Functional characterization of DNase II in immunity of *Drosophila melanogaster*

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by

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Certificate

This is to certify that this dissertation entitled “Functional characterization of DNase II in immunity of Drosophila melanogaster” towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research represents study/work carried out by Akshata Kotwal at the Global Health Institute, École Polytechnique Fédérale Lausanne (EPFL) under the supervision of Prof. Bruno Lemaitre, Professor, Global Health Institute, EPFL, during the academic year 2023-2024.

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Lausanne May 14 2024

Prof. Bruno Lemaitre
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Declaration

I hereby declare that the matter embodied in the report entitled “Functional characterization of DNase II in immunity of Drosophila melanogaster” are the results of the work carried out by me at the École Polytechnique Fédérale Lausanne (EPFL) under the supervision of Prof. Bruno Lemaitre. This work has not been submitted elsewhere for any other degree. Wherever others contribute, every effort is made to indicate this clearly, with due reference to the literature and acknowledgement of collaborative research and discussions.

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Abstract

DNases are enzymes that play a crucial role in degradation of DNA, essential for innate immune responses of mammals. While the biochemical properties of DNase II have been extensively elucidated in mammals, there is little characterization of its functional role in immunological mechanisms. DNases possess antimicrobial activity, and their absence causes susceptibility to infections, but the molecular pathways underlying this susceptibility are yet to be delineated. In *DNase II* null mutants we discovered that absence of DNase II causes accumulation of apoptotic DNA, developmental defects, increased susceptibility to infection and dysregulation of known immune pathways upon injury. We also further expand on the properties of this enzyme in insects with respect to its subcellular localisation and non-cell autonomous function. We tried to assess the humoral and cellular immune pathways that are classically associated with damage responses to understand the effects of DNA accumulation. In the *DNase II* mutant, hemocytes have regular phagocytic activity and there is no aberration in the JNK pathway, however, we noticed some discrepancies in the IMD pathway. We also tried identifying if a cytosolic sensor of DNA homologous to mammalian cGAS exists in flies and if accumulation of DNA has effects on the cGAS-STING pathway. Lastly, we also explored the role of another DNase, Stress Induced DNase and the possible synergy between SID and DNase II.

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Contributions

Contributor name	Contributor role
Prof. Bruno Lemaitre, Akshata Kotwal	Conceptualization Ideas
Prof. Bruno Lemaitre, Akshata Kotwal	Methodology
-	Software
Akshata Kotwal	Validation
Akshata Kotwal	Formal analysis
Akshata Kotwal	Investigation
EPFL, PT-BIOP and PTCF	Resources
-	Data Curation
Akshata Kotwal	Writing - original draft preparation
-	Writing - review and editing
Prof. Bruno Lemaitre	Visualization
Prof. Bruno Lemaitre	Supervision
Prof. Bruno Lemaitre	Project administration
Prof. Bruno Lemaitre	Funding acquisition

1. Introduction

1.1 Deoxyribonucleases

Enzymes responsible for degradation of nucleic acids are termed nucleases and can be categorized based on their substrate as deoxyribonucleases (DNases) and ribonucleases (RNases) or based on their activity as endonucleases and exonucleases.

Nucleases play integral roles in cellular processes such as DNA replication, recombination, and repair all of which require structural alterations to nucleic acids. Additionally, degradation of nucleic acids is also a major microbial defence strategy and is vital in programmed cell death pathways (Parrish and Xue, 2006). Inefficient removal of either extracellular or endogenously produced nucleic acids can lead to DNA accumulation, which has been associated with autoimmune diseases (Crow and Rehwinkel, 2009).

DNase II, an endonuclease, is responsible for degrading DNA by hydrolysing its phosphodiester backbone (Laskowski Sr., 1967). It functions without apparent sequence specificity for the cleavage site and uses single strand nicking mechanism to fragment DNA (Harosh *et al.*, 1991). Biochemically, mammalian DNase II has a signal peptide and a catalytic domain and yields 5´ hydroxyl and 3´ phosphorylated products(Fig 1.1) (Baker et al., 1998; Krieser & Eastman, 1998). Known as an acid deoxyribonuclease due to its optimal activity in low pH conditions, DNase II localizes in lysosomes and phagolysosomes in vertebrates, owing to their acidic environment (Shpak *et al.*, 2008). Expressed in most tissues, DNase II plays a critical role in phagocytosis-mediated DNA digestion of apoptotic cells (Krieser *et al.*, 2002).

1.1.1 DNases in Mammals

Deoxyribonucleases in mammals are categorized in two families as the DNase I family consisting of DNase I, DNase1L1, DNase1L2 and DNase1L3, and the DNase II family consisting of DNase II α and DNase II β. DNase I and DNase II have distinct mechanisms of action and separate subcellular localisation. The former is suspected to be secreted and performs extracellular roles while the latter performs cellular roles

inside the lysosomal compartment. (Fig 1.1) Due to its optimal activity in lower pH, DNase II is referred to as an acid endonuclease that functions inside the lysosomal vesicles. Additionally, there are other exonucleases like TREX1 and TREX2 enzymes that do not belong to the DNase family but are important in DNA degradation as well.

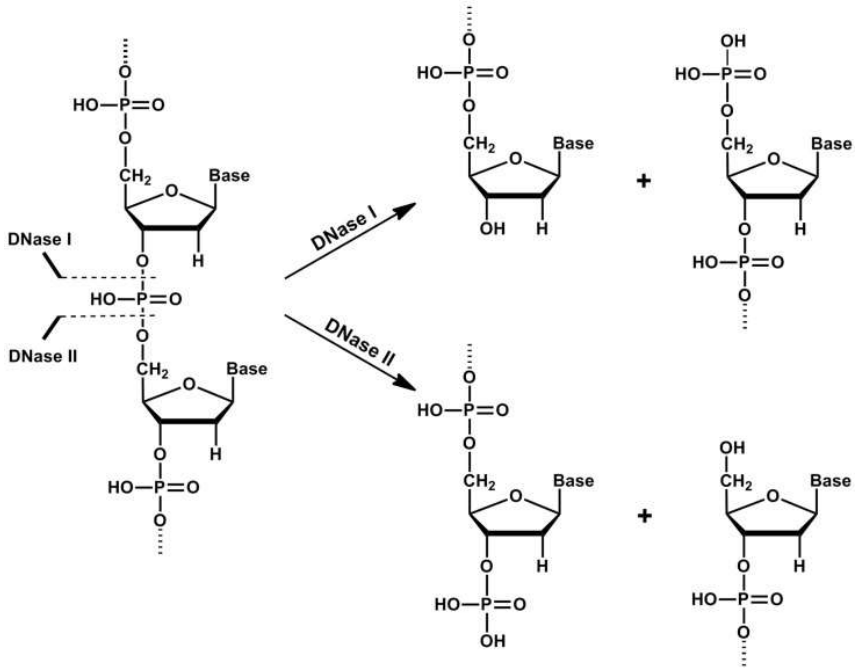

Figure 1.1:Mechanism of Action of DNase I and DNase II (Lauková *et al.*, 2020).

Caspase-Activated DNase (CAD), another DNase present in mice, is said to be complexed in the cytoplasm with its inhibitor ICAD. During apoptotic events, caspases (3 and 7) cleave ICAD, leading to the release of CAD. CAD then translocates into the nucleus to degrade DNA into nucleosomal units. The apoptotic cell is subsequently phagocytosed by macrophages, where the caspase-independent deoxyribonuclease, DNase II, completes the digestion of DNA from the engulfed apoptotic cell in the endo-lysosomal compartment (McIlroy *et al.*, 2000).

Ineffective degradation of apoptotic bodies can activate the innate immune system if the DNA is found in circulation (Hanayama *et al.*, 2004). There can be accumulation of DNA in the cells that can stimulate production of the INF-β response pathway in mice (Yoshida *et al.*, 2005). The resulting higher inflammatory response in absence of pathogens is mediated through the cGAS-STING pathway, and can cause developmental issues and autoimmune diseases (Ahn *et al.*, 2012). Therefore, DNases that digest intra- and extra-cellular DNA are crucial to maintain homeostasis and prevent a sterile activation of innate immunity in mammals.

1.1.2 DNases in *C. elegans*

Human DNase II was well characterised as an enzyme in the 1980s, but its role *in vivo* and its physiological importance was first indicated in *Caenorhabditis elegans* (Lyon *et al.*, 2000; Wu *et al.*, 2000). Identification of *nuc-1,* which encodes the acid endonuclease proved the conservation of DNase II enzyme in invertebrates. Genetic complementation studies, backed up by biochemical and molecular studies revealed that this DNase II homolog was responsible for degrading apoptotic DNA in the phagocytic cells. *nuc-1* mutants showed accumulation of apoptotic DNA in the phagocytosing cells (Sulston and Brenner, 1997). Interestingly, in the mutants with engulfment defect, but wild-type NUC-1 activity, the nuclei of dead cells remained undegraded (Hedgecock *et al.*, 1983). This led to the conclusion that DNase II plays a cell autonomous role and performs DNA degradation only inside phagocytic cells.

1.1.3 DNases in *D. melanogaster*

Drosophila genome encodes for various DNases including Caspase activated DNase (CAD), Stress Induced DNase (SID) and DNase II that function as endonucleases. *CG7780*(dDNase II) was identified as a *Drosophila* homologue of DNase II and is further referred as DNase II in this thesis. Focusing on *Drosophila melanogaster* immunity, Mukae et al. 2002, identified the possible roles of DNase II. They show that in *Drosophila,* upon apoptosis, CAD, and DNase II work independently to degrade chromosomal DNA. There was absence of DNA fragments in CAD/ICAD mutants while there was accumulation of chromosomal DNA in hypomorphic mutants of *DNase II*. As described in mammalian models, apoptotic signals trigger CAD to digest nuclear DNA of the apoptotic cells into nucleosomal units while further phagocytosis of apoptotic cells leads to degradation of DNA by DNase II, activated in the phagocytic cell. Alongside this, the hypomorphic mutant DNase II^{lo} also showed constitutive expression of Diptericin and Attacin, two AMPs regulated by the Imd pathway (Further discussed in signalling pathways). This study suggests that if endogenous DNA is not degraded, the escape of DNA in *DNase II* mutants can be recognised and activate the immune system.

The predicted AlphaFold structure of DNase II (Fig1.2) and sequence analysis methods annotate amino acids 1-25 as a signal peptide, indicating potential targeting towards secretory pathway. However, due to its lysosomal role, it remains uncertain whether dDNase II is secreted or functions autonomously in cells. DNase I in mammals is known to be secreted as an extracellular protein but does not have a homologue in *Drosophila*. Hence, we need to explore further to determine if DNase II plays a dual role in flies.

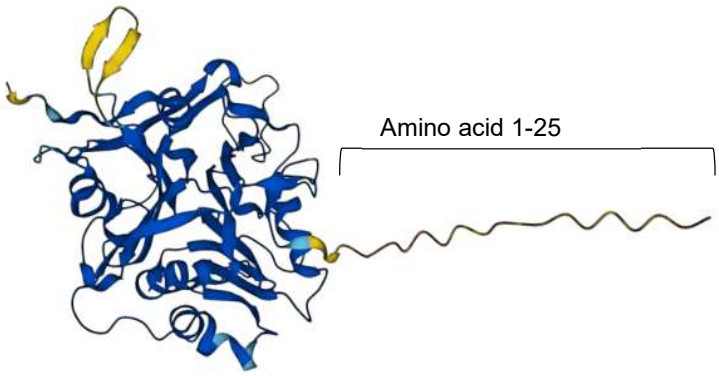

Figure 1.2: AlphaFold predicted structure of CG7780 dDNase II. The structure resembles the structure of mammalian DNase II, hence has been predicted to have a similar mechanism of action. It contains a twenty-five amino acid long signal peptide at the N-terminus.

1.2 *Drosophila* Immunity

1.2.1 Immune defence mechanisms

Drosophila employs several mechanisms in its host defence system, as described below.

A) Disease Avoidance

Natural habitats of fruit flies are microbe rich. Given the cost of mounting an immune response, behavioural mechanisms serve as the first line of defence against microbes. Grooming behaviour not only helps *Drosophila* to remove dust particles and harmful environmental pathogens but also plays a role in antennal cleaning maintains the olfactory cues for protective function. Modulation in feeding behaviour and other physiological adaptations allow *Drosophila* to avoid re-ingestion of food from a pathogen-rich source.

B) Immune Resistance

Behavioural mechanisms are valuable but often insufficient to prevent infection by pathogens and immune responses play a critical role to resist microbial infections. Unlike vertebrates which possess both adaptive and innate immune responses, *Drosophila melanogaster* relies only on innate immunity to combat diseases. Epithelial barriers prevent the physical entry of pathogens (Davis and Engström, 2012) but evasion of these barriers by the pathogen, results in its entry in the body cavity. This leads to activation of the cellular and humoral responses due to the presence of Pathogen-Associated Molecular Patterns(PAMPs) (Lemaitre and Hoffmann, 2007). Janeway & Medzhitov, 2002 define PAMPs as conserved, microbes-specific motifs that can help distinguish between infectious non-self and self. Bacterial peptidoglycan, lipopolysaccharides, viral nucleic acids and fungal β-glucans (Gottar et al., 2002, Imler et al., 2000, Dostert et al., 2005, Brown & Gordon, 2005) are examples of well recognised PAMPs in *Drosophila*. The detection of pathogens mounts an immune response by activating downstream signalling pathways that can collectively help in decreasing pathogen load. Overall, the elimination of pathogens from the host as a result of activation of immune system can be defined as immune resistance.

C) Disease Tolerance

While mechanisms of immune resistance are effective, they can be metabolically costly and have detrimental effects on the host tissues. Hence, disease tolerance mechanisms occur concurrently to minimize host damage and maintain homeostasis (Råberg *et al.*, 2007). Sensing of damage associated molecular patterns (DAMPs) and homeostatic parameters such as osmolarity, temperature, oxygen concentration can trigger downstream pathways like JAK/STAT pathway(Chakrabarti *et al.*, 2016) and induce expression of heat shock proteins (Ritossa, 1962). Additionally, organisms undergo physiological adaptation to maintain metabolism in conditions of stress such as starvation and dehydration. Ultimately, the immune response of the organism and the pathogen load itself can inflict considerable damage on the host, which is combated by an induction of stress response pathways. Some such pathways converge in response to physiological activities such as apoptosis, necrosis and in unfavourable physiological conditions. An example of such a mechanism is Turandot proteins which are induced in different physiological stresses and after infections to protect the host from effects of AMPs. (Rommelaere *et al.*, 2024)

There is often a trade-off involved in biology, with one trait increasing fitness at the expense of decreased fitness in another trait. Immune resistance often entails metabolic and energetic expenses and can lead to tissue-damage; but it increases overall fitness by reducing pathogen load. The trade-off shifts progressively from resistance to tolerance over the course of the infection. However, some pathways may have a role in both processes. We suspect DNase activity to be one such pathway, which might help in clearance of foreign DNA as an immune resistance mechanism and clearance of apoptotic DNA as a disease tolerance mechanism.

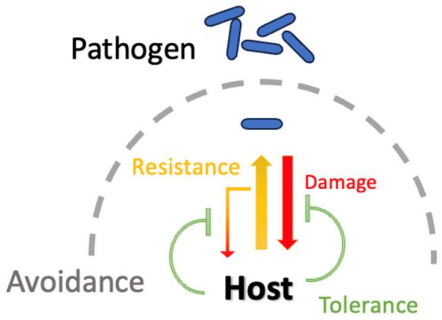

Figure 1.3: The three complementary defence mechanisms shaping the innate immune response. Avoidance limits pathogen exposure, resistance lowers pathogen number and tolerance limits damage caused by the infection and resistance mechanisms.

1.2.2 Immune Responses in *Drosophila Melanogaster*

A) The epithelial immune response

The first line of non-specific immune responses involves physical barriers like the outer cuticle, peritrophic matrix and mucus lining in the gut, which limit the invasion of pathogens into the hemocoel (Chihara and Kimbrell, 1986; Capo *et al.*, 2019). Although the tracheal system in *Drosophila* allows for the exchange of gases, it can also become accessible to pathogens. Hence, localised secretion of AMPs by the barrier epithelia, both constitutively and inducible, has evolved as a host defence mechanism (Tzou et al., 2000, Gendrin et al., 2013).

Local generation of Reactive Oxygen Species (ROS) upon detection of pathogens in the gut is another mechanism to eliminate microbes. Duox and Nox are two NADPH oxidases involved in the rapid production of ROS to decrease pathogen load in the gut (Benguettat *et al.*, 2018; Iatsenko *et al.*, 2018).

B) The humoral immune response

Systemic infection in flies leads to secretion of effectors into the hemolymph upon detection of PAMPs. The fat body of the fly, analogous to mammalian liver, synthesizes and secretes various peptides and proteins into the hemolymph to eliminate microbes through antimicrobial activity, nutrient sequestration, and fulfilling host's metabolic requirements (Lemaitre and Hoffmann, 2007). Iron sequestration involves leaching of free iron from hemolymph and its relocation to the fat body. This is another adaptation to reduce microbial load, as iron is required by the pathogen for its metabolic processes (Iatsenko *et al.*, 2020). Several innate immune responses exist in flies and some of them are further discussed in Section 1.2.3.

C) The cellular immune response

In addition to the humoral immune responses, the host also mounts a cell-mediated defence mechanism through circulating immune cells called hemocytes that are the "blood cells" of arthropods.

Plasmatocytes: 90-95% population of hemocytes is represented by plasmatocytes throughout different developmental stages(Lanot *et al.*, 2001). They are analogous to mammalian macrophages and mediate the process of phagocytosis wherein large particles (>0.5 microns) are engulfed by the cell, a conserved mechanism across evolution for clearance of pathogens and apoptotic debris. Similar to mammalian macrophages, plasmatocytes undergo receptor clustering, activation and zippering of plasma membrane around the microbe (Pearson *et al.*, 2003). These phagocytic cells are responsible for engulfing and digesting not only circulating pathogens but also of self-debris such as apoptotic and necrotic particles. A plethora of receptors on the plasma membrane of plasmatocytes activate downstream signalling cascades upon ligand binding (Melcarne *et al.*, 2019; Ramond *et al.*, 2020; Petrignani *et al.*, 2021). An uptake machinery assembles for engulfment and phagosome formation. To acquire bactericidal activity, these phagosomes undergo maturation by fusion with endosomes and lysosomes. Digestive hydrolases, such as proteases like Cathepsin and nucleases like DNase II, require an acidic pH for their activity and are released in the lumen of phagolysosome, to degrade proteins and nucleic acids. Phagolysosomes maintain a low pH of 4.5-5 for the optimal enzymatic activity and pathogen clearance (Kocks *et al.*, 2003; Seong *et al.*, 2014).

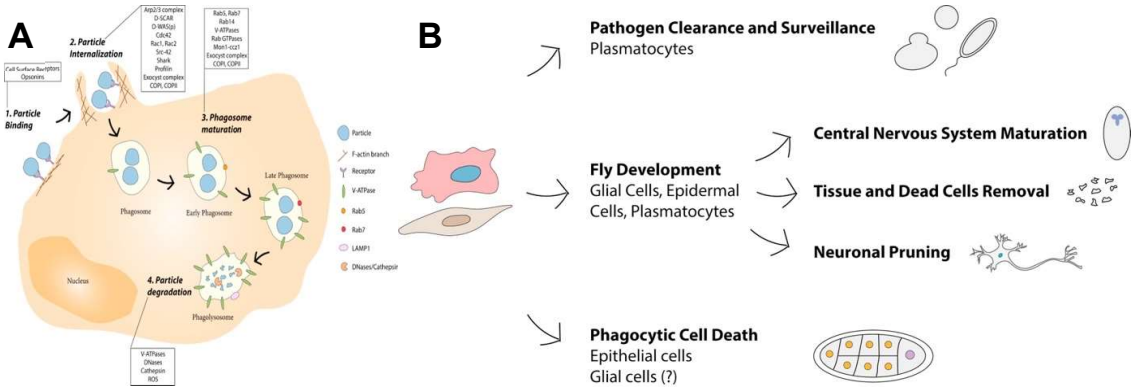

Figure 1.4: Phagocytosis in *Drosophila* (Melcarne *et al.*, 2019) A)Engulfment and digestion of unwanted cellular debris, performed by hemocytes. Phagolysosome formation is important to create a suitable environment for the enzymes to perform degradation. B) It plays major roles to maintain host physiology and disruption in any of the steps can lead to developmental defects and susceptibility to infection.

Crystal cells: Constituting 5-10% of the blood cell population, these are non-phagocytic cells responsible for an innate immune process called melanisation, triggered upon injury. This is a key process for effective wound healing, pathogen elimination, microbial sequestration and parasite encapsulation (Dudzic *et al.*, 2019).
Lamellocytes: Rarely found in healthy organisms, these are large cells exclusive to larvae. They mediate the process of encapsulation upon wasp infestation. Wasps are parasites that use *Drosophila* larvae to lay their eggs. Hemocytes differentiate into lamellocytes upon such an infection and these cells are responsible for containing and eliminating wasp eggs (Binggeli *et al.*, 2014).

1.2.3 Steps of *Drosophila* Immune Response

A) Detection

The recognition of molecular patterns associated with infectious non-self by pattern recognition receptors (PRR) initiates the first step in mounting an immune resistance response. Gram-positive bacteria are recognised through their characteristic Lysine-type peptidoglycan by peptidoglycan recognition protein (PGRP)-SA, while gram-negative bacteria are recognised by various receptors like PGRPSD, PGRP-LC and PGRP-LE through their DAP-type peptidoglycan cell wall (Gottar *et al.*, 2002, 2006; Leulier *et al.*, 2003).

Sensing of viral nucleic acids occurs through intracellular protein DICER, that activates RNAi response or by the cytosolic sensor cGAS-like receptor (CGLR) (Holleufer *et al.*, 2021).

DAMPs, released by self-cells can also trigger an immune response upon recognition as they are perceived as “danger” signals by the organism. These signals are often released by apoptotic or necrotic cells following tissue damage, either from sterile injuries, post infection with a pathogen or also by tumour cells. An example of such a DAMP would be α-actinin which activates the JAK-STAT pathway in *Drosophila* (Brown and Gordon, 2005). Self-DNA has also been implicated as a DAMP that can lead to sterile inflammation and requires digestion by DNase (Mukae *et al.*, 2002).

B) Signalling Cascade

Upon detection, different receptors trigger various immune responses. These responses have been comprehensively reviewed by Lemaitre & Hoffmann, 2007. Depending on the recognised ligand, the signal cascade is used to mount a specific immune response by secretion of effector molecules.

Upon detection of gram positive and negative bacteria by different receptors, they activate either the Toll or Imd signalling pathways using various molecules as depicted in the Fig 1.5. The transcriptional factor Relish mediates downstream Imd signalling for the transcription of antibacterial peptide, Diptericin. The Toll and Imd pathway are essential immune pathways in flies, and disruption in any of these pathways renders flies immunocompromised. The Imd pathway, critical in response to Gram negative bacteria, interacts with the JNK and cGAS-STING pathway. Therefore, the upregulation and downregulation of the Imd pathway offers insights into multiple signalling cascades that are activated in case of any dysregulation.

Different signalling cascades lead to transcription of distinct set of genes based on required immune activity, although these signalling cascades are often intertwined.

This study particularly focuses on JNK pathway and cGAS-STING pathway in *Drosophila* to potentially elucidate the molecular mechanisms of DNase II and its role in innate immunity of the fly.

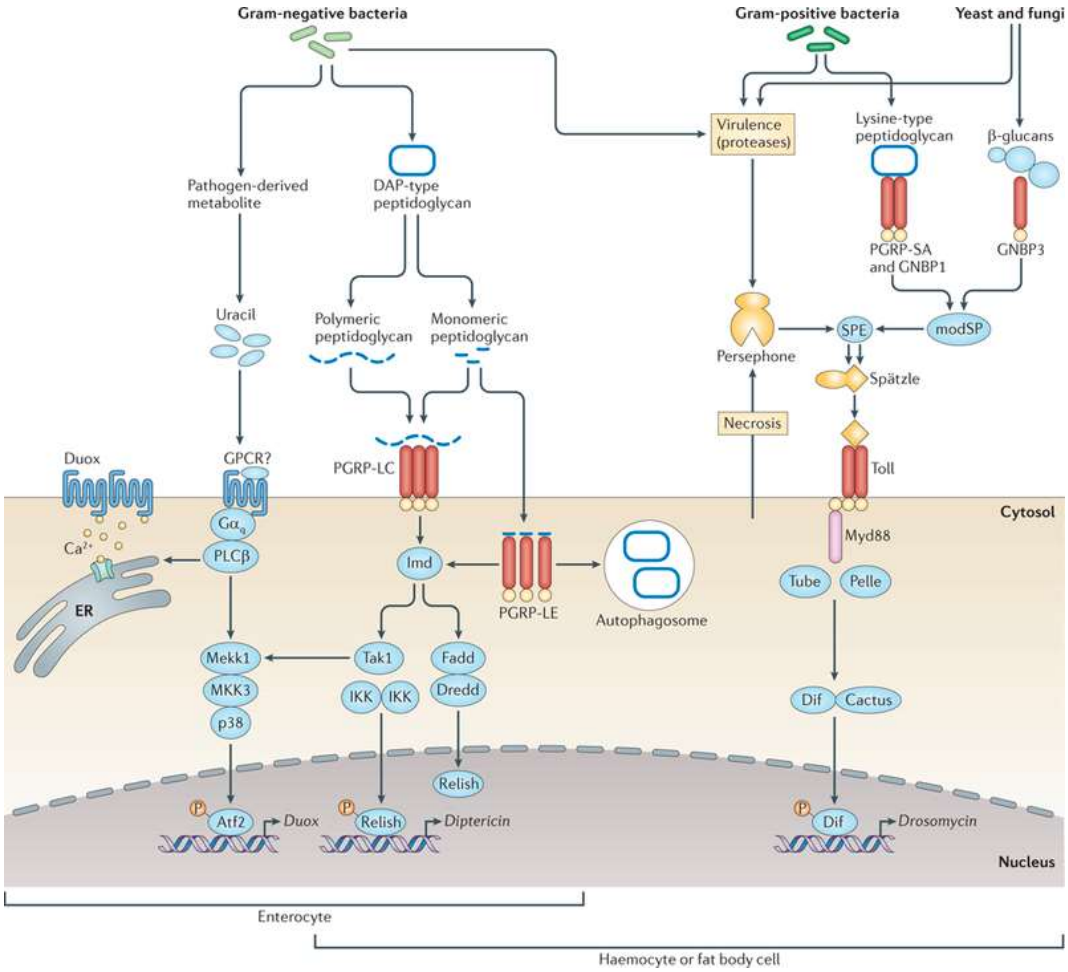

Figure 1.5: Detection, Signal Cascade and transcription of Immune effectors upon entry of Gram positive or Gram negative bacteria.(Buchon *et al.*, 2014)

The JNK pathway

The c-Jun N-terminal Kinase (JNK) pathway belongs to the mitogen-activated protein kinase (MAPK) family and is evolutionarily conserved. Activation of this pathway can be triggered by Eiger (Egr), the extracellular ligand binds to one of the two TNF receptors, Grindelwald (Grnd), or Wengen (Wgn). This binding activates Tak1, which in turn activates hemipterous (hep). Hep triggers the phosphorylation of Basket (Bsk), the sole *Drosophila* JNK protein, leading to the phosphorylation of d-Fos and d-Jun (AP-1) transcription factors (Weston and Davis 2002). AP1 promotes the expression of the family of Upd cytokines and matrix metalloproteinases MMP1/2. Another target is Puckered (Puc), a JNK phosphatase that establishes a negative feedback loop and is often used as a readout for activity in the JNK pathway.

The JNK pathway also intersects with the Imd pathway through Tak1 and participates in diverse biological processes (Davis 2000). In *Drosophila*, it plays roles in development, cell proliferation and differentiation, wound healing, stress induced responses, apoptotic processes, DNA damage, and immunity (Yoshida et al. 2005; Shen and Liu 2006; Delaney et al. 2006; Arthur and Ley 2013). Relish-mediated Tak degradation inhibits JNK signalling, regulates immune mediated apoptosis (Park *et al.*, 2004). Some in vitro studies have also highlighted an immunostimulatory role for this pathway, by identifying the JNK pathway as a crucial regulator of the AMP response, but this remains speculative (Kallio et al. 2005).

The cGAS/STING pathway

Extensive research over the past decade has led to thorough understanding of the cGAS-STING pathway, a highly conserved mechanism to detect foreign DNA (Morehouse *et al.*, 2020). In mammals, this function is carried out by the cyclic GMP-AMP synthase(cGAS)-stimulator of interferon genes (STING) pathway. The binding of cGAS to double-stranded DNA (dsDNA) in the cytoplasm, initiates the cascade, resulting in the production of 2'3' cyclic GMP-AMP (cGAMP). cGAMP then activates STING, which is present in the endoplasmic reticulum(Ishikawa and Barber, 2008; Sun *et al.*, 2013) which in turn, activates IRF3 and TBK1, producing a robust type I interferon response and inducing various inflammatory mediators(Decout *et al.*, 2021). The high conservation of this immune pathway from bacteria to mammals has spurred interest in assessing its relevance in insects (Fig. 1.6B).

dmSTING(CG1667) is the *Drosophila* ortholog of human STING with a structurally conserved domain essential for binding cyclic dinucleotides (CDNs) (Martin *et al.*, 2018). Subsequently, a study revealed the transcriptional induction of dmSTING post ZIKA virus infection via the IMD pathway, pointing to an antiviral role for dmSTING (Liu et al. 2018). dmSTING was also suggested to act upstream of Relish, stimulating the induction of AMPs while studying the antimicrobial activity of dmSTING against Listeria monocytogenes (Martin et al. 2018).

Finally, a genetic approach was used to identify cGAS-like receptors (cGLRs) in *Drosophila*, revealing that cGLR1 and cGLR2 are both involved in sensing foreign RNA upon viral infection((Holleufer *et al.*, 2021; Slavik *et al.*, 2021)). It was then delineated that the antiviral response was mounted through a cGLR-CDN-STING mediated pathway, very similar to that found in mammals (Cai *et al.*, 2023).

Collectively, the recent discovery of the cGAS-STING pathway in *Drosophila* underscores the significance of a novel aspect in insect innate immune pathways playing a role in DNA sensing.

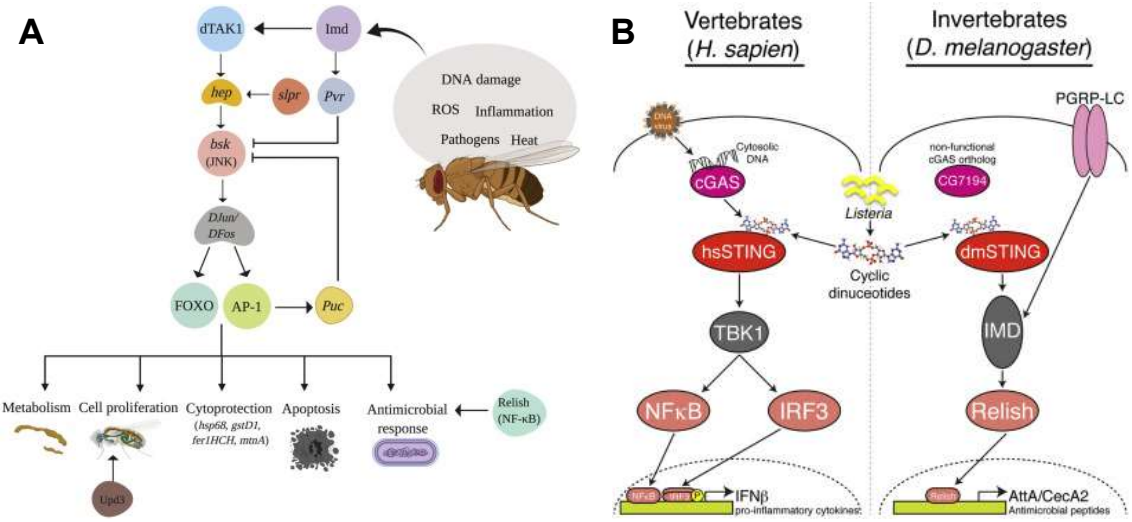

Figure 1.6: Immune Responses in the Fly A)JNK pathway triggered by DAMPs(Tafesh-Edwards and Eleftherianos, 2020). B)cGAS-STING pathway in mammals and *Drosophila*(Martin *et al.*, 2018).

C) Immune effectors

Upon pathogen recognition, activation of various signalling cascades leads to transcription of effector molecules that assist in fighting off the infection. Some examples include antimicrobial peptides (AMPs) for immune resistance, Turandots for disease tolerance and potentially DNase II for both roles. These effector molecules are transcribed in response to specific cues and play key roles in survival of the fly. Microarray studies in the late 1990s helped profile gene expression changes in response to stimuli, uncovering more than 400 genes called "*Drosophila* immune-regulated genes" (DIRGs) that have differential expression following microbial challenges(De Gregorio *et al.*, 2001). The Toll and Imd pathways are the main regulators of this response, while other immune signalling pathways discussed above also contribute to the production of various effectors. While most of these genes encode proteins with well-defined functions, like AMPs, transferrins in iron sequestration, catalases for detoxifying reactive oxygen species (ROS), and serine proteases for melanisation, many of the identified genes and the proteins they encode remain uncharacterized.

1.3 Role of DNase II in *Drosophila*

DNase II is the enzyme responsible for degrading DNA in *Drosophila*.

An exposure to self-DNA can lead to reduced fecundity and developmental defects (Colombo *et al.*, 2023). Particularly, lethality was observed at the larval stage during development, accompanied by a significant delay in development of adults from the eggs and a decrease in number of eggs laid when *Drosophila* was fed with self-DNA. Undigested DNA is suspected of being one of the immune elicitors, fitting into the category of both PAMPs and DAMPs. It activates the cytosolic receptor cGAS in mammals, and with the recent discovery of CGLRs in *Drosophila*, there is a need to explore the molecular basis of DNA recognition in fruit flies. While the biochemical properties of DNase II have been extensively elucidated, there is little characterization of its functional role in immunological mechanisms. DNases possess antimicrobial activity (Deng *et al.*, 2022) and hypomorphs of *DNase II* were previously found to be more susceptible to microbes (Seong *et al.*, 2006). We seek to determine if DNase II plays a role in disease tolerance or immune resistance, or both, and the molecular pathways involved and choose *Drosophila* as our model organism due to the ease in genetic manipulation.

DNase II's involvement in *Drosophila* innate responses was first suggested by Seong *et al.*, 2006. They used the hypomorphic mutant of CG7780 called *DNase II*^{lo} and observed increased susceptibility to infection by both Gram-positive and Gram-negative bacteria. However, the specific role of DNase II in immune responses remained unclear. Transcriptomic analysis by De Gregorio *et al.*, 2001, revealed upregulation of *dnase II* and *sid* upon infection, pointing to a potential role of DNase II and SID as immune effectors.

Our lab generated *DNase II*^{SK4}, a null mutant line of DNase II and Dr. Alexia Carboni characterised it. Again, the flies lacking DNase II showed increased susceptibility to bacterial infections. However, examination of the Toll and IMD pathways in adult flies post-infection revealed normal expression of antimicrobial peptides (AMPs), and the pathogen load decreased normally in the mutants. These findings suggest that death of infected DNase II mutants may not result from an inability to combat pathogen itself, but rather from ineffective degradation of debris generated during pathogen clearance.

A more recent study also identified a novel stress induced DNase (SID) in *Drosophila*, and suggested role of SID in protecting flies from the toxicity of accumulated DNA (Seong *et al.*, 2014).

The recent discoveries of cGAS-STING pathway in flies have focussed on their role in innate immunity against viruses. Among key players in this pathway are cGLRs, with cGLR1(CG12970) and cGLR2(CG30424) identified as PRR for nucleic acids (Holleufer *et al.*, 2021). In *Drosophila melanogaster,* CG7194 is the third identified cGLR but lacks a known ligand and is a compelling candidate for a PRR involved in DNA sensing. Given DNase II is responsible for degradation of DNA, its dysfunction can lead to stimulation of such a pathway.

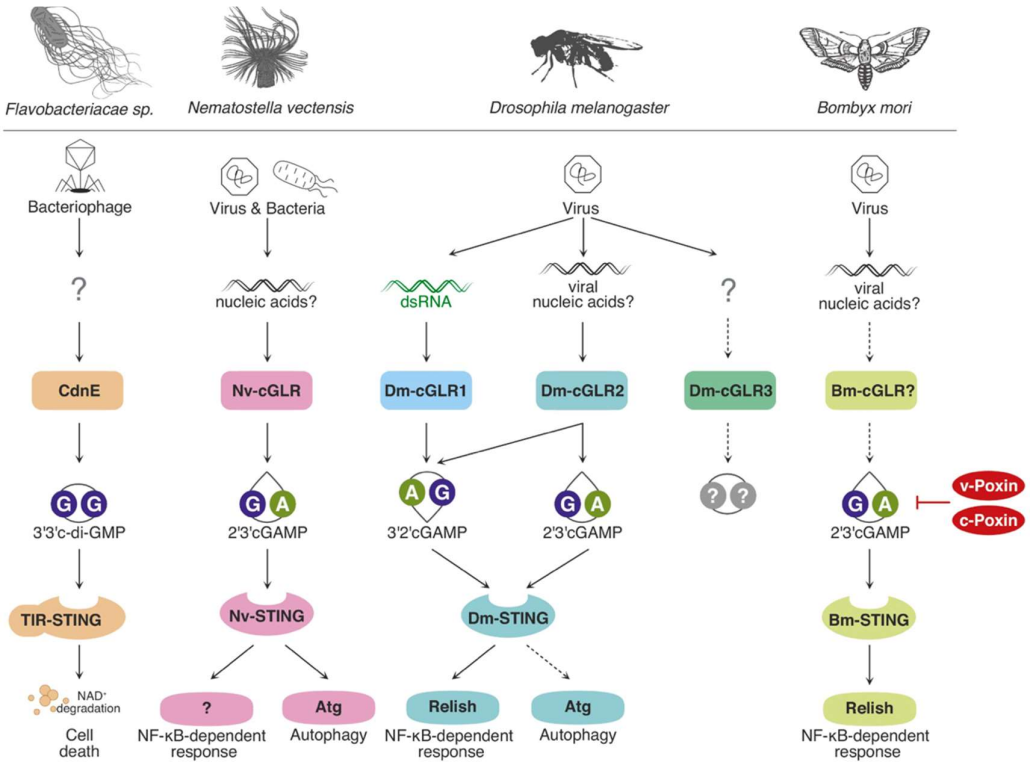

Figure 1.7: Conservation of cGAS-STING pathway in invertebrates. Adapted from (Cai *et al.*, 2022)

The gene *dnase II* is strongly expressed in the larval fat body (Supplementary Fig 9), while hemocytes serve as the primary phagocytosing cells. This raises the question whether DNase II functions as a non-cell autonomous factor in *Drosophila* contrary to its mammalian counterpart. Absence of other deoxyribonucleases also raises futher question regarding multifunctional roles of DNase II.

1.4 Hypothesis and Aims

My goal is to characterise the role of DNase II in the Drosophila immune response.

1.4.1 Hypothesis

DNase II is responsible for degradation of pathogen associated and apoptotic DNA. It is a lysosomal enzyme, involved in the process of efferocytosis and its deficiency can lead to incomplete digestion of debris and hence accumulation of DNA. Apoptosis often occurs during different developmental stages and also after an injury, pertaining to the role of DNase II during both these processes. Ineffective clearance of DNA can result in phagocytic defects and activation of immune pathways pathway as the accumulated DNA can function as a DAMP.

If these pathways cause inflammation in the fly, then knocking down different genes from these cascades can help rescue the phenotypic abnormalities.

1.4.2 Aims

1. To characterize expression and activity of DNase II.
2. Examine the effect of absence of DNase II on life history traits of the fly.
3. Examine its effect on humoural and cellular response pathways.

2. Materials and methods

2.1 Fly stocks and Genetics

Yeast-cornmeal food (6% yeast, 6% cornmeal, 0.62% agar, 0.1% fruit juice supplemented with propionic acid at 4.9mL/L and Moldex at 10.6g/L) was used to raise fly stocks at 25° C. Unless stated otherwise, experiments were performed at wandering late stage L3 larvae(6-7 days after egg laying) and 4-6 day old adult flies at 25° C. In case of all mutations, homozygous organisms were used and isogenised *w*^{1118} Drosdel flies (w^{iso}) were used as control. CRISPR-Cas9 was performed as described in Kondo & Ueda, 2013. A list of all the fly lines used is mentioned in the Supplementary Table 6.1. Except the DNaseII^{SK4} mutant line, all lines used in the study have been isogenised.

Table 2.1: Genotypes of the Fly Lines majorly used.

Stock Genotype	**Chromosome number**	**Description**
DptB GFP	3	Reporter of IMD pathway
;;DptB GFP, DNaseII/ TM6 c	3	Reporter combined with mutation
;;DNaseII^{Sk4}	3	1bp deletion, frameshift, null mutation
;;SID^{HDR22}	3	CRISPR mutant, deletion, null mutant
+ ; Sting^{JL} ; +	2	4 bp deletion in the Sting gene
;Sting^{JL} ; Dnase II/ TM6c	2 & 3	Sting + DNase II mutation
;Sting ^{JL} ; DptB GFP, DNaseII^{Sk4}/ TM6c	2 & 3	Sting + DNase II mutation with DptB reporter
Tre-RFP	2	JNK Reporter
Tre-RFP/CyO; DNaseII^{Sk4}/TM6b	2 & 3	JNK Reporter combined with DNase II mutation
; CGLR1&2/Cyo ; DNaseII^{Sk4}/TM6B	2 & 3	Combined triple mutant: CGLR 1 and 2 Knockout+ DNase II mutation
w1118 ; hmlGal4, UAS GFP	2	hemocyte driver
hml GAl4 , UAS GFP/ CyO ; DNase II sk4	2 & 3	hemocyte driver combined with Dnase II mutation
ptwR UAS DNase II-RFP	1	Insertion with DNase II-RFP
lppGal4 (TS)	3	fat body driver (thermo sensitive)

2.1.1 Egg laying and tracking developmental cycle.

Equal number of newly eclosed (virgin) males and females are kept in a cage and made to lay eggs on a grape juice plate over the span of 6 days and number of eggs laid are counted each day. Due to deficient media, the flies lose their capacity to lay eggs much faster.

To track development, 100 eggs of each genotype, laid within a span of 4-5 hours are collected in a tube. These are then monitored to calculate number of L3 larvae, pupae, and adults at different days during the life cycle. As the L3 larvae become pupae within a day, the graph shows number of larvae on each day whereas in case of pupae, it is cumulative. The number of adults eclosing each day is also observed and is cumulative, however, even in case the adult dies soon after, it is counted as we are tracking the development in this experiment and not longevity.

2.2 Microbial culture

Bacteria were cultured overnight on a shaking plate at 180 RPM. The following morning, they were pelleted by centrifugation (4000 RPM at 4°C) and the bacterial pellets were diluted to the desired optical density (OD)600 at 600 nm. *Pectobacterium carotovorum carotovorum 15* (Ecc15) and *Micrococcus luteus* were grown in LB medium at 29°C.

2.3 RT-qPCR

RT-qPCR was used to measure mRNA content that shows gene expression levels. Five larvae/wholes flies were taken and frozen at -20°C, 2 or 4 or 6 hours after the infection/ injury/ pinching as specified in the experiment. They were then homogenized and TRIzol reagent was used to extract their RNA. This was resuspended in RNase-free water and 50mg RNA in 10 µl reaction mix containing PrimeScript RT TAKARA mix with random hexamers and Oligo dTs was used to perform reverse transcription. Quantitative PCRs were performed on a LightCycler 480 (Roche) using PowerUp SYBR Green Master Mix.

2.4 Phagocytosis assay

2.4.1 Apoptotic Bodies Preparation

50µg/mL cycloheximide is added to S2 cells cultured in Schneider media to induce apoptosis. 24 hours later, it is centrifuged at 400g at 4°C for 5 min. The supernatant is stained with CFSE (5(6)-CFDA/SE Molecular Probes™) at concentration of 5µM and incubated for 15 min in the dark at RT. The apoptotic bodies are collected and washed by two centrifugation steps at 3000g for 20 min at 4°C. After each centrifugation, the pellet is re-suspended in Schneider medium while the supernatant is discarded. Concentration of apoptotic bodies is measured in CytoFLEX.

2.4.2 In-Vitro Assay

Five 3rd instar larvae are cleaned and vortexed briefly to detach all hemocytes. They are then bled into 150 µL Schneider media for exactly 2 minutes, and then collected in Protein LoBind eppendorfs. Approximately 20,000 apoptotic bodies are added per sample and incubated at room temperature for 1 hour, after which they are immediately shifted on ice and cells are counted in CytoFLEX, Beckman Coulter.

2.4.3 Cell counting

HmlGal4>UAS GFP larvae are used to gate hemocyte population. As hemocytes from these larvae express GFP, the cells are gated using B525 channel. GFP- positive cells that show up are marked in a gate(red) as shown in Figure 2.1. The rest is debris or other cells that are not hemocytes(black). This gating is further used in all genotypes to analyse the hemocyte population by counting the total number of hemocytes, the number of cells in positive gate for B525(to account for presence of CFSE) and the intensity of fluorescence.

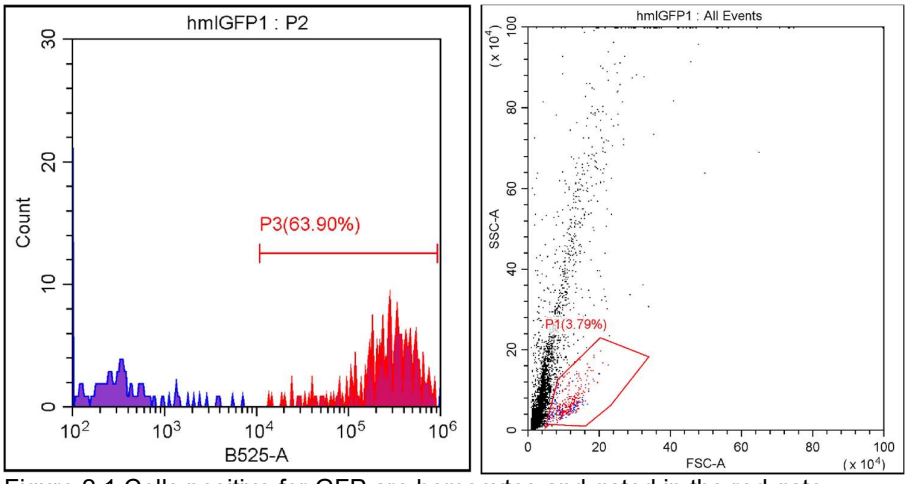

Figure 2.1 Cells positive for GFP are hemocytes and gated in the red gate

Using the information obtained from above, fraction of phagocytosing cells and phagocytic index is calculated to assess the phagocytic ability. The same also gives us a total number of hemocytes in each sample. Here, 60µL of sample is read at a medium speed 30µL/min to calculate all three aforementioned readings.

$$Fraction\ of\ phagocytosing\ cells(f) = \frac{Number\ of\ cells\ positive\ for\ B525A\ signal}{Total\ number\ of\ cells}$$

$$Phagocytic\ Index = Mean\ fluorescence\ Intensity\ of\ hemocytes\ x\ f$$

2.5 Microscopy

2.5.1 Hemocyte collection

5-10 cleaned 3rd instar larvae, depending on the experiment are taken per sample and opened in 150 µL Schneider media supplemented with 1 µM PTU, making sure that none of the vital organs are damaged. They are incubated in a humid chamber for 40 min and allowed to settle and adhere to the slides.

2.5.2 TUNEL staining.

Ten larvae are used. 4% PFA is used for 15-20 min to fix cells, followed by washes in PBS. They were then permeabilised for exactly 2 min with freshly prepared 0.1%PBS-Triton, 0.1% sodium citrate tribasic dihydrate solution and rinsed in PBS. It was incubated with 50 µL of reagents (5:45) of In Situ Cell Death Detection Kit, TMR red (Roche) for 1 hour at 37°C. Phalloidin-Alexa Fluor 488 was used at dilution 1/100 (Thermo Fisher Scientific, A12379) for 1-2 hours. During the 3rd wash of PBS-T, DAPI was used at 1:20000 dilution for 10 min. It is then washed in PBS thrice before mounting it with Dako Fluorescence Mounting Medium (Agilent).

2.5.3 Immunofluorescence

6-7 larvae are used to collect hemocytes and cells are fixed with 4% PFA as described above and are then washed thrice with 0.1%PBS-Triton for 5 min. Blocking is done with 2%BSA for 1 hour at RT, followed by incubation with primary antibody overnight at 4°C. It is then incubated with secondary antibody for 1 hour, followed by staining with Phalloidin and DAPI as described above.

Primary Antibodies: Invitrogen rat Anti-mCherry(M11217) is used at 1:100 to detect RFP as it shows reactivity towards RFP as well as mCherry. Rabbit anti-LAMP(Ab30687) was used at 1:500 to detect matured lysosomes.

Secondary Antibodies: Goat anti-rabbit AlexaFluor 555 and goat anti-rat AlexaFluor 555 are used at 1:1500.

2.5.4 Live imaging

Lysosomal Compartments: Collected hemocytes are exposed to LysoTracker™ Green DND-26(1:1000 in PBS) for exactly 2 minutes and cells are immediately imaged. As the cells express RFP, they did not need to be stained.

Apoptotic Bodies: 50µL of stained apoptotic bodies are added as soon as hemocytes are collected. The one-hour incubation ensures their settling, and they are then washed with PBS once and imaged.

2.5.4 Imaging and Visualisation

Leica SP8 is used for imaging and hemocytes are imaged using 60x oil immersion lens. FIJI- Image J is used for visualization of the images. Maximum Z- projections are taken in most cases except for live imaging and unless specified.

2.6 Western blot

6-7 3rd instar larvae are crushed in 300µL RIPA buffer with protease inhibitor (Complete Inhibitor Roche, diluted 1/50) and PMSF(1mM) by homogenisation. It was then on maintained on ice or at 4° C. A 5-minute centrifugation at 1500rpm, followed by 15 min centrifugation at 15000rpm is done to remove debris and lipids, respectively. The supernatant was collected in both cases and was used for BCA analysis to equalise total amount of protein in all samples. 2µg/µL protein concentration was achieved and 30µL of this was then mixed with 1x NuPAGE® LDS buffer and 10mM Dithiothreitol (DTT). Samples were incubated at 95^{o}C for 5 minutes and then 10 µL was loaded on a Novex 8% precast Glycine gel to separate proteins through SDS-PAGE at 100V. Invitrogen iBlot was used to transfer proteins onto a nitrocellulose membrane. The membrane was blocked for 1 hour in 5% non-fat dry milk in PBST (0.1% Tween in PBS), followed by an incubation overnight with primary antibody at 4°C. Anti-RFP (1:1000 in blocking) is conjugated with HRP, so this was followed by 3 washes with PBST and the antibody was detected using SURESIGNAL Western Substrate (lubioScience). ChemiDoc XRS+ (Bio-Rad) was used to image the membrane. To equalize for housekeeping gene, mouse anti-tubulin (1:2000) was used as primary antibody and the three washes were followed by incubation with Goat anti-mouse-HRP secondary antibody (Jackson ImmunoResearch) diluted at 1:15000 at

room temperature (RT) for 1h. The signal was detected same as before after 3 washes. The antibodies were diluted in blocking solution.

2.7 Infection and Survival

Infection: 4 to 6-day old adult flies or 3rd instar larvae were pricked in the thorax or at the posterior end respectively with a 100µm thick needle dipped into a concentrated pellet of bacteria at desired OD_{600}. Infected flies and larvae were then either frozen at -20 for RT-qPCR after as many hours as desired or were maintained at 25 were then maintained at 25°C to note survivals. Systemic infection with DNA was done by injecting 23.5nL *Drosophila* DNA (250ng/µL) using a nanoinjector and glass capillary needles.

Survival: To analyse survival during stresses, the number of flies were scored every 2-4 hours except for 12 hours during the night. 20 flies of each sex, each genotype was kept in a single tube and two such vials were maintained. They were maintained with steady circadian rhythm and stable oxygen supply to minimize effects of any other stress response pathways. Flies were also flipped every two days to control microbiota load. Survival experiments were performed using a Cox proportional hazards (CoxPH) regression model in R 1.4.1103.

2.8 Statistical analysis and Software

All experiments were performed in triplicates unless otherwise indicated. All data represented as mean ± SD (standard deviation) with error bars and individual values indicated, calculated using Microsoft Excel and GraphPad Prism v.9. Each point in the graph represents one biological replicate, unless otherwise said so. Unpaired student's t-test and one-way ANOVA test was used to determine significance (ns = not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) and is specified in the legends.

2.8.1 Table 2.2: Software used for data analysis and visualization.

Software	**Source**
BioRender®	BioRender
Fiji (Fiji is Just ImageJ)	Open source
GraphPad Prism v9	GraphPad Software Inc
SnapGene Viewer	Dotmatics
Leica Application Suite x (LAS X)	Leica Microsystems

3.Results

3.1 DNase II is *Drosophila* Immune Regulated Gene (DIRG)

Microarray analysis of adult *Drosophila* revealed that infection with a mix of Gram negative and positive bacteria led to the upregulation of 230 genes (De Gregorio et al., 2001). *DNase II* and *SID* were two such genes upregulated in female adult flies upon infection (Fig.3.1A-B). RT-qPCR was used to check the mRNA levels of *dnaseII* and *sid* upon pricking late L3 larvae with a mix (OD=200) of Gram-negative (*Ecc 15*) and positive bacteria (*M. luteus*). Larvae were collected at different time points post-infection and also pricked with a sterile needle to serve as a control for injury since sterile injury can also mount immune responses in the fly. There was an induction of *dnase II*, 2 hours after infection with sustained upregulation until 6 hours post-infection coinciding with the onset of pupariation (Fig 3.1 C). This indicates a consistent immune response between larvae and adults, albeit clean injury also elicited a similar response so the response may have been an effect of injury rather than infection. Interestingly, unlike in adults, SID was not induced upon injury in L3 larvae (Fig 3.1 D).

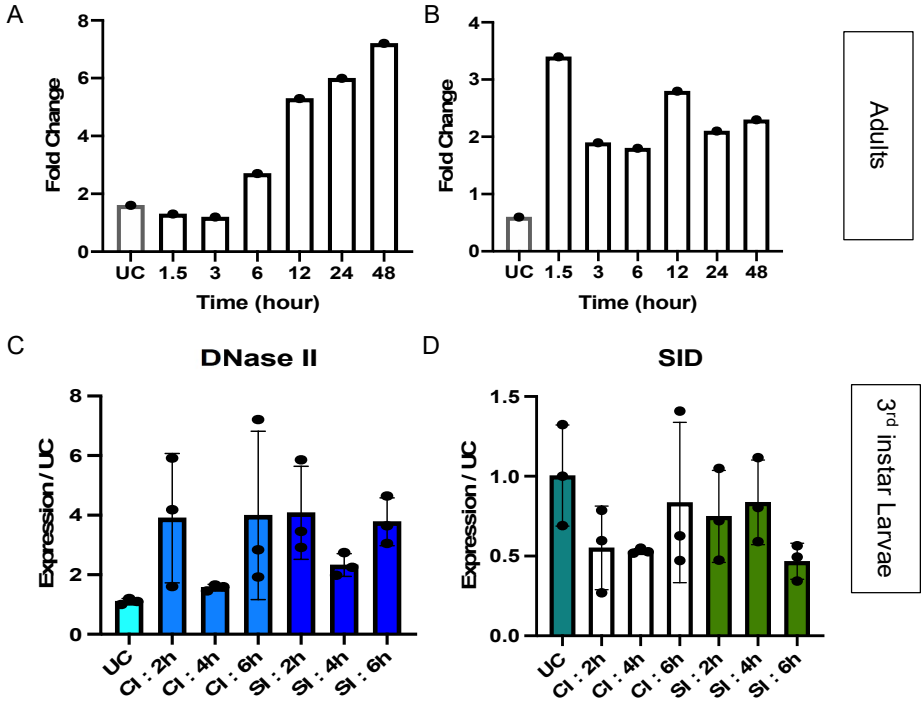

Figure 3.1: Induction of DNase II and SID upon bacterial infection. (A&B) Transcriptomics data showing induction of DNase II and SID in adult flies upon infection with a mix of *Ecc15* and *M. Luteus*. (De Gregorio et al., 2001) (C&D) RT-qPCR of 3rd instar larvae at different time points (2h, 4h and 6h) after clean injury (CI): ethanol cleaned needle and septic injury (SI): Equal mix of *Ecc15* and *M. luteus* (OD)

3.2 UAS-DNase II^{RFP} as a tool to check properties of DNase II

3.2.1 DNase II is secreted and localised in lysosomes.

The construct of UAS-DNase II, with the protein tagged with RFP at the C-terminus, was generated using a transposable element inserted in the first chromosome. To validate the construct, we induced its overexpression in the fat body (LppGal4>UAS DNase II^{RFP}) and performed Western Blot analysis using HRP-conjugated anti-RFP. Positive control lines were included and the UAS DNase II^{RFP} line (lacking Gal4) served as a negative control. Given that molecular weight of DNase II is 41.3kDa and RFP is 27kDa, the observed band's weight confirmed the successful fusion of RFP tag with DNase II (Fig3.2.1 A). Interestingly, upon overexpression from hemocytes, DNase II^{RFP} was being secreted. Nephrocytes of *Drosophila* larvae were positive for RFP when DNase II^{RFP} expression was driven by fat body, hemocyte & salivary gland drivers, connoting its secretion (Fig3.2.1B-D).

Given the similarity with mammalian DNase II, we hypothesized its presence in the lysosome. DNase II tagged with RFP was overexpressed in hemocytes using hmlGal4> UAS DNase II^{RFP} and the subcellular localisation of RFP was vesicular (Fig 3.6.1 G) and not cytosolically distributed. Subsequently, we checked its co-localisation with lysosomal compartments. Lysotracker, that labels acidic compartments in cells during live imaging and Drosophila anti-LAMP1 antibodies, targeting lysosomal proteins, were utilized. Some co-localisation was detected using the *Drosophila* anti-LAMP1 and anti-RFP antibodies along with live imaging with Lysotracker (Figure 3.6.1 E and F-H respectively).

1.2.1 Activity DNase II in hemocytes

We drove overexpression of DNase II^{RFP} in hemocyte using hmlGal4> UAS DNase II^{RFP} and incubated the hemocytes with CFSE stained apoptotic bodies and after 1 hour of incubation, there was only little co-localisation of the apoptotic bodies with the DNase II-RFP (Fig 3.2.2 A, B). But with longer incubation, co-localisation increased. As this was an *ex vivo* phagocytosis assay with live imaging, the RFP signal fainted with time. Video 1 is a run through the z stacks after 1.5 h of incubation and shows the co-localisation of apoptotic body and DNase II^{RFP} possibly inside a vesicle (Fig 3.2.2 C: snapshot from the video). It can hence be speculated that as phagolysosome matures after engulfment of apoptotic corpses, DNase II might play an active role.

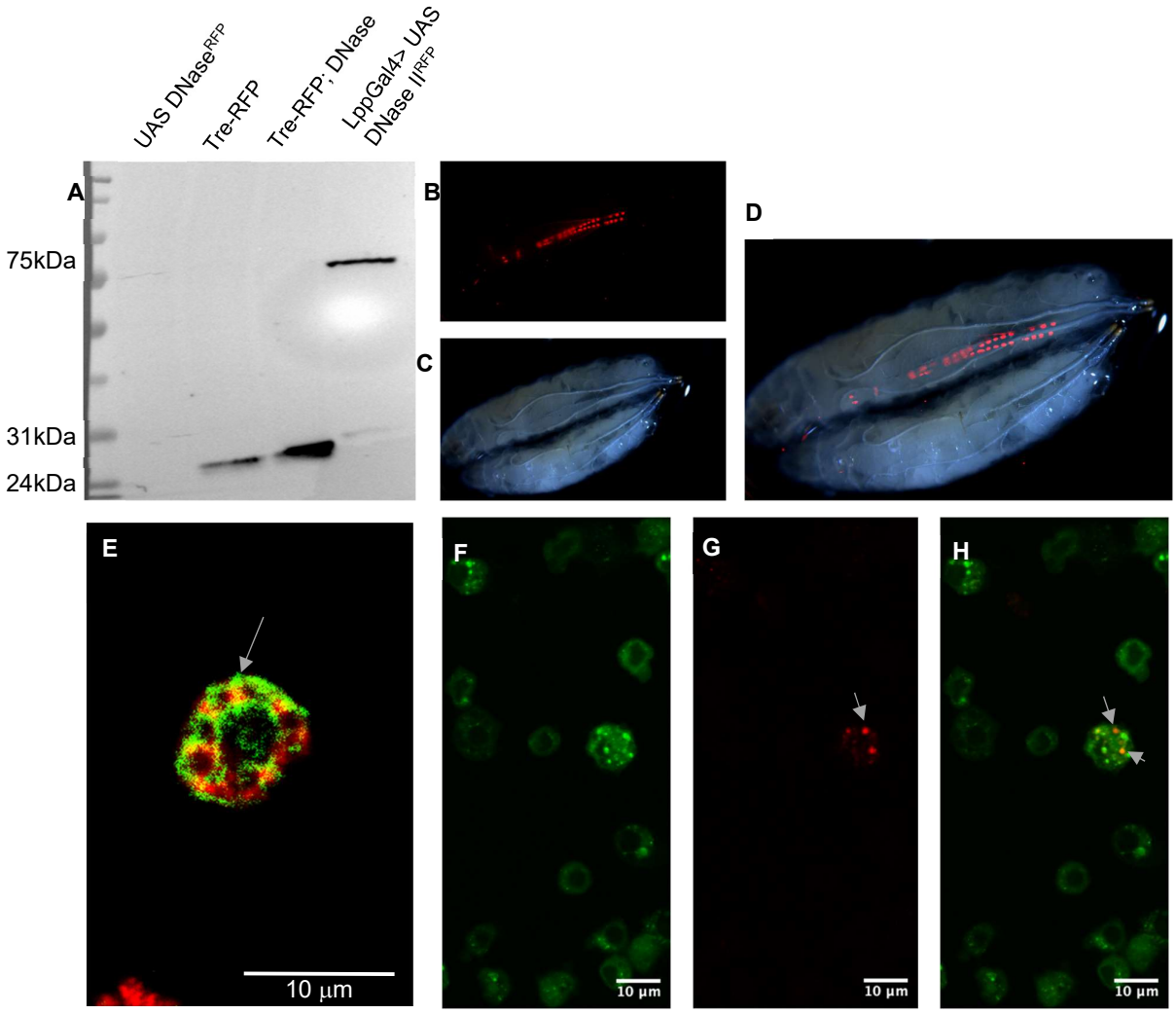

Figure 3.2.1 DNase II- RFP tagged protein and its subcellular localisation A) Western Blot confirming the tag attached to DNase II. B) DNAse II-RFP: red C) larvae D)Merged;presence of RFP in nephrocytes E) co-localisation of anti-LAMP1(green) anti-RFP (red) creating yellow vesicles F) Lysotracker (Green) on live cells G) DNase II^{RFP} expression in live cells H) merged, showing colocalization of protein in an acidic vesicle.

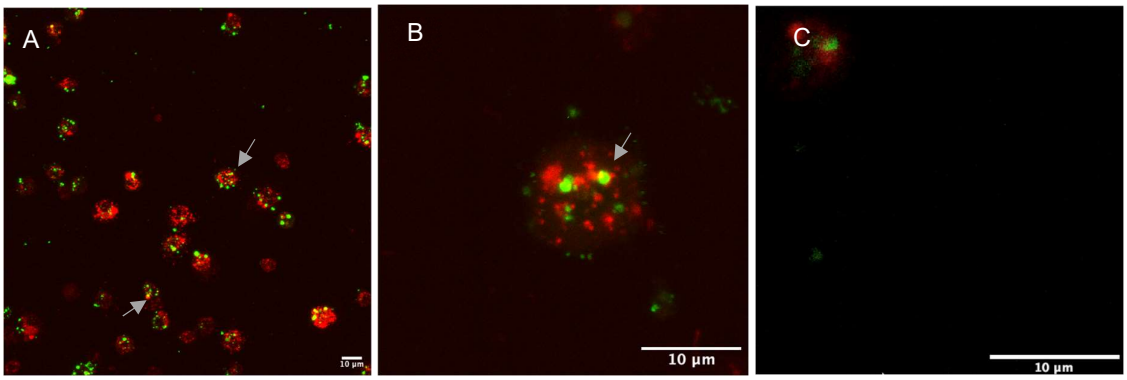

Figure 3.2.2 Role of DNase IIRFP during efferocytosis (A) Apoptotic Bodies(green) incubated with hemocytes for 50 min (hmlGal4>UAS-DNase II^{RFP}) B) After 1 hour 10 min of incubation C) Screenshot from Video 1 after incubation for 1 hour 30 min.

3.3 Loss of DNase II causes defects in certain life history traits

To investigate the role of DNase II in the host defence, we generated a null mutant line. *DNase II*^{SK4} is a CRISPR-Cas9 generated DNase II-null line with one base pair deletion in *dnase II(CG7780)* gene that introduces a stop codon, stalling the translational of DNase II protein (Sup. Fig 1). The flies are homozygous viable but show strong defects in some life-history traits.

DNase II deficient flies lay lesser eggs compared to their wild-type counterparts overall (Fig 3.3 A). Generally, very young females lay lesser eggs, and the egg laying capacity increases with age, achieves a peak and reduces with further ageing. There is no disruption in this cycle of egg laying in the mutant (Sup. Fig 2).

Using synchronised embryos, we noticed a significant delay in development of DNase II mutants. The delay in development increases during progressive stages of life cycle (Fig 3.3 B- D).

DNase II^{SK4} flies have developmental defects leading to lethality and much lesser progeny eclosing from equal number of eggs. To check the stage of development that is affected, we assess individuals at different steps and find that number of larvae hatching from eggs is not the determinant step and equal number of larvae emerge from the eggs. However, the fraction of larvae that convert into pupae shows a stark difference (Figure 3.3 C). The number of pupae were calculated cumulatively while the number of larvae were not but the developmental defects are likely occurring during pupariation, leading to lesser adults emerging from larvae. The number of adults eclosing from the pupae does not show significant difference, either. However, the adults that emerge are much weaker.

A CRISPR mutant of SID(CG9989): SID^{HDR} with a deletion in the gene is used along with DNase II^{SK4} for life span analysis. The *DNase II* mutants were significantly weaker than control flies while the *SID* mutants were not. The life span of *DNase II*^{sk4} was much lesser than normal (Fig 3.3 E).

Overall, absence of DNase II deficiency leads to lesser egg laying, developmental delay, lethality during development as well as lower life span of the adult flies. We conclude that during development, this lethality is at the larval stage.

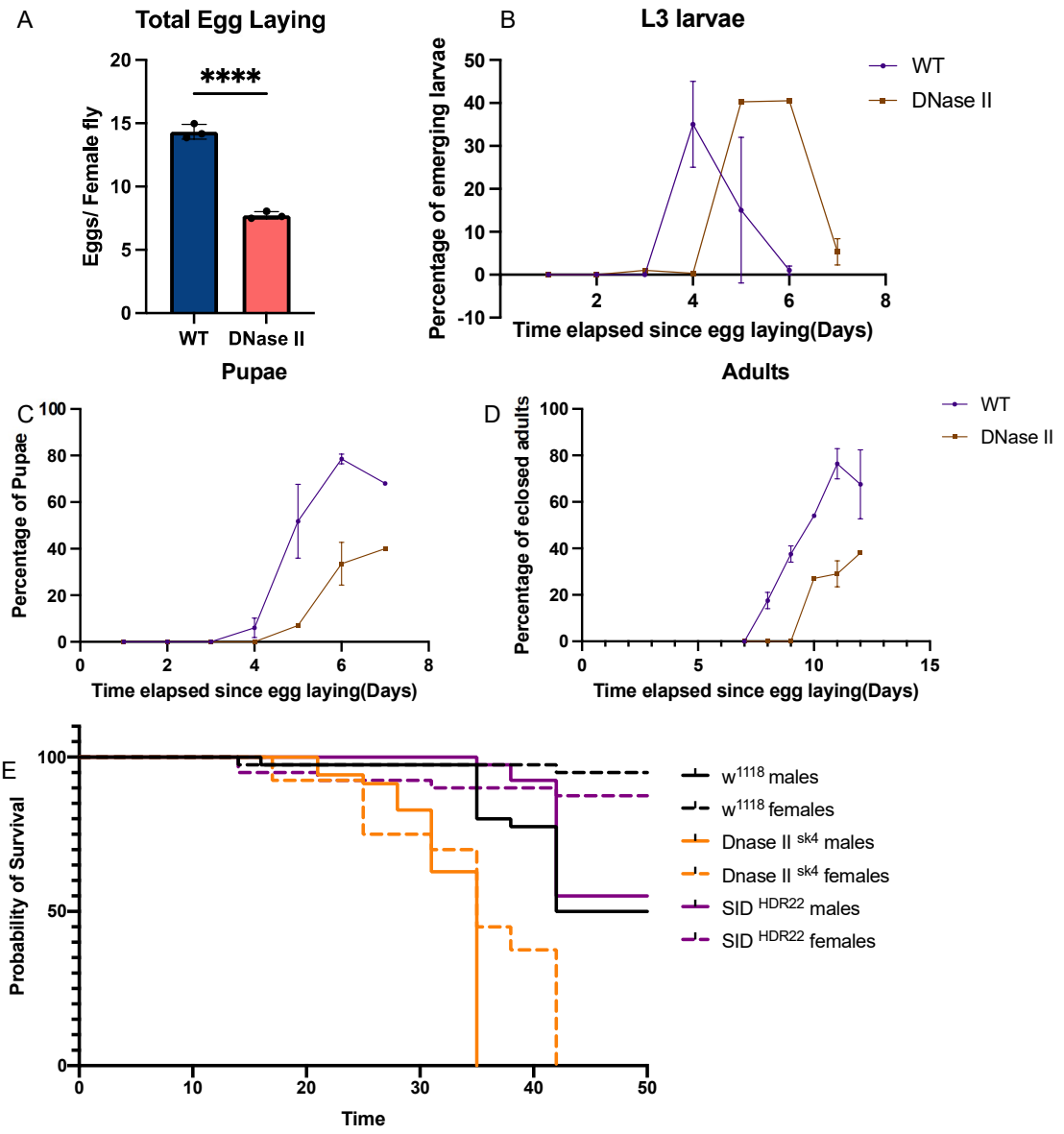

Figure 3.3: Developmental defects due to DNase II deficiency. A) Egg laying capacity is reduced in the mutants B) Eggs take longer to develop into L3 larvae. 24 hours of delay is observed C) The delay in development continues to pupae; also, much fewer pupae develop from equal number of eggs. By day 7, there are no more L3 larvae. D) Much lesser adults emerge from equal number of eggs; however, this percentage is the same as percentage of pupae emerged, hence, there is no pupal lethality while eclosing from pupae to adults. The delay in development increases to 36-48 hours. No more adults emerge after day 13. Three independent experiments were done with eggs collected from the same mothers in 2 experiments, and with new mothers in the 3rd experiment to avoid handling biases. E) Longevity of DNase II^{SK4} and SID^{HDR} mutants.

3.4 Effects of deficiency in these DNases

3.4.1 DNase II^{SK4} is susceptible to bacteria and has Motor Defects

DNase II deficient flies were checked for susceptibility to bacterial infections and showed susceptibility to both Gram positive and negative bacteria (Fig 3.4.1 A). Only results from *Ecc 15(Gram -)* are shown, but equivalent results were obtained for *E. faecalis (Gram +)* as well. This was previously also demonstrated using DNase II^{lo} (by Seong *et al.*, 2006) i.e. the hypermorphic mutants, hence it establishes DNase II^{SK4} as a good mutant line to study DNase II deficiency. The DNase II flies were also observed to be weak and would rarely climb unlike other flies. Climbing assay revealed motor defects in DNase II^{SK4} flies as they showed poor climbing compared to the control (Fig 3.4.1 B). As the high bacterial load could have been a source of this defect, we also checked axenic flies, but they did not show any improvement in the same.

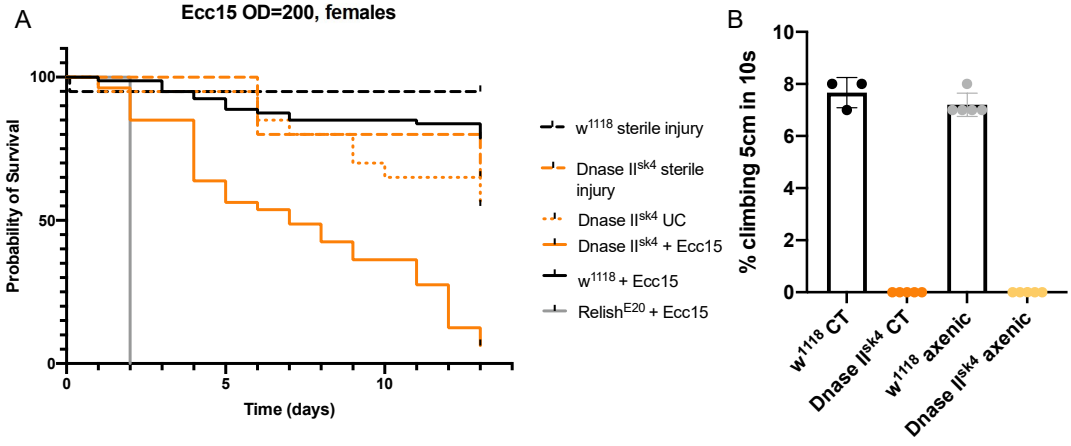

Figure 3.4.1: DNase II mutants are susceptible to bacterial infections and show climbing defects. Experiments performed by Dr. Alexia Carboni during her PhD.

3.4.2 Stress Induced DNase null mutants do not succumb to stress.

SID^{HDR} mutants containing a deletion in SID were used to assess its susceptibility to stress as null mutants.

Thermal stress was given by keeping the flies at 34° C. Normally, flies perform optimally at temperatures between 18° C and 29° C. Thermal stress causes the flies to die in 4-5 days (Fig 3.4.2A).

Osmotic stress was given by feeding the flies food containing 4% NaCl. Higher salt concentration causes osmotic stress killing the flies within 2-3 days. (Fig 3.4.2B)

Dehydration stress was given by putting the flies in empty vials, so there was no food and water for the flies. This stress comprised of both dehydration and starvation stresses. Parallelly, only starvation stress was also given to the flies by raising them on medium made with only 1.8% agar. As starvation alone also did not show any significant aberrations between the mutant and control flies, only dehydration stress is shown here which would encompass both. (Fig 3.4.2C)

None of the stresses increased lethality in the SID mutants. Generally, males die sooner than females and the same trend was followed but there was no apparent defect in the SID mutants.

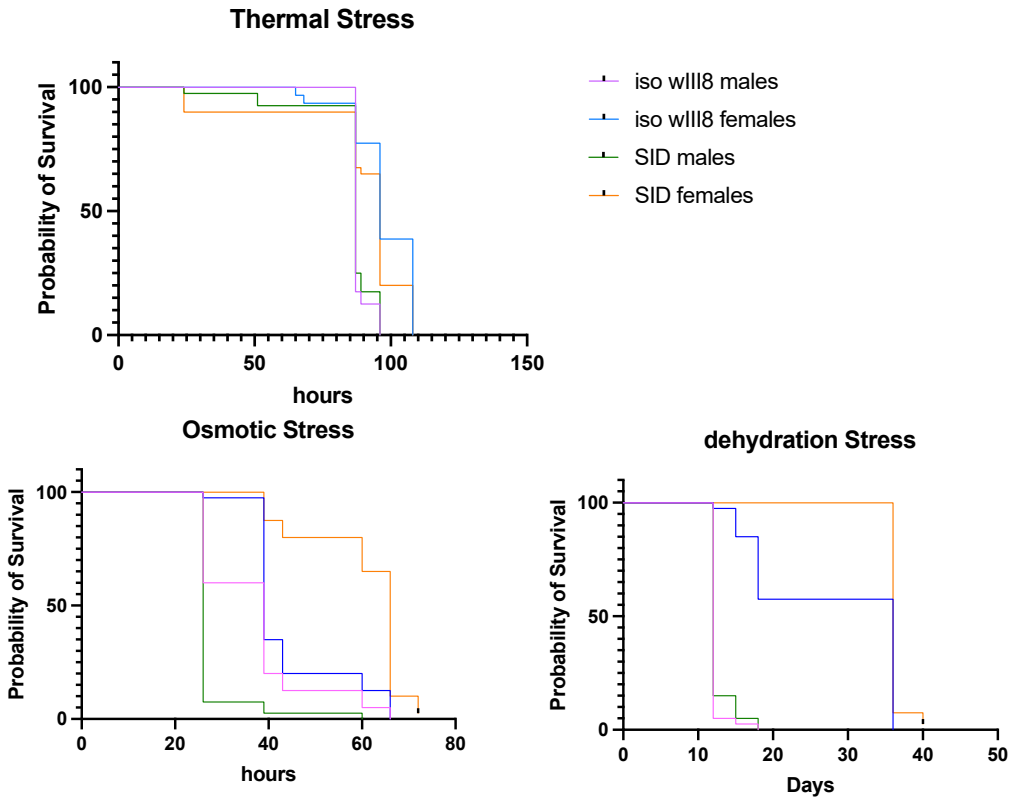

Figure 3.4.2. Stress Response in SID mutant. A) Thermal Stress, 34°C B) Osmotic Stress: 4% NaCl and C) Dehydration stress: Flies kept in empty vials, so simultaneous starvation. SID here refers to SID^{HDR} i.e. the mutant. Two sets of 20 flies/genotype were used for each sex in each condition and survival was plotted.

So, loss of DNase II rendered flies more susceptible to infections and motor-neuron impairments suggesting its role in one of the two processes but we could not find the potential role of SID in osmotic, starvation and thermal stresses or life span of a fly.

3.5 DNase II deficiency causes anomalies in hemocytes.

As previously indicated, DNase II might be essential in digestion of phagocytosed DNA; thus, we investigated defects in the macrophages of *Drosophila*, i.e. the plasmatocytes. We used hmlGal4>UAS GFP line to label the plasmatocytes. Hemocytes in the DNase II mutants were less sessile. Typically, late 3rd instar larval hemocytes are evenly distributed in sessile pockets within each segment and in the lymph gland, the primary hematopoietic organ in larvae. These pockets are usually attached to the cuticular epidermis. In our *DNase II* mutant, these pockets were entirely absent except in the posterior section of larvae and the lymph gland to be much more densely packed compared to that of wild type larvae. (Fig 3.5 A and B)

$Sting^{JL}$, is a null mutant of CG1667 i.e. dmSTING (described in Cai *et al.*, 2020) and we generated a double mutant by combining it with *DNase* II^{SK4}. If inflammation through cGAS-STING pathway led to any phenotypic abnormalities, disrupting the STING pathway would potentially rescue them.

Using flow cytometry-based assays, we compared the total number of hemocytes in DNase II^{SK4} mutants and observed no significant difference between wild-type and mutant larvae (Fig 3.5 C). We initially gated the line using hmlGal4>UAS GFP line, with *NimC1;Eater* mutant serving as a positive control, with known hematopoietic defect, characterized by a high number of hemocytes (Petrignani *et al.*, 2021).

However, during microscopy experiments, we found the number of hemocytes a significantly lower number of hemocytes in the slides of *DNase* II^{SK4}. Interestingly, the double mutant: *Sting*^{JL} *; DNase* II^{SK4} has normal number of hemocytes. As we observed higher number of hemocytes in the *Sting*^{JL} mutants, it prompted speculation about potential compensatory or rescue mechanism at play. An example of individual frames is provided in the Supp. Fig. 4. Illustrating this observation which remained consistent across independent experiments.

Due to contradictory but reproducible results, we did not conclusively determine the exact defect in hemocytes. We considered the reasons for these differences. Our microscopy techniques involve the settling of plasmatocytes, requiring their adhesion to the slides to be visible in imaging. Also, during cytometry experiments, larvae are briefly vortexed to break their sessile pockets, a step avoided during microscopy where only circulating hemocytes are collected. These differences may explain discrepancies in the two results and give a clue regarding the real defect in haematopoiesis.

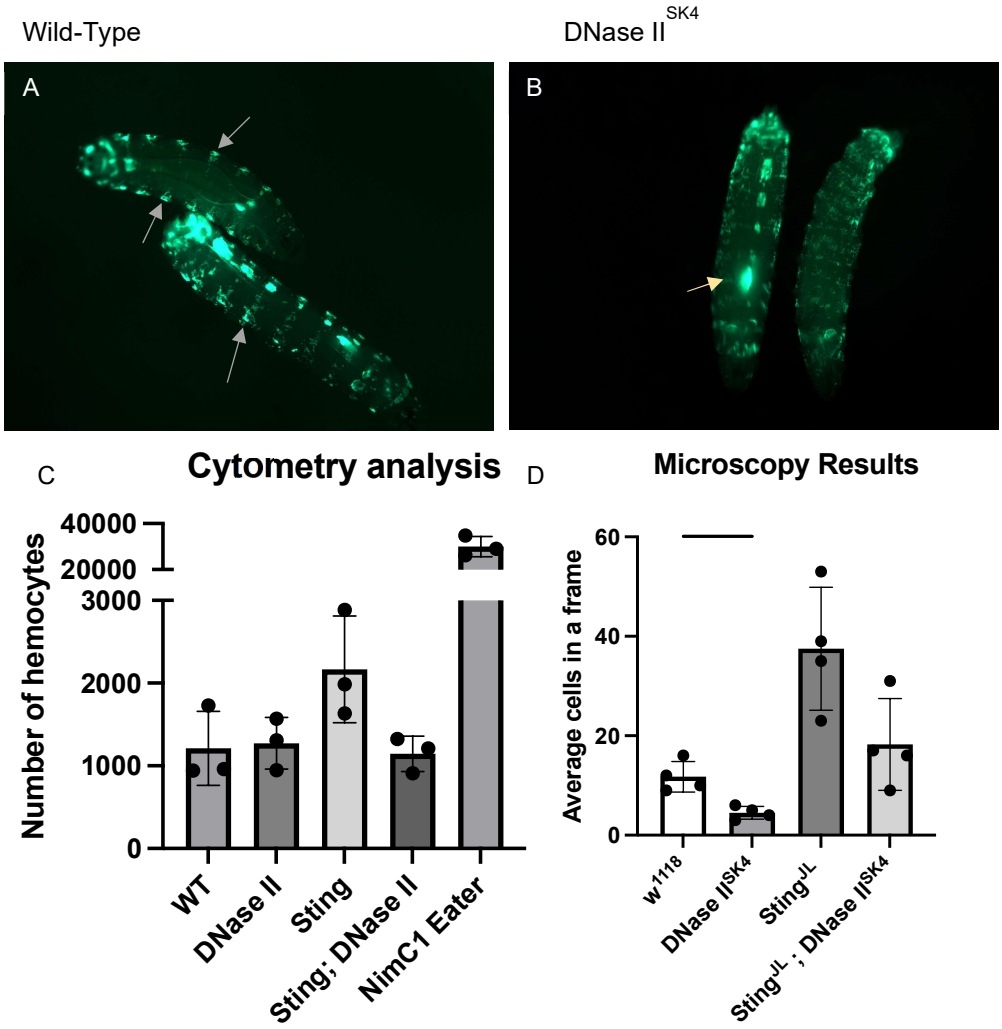

Figure 3.5 Defects in hemocytes of DNase II^{SK4}. (A and B) Comparing WT and DNase II^{SK4} larvae, both with hmlGal4>UAS GFP construct. Grey arrows in WT indicate the stereotypical sessile pockets in segments of WT larvae, absent in the mutant larvae B) the yellow arrow indicates the highly fluorescent lymph gland in DNase II mutant (C) Flow Cytometry used to check total number of cells and no significant difference is found between WT and DNase II (D) Number of plasmatocytes on average in one frame after staining with Phalloidin 488 and DAPI. ~10 frames per experiment and n=4 independent experiments. Equal number of larvae were taken in the beginning and number of hemocytes in one frame were counted manually by considering stained actin filaments (Phalloidin) of the cells with stained nucleus (DAPI). Difference between WT and DNase II is statistically significant (**, $p<0.005$)

3.6 Phagocytic Ability of Hemocytes

Injury, infection, and developmental cycle, all involve major apoptotic events with plasmatocytes serving as *Drosophila* macrophages responsible for the phagocytosis and digestion of apoptotic material. Given the observed anomalies in hemocytes, we next investigated whether *DNase II* mutants exhibit defects in phagocytosis of apoptotic bodies. We used Flow Cytometry based *in vitro* assay with CSFE stained apoptotic bodies to assess the ability of hemocytes to engulf apoptotic corpses (Detailed in Materials and Methods). It detects fluorescent signal within single cells, providing counts of cells with and without the signal, and the signal intensity. The fraction of hemocytes performing phagocytosis offers insight into the functional ability of hemocytes to perform phagocytosis (Fig 3.5A). Phagocytosis index, which measures the intensity of signal from hemocytes, is biologically interpreted as the capacity to phagocytose (Fig3.5B).

We hypothesised that hemocytes from *DNase II*^{SK4} mutants would have reduced phagocytosis due to inefficient digestion of DNA from apoptotic bodies. While these hemocytes might initially engulf apoptotic corpses but their inability to digest DNA, could trigger a negative feedback loop inhibiting any further engulfment, lowering phagocytic index in the *DNase II*^{SK4} mutants. Contrary to our hypothesis, DNase II deficiency did not result in any defect in phagocytosis. We also evaluated Sting null mutants and the double mutants and used *NimC1; Eater* as a positive control, due to its known phagocytic defects. Unchallenged hemocytes (i.e. those without the addition of apoptotic bodies) served as negative control.

To check whether the concentration of apoptotic bodies could impact the ability to phagocytose, we independently conducted the same experiment with higher and lower concentration of apoptotic bodies, yielding equivalent results. Consequently, data from experiments with same concentration of apoptotic bodies, as outlined in Materials and Methods, were pooled from independent experiments revealing no significant difference between the control and mutant groups.

Overall, we did not notice any defect in phagocytosis in the *DNase II* mutants. While the initial efferocytosis seems to function well, there might still be a defect in the later stages during digestion of the corpses.

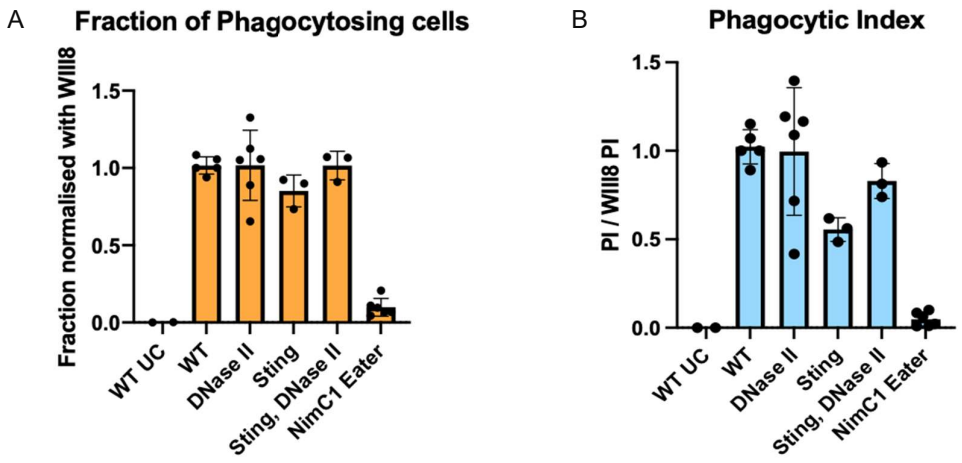

Figure 3.6 Phagocytic Assay A) Fraction of Phagocytosing cells. B) Phagocytic index. Each value has been normalised with that of WT to account for handling errors across experiments. Each dot represents one biological replicate of the genotype, and the data has been pooled from multiple independent experiments. The difference between WT and mutant is not significant in either one of them.

3.7 TUNEL assay: accumulation of apoptotic DNA

As plasmatocytes perform normal phagocytosis, our subsequent question focussed on whether a defect in the digestion of phagocytosed material occurs in the absence of DNase II. To visualise the phagocytosed apoptotic DNA, we used Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) which identifies fragmented DNA, characteristic of apoptosis. For the *in vivo* assay, larvae were gently pinched at the posterior end, ensuring that cuticle is intact, and the larvae are left undisturbed for 2 hours (Fig 3.7.1 A). Following this, larvae were collected, and hemocytes were bled into Schneider media and immunostained as described in Materials and Methods. Visualization was performed using FIJI, with ~10 frames used for quantification of TUNEL positive cells (Fig 3.7.1 B). DAPI stained the apoptotic DNA along with the nuclear DNA, so only the cells containing a colocalised pink signal are counted as TUNEL positive (Fig 3.7.1 C-F). Cells such as the one marked by the arrow in Fig 3.7.2 (H) were not considered TUNEL-positive. For enhanced visualization of apoptotic DNA, images at higher magnification were captured, with one representative frame shown for different genotypes (Figure 3.7.2 A-H).

Overall, observations and quantification confirmed the accumulation of apoptotic DNA in hemocytes from larvae deficient in DNase II. Microscopy analysis revealed only slight increase in accumulation after pinching, which was not significant, thus excluded from quantification for simplicity. Furthermore, this accumulation was independent of the Sting pathway. During larval developmental stages, organisms typically undergo considerable apoptosis, therefore pinching might increase apoptotic DNA only minimally. Speculatively, the DNase II-deficient hemocytes appeared less healthy with nuclear DNA potentially beginning to show signs of apoptosis in some instances.

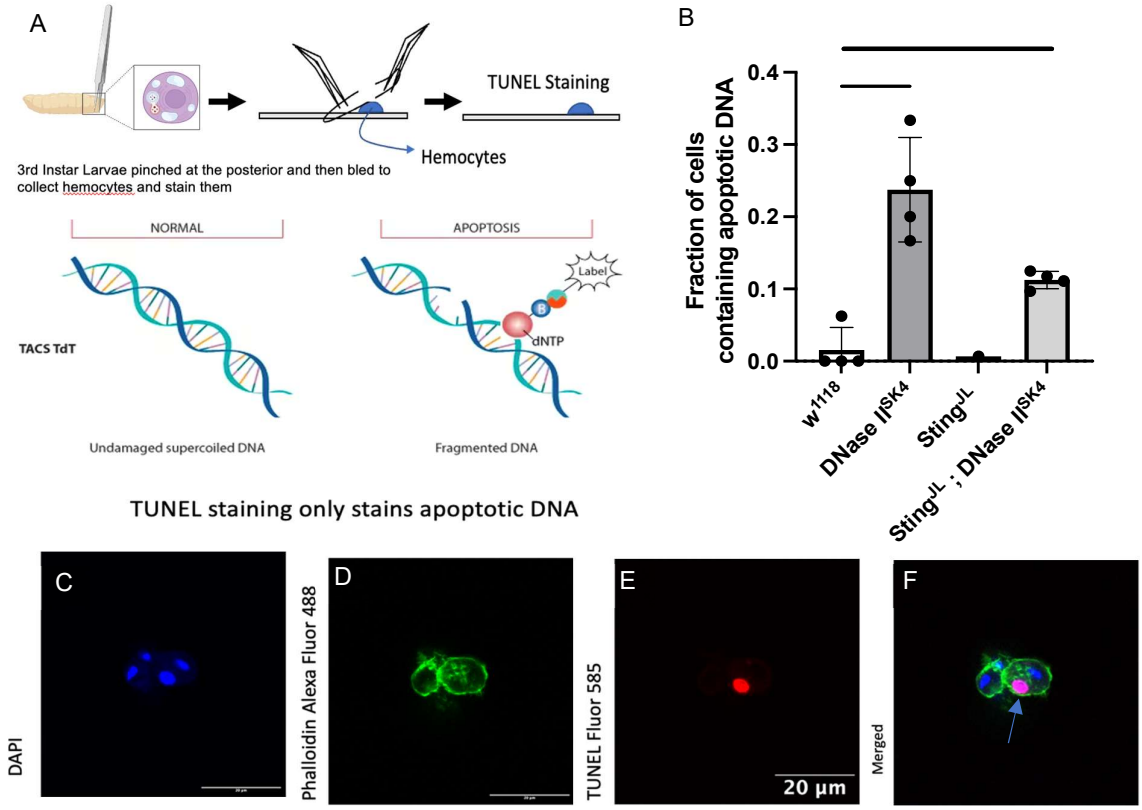

Figure 3.7.1: TUNEL Staining. A) Protocol of the in-vivo assay and TUNEL staining. B) Quantification of fraction of TUNEL positive cells with both DNase II^{SK4} and the $Sting^{JL}$; DNase II^{SK4} double mutant with significant difference between the DNase II deficient larvae (**, $p<0.005$) in both cases C), D), E), F) show a representative of DAPI (blue)- all DNA, Phalloidin(green)- whole cell, TUNEL (red)- apoptotic DNA staining and a merged image respectively. Pink undegraded DNA shown by the arrow is considered accumulated undigested DNA.

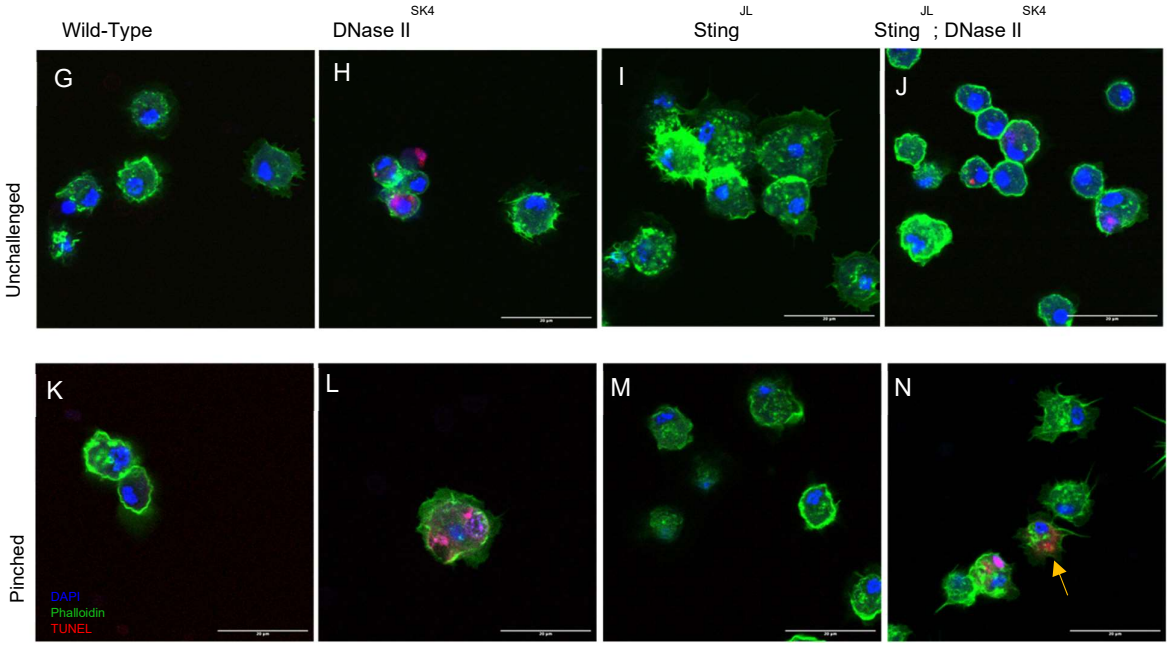

Figure 3.7.2: TUNEL Staining. A), B), C), D) merged images of hemocytes from unchallenged larvae E), F), G), H) are images of hemocytes from pinched larvae. Genotypes have been mentioned between the two images. Scale bar: 20µm.

Overall, there was significantly higher undigested apoptotic DNA in the hemocytes of *DNase II*^{SK4}. Sting pathway does not seem to play a role in the digestion as the double mutants also accumulate DNA. Also, pinching does not increase this, so it is likely that the fragmented DNA is naturally generated during developmental processes of larvae.

3.8 Immune Pathways during DNase II deficiency

3.8.1 IMD pathway: Induction of Diptericin B upon Tissue Damage

Given DNase II's role in DNA digestion and the observed accumulation of DNA in its absence, we explored whether this accumulated DNA could trigger an immune response, focusing on Diptericin B (DptB) as a readout of the Imd pathway. Pinching, speculated to induce apoptosis, was expected to heighten the immune response. If mediated through the cGAS-Sting pathway, a Sting mutation would be anticipated to rescue it. We used RT-qPCR to assess mRNA levels and visualized the tissues inducing this response using a DptB-GFP reporter line, expressing GFP under the Dpt-B promoter. The DNase II^{SK4} mutation was recombined with this reporter, followed by a combination with Sting^{JL}.

Pinching induces a robust DptB response in DNase II mutants (Fig3.8.1 A), unaffected by *Sting* mutation. Visualizing the tissues inducing this response in mutants combined

with DptB-GFP revealed strong responses in the proventriculus and fat body (Fig 3.8.1 D), contrary to expectations given the posterior location of the pinch. Proventriculus is in the anterior part of the gut connecting the foregut and midgut. As positive controls, larvae were pricked with Ecc15 known to induce the Imd pathway and with a sterilized needle. Both induced strong DptB expression in the fat body. DptB induction was significantly higher after pinching in DNase II^{SK4} larvae compared to WT larvae. This heightened response is consistent with the RT-qPCR findings of elevated Imd pathway in *DNase II* mutant larvae after pinching. Supplementary Figure 6 shows more details about the induction of DptB in the double mutant as well as the different expression levels in fat body and proventriculus.

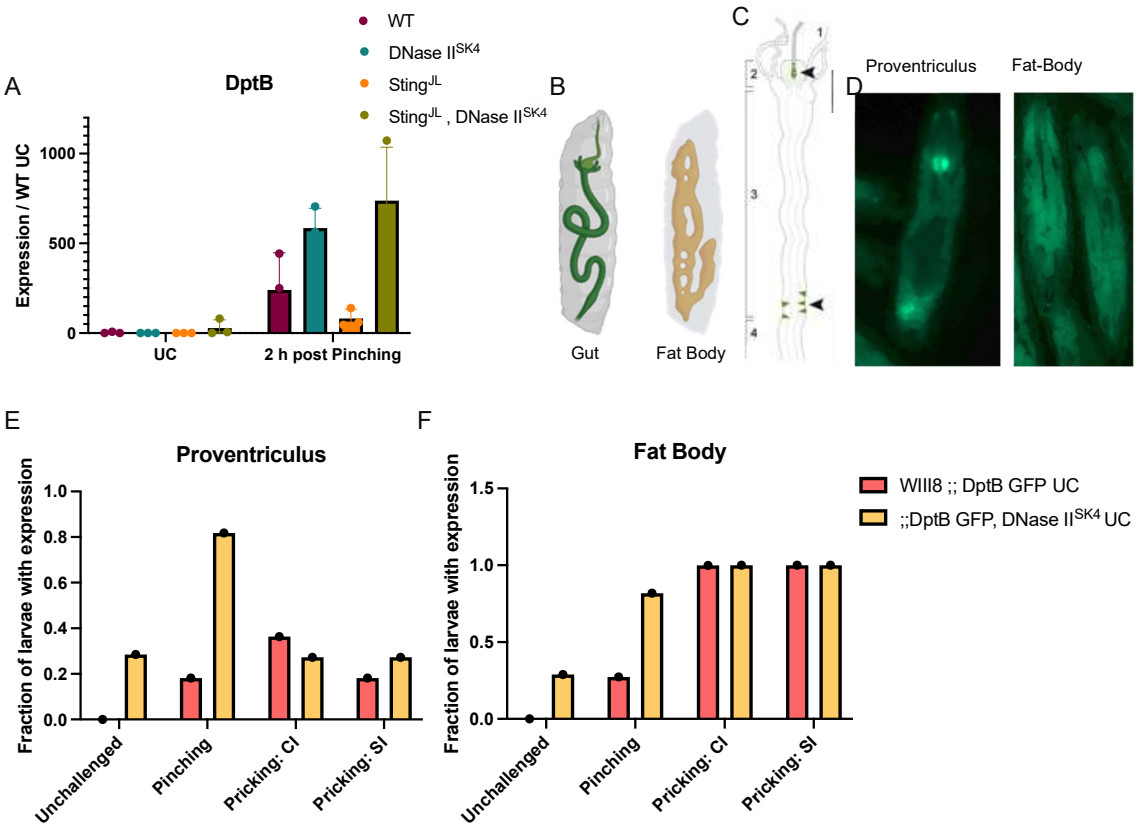

Figure 3.8.1: IMD pathway. A) Pinching induces the Imd pathway very highly in DNase II mutants, with Sting unable to rescue the same. B) The gut and fat body in 3rd instar larvae C) Hemocytes marked by arrows in different parts of the gut; 2 marks Proventriculus adapted from (Zaidman-Rémy *et al.*, 2012) D) Induction of DptB-GFP in proventriculus and fat body respectively in DNase II mutants E) and F) Quantification of the tissue specific response by taking fraction of larvae amongst 10 larvae that show an induction of DptB.

Findings from microscopy and RT-qPCR corroborate the upregulation of Imd pathway after pinching due to DNase II deficiency, regardless of the STING pathway.

3.8.2 JNK pathway dysregulation in DNase II mutant

Due to the association of the JNK pathway with damage-associated molecular patterns (DAMPs), we hypothesized that the JNK pathway could be activated in the *DNase II* mutant. Puckered, a commonly used target of the JNK pathway, was assessed using RT-qPCR. Initially, our results showed some promising signs of upregulation of *puc* in mutant larvae under unchallenged conditions. However, due to high variation in the levels of *puc* in DNase II larvae, these results were not consistently reproducible. Subsequently, adult flies were infected with bacteria, and the expression of *puc* was examined, but no notable differences were observed (Supp Fig 7). Overall, the RT-qPCR results did not indicate any significant upregulation of *puc* (Fig 3.8.2A). To further confirm whether the JNK pathway was indeed not upregulated, we use TRE-RFP, an extremely sensitive reporter of the JNK pathway. TRE-RFP contains four copies of the Jun/Fos (AP-1 transcription factor) binding site fused with RFP and serves as a reporter line that expresses RFP when the JNK pathway is activated, as described by Chatterjee & Bohmann, 2012. The larvae from wild-type line of TRE-RFP express elevated levels of RFP. Hence, comparing the DNase II^{SK4} combined with TRE-RFP line to the wild type proved challenging using microscopy with high expression of RFP. So, we opted to do a western blot quantifying the amount of RFP in our mutant and wild-type lines. Upon examination of all three replicates, no striking difference was observed between the mutant and wild-type lines, hence it was not quantified (Fig 3.8.2 B).

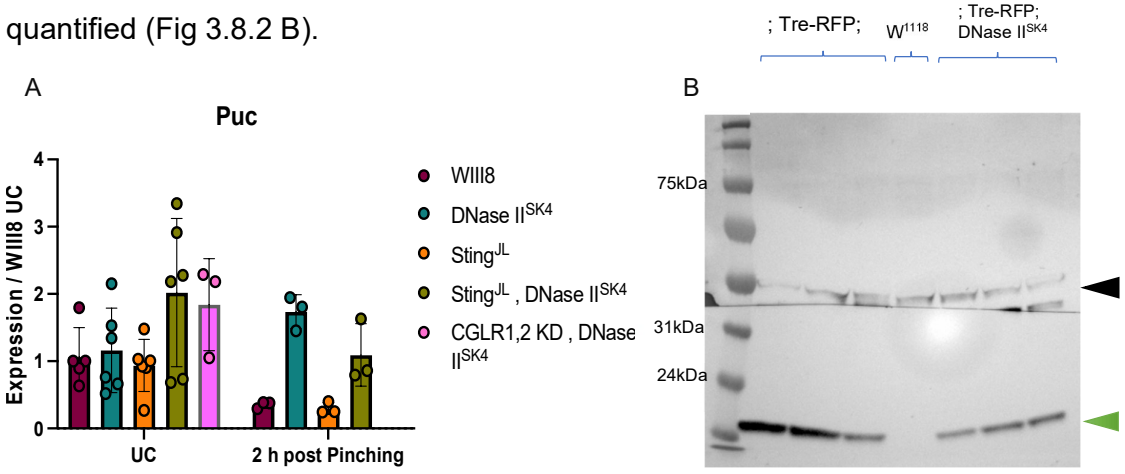

Figure 3.8.2: JNK pathway. A) RT-qPCR checking *puc* mRNA B) Western blot comparing RFP levels in DNase II mutant and control, combined with TRE-RFP, a JNK pathway reporter. Tubulin: black arrow, RFP: Green arrow.

Hence, it can be concluded that JNK pathway is not upregulated in case of DNase II deficiency.

3.8.3 cGAS-Sting pathway upregulation in DNase II^{SK4}

As previously described, cGAS-Sting pathway recognises nucleic acids and activates the immune system in mammals. With DNA accumulation being a major phenotype found the *DNase II* mutants, we aimed to study the activity of this pathway, which is a recently discovered in invertebrates and is poorly characterised in *Drosophila.* Three Sting-regulated genes *srg 1, srg2* and *srg3* were chosen as reporters of its activation. We infected adult male flies with a mix of Gram positive (*M. luteus*) and negative (*Ecc15*) bacteria and assessed induction of the *srg2.* Surprisingly, *srg2* was upregulated in *DNase II* mutant not only upon pricking but also under unchallenged conditions (Fig 3.8.3 A). However, the degree of upregulation of *srg2* was very slight. We also investigated whether DNA itself could be immunogenic for the flies. Bacterial and self-DNA did not result in death of the mutant flies (Supp Fig 10), but we also wanted to check its effect on the flies. Upon injection of *Drosophila* DNA in adult females, *srg2* was upregulated in both w^{1118} and *DNase II^{SK4}* flies but the activation was not higher in the mutant (Fig 3.8.3C). This differed from the response observed upon infection with bacteria or sterile injury. As expected, this upregulation was rescued in the double mutant, indicating that Sting is required for this upregulation of *srg2*.

Further investigation in larvae revealed a similar upregulation of *srg2* upon pinching DNase II mutants (Fig 3.8.3B), which was again rescued with a Sting mutation. Subsequently, we examined the upregulation of *srg3* upon pinching 3rd instar larvae and found that this upregulation was not rescued in the cGLR 1,2 knockout mutants (Fig 3.8.3D). The upregulation of SRGs was subtle compared to the typically observed upregulations upon disruption of immune pathways, but it formed a discernible pattern and warrants further investigation. Interestingly, the third newly discovered cGLR, i.e. cGLR3, does not have any known ligands and is hypothesised to be binding to DNA. cGLR 1 and 2 have previously been associated for sensing viral nucleic acids, with cGLR2 recognising viral RNA. Hence, cGLR 1 and 2 unable to rescue the upregulation of SRGs is not surprising. Three mutants for cGLR3, a potential DNA binding receptor, were assessed for cGLR3 expression, and the mutant with null expression is currently being utilized to further characterize molecular pathways associated with DNase II

deficiency (Supp Fig 8). This represents the latest avenue of study in our research, with ongoing work in this area.

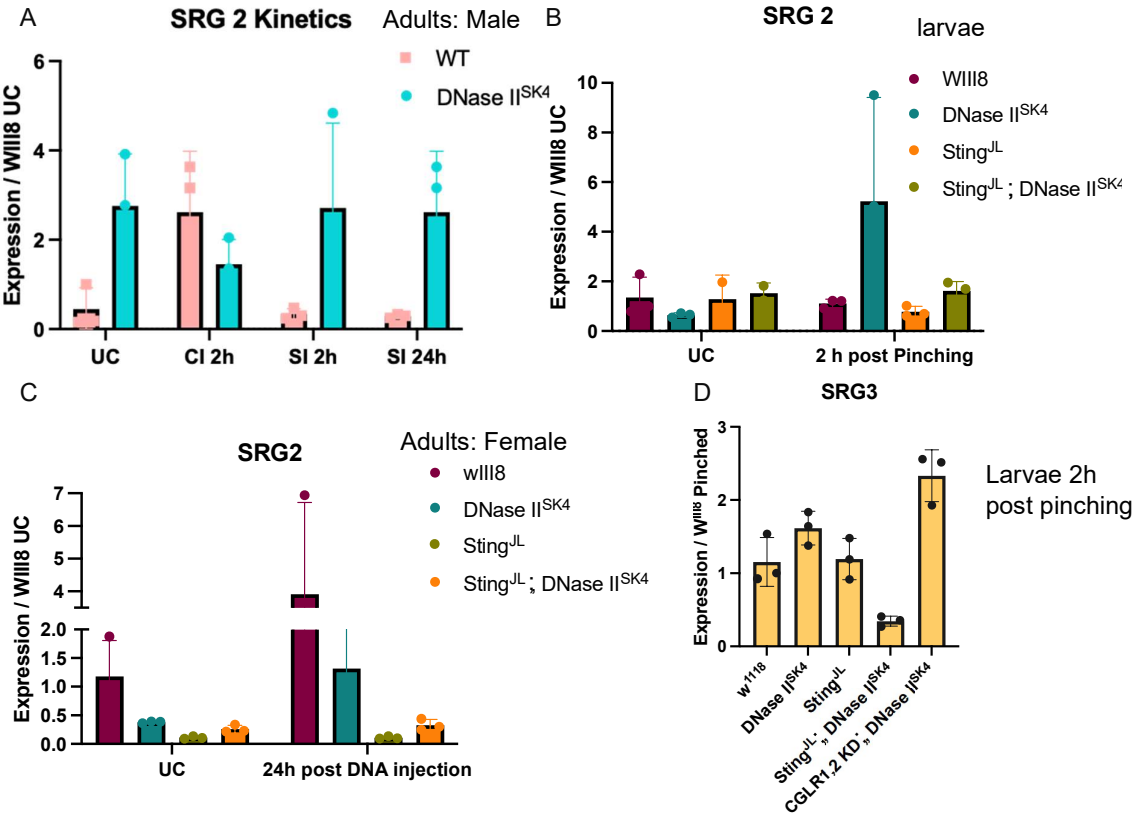

Figure 3.8.3: Upregulation of cGAS-Sting pathway, expression of *srg*s. A) Adult males infected using a needle CI: Clean injury, SI: Septic Injury (OD=200 *Ecc15+M.luteus)* B) Pinching 3rd instar larvae. C) Injecting 23.5nL *Drosophila* DNA (250ng/µL) per fly and checking the immune response 24h later. D) *srg3* is also checked as a reporter after pinching 3rd instar larvae and CGLR1,2 KD; DNase II^{SK4} is a combined line with 3 mutations: deficient in CGLR1, CGLR 2 and DNase II. Note: In A, B and C normalisation is done with respect to Unchallenged w^{1118} larvae, whereas in D, normalisation is done with respect to pinched w^{1118} larvae

While there is indeed some Sting dependent upregulation of the cGAS-STING pathway possibly due to excessive DNA, there needs to be further investigation to understand the exact mechanism of DNA sensing and the role of DNase II in the process. Hence, some of the results are consistent with our hypothesis but need to be further studied to underscore the exact functions of DNase II.

4. Discussion

The induction of DNase II not only upon infection with bacteria in both adults and larvae, but also upon sterile injury in larvae, suggests its involvement in disease tolerance mechanisms in flies. However, despite its induction in response to injury, the susceptibility of DNase II mutants to bacterial infections implies a dual role in both disease tolerance and resistance mechanisms. Although DNase II deficiency does not lead to strong lethality upon bacterial infections, it may contribute to compromised pathogen clearance, indicating its importance in disease resistance.

An overexpression line with the DNase II protein tagged with RFP offers a valuable tool for investigating the involvement of DNase II in cellular processes. Given its structural similarity to its mammalian homolog, lysosomes appeared to be the most likely subcellular localization. We demonstrated the colocalization of RFP with acidic compartments using LysoTracker and with lysosomal compartments using dLAMP1 (Drosophila) antibodies. However, further scrutiny is necessary regarding the specificity of the LAMP1 antibodies, to confirm that this colocalization was not an artifact of microscopy techniques. This is particularly important considering that DNase II could be a secreted protein in Drosophila, as secretion is observed upon its overexpression. Given the absence of DNase I, there arises the question of whether DNase II also digests nucleic acids extracellularly. Tissue specificity of DNase II was previously explored using tissue-specific RNAi by Dr. Alexia Carboni, but it was inconclusive, suggesting the possibility of non-cell autonomous role of DNase II.

The presence of DNase II-RFP with apoptotic bodies suggests its role in the digestion of apoptotic corpses, as predicted. However, the secretion of DNase II raises biochemical questions, as it was previously assumed to require an acidic environment for its function. Importantly, it remains to be investigated whether its expression of from specific tissues can rescue the phenotypic peculiarities associated with its deficiency.

DNase II also plays a significant role in developmental cycle and fertility of the fly. FlyBase data indicates its high expression in the spermatheca, while previous studies suggest their relevance in nurse cells, suggesting roles in fertility and embryogenesis. In our study, we observed pronounced developmental defects in the null mutant.

Firstly, DNase II mutant flies exhibited a reduced overall number of eggs laid per female, and among the eggs laid, there was high lethality during early stages of development. We observed a lower percentage of adults eclosing from eggs in our mutant flies, although there was little defect in the number of flies eclosing from equal numbers of pupae. While the number of emerging L3 larvae was comparable, there was a significant difference in the number of pupae formed, indicating a role for DNase II during this stage of metamorphosis. As was previously observed, higher DNA content might be the cause of larval lethality. During pupariation in Drosophila, larval tissues undergo destruction as new adult tissues emerge. Pre-pupal ecdysone peaks trigger degeneration of certain tissues, which are typically cleared by circulating hemocytes. Defects in this clearance process may disrupt pupal formation. Overall, DNase II deficiency compromises egg laying, development of the third instar larval stage and shows notable delays in developmental processes, which increase across distinct stages of metamorphosis. Interestingly, our collaborators recently discovered DNA accumulation in embryos of *DNase II* mutant flies, suggesting that apoptotic DNA may also affect hemocytes in these embryos. This unpublished study underscores the importance of DNase II in embryonic development and highlights its potential role in hemocyte function.

The emerged adults of the mutant also show reduced lifespan and climbing defects. The role of phagocytic clearance, as suggested by Fig 1.4 B, is essential for overall development and specifically for neuronal tissue development. Thus, climbing defects in DNase II mutant flies may indicate motor neuron defects or inflammation in the nervous system.

Hemocytes, being the primary tissue responsible for clearance of apoptotic and bacterial corpses, might have major roles of DNase II enzyme. *DNase II* mutants did not have the sessile pockets that are considered as major hematopoietic tissues(Leitão and Sucena) and instead had an enlarged concentration of hemocytes in the lymph gland. While flow cytometry data indicated no significant difference in the total number of hemocytes, it is conceivable that the properties of hemocytes were altered in *DNase II* mutants. Observationally, hemocytes from DNase II mutants appeared to have a larger surface area and seemed less healthy, but these were observational characteristics across multiple experiments that were not quantified.

These alterations might explain their reduced adherence to slides, potentially indicating an "activated" state induced by DNA accumulation or inflammation.

Hemocytes from DNase II mutants did not show a phagocytic defect, as evidenced by their ability to engulf apoptotic bodies. However, there was a notable accumulation of apoptotic DNA within these hemocytes, indicating that while their ability to phagocytose remains unaffected, their capacity to digest DNA is impaired. This suggests that the accumulation of DNA does not trigger a negative feedback loop that restricts further phagocytosis. However, this impairment in DNA digestion could potentially lead to activation of immune responses in flies.

The JNK and cGAS-Sting pathways emerged as primary targets in our investigation due to their known associations with DNA damage and nucleic acid sensing, respectively. Both pathways intersect with the Imd pathway, making them key focuses of our study. Interestingly, we did not observe any deviations in the JNK pathway despite DNA being a known DAMP. However, the Imd pathway displayed significant upregulation upon pinching, particularly in the proventriculus, a compartment known for its high microbial activity. This upregulation was consistent in double mutants lacking Sting, indicating independence from this pathway.

Furthermore, Sting dependent upregulation of Sting-Regulated Genes (SRGs) in our mutant line suggests potential activation of the cGAS-STING pathway in the absence of DNase II.

Stress Induced DNase was recently identified to be a deoxyribonuclease present in *Drosophila* that was involved in stress response pathways (Seong *et al.*, 2014). There has not been follow up studies on this and we did not find our mutant flies to be susceptible to dehydration, osmotic and thermal stresses in either sex. It also did not show any developmental or life-span defects, so it was concluded to not play a role in development of flies. A DNase II^{SK4}, SID^{HDR} double mutant is not homozygous viable, which raises questions if SID plays some redundant role in case of absence of DNase II? the lack of induction of SID in larvae upon infection, coupled with the absence of increased susceptibility to stresses or bacterial infections in SID mutants, suggests a predominant role for SID in adult flies, which suggests a need for deeper investigation into its function. Due to the absence of DNase I and other exonucleases in flies, we

can speculate that DNase II and SID might need to play multiple roles in innate immunity of the fly.

4.1 Conclusion

Altogether, our study highlights the pivotal role of DNase II in Drosophila, implicating its involvement in various facets of innate immunity and developmental processes. The absence of DNase II results in shortcomings including developmental defects, reduced fecundity, heightened susceptibility to infections, and diminished life span in adult flies.

The inability of DNase II mutants to effectively digest DNA leads to the accumulation of apoptotic DNA in phagocytic cells, contributing to aforementioned defects. Our study also implicates DNase II in the developmental transition from larvae to pupae and confirms the previous finding of excess DNA hampering this morphological process. The induction of DNase II upon infection suggests its involvement in disease tolerance or resistance mechanisms, further emphasizing its significance in host defence. The observed activation of the IMD and cGAS-STING pathways in DNase II-deficient larvae, particularly in response to apoptosis, underscores the intricate interplay between DNA accumulation and immune signalling pathways. Overall, our findings shed light on the complex molecular mechanisms underlying the phenotypic defects observed in DNase II mutant flies. Further exploration of immune signalling pathways in the context of DNA accumulation is needed to unravel the intricate molecular pathways that govern the physiological and immunological responses mediated by DNase II in Drosophila.

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Supplementary Information

Figure 1. DNase II^{SK4} mutation

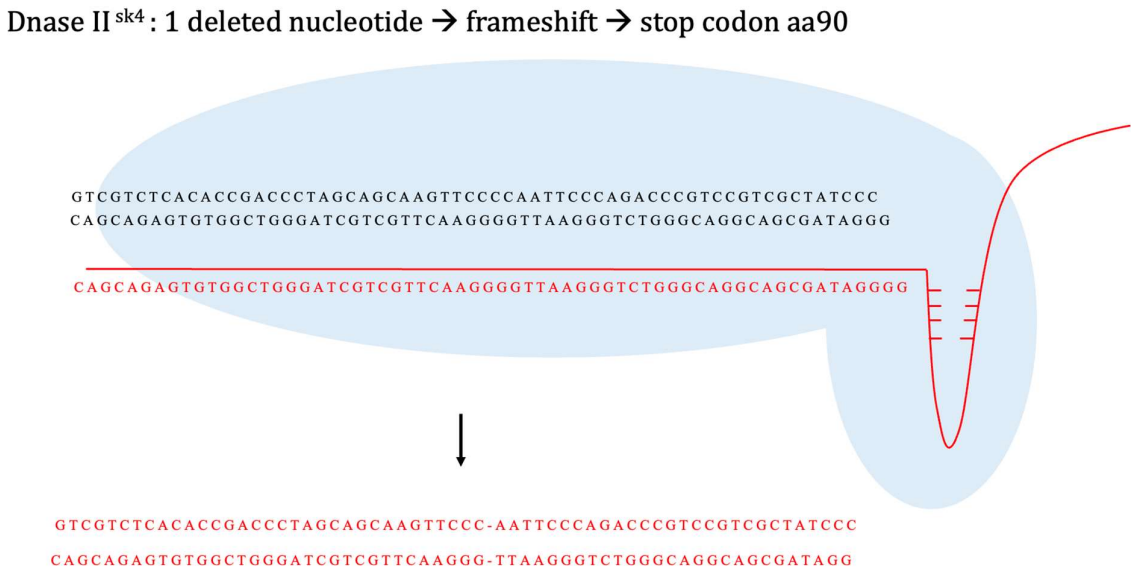

Figure 2. Life history Traits in Drosophila

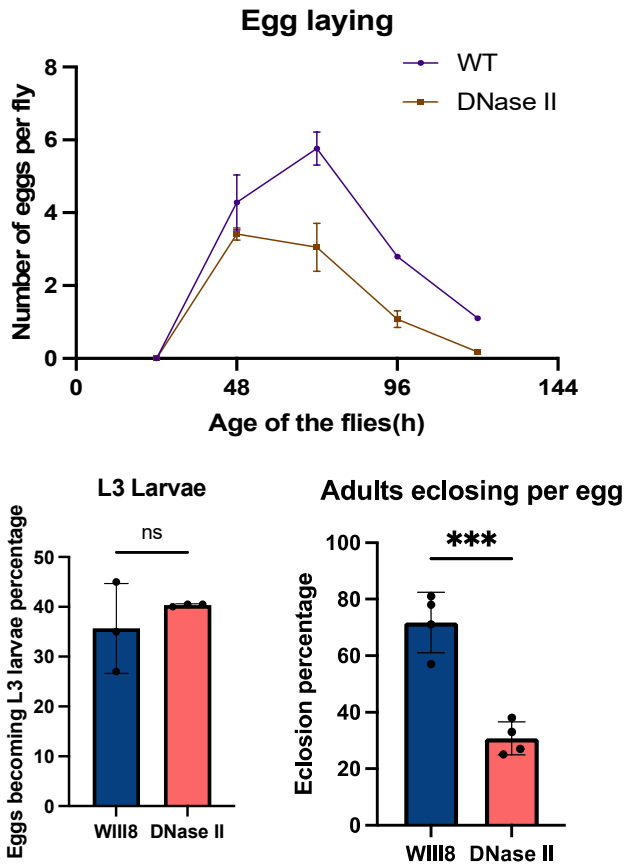

Figure 3. No defect in hemocyte movement post-injury

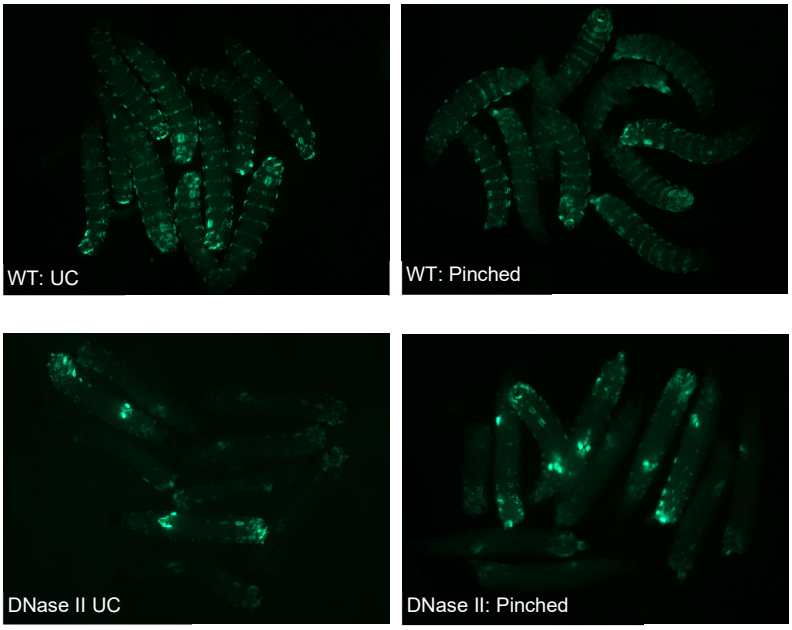

Figure 4. Frames used in microscopy to count plasmatocytes.

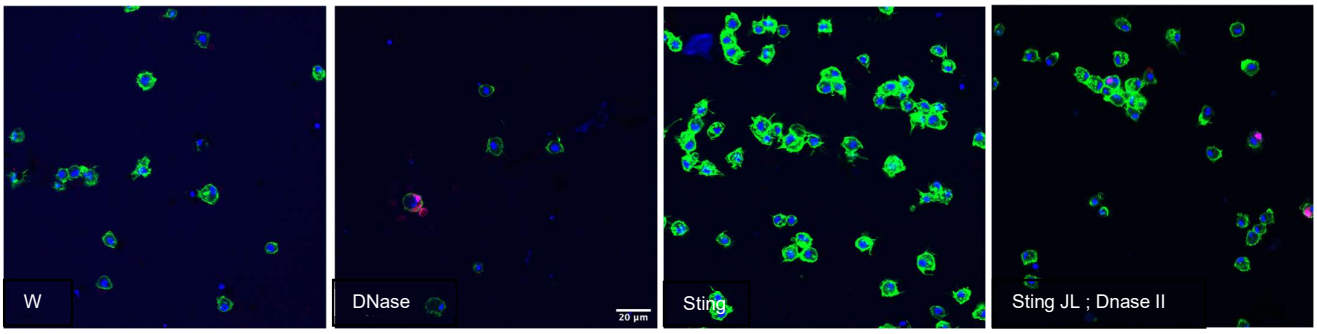

Figure 5. UAS- DNase II- RFP: Vesicular expression in hemocytes

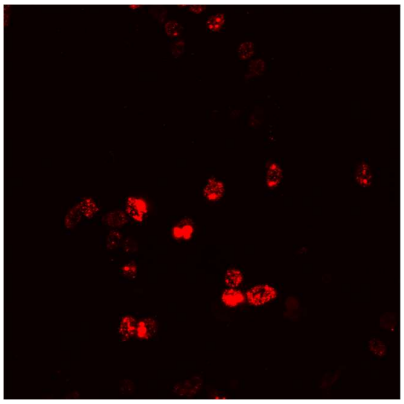

Figure 6. DptB-GFP Reporter expression

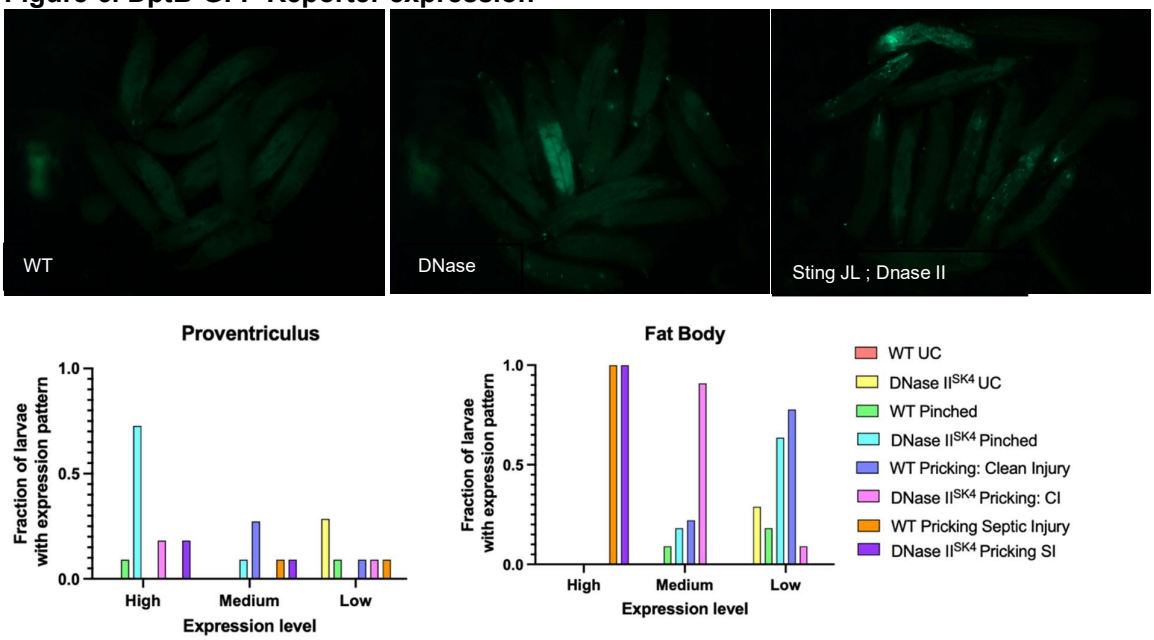

Fig 7 JNK pathway: Adults Pricking with *Ecc15+M. luteus.*

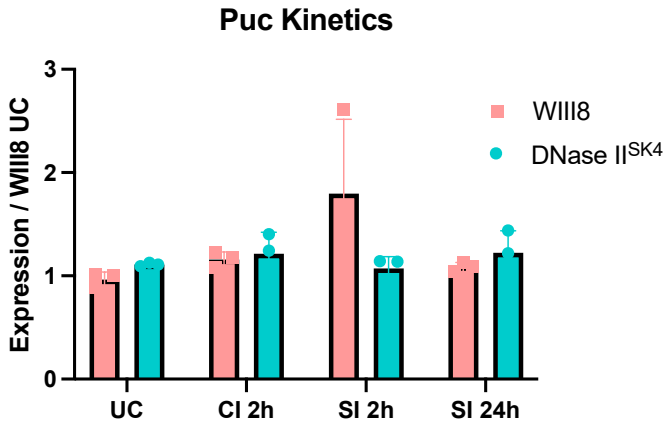

Fig 8. Choosing the CGLR3 mutant

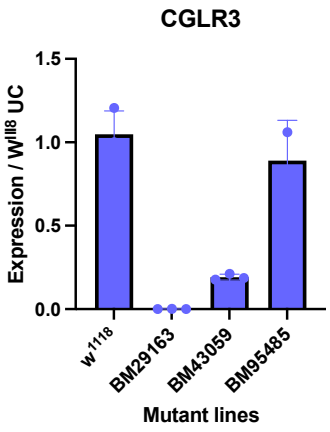

Figure 9: Expression of *dnase II* in *Drosophila.* Adapted from FlyBase

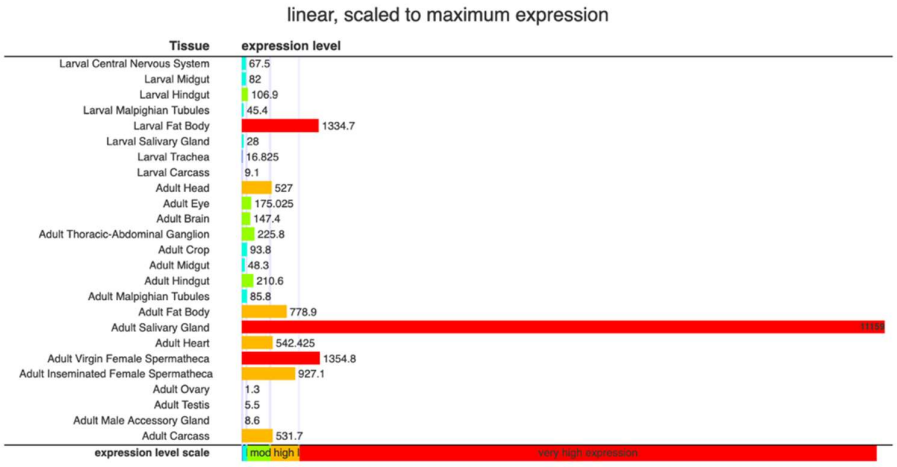

Figure 10. DNA is not immunogenic to the fly.