused by Toll activation?

We observed that on activating Toll pathway, the cultivable bacterial density increased. This is counterintuitive as Toll activation increases AMP synthesis and secretion. We hypothesise that this increase in chronic AMP secretion favors some bacteria and extinguishes others. The former should be the ones that represent the bacterial load increase. Research shows that changes in microbiota in the gut, as in the case of dysbiosis, impacts the epithelial homeostasis. Therefore exploring whether Toll activation-mediated increase in bacterial density has a role to play in causing dysplasia, needs to be studied. One way of analysing this would be to use germ-free flies with activated Toll signalling to determine whether Toll is able to induce dysplasia even in the absence of gut bacteriome.

5 APPENDIX

5.1 Fly food recipe

Reagent	Amount (mass/vol)
Maize Flour (Brian Drewitt)	1.8kg
Malt Extract (Brian Drewitt)	1.8kg
Molasses (Rayners, Brian Drewitt)	500g
Soya Flour (Brian Drewitt)	216g
Yeast Powder (Brian Drewitt)	366g
Agar (Fisher Scientific, BPE2641-1)	169g
Methyl 4-hydroxybenzoate (Nipagin, Sigma-Aldrich W271004)	74g
Ethanol (VWR)	703ml
95% propionic acid (Sigma-Aldrich P5561), 5% phosphoric acid (Sigma-Aldrich P5811) solution	140ml
Water	28L

Table 5.1: Composition of the conventional fly food used in this project.

5.2 De Man, Rogosa and Sharpe (MRS) media composition

Reagent	Amount (gm/litre)
Peptone	10g
'Lab-Lemco' powder	8g
Yeast extract	4g
Glucose	20g
Sorbitan mono-oleate	1ml
Dipotassium hydrogen phosphate	2g
Sodium acetate 3H ₂ O	5g
Triammonium citrate	2g
Magnesium sulphate 7H ₂ O	0.2g
Manganese sulphate 4H ₂ O	0.05g
Agar	10g
pH 6.2 ± 0.2 @ 25°C	

Table 5.2: Composition of de Man, Rogosa and Sharpe (MRS) agar for analyzing the cultivable gut microbial load of flies.

Bibliography

- [1] B. Lemaitre, E. Nicolas, L. Michaut, J. M. Reichhart, and J. A. Hoffmann, "The dorsoventral regulatory gene cassette spatzle/Toll/Cactus controls the potent antifungal response in *Drosophila* adults," *Cell*, vol. 86, no. 6, 1996, doi: 10.1016/S0092-8674(00)80172-5.
- [2] A. Poltorak *et al.*, "Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: Mutations in Tlr4 gene," *Science* (1979), vol. 282, no. 5396, 1998, doi: 10.1126/science.282.5396.2085.
- [3] R. Medzhitov and C. Janeway, "Innate immune recognition: mechanisms and pathways," *Immunol Rev*, vol. 173, p. 89–97, Feb. 2000, doi: 10.1034/j.1600-065x.2000.917309.x.
- [4] K. Takeda and S. Akira, "Toll-Like Receptors," *Curr Protoc Immunol*, vol. 109, no. 1, pp. 14.12.1-14.12.10, 2015, doi: <https://doi.org/10.1002/0471142735.im1412s109>.
- [5] A. S. Sameer and S. Nissar, "Toll-Like Receptors (TLRs): Structure, Functions, Signaling, and Role of Their Polymorphisms in Colorectal Cancer Susceptibility," *Biomed Res Int*, vol. 2021, p. 1157023, 2021, doi: 10.1155/2021/1157023.
- [6] A. Iwasaki and R. Medzhitov, "Regulation of Adaptive Immunity by the Innate Immune System," *Science* (1979), vol. 327, no. 5963, pp. 291–295, Jan. 2010, doi: 10.1126/science.1183021.
- [7] K. A. Zarembek and P. J. Godowski, "Tissue Expression of Human Toll-Like Receptors and Differential Regulation of Toll-Like Receptor mRNAs in Leukocytes in Response to Microbes, Their Products, and Cytokines," *The Journal of Immunology*, vol. 168, no. 2, pp. 554–561, Jan. 2002, doi: 10.4049/jimmunol.168.2.554.
- [8] K. Miyake, "Innate immune sensing of pathogens and danger signals by cell surface Toll-like receptors," *Semin Immunol*, vol. 19, no. 1, pp. 3–10, 2007, doi: <https://doi.org/10.1016/j.smim.2006.12.002>.
- [9] S. Akira, S. Uematsu, and O. Takeuchi, "Pathogen Recognition and Innate Immunity," *Cell*, vol. 124, no. 4, pp. 783–801, Feb. 2006, doi: 10.1016/j.cell.2006.02.015.
- [10] C. A. Leifer and A. E. Medvedev, "Molecular mechanisms of regulation of Toll-like receptor signaling," *J Leukoc Biol*, vol. 100, no. 5, pp. 927–941, Nov. 2016, doi: 10.1189/jlb.2MR0316-117RR.
- [11] R. McClure and P. Massari, "TLR-dependent human mucosal epithelial cell responses to microbial pathogens," *Frontiers in Immunology*, vol. 5, no. AUG. 2014, doi: 10.3389/fimmu.2014.00386.
- [12] A. Semlali, C. Witoled, M. Alanazi, and M. Rouabhia, "Whole Cigarette Smoke Increased the Expression of TLRs, HBDs, and Proinflammatory Cytokines by Human Gingival Epithelial Cells through Different Signaling Pathways," *PLoS One*, vol. 7, no. 12, 2012, doi: 10.1371/journal.pone.0052614.
- [13] A. Beklen, M. Hukkanen, R. Richardson, and Y. T. Konttinen, "Immunohistochemical localization of Toll-like receptors 1-10 in periodontitis," *Oral Microbiol Immunol*, vol. 23, no. 5, 2008, doi: 10.1111/j.1399-302X.2008.00448.x.
- [14] Q. Lu, R. P. Darveau, L. P. Samaranayake, C. Y. Wang, and L. Jin, "Differential modulation of human β -defensins expression in human gingival epithelia by *Porphyromonas gingivalis* lipopolysaccharide with tetra- And penta-acylated lipid A structures," *Innate Immun*, vol. 15, no. 6, 2009, doi: 10.1177/1753425909104899.
- [15] J. Liu, J. Chen, X. Du, L. Hu, and L. Chen, "The expression of hBDs in the gingival tissue and keratinocytes from healthy subjects and periodontitis patients," *Arch Oral Biol*, vol. 59, no. 2, 2014, doi: 10.1016/j.archoralbio.2013.11.007.
- [16] I. Ioannidis, F. Ye, B. McNally, M. Willette, and E. Flaño, "Toll-Like Receptor Expression and Induction of Type I and Type III Interferons in Primary Airway Epithelial Cells," *J Virol*, vol. 87, no. 6, 2013, doi: 10.1128/jvi.01956-12.
- [17] Q. Sha, A. Q. Truong-Tran, J. R. Plitt, L. A. Beck, and R. P. Schleimer, "Activation of airway epithelial cells by toll-like receptor agonists," *Am J Respir Cell Mol Biol*, vol. 31, no. 3, 2004, doi: 10.1165/rcmb.2003-0388OC.
- [18] L. Armstrong *et al.*, "Expression of functional toll-like receptor-2 and -4 on alveolar epithelial cells," *Am J Respir Cell Mol Biol*, vol. 31, no. 2 I, 2004, doi: 10.1165/rcmb.2004-0078OC.
- [19] V. Regueiro, D. Moranta, M. A. Campos, J. Margareto, J. Garmendia, and J. A. Bengoechea, "Klebsiella pneumoniae increases the levels of toll-like receptors 2 and 4 in human airway epithelial cells," *Infect Immun*, vol. 77, no. 2, 2009, doi: 10.1128/IAI.00852-08.
- [20] K. Ueno *et al.*, "MUC1 mucin is a negative regulator of toll-like receptor signaling," *Am J Respir Cell Mol Biol*, vol. 38, no. 3, 2008, doi: 10.1165/rcmb.2007-0336RC.

- [21] M. V. Bautista, Y. Chen, V. S. Ivanova, M. K. Rahimi, A. M. Watson, and M. C. Rose, "IL-8 Regulates Mucin Gene Expression at the Posttranscriptional Level in Lung Epithelial Cells," *The Journal of Immunology*, vol. 183, no. 3, 2009, doi: 10.4049/jimmunol.0803022.
- [22] X. Wang, Z. Zhang, J. P. Louboutin, C. Moser, D. J. Weiner, and J. M. Wilson, "Airway epithelia regulate expression of human beta-defensin 2 through Toll-like receptor 2.," *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, vol. 17, no. 12, 2003, doi: 10.1096/fj.02-0616fje.
- [23] C. A. Wu, J. J. Peluso, L. Zhu, E. G. Lingenheld, S. T. Walker, and L. Puddington, "Bronchial epithelial cells produce IL-5: Implications for local immune responses in the airways," *Cell Immunol*, vol. 264, no. 1, 2010, doi: 10.1016/j.cellimm.2010.04.008.
- [24] A. S. Vamadevan, M. Fukata, E. T. Arnold, L. S. Thomas, D. Hsu, and M. T. Abreu, "Regulation of Toll-like receptor 4-associated MD-2 in intestinal epithelial cells: A comprehensive analysis," *Innate Immun*, vol. 16, no. 2, 2010, doi: 10.1177/1753425909339231.
- [25] T. Eaves-Pyles *et al.*, "Luminal-applied flagellin is internalized by polarized intestinal epithelial cells and elicits immune responses via the TLR5 dependent mechanism," *PLoS One*, vol. 6, no. 9, 2011, doi: 10.1371/journal.pone.0024869.
- [26] S. Akira and K. Takeda, "Toll-like receptor signalling," *Nat Rev Immunol*, vol. 4, no. 7, pp. 499–511, 2004, doi: 10.1038/nri1391.
- [27] J. L. Slack *et al.*, "Identification of two major sites in the type I interleukin-1 receptor cytoplasmic region responsible for coupling to pro-inflammatory signaling pathways," *Journal of Biological Chemistry*, vol. 275, no. 7, 2000, doi: 10.1074/jbc.275.7.4670.
- [28] A. Dunne and L. A. J. O'Neill, "The interleukin-1 receptor/Toll-like receptor superfamily: signal transduction during inflammation and host defense.," *Science's STKE : signal transduction knowledge environment*, vol. 2003, no. 171, 2003, doi: 10.1126/stke.2003.171.re3.
- [29] K. Takeda, T. Kaisho, and S. Akira, "Toll-like receptors," *Annual Review of Immunology*, *Annu Rev Immunol*, vol. 21, 2003.
- [30] M. Karin and Y. Ben-Neriah, "Phosphorylation meets ubiquitination: The control of NF- κ B activity," *Annual Review of Immunology*, vol. 18, 2000, doi: 10.1146/annurev.immunol.18.1.621.
- [31] E. Meylan *et al.*, "RIP1 is an essential mediator of Toll-like receptor 3-induced NF- κ B activation," *Nat Immunol*, vol. 5, no. 5, pp. 503–507, 2004, doi: 10.1038/ni1061.
- [32] S. Sharma, B. Tenover, N. Grandvaux, G.-P. Zhou, R. Lin, and J. Hiscott, "Triggering the Interferon Antiviral Response Through an IKK-Related Pathway," *Science*, vol. 300, pp. 1148–1151, Jun. 2003, doi: 10.1126/science.1081315.
- [33] A. K. Perry, E. K. Chow, J. B. Goodnough, W.-C. Yeh, and G. Cheng, "Differential Requirement for TANK-binding Kinase-1 in Type I Interferon Responses to Toll-like Receptor Activation and Viral Infection," *Journal of Experimental Medicine*, vol. 199, no. 12, pp. 1651–1658, Jun. 2004, doi: 10.1084/jem.20040528.
- [34] G. Oganessian *et al.*, "Critical role of TRAF3 in the Toll-like receptor-dependent and -independent antiviral response," *Nature*, vol. 439, no. 7073, pp. 208–211, 2006, doi: 10.1038/nature04374.
- [35] G. Sellge and T. A. Kufer, "PRR-signaling pathways: Learning from microbial tactics," *Semin Immunol*, vol. 27, no. 2, pp. 75–84, 2015, doi: <https://doi.org/10.1016/j.smim.2015.03.009>.
- [36] Y. Nouri, R. Weinkove, and R. Perret, "T-cell intrinsic Toll-like receptor signaling: Implications for cancer immunotherapy and CAR T-cells," *Journal for ImmunoTherapy of Cancer*, vol. 9, no. 11, 2021, doi: 10.1136/jitc-2021-003065.
- [37] P. Bergman, S. Seyedoleslami Esfahani, and Y. Engström, "Chapter Two - Drosophila as a Model for Human Diseases—Focus on Innate Immunity in Barrier Epithelia," in *Current Topics in Developmental Biology*, vol. 121, L. Pick, Ed., Academic Press, 2017, pp. 29–81. doi: <https://doi.org/10.1016/bs.ctdb.2016.07.002>.
- [38] C. J. Evans, V. Hartenstein, and U. Banerjee, "Thicker than blood: Conserved mechanisms in Drosophila and vertebrate hematopoiesis," *Developmental Cell*, vol. 5, no. 5, 2003, doi: 10.1016/S1534-5807(03)00335-6.
- [39] U. Tepass, L. I. Fessler, A. Aziz, and V. Hartenstein, "Embryonic origin of hemocytes and their relationship to cell death in Drosophila," *Development*, vol. 120, no. 7, 1994, doi: 10.1242/dev.120.7.1829.
- [40] R. Lanot, D. Zachary, F. Holder, and M. Meister, "Postembryonic hematopoiesis in Drosophila," *Dev Biol*, vol. 230, no. 2, 2001, doi: 10.1006/dbio.2000.0123.

- [41] M. A. Hanson and B. Lemaitre, "New insights on *Drosophila* antimicrobial peptide function in host defense and beyond," *Current Opinion in Immunology*, vol. 62, 2020. doi: 10.1016/j.coi.2019.11.008.
- [42] S. Stöven, I. Ando, L. Kadalayil, Y. Engström, and D. Hultmark, "Activation of the *Drosophila* NF- κ B factor Relish by rapid endoproteolytic cleavage," *EMBO Rep*, vol. 1, no. 4, pp. 347–352, Oct. 2000, doi: <https://doi.org/10.1093/embo-reports/kvd072>.
- [43] M.-L. Wiklund, S. Steinert, A. Junell, D. Hultmark, and S. Stöven, "The N-terminal half of the *Drosophila* Rel/NF- κ B factor Relish, REL-68, constitutively activates transcription of specific Relish target genes," *Dev Comp Immunol*, vol. 33, no. 5, pp. 690–696, 2009, doi: <https://doi.org/10.1016/j.dci.2008.12.002>.
- [44] T. Kim and Y. J. Kim, "Overview of innate immunity in *Drosophila*," *Journal of Biochemistry and Molecular Biology*, vol. 38, no. 2, 2005. doi: 10.5483/bmbrep.2005.38.2.121.
- [45] H. Myllymäki, S. Valanne, and M. Rämet, "The *Drosophila* Imd Signaling Pathway," *The Journal of Immunology*, vol. 192, no. 8, 2014, doi: 10.4049/jimmunol.1303309.
- [46] T. Kuraishi, A. Hori, and S. Kurata, "Host-microbe interactions in the gut of *Drosophila melanogaster*," *Frontiers in Physiology*, vol. 4 DEC. 2013. doi: 10.3389/fphys.2013.00375.
- [47] N. Buchon, N. A. Broderick, M. Poidevin, S. Pradervand, and B. Lemaitre, "Drosophila Intestinal Response to Bacterial Infection: Activation of Host Defense and Stem Cell Proliferation," *Cell Host Microbe*, vol. 5, no. 2, pp. 200–211, Feb. 2009, doi: 10.1016/j.chom.2009.01.003.
- [48] D. Ferrandon, J.-L. Imler, and J. A. Hoffmann, "Sensing infection in *Drosophila*: Toll and beyond," *Semin Immunol*, vol. 16, no. 1, pp. 43–53, 2004, doi: <https://doi.org/10.1016/j.smim.2003.10.008>.
- [49] I.-H. Jang *et al.*, "A Spätzle-Processing Enzyme Required for Toll Signaling Activation in *Drosophila* Innate Immunity," *Dev Cell*, vol. 10, no. 1, pp. 45–55, 2006, doi: <https://doi.org/10.1016/j.devcel.2005.11.013>.
- [50] A. N. R. Weber, M. Gangloff, M. C. Moncrieffe, Y. Hyvert, J. L. Imler, and N. J. Gay, "Role of the spätzle pro-domain in the generation of an active toll receptor ligand," *Journal of Biological Chemistry*, vol. 282, no. 18, 2007, doi: 10.1074/jbc.M700068200.
- [51] C. J. Arnot, N. J. Gay, and M. Gangloff, "Molecular mechanism that induces activation of Spätzle, the ligand for the *Drosophila* toll receptor," *Journal of Biological Chemistry*, vol. 285, no. 25, 2010, doi: 10.1074/jbc.M109.098186.
- [52] A. N. R. Weber, M. C. Moncrieffe, M. Gangloff, J. L. Imler, and N. J. Gay, "Ligand-receptor and receptor-receptor interactions act in concert to activate signaling in the *Drosophila* toll pathway," *Journal of Biological Chemistry*, vol. 280, no. 24, 2005, doi: 10.1074/jbc.M502074200.
- [53] M. Gangloff *et al.*, "Structural insight into the mechanism of activation of the toll receptor by the dimeric ligand Spätzle," *Journal of Biological Chemistry*, vol. 283, no. 21, 2008, doi: 10.1074/jbc.M800112200.
- [54] T. Horng and R. Medzhitov, "Drosophila MyD88 is an adapter in the Toll signaling pathway," *Proc Natl Acad Sci U S A*, vol. 98, no. 22, 2001, doi: 10.1073/pnas.231471798.
- [55] T. Xiao, P. Towb, S. A. Wasserman, and S. R. Sprang, "Three-dimensional structure of a complex between the death domains of pelle and tube," *Cell*, vol. 99, no. 5, 1999, doi: 10.1016/S0092-8674(00)81542-1.
- [56] M. C. Moncrieffe, J. G. Grossmann, and N. J. Gay, "Assembly of oligomeric death domain complexes during toll receptor signaling," *Journal of Biological Chemistry*, vol. 283, no. 48, 2008, doi: 10.1074/jbc.M805427200.
- [57] H. Sun, B. N. Bristow, G. Qu, and S. A. Wasserman, "A heterotrimeric death domain complex in toll signaling," *Proc Natl Acad Sci U S A*, vol. 99, no. 20, 2002, doi: 10.1073/pnas.202396399.
- [58] L. P. Wu and K. V. Anderson, "Regulated nuclear import of Rel proteins in the *Drosophila* immune response," *Nature*, vol. 392, no. 6671, 1998, doi: 10.1038/32195.
- [59] B. A. Stokes, S. Yadav, U. Shokal, L. C. Smith, and I. Eleftherianos, "Bacterial and fungal pattern recognition receptors in homologous innate signaling pathways of insects and mammals," *Front Microbiol*, vol. 6, no. JAN, 2015, doi: 10.3389/fmicb.2015.00019.
- [60] M. Farrugia and B. Baron, "The Role of Toll-Like Receptors in Autoimmune Diseases through Failure of the Self-Recognition Mechanism," *Int J Inflamm*, vol. 2017, p. 8391230, 2017, doi: 10.1155/2017/8391230.
- [61] Y. Zhang, R. Thompson, H. Zhang, and H. Xu, "APP processing in Alzheimer's disease," *Mol Brain*, vol. 4, no. 1, p. 3, 2011, doi: 10.1186/1756-6606-4-3.
- [62] Z. Sepehri, Z. Kiani, A. A. Nasiri, and F. Kohan, "Toll-like receptor 2 and type 2 diabetes," *Cell Mol Biol Lett*, vol. 21, no. 1, p. 2, 2016, doi: 10.1186/s11658-016-0002-4.
- [63] M. Y. Donath and S. E. Shoelson, "Type 2 diabetes as an inflammatory disease," *Nat Rev Immunol*, vol. 11, no. 2, pp. 98–107, 2011, doi: 10.1038/nri2925.

- [64] N. Javaid and S. Choi, "Toll-like Receptors from the Perspective of Cancer Treatment," *Cancers (Basel)*, vol. 12, no. 2, 2020, doi: 10.3390/cancers12020297.
- [65] S. R. El-Zayat, H. Sibaii, and F. A. Mannaa, "Toll-like receptors activation, signaling, and targeting: an overview," *Bull Natl Res Cent*, vol. 43, no. 1, p. 187, 2019, doi: 10.1186/s42269-019-0227-2.
- [66] T. Duan, Y. Du, C. Xing, H. Y. Wang, and R.-F. Wang, "Toll-Like Receptor Signaling and Its Role in Cell-Mediated Immunity," *Front Immunol*, vol. 13, 2022, doi: 10.3389/fimmu.2022.812774.
- [67] X. He, Z. Jing, and G. Cheng, "MicroRNAs: New Regulators of Toll-Like Receptor Signalling Pathways," *Biomed Res Int*, vol. 2014, p. 945169, 2014, doi: 10.1155/2014/945169.
- [68] N. Shen, D. Liang, Y. Tang, N. de Vries, and P.-P. Tak, "MicroRNAs—novel regulators of systemic lupus erythematosus pathogenesis," *Nat Rev Rheumatol*, vol. 8, no. 12, pp. 701–709, 2012, doi: 10.1038/nrrheum.2012.142.
- [69] R. Sender, S. Fuchs, and R. Milo, "Revised Estimates for the Number of Human and Bacteria Cells in the Body," *PLoS Biol*, vol. 14, no. 8, 2016, doi: 10.1371/journal.pbio.1002533.
- [70] B. Greenwood-Van Meerveld, A. C. Johnson, and D. Grundy, "Gastrointestinal physiology and function," *Handb Exp Pharmacol*, vol. 239, 2017, doi: 10.1007/164_2016_118.
- [71] J. M. Allaire, S. M. Crowley, H. T. Law, S. Y. Chang, H. J. Ko, and B. A. Vallance, "The Intestinal Epithelium: Central Coordinator of Mucosal Immunity," *Trends in Immunology*, vol. 39, no. 9, 2018, doi: 10.1016/j.it.2018.04.002.
- [72] M. G. Rooks and W. S. Garrett, "Gut microbiota, metabolites and host immunity," *Nat Rev Immunol*, vol. 16, no. 6, pp. 341–352, 2016, doi: 10.1038/nri.2016.42.
- [73] J. F. Burgueño and M. T. Abreu, "Epithelial Toll-like receptors and their role in gut homeostasis and disease," *Nat Rev Gastroenterol Hepatol*, vol. 17, no. 5, pp. 263–278, 2020, doi: 10.1038/s41575-019-0261-4.
- [74] T. Clavel *et al.*, "Intestinal microbiota in metabolic diseases," *Gut Microbes*, vol. 5, no. 4, pp. 544–551, Jul. 2014, doi: 10.4161/gmic.29331.
- [75] M. Rajilić-Stojanović *et al.*, "Development and application of the human intestinal tract chip, a phylogenetic microarray: analysis of universally conserved phylotypes in the abundant microbiota of young and elderly adults," *Environ Microbiol*, vol. 11, no. 7, pp. 1736–1751, Jul. 2009, doi: <https://doi.org/10.1111/j.1462-2920.2009.01900.x>.
- [76] S. Lamichhane, P. Sen, A. M. Dickens, M. Orešić, and H. C. Bertram, "Gut metabolome meets microbiome: A methodological perspective to understand the relationship between host and microbe," *Methods*, vol. 149, pp. 3–12, 2018, doi: <https://doi.org/10.1016/j.ymeth.2018.04.029>.
- [77] M. A. R. Vinolo, H. G. Rodrigues, E. Hatanaka, F. T. Sato, S. C. Sampaio, and R. Curi, "Suppressive effect of short-chain fatty acids on production of proinflammatory mediators by neutrophils," *J Nutr Biochem*, vol. 22, no. 9, pp. 849–855, 2011, doi: <https://doi.org/10.1016/j.jnutbio.2010.07.009>.
- [78] M. Levy, E. Blacher, and E. Elinav, "Microbiome, metabolites and host immunity," *Curr Opin Microbiol*, vol. 35, pp. 8–15, 2017, doi: <https://doi.org/10.1016/j.mib.2016.10.003>.
- [79] N. Ottman, H. Smidt, W. de Vos, and C. Belzer, "The function of our microbiota: who is out there and what do they do?," *Front Cell Infect Microbiol*, vol. 2, 2012, [Online]. Available: <https://www.frontiersin.org/articles/10.3389/fcimb.2012.00104>
- [80] J. Ni, G. D. Wu, L. Albenberg, and V. T. Tomov, "Gut microbiota and IBD: causation or correlation?," *Nat Rev Gastroenterol Hepatol*, vol. 14, no. 10, pp. 573–584, 2017, doi: 10.1038/nrgastro.2017.88.
- [81] E. A. Alhina, G. E. Walton, and D. M. Commane, "The Role of the Gut Microbiota in Colorectal Cancer Causation," *Int J Mol Sci*, vol. 20, no. 21, 2019, doi: 10.3390/ijms20215295.
- [82] P.-X. Wang, X.-R. Deng, C.-H. Zhang, and H.-J. Yuan, "Gut microbiota and metabolic syndrome," *Chin Med J (Engl)*, vol. 133, no. 7, 2020, [Online]. Available: https://journals.lww.com/cmj/Fulltext/2020/04050/Gut_microbiota_and_metabolic_syndrome.9.aspx
- [83] M. A. Conlon and A. R. Bird, "The Impact of Diet and Lifestyle on Gut Microbiota and Human Health," *Nutrients*, vol. 7, no. 1, pp. 17–44, 2015, doi: 10.3390/nu7010017.
- [84] A. Agus, K. Clément, and H. Sokol, "Gut microbiota-derived metabolites as central regulators in metabolic disorders," *Gut*, vol. 70, no. 6, pp. 1174–1182, 2021, doi: 10.1136/gutjnl-2020-323071.
- [85] V. Caputi and M. C. Giron, "Microbiome-Gut-Brain Axis and Toll-Like Receptors in Parkinson's Disease," *Int J Mol Sci*, vol. 19, no. 6, 2018, doi: 10.3390/ijms19061689.
- [86] R. Ding *et al.*, "Revisit gut microbiota and its impact on human health and disease," *J Food Drug Anal*, vol. 27, no. 3, pp. 623–631, 2019, doi: <https://doi.org/10.1016/j.jfda.2018.12.012>.
- [87] A.-M. Baker *et al.*, "Quantification of Crypt and Stem Cell Evolution in the Normal and Neoplastic Human Colon," *Cell Rep*, vol. 8, no. 4, pp. 940–947, Aug. 2014, doi: 10.1016/j.celrep.2014.07.019.

- [88] H. Clevers, "The Intestinal Crypt, A Prototype Stem Cell Compartment," *Cell*, vol. 154, no. 2, pp. 274–284, Jul. 2013, doi: 10.1016/j.cell.2013.07.004.
- [89] J. R. De Mey and J.-N. Freund, "Understanding epithelial homeostasis in the intestine," *Tissue Barriers*, vol. 1, no. 2, 2013, doi: 10.4161/tisb.24965.
- [90] J. Chang, R. W. Leong, V. C. Wasinger, M. Ip, M. Yang, and T. G. Phan, "Impaired Intestinal Permeability Contributes to Ongoing Bowel Symptoms in Patients With Inflammatory Bowel Disease and Mucosal Healing," *Gastroenterology*, vol. 153, no. 3, pp. 723–731.e1, Sep. 2017, doi: 10.1053/j.gastro.2017.05.056.
- [91] M. A. Odenwald and J. R. Turner, "The intestinal epithelial barrier: a therapeutic target?," *Nat Rev Gastroenterol Hepatol*, vol. 14, no. 1, pp. 9–21, 2017, doi: 10.1038/nrgastro.2016.169.
- [92] T. K. Noah, B. Donahue, and N. F. Shroyer, "Intestinal development and differentiation," *Exp Cell Res*, vol. 317, no. 19, pp. 2702–2710, 2011, doi: <https://doi.org/10.1016/j.yexcr.2011.09.006>.
- [93] Y. S. Kim and S. B. Ho, "Intestinal Goblet Cells and Mucins in Health and Disease: Recent Insights and Progress," *Curr Gastroenterol Rep*, vol. 12, no. 5, pp. 319–330, 2010, doi: 10.1007/s11894-010-0131-2.
- [94] C. L. Bevins and N. H. Salzman, "Paneth cells, antimicrobial peptides and maintenance of intestinal homeostasis," *Nat Rev Microbiol*, vol. 9, no. 5, pp. 356–368, 2011, doi: 10.1038/nrmicro2546.
- [95] H. C. Clevers and C. L. Bevins, "Paneth Cells: Maestros of the Small Intestinal Crypts," *Annu Rev Physiol*, vol. 75, no. 1, pp. 289–311, Feb. 2013, doi: 10.1146/annurev-physiol-030212-183744.
- [96] T. Sato *et al.*, "Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts," *Nature*, vol. 469, no. 7330, pp. 415–418, 2011, doi: 10.1038/nature09637.
- [97] H. M. Cox, "Neuroendocrine peptide mechanisms controlling intestinal epithelial function," *Curr Opin Pharmacol*, vol. 31, pp. 50–56, 2016, doi: <https://doi.org/10.1016/j.coph.2016.08.010>.
- [98] G. RINDI, A. B. LEITER, A. S. KOPIN, C. BORDI, and E. SOLCIA, "The 'Normal' Endocrine Cell of the Gut: Changing Concepts and New Evidences," *Ann N Y Acad Sci*, vol. 1014, no. 1, pp. 1–12, Apr. 2004, doi: <https://doi.org/10.1196/annals.1294.001>.
- [99] N. A. Mabbott, D. S. Donaldson, H. Ohno, I. R. Williams, and A. Mahajan, "Microfold (M) cells: important immunosurveillance posts in the intestinal epithelium," *Mucosal Immunol*, vol. 6, no. 4, pp. 666–677, Jul. 2013, doi: 10.1038/mi.2013.30.
- [100] B. D. Simons and H. Clevers, "Stem cell self-renewal in intestinal crypt," *Exp Cell Res*, vol. 317, no. 19, pp. 2719–2724, 2011, doi: <https://doi.org/10.1016/j.yexcr.2011.07.010>.
- [101] N. Takeda, R. Jain, M. R. LeBoeuf, Q. Wang, M. M. Lu, and J. A. Epstein, "Interconversion Between Intestinal Stem Cell Populations in Distinct Niches," *Science (1979)*, vol. 334, no. 6061, pp. 1420–1424, Dec. 2011, doi: 10.1126/science.1213214.
- [102] Z. Kabiri *et al.*, "Stroma provides an intestinal stem cell niche in the absence of epithelial Wnts," *Development*, vol. 141, no. 11, pp. 2206–2215, Jun. 2014, doi: 10.1242/dev.104976.
- [103] P. Ma, P. Fang, T. Ren, L. Fang, and S. Xiao, "Porcine Intestinal Organoids: Overview of the State of the Art," *Viruses*, vol. 14, no. 5, 2022, doi: 10.3390/v14051110.
- [104] D. H. Reikvam *et al.*, "Depletion of Murine Intestinal Microbiota: Effects on Gut Mucosa and Epithelial Gene Expression," *PLoS One*, vol. 6, no. 3, pp. e17996–, Mar. 2011, [Online]. Available: <https://doi.org/10.1371/journal.pone.0017996>
- [105] F. Sommer and F. Bäckhed, "The gut microbiota — masters of host development and physiology," *Nat Rev Microbiol*, vol. 11, no. 4, pp. 227–238, 2013, doi: 10.1038/nrmicro2974.
- [106] A. H. Broquet, Y. Hirata, C. S. McAllister, and M. F. Kagnoff, "RIG-I/MDA5/MAVS are required to signal a protective IFN response in rotavirus-infected intestinal epithelium," *J Immunol*, vol. 186, no. 3, pp. 1618–1626, 2011, doi: 10.4049/jimmunol.1002862.
- [107] M. T. Abreu, "Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function," *Nat Rev Immunol*, vol. 10, no. 2, pp. 131–144, 2010, doi: 10.1038/nri2707.
- [108] G. Y. Chen and G. Núñez, "Inflammasomes in Intestinal Inflammation and Cancer," *Gastroenterology*, vol. 141, no. 6, pp. 1986–1999, Dec. 2011, doi: 10.1053/j.gastro.2011.10.002.
- [109] L. W. Peterson and D. Artis, "Intestinal epithelial cells: regulators of barrier function and immune homeostasis," *Nat Rev Immunol*, vol. 14, no. 3, pp. 141–153, 2014, doi: 10.1038/nri3608.
- [110] W. A. R. li, K. Sakamoto, and C. A. Leifer, "TLR9 is important for protection against intestinal damage and for intestinal repair," *Sci Rep*, vol. 2, 2012, doi: 10.1038/srep00574.
- [111] K. Kamdar *et al.*, "Innate Recognition of the Microbiota by TLR1 Promotes Epithelial Homeostasis and Prevents Chronic Inflammation," *The Journal of Immunology*, vol. 201, no. 1, 2018, doi: 10.4049/jimmunol.1701216.

- [112] S. Rakoff-Nahoum, J. Paglino, F. Eslami-Varzaneh, S. Edberg, and R. Medzhitov, "Recognition of Commensal Microflora by Toll-Like Receptors Is Required for Intestinal Homeostasis," *Cell*, vol. 118, no. 2, pp. 229–241, Jul. 2004, doi: 10.1016/j.cell.2004.07.002.
- [113] T. Naito *et al.*, "Lipopolysaccharide from crypt-specific core microbiota modulates the colonic epithelial proliferation-to-differentiation balance," *mBio*, vol. 8, no. 5, 2017, doi: 10.1128/mBio.01680-17.
- [114] M. D. Neal *et al.*, "Toll-like receptor 4 is expressed on intestinal stem cells and regulates their proliferation and apoptosis via the p53 up-regulated modulator of apoptosis," *Journal of Biological Chemistry*, vol. 287, no. 44, 2012, doi: 10.1074/jbc.M112.375881.
- [115] R. Santaolalla *et al.*, "TLR4 Activates the β -catenin Pathway to Cause Intestinal Neoplasia," *PLoS One*, vol. 8, no. 5, 2013, doi: 10.1371/journal.pone.0063298.
- [116] C. P. Sodhi *et al.*, "Intestinal epithelial toll-like receptor 4 regulates goblet cell development and is required for necrotizing enterocolitis in mice," *Gastroenterology*, vol. 143, no. 3, 2012, doi: 10.1053/j.gastro.2012.05.053.
- [117] Q. Yang, N. A. Bermingham, M. J. Finegold, and H. Y. Zoghbi, "Requirement of Math1 for secretory cell lineage commitment in the mouse intestine," *Science (1979)*, vol. 294, no. 5549, 2001, doi: 10.1126/science.1065718.
- [118] S. Fre, M. Huyghe, P. Mourikis, S. Robine, D. Louvard, and S. Artavanis-Tsakonas, "Notch signals control the fate of immature progenitor cells in the intestine," *Nature*, vol. 435, no. 7044, 2005, doi: 10.1038/nature03589.
- [119] R. Dheer *et al.*, "Intestinal epithelial Toll-like receptor 4 signaling affects epithelial function and colonic microbiota and promotes a risk for transmissible colitis," *Infect Immun*, vol. 84, no. 3, 2016, doi: 10.1128/IAI.01374-15.
- [120] J. V. Troll *et al.*, "Microbiota promote secretory cell determination in the intestinal epithelium by modulating host Notch signaling," *Development*, vol. 145, no. 4, 2018, doi: 10.1242/dev.155317.
- [121] E. Cario, G. Gerken, and D. K. Podolsky, "Toll-Like Receptor 2 Controls Mucosal Inflammation by Regulating Epithelial Barrier Function," *Gastroenterology*, vol. 132, no. 4, 2007, doi: 10.1053/j.gastro.2007.02.056.
- [122] D. L. Gibson, C. Ma, K. S. B. Bergstrom, J. T. Huang, C. Man, and B. A. Vallance, "MyD88 signalling plays a critical role in host defence by controlling pathogen burden and promoting epithelial cell homeostasis during *Citrobacter rodentium*-induced colitis," *Cell Microbiol*, vol. 10, no. 3, 2008, doi: 10.1111/j.1462-5822.2007.01071.x.
- [123] A. L. Frantz *et al.*, "Targeted deletion of MyD88 in intestinal epithelial cells results in compromised antibacterial immunity associated with downregulation of polymeric immunoglobulin receptor, mucin-2, and antibacterial peptides," *Mucosal Immunol*, vol. 5, no. 5, 2012, doi: 10.1038/mi.2012.23.
- [124] M. Nighot *et al.*, "Lipopolysaccharide-Induced Increase in Intestinal Epithelial Tight Permeability Is Mediated by Toll-Like Receptor 4/Myeloid Differentiation Primary Response 88 (MyD88) Activation of Myosin Light Chain Kinase Expression," *American Journal of Pathology*, vol. 187, no. 12, 2017, doi: 10.1016/j.ajpath.2017.08.005.
- [125] S. Guo, R. Al-Sadi, H. M. Said, and T. Y. Ma, "Lipopolysaccharide causes an increase in intestinal tight junction permeability in vitro and in vivo by inducing enterocyte membrane expression and localization of TLR-4 and CD14," *American Journal of Pathology*, vol. 182, no. 2, 2013, doi: 10.1016/j.ajpath.2012.10.014.
- [126] S. M. Chabot, M. Shawi, T. Eaves-Pyles, and M. R. Neutra, "Effects of flagellin on the functions of follicle-associated epithelium," *Journal of Infectious Diseases*, vol. 198, no. 6, 2008, doi: 10.1086/591056.
- [127] S. Chabot, J. S. Wagner, S. Farrant, and M. R. Neutra, "TLRs Regulate the Gatekeeping Functions of the Intestinal Follicle-Associated Epithelium," *The Journal of Immunology*, vol. 176, no. 7, 2006, doi: 10.4049/jimmunol.176.7.4275.
- [128] M. Rimoldi, M. Chieppa, P. Larghi, M. Vulcano, P. Allavena, and M. Rescigno, "Monocyte-derived dendritic cells activated by bacteria or by bacteria-stimulated epithelial cells are functionally different," *Blood*, vol. 106, no. 8, 2005, doi: 10.1182/blood-2004-11-4321.
- [129] M. Rimoldi *et al.*, "Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells," *Nat Immunol*, vol. 6, no. 5, 2005, doi: 10.1038/ni1192.
- [130] B. He *et al.*, "Intestinal Bacteria Trigger T Cell-Independent Immunoglobulin A2 Class Switching by Inducing Epithelial-Cell Secretion of the Cytokine APRIL," *Immunity*, vol. 26, no. 6, 2007, doi: 10.1016/j.immuni.2007.04.014.

- [131] L. T. Reiter, L. Potocki, S. Chien, M. Gribskov, and E. Bier, "A systematic analysis of human disease-associated gene sequences in *Drosophila melanogaster*," *Genome Res*, vol. 11, no. 6, 2001, doi: 10.1101/gr.169101.
- [132] F. Capo, A. Wilson, and F. Di Cara, "The intestine of *Drosophila melanogaster*: An emerging versatile model system to study intestinal epithelial homeostasis and host-microbial interactions in humans," *Microorganisms*, vol. 7, no. 9. 2019. doi: 10.3390/microorganisms7090336.
- [133] I. Miguel-Aliaga, H. Jasper, and B. Lemaitre, "Anatomy and Physiology of the Digestive Tract of *Drosophila melanogaster*," 2018, doi: 10.1534/genetics.118.300224.
- [134] N. Buchon and D. Osman, "All for one and one for all: Regionalization of the *Drosophila* intestine," *Insect Biochem Mol Biol*, vol. 67, 2015, doi: 10.1016/j.ibmb.2015.05.015.
- [135] C. A. Micchelli and N. Perrimon, "Evidence that stem cells reside in the adult *Drosophila* midgut epithelium," *Nature*, vol. 439, no. 7075, 2006, doi: 10.1038/nature04371.
- [136] E. Cohen, J. K. Sawyer, N. G. Peterson, J. A. T. Dow, and D. T. Fox, "Physiology, development, and disease modeling in the *Drosophila* excretory system," *Genetics*, vol. 214, no. 2. 2020. doi: 10.1534/genetics.119.302289.
- [137] A. Bonfini, X. Liu, and N. Buchon, "From pathogens to microbiota: How *Drosophila* intestinal stem cells react to gut microbes," *Dev Comp Immunol*, vol. 64, 2016, doi: 10.1016/j.dci.2016.02.008.
- [138] Z. Sadaqat, S. Kaushik, and P. Kain, " Gut Feeding the Brain: *Drosophila* Gut an Animal Model for Medicine to Understand Mechanisms Mediating Food Preferences ," in *Preclinical Animal Modeling in Medicine*, 2022. doi: 10.5772/intechopen.96503.
- [139] A. Šarić, M. Kalafatić, G. Rusak, G. Kovačević, D. Franjević, and H. O. Gutzeit, "Postembryonic development of *Drosophila melanogaster* meigen, 1830 under the influence of quercetin," *Entomol News*, vol. 118, no. 3, 2007, doi: 10.3157/0013-872X(2007)118[235:PDODMM]2.0.CO;2.
- [140] B. Biteau and H. Jasper, "Slit/Robo Signaling Regulates Cell Fate Decisions in the Intestinal Stem Cell Lineage of *Drosophila*," *Cell Rep*, vol. 7, no. 6, 2014, doi: 10.1016/j.celrep.2014.05.024.
- [141] J. Chen *et al.*, "Transient Scute activation via a self-stimulatory loop directs enteroendocrine cell pair specification from self-renewing intestinal stem cells," *Nat Cell Biol*, vol. 20, no. 2, 2018, doi: 10.1038/s41556-017-0020-0.
- [142] Z. Guo and B. Ohlstein, "Bidirectional Notch signaling regulates *Drosophila* intestinal stem cell multipotency," *Science (1979)*, vol. 350, no. 6263, 2015, doi: 10.1126/science.aab0988.
- [143] B. Ohlstein and A. Spradling, "Multipotent *Drosophila* Intestinal Stem Cells Specify Daughter Cell Fates by Differential Notch Signaling," *Science (1979)*, vol. 315, no. 5814, pp. 988–992, Feb. 2007, doi: 10.1126/science.1136606.
- [144] B. Ohlstein and A. Spradling, "The adult *Drosophila* posterior midgut is maintained by pluripotent stem cells," *Nature*, vol. 439, no. 7075, 2006, doi: 10.1038/nature04333.
- [145] M. Strand and C. A. Micchelli, "Quiescent gastric stem cells maintain the adult *Drosophila* stomach," *Proc Natl Acad Sci U S A*, vol. 108, no. 43, 2011, doi: 10.1073/pnas.1109794108.
- [146] L. E. O'Brien and D. Bilder, "Beyond the niche: Tissue-level coordination of stem cell dynamics," *Annual Review of Cell and Developmental Biology*, vol. 29. 2013. doi: 10.1146/annurev-cellbio-101512-122319.
- [147] L. E. O'Brien, "Regional specificity in the drosophila midgut: Setting boundaries with stem cells," *Cell Stem Cell*, vol. 13, no. 4. 2013. doi: 10.1016/j.stem.2013.09.008.
- [148] J. Liang, S. Balachandra, S. Ngo, and L. E. O'Brien, "Feedback regulation of steady-state epithelial turnover and organ size," *Nature*, vol. 548, no. 7669, 2017, doi: 10.1038/nature23678.
- [149] Z. A. Kopp, U. Jain, J. Van Limbergen, and A. W. Stadnyk, "Do antimicrobial peptides and complement collaborate in the intestinal mucosa?," *Frontiers in Immunology*, vol. 6, no. JAN. 2015. doi: 10.3389/fimmu.2015.00017.
- [150] E. Martini, S. M. Krug, B. Siegmund, M. F. Neurath, and C. Becker, "Mend Your Fences: The Epithelial Barrier and its Relationship With Mucosal Immunity in Inflammatory Bowel Disease," *CMGH*, vol. 4, no. 1. 2017. doi: 10.1016/j.jcmgh.2017.03.007.
- [151] D. Mathur, A. Bost, L. Driver, and B. Ohlstein, "A transient niche regulates the specification of drosophila intestinal stem cells," *Science (1979)*, vol. 327, no. 5962, 2010, doi: 10.1126/science.1181958.
- [152] K. Beebe, W. C. Lee, and C. A. Micchelli, "JAK/STAT signaling coordinates stem cell proliferation and multilineage differentiation in the *Drosophila* intestinal stem cell lineage," *Dev Biol*, vol. 338, no. 1, 2010, doi: 10.1016/j.ydbio.2009.10.045.
- [153] W. Reiher, C. Shirras, J. Kahnt, S. Baumeister, R. E. Isaac, and C. Wegener, "Peptidomics and peptide hormone processing in the drosophila midgut," *J Proteome Res*, vol. 10, no. 4, 2011, doi: 10.1021/pr101116g.

- [154] S. Shanbhag and S. Tripathi, "Epithelial ultrastructure and cellular mechanisms of acid and base transport in the *Drosophila* midgut," *Journal of Experimental Biology*, vol. 212, no. 11, 2009. doi: 10.1242/jeb.029306.
- [155] G. Overend, Y. Luo, L. Henderson, A. E. Douglas, S. A. Davies, and J. A. T. Dow, "Molecular mechanism and functional significance of acid generation in the *Drosophila* midgut," *Sci Rep*, vol. 6, 2016, doi: 10.1038/srep27242.
- [156] A. M. O'Hara and F. Shanahan, "The gut flora as a forgotten organ," *EMBO Reports*, vol. 7, no. 7, 2006. doi: 10.1038/sj.embor.7400731.
- [157] F. Staubach, J. F. Baines, S. Künzel, E. M. Bik, and D. A. Petrov, "Host Species and Environmental Effects on Bacterial Communities Associated with *Drosophila* in the Laboratory and in the Natural Environment," *PLoS One*, vol. 8, no. 8, 2013, doi: 10.1371/journal.pone.0070749.
- [158] I. S. Pais, R. S. Valente, M. Sporniak, and L. Teixeira, "*Drosophila melanogaster* establishes a species-specific mutualistic interaction with stable gut-colonizing bacteria," *PLoS Biol*, vol. 16, no. 7, 2018, doi: 10.1371/journal.pbio.2005710.
- [159] H. Jiang and B. A. Edgar, "Intestinal stem cell function in *Drosophila* and mice," *Current Opinion in Genetics and Development*, vol. 22, no. 4, 2012. doi: 10.1016/j.gde.2012.04.002.
- [160] P. H. Patel, D. Dutta, and B. A. Edgar, "Niche appropriation by *Drosophila* intestinal stem cell tumours," *Nat Cell Biol*, vol. 17, no. 9, 2015, doi: 10.1038/ncb3214.
- [161] H. Li, Y. Qi, and H. Jasper, "Preventing Age-Related Decline of Gut Compartmentalization Limits Microbiota Dysbiosis and Extends Lifespan," *Cell Host Microbe*, vol. 19, no. 2, 2016, doi: 10.1016/j.chom.2016.01.008.
- [162] F. Landmann, "The Wolbachia Endosymbionts," *Microbiol Spectr*, vol. 7, no. 2, 2019, doi: 10.1128/microbiolspec.bai-0018-2019.
- [163] A. C. N. Wong, Y. Luo, X. Jing, S. Franzenburg, A. Bost, and A. E. Douglas, "The host as the driver of the microbiota in the gut and external environment of *Drosophila melanogaster*," *Appl Environ Microbiol*, vol. 81, no. 18, 2015, doi: 10.1128/AEM.01442-15.
- [164] W. B. Ludington and W. W. Ja, "*Drosophila* as a model for the gut microbiome," *PLoS Pathog*, vol. 16, no. 4, 2020, doi: 10.1371/journal.ppat.1008398.
- [165] A. C. N. Wong, J. M. Chaston, and A. E. Douglas, "The inconstant gut microbiota of *Drosophila* species revealed by 16S rRNA gene analysis," *ISME Journal*, vol. 7, no. 10, 2013, doi: 10.1038/ismej.2013.86.
- [166] N. A. Broderick and B. Lemaitre, "Gut-associated microbes of *Drosophila melanogaster*," *Gut Microbes*, vol. 3, no. 4, 2012. doi: 10.4161/gmic.19896.
- [167] G. Tafesh-Edwards and I. Eleftherianos, "The role of *Drosophila* microbiota in gut homeostasis and immunity," *Gut Microbes*, vol. 15, no. 1, 2023. doi: 10.1080/19490976.2023.2208503.
- [168] M. O. Fauvarque, "Small flies to tackle big questions: Assaying complex bacterial virulence mechanisms using *Drosophila melanogaster*," *Cellular Microbiology*, vol. 16, no. 6, 2014. doi: 10.1111/cmi.12292.
- [169] S. Limmer, J. Quintin, C. Hetru, and D. Ferrandon, "Virulence on the Fly: *Drosophila melanogaster* as a Model Genetic Organism to Decipher Host-Pathogen Interactions," *Curr Drug Targets*, vol. 12, no. 7, 2011, doi: 10.2174/138945011795677818.
- [170] N. Buchon, N. A. Broderick, S. Chakrabarti, and B. Lemaitre, "Invasive and indigenous microbiota impact intestinal stem cell activity through multiple pathways in *Drosophila*," *Genes Dev*, vol. 23, no. 19, 2009, doi: 10.1101/gad.1827009.
- [171] S. C. Herrera and E. A. Bach, "JAK/STAT signaling in stem cells and regeneration: From *drosophila* to vertebrates," *Development (Cambridge)*, vol. 146, no. 2, 2019, doi: 10.1242/dev.167643.
- [172] H. Jiang, P. H. Patel, A. Kohlmaier, M. O. Grenley, D. G. McEwen, and B. A. Edgar, "Cytokine/Jak/Stat Signaling Mediates Regeneration and Homeostasis in the *Drosophila* Midgut," *Cell*, vol. 137, no. 7, 2009, doi: 10.1016/j.cell.2009.05.014.
- [173] S. J. F. Cronin *et al.*, "Genome-wide RNAi screen identifies genes involved in intestinal pathogenic bacterial infection," *Science (1979)*, vol. 325, no. 5938, 2009, doi: 10.1126/science.1173164.
- [174] A. Cammarata-Mouchtouris, A. Acker, A. Goto, D. Chen, N. Matt, and V. Leclerc, "Dynamic Regulation of NF- κ B Response in Innate Immunity: The Case of the IMD Pathway in *Drosophila*," *Biomedicines*, vol. 10, no. 9, 2022. doi: 10.3390/biomedicines10092304.
- [175] M. Boutros, H. Agaisse, and N. Perrimon, "Sequential activation of signaling pathways during innate immune responses in *Drosophila*," *Dev Cell*, vol. 3, no. 5, 2002, doi: 10.1016/S1534-5807(02)00325-8.
- [176] X. Liu *et al.*, "Microbes affect gut epithelial cell composition through immune-dependent regulation of intestinal stem cell differentiation," *Cell Rep*, vol. 38, no. 13, 2022, doi: 10.1016/j.celrep.2022.110572.

- [177] G. Tafesh-Edwards and I. Eleftherianos, "JNK signaling in Drosophila immunity and homeostasis," *Immunology Letters*, vol. 226, 2020. doi: 10.1016/j.imlet.2020.06.017.
- [178] T. Gan *et al.*, "Jnk signaling in drosophila aging and longevity," *International Journal of Molecular Sciences*, vol. 22, no. 17, 2021. doi: 10.3390/ijms22179649.
- [179] S. C. Herrera and E. A. Bach, "The emerging roles of jnk signaling in drosophila stem cell homeostasis," *Int J Mol Sci*, vol. 22, no. 11, 2021, doi: 10.3390/ijms22115519.
- [180] B. Biteau, J. Karpac, D. S. Hwangbo, and H. Jasper, "Regulation of Drosophila lifespan by JNK signaling," *Exp Gerontol*, vol. 46, no. 5, 2011, doi: 10.1016/j.exger.2010.11.003.
- [181] B. Biteau, C. E. Hochmuth, and H. Jasper, "JNK Activity in Somatic Stem Cells Causes Loss of Tissue Homeostasis in the Aging Drosophila Gut," *Cell Stem Cell*, vol. 3, no. 4, 2008, doi: 10.1016/j.stem.2008.07.024.
- [182] B. Biteau, J. Karpac, S. Supoyo, M. DeGennaro, R. Lehmann, and H. Jasper, "Lifespan extension by preserving proliferative homeostasis in Drosophila," *PLoS Genet*, vol. 6, no. 10, 2010, doi: 10.1371/journal.pgen.1001159.
- [183] J. Cho, J. H. Hur, and D. W. Walker, "The Role of Mitochondria in Drosophila Aging," *Exp Gerontol*, vol. 46, no. 5, 2011, doi: 10.1016/j.exger.2010.08.010.
- [184] V. J. Milton and S. T. Sweeney, "Oxidative stress in synapse development and function," *Dev Neurobiol*, vol. 72, no. 1, 2012, doi: 10.1002/dneu.20957.
- [185] A. R. Brock, M. Seto, and R. K. Smith-Bolton, "Cap-n-Collar promotes tissue regeneration by regulating ROS and JNK signaling in the drosophila melanogaster wing imaginal disc," *Genetics*, vol. 206, no. 3, 2017, doi: 10.1534/genetics.116.196832.
- [186] B. G. Lindberg *et al.*, "Nubbin isoform antagonism governs Drosophila intestinal immune homeostasis," *PLoS Pathog*, vol. 14, no. 3, 2018, doi: 10.1371/journal.ppat.1006936.
- [187] D. N. A. Suong *et al.*, "Overexpression of dJmJ differentially affects intestinal stem cells and differentiated enterocytes," *Cell Signal*, vol. 42, 2018, doi: 10.1016/j.cellsig.2017.10.017.
- [188] J. E. La Marca and H. E. Richardson, "Two-Faced: Roles of JNK Signalling During Tumourigenesis in the Drosophila Model," *Frontiers in Cell and Developmental Biology*, vol. 8, 2020. doi: 10.3389/fcell.2020.00042.
- [189] J. Zhou and M. Boutros, "JNK-dependent intestinal barrier failure disrupts host-microbe homeostasis during tumorigenesis," *Proc Natl Acad Sci U S A*, vol. 117, no. 17, 2020, doi: 10.1073/pnas.1913976117.
- [190] A. S. Nateri, B. Spencer-Dene, and A. Behrens, "Interaction of phosphorylated c-Jun with TCF4 regulates intestinal cancer development," *Nature*, vol. 437, no. 7056, 2005, doi: 10.1038/nature03914.
- [191] D. J. K. Hu and H. Jasper, "Control of Intestinal Cell Fate by Dynamic Mitotic Spindle Repositioning Influences Epithelial Homeostasis and Longevity," *Cell Rep*, vol. 28, no. 11, 2019, doi: 10.1016/j.celrep.2019.08.014.
- [192] J. Mundorf, C. D. Donohoe, C. D. McClure, T. D. Southall, and M. Uhlirova, "Ets21c Governs Tissue Renewal, Stress Tolerance, and Aging in the Drosophila Intestine," *Cell Rep*, vol. 27, no. 10, 2019, doi: 10.1016/j.celrep.2019.05.025.
- [193] J. Zhou *et al.*, "Dpp/Gbb signaling is required for normal intestinal regeneration during infection," *Dev Biol*, vol. 399, no. 2, 2015, doi: 10.1016/j.ydbio.2014.12.017.
- [194] J. Chen, N. Xu, H. Huang, T. Cai, and R. Xi, "A feedback amplification loop between stem cells and their progeny promotes tissue regeneration and tumorigenesis," *Elife*, vol. 5, no. MAY2016, 2016, doi: 10.7554/eLife.14330.
- [195] S. Bahuguna *et al.*, "Bacterial recognition by PGRP-SA and downstream signalling by Toll/DIF sustain commensal gut bacteria in Drosophila," *PLoS Genet*, vol. 18, no. 1, 2022, doi: 10.1371/journal.pgen.1009992.
- [196] A. H. Brand and N. Perrimon, "Targeted gene expression as a means of altering cell fates and generating dominant phenotypes," *Development*, vol. 118, no. 2, 1993, doi: 10.1242/dev.118.2.401.
- [197] M. L. Suster, L. Seugnet, M. Bate, and M. B. Sokolowski, "Refining GAL4-driven transgene expression in Drosophila with a GAL80 enhancer-trap," *Genesis (United States)*, vol. 39, no. 4, 2004, doi: 10.1002/gene.20051.
- [198] N. Buchon *et al.*, "Morphological and Molecular Characterization of Adult Midgut Compartmentalization in Drosophila," *Cell Rep*, vol. 3, no. 5, 2013, doi: 10.1016/j.celrep.2013.04.001.
- [199] H. Jiang and B. A. Edgar, "EGFR signaling regulates the proliferation of Drosophila adult midgut progenitors," *Development*, vol. 136, no. 3, 2009, doi: 10.1242/dev.026955.

- [200] A. K. H. Shia, M. Glittenberg, G. Thompson, A. N. Weber, J. M. Reichhart, and P. Ligoxygakis, "Toll-dependent antimicrobial responses in *Drosophila* larval fat body require Spätzle secreted by haemocytes," *J Cell Sci*, vol. 122, no. 24, 2009, doi: 10.1242/jcs.049155.
- [201] Y. S. Cho, L. M. Stevens, and D. Stein, "Pipe-dependent ventral processing of easter by snake is the defining step in *drosophila* embryo DV axis formation," *Current Biology*, vol. 20, no. 12, 2010, doi: 10.1016/j.cub.2010.04.056.
- [202] J. Zhou, B. A. Edgar, and M. Boutros, "ATF3 acts as a rheostat to control JNK signalling during intestinal regeneration," *Nat Commun*, vol. 8, 2017, doi: 10.1038/ncomms14289.
- [203] P. Ligozygakis, P. Bulet, and J. M. Reichhart, "Critical evaluation of the role of the Toll-like receptor 18-Wheeler in the host defense of *Drosophila*," *EMBO Rep*, vol. 3, no. 7, 2002, doi: 10.1093/embo-reports/kvf130.
- [204] P. Virtanen *et al.*, "SciPy 1.0: fundamental algorithms for scientific computing in Python," *Nat Methods*, vol. 17, no. 3, 2020, doi: 10.1038/s41592-019-0686-2.
- [205] C. Stringer, T. Wang, M. Michaelos, and M. Pachitariu, "Cellpose: a generalist algorithm for cellular segmentation," *Nat Methods*, vol. 18, no. 1, 2021, doi: 10.1038/s41592-020-01018-x.
- [206] S. Van Der Walt *et al.*, "Scikit-image: Image processing in python," *PeerJ*, vol. 2014, no. 1, 2014, doi: 10.7717/peerj.453.
- [207] N. Vodovar *et al.*, "*Drosophila* host defense after oral infection by an entomopathogenic *Pseudomonas* species," *Proc Natl Acad Sci U S A*, vol. 102, no. 32, 2005, doi: 10.1073/pnas.0502240102.
- [208] A. M. Huang, J. Rusch, and M. Levine, "An anteroposterior dorsal gradient in the *Drosophila* embryo," *Genes Dev*, vol. 11, no. 15, 1997, doi: 10.1101/gad.11.15.1963.
- [209] Z. Jin, M. Che, and R. Xi, "Identification of progenitor cells and their progenies in adult *Drosophila* midgut," in *Methods in Cell Biology*, vol. 170, 2022. doi: 10.1016/bs.mcb.2022.02.013.
- [210] X. Zeng, C. Chauhan, and S. X. Hou, "Stem cells in the *Drosophila* digestive system," *Adv Exp Med Biol*, vol. 786, 2013, doi: 10.1007/978-94-7-6621-1_5.
- [211] S. Govind, A. M. Whalen, and R. Steward, "In vivo self-association of the *Drosophila* rel-protein dorsal," *Proc Natl Acad Sci U S A*, vol. 89, no. 17, 1992, doi: 10.1073/pnas.89.17.7861.
- [212] A. N. R. Weber *et al.*, "Binding of the *Drosophila* cytokine Spätzle to Toll is direct and establishes signaling," *Nat Immunol*, vol. 4, no. 8, 2003, doi: 10.1038/ni955.
- [213] A. Casali and E. Batlle, "Intestinal Stem Cells in Mammals and *Drosophila*," *Cell Stem Cell*, vol. 4, no. 2, 2009. doi: 10.1016/j.stem.2009.01.009.
- [214] B. Biteau, C. E. Hochmuth, and H. Jasper, "Maintaining tissue homeostasis: Dynamic control of somatic stem cell activity," *Cell Stem Cell*, vol. 9, no. 5, 2011. doi: 10.1016/j.stem.2011.10.004.
- [215] M. Kim, H. Ashida, M. Ogawa, Y. Yoshikawa, H. Mimuro, and C. Sasakawa, "Bacterial interactions with the host epithelium," *Cell Host and Microbe*, vol. 8, no. 1, 2010. doi: 10.1016/j.chom.2010.06.006.
- [216] A. Amcheslavsky, J. Jiang, and Y. T. Ip, "Tissue Damage-Induced Intestinal Stem Cell Division in *Drosophila*," *Cell Stem Cell*, vol. 4, no. 1, 2009, doi: 10.1016/j.stem.2008.10.016.
- [217] P. Irving *et al.*, "New insights into *Drosophila* larval haemocyte functions through genome-wide analysis," *Cell Microbiol*, vol. 7, no. 3, 2005, doi: 10.1111/j.1462-5822.2004.00462.x.
- [218] H. Jiang and B. A. Edgar, "Intestinal stem cells in the adult *Drosophila* midgut," *Experimental Cell Research*, vol. 317, no. 19, 2011. doi: 10.1016/j.yexcr.2011.07.020.
- [219] T. Takahashi and A. Shiraishi, "Stem cell signaling pathways in the small intestine," *International Journal of Molecular Sciences*, vol. 21, no. 6, 2020. doi: 10.3390/ijms21062032.
- [220] R. Sancho *et al.*, "JNK signalling modulates intestinal homeostasis and tumourigenesis in mice," *EMBO Journal*, vol. 28, no. 13, 2009, doi: 10.1038/emboj.2009.153.
- [221] P. K. Roy, F. Rashid, J. Bragg, and J. A. Ibdah, "Role of the JNK signal transduction pathway in inflammatory bowel disease," *World J Gastroenterol*, vol. 14, no. 2, 2008, doi: 10.3748/wjg.14.200.
- [222] B. Biteau and H. Jasper, "EGF signaling regulates the proliferation of intestinal stem cells in *Drosophila*," *Development*, vol. 138, no. 6, 2011, doi: 10.1242/dev.056671.
- [223] Z. Li, C. Wu, X. Ding, W. Li, and L. Xue, "Toll signaling promotes JNK-dependent apoptosis in *Drosophila*," *Cell Div*, vol. 15, no. 1, 2020, doi: 10.1186/s13008-020-00062-5.
- [224] C. N. Perdigoto, F. Schweisguth, and A. J. Bardin, "Distinct levels of Notch activity for commitment and terminal differentiation of stem cells in the adult fly intestine," *Development*, vol. 138, no. 21, 2011, doi: 10.1242/dev.065292.
- [225] A. J. Bardin, C. N. Perdigoto, T. D. Southall, A. H. Brand, and F. Schweisguth, "Transcriptional control of stem cell maintenance in the *Drosophila* intestine," *Development*, vol. 137, no. 5, 2010, doi: 10.1242/dev.039404.

- [226] K. Petkau, M. Ferguson, S. Guntermann, and E. Foley, "Constitutive Immune Activity Promotes Tumorigenesis in *Drosophila* Intestinal Progenitor Cells," *Cell Rep*, vol. 20, no. 8, 2017, doi: 10.1016/j.celrep.2017.07.078.
- [227] S. Zaffran and M. Frasch, "Barbu: An E(spl) m4/m α -related gene that antagonizes Notch signaling and is required for the establishment of ommatidial polarity," *Development*, vol. 127, no. 5, 2000, doi: 10.1242/dev.127.5.1115.