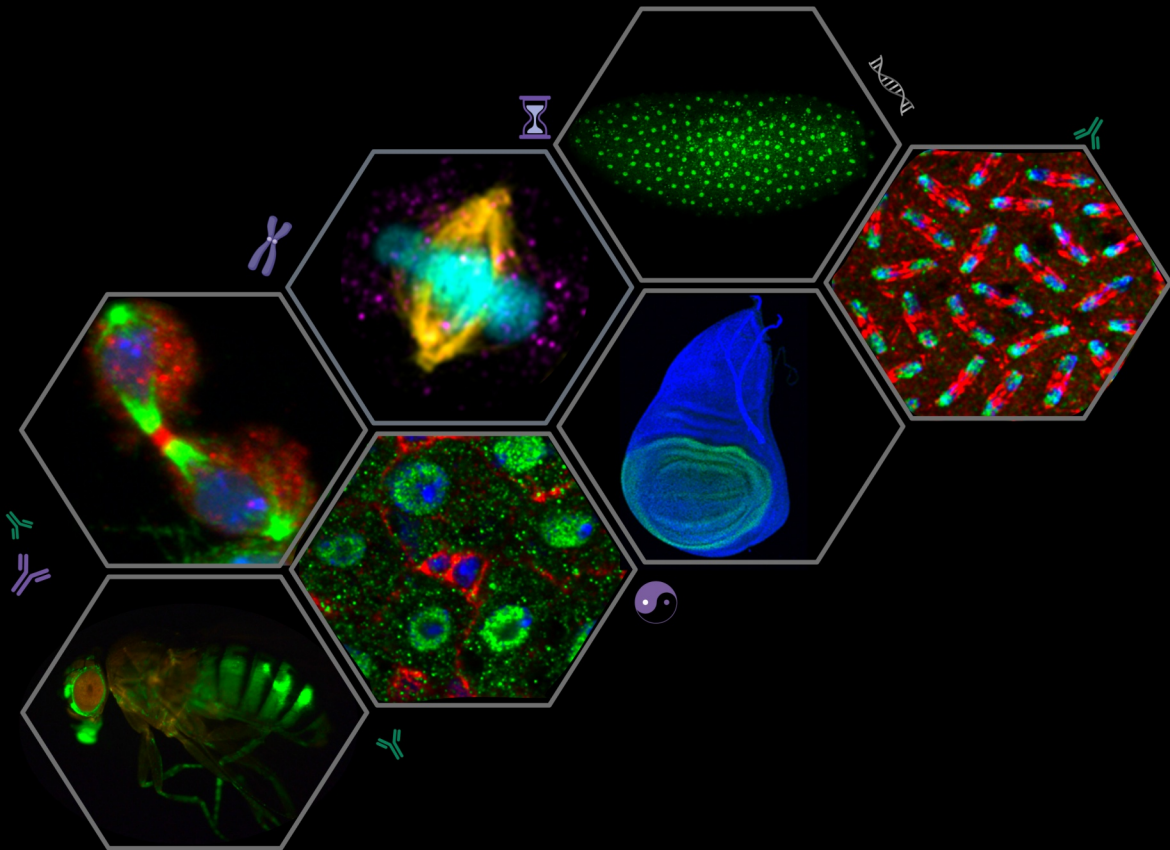


The *Drosophila* POU/Oct factors: multifaceted proteins

Priya Gohel



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Priya Gohel

Academic dissertation for the Degree of Doctor of Philosophy in Molecular Bioscience at Stockholm University to be publicly defended on Friday 22 March 2024 at 10.00 in Magnélisalen, Kemiska övningslaboratoriet, Svante Arrhenius väg 16 B.

Abstract

Dysregulation of physiological and cellular processes underlies various pathological conditions, including cancer and inflammatory disorders. Unraveling the molecular mechanisms driving these processes is crucial. The aim of this thesis was to investigate the roles of evolutionarily conserved POU/Oct transcription factors using *Drosophila melanogaster* as a model organism. The thesis highlights the functions of Nubbin (Nub) protein isoforms (Nub-PB and Nub-PD) in the regulation of cellular proliferation and mitosis, epithelial regeneration, and innate immune responses.

In paper I, we demonstrate that Nub-PB acts as a potent transcriptional activator of immune and stress response genes, while Nub-PD represses their expression, indicating transcriptional antagonism by these Nub isoforms. Overexpression of Nub-PB in midgut cells effectively cleared local infections. However, prolonged Nub-PB overexpression caused a hyperactive immune response, leading to pro-inflammatory reactions, apoptosis, and reduced adult lifespan. These findings emphasize the importance of Nub protein isoforms in fine-tuning immune responses.

In Paper II, we generated and phenotypically characterized a Nub-PB-specific mutant revealing impaired gut morphology, disorganized visceral muscles, and aberrant lineage specification in the midgut. In addition, it displays impaired immune gene activation, shortened lifespan, and enhanced reactive oxygen species (ROS) expression, which correlates with increased numbers of gut microbiota, featuring an important role of Nub-PB in intestinal epithelium homeostasis.

In Paper III, we show that Nub-PD is necessary for proper nuclear divisions in transcriptionally silent pre-blastoderm embryos. The Nub-PD protein is enriched around the mitotic spindles in metaphase, requiring intact spindle microtubules. Live imaging of mitotic divisions revealed that Nub-PD is involved in the maintenance of spindle organization and its dynamics. We also infer similar mitotic roles for Nub-PD in S2 cells and for Oct1/POU2F1 in human cell culture. Our findings unveil a direct role of POU/Oct factors in proper mitotic progression, which may be evolutionarily preserved among insects and mammals.

In Paper IV, we study how the loss of Nub and Pdm2 proteins affects wing growth and development. We found that Nub-PD is specifically required for cell proliferation, while balanced Nub-PB and Nub-PD expression levels at the dorso-ventral boundary are essential for correct wing margin formation.

Overall, this thesis elucidates crucial roles of *Drosophila* POU proteins in maintaining immune and tissue homeostasis and additionally uncovers mitotic roles of POU/Oct factors, suggesting new functions in regulation of cell proliferation and development.

Keywords: *POU/Oct factors, transcriptional regulation, innate immunity, developmental processes, mitotic spindles, chromosome segregation.*

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PROTEINS

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To my beloved family

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Abstract:

Dysregulation of physiological and cellular processes underlies various pathological conditions, including cancer and inflammatory disorders. Unraveling the molecular mechanisms driving these processes is crucial. The aim of this thesis was to investigate the roles of evolutionarily conserved POU/Oct transcription factors using *Drosophila melanogaster* as a model organism. The thesis highlights the functions of Nubbin (Nub) protein isoforms (Nub-PB and Nub-PD) in the regulation of cellular proliferation and mitosis, epithelial regeneration, and innate immune responses.

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proper mitotic progression, which may be evolutionarily preserved among insects and mammals.

In Paper IV, we study how the loss of Nub and Pdm2 proteins affects wing growth and development. We found that Nub-PD is specifically required for cell proliferation, while balanced Nub-PB and Nub-PD expression levels at the dorsoventral boundary are essential for correct wing margin formation.

Overall, this thesis elucidates crucial roles of *Drosophila* POU proteins in maintaining immune and tissue homeostasis and additionally uncovers mitotic roles of POU/Oct factors, suggesting new functions in regulation of cell proliferation and development.

Sammanfattning

Många sjukdomar och patologiska tillstånd, t.ex. cancer och inflammatoriska sjukdomar beror på felaktig reglering av celldelning, vävnadsregenerering och immunsvaret. Genom att studera de molekylära mekanismerna för dessa processer kan vår förståelse för livets grundläggande processer öka. Denna avhandling syftar till att undersöka betydelsen av evolutionärt besläktade POU/Oct transkriptionsfaktorer med bananflugan *Drosophila melanogaster* som modellsystem. Studierna belyser specifikt funktionerna hos Nubbin (Nub) proteinerna Nub-PB och Nub-PD i reglering av celldelning och mitos, epitelial regenerering och medfödda immunreaktioner.

I arbete I visar vi att Nub-PB fungerar som en potent transkriptionell aktivator av gener för immun- och stressresponser, medan Nub-PD hämmar samma geners uttryck, vilket visar på transkriptionell antagonism mellan dessa Nub-proteiner. Överproduktion av Nub-PB i tarmceller utlöser effektiva lokala infektioner. Men detta orsakade också ett hyperaktivt immunsvaret vilket slutligen ledde till ökade proinflammatoriska reaktioner, apoptos i tarmslemhinnan och en negativ påverkan på flugornas livslängd. Dessa resultat belyser vikten av att finjustera immunreaktionen och att avbryta immunaktivering då infektionen är över så att vävnadsbalansen upprätthålls.

I arbete II skapade vi en Nub-PB-specifik flugmutant och i den efterföljande karakteriseringen identifierades avvikande morfologi i tarmslemhinnan, i det visceral muskelsystemet och i cellulär organisation i den främre tarmen. Nub-PB-mutanten uppvisar dessutom påverkan på stamcellernas celldelning och differentiering i tarmen, och fördelningen mellan enterocyter och enteroendokrina celler är förändrad. Dessutom visar Nub-PB-mutanten nedsatt aktivering av immungener, kort livslängd och förhöjda uttryck av reaktiva syreföreningar (ROS), vilket korrelerar med ökat antal tarmbakterier. Dessa resultat visar sammantaget att Nub-PB spelar en viktig roll för den cellulära och immunologiska homeostasen i flugans tarmslemhinna.

I arbete III visar vi att POU-faktorer krävs för välfungerande celldelning och mitos, framförallt för rätt hastighet och precision i dessa processer i tidig

embryonalutveckling hos bananfluga och i odlade celler. Under bananflugans tidigaste embryonalstadium delar sig cellkärnor utan omslutande cellmembran och i dessa cellkärnor sker ingen transkription. POU-proteinet Nub-PD, är nödvändigt för korrekt mitos hos dessa cellkärnor och för organisationen av den mitotiska spindeln. Nub-PD-proteinet ansamlas kring den mitotiska spindeln i metafasen och för detta krävs intakta spindelmikrotubuli. Mikroskopisk avbildning i realtid av mitotiska kärndelningar påvisade att Nub-PD behövs för att bevara organisationen av den mitotiska spindeln och dess dynamik. Dessa resultat belyser funktionen och betydelsen av POU-proteiner för korrekt progression av mitosen i *Drosophila* embryon och i odlade mänskliga celler.

I arbete IV studerar vi hur förlusten av Nub- och Pdm2-proteiner påverkar utveckling och tillväxt av bananflugans vingar. Nub-PD krävs specifikt för celldelning i vingeanlagen. Experimentell manipulering av koncentrationen av Nub-PB och Nub-PD-proteiner orsakade förändringar i vingens storlek och form, och i de tvärgående venerna.

Dessa studier belyser väsentliga roller som *Drosophila* POU-proteiner spelar för att upprätthålla immun- och vävnadshomeostas. Dessutom upptäckte vi att POU-faktorer har funktioner under mitosen, som kan vara evolutionärt bevarade mellan insekter och däggdjur.

Abbreviations:

| | |
|--------------|--|
| Acj6 | Abnormal chemosensory jump 6 |
| AMPs | Antimicrobial peptides |
| A-P | Anterior-Posterior |
| AstC | Allatostatin C |
| Att | Attacin |
| BM | Basement membrane |
| BOM | Bomanin |
| Cas | Castor |
| CDK | Cyclin-dependent kinase |
| Cec | Cecropin |
| CIN | Chromosomal instability |
| CPC | Chromosome passenger complex |
| DAP-PGN | Diaminopimelic acid type peptidoglycan |
| Def | Defensin |
| dFADD | <u>F</u> as- <u>a</u> ssociated <u>d</u> eath <u>d</u> omain |
| Dfr/vvl | Drifter/ ventral veins lacking |
| Dl | Delta |
| Dpp | Decapentaplegic |
| Dpt | Diptericin |
| Dredd | Death-related ced-3/Nedd2-like caspase |
| Dro | Drosocin |
| Drs | Drosomycin |
| dTAK1 | <i>Drosophila</i> TGF- β activated kinase 1 |
| D-V | Dorsal-Ventral |
| E(Spl) | Enhancer of split |
| EB | Enteroblast |
| EC | Enterocyte |
| E-cad | E-cadherin |
| <i>Ecc15</i> | <i>Erwinia carotovora carotovora 15</i> |
| EE | Enteroendocrine |
| EEP | Enteroendocrine progenitor |

| | |
|------------------|--|
| EGFR | Epidermal growth factor receptor pathway |
| En | Engrailed |
| Esg | Escargot |
| Fkh | Fork head |
| GBE | <i>grh</i> protein binding element |
| GMC | Ganglion mother cells |
| Hb | Hunchback |
| Hh | Hedgehog |
| HTH | Helix-Turn-Helix |
| IMD | Immune deficiency pathway |
| ISC | Intestinal stem cell |
| JAK-STAT | Janus kinases (JAKs), signal transducers, and activators of transcription proteins (STATs) |
| JNK | c-Jun N-terminal kinase |
| Klu | Klumpfuss |
| Kr | Krüppel |
| Lys-PGN | Lysine type peptidoglycan |
| MAP | MTs-associated proteins |
| MORE | More palindromic Oct factor Recognition Element |
| MT | Microtubule |
| Mtk | Metchnikowin |
| Myo1A | Myosin 31DF |
| NB | Neuroblast |
| NC | Nuclear Cycle |
| NEB | Nuclear envelop breakdown |
| NFκB | Nuclear factor kappa B |
| NICD | Notch intracellular domain |
| NL | Nuclear lamina |
| Nub | Nubbin |
| Oct | Octamer binding protein |
| Pdm 1 | POU domain protein 1 |
| Pdm 2 | POU domain protein 2 |
| PGN | Peptidoglycan |
| PGRPs | Peptidoglycan recognition proteins |
| PM | Peritrophic membrane |
| PORE | Palindromic Oct factor Recognition Element |
| POU _H | POU homeodomain |
| POU _S | POU specific domain |
| PPO | prophenoloxidasases |

| | |
|--------|--|
| Pros | Prospero |
| ROS | Reactive oxygen species |
| SAC | Spindle assembly checkpoint proteins |
| SAF | Spindle assembly factors |
| Sc | Scute |
| sfGFP | Super folder Green Fluorescent Protein |
| Sox21a | Sox family transcription factor |
| Su(H) | Suppressor of Hairless |
| TF | Transcription factors |
| TLR | Toll-like receptor |
| TNFR | Tumor necrosis factor receptor |
| TotA | Turandot A |
| Upd | Unpaired |
| Wg | Wingless |
| Zfh2 | Zink finger/homeodomain 2 |

List of papers:

Paper I

Lindberg BG, Tang X, Dantoft W, **Gohel P**, Seyedoleslami Esfahani S, Lindvall JM, Engström Y., (2018) Nubbin isoform antagonism governs *Drosophila* intestinal immune homeostasis. PLoS Pathog 14(3): e1006936.

Paper II

Tang X, **Gohel P**, Engström Y., (2024) The *Drosophila* POU protein, Nub-PB, preserves intestinal epithelial homeostasis (*manuscript*)

Paper III

Gohel P, Tsarouhas V, Kansara L, Sajwan S, Engström Y., (2024) A direct role of POU/Oct factors in mitotic progression (*manuscript*)

Paper IV

Gohel P, Engström Y., (2024) Independent roles of POU proteins in patterning and growth of *Drosophila* wings (*manuscript*)

Introduction:

The development and health of multicellular organisms involve a complex interplay among diverse cellular processes, including cell proliferation, tissue growth, regeneration, and physiological responses such as immune and stress responses. However, an excessive immune response can result in chronic inflammation and tissue damage, while uncontrolled cell proliferation can promote cancer. Thus, a balance between these processes is essential. To gain insights into how tissue and immune homeostasis are regulated, it is essential to explore the factors that affect tissue regeneration and cell division rates and the factors involved in immune signaling cascades in response to infection. The aim of the thesis work is to unravel some of the molecular mechanisms that govern these fundamental cellular processes. The thesis highlights the roles played by the POU proteins, which contribute to cellular proliferation and mitosis (Paper III and IV), tissue growth and regeneration (Paper II and IV), and innate immune responses (Paper I and II).

The work presented in this thesis takes advantage of *Drosophila melanogaster* (fruit fly) as a model organism. *Drosophila* has proven to be a powerful model organism with its well-characterized genome, short generation time, and a wealth of genetic tools. *Drosophila* is amenable to various targeted gene manipulation strategies, such as RNA interference (RNAi) and CRISPR/Cas9 gene editing techniques, in a tissue-specific and developmental stage-specific manner. The evolutionary conservation of crucial cellular processes between *Drosophila* and humans makes it an invaluable tool for a mechanistic understanding of various diseases, including neurodegenerative disorders, inflammatory bowel disease, and tumorigenesis.

Drosophila undergoes four developmental stages: embryo, larva, pupa, and adult, with a life cycle of approximately ten days (Figure 1). Each developmental stage offers unique experimental advantages, making it possible to study various aspects of biological processes. Since many detailed reviews are available on *Drosophila* as a model, the following sections will briefly discuss

specific points regarding the different developmental stages covered in the thesis work (Hales et al., 2015) (Myasnikova et al., 2021). (Figure 1)

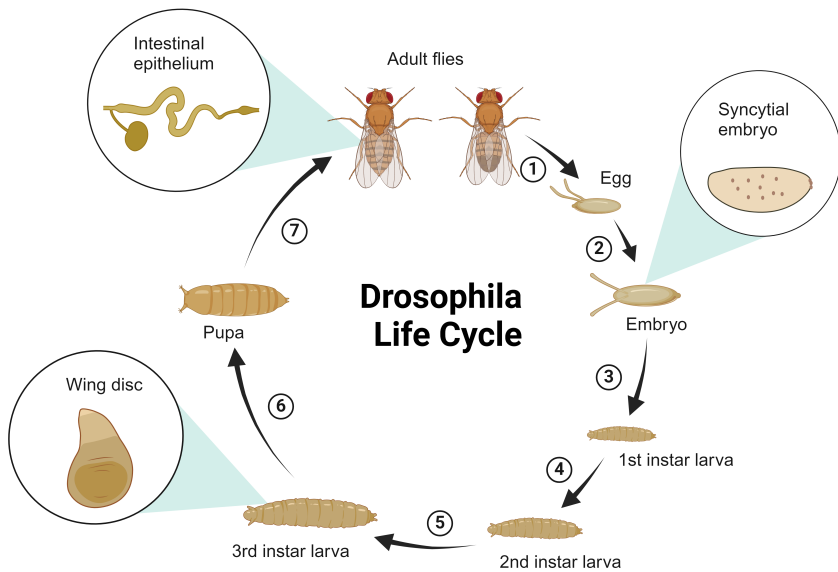


Figure 1: Illustration indicates the life cycle of *Drosophila melanogaster* and highlights the tissues/organs used as experimental systems in this thesis: the syncytial embryo, the wing disc, and the intestinal epithelium [Created with BioRender.com].

Embryonic stage: *Drosophila* embryo (~500 μm long) undergoes rapid development from one-cell embryo to gastrulation. The initial two hours of embryonic development are directed by maternal mRNA and proteins. This offers an excellent opportunity to investigate the roles of maternal factors during embryogenesis. *Drosophila* embryos are a perfect model to visualize early developmental processes, allowing for high-resolution live imaging of mitotic nuclear divisions and cytoskeletal arrangements, which enable accurate and quantifiable measurements of these processes *in vivo*. (Studied in Paper III) (Figure 1).

Larval stage: *Drosophila* larvae develop through three distinct larval stages (L1, L2, and L3). The precursor cells for most of the adult structures are set aside during embryogenesis and begin to develop in imaginal tissues during these larval stages. The larval imaginal discs undergo extensive cell prolifer-

ation and growth, providing a proper model system for understanding the precise coordination of numerous cellular processes, including cell-cell communication, cell division, gene expression, and cell shape changes. Furthermore, genetic mosaics are often experimentally induced in imaginal discs to gain insight into cell proliferation mechanisms (Paper IV) (Figure 1).

Pupal stage: Metamorphosis involves the transformation of larval tissues into adult structures through tissue remodeling and transitional changes. Finally, a fly eclosed from pupal case to begin the final stage of the life cycle.

Adult stage: The fly comprises well-developed structures or organs, for example, compound eyes, ovaries, and gut. The adult midgut exhibits physiological and functional similarities with their mammalian counterparts. Thus, it is a valuable model for understanding the molecular and cellular details of midgut epithelium regeneration and immune responses to commensal and pathogenic microbes (studied in papers I and II) (Figure 1).

In the subsequent sections, I will introduce various facets of *Drosophila* development, with a particular focus on early embryogenesis, larval wing disc development, and adult midgut epithelium. Additionally, I have included a brief discussion of the process of mitosis, innate immune regulation, and *Drosophila* POU/Oct factors.

Chapter 1 | Early embryogenesis:

In *Drosophila*, early embryogenesis is initiated with the activation/fertilization of an egg, followed by cleavage divisions, cellular blastoderm formation, and gastrulation. Egg activation is necessary for releasing the meiotic arrest (metaphase I) and is sufficient to reset the mature oocyte/egg for embryogenesis (Horner and Wolfner, 2008). Detailed in vivo analysis of *Drosophila* oogenesis has revealed that egg activation begins as the egg passes through the lateral oviduct, where mechanical forces stimulate molecular changes, including the translation of many maternal proteins and subsequent increase in intracellular Ca^{2+} concentration (Heifetz et al., 2001; Sartain and Wolfner, 2013; Tadros et al., 2003). However, in *Drosophila* and some other insects, egg activation can occur without fertilization (Heifetz et al., 2001; Kaneuchi et al., 2015; Sackton et al., 2007). This distinctive feature offers a convenient experimental system to understand how a differentiated oocyte becomes a developmentally competent embryo (the oocyte-to-embryo transi-

tion) as well as the contribution of various maternal factors during these developmental processes (reviewed in (Avilés-Pagán and Orr-Weaver, 2018; Krauchunas and Wolfner, 2013)).

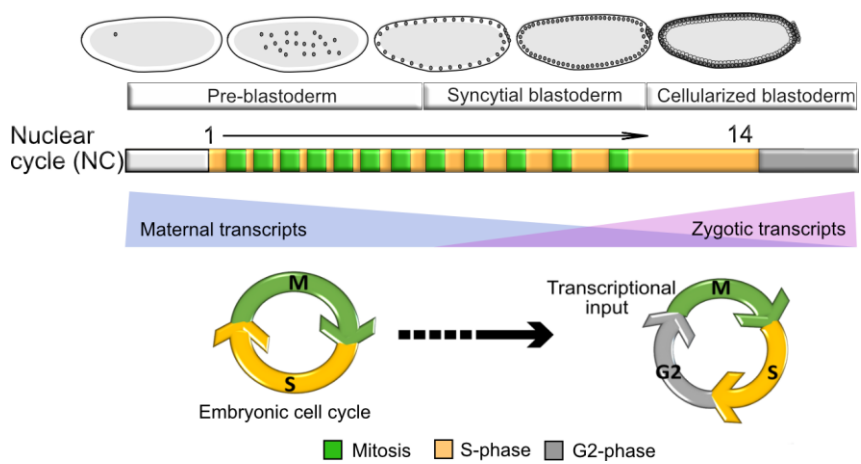


Figure 2: Illustration shows stages of early embryogenesis and timing of the nuclear division cycles (NC), highlighting the maternal to zygotic switch (MTZ) in the embryonic cell cycle. This also involves the introduction of gap phases (G1 and G2 phases) in two distinct steps during embryogenesis. The G1 phase appeared in NC 14, and the G2 phase in NC 17 (Du and Dyson, 1999)

Cleavage divisions are set to start after ‘pronuclear apposition’ in the newly fertilized embryo (Avilés-Pagán and Orr-Weaver, 2018). Remarkably, within two hours, the *Drosophila* embryo undergoes 13 rounds of nuclear division cycles (NC) before entering the gastrulation phase (Figure 2). These rapid nuclear divisions are facilitated by an altered/ simplified embryonic cell cycle with an exceptionally rapid DNA replication phase (S phase) without intervening gap phases (G1 and G2 phases). Thus, dividing nuclei alternate between the S and M phases (mitosis) (Foe, 1989) (Figure 2). The rapid and synchronized cleavage divisions occur within a single cytoplasmic compartment, forming a syncytial blastoderm (Farrell and O’Farrell, 2014). These nuclear cycles are regulated by changes in the activity of cyclin-dependent kinase 1 (Cdk1) and mitotic phosphatases PP1 and PP2A (Morgan, 2007). The cytoskeletal structures, such as F-actin, microtubules, and intermediate filaments, undergo significant reorganization in a cell cycle-dependent manner within minutes. This is crucial for maintaining the integrity and facilitating

proper nuclear division during early embryogenesis (Sullivan and Theurkauf, 1995)

The earliest nuclear cycles occur in the interior of the embryo (NC 1-3). In NC 4-7, the nuclei undergo axial expansion and are distributed along the anterior-posterior axis, driven by an actomyosin gradient (Deneke et al., 2019). Subsequently, in NC 8-9, nuclei progressively migrate towards the surface of the embryo and acquire spatial organization and uniform distribution on the cortex of the embryo, and by NC 10, forming the blastoderm (Foe and Alberts, 1983). The cortical movements and uniform positioning of nuclei at the cortex are facilitated by astral microtubules that attach to neighboring nuclei (Baker et al., 1993; Raff and Glover, 1989). During the transition from NC13 to NC14, the previously rapid cell cycle gradually slows down, and the S phase undergoes elongation. Additionally, the first gap phase (G2 phase) emerges, which marks a significant shift in the progression of the cell cycle. The cellularization stage starts in NC14, which is characterized by the formation of a plasma membrane around each of the more than 6000 nuclei. (Edgar and O'Farrell, 1990; Foe, 1989). The early nuclear divisions are transcriptionally silent and directed by maternal mRNA and proteins. As the embryo development processes, the regulatory control shifts from maternal to the zygotic genome (MZT), coinciding with a major wave of zygotic gene activation (ZGA) at NC 14. However, minor transcriptional waves from NC 8 have been observed prior to the major wave of ZGA (Ali-Murthy et al., 2013; Lécuyer et al., 2007) (Figure 2). Subsequently, gastrulation takes place, which involves the formation of the embryonic germ layers, mesoderm, endoderm, and ectoderm, to establish the basic embryonic structures.

Chapter 2 | Mitosis:

Mitosis intricately coordinates the faithful segregation of genetic material in daughter cells, marked by dramatic changes in the nuclear architecture, nuclear lamina (NL), spindle microtubules, and actin cytoskeletal networks. The fast and coordinated mitotic divisions in early *Drosophila* embryos provide an ideal system to study molecular components and regulatory mechanisms governing mitosis. The key mitotic regulators are highly conserved between *Drosophila* and other organisms, including humans. Cyclin-dependent kinases (Cdks), along with regulatory cyclins, play a crucial role in the entry into mitosis. Conversely, protein phosphatases (PPs) dephosphorylate Cdks to facilitate mitotic exit. Briefly, mitosis encompasses several stages: Prophase is the stage where chromosomes condense, and the nuclear envelope undergoes breakdown. In Metaphase, chromosomes are aligned at the cell's equator. During Anaphase, sister chromatids separate and migrate towards opposite ends, contributing to the formation of new cells. Telophase marks the formation of new nuclear envelopes around each set of chromosomes, and finally, cytokinesis leads to the division of the cytoplasm and the creation of two new cells (Figure 3)

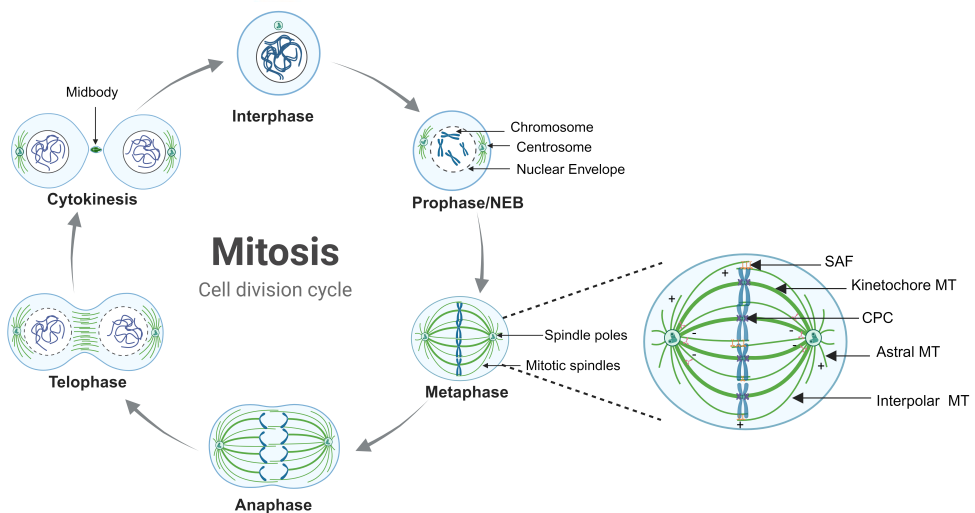


Figure 3: Illustration representing stages of mitosis and key mitotic components. Subset of metaphase show different types of microtubules (MT) present in the mitotic spindle, spindle assembly factors (SAF) and proteins in Chromosome passenger complex (CPC). [Created with BioRender.com]

Different modes of mitosis exist, such as open, semi-open, and closed mitosis (De Souza & Osmani, 2007; Webster et al., 2009). Open mitosis involves complete nuclear envelope breakdown (NEB), and spindle microtubules (MTs) form a connection with kinetochores. While in closed mitosis, the nuclear envelope remains intact. *Saccharomyces cerevisiae* display closed mitosis where spindle poles stay within the nuclear envelope. The semi-open mitosis is documented in *Drosophila* syncytial embryos where restricted nuclear envelope breakdown occurs at the poles (proximal to centrosomes), enabling centrosome-derived spindle MTs access to chromosomes (Paddy et al., 1996).

The mitotic spindles ensure the positioning of the chromosomes and movement of sister chromatids, as well as regulate the position of central spindles and the cytokinetic furrow. The mitotic spindle is formed by MT bundles composed of two conserved proteins, α - and β -tubulins and γ -tubulin is located at the microtubule organization centers and promotes the spindle polymerization (McDonald et al., 1992; McIntosh and Euteneuer, 1984). Three different modes of microtubule nucleation pathways have been proposed: microtubule-microtubule nucleation, Centrosome-mediated microtubule nucleation, and Chromosome-mediated microtubule nucleation. Centrosome and chromatin-mediated pathways drive spindle formation predominantly from centrosomes, and the kinetochore facilitates chromosome reorganization in early prometaphase (Kamasaki et al., 2013).

The spatial and temporal modulation of MT dynamics and assembly depends on the spindle assembly checkpoint proteins (SAC), the chromosome passenger complex proteins (CPC), spindle assembly factors (SAF), and MTs-associated proteins (MAPs) (Figure 4). Loss of mitotic activity of these factors delays the transition through metaphase-to-anaphase, consequently leading to chromosome segregation errors. Mitosis errors or abnormalities can lead to chromosomal instability (CIN) and contribute to diseases such as cancer (Foley and Kapoor, 2013; Holland et al., 2010; Vitre et al., 2014).

Chapter 3 | *Drosophila* wing growth and patterning

During embryonic stage 11, the precursor cells (~ 50 cells) for the wing imaginal discs are set aside (named wing primordium) from the embryonic ectoderm (Campos-Ortega, 1997; Cohen et al., 1993; Kubota et al., 2000). During the three larval stages, the discs grow exponentially in size as the disc cells undergo massive proliferation to generate >30,000 cells (Martín et al., 2009). At the third larval stage, wing discs achieve a characteristic shape, and the wing disc cells acquire different positional identities. During the pupal stage (metamorphosis), cells in the wing discs undergo two rounds of cell division and differentiate. The pupal wing will give rise to adult wing structures, including the wing blade, hinge, and notum (Reviewed by (Klein, 2001; Tripathi and Irvine, 2022)). Wing development in *Drosophila* is a precisely regulated and coordinated process. This involves a dynamic interplay of cell proliferation, differentiation, and patterning. The *Drosophila* wing discs are a powerful experimental system for studying conserved molecular mechanisms that underlie cell proliferation, patterning, and planar cell polarity. The method of creating genetic mosaics within the disc is facilitated by rapid cellular proliferation and has contributed to the identification of molecular factors that regulate cell proliferation (Xu and Rubin, 1993).

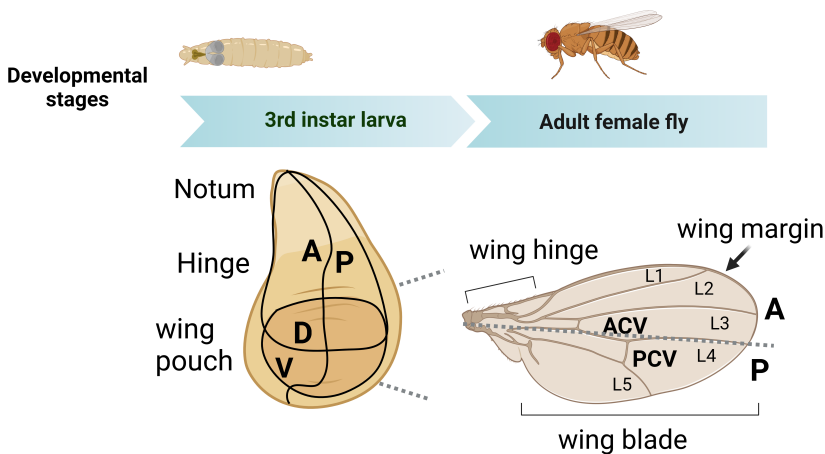


Figure 4: Illustration representing selected stages of *Drosophila* wing development. Different wing disc compartments are denoted as A: Anterior, P: Posterior, D: dorsal, and V: ventral, L1-L5: longitudinal veins, ACV: anterior cross vein, PVC: posterior cross vein. [Created with BioRender.com]

The growth of larval wing discs is influenced by intrinsic signals, such as morphogens, and extrinsic factors, such as nutrients and temperature (Truman et al., 2006). The intrinsic factors ensure the correct shape of the tissue/organ for optimal functions of the adult wing, and the extrinsic factors maintain the critical size of the organ/tissue in relation to other organs/tissues and to developmental timing. The morphogens Wingless (Wg), Decapentaplegic (Dpp), and Hedgehog (Hh) create concentration gradients to regulate the growth and patterning of wing discs in *Drosophila*. Furthermore, morphogens have critical functions in specifying different cell fates and in establishing boundaries between the anterior-posterior (A-P) and the dorsal-ventral (D-V) compartments (Garcia-Bellido et al., 1976). The A-P axis of the wing disc is characterized by the expression of Engrailed (En) and Hh. Dpp marks the A-P compartment boundary in response to Hh signaling (Garcia-Bellido et al., 1976). Along the D-V axis, Notch signaling is required to regulate Wg and Vestigial (Vg) expression (Rulifson and Blair, 1995). In addition to the A-P and D-V compartments, the larval disc exhibits a proximo-distal (P-D) axis where Wg and Dpp collaboratively regulate gene expression and growth (Garcia-Bellido et al., 1976).

Chapter 4 | The *Drosophila* gut:

The digestive tract is one of the largest organs in the body cavity of *Drosophila* (Figure 5). The adult digestive tract shares structural and functional similarities to the mammalian digestive system (Bergman et al., 2017; Lemaitre and Miguel-Aliaga, 2013). The *Drosophila* gut is divided into three main parts based on its developmental origin: foregut, midgut, and hindgut. The foregut and hindgut are derived from the ectoderm, while the midgut is derived from the endoderm. (King, 1988). Based on different metabolic and digestive functions, the midgut is further subdivided into six regions (R0 to R5) (Figure 5). Each anatomical region is characterized by unique gene expression patterns, cellular characteristics, and morphology (Buchon et al., 2013; Marianes and Spradling, 2013). The peritrophic matrix (PM) acts as a protective layer and limits the contact of the cellular layer with damaging agents and microbes (Lemaitre and Miguel-Aliaga, 2013). Circular and longitudinal visceral muscles are present throughout the intestine (Sandborn et al., 1967). The intestinal tract is also surrounded by the branched trachea and en-

teric neurons. The cellular layer of the midgut epithelium is composed of proliferating intestinal stem cells (ISCs), lineage-committed progenitors, enteroblast (EBs) and enteroendocrine progenitors (EEPs), and differentiated cell types: absorptive enterocytes (ECs) and secretory enteroendocrine cells (EEs) (Lemaitre and Miguel-Aliaga, 2013) (Figure 5).

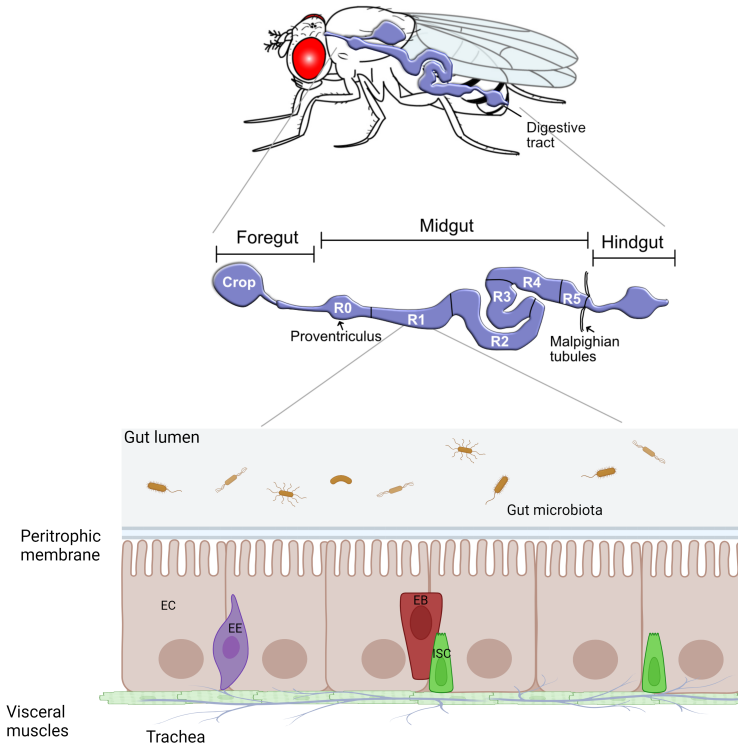


Figure 5: The illustration of the *Drosophila* adult midgut epithelium. The adult digestive tract is made up of three parts: foregut, midgut, and hindgut. The midgut is divided into six regions labeled R0 to R5. A schematic representation of different cell types such as enterocytes (EC), enteroblast (EB), intestinal stem cells (ISC), enteroendocrine cells (EE) and other parts, basal membrane (BM), visceral muscle (VM) and trachea of the midgut epithelium. [Created with BioRender.com]

Lineages and Markers for cell types:

Midgut ISCs are located close to visceral muscles and are uniformly distributed along the midgut, interspersed between the differentiates ECs and EEs. Under normal regenerative conditions, ISCs divide asymmetrically into one daughter cell with ISC characteristics and one EB, further differentiating into polyploid ECs (Ohlstein and Spradling, 2007). In a proposed model for

the specification of EEs, ISC become committed to enteroendocrine progenitors (EEP) and then terminally differentiate into mature EEs (Beehler-Evans and Micchelli, 2015). In genetic studies, each cell type can be specifically identified using antibodies or from the expression of reporter constructs of marker genes (Jin et al., 2022), summarized in Table 1.

Table 1: Marker genes for identification of specific cell types in *Drosophila* midgut

| Cell types in the adult gut | Marker to identify specific cell types |
|----------------------------------|--|
| Intestinal stem cells (ISC) | Dl, Esg, Zfh2, Fkh, Sox21a |
| Enteroblast (EB) | Su(H)GBE, Esg, Klu, Sox21a, zfh2, fkh, Nub |
| Enterocytes (EC) | Myo1A, Nub |
| Enteroendocrine Progenitor (EEP) | Sc, Piezo |
| Enteroendocrine (EE) | Pros |

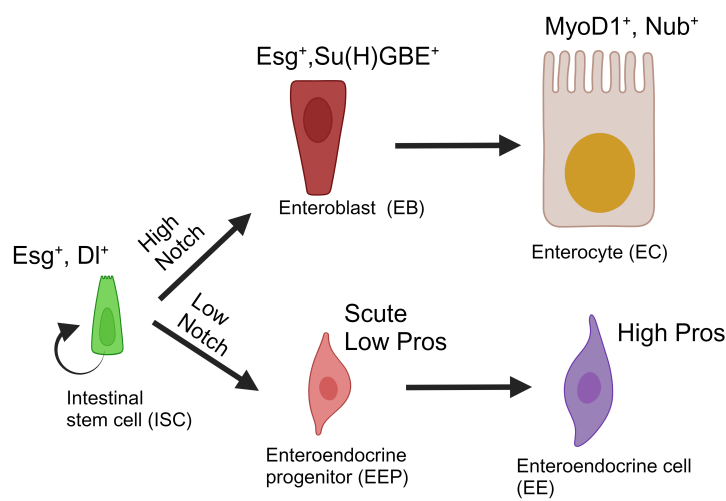


Figure 6: The illustration detailed known lineages of adult midgut ISCs (intestinal stem cells) undergoing asymmetrical division. An ISC divides to form either one ISC and one EB (enteroblast) or one ISC and a Pre-EE (precursor enteroendocrine) cell. In the ISC/EB lineage, the EB further differentiates into an EC (enterocyte). On the other hand, in the ISC/Pre-EE branch, the Pre-EE differentiates into a mature EE (enteroendocrine). [Created with BioRender.com]

Intestinal stem cell regulation: signaling pathways:

The ISC is the main proliferating cell type in the adult *Drosophila* midgut, which divides asymmetrically in homeostatic conditions or symmetrically in response to infection and stress (Biteau et al., 2008; Choi et al., 2008). Many signaling pathways influence ISC activity in the gut of *Drosophila*. The Notch

signaling pathway is an evolutionarily conserved pathway that regulates critical processes such as ISC specification, proliferation, and differentiation (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Ohlstein and Spradling, 2007). The Notch ligand, Delta (Dl), interacts with the extracellular part of Notch to induce proteolytic cleavages, which release the Notch intracellular domain (NICD) from the plasma membrane. NICD translocates to the nucleus, where it forms a complex with the DNA binding protein, Suppressor of Hairless (Su (H)), to start transcription of Notch target genes, such as the Enhancer of Split complex (*E(Spl)-C*). High levels of the Notch ligand Dl in ISCs inhibit Notch activity and maintain ISC identity (Ohlstein and Spradling, 2007). ISCs express Dl to activate high Notch activity in neighboring EBs to differentiate into ECs. This E-cadherin (E-Cad)-mediated direct interaction of Dl-Notch is called lateral inhibition (Maeda et al., 2008). In addition to Dl, the Notch pathway inhibitor Numb is also important in maintaining ISC fate and lineage specification. The symmetric distribution of Numb maintains low Notch activity and specifies the EEP lineage. The differentiation of EEPs into EE cells is also regulated by the *de novo* synthesis of Numb and the expression of Scute and Prospero (Pros) transcription factors. Upon asymmetric cell division, the daughter cell without Numb shows high Notch activity and differentiates into an EC (Sallé et al., 2017) (Figure 6). In addition to the Notch pathway, JAK/STAT, insulin, epidermal growth factor receptor (EGFR), and other signaling pathways also influence ISC proliferation and differentiation (Ohlstein and Spradling, 2006)

Chapter 5 | Innate Immunity in *Drosophila*

In nature, insects encounter various pathogenic microorganisms, including bacteria, fungi, and viruses, as well as other insects, such as parasitic wasps. *Drosophila* and other insects, which lack an adaptive immune response of the type present in vertebrates and mammals, rely on innate immune reactions for protection against infection. The cuticle of the fly serves as a first line of defense and a potent physical barrier against microbes (Schaefer et al., 1987). *Drosophila* is able to invoke a variety of innate immune responses, often categorized into cellular and humoral responses.

Cellular immune responses:

Upon infection, hemocyte-mediated immune responses are initiated in the insect hemolymph. The mature hemocytes (blood cells), released from lymph glands in response to infection, accumulate at the site of infection/wounds. Three types of hemocytes have been identified in *Drosophila* larvae, which can be divided on the basis of morphological and function properties: plasmatocytes (small and spherical), crystal cells (small and circular), and lamellocytes (large and flat). Most circulating hemocytes (95%) are plasmatocytes. Plasmatocytes, similar to mammalian macrophages, can phagocytose small pathogens (Evans et al., 2003; Lanot et al., 2001). The crystal cells make up about 5% of the circulating hemocyte population. They produce prophenoloxidas (PPOs), which are responsible for the melanization reaction, which helps to heal wounds and can immobilize pathogens (Bidla et al., 2007; Meister, 2004). Lamellocytes are only observed in larval hemolymph upon parasitic wasp infection. Lamellocytes mediate the encapsulation of large particles, such as wasp eggs (Rizki and Rizki, 1992).

Humoral immune responses:

The humoral responses in *Drosophila* are attributed by expression of different effector molecules, primarily exemplified by the antimicrobial peptides (AMPs). Upon systemic infection, the expression of AMPs is induced in the fat body (a functional equivalent of the mammalian liver) and secreted into the hemolymph to control infection (Bergman et al., 2017; Uvell and Engström, 2007). Furthermore, other humoral reactions, such as coagulation and melanization, are important responses to wounding. The coagulation system and prophenoloxidase (PPO) cascade work together in the clot formation to seal the wound (Theopold et al., 2002). In addition to AMPs, the production

of reactive oxygen species (ROS) by epithelial tissues also contributes to mucosal immunity (Ha et al., 2005).

Antimicrobial peptides:

A significant hallmark of the innate immune response in *Drosophila* is a rapid increase in the production of AMPs. AMPs were first identified and characterized by Hans Boman and colleagues in 1972 (Boman et al., 1972). AMPs are small, positively charged (4-20 kDa) peptides with antibacterial and antifungal activity. Many classes of AMPs have been identified in *Drosophila*, such as Attacins (Att), Cecropins (Cec), Defensins (Def), Diptericins (Dpt), Drosocins (Dro), Drosomycin (Drs), Metchnikowin (Mtk) and Bomanins (Hultmark, 2003; Lemaitre and Hoffmann, 2007; Uttenweiler-Joseph et al., 1998). Several studies have been conducted with purified and chemically synthesized Cecropin to understand the antimicrobial mechanisms of AMPs (Andersons et al., 1991; Steiner et al., 1988). Briefly, these cationic (positively charged) peptides interact with the negatively charged bacterial or fungal cell wall and induce pore formation in the membranes, leading to membrane collapse and death of the microbes (Andreu et al., 1985; Lockey and Ourth, 1996). AMPs can be further classified into three families based on their targets: Cecropins, Attacins, Drosocin, Diptericin acts against Gram-negative bacteria, Defensin acts against Gram-positive bacteria, and Drosomycin, Metchnikowin against fungi. Cecropins act in combination with other AMPs to restrict both Gram-negative and fungi (Hergannan and Rechhart, 1997; Hultmark, 1993; Imler and Bulet, 2005). Hanson, MA. et al. recently used CRISPR-based systematic knockdown of all known AMP genes to demonstrate pathogen-specific roles of individuals and groups of AMPs in an in vivo setup (Hanson et al., 2019).

In addition to the infection-induced AMP expression, a basal/constitutive expression of AMPs was also reported. The barrier epithelia, such as the midgut, trachea, and male and female reproductive tracts, are constantly exposed to the outer environment, encounter frequent contact with microbes, and display constitutive expression of AMPs. The presence of AMPs in barrier epithelia may provide protection against invading pathogens (Zasloff, 2006). Constitutive expression of *Defencins* and *CecAI* was reported in the male and female reproductive tracts (Ferrandon et al., 1998), and Attacins and Diptericin were detected in the midgut epithelium (Buchon et al., 2009; Tzou et al., 2000). In addition, IMD pathway-dependent *CecAI* production was reported

in embryonic and larval epidermis upon wounding (Esfahani and Engström, 2011; Onfelt Tingvall et al., 2001).

Transcriptional regulation of *Drosophila* innate immunity:

In *Drosophila*, κ B-like motifs in upstream regions of several AMP genes were shown to be required for their transcriptional activation by NF κ B-like transcription factors, Relish, Dif, and Dorsal (Engström et al., 1993; Reichhart et al., 1992). The production of AMPs relies on two well-conserved NF- κ B signaling pathways, the Toll pathway and the immune deficiency (IMD) pathway (Lemaitre et al., 1995; Lemaitre et al., 1996; Wasserman, 1993). The Toll pathway regulates the nuclear translocation of Dif and Dorsal, whereas the nuclear translocation of Relish depends on the IMD signaling pathway. In parallel to Relish, the GATA factor Serpent was shown to be needed for *CecA1* expression in embryos and the larval fat body (Petersen et al., 1999; Tingvall et al., 2001). Furthermore, the homeodomain protein Caudal (Cad) and the POU protein Drifter (Dfr) were shown to be necessary for constitutive expression of *Drs* and *CecA1* in the male ejaculatory duct (Junell et al., 2010; Ryu et al., 2004).

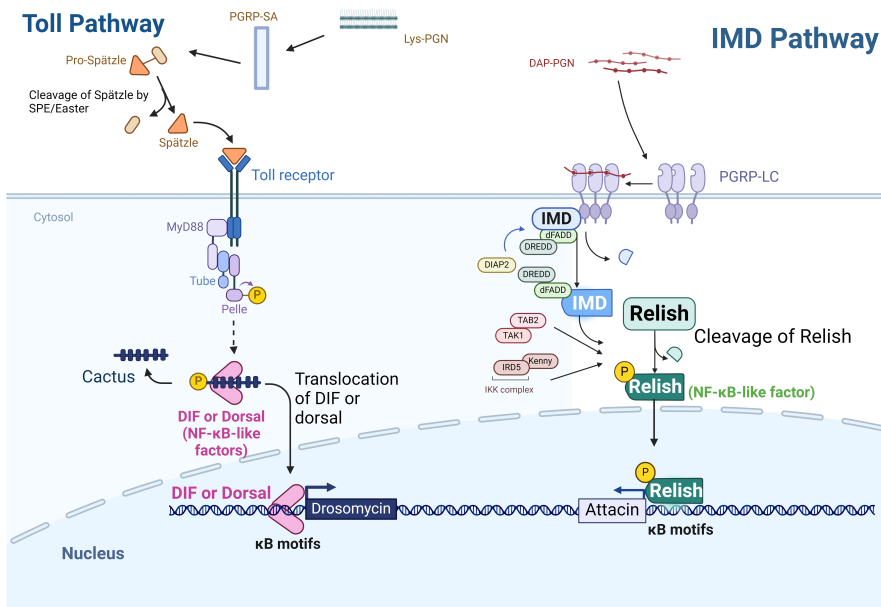


Figure 7: Illustration of key components of the core *Drosophila* innate immune pathways, the Toll and IMD pathways (recreated from (Uvell and Engström, 2007) [Created with BioRender.com])

Pattern recognition and signaling:

The peptidoglycan recognition proteins (PGRPs) are sensors in host cells that detect the peptidoglycan (PGN) from different types of microbes. Two different kinds of PGN are produced by bacteria. Diaminopimelic acid (DAP) type PGN is displayed by Gram-negative bacteria, and Lysine (Lys) type PGN by Gram-positive bacteria (Mengin-Lecreulx and Lemaitre, 2005). Upon recognition and binding of microbial PGNs, the PGRPs stimulate downstream signaling cascades of both the Toll and IMD pathways (PGRPs are reviewed in (Royet and Dziarski, 2007; Steiner, 2004). In addition, there are catalytically active PGRPs that cleave PGN, some that produce monomeric PGN that further trigger signaling pathways, and other PGRPs that are modeling and turning off immune responses once the infection has been eradicated (Costechareyre et al., 2016).

The Toll pathway

The *Drosophila* Toll pathway shows some similarity to the mammalian Toll-like receptor (TLR) signaling. In the Toll signaling pathway, PGRP-SA mediates recognition of lysine-type PGN from Gram-positive bacteria, which activates extracellular proteolytic cascades and leads to the processing of pro-Spätzle protein into the mature Spätzle protein (Bischoff et al., 2004; Michel et al., 2001; Valanne et al., 2011). Spätzle binds to the Toll receptor, and this leads to the recruitment of a protein complex consisting of MyD88, Tube, and Pelle (DEATH-domain proteins) and subsequent dissociation of the inhibitory protein Cactus (IkB-like protein) from the Rel/ NFκB proteins, Dif and Dorsal (Ip et al., 1993; Rushlow et al., 1989). Dif and Dorsal both translocate into the nucleus upon Toll signaling to activate the expression of downstream effectors, such as the genes for the AMPs Drosomycin, Metchnikowin, and Bomanins. (The Toll pathway is extensively reviewed by (Valanne et al., 2022; Valanne et al., 2011) (Figure 7).

The IMD pathway:

The IMD pathway shows partial similarity to the mammalian tumor necrosis factor receptor (TNFR) pathway (Kleino and Silverman, 2014). The IMD pathway is activated by DAP-type PGN from Gram-negative and some Gram-positive bacteria. PGRP-LC, a transmembrane protein, mediates PGN detection and activates cytoplasmic Imd protein. Activated Imd interacts with the *Drosophila* Fas-associated death domain (dFADD) and Death related ced-

3/Nedd2-like caspase (Dredd) complex and *Drosophila* TGF- β activated kinase 1 (dTak1) (Kaneko et al., 2006; Takehana et al., 2002). The I κ B kinase (IKK) complex and the dFADD-Dredd complex can activate the proteolytic cleavage of the NF κ B-like protein Relish. The N-terminal part of Relish, including the DNA-binding domain, is translocated into the nucleus to activate IMD-responsive effector genes, such as AMP genes (Gesellchen et al., 2005; Kleino et al., 2005). The IMD pathway shows crosstalk with the c-Jun N-terminal kinases (JNK) pathway. The dTak1-mediated activation of JNK signaling is required in parallel to IMD/Relish signaling to activate AMP gene expression (Kim et al., 2007; Kim et al., 2005) (Figure 7).

The JAK-STAT pathway:

The JAK-STAT pathway also contributes to the transcriptional activation of immune and stress response genes upon infection (Agaisse and Perrimon, 2004). The recognition of bacteria or viruses by hemocytes, fat body cells, or enterocytes leads to the secretion of Unpaired (Upd, Upd 2, and Upd3), which are ligands that bind and activate the Domeless receptor (related to IL-6 receptor in vertebrates). This triggers activation of the Janus kinase (JAK) Hopscotch (Hop), which phosphorylates the receptor-bound transcription factor STAT92E (signal transducer and activator of transcription). STAT92E forms dimers upon phosphorylation and translocates to the nucleus to activate effector genes, such as Turandot A (tot A) (Agaisse and Perrimon, 2004). The JAK-STAT pathway is also involved in conferring intestinal immunity, ISC proliferation, and epithelial cell renewal (Ohlstein and Spradling, 2006). The JNK and JAK-STAT pathways also contribute to cellular immunity, promoting the differentiation of plasmatocytes and lamellocytes under stress and after cell damage (Rodrigues et al., 2021; Tokusumi et al., 2009).

Chapter 6 | POU/Oct transcription factors

The POU/Oct protein family comprises a group of evolutionarily conserved transcription factors (TF) that have been identified in all metazoans studied so far, including *Caenorhabditis elegans*, *Drosophila melanogaster*, *Xenopus laevis*, humans and other mammals, and (Ilia, 2004). The nomenclature of the members of the **POU** family (**Pit-Oct-Unc**) was defined from the mammalian **P**ituitary-specific transcription factor 1 (Pit-1) and the **O**ctamer-binding proteins (Oct1/Oct2), and the nematode gene **U**nc-86 (Clerc et al., 1988; Finney et al., 1988; Fletcher et al., 1987; Herr et al., 1988; Sturm et al., 1988). All three members share high sequence similarity over the POU domain, the DNA-binding domain. Members of the POU/Oct TF family are classified into six classes (from POU I to POU VI) based on sequence similarity throughout the POU domain and the length of the hypervariable linker (Bürglin and Affolter, 2016) (Table 2).

Table 2: Mammalian and *Drosophila* POU/Oct factors

| Class | Mammalian POU/Oct factors | <i>Drosophila</i> POU/Oct factors |
|---------|--|--|
| POU I | <i>POU1F1 (Pit-1)</i> | None |
| POU II | <i>POU2F1 (Oct-1)</i> <i>POU2F2 (Oct-2)</i> <i>POU2F3 (Oct-11)</i> | <i>nub (pdm1)</i> , <i>pdm2 (miti-mere)</i> |
| POU III | <i>POU3F1 (Oct-6)</i> <i>POU3F2 (Brn-2)</i> <i>POU3F3 (Brn-1)</i> <i>POU3F4 (Brn-4)</i> | <i>drifter (vvl, cfla)</i> |
| POU IV | <i>POU4F1 (Brn-3a)</i> <i>POU4F2 (Brn-3b)</i> <i>POU4F3 (Brn-3c)</i> | <i>acj6</i> |
| POU V | <i>POU5F1 (Oct-4)</i> <i>POU5F2 (SPRM-1)</i> | None |
| POU VI | <i>POU6F1 (Brn-5)</i> | <i>pdm3</i> |

Structural and DNA binding properties of POU/Oct TFs:

The POU/Oct TFs are characterized by bipartite DNA binding domains, namely the POU-specific domain (POU_S) and the POU-homeo domain (POU_H). The POU_S domain, which spans 74-82 amino acids, is distinctive for POU/Oct factors. Comparative sequence analysis has revealed higher conservation of POU_S domains across species compared to the POU_H domain (60 amino acids). A variable-length linker (14-26 amino acids) connects the POU

and Homeo domains, providing flexibility in interactions with the octamer and other target sequences (Herr et al., 1988; Klemm and Pabo, 1996).

POU/Oct TFs recognize and bind to the canonical octamer sequence motif (5'-ATGCAAAT-3') and related sequences in the promoter and enhancer regions of various target genes (Ingraham et al., 1990). The POU and homeodomains fold independently to form Helix-turn-Helix (HTH) motifs, displaying high-affinity cooperative binding to each half-site on the octamer sequence. Specifically, the POU_S domain is associated with 5'-ATGC-3' and the POU_H domain with 5'-AAAT-3' (Phillips and Luisi, 2000; Tang and Engström, 2019; Verrijzer et al., 1990).

In addition to the canonical octamer motif, POU/Oct TFs demonstrate the ability to recognize diverse Oct-like target sequences, such as the More palindromic Oct factor Recognition Element (MORE) and the Palindromic Octamer Recognition Element (PORE) (Botquin et al., 1998; Tang and Engström, 2019). In summary, structurally unique POU and homeodomains, coupled with flexibility in interactions with the target site, confer POU proteins with immense diversity in gene regulation properties, playing a critical role in development.

***Drosophila* POU/Oct transcription factors:**

Several POU/Oct transcription factor genes have been identified in the genome of *Drosophila melanogaster*, and these belong to four distinct classes (Bürglin and Affolter, 2016; Tantin, 2013) (Table 2). *Drosophila* POU/Oct TFs show developmental stage and tissue-specific expression patterns. They are essential regulators of development processes. Nubbin (Nub) / POU domain protein 1 (Pdm1) has been implicated in wing development, stem cell division and differentiation, and innate immunity (Bhat and Schedl, 1994; Billin et al., 1991; Dantoft et al., 2013; Lindberg et al., 2018; Ng et al., 1995; Tang et al., 2018). The *pdm2* (*miti-mere*) gene is required for neurogenesis in the embryo and in neuroblast specification and differentiation (Bhat et al., 1995; Bhat and Schedl, 1994; Billin et al., 1991). The *drifter* (*dfr*) gene (also named *ventral veins lacking*, *vvl*) is involved in the development of the embryonic brain and nervous system, the development of the trachea, and the determination of the wing veins. It was reported that Dfr is involved in the transcriptional regulation of adult epithelial immunity and of genes involved in steroid hormone biosynthesis (Anderson et al., 1995; Danielsen et al., 2014; de Celis et al., 1995; Junell et al., 2010; Zhao et al., 2021). The *pdm3* and *acj6*

genes are involved in targeting within the olfactory neuron axons and in odor detection (Jafari and Alenius, 2015; Tichy et al., 2008)

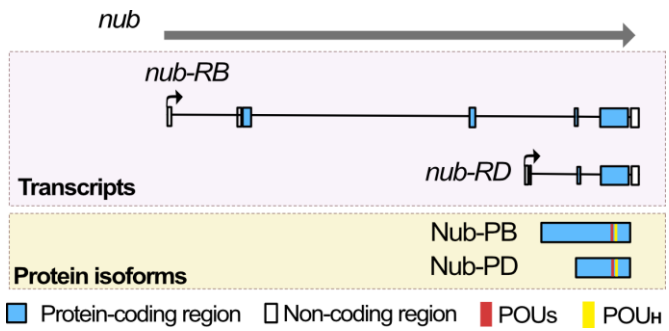


Figure 8: Schematic representation of *nub* gene organization, the two transcription units *nub-RB* and *nub-RD*, and two independent protein isoforms, Nub-PB and Nub-PD. (Arrows indicate transcription start sites)

Several studies have reported the expression of Nub/Pdm1 in *Drosophila* embryos, imaginal discs, and nervous systems (Billin et al., 1991; Dick et al., 1991; Ng et al., 1995). Our research group has demonstrated that the *Drosophila nub* gene encodes two functional proteins, Nub-PB (104 kDa) and Nub-PD (65 kDa), which exhibit tissue-specific expression patterns during different developmental stages (Dantoft et al., 2013; Lindberg et al., 2018)(Figure 8). Nub-PB and Nub-PD are expressed in larval imaginal discs, adult gut, wing veins, and leg joints. However, Nub-PD is specifically expressed in syncytial embryos, embryonic nervous systems, and larval brains (Paper I and Paper III). The classical *nub^l* allele is associated with an insertion of a transposon element within the promoter region of *nub-RD*, leading to generally reduced expression of Nub-PD protein and small wings. However, further characterization revealed the loss of both Nub-PB and Nub-PD proteins in *nub^l* mutant wing discs (Loker and Mann, 2022)(Paper III and IV) Table 3.

Table 3: Expression of Nub isoforms in wild type and *nub^l* mutants.

| | Wild type | | | <i>nub^l</i> | | |
|-----------|-----------|-----------|---------|------------------------|-----------|---------|
| | Embryo | Wing disc | Midgut | Embryo | Wing disc | Midgut |
| Nub-PD | Present | Present | Present | Absent | Absent | Absent |
| Nub-PB | - | Present | Present | - | Reduced | Present |
| Pdm2PA/PB | Present | Present | Present | Present | Absent | Present |

The paralogous genes, *nub* and *pdm2* are located adjacently on the left arm of chromosome 2 (cytology map position 33F1). Both genes show similar exon-intron organization and a high degree of conservation of the POU and homeodomains, and it has been suggested that *nub* and *pdm2* arose from a recent gene duplication event (Loker and Mann, 2022; Ross et al., 2015). Both Nub and Pdm2 proteins show overlapping expression patterns in a subset of embryonic neural precursor cells, neuroblasts (NB), and ganglion mother cells (GMC) (Dick et al., 1991). Pioneering studies in *Drosophila* neuroblasts demonstrated that Nub/Pdm2 proteins (collectively called Pdm) are part of a highly conserved temporal expression cascade (Hunchback (Hb) → Krüppel (Kr) → Nub/Pdm2 (Pdm) → Castor (Cas) for specific neuroblast lineages (NB4-2>GMC-1) that generate RP2/Sib⁺ neurons during embryogenesis (Cleary and Doe, 2006; Kambadur et al., 1998). Each member of the temporal identity cascade is necessary to generate neurons in a specific temporal developmental window. The embryo mutant, *nub*^{E37} (loss of Nub only), exhibits partial loss of RP-2 neurons and mild defects in NB differentiation due to a *nub* null allele. Loss of *nub* and *pdm2* in double mutant embryos (*Df(2L) Gr4*) results in a significant reduction or loss of RP2/sib⁺ neurons, which implies that both Nub and Pdm2 contribute to the specification and differentiation of NBs in *Drosophila* embryos (Yeo et al., 1995).

Aim of the thesis

The primary objective of this thesis was to study the specific functions of the *Drosophila* POU protein, Nubbin (Nub), in essential biological processes, including cell proliferation and mitosis, tissue regeneration, and immune responses.

The specific aims of each paper:

Paper I: To investigate distinct roles of Nub isoforms in the regulation of immune genes.

Paper II

To identify the specific function of Nub-PB in the intestinal epithelium.

Paper III

To investigate the particular involvement of Nub isoforms in cellular proliferation, with a focus on the cell cycle.

Paper IV

To assess the isoform-specific involvement of Nub and Pdm2 proteins in growth and patterning of the wing disc.

Results and discussion:

Paper I: Nubbin isoform antagonism governs *Drosophila* intestinal immune homeostasis

Background:

Our group has previously identified three POU domain transcription factors, Dfr/Vvl, Nub/Pdm1, and Pdm2, as regulators of the AMP gene *CecA1* in a yeast screen (Junell et al., 2007). A subsequent study showed that Dfr/Vvl is needed for the constitutive expression of *CecA1* in the adult male ejaculatory duct[94]. Based on genome annotation prediction, the *nub* gene encodes two protein isoforms, Nub-PB and Nub-PD. Dantoft *et al.* (2013) confirmed the existence of two independent protein isoforms, Nub-PB (104 kDa) and Nub-PD (65 kDa) (Figure 8). Furthermore, this work showed that Nub-PD binds directly to octamer sequence clusters in AMP genes and negatively regulates NF- κ B/Relish-dependent AMP gene activation. The gene expression profiling in *nub^l* mutant flies display that many immune and stress response genes were up-regulated, confirming that Nub-PD acts as a transcriptional repressor of these genes. The overactive immune responses in *nub^l* mutants led to an atypical composition of the commensal gut microbiota (Dantoft et al., 2013; Dantoft et al., 2016). However, the functional roles of the Nub-PB isoform in the regulation of immune genes and other processes/genes have not yet been defined.

Results and discussion:

The mRNA expression profile was examined to investigate the function of Nub-PB in immune regulation. Microarray analysis indicated that overexpression of Nub-PB in the fat body and gut was sufficient to induce the expression of a battery of genes involved in immune and stress responses, cytokine production, cell differentiation, and metabolism. A comparison was made between the differentially expressed AMP genes identified in microarray analy-

sis in response to Nub-PB overexpression and the results from a previous analysis of the *nub¹* mutant, lacking Nub-PD expression specifically (Dantoft et al., 2013). This comparison led to identifying 65 immune genes that were up-regulated in both data sets, indicating that these genes were positively regulated by Nub-PB and negatively regulated by Nub-PD. The expression levels of selected AMP genes were further validated by RT-qPCR after co-overexpression of *nub-RB* and *nub-RD* in the midgut. Nub-PB overexpression activated the expression of AMPs, while co-overexpression of *nub-RD*, along with *nub-RB*, dampened the expression of the same AMPs. These findings indicate that Nub-PB and Nub-PD regulate the expression of the same set of AMP genes in an antagonistic manner.

Nub-PB and Nub-PD share the same C-terminal region, containing the DNA binding POU and homeodomains known to interact with octamer sequence motifs. We asked whether Oct-like motifs in the upstream regulatory region of *CecAI* were necessary for NubPB-mediated activation. To examine this, we generated *CecAI ΔOct-lacZ* reporter fly lines and found that the Oct cluster was required for full activation of the reporter gene by Nub-PB. The results further suggested that Nub-PB and Nub-PD bind to the Oct cluster to regulate the expression of *CecAI* in a positive and negative manner.

Next, we performed oral infections with *Erwinia carotovora carotovora 15* (*Ecc15*) bacteria to examine the function of Nub-PB after bacterial challenge. Although Nub-PB overexpressing flies eliminated the *Ecc15* bacteria, the flies died shortly after infection, suggesting that bacterial overgrowth was not the direct cause of the increased mortality. Furthermore, we discovered that prolonged overexpression of Nub-PB in enterocytes triggered pro-inflammatory reactions, including activation of the Jak-STAT and JNK pathways, increased intestinal stem cell proliferation, and apoptosis. However, this also negatively affected the adult lifespan. This indicates that overexpression of Nub-PB leads to excessive immune activation and consequently induces gut pathology. Taken together, our results highlight the importance of fine-tuning between immune regulators, such as Nub-PB (activator) and Nub-PD (repressor), to achieve effective eradication of pathogens but not elicit too strong immune responses, which may have detrimental consequences for the host.

Paper II: The *Drosophila* POU protein, Nub-PB, preserves intestinal epithelial homeostasis (manuscript)

Background:

In paper I, we show that Nub-PB and Nub-PD play opposite roles in the transcriptional regulation of immune genes in the *Drosophila* gut. Furthermore, Tang et al. (2018) demonstrated that Nub-PB and Nub-PD act antagonistically in midgut progenitor cells to regulate intestinal stem cell (ISC) proliferation and that Nub-PD is required for ISC proliferation. On the contrary, Nub-PB acted as a differentiation factor. The downregulation of *nub-RB* by RNAi led to a hyperproliferation phenotype in the midgut, further supporting the role of Nub-PB as a differentiation factor (Tang et al., 2018). Here, we aimed to gain further insights into the role of Nub-PB in the regulation of midgut epithelium homeostasis.

Results and discussion:

To investigate the specific role of Nub-PB, we generated a Nub-PB-specific mutant, *nub^{PB-3}*, using CRISPR/Cas9 gene editing. We examined the survival of adult *nub^{PB-3}* mutants after *Ecc15* infection. This showed that *nub^{PB-3}* flies were highly susceptible to oral infection. Moreover, RT-qPCR for AMP genes clearly showed that AMP gene activation was significantly reduced in the *nub^{PB-3}* mutant gut. These results suggest that *nub^{PB-3}* flies failed to fully activate AMP genes in response to infection, leading to a decrease in adult survival.

Further characterization revealed defects in the midgut morphology of *nub^{PB-3}* mutants, including an enlarged anterior midgut, short midgut length with increased epithelial delamination, and defects in visceral muscle organization. This indicates that Nub-PB is required for maintaining normal adult midgut morphology. Furthermore, the *nub^{PB-3}* mutant showed an impact on normal midgut regeneration. We observed increased ISC proliferation and accumulation of GBE-GFP positive cells in *nub^{PB-3}* mutant guts, indicating impaired progenitor differentiation. In addition, an increased number of paired EE cells were scored in the *nub^{PB-3}* mutant guts, suggesting an enhancement in ISC lineage commitment towards EEP-EE lineage and EE differentiation. Together, these findings emphasize a significant contribution of Nub-PB in maintaining immune and tissue homeostasis in the intestinal epithelium.

Paper III: Direct role of POU/Oct factors in mitotic progression. (manuscript)

Background:

The intricate balance between stem cell proliferation and differentiation is essential for tissue homeostasis and development. The *Drosophila nub* and *pdm2* genes have been identified as crucial players in neuroblast division and fate specification during embryogenesis (Yang et al., 1993). Tang et al.(2018) highlight the antagonistic roles of Nub-PB and Nub-PD proteins in intestinal stem cell (ISC) proliferation and differentiation, where Nub-PD promotes proliferation while Nub-PB drives differentiation (Tang et al., 2018). However, the precise mechanisms by which Nub isoforms regulate proliferative activity during the cell cycle remain poorly understood.

Results and discussion:

To investigate the role of Nub proteins during proliferation, we performed isoform-specific knockdown in *Drosophila* S2 cells. Downregulation of Nub-PD, but not of Nub-PB, had negative effects on progression through the G2/M phases, suggesting an isoform-specific involvement in control of the cell cycle. Further analyses revealed aberrant mitotic defects, including defective spindle organization and delayed progression through mitosis in S2 cells. Mitotic defects after Nub-PD knockdown suggest two possible modes of regulation. Either Nub-PD may control mitosis by transcriptional regulation of cell cycle genes or possibly regulate mitosis in a more direct fashion. To distinguish between these two alternative modes of action of Nub-PD, we used *Drosophila* syncytial embryos, which undergo fast, synchronous, and transcriptionally silent mitotic divisions. Notably, we show that only Nub-PD, but no Nub-PB protein, is present in syncytial embryos.

To assess the direct impact of the loss of Nub-PD on mitotic divisions, we used *nub^l* homozygous mutant embryos, which lack the Nub-PD protein but still express the Pdm2 protein. Live imaging analysis of early nuclear divisions revealed various mitotic phenotypes in *nub^l* mutant embryos, classified as fused nuclei, defective spatial distribution of nuclei, cortical gaps with nuclear fallout (NUF), asynchronous nuclear divisions, and delayed progression through mitotic phases during nuclear cycle 10-13 (NC 10-13). These mitotic defects were also present, and even enhanced, in embryos injected with anti-Nub antibodies, demonstrating that Nub-PD is essential for nuclear divisions

in early embryos. Although the mitotic defects in *nub*¹ embryos were generally present at NC 10-13, the penetration of the phenotypes was relatively variable between individual embryos, suggesting that *nub*¹ is a hypomorph with varying levels of Nub-PD protein. To address this, we performed *nub* RNAi in *nub*¹ mutant background to minimize the Nub-PD protein levels. As expected, further reduction of Nub-PD led to more severe mitotic defects, which were visible in earlier nuclear cycles (NC 5). This further validated a non-transcriptional role of Nub-PD in mitosis, as there is no transcription taking place at these early nuclear division cycles. These results confirm that the Nub-PD protein is crucial for early nuclear divisions in a direct and non-transcriptional manner.

To ensure that mitotic failures upon downregulation of Nub-PD using maternal Gal4 drivers, was not causing defects already in the oocyte and during egg maturation, we carried out targeted degradation of maternal GFP-tagged Nub-PD protein in embryos, using the deGradFP system. This exhibited severe defects in nuclear divisions and spindle organization as well, confirming that Nub-PD protein is necessary specifically in the embryo, during early mitotic divisions.

To elucidate the cause of nuclear division defects, we analyzed mitotic spindles by live imaging using the microtubule plus the end-binding protein EB1 (tagged with GFP) (EB1-GFP) in *nub*¹ mutant embryos. This revealed that the loss of Nub-PD mounted a significant increase in abnormal spindles, and this was accompanied by defective spindle dynamics, suggesting that Nub-PD is involved in the maintenance of mitotic spindle dynamics in the early embryo. In particular, mitotic defects, including spindle organization, were partially rescued after the restoration of Nub levels from a genetic translocation/duplication. This further confirmed that Nub-PD contributes to spindle organization during nuclear divisions.

Next, we examined the localization of Nub-PD in early embryos and by live imaging of S2 cells during the mitotic phases. In prophase, Nub-PD showed nuclear localization, and during metaphase, it showed enrichment around mitotic spindles. At the end of mitosis (telophase), Nub-PD was localized in the nuclei (syncytial embryo) and at the midbody during cytokinesis (live S2 cells), indicating that Nub-PD exhibits dynamic localization patterns in each mitotic phase. The enrichment of Nub-PD around mitotic spindles during metaphase further supports a role in spindle organization.

To identify molecular factors that regulate the location of Nub-PD during mitosis, we performed a targeted RNAi screen for components involved in the assembly and function of the mitotic spindle. Our analysis revealed nine genes, including components of the Chromosome passenger complex (CPC), kinesin motor proteins (Klp61F and Klp3A), Crumbs and kinases (Cdk1, Nek1, and Niki), that upon RNAi affected Nub-PD localization during mitosis. These results indicate that the dynamic localization of Nub-PD is regulated by these mitotic factors.

As mentioned above, Nub-PD was found to be enriched around the mitotic spindles. We investigated whether this enrichment of Nub-PD is dependent on intact microtubules. This was addressed by cold-induced microtubule depolymerization in the syncytial embryo and colchicine drug-induced depolymerization in S2 cells. Our analysis demonstrated loss of Nub-PD enrichment upon microtubule depolymerization, suggesting that intact microtubules are required for the localization of Nub-PD during metaphase.

Additionally, the knockdown of POU2F1/Oct1, a human homolog of Nub, in HeLa cells displayed delayed mitosis and defective spindle organization, suggesting that POU2F1/Oct1 may perform a similar mitosis-specific role in human cells.

In summary, we demonstrate that loss of Nub-PD, both in the syncytial embryo and in cell culture, leads to a multitude of mitotic defects, including defective segregation of chromosomes, delayed mitotic progression, and defective spindle organization. The analysis of the syncytial embryo suggests that Nub-PD can play a non-transcriptional role during mitotic. Together, we propose a direct role of POU factors in mitotic spindle organization and for timely mitotic progression.

Paper IV: Independent roles of POU proteins in patterning and growth of *Drosophila* wings (manuscript)

Background:

The *Drosophila* POU transcription factors, Nub and Pdm2, have been reported to be involved in the regulation of wing development. Loss of Nub and Pdm2 during wing disc development causes striking wing phenotypes involving small, opaque, and curved wings with irregular wing margins and partial loss of wing hinge (Cifuentes and García-Bellido, 1997; Ng et al., 1995). A recent study demonstrated that Pdm2 shows a redundant function to Nub during wing formation (Loker and Mann, 2022). However, little is known about the isoform-specific roles of Nub during the development of adult appendages. In this study, we investigated the involvement of Nub and Pdm2 protein during the growth of larval wing discs.

Results and discussion:

To analyze the expression of Nub and Pdm2 in discs of 3rd instar larvae, we used immunostaining as well as endogenously targeted fly lines (sfGFP knock-in flies). Nub antibody staining confirmed expression of Nub protein in wing, haltere, and leg discs. Both *nub^{sfGFP-NubPB}* and *nub^{sfGFP-NubPD}*, as well as a Pdm2-reporter line show expression in the wing pouch and hinge region. This confirms that Nub isoforms and Pdm2 are present in wing discs and are likely to be involved in their development.

Next, we investigated the role of Nub isoforms in wing disc development using transcript-specific RNAi against *nub-RB* and *nub-RD* in the wing disc pouch. Downregulation of *nub-RD* and *nub-RB* caused a reduction of the size of the wing disc pouch compared to controls. Double knockdown of *nub-RB* and *nub-RD* showed a stronger reduction of disc size, not only in the wing pouch but also in the hinge and notum regions, suggesting Nub isoforms are required for growth of wing disc. Analysis of the mitotic index revealed a significant reduction in the number of mitotic cells (PH3 positive cells) upon *nub-RD* RNAi and in double *nub-RB/RD* RNAi, but not after *nub-RB* RNAi alone. These results suggest a specific role for Nub-PD in the regulation of cell proliferation within the wing disc.

Furthermore, we analyzed the size and shape of adult wings after the downregulation of Nub and Pdm2 proteins. RNAi of *nub-RD* caused a severe reduction in the size of the wing blade and hinge, including defects at the wing

margin and the anterior and posterior cross veins (ACV and PCV). Interestingly, these defects were partially rescued by *nub-RD* overexpression. Next, downregulation of *nub-RB* showed a slightly reduced wing size and mild defects in the ACV, and it also caused melanin deposition in the wing blade, suggestive of an apoptosis. Double knockdown of *nub-RD* and *nub-RB* showed an additive effect on wing size, cross veins, and melanin deposition. However, the wing margin phenotype triggered by *nub-RD* RNAi was instead rescued by *nub-RD/RB* RNAi. Together, these results indicate that Nub-PB and Nub-PD are involved in the regulation of growth and patterning of wings and that a balanced expression of Nub-PD and Nub-PB is required for the correct formation of the wing margin.

To investigate the specific role of Nub and Pdm2 proteins in cell proliferative activity in wing discs, mitotic clones were analyzed using the MARCM technique. The analysis involved the *nub^l* mutant allele, which is almost devoid of both Nub and Pdm2 proteins in wing discs and the *nub^{E37}* mutant allele, which lacks both Nub-PB and Nub-PD but still expresses Pdm2. The *nub^l* mutant clones were small (primarily single cell) and showed strongly reduced mitotic activity, indicating that Nub and Pdm2 proteins are required for cell proliferation. The *nub^{E37}* mutant clones had an intermediate pattern, with fewer cells in the clones but more than in the *nub^l* clones, suggesting a functional compensation of Pdm2 in cell proliferation. Taken together, the results suggest that Nub and Pdm2 play a crucial role in the regulation of cell proliferation and growth of the wing discs in isoform-specific manner.

Conclusions and Future Perspectives:

This thesis work has highlighted some of the pivotal roles played by Nub protein isoforms. We demonstrate the antagonistic functions of Nub protein isoforms in the transcriptional regulation of immune genes. We propose that tuning the activity of these factors is important for the elimination of infections and for regaining and maintaining immune homeostasis. Next, we show that Nub-PB plays important roles in lineage specification and differentiation during intestinal epithelial regeneration. Further, we highlight a novel feature of POU protein function by revealing a direct role of Nub-PD in mitotic cell division, specifically in the maintenance of mitotic spindle organization and dynamics. Finally, we show that the Nub protein isoforms are indispensable in wing development by regulating cell proliferation and patterning in wing discs. Collectively, our findings suggest that Nub protein isoforms are multifaceted proteins that are involved in various processes such as mitosis, tissue growth and regeneration, and immunity.

To better understand the role of Nub-PD in mitosis, it will be important to investigate further how it regulates spindle organization and dynamics at the molecular level. A promising approach would be to identify Nub-PD protein interactors during mitosis and study protein complexes using affinity pull-down and mass spectrometry techniques. That could possibly also provide new insights into evolutionarily conserved functions of POU factors and their roles in pathogenesis and cancer.

Many posttranslational modifications have been documented for mammalian POU proteins affecting their activity and subcellular localization. The Nub-PD protein shows dynamic localization patterns in each phase of mitosis, but how this is regulated needs further investigation. Our preliminary results suggest that Nub-PD is a labile protein, which may undergo degradation in response to infection/stress by yet unknown signals. In the future, it would be valuable to identify the specific protein domains and possible post-

translational modifications of Nub-PB and Nub-PD proteins, to further understand the molecular mechanisms that regulate Nub protein isoform localization and activity in different biological settings.

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