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The role of CG18446 gene in immune response in *Drosophila melanogaster*

Master thesis

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Cross-Border joint Master's Program Biological Chemistry

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Annotation

The aim of this thesis was to investigate the role of CG18446 gene in hematopoietic organs of *Drosophila melanogaster*, more specifically in lymph gland, circulating and sessile cells. The gene role was examined under normal and infection conditions. Additionally, the possible regulation of CG18446 by Notch signaling pathway was inspected.

Affirmation

I hereby declare that I have worked on my master thesis independently and used only the sources listed in the bibliography.

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Dajana Tanasić

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List of abbreviations

Su(H)	Suppressor of Hairless, transcription factor of Notch receptor signaling
ORF	Open reading frame
PSC	Posterior signaling center, niche of *Drosophila*'s lymph gland
Antp	Antennapedia, marker of PSC
Hml	Hemolectin, marker of mature immune cells
P1	Marker of plasmatocytes
Lz	Lozenge, marker of crystal cells
ROS	Reactive oxygen species
NRE	Notch activity-sensing reporter
AEL	After eggs laying
PBS	Phosphate-buffered saline
PBX	PBS buffer with Triton X-100 detergent
PBT	PBX buffer with bovine serum albumin (BSA)

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1. INTRODUCTION

1.1. NOTCH SIGNALING PATHWAY

Cell to cell communication is crucial for the survival and the proper development of the organism. There are multiple ways for the transduction of signals and one of them is via the Notch signaling pathway, influencing cells' differentiation, proliferation and apoptosis. Notch pathway is an evolutionarily conserved way of signaling, allowing its study in lower species such as *C. elegans* and *Drosophila melanogaster*, but at the same time the results of such a research are applicable to higher organisms.

Main components of the pathway are Notch receptors, Notch ligands and intracellular proteins spreading the Notch signal to the nucleus (Bi & Kuang 2015). The cascade starts with the cleavage in the Golgi apparatus that produces a heterodimer (Figure 1). This cleavage is so called Furin-dependent cleavage and heterodimer produced is a Notch extracellular domain (NECD) (Hori et al. 2013). Triggering of the receptor occurs by the contact with neighbouring cell-surface ligands of DSL family (Delta, Serrate, LAG-2, Delta-like proteins and Jagged). The binding causes the formation of activated membrane-bound form Notch Extracellular Truncation (NEXT). Third cleavage releases the cytoplasmic tail of the Notch receptor, Notch intracellular domain (NICD) that then travels to the nucleus (Bray 2006). In the nucleus, NICD binds to the CSL transcription factor. In the presence of NICD, it recruits other coactivators required for the transcription (MAM); in NICD absence, however, it binds to the DNA and, together with other corepressors, works as a transcription repressor (Kopan 2012).

Besides mentioned the canonical way of Notch signaling cascade that involves activation by Delta or Serrate/Jagged ligands, ligand-independent manner of triggering Notch pathway has also been discovered. This way of activation is dependent on genes in charge of endosomal sorting and ubiquitination. Namely, Notch receptor expressed on the cell surface will be marked for the degradation if there is no ligand present or it will be recycled back to the plasma membrane. Another possibility is that the cleavage of Notch will be initiated without the presence of a ligand, inside the vesicles. The endocytic route is initiated by the E3 ubiquitin ligase, Deltex (Dx). Another E3 ubiquitin ligase, Kurtz (Krz), makes a complex with Notch and Dx allowing to be recognized by The Endosomal Complex Required for Transport (ESCRT). In the case Notch stays trapped on the outer endosome membrane, instead of being

sent to lysosome for the degradation, NICD can be cleaved and sent to nucleus to initiate the transcription (Palmer & Deng 2015).

When compared to other signaling pathways, one of the most remarkable specificities of the Notch is the signaling control. Notch is very sensitive to gene dosage and, in the case of less (or more) than two gene copies, malfunction is observed. The dose sensitivity in mammals is linked to aortic and oncogenic diseases. Another specificity of Notch, being derived from previous one, is the absence of the enzymatic amplification step during signaling. Namely, Notch functions based on the stoichiometric relationship between pathways' components, providing a mean of very tight regulation. Additionally, so called in *cis* and in *trans* plays an important role in the pathway regulation as well. *Trans* interaction between the ligand on the surface of the signal sending cell and the receptor of the signal receiving cell will activate the signal cascade. On the other hand, *cis* interaction causes signal inhibition. The number of *cis* and *trans* receptors can modulate not only small amplification differences between the signals but also whether a certain cell will be signal sending or signal receiving one. Finally, post-transitional modifications also have a profound effect on the regulation of the pathway (Guruharsha et al. 2012).

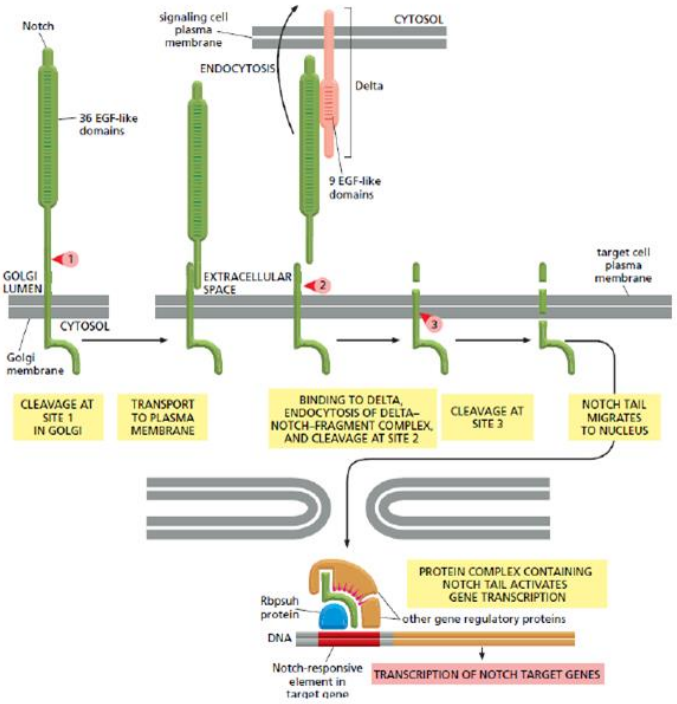


Figure 1: Notch signaling cascade (Alberts et al. 2007)

1.2. CG18446 GENE

Two pioneer experiments pointing to the potential significance of CG18446 were measuring the level of mRNA after inducing Notch activation in S2N and D8 cells and chromatin immunoprecipitation experiments (performed in order to locate the binding sites for the key transcription factor of Notch. Suppressor of Hairless Su(H)). Several hundred genes were upregulated after the activation of Notch receptor (Krejci et al. 2009) and CG18446 gene was amongst the genes that showed a robust Notch response (Fig. 2). Moreover, CG18446 also had a Su(H) peak in its vicinity (in the open reading frame, Fig. 3) suggesting that it is a primary target of the Notch pathway. Further research concerning the expression pattern of CG18446 was continued during the work on my bachelor thesis (Tanasić 2014). As already suggested by FlyBase database (Anon n.d.), the expression profile is very weak due to very low expression signal. The expression has been noticed in adult ovaries and larval fat body (in accordance to the FlyBase), but also in larval testes, larval ovaries as well as in the lymph gland. As the role of Notch has already been reported in *Drosophila*'s hematopoiesis, we have decided to concentrate on the expression we observed in the lymph gland and to try to dissect the role of CG18446 in the fruit fly immune system.

For the purposes of our experiments, two types of mutants were created: $CG18446^{exD}$ (referred to as exD in my thesis) and $CG18446^{MI02952}$ (referred to as "mimic" in my thesis). The exD mutant was created by deletion of specific transposons from the Exelixis collection located before and after the CG18846 gene, taking away the CG18446 gene but also genes or their parts that are found in the nearby gene rich region (cbx, CG12744, CR45138, Ntmt, CG30010). Mimic mutant, on the other hand, involves the insertion of so called Minos transposon in the coding region of the gene (Venken et al. 2011). This insertion disorders the transcription only of the CG18446 gene, although we cannot entirely exclude that other genes in this gene rich regions would be affected. Neither of CG18446 mutants showed obvious phenotype in homozygous state.

Since CG18446 antibodies do not exist, we could only rely on the CG18446-GFP tagged flies instead to see CG18446 expression. Here we used the advantage of the FlyFos method that can be used to create an EGFP fusion of a protein of interest in the context of large BAC genomic construct. The GFP expression of these CG18446-GFP transgenic flies

should mirror the endogenous expression pattern of CG18446, as the large BAC construct most probably contains all the regulatory regions of the gene.

The function of the CG18446 gene is unknown and there are no published data regarding this gene. What is known according to the FlyBase is that it is predicted to be a zinc finger protein (Fig. 4). It localizes to the nucleus and it is conserved amongst all *Drosophila* species. The presence of zinc finger regions that make majority of the CG18446 protein structure makes it difficult to identify a mammalian orthologue.

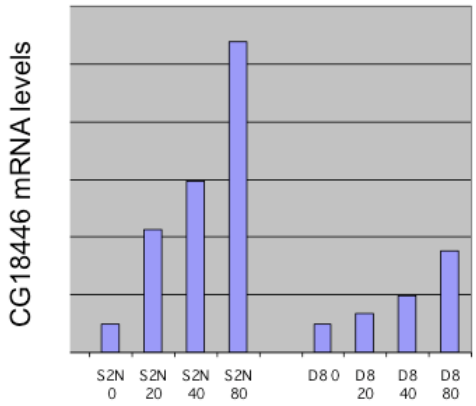


Figure 2: Temporal response of CG18446 following activation of Notch receptor in S2N and DmD8 cells (The y axis shows time in minutes).

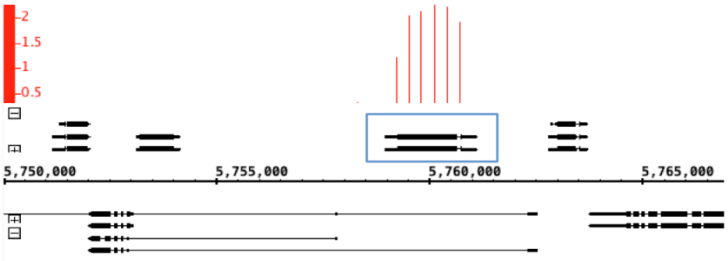


Figure 3: The ChIP-chip data of Su(H) binding in DmD8 cells. Su(H) binds to the whole open reading frame of CG18446 gene, forming a strong and rather large peak. CG18446 gene is in the blue frame.

Figure 4: Predicted structure of the CG18446 gene

1.3. IMMUNITY

1.3.1. Introduction to immunity

Immune system is a crucial mechanism for survival in the living world. There are two different types of the immunity: innate and adaptive immune system. Innate immune system is evolutionarily conserved across all species. It is a primitive form of the protection, having no immunological memory and using defensive mechanisms in the form of physical and chemical barriers. Adaptive or acquired immunity is a more specialized way of fighting invaders, since it is involved the production of antibodies designed to specifically impair targeted pathogen. Additionally, this type of immunity is capable of producing memory cells, being much faster in killing the pathogen in the case of a second attack. In accordance to the published data, the most primitive form of adaptive immune system was described in jawless fish (Martin Flajnik and Masanori Kasahara 2010).

Drosophila melanogaster is a well-studied model organism of innate immunity due to its simplicity but still enough conserved similarity to more advanced species. Fruit flies are considered to have humoral (mediated by body fluids) and cellular (mediated by cells' action) immune response. Humoral immunity is regarded to be the production of antimicrobial peptides and melanization whereas cell-mediate immunity involves phagocytosis and encapsulation of the invader (Lemaitre & Hoffmann 2007). Particularly significant is the cellular immunity due to its conserved feature when compared to vertebrates (Parsons & Foley 2016). There are two different origins of all heamatopoietic organs in *Drosophila melanogaster*, procephalic and cardiogenic (Holz et al. 2003). The procephalic tissue gives rise to sessile and circulating cells, whereas cardiogenic tissue generates lymph gland (Holz et al. 2003).

1.3.2. *Drosophila*'s immunity

1.3.2.1. Embryonic hematopoiesis

The very first immune cells are formed at the stage 7 of embryonic development. Early fate of blood cells has been decided by the activity of two transcription factors: the GATA factor Serpent (Srp) and the friend of GATA (FOG) U-shaped (Ush) (Waltzer et al. 2002).

Head mesoderm is the place from which hemocytes start their migration through the embryo. On their way towards the posterior part of the embryo, blood cells need to be guided and also need to penetrate an epithelial DE-cadherin-based tissue barrier. Guidance is achieved by platelet-derived growth factor/vascular endothelial growth factor-receptor homolog (PDGF/VEGFR) together with their ligandsPvf2 and Pvf3 (Parsons & Foley 2013). Dislocation of Rap1 during penetration of the epithelial barrier and engagement of RhoL in control of this process are both reported to be required for the proper hemocytes transmigration (Siekhaus et al. 2010). While populating embryos, hemocytes' mobility and polarized behavior have been kept under the control by variety of membrane and cytoskeletal factors. Immune cells start migrating at the stage 12 and by the stage 15 of the embryogenesis the embryo is almost evenly occupied (Fig. 5A) (Tepass et al. 1994).

1.3.2.2. Larval hematopoiesis

Larval hematopoiesis has also been defined in the literature as "a wave of macrophage expansion initiated by blood cell colonization" (Makhijani & Brueckner 2012). Hemocytes formed during the embryonic development migrate towards the so-called "hematopoietic pockets" placed along the aorta (Fig. 5Bii). They are also capable of proliferating in these "sacks", termed sessile cells (Holz et al. 2003). This wave of hematopoiesis is characterized by the expansion in the number of immune cells, starting with 300 cells at the onset of the larval stage and increasing to more than 5000 cells in the third larval stage (Tepass et al. 1994). Besides hemocytes in these pockets, certain number of circulating hemocytes during the larval development exists as well. It has been reported that there is a constant exchange between hematopoietic pockets and circulating immune cells that tends to become more intensive with the later stage of larval development (Makhijani et al. 2011). Under the challenged conditions, this interaction becomes even more dynamic, accumulating circulating cells on the place of injury or the pathogen attack (Pastor-Pareja et al. 2008). Additionally, the importance of the peripheral nervous system (PNS) in the microenvironment of the hemapotpoesis has been reported, as it is recognized to support the hemocyte attraction and adhesion (Makhijani et al. 2011).

1.3.2.3. Lymph gland hematopoiesis

Lymph gland is the organ of immune system that arises along the anterior part of the dorsal vessel, consisted of immune cells of cardiogenic origin (Fig. 5Bi). In the first two stage of larval development, the growth of the lymph gland is rather slow and it completely develops during the second instar larval stage (Vlisidou & Wood 2015). However, the growth significantly increases by the late third instar stage, having all hemocytes differentiated. At the end of larval stage, lymph gland starts disintegrating, releasing all immune cells in the circulation (Vlisidou & Wood 2015).

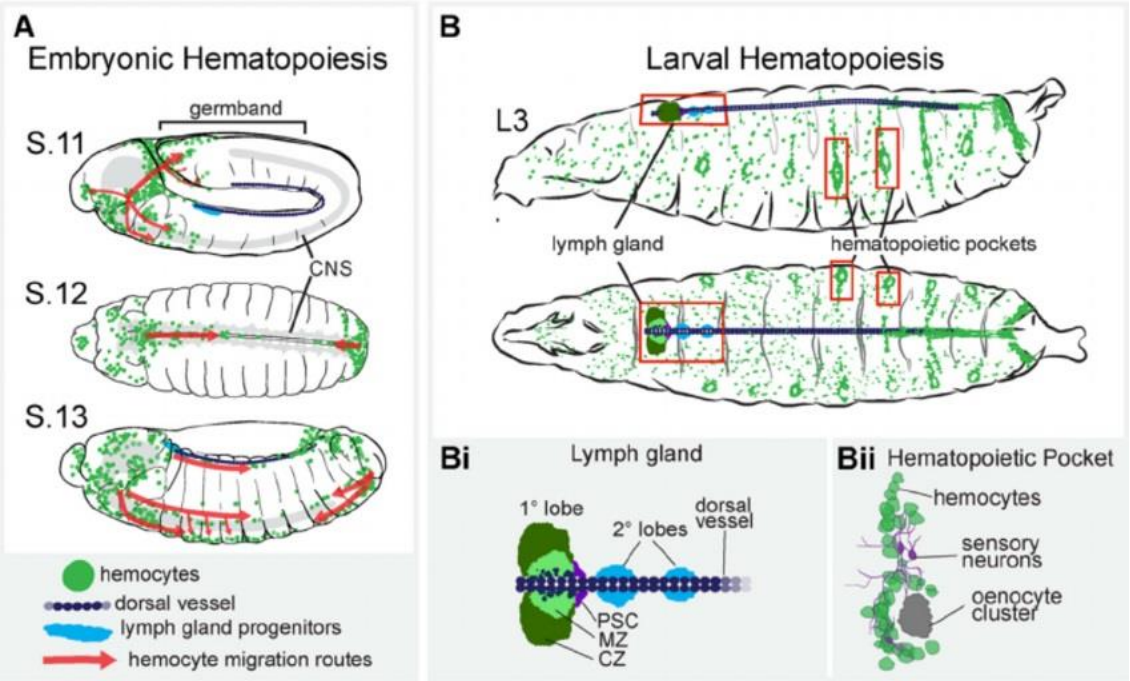


Figure 5: Three waves of *Drosophila* hematopoiesis (Parsons & Foley 2016)

1.3.2.4. Type of *Drosophila*'s immune cells

Innate immune system of *Drosophila* can be divided into the following hematopoietic tissues: lymph gland (main organ), sessile cells, circulating hemocytes and antimicrobial peptides in fat body. Three types of *Drosophila* blood cells can be differentiated in all these hematopoietic tissues.

Plasmatocytes are the most abundant type of hemocytes, making 95% of the total number of immune cells. Besides having a crucial role in the defensive mechanism of the organism, plasmatocytes can influence shape and positioning of organs (Bunt et al. 2010), proper development of CNS by cleanance of dead cells (Sears et al. 2003) and metabolism (Woodcock et al. 2015). These primitive macrophages are highly motile cells and have distinct migration paths during embryonic development, resembling the ones found in vertebrates (Ratheesh et al. 2015). Plasmatocytes play an important role in removing pathogens and apoptic cells by encapsulation and phagocytosis. Especially important is their phagocytic activity, since they are considered one of the oldest macrophages. NimrodC1 is a single-pass transmembrane protein expressed on the surface of plasmatocytes and is found to be responsible for phagocytosis of bacteria (Kurucz, Markus, et al. 2007). Another protein originally described to be involved in the phagocytosis of Gram-positive and Gram-negative bacteria is Eater (Kocks et al. 2005). It has also been reported that Eater plays a role during plasmatocytes entering the sessile state (Bretscher et al. 2015). Eater deletion has led to the lack of sessile compartments. This has been explained by the importance of this ligand in adhesion of hemocytes. Finally, the removal of apoptic cells has been performed via a phagocytic receptor named Draper, whose homologue was first recognized in *C. elegans* (Manaka et al. 2004). NimC1, Eater and Draper all belong to Nimrod protein family, characterized by the presence of specific EGF repeat. Together with certain proteins encoded in the human genome, they create a protein superfamily (Somogyi et al. 2008).

Crystal cells are the second type of immune cells present in *Drosophila*'s hemolymph differentiated under the normal conditions. They represent around 5% of total number of mature hemocytes and they are in charge of encapsulation and coagulation. According to the published data (Duvic et al. 2002), Notch pathway performs an important role in the specification of the crystal cells during the larval development. Binding of the transcription factor Lozenge (Lz), that contains Runx motif, addresses Notch to properly lock crystal cells fate (Terriente-Felix et al. 2013). This pathway is needed at several stages during the lineage specification, with targets of regulation being different at each stage (Terriente-Felix et al. 2013). It has also been reported that this Notch crystal cell differentiation role is performed via Serrate activity through the posterior signaling center (Duvic et al. 2002). Two another binding partners, Yorkie (Yki) and Scalloped (Sd) have been found as Notch downstream

crystal cell fate and maturation contributors (Ferguson & Martinez-Agosto 2014). Suggested mechanism involves Notch being most upstream, activating Yki that then regulates the expression of Lz. (Ferguson et al. 2014). The same study suggests that Sd is needed in the early development of crystal cells whereas it is actually down-regulated in the later course of cells' maturation. Furthermore, crystal cells differentiation is blocked upon the parasitic immune challenge (Small et al. 2014). The group from Los Angeles has also shown that infection by the wasp parasite blocks Yki and reduces Sd, influencing Serrate expression that finally leads to crystal cell differentiation inhibition (Ferguson et al. 2014). Besides Notch, other pathways can influence the number of crystal cells. Despite the fact that both Yki and Sd are Hippo pathway activators, it was proven that they influence crystal cells independently of this signaling pathway (Ferguson et al. 2014). However, the activation of kinase AKT of TORC1 pathway boosts the number of crystal cells (Dragojlovic-Munther & Martinez-Agosto 2012). Whereas the specification of crystal cells has shown to be regulated by Notch/Serrate, maintenance of crystal cells has been discovered to be controlled via non-canonical Notch and its interaction with Sima, *Drosophila* orthologue of hypoxia-inducible factor (Mukherjee et al. 2011). This Sima/Notch signaling has reported to be important under both normal and stress conditions. All of previous findings are in regard to the crystal cells in the lymph gland. Concerning the hematopoietic niche outside the lymph gland, sessile cells, there are findings showing that Serrate is necessary for crystal cells specification from the sessile plasmatocytes (Leitão & Sucena 2015). Whether both Yki and Sd are also involved in controlling the content of sessile hematocytes and circulating cells remains to be investigated.

Crystal cells contain two out of three types of enzymes that can induce melanization – prophenoloxidase 1 (PPO1) and prophenoloxidase 2 (PPO2) (Irving et al. 2005). Melanization is a rapid reaction of the immune system, induced within a few minutes after the challenge (Tang 2009). One of the functions of melanin is a physical inhibition of the growth of the intruder. Second function involves the formation of highly reactive and toxic quinone intermediates during its production that are capable of killing the pathogen (Christensen et al. 2005). Prophenoloxidase is the enzyme in the serine proteinase cascade that leads to melanin formation (Fig. 6). Common components present among fungi and bacteria, like lipopolysaccharides and peptidoglycans, serve as the activator of the serine protease cascade. Nevertheless, it is interesting to mention that additional factors that could also trigger the

cascade are calcium ions, sodium dodecyl sulfate (SDS), trypsin and high temperature (Cerenius & Söderhäll 2004).

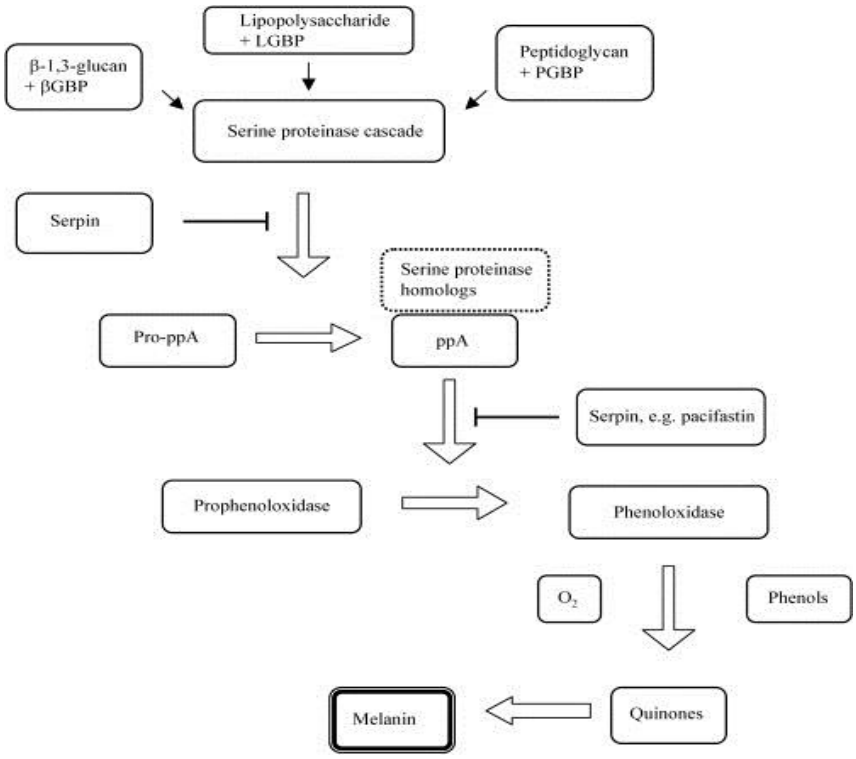


Figure 6: Serine proteinase cascade leading to the formation of melanin (Cerenius & Söderhäll 2004)

Lamellocytes are the type of immune cells that appear in hemoplymph of *Drosophila* only in the case of infection. They are the biggest blood cells and they have adhesive properties, encapsulating pathogens too big for phagocytosis (Jung et al. 2005). In accordance to the published data, increased level of reactive oxygen species (ROS) species in the posterior signaling center results in lamellocytes' differentiation in circulation and the lymph gland (Sinenko et al. 2011). The same is not observed in the case of oxidative stress in any other tissue such as fat body, the neurons or wing discs (Sinenko et al. 2011). Nevertheless, the expression of collier in the posterior signaling center (the regulatory part of the lymph gland, shortly PSC) is crucial for the lamellocyte differentiation, as in it absence this process is blocked (Crozatier et al. 2004). Silencing of the EGFR ligand Spitz (Spi) also blocks lamellocyte response (Sinenko et al. 2011). Therefore, following mechanism has been suggested: parasitic wasp infection increases levels of ROS in the posterior signaling center,

that then induces Spitz expression resulting in the lamellocytes differentiation (Sinenko et al. 2011). Nevertheless, Notch pathway has also been discovered to play a role in the immune-challenged *Drosophila* larvae where Notch activity was shown to be necessary for lamellocyte differentiation after wasp infection (Duvic et al. 2002). Other authors suggest that moderate level of Notch activity followed by its decrease is necessary for lamellocyte development, coinciding with less crystal cell production (Small et al. 2014). Notch activity is low or completely absent in lamellocytes (Small et al. 2014). Infection seems to lower the Notch in lamellocyte precursors that in turns makes them responsive to oxidative stress challenge. This eventually leads to the lamellocyte differentiation (Small et al. 2014). Therefore, Notch is necessary to keep the ROS levels in the anterior lobes low, retain lamellocyte progenitors silent and stimulate crystal cells development (Small et al. 2014). Additionally, since very few crystal cells are observed under the infection conditions, it has been reported that lamellocytes differentiate at the expense of crystal cells (Krzemien et al. 2010). What remains to be dissected is the exact mechanism behind Collier, Spitz and Notch interaction. Lamellocytes have also been found to be capable of releasing prophenoloxidase 3 (PPO3), enzyme responsible for the melanization (Irving et al. 2005). Lamellocytes kill pathogens in two steps: first one includes encapsulation of the foreign object and second one leads to melanization of the capsules (Dudzic et al. 2015). The expression of PPO3 has found to be specific to lamellocytes (Dudzic et al. 2015). It is also interesting to mention that PPO3 is found only in *Drosophila melanogaster* group, proposing that the ability of lamellocytes to produce this enzyme arose evolutionarily recently via gene duplication process (Dudzic et al. 2015).

Lamellocytes differentiation can also be induced by genetic mutations (Sorrentino et al. 2002). These genetic mutations usually involve perturbations in pathways such as JAK/STAT, JNK, Toll and Wingless (Zettervall et al. 2004). One of the fly strains we used in our experiments has the mutation of the Hopscotch gene that encodes a JAK protein tyrosine kinase (Binari & Perrimon 1994). Hopscotch gene is a *Drosophila* oncogene located at the X chromosome and the protein that it encodes contains 1177 amino acids (Binari & Perrimon 1994; Dearolf 1999). Hopscotch$^{\text{Tumorous-lethal}}$ (Hop$^{\text{tum}}$) is a dominant and temperature-dependent mutation resulting from a single amino acid substitution of glycine to glutamic acid at the residue 341 (Luo et al. 1995). As a consequence, hematopoietic defects including lamellocytes differentiation and melanotic black tumors formation are observed (Luo et al. 1995).

One recent elegant study lead to the discovery of all possible differentiation states from plasmatocytes to lamellocytes induced by wasp infection (Anderl et al. 2016). The group has reported six different blood cell types in the circulation under the immune-challenged conditions. Main tool in the study was flow cytometry and reporters for plasmatocytes and lamellocytes. Besides already described plasmatocytes and lamellocytes, activated plasmatocytes, prelamellocytes, lamellocytes type II and the new population of the infection-induced cells called lamelloblasts are reported (Fig. 7). There have been some previous reports on the different states of plasmatocytes in the case of the infection (Krzemien et al. 2010), however this is the first study completely elucidating the morphology and time parameter of the transdifferentiation of sessile plasmatocytes into lamellocytes. Particularly interesting are lamelloblasts, being characterized by the expression of lamellocytes reporter but having plasmatocyte-like morphology. However, their origin has found to be not connected to plasmatocytes, but to the sessile tissue. Since lamelloblasts are found in the circulation 8 hours after the infection, this could be an additional support of already proposed model saying that lymph gland hemocytes contribute to the number of lamellocytes only at the later course of the infection (Márkus et al. 2009). In other words, this would contribute to the importance of the sessile cells, since they could actually be the main source of lamellocytes in the hemoplymph.

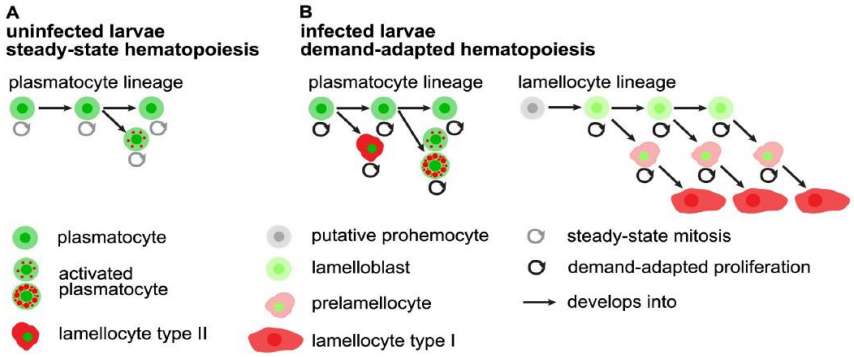


Figure 7: Two-lineage model for lamellocytes formation. A) Hematopoesis under the normal condition. B) Infection triggers the increase in the plasmatocytes' proliferation rate. This results in the trasndifferentiation of plasmatocytes into lamellocytes type II on the wasp egg. Infection also triggers a lamellocyte hematopoiesis, starting from the sessile tissue precursor. Via lamelloblasts and prolamelloblasts, the final results is the formation of lamellocytes type I. (Anderl et al. 2016)

1.3.2.5. Lymph gland

Lymph gland is the main organ of the immune system. It originates from a mesodermal tissue (the same one as heart-like organ dorsal vessel) and arises within first and third thoracic segments from the fusion of three paired clusters of cells expressing Odd-skipped (Odd) (Mandal et al. 2004). Lymph gland fully develops during second instar larval stage. Mature organ consists of paired lobes (from two to four pairs) oriented along the dorsal vessel. Three distinct regions can be differentiated in primary lobes (Fig. 8). Cortical zone (CZ) is the zone covering the edges of lobes; mature hemocytes can be found in this region. Mature and fully differentiated hemocytes are characterized by the expression of hemolectin (hml), a protein that is engaged in the coagulation of *Drosophila*'s larvae and has conserved domains with human von Willebrand coagulation factor (Goto et al. 2003; Goto et al. 2001). The inner zone is so called medullary zone (MZ). Non-differentiated hemocytes in medullary zone receive signals about their fate and, as they maturate, move to the cortical zone. This region is distinguishable by the expression of JAK/STAT receptor Domeless and JAK/STAT reporter dome-MESO (Krzemień et al. 2007). Progenitors maturing in MZ are found to be destined to become either plasmatocytes or crystal cells earlier during the development, even though crystal cells differentiation actually occurs in third instar larval stage (Krzemien et al. 2010). Finally, at connection site towards the other pair of lobes is the area of posterior signaling center (PSC). PSC is crucial in terms of keeping the equilibrium between mature and immature blood cells, what can play an important role during the infection. Cells of this region, termed as niche, rarely divide (Jung et al. 2005; Krzemien et al. 2010). Precursors of the completely differentiated hemocytes are called prohemocytes and they start to mature during the period of mid-2nd larval stage. This results in an approximate number of 2000 to 3000 mature hemocytes, of which more than 90% are plasmatocytes (Jung et al. 2005). Lymph gland persists only during the larval period, as 12 hours after puparium formation it disintegrates and releases blood cells into the circulation (Grigorian et al. 2011). According to the literature, posterior signaling center plays a role of the niche of the lymph gland (Krzemień et al. 2007). This region of the lymph gland has been specified early in the embryonic state by the homeotic gene Anetennapedia (Antp) (Mandal et al. 2010) and EBF transcription factor Collier (Col) (Krzemień et al. 2007). The absence of PSC results in the distribution of the mature hemocytes across the whole lymph gland (Mandal et al. 2010). The

increase of the PSC area, on the other hand, leads to the decrease of area containing mature hemocytes (Mandal et al. 2010). In order to coordinate the differentiation state of the organ, it uses several signaling pathways such as Notch, Hedgehog and Wingless (Lebestky et al. 2003; Mandal et al. 2010; Sinenko et al. 2009). It has already been mentioned that Notch signaling pathway stimulates crystal cell differentiation via Serrate through PSC (Duvic et al. 2002). Hedgehog and Wingless signaling have been discovered to be important for maintaining the population of prohemocytes either by regulating PSC (in the case of Hedgehog) or medullary zone (Wingless) (Mandal et al. 2010; Sinenko et al. 2009). Decapentapiegic (Dpp) is the signaling reported to be required for the size of PSC and Pvf1 is necessary for prohemocytes maintenance in the cortical zone (Pennetier et al. 2012; Mondal et al. 2015). JAK/STAT signaling has shown to be crucial in the case of immune-challenged conditions. Namely, JAK/STAT activity is maintained in prohemocytes as this gives them multipotent character that can be used for adopting the fate of infection-responsive lamellocytes (Krzemień et al. 2007).

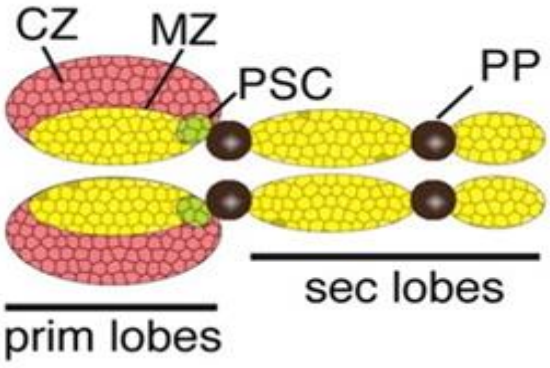


Figure 8: *Drosophila*'s lymph gland (Benmimoun et al. 2012)

1.3.2.6. Sessile cells

Sessile cells are the largest immune cells compartment in the *Drosophila* larva. They consist of clusters of cells evenly distributed along the aorta (Fig. 9) (Crozatier & Vincent 2011). Additionally, many sessile cells are also found on the larval imaginal discs (Kurucz, Vaczi, et al. 2007). As in the case of the lymph gland, these hematopoietic compartments develop during the larval stage. In the second instar larval stage, pockets are mostly positioned around the posterior end whereas in the third instar larval stage the pattern of the sessile

tissues is completely formed (Márkus et al. 2009). According to the published data, sessile cells are directed towards their compartments by peripheral nervous system (Makhijani et al. 2011). The crucial aspect of this hematopoietic tissue is the contact that cells in these pockets are capable of establishing. Namely, all cells in lymph gland are pre-destined to become either plasmatocytes or crystal cells, even if they are still in the medullary zone. In sessile compartments, however, differentiation of plasmatocytes into crystal cells can easily occur due to "tight" environment and opportunity for cell-cell signaling. Moreover, this process has found to be Notch-dependent (Leitão & Sucena 2015). As already mentioned, sessile cells have reported to be highly responsive under immune-challenged conditions. Lower number of sessile cells and higher number of circulating cells are detected in the infected *Drosophila* larva. As already mentioned, lamellocytes found at the onset of infection are shown to be differentiated from immune cells of sessile compartments (Anderl et al. 2016; Márkus et al. 2009). All of this suggests crucial role of sessile cells in the defense of the organism.

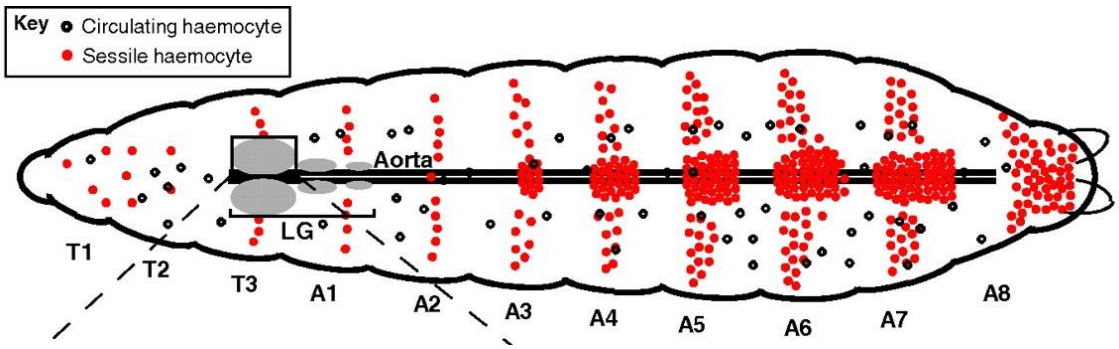


Figure 9: Schematic representation of sessile and circulating hemocytes (Crozatier & Vincent 2011)

1.3.2.7. Circulating cells

Circulating hemocytes are hemocytes present in the hemolymph of *Drosophila*. Since they are capable of dividing, number of circulating blood cells constantly increases during the larval development (Lanot et al. 2001). They dynamic movement is partially due to the actin network. This actin network depends on the so-called Arp2/3 complex and certain nucleation-promoting factors, being responsible for polymerization and depolymerization of actin filaments (Campellone & Welch 2010). Proteins from Rho-family, further instructing the formation of membrane protrusions with the narrow actin spikes, assist movement (Nobes &

Hall 1995). Many cell receptors with variety of different functions are expressed on the surface of these cells. These functions include cell-adhesion, cell-cell identification, phagocytosis, etc. There is a constant exchange of hemocytes between the ones in the circulation and the ones sitting in the sessile compartments. Parasite infection or any mechanical stimuli can mobile the cells from the sessile pockets to enter the hemolymph (Fauvarque & Williams 2011). As in the case of sessile pockets, circulating plasmatocytes are reported to be capable of transdifferentiation into lamellocytes upon wasp infection (Márkus et al. 2009). EGF receptor ligand Spitz in the PSC has been reported to be important for the formation of circulating lamellocytes, since in its absence the lack of circulating lamellocytes is observed (Meister & Ferrandon 2012).

1.3.2.8. Antimicrobial peptides

Antimicrobial peptides (AMP) were discovered to have the conserved sequences from insects to mammals. Additionally, they regulation in both lower and higher species has also shown to be conserved and performed by NF-κB signaling pathway. Other pathways involved in regulation of antimicrobial peptides are Toll signaling cascade, being comparable to Toll-like receptor pathway in mammals, and the immune deficiency pathway (Imd). The Imd pathway is activated by diaminopomelic acid (DAP) – type peptidoglycan, produced by Gram-negative bacteria. This triggers both NF-κB and Toll pathway, leading finally to the expression of antimicrobial peptides (Buchon et al. 2014). Fat body tissues are mostly responsible for the production of antimicrobial peptides, but it is interesting to point out that embryonic plasmatocytes are also capable of producing and secreting them (Fauvarque & Williams 2011).

2. MATERIALS AND METHODS

2.1. Fly techniques

All fly strains were raised on the standard food, that is agar food enriched with a cornmeal, glucose and yeast (agar 9 g, glucose 150 g, cornmeal 160 g, yeast 30 g, methylparaben 50 mL, water 1900 mL) having additional grains of dry yeast on the top of the food. They were kept in incubators at temperature of either 18°C or 25°C having natural light/dark cycles. The following *Drosophila* strains were used:

- CG18446 – GFP (II chromosome) (FlyFos construct containing about 30kbp of genomic DNA surrounding the CG18446 gene tagged with GFP on its C terminus, inserted on the III chromosome. These flies also carry a dsRED selection marker that is visible in the adult eyes under a fluorescent microscope)
- Hop^{tum} / (FM7); CG18446 – GFP (point mutation of Hopscotch gene on the X chromosome, mimicking the infection)
- $CG18446^{exD}$, referred simply as ExD (II chromosome, mutant for CG18446 and surrounding genes, see Introduction)
- $CG18446^{MI02952}$ referred simply as Mimic (Minos transposon insertion on II chromosome, mutant for CG18446 gene)
- Hop^{tum} / (FM7); exD
- hmlGFP (Hemolectin, marker of mature hemocytes, tagged with GFP on the III chromosome)
- Mimic; hmlGFP
- whiteRabbit - CG18446 II (flies having plasmid construct containing CG18446 open reading frame in front of the LacZ reporter and inserted on the II chromosome)
- whiteRabbit – CG18446 III (flies having plasmid construct containing CG18446 open reading frame in front of the LacZ reporter and inserted on the III chromosome)
- mutated whiteRabbit – CG18446 II (flies having mutated plasmid construct containing CG18446 open reading frame in front of the LacZ reporter inserted on the II chromosome (possibility of the double insertion on III chromosome). There are two point mutations in the predicated Su(H) binding sites)

- yw (used as a control)
- w^{1118} (used as a control)

2.2. Immunohistochemistry

For lymph gland dissections, third instar stage larvae were dissected. Protocol for immunostainings of lymph glands used was following:

1. Larvae were dissected in PBS at room temperature and collected into a dissecting dish with PBS on ice within 15 minutes. Larvae were cut into half, anterior part was carefully turned inside out and some fat tissue and gut were removed. Larvae cuticle with brain, discs and remaining parts of gut were basically all fixed. At this point lymph glands were left attached to brains;
2. Afterwards, fixation was performed for 40 minutes in 4% formaldehyde/PBS (room temperature);
3. First two washings for 10 minutes were done with PBX;
4. Second two washings were done with PBT, for 10 minutes and 1 hour. While having tissues in PBT for 1 hour, further dissections were performed. Now only brains having lymph glands attached were completely dissected. The rest of the tissue was removed;
5. Incubation with primary antibody (50 mL) was performed in a small Eppendorf tube, overnight in a cold room with slightly moving;
6. After being in primary antibody, three washings for 20 minutes were performed in the glass dish with PBT;
7. Afterwards, larvae were incubated with secondary antibody (80-100 mL) in the glass dish for 1 hour 30 minutes at room temperature, with slightly moving and protected from light (all secondary antibodies were used in 1:500 dilution);
8. Final three washings were done for 20 minutes in PBT, at room temperature with slightly moving and protected from light. If necessary, additional dissections were performed during these last washes;
9. Larvae were then transferred in 70% glycerol/PBS and left for at least 20 minutes;
10. Finally, lymph glands were prepared for confocal imaging by being mounted with CitiFluor on the microscope slide, covered with a coverslip and sealed with a nail

polisher. Before placing a coverslip, brains were carefully detached from lymph glands and removed from the microscope slide.

For immunostaining of circulating and sessile, third instar stage larvae were dissected. Protocol used was following:

1. Sessile cells: 6 larvae was washed in 20 µL PBS. Posterior part of the larva was cut and larva was inverted inside out. Then it was gently washed in PBS to get rid of any residual circulating cells. 60 µL of PBS was put on a coverslip and then sessile cells from all six larvae were detached by passing forceps through the inside out cuticle gently;

   Circulating cells: Larva was bled in 20 µL Schneider medium. The solution was immediately mixed and placed to the coverslip. Another larva was bled in 20 µL Schneider medium, mixed and added to the cover slip;
2. Coverslip is placed on a microscope slide in a humidified chamber and cells were allowed to attach for 20 minutes;
3. PBS/Medium was then sucked off and 1,5 mL 4% formaldehyde/PBS was added. Cells were fixed for 20 minutes at room temperature;
4. Two washes for 5 minutes in PBS were performed in a 6-well dish;
5. One wash in PBX and one wash in PBT were then done. Wash in PBT lasted for 45 minutes;
6. Humidified chamber with a microscope slide was prepared again. 15 µL of primary antibody was then put on a cover slip placed on the slide in a chamber and left for 1 hour 30 minutes at room temperature;
7. Coverslip was then transferred back to the 6-well dish and washed 3 times for 10 minutes with PBT;
8. 15 µL of secondary antibody was added in the same way as primary (coverslip on microscope slide in a humidified chamber) and left at the room temperature for 1 hour.
9. Final washings were then done again in a 6-well dish, 3 times for 10 minutes in PBT;
10. A small drop of mounting media was added to a clean microscope slide, coverslip was placed onto it (with the side with cells towards media), left for 15 minutes and sealed with nail polish;

Following primary antibodies were used: α-GFP Rabbit (Life technologies, 1:500), P1 Mouse (a generous gift from the laboratory of I. Ando, 1:100), Lz Mouse (Developmental Studies Hybridoma Bank, 1:20), Antp Mouse (Developmental Studies Hybridoma Bank, 1:100).

2.3. Staging

Staging of larvae was performed in the following way (in accordance to the literature Linford, Bilgir, Ro, & Pletcher, 2013). 100 females and males were put in small cages on juice plates with a yeast paste and left for one to two days to adapt to the environment. Plates were changed every day. Before collecting the embryos of the same age within 2 hours period, eggs laid during pre-lays period should be excluded (Tran & Welte 2010). Flies endeavor to keep embryos in their oviduct and fresh food stimulates their deposition. Therefore a plate was changed once in an hour to eliminate all preserved embryos. Finally, fresh juice plate with a very few amount of yeast paste was put and flies were laying embryos for 2 hours. Embryos from the plates were collected, carefully washed in PBS three times and seeded in vials with the food. Dissections were then performed at specific time points (between 98^{th} to 145^{th} hour) after eggs laying (AEL). Immunostaining was performed as previously described.

2.4. Temperature-induced melanization

The protocol was modified from the protocol found in the literature (Dudzic et al. 2015) and performed in a following way: larvae were washed in PBS, dried gently on the tissue paper and placed separately in PCR tubes (one larva per tube). Tubes with larvae were then placed in PCR machine at the temperature 60°C for 10 minutes. Larvae were then placed onto microscopic slide and pictures were taken using bright field microscope.

2.5. X-gal staining

X-gal staining was performed using either third instar stage larvae (for staining larval tissues) or 3-5 days old males/virgins (for staining adult teste/ovaries). Protocol used was following:

1. Larvae/adult ovaries/adult testis were dissected in PBS within 15 minutes;
2. Fixation was performed with 2,5% glutaraldehyde/PBS for 7 minutes at room temperature;

3. Tissues were washed three times in PBS at room temperature;
4. Second two washings were performed in PT solution (1 mL 10xPBS, 100 µL 1M $MgCl_2$ x $6H_2O$, 300 µL 10% Triton, 8 mL H_2O, 320 µL 0,1M $K_4[Fe(CN)_6]$, 320 µL $K_3[Fe(CN)_6]$) for 10 minutes;
5. Afterwards, PT solution was changed for the third time and few grains of X Gal were added. Solutions with grains and tissues were properly mixed and placed at 37°C to wait for a color to develop. Tubes at 37°C were occasionally mixed.
6. Reaction was stopped at the same time for all tissues and final (at least three) washings were performed in PBS;
7. Finally, 70% glycerol/PBS was added for at least 20 minutes and tissues were mounted and preserved with CitiFluor on a microscope slide covered with a coverslip;
8. Pictures were taken using bright-field microscope;

2.6. Site-directed mutagenesis

Plasmid construct 'whiteRabbit-CG18446' containing CG18446 coding region in front of the LacZ reporter was point-mutated at the putative Su(H) binding sites in order to elucidate whether the expression of the construct is regulated by Notch. Firstly, plasmid was purified using QIAGEN Plasmid Midi Kit. After the purification, plasmid was amplified using Pfx polymerase and first set of primers designed to include the point mutation. In order to destroy the non-mutated, parental plasmid, digestion was performed using DpnI enzyme (since the original plasmid isolated from bacteria is methylated and hence recognized by DpnI but the PCR product is not). Then plasmid was transformed into the competent bacteria cells using heat-shock transformation. After the successful transformation, plasmid was purified using High-Speed Plasmid Mini Kit and checked by sequencing verification. Once the mutation was confirmed, the whole procedure was repeated using another set of primers, to mutate another Su(H) binding site. After mutating the plasmid in the desired way, it was sent for injection to generate transgenic flies. It is important to mention that plasmid contained LacZ region, enabling X-gal staining.

2.7. DNA extraction and flies verification

Flies used in the experiments were verified by PCR. Single-fly genomic DNA extraction provided DNA clean enough for the PCR. However, in the case of qPCR, genomic DNA extraction was performed by a cleaner method. For both PCR methods the same set of primers was used, spanning the size of 94 bp and having following sequences:

- 18446 mimic real a: CATGTTGGGTGAGGTGCTC
- 18446 mimic real s: GCTGGTCAAGGAGATTCTGG

Single Fly Extraction

1. 1 female or male fly (better male due to no possibility of recombination) in 50 µL of squashing buffer (add 1/100 dilution of 20 mg/mL stock proteinase K to make final concentration of 200 µg/mL),
2. Mash flies with yellow pipette tip (suck a little bit liquid into tip first in order to avoid the stuck of flies pieces), mash flies until solution becomes cloudy.
3. 37°C for 30 minutes (water bath)
4. 95°C for 2 minutes (heat blocker)
5. Suck of the supernatant for precipitation or store samples at -20°C (use the top for PCR immediately)

SB (squashing buffer) solution

10 mM Tris HCl pH 8.2
1 mM EDTA
25 mM NaCl
0,2% Triton x-100

Genomic DNA extraction

1. select 5 males and put them in a 1,5mL tube
2. add 100µL of squashing buffer
3. homogenize the flies

4. add 12,5µL of 20mg/mL proteinase K
5. add 50µL of SDS 10%
6. incubate over night at 55°C
7. add H_2O till 500µl
8. add 500µL of phenol and mix the two phases with vortex
9. centrifuge 7' at 13.500 rcf
10. take the supernatant and put it in a fresh tube
11. add 500µL of chloroform and mix the two phases with vortex
12. centrifuge 7' at 13.500 rcf
13. take the supernatant and put it into a fresh tube
14. add 1mL of 100% Et-OH
15. add 40µl of 3M sodium acetate
16. incubate over night at -20°C
17. centrifuge 20' at 17000 rcf at 4°C
18. discard the supernatant
19. add 1mL of 70% Et-OH
20. centrifuge 10' at 17000 rcf at 4°C
21. discard supernatant
22. let dry all the ethanol
23. resuspend the pellet with the right amount of H_2O (depending of the size of the pellet)

At the end the DNA must be read on Nanodrop in order to know its concentration and its purity from proteins and from phenols.

2.8. Statistics and microscopy

Graphs were done in Microsoft Excel and RStudio. Statistics was done in RStudio and significance of results was calculated using two-tailed Student's t test. Statistical significance was presented on graphs in a following manner: for p-value ≤ 0,05 one asterisk (*), p-value ≤ 0,01 two asterisks (**), p-value ≤ 0,001 and p-value ≤ 0,0001 three asterisks (***).

Pictures of lymph glands, circulating and sessile cells were taken on Olympus confocal microscope and analyzed using Fluorview ver 3.1 (Olympus).

3. RESULTS

3.1. CG18446 expression in the lymph glands of CG18446-GFP flies

The expression of CG18446 gene in the both wild type and Hop^{tum}/(FM7) flies has been observed in the cortical zone of the lymph gland. This zone is characterized by the presence of mature hemocytes, more specifically plasmatocytes and crystal cells. Therefore, we used CG18446-GFP flies and stained them with α-GFP antibody, P1 (marker of plasmatocytes) and Lz (marker of crystal cells). According to the results, CG18446 is expressed in plasmatocytes under normal conditions (Fig. 10). Namely, P1 staining colocalized with cells expressing CG18446. Picture shown on the figure represents one optical slice, giving the impression of a not completely overlapping patterns (since CG18446 is a nuclear protein and P1 is in cytoplasm). Nevertheless, once all pictures throughout the whole tissue are checked, the expression of the gene in plasmatocytes is clear.

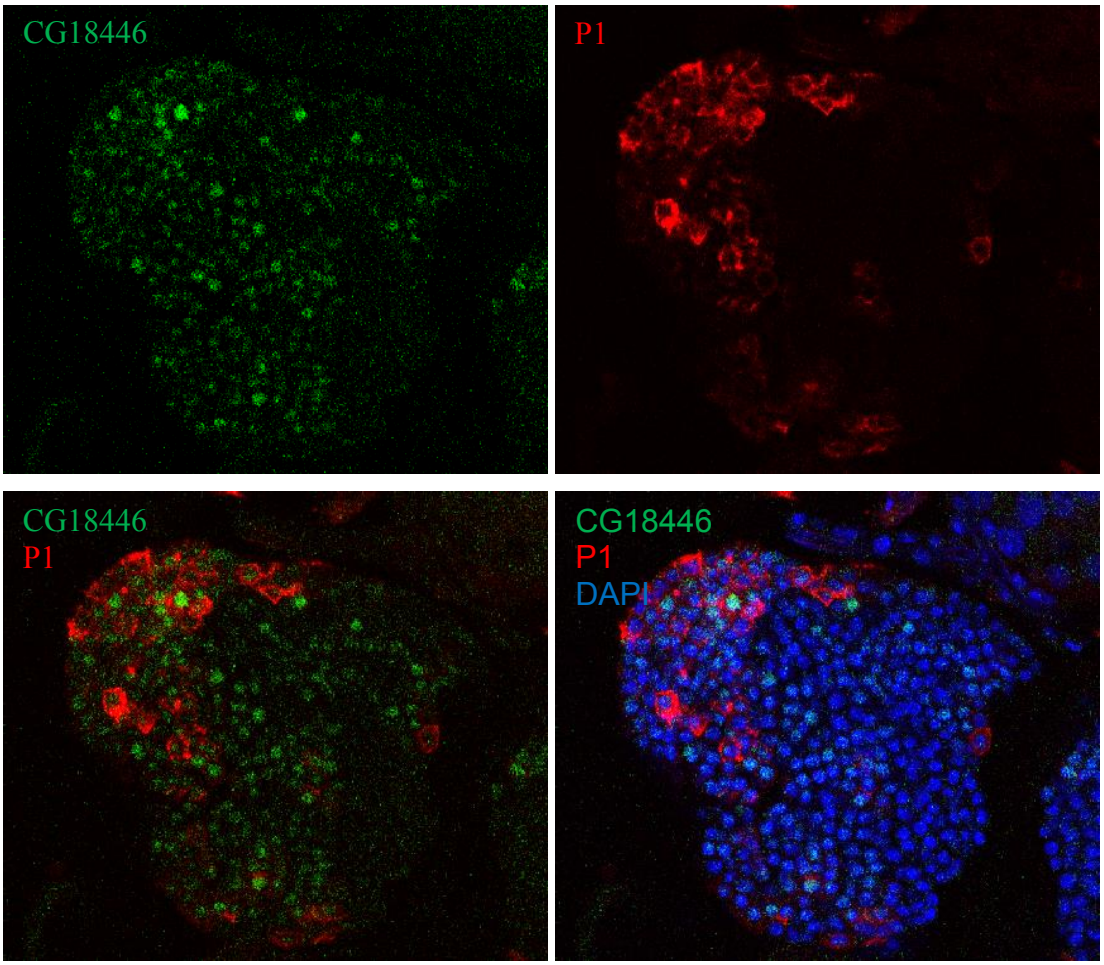


Figure 10: The expression of CG18446 (GFP, green) and plasmatocytes (P1, red) in the lymph gland of CG18446-GFP flies.

The expression pattern of crystal cells and CG18446 has been compared as well. As it can be seen on the Figure 11, there are no cells expressing both CG18446 and Lz, marker for crystal cells.

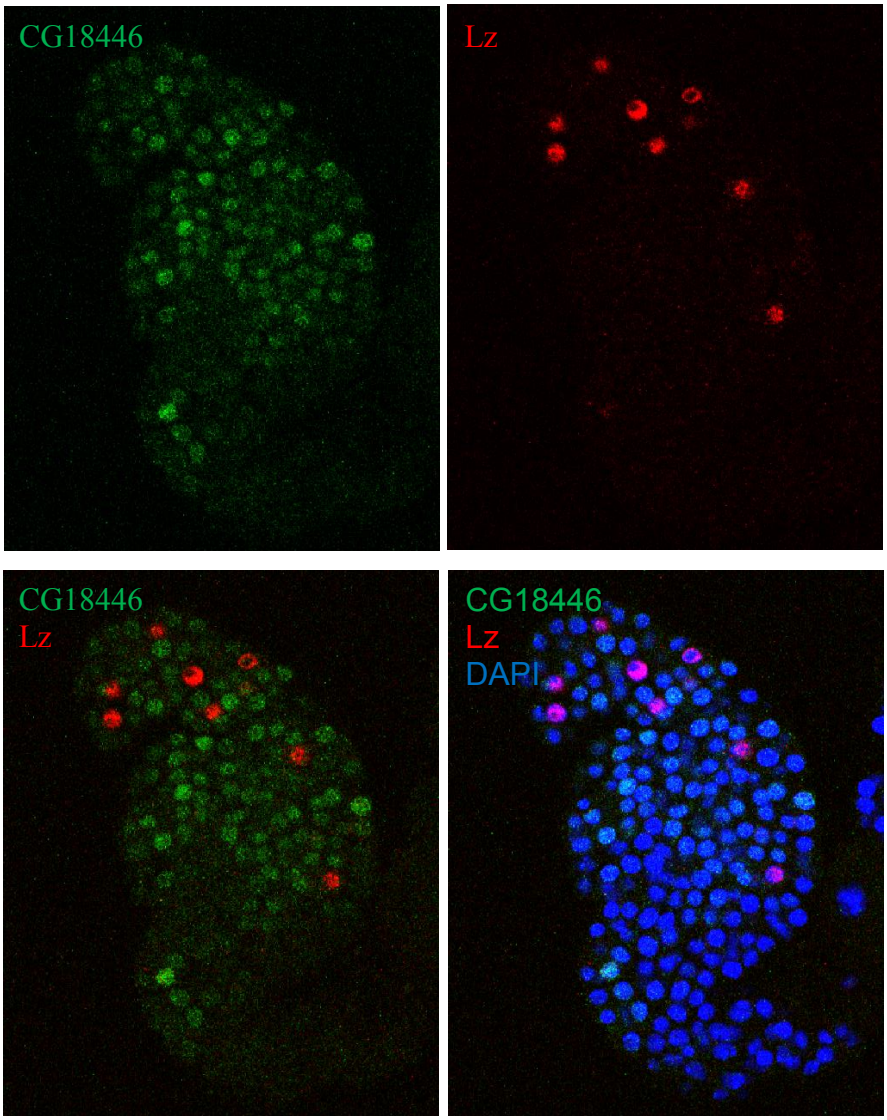


Figure 11: The expression of CG18446 (GFP, green) and crystal cells (Lz, red) in the lymph gland of CG18446-GFP flies.

Besides the cortical zone, we noticed that the CG18446 signal was occasionally present in the posterior signaling center of the lymph gland (PSC) under the normal uninfected conditions as well as in Hop^{tum} lymph glands (Fig. 12). This is very interesting to mention, as this area is believed to be a master regulator of the pool of mature and immature hemocytes in the lymph gland and a niche for the specification of lymph gland immune cells (Jung et al. 2005; Krzemien et al. 2010). PSC is the essential part of the lymph gland, since all the pathways influencing hemocytes situated in either cortical or medullary zone are functioning via PSC (Duvic et al. 2002; Lebestky et al. 2003; Mandal et al. 2010; Sinenko et al. 2009).

Antennapedia (Antp) is a gene known to be expressed in PSC (Mandal et al. 2010) and we found that some cells express both CG18446 and Antp. However, the CG18446 signal is only transient and turned to be very difficult to catch by immunostaining.

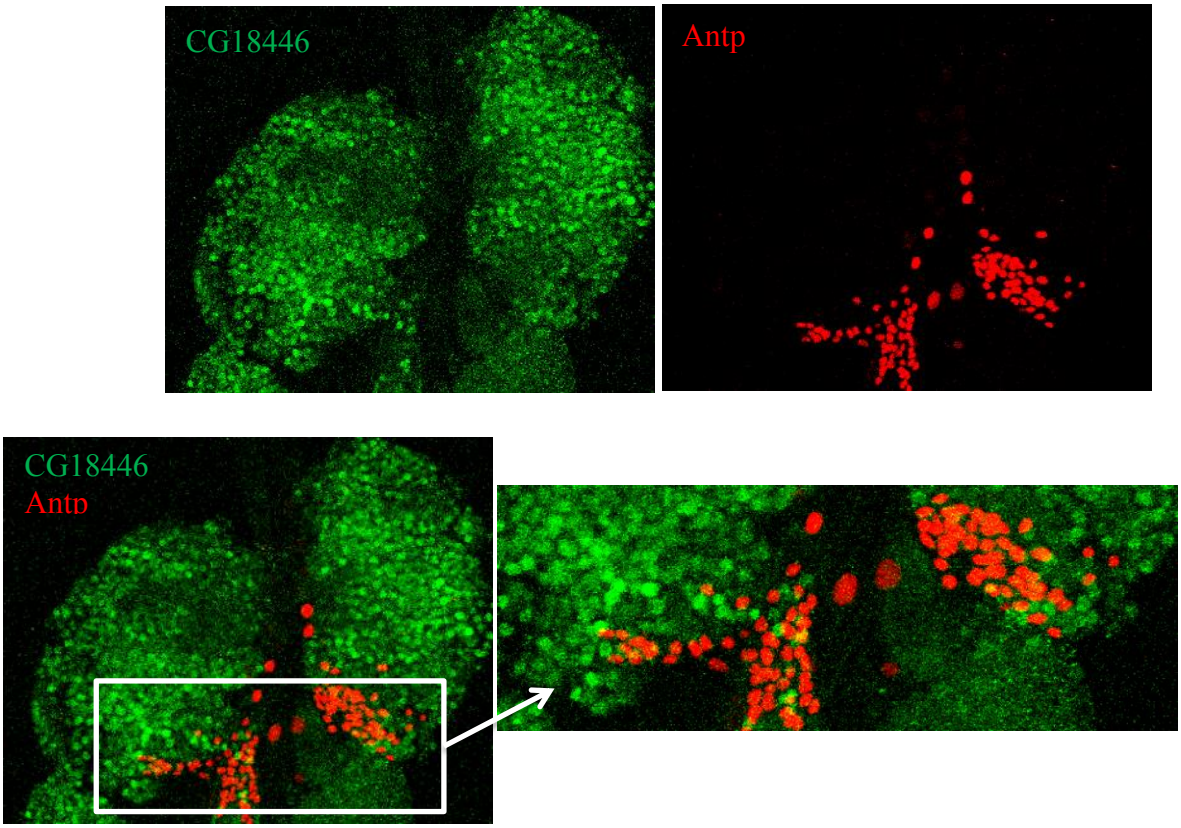


Figure 12: The expression of CG18446 (GFP, green) in PSC marker Antennapedia (Antp, red) in the lymph gland of the CG18446-GFP flies.

As we mentioned earlier, the expression of CG18446-GFP in the lymph gland is very low and at the beginning of our experiments we encountered problems with the reproducibility of the staining; there were lymph glands that stained well with GFP and lymph glands where we did not see any GFP signal. This could have been a technical issue or it could reflect a dynamic expression pattern of CG18446 during the lymph gland development (we stained lymph glands of various ages in the same batch). To distinguish between these two scenarios we decided to determine the expression of CG18446 gene in the lymph gland throughout the larval development, carefully staging the larvae the way explained in the 2.3 Materials and Methods section. Results suggested that the expression of CG18446 in the cortical region of

the lymph gland does not change significantly during the development and the lack of GFP signal after immunostaining is probably due to the technical issue when we detect signal close to the threshold detecting limits (Fig. 13).

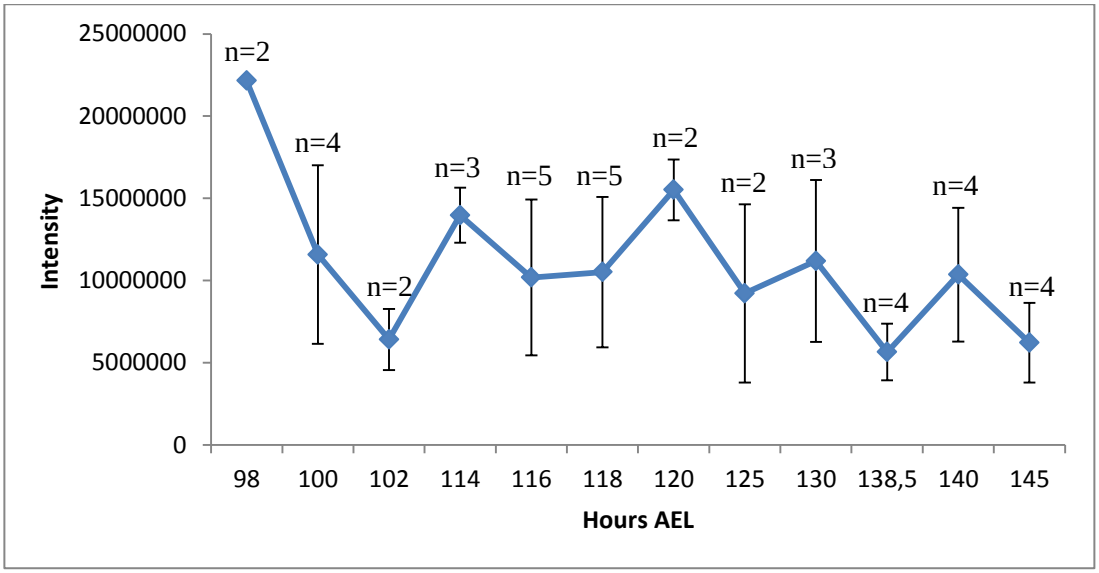


Figure 13: The expression of CG18446 throughout larval development (from 98th hour until 145th hour AEL). The graph is constructed by measuring the intensity of the CG18446 signal in the cortical zone of the lymph gland divided by the area where the signal was measured. Error bars represent standard deviation per each time point.

3.2. CG18446 expression in the Hop^{tum} flies

The expression of CG18446 has also been checked in the lymph gland of Hop^{tum}/(FM7); CG18446-GFP flies that are considered to be mimicking the infection conditions. As it has been done in the case of CG18446-GFP flies without Hop^{tum}, markers of both plasmatocytes and crystal cells have been used simultaneously with α-GFP on Hop^{tum}/(FM7); CG18446-GFP flies. It has been observed that none of the expression patterns significantly differs from the ones noticed in wild type (Fig. 14). In other words, CG18446 has been expressed only in plasmatocytes even under infection conditions. It would have been interesting to check the colocalization of CG18446 with any marker of lamellocyte differentiation in these flies but we did not succeed in making the lamellocyte specific L1 antibody work in our hands. It should also be pointed out that the dissection of lymph glands

in Hop^{tum}/(FM7) flies was rather tricky, since they start disintegrating during early third larval stage as a response to the "infection".

It is also important to mention that Hop^{tum}; CG18446-GFP stock is balanced with FM7 on I chromosome. The marker can be checked in both larvae (green dots in antennae under the GFP microscope) and adults (heart-like eyes). However, all larvae used for dissections were not FM7 (even though the marker was sometimes missing in adults, but constantly present in larval state). Additionally, these flies are only possible to be kept in heterozygotes state on II chromosome, probably due to possible synthetic lethality of the homozygotes state.

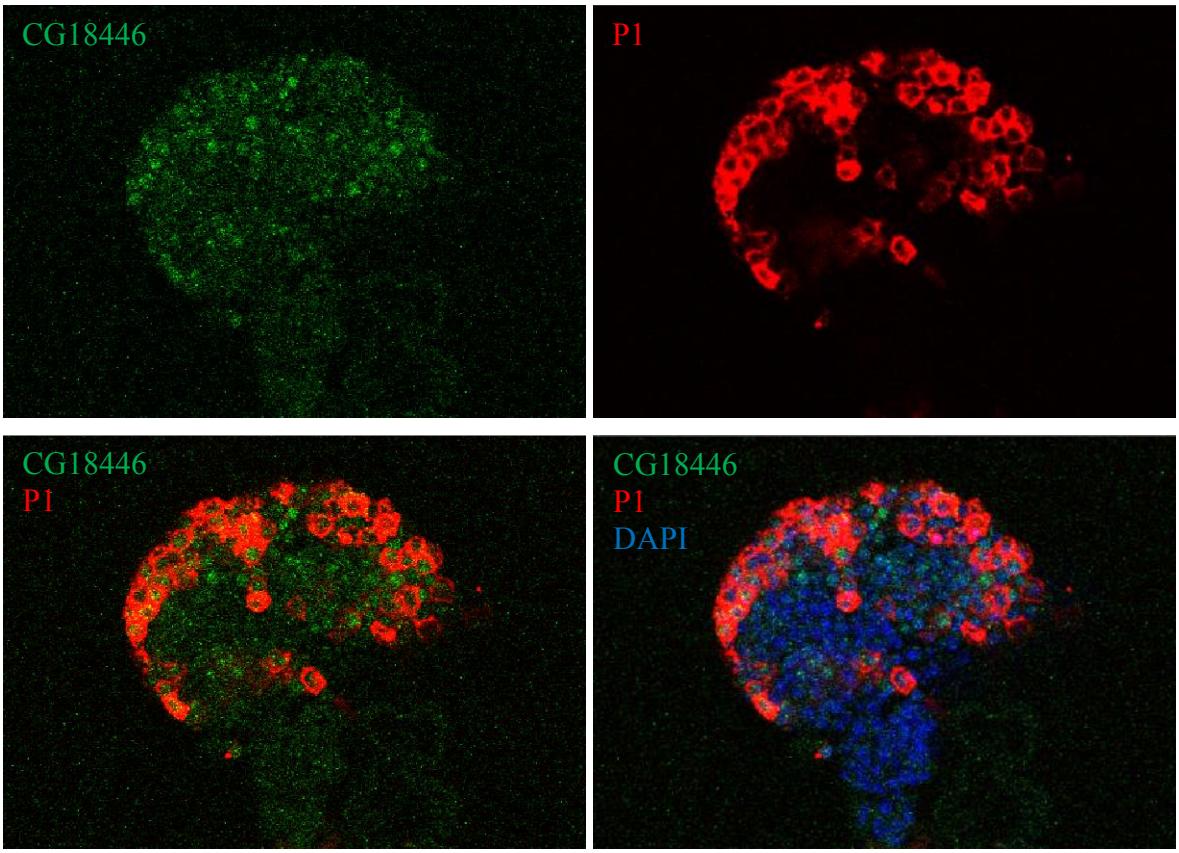


Figure 14: The expression of CG18446 (GFP, green) and P1 (plasmatocyte marker, red) in Hop^{tum}/(FM7); CG18446-GFP flies.

As expected, less crystal cells are noticed in Hop^{tum}/(FM7); CG18446-GFP flies when compared to wild types (Small et al. 2014). Additionally, the expression pattern of Lz and CG18446 did not overlap in those flies (Fig. 15).

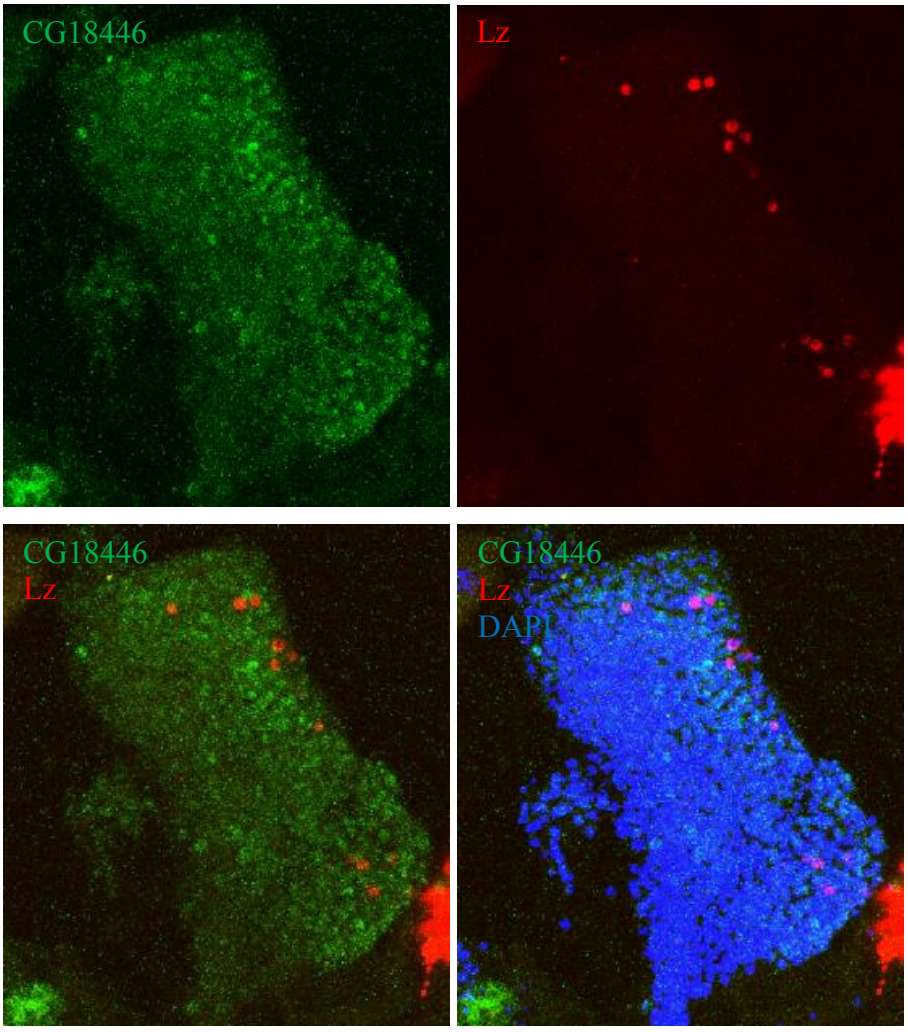


Figure 15: The expression of CG18446 (GFP, green) and Lz (crystal cell marker, red) in Hop^{tum}/ (FM7); CG18446-GFP flies

We also checked whether the interesting observation of CG18446 signal in PSC under normal conditions is true also under the immune-challenged conditions – in Hop^{tum} flies. The very same results are obtained (Fig. 16), suggesting that the expression of CG18446 in plasmatocytes and in PSC is the same in normal situation as well as in the condition of an immune challenge. What can also be suggested from the images of immunostainings is that the expression pattern of CG18446 in PSC is increased in the case of the infection. However, this hypothesis should be further investigated.

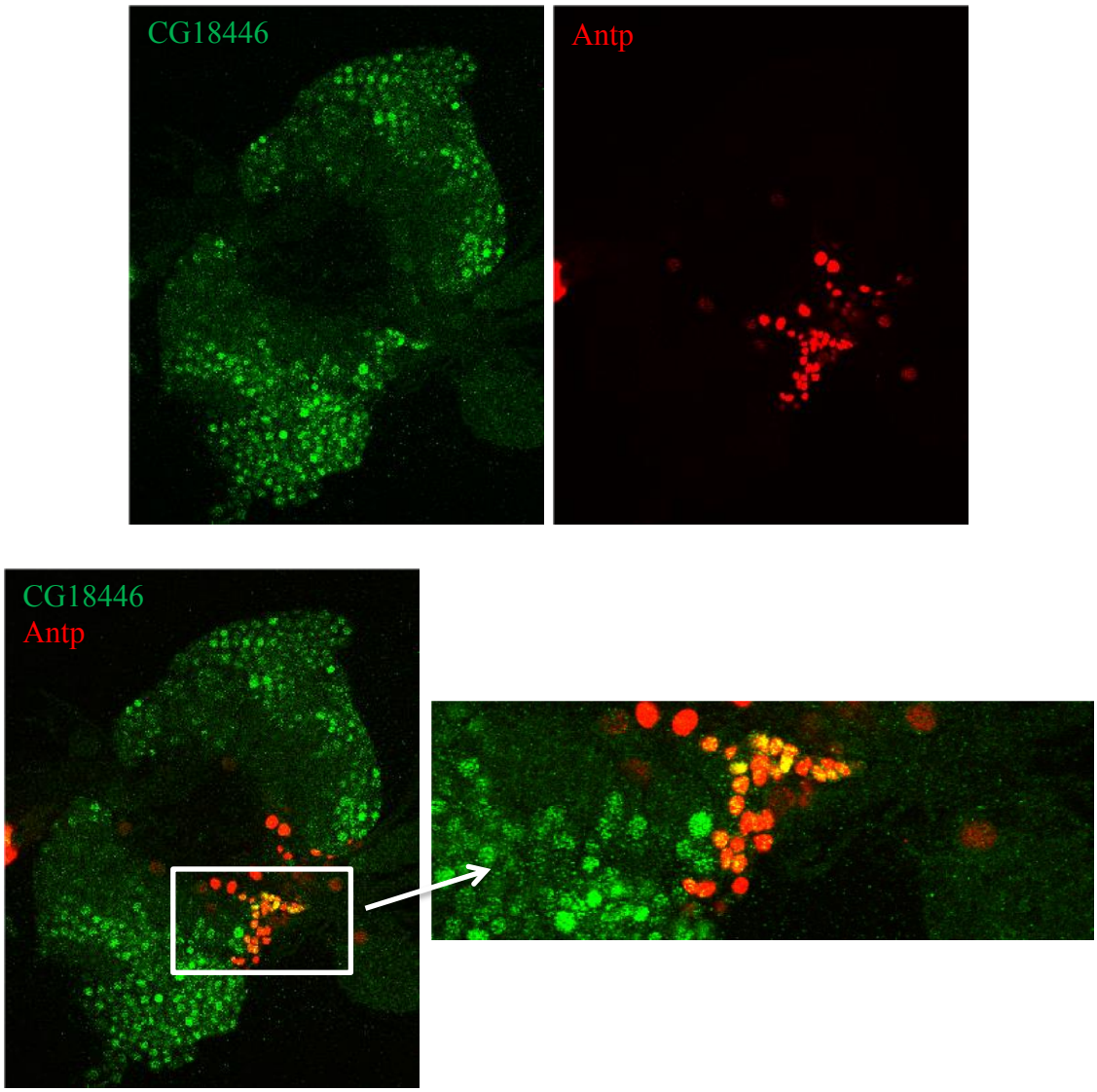


Figure 16: The expression of CG18446 (GFP, green) in Antennapedia (Antp, marker of PSC, red) in Hop^{tum}/ (FM7); CG18446-GFP flies.

3.3. Influence of CG18446 on the number and the class of circulating and sessile hemocytes under normal conditions

Before being able to compare the number of circulating immune cells in wild type versus CG18446 mutant flies we had to construct the mimic;HemGFP stock (HemGFP is a marker of mature hemocytes). To verify the stock we performed a real time PCR analysis of the CG18446 gene in these flies with primers described in 2.7. section of Materials and Methods. The primers span the insertion site of the mimic transposon. This means that no

signal should be seen from amplification from the genomic DNA of the mimic flies as the potential 3kb long PCR product will not be amplified by the real time PCR settings optimized for the amplification of only very short DNA fragments (Fig. 17).

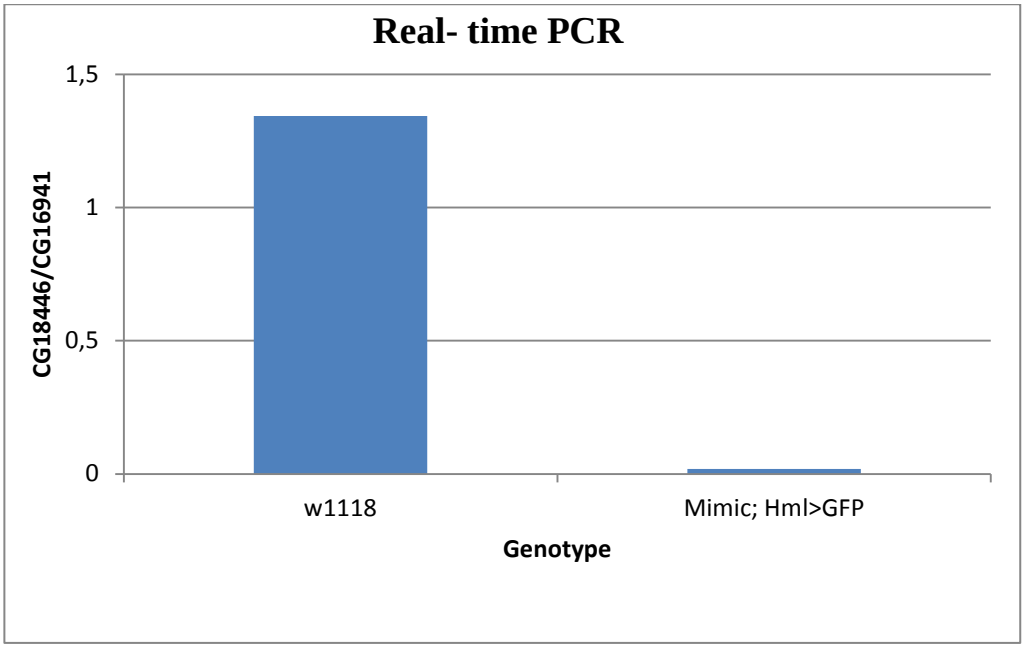


Figure 17: qPCR data for w^{1118} and Mimic; Hml>GFP genomic DNA, using primers described in 2.7. section of Materials and Methods. It is normalized to signal from CG16941 as a positive control.

In order to test whether CG18446 has an effect on the number of circulating hemocytes, we have counted the total number of immune cells in wild type and CG18446 mimic mutants and compared it to wild type situation. We showed that CG18446 mutants have lower number of circulating hemocytes when compared to the wild type (Fig. 18). The decrease was about 50% when comparing the median values.

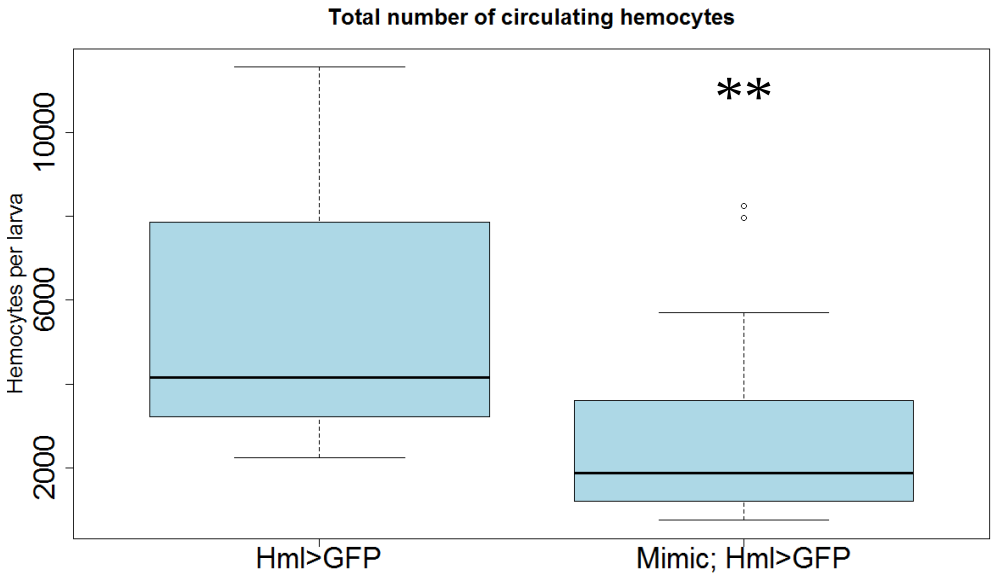


Figure 18: Total number of circulating hemocytes in third instar larvae counted in CG18446 mutants (Mimic; Hml>GFP) and wild type (Hem>GFP). Error bars represent standard deviation, 15 larvae per genotype were counted. Student t test has been run to obtain p-value = 0,009734.

After detecting the lower number of circulating immune cells in CG18446 mutants, we were wondering whether there is any difference in the number of mature and immature hemocytes. Since hemolectin is reported to be a marker of mature hemocytes (Goto et al. 2003; Goto et al. 2001), it is straightforward to make a distinction between differentiated and undifferentiated immune cells. Our results revealed that CG18446 mutants manage to maintain roughly the same number of differentiated hemocytes, despite having smaller total number of cell in the hemolymph. However, they have difficulties in maintaining the pool of immature hemocytes (Fig. 19).

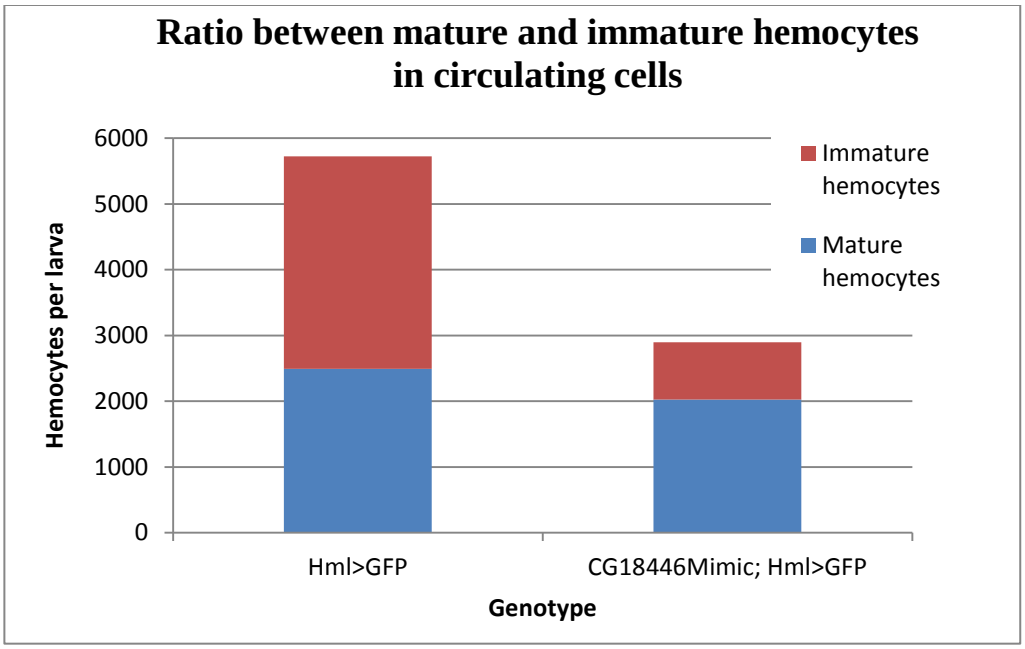


Figure 19: The number of mature (GFP positive) and immature (GFP negative) hemocytes in circulating cells in wild type and CG18446 mutant.

The actual number of circulating mature hemocytes in CG18446 mutants is the same as in wild type flies. However, if we compare the ratios of mature hemocytes to the total number of cells in wild type and CG18446 mutants, we can say that there are less immature immune cells in CG18446 mutants than in wild type. The same trend is observed in sessile cells (Figure 20). It should be mentioned that the total number of sessile cells could not be obtained due to technical difficulties, therefore the ratios are acquired by counting hemolectin positive cells and all cells on different spots on the microscope slides. Student t tests were ran for both circulating and sessile cells results.

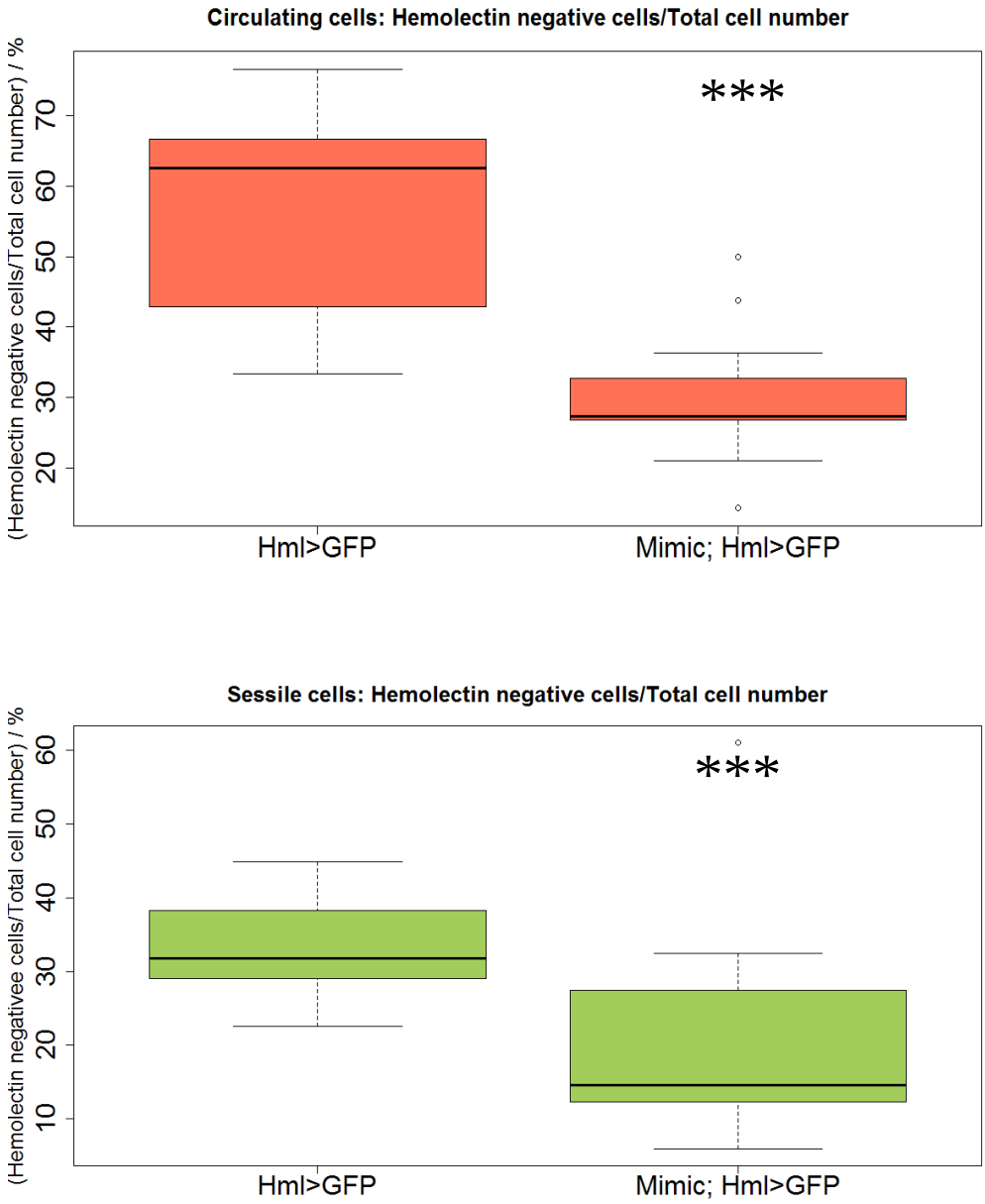


Figure 20: Ratio of the number of immature hemocytes (GFP negative) to the total number of immune cells (GFP positive + GFP negative) per one picture taken on microscope, in CG18446 mutant (Mimic; Hml>GFP) and wild type (Hml>GFP) calculated in circulating cells and sessile cells. Error bars represent standard deviation. 10 repeats (10 pictures from how many larvae in total) were counted for circulating cells/20 repeats were counted for sessile cells. In the case of circulating cells, p-value = 0,0002962 and in the case of sessile cells p-value = 0,0002966.

3.4. Influence of CG18446 on the class of hemocytes under immune-challenged conditions (Hop^{tum} flies)

Under immune-challenged conditions, besides plasmatocytes and crystal cells, differentiation of lamellocytes occurs as well. As already stated, both crystal cells and lamellocytes are capable of producing melanin, enzyme used as a defensive mechanism against parasites (Dudzic et al. 2015). Melanization also enables a visualization of these cells. Therefore we have exposed Hop^{tum}/(FM7) flies and Hop^{tum}/(FM7); exD flies to 60°C for 10 minutes (2.4. section of Materials and Methods). We have assumed that any difference observed would be due to the difference in the number of lamellocytes (or crystal cells even if it is not likely under immune challenge conditions). Additionally, heating of CG18446 mutants in non-infected conditions (without Hop^{tum}) has also been done and no significant difference was observed (Fig. S1, Supplementary Data, compared just by eye since quantification of the signal is difficult). According to the outcome of this experiment, the difference is striking (Fig. 21). It is rather hard to spot the melanotic tumors caused by boiling at this magnification in the case of Hop^{tum}/(FM7) flies. As already emphasized earlier, larvae used for the experiments were homozygous on I, but heterozygous on II chromosome. Therefore the heating was performed on flies containing the cyo balancer.

Figure 21: Hop^{tum}/ (FM7) and Hop^{tum}/ (FM7); CG18446^{exD}/ cyo larvae after the heat treatment at 65°C for 10 minutes. Number of larvae tested per genotype tested was 20.

3.5. CG18446 expression regulation

As already mentioned in the Introduction section 1.2., chromatin immunoprecipitation experiments showed that the binding of the key transcription factor of Notch, Su(H), to the CG18446 gene within its open reading frame. Therefore, we cloned a plasmid with open reading frame of CG18446 in front of the lacZ construct and expected that if the ORF contains regulatory region for CG18446 expression we should see blue staining (lacZ signal revealed by X-gal staining) in tissues where C18446 is normally expressed (as we know from the CG18446-GFP stainings). Then, we wanted to mutate the putative binding sites of Su(H) within the open reading frame and test whether the expression of CG18446 lazC reporter is abolished, revealing a subset of tissues where CG18446 is regulated by the Notch pathway. Before the experiment with X-gal staining was performed, we verified that the transgenic flies indeed contained the whiteRabbit plasmid with CG18446 open reading frame (Fig. 22).

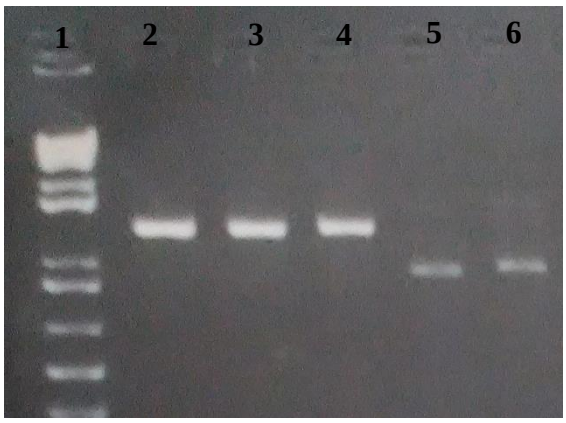
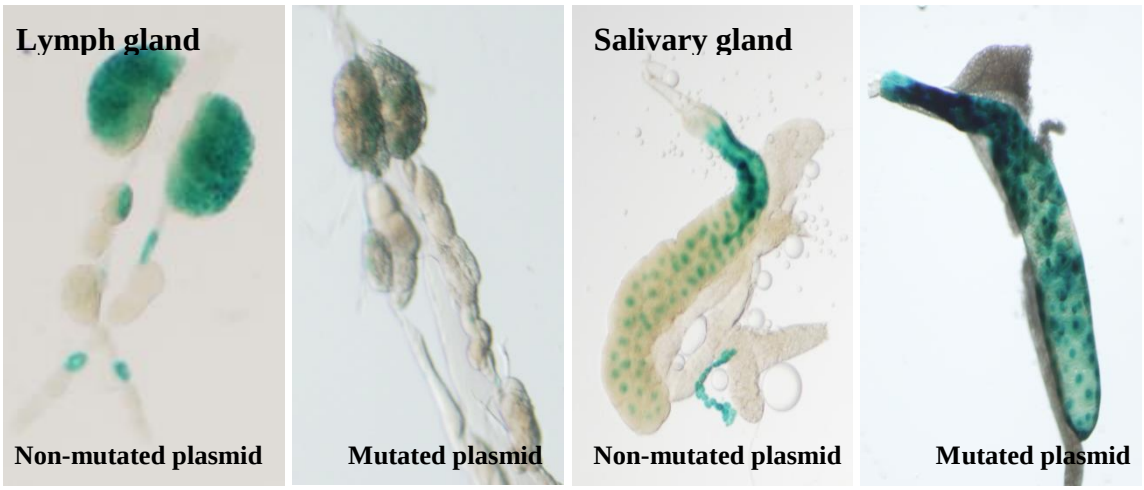
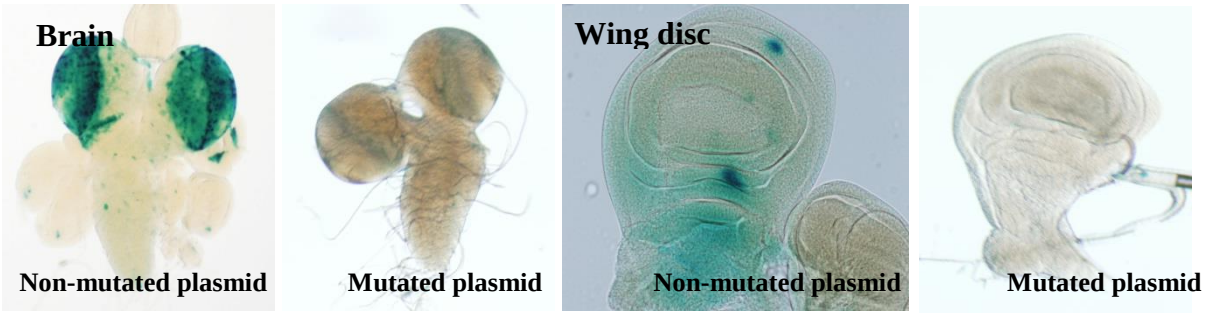


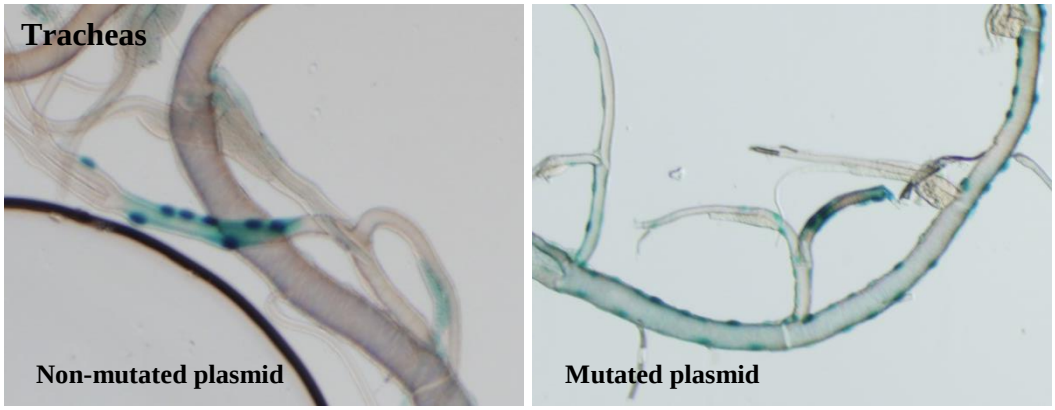
Figure 22: Agarose gel with PCR samples containing DNA from following flies' strains: 1. Ladder, 2. With mutated plasmid, 3. With non-mutated plasmid on II chromosome, 4. With non-mutated plasmid on III chromosome, 5. w^{1118} (control I), 6. yw (Control II). Primers used were described in 2.7. section of Materials and Methods.

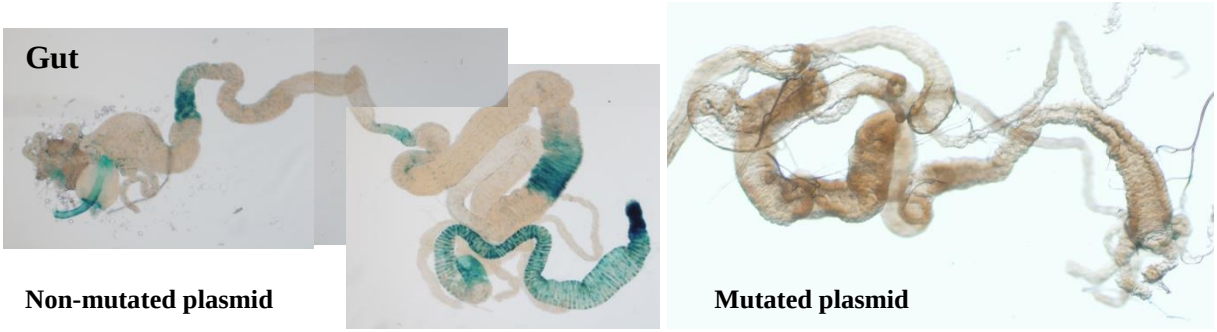
We tested Notch dependent regulation of CG18446 in larval tissues and adults ovaries and testes. We observed that the blue color of X-gal staining corresponded to the antibody staining of CG18446 so we can conclude that the regulatory regions for CG18446 expression lie within its ORF. As it can be observed on the Figure 23, expression of both the non-mutated and mutated constructs is present in the lymph gland, salivary gland, brain, tracheas, testes

and ovaries. Nevertheless, in many of these tissues the expression of the mutated construct is weaker than in the case of the non-mutated plasmid construct and it is completely missing in the gut. This could be due to Notch dependent regulation of CG18446 but the mutations we introduced did not fully abolish the Su(H) binding; alternatively, the differences could be only due to different strengths of the insertion sites for the mutated and non-mutated constructs without a proof of Notch dependent regulation is weaker than in the case of flies having non-mutated plasmid construct. A note should be made regarding the X-gal staining of adult testes. The most interesting part of the tissue is considered to be the very tip of testes (marked with a square on the Figure 23), a place of non-dividing so called hub cells, surrounded by germline stem cells (Hardy et al. 1977).


Lymph gland
Non-mutated plasmid
Mutated plasmid
Salivary gland
Non-mutated plasmid
Mutated plasmid


Brain
Non-mutated plasmid
Mutated plasmid
Wing disc
Non-mutated plasmid
Mutated plasmid


Tracheas
Non-mutated plasmid
Mutated plasmid


Gut
Non-mutated plasmid
Mutated plasmid

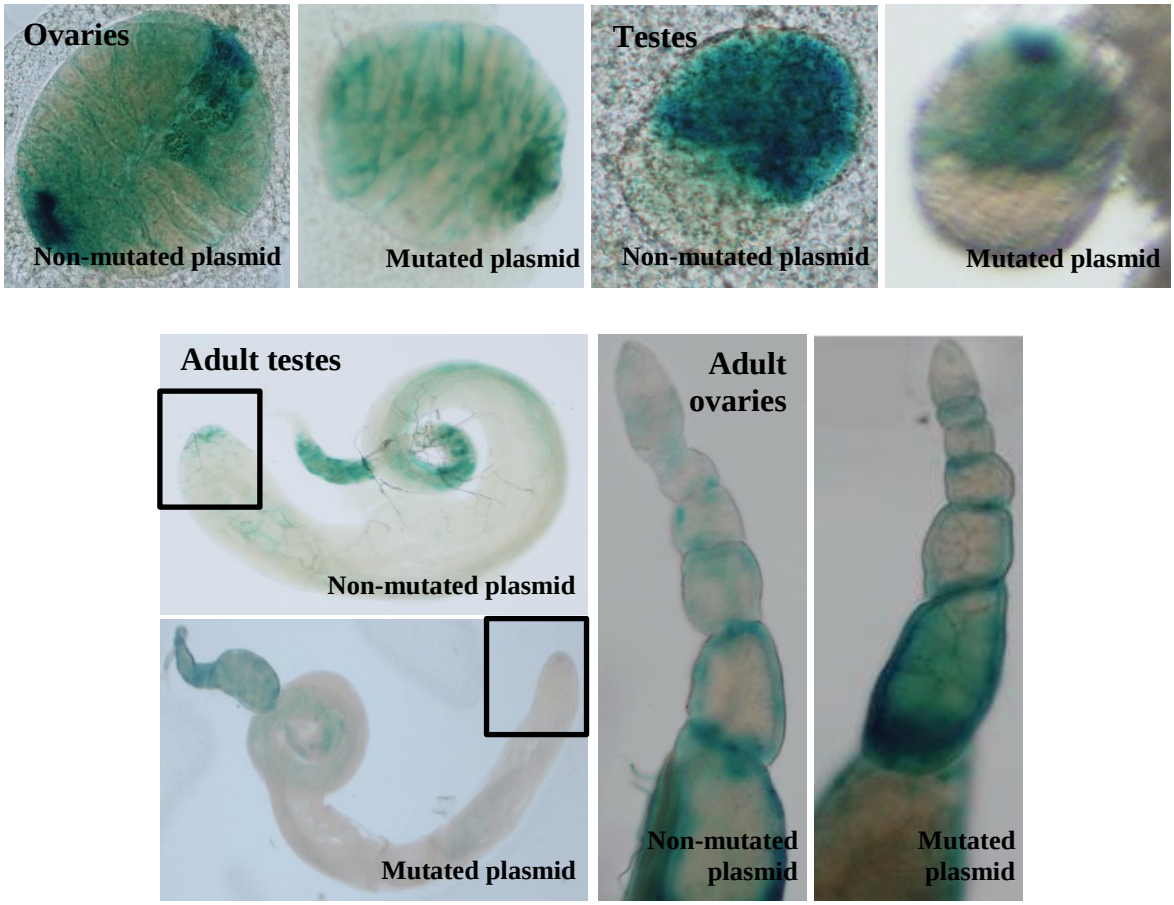


Figure 23: X-gal staining of flies containing the non-mutated construct with CG18446 ORF in front of the LacZ reporter (non-mutated whiteRabbit-CG18446) and of flies where the mutated putative binding sites of Su(H) within CG18446 ORF - LacZ construct were mutated (mutated whiteRabbit-CG18446).

Table I summarizes all the tissues that showed the expression pattern observed using antibody and performing X-gal staining. There are two lines of flies containing non-mutated plasmid since in one case it was inserted on the second and in other case on the third chromosome. However, the expression localization is almost identical, with slight differences in the intensity or pattern in few tissues.

Larva	Non-mutated plasmid (III)	Non-mutated plasmid (II)	Mutated plasmid	Antibody
Brain	yes strong	yes strong	very weak	*n.a.*
Lymph gland	yes	yes	Yes	yes
Gut	yes	yes weak	No	*n.a.*
Ovaries	yes	yes	Yes	yes
Testes	yes	yes	Yes	yes
Cells in cuticule	yes	yes	yes	*n.a.*
Fat tissue	no	no	no	yes
Wing disc	yes stripe	yes dots	no	no
Eye disc	yes	yes	yes	*n.a.*
Tracheas	yes	yes	yes	*n.a.*
Salivary gland	yes	yes	yes	*n.a.*
Adults	Non-mutated plasmid (III)	Non-mutated plasmid (II)	Mutated plasmid	Antibody
Adult testes	yes	yes	yes weak	yes
Adult ovaries	yes	yes	yes	yes

Table I: The expression pattern observed using X-gal staining on flies with non-mutated and mutated whiteRabbit-18446 constructs and α-GFP antibody on CG18446-GFP flies.

4. DISCUSSION

At the very beginning of the discussion, the reasoning behind the use of CG18446 mutants should be explained. First set of experiments were done with $CG18446^{exD}$ mutants, a mutant having the complete deletion of the CG18446 gene as well as in a few surrounding genes present in this gene rich region. Another mutant, $CG18446^{Mimic}$, has a 3 kbp long sequence inserted in its ORF, presumably disturbing only the expression of the CG18446 gene (although we cannot entirely exclude indirect, regulatory, effects on the expression of other genes in this gene rich region). All experiments shown in Supplementary Data are conducted with both types of mutants, since we were hoping to see the same trends in the results using either of the mutants. Except the fecundity assays, exD and Mimic mutants behaved indeed very similarly suggesting that the phenotypes observed is due to CG18446 disruption.

4.1. The role of CG18446 gene in immune response

We observed the expression pattern of CG18446 gene in plasmatocytes under both normal and immune-challenged conditions (Fig. 10 and Fig. 14) but we did not notice the expression in crystal cells under neither conditions (Fig. 11 and Fig. 15). Due to lack of antibody, expression of CG18446 in lamellocytes under infection conditions was not checked by immunostaining. As it is shown in Figure 13, CG18446 is relatively constantly expressed in the lymph gland throughout larval development. This suggests the need for this gene during the growth of *Drosophila* larval lymph gland. Besides the expression in mature plasmatocytes that are situated in the cortical zone of the lymph gland, CG18446 was also detected in the posterior signaling center under both normal and infection-like conditions (Fig. 12 and Fig. 16). This is very interesting to notice, since posterior signaling center (PSC) was reported to be crucial in keeping the reservoir of mature and immature hemocytes (Jung et al. 2005). Once we elucidated the expression pattern in the lymph gland, we wanted to closer investigate the role of CG18446 in the immune system and its components in *Drosophila* by comparing the lymph glands of wild type and CG18446 mutant flies. However, no significant difference was observed between them. Lymph glands of CG18446 mutants did not differ from lymph glands dissected from wild type *Drosophila* in terms of shape, size nor fragility. The Hop^{tum} and Hop^{tum}; CG18446 mutants showed much fragile lymph glands (as expected, since it is known

that hemocytes from the lymph gland start entering the circulation upon the infection (Sorrentino et al. 2002)), but again, we did not see any obvious difference in terms of size or shape between them. According to the literature, immune cells from the lymph gland contribute to the defense of the organism once the organism is under the immunologically challenged conditions, however only at the later point of the infection (Anderl et al. 2016; Márkus et al. 2009). Therefore, we have first decided to describe the role of CG18446 at the onset of the infection. For this we turned to the investigation of another hematopoietic tissues – circulating and sessile cells. Firstly, we calculated the difference between the total number of hemocytes in wild type and CG18446 mutants. As it can be seen in the Figure 18, there is a significant decrease in this number in the case of CG18446 mutant. In order to detect any differences in the type of the circulating hemocytes, we calculated the number of mature and immature hemocytes. This was possible by usage of Hemolectin, marker of mature hemocytes, endogenously tagged with GFP. The total number of mature hemocytes was very similar between wild type and CG18446 mutant, but significantly less immature hemocytes have been counted in CG18446 mutant (Fig. 19). We noticed the same trend also in sessile cells (Fig. 20). These results showed that CG18446 mutants have troubles maintaining the pool of immature hemocytes under normal conditions. As it was reported that differentiation of lamellocytes in circulating and sessile cells occurs from plasmatocytes (Anderl et al. 2016), we wanted to see whether the detected impairment of CG18446 mutants can influence further differentiation of hemocytes. In other words, we wanted to see if there is any variation in the number of lamellocytes between wild type and CG18446 mutants under infection-like conditions. Since we were not able to use any marker or reporter for lamellocytes, we decided to try to estimate their number using different approach. We have induced melanization in L3 larvae by temperature and we searched for any differences between normal and infection-like conditions. Under normal conditions, we saw no apparent difference between wild type and CG18446 mutants (Fig. S1, Supplementary Data). It is difficult to quantify the melanization signal but based on a simple visual impression the CG18446 mutants may have slightly more black cells on average. This could be due to the presence of more crystal cells, as CG18446 expression is normally missing in the Lz positive crystal cells of wild type flies (Fig. 11). The missing CG18446 in the mutant fly may support crystal cells development at the expense of other cell types. Furthermore, we have induced melanization in the infection-like conditions

(Hop^{tum} larvae) and we have presumed that any difference observed in this case would be due to different number of lamellocytes present. As it can be seen on the Figure 20, there is a significantly higher number of melanotic spots present in CG18446 mutant larvae, suggesting higher number of lamellocytes. Alternatively however, the high number of melanotic spots could be also due to crystal cells that normally do not differentiate after infection but maybe in the CG18446 mutant they do.

From all previous results the following hypothesis can be established. (1) We suggest that CG18446 plays a role in maintaining the pool of undifferentiated hemocytes, based on the analysis of circulating hemocytes in CG18446 mutant. The expression pattern of CG18446 in posterior signaling center also favors this idea. (2) Additionally, CG18446 may play a role in preventing lamellocyte and possibly also crystal cell differentiation, based on the Hop^{tum} CG18446 mutant boiling experiment. In other words, maybe the expression of CG18446 in the plasmatocytes in the lymph gland that we observed is to prevent their differentiation into crystal cells or lamellocytes. According to literature, Notch signaling pathway needs to be blocked for production of lamellocytes (Small et al. 2014). If CG18446 is indeed a Notch target gene, then the blockage of CG18446 could phenocopy the blockage of Notch pathway and support lamellocyte differentiation. As this is exactly what we probably observed in the Hop^{tum} boiled larvae, we favour the hypothesis that CG18446 is a Notch target in some of the circulating/sessile cells. It certainly is not a Notch target in the crystal cells in the lymph gland where we did not observe any CG18446 coexpression with Lz. We can express a hypothesis (poor speculation) that the lymph gland P1 positive population also needs Notch signal but this is distinct from the Notch signal in Lz cells; from our preliminary observations, we know that Delta is expressed in the cortical region of the lymph gland and activation by Delta may give a different output of Notch pathway than activation by Serrate from the PSC.

Ideally, expression pattern of CG18446 and lamellocytes should be compared in order to confirm the previous statement. This could be accomplished either by using L1 antibody (marker of lamellocytes) or the Misshapen reporter line (F9 enhancer driven the expression of RFP). The above mentioned assumptions would suggest that, in the case of an infection, CG18446 expression should be downregulated. According to the screening performed in *Drosophila* Kc cells in order to find genes affected by JAK/STAT activation, CG18446 is

indeed downregulated with first 4 hours of JAK/STAT (Bina et al. 2010). This could mean that CG18446 is a target of JAK/STAT signaling but does not exclude that it is a target of Notch, as Notch components were also downregulated in the JAK/STAT activation in Kc cells (Delta, Mam) (Bina et al. 2010). JAK/STAT signaling pathway is evolutionarily conserved and is highly active upon the trigger of the immune system (Morin-Poulard et al. 2013).

For further investigation, the expression pattern of CG18446 should be tested not only in Hop^{tum} flies, but also in flies that have been physically infected by parasite or bacteria. It would be interesting to obtain the total number of circulating hemocytes in CG18446 mutants and analyze their content under these real infection conditions. In the case of correctly suggested hypothesis, the total number of circulating hemocytes would be still low and possibly even fewer immature hemocytes would be detected. Of a special interest would be to monitor the expression of CG18446 throughout the larval development under the infection condition. A decrease in the expression should be noticed at the specific course of the infection, what would correspond to the onset of lamellocytes differentiation.

We discovered that the open reading frame of CG18446 gene contains its regulatory sequences, because its X-gal expression patterns correlates with antibody staining of the Flyfos-GFP construct (Fig. 23 and Table I). However, the Su(H) binding sites we mutated are probably not responsible for Notch dependent regulation of the gene in vivo, at least in the majority of tissues. However, there were two stronger predicted Su(H) sites that we mutated but there are still less good Su(H) sites that were left untouched. The ChIP peak is unusually broad what could suggest more than one Su(H) binding site present. In this case, Su(H) can still bind to the construct in certain places that is reflected in the weaker but still detectable X-Gal signal in the mutant construct. The weakest signal was in the brain and in the gut but also the lymph gland did not stain as strong. However, the fact that some signal is weaker in the mutated construct can also simply reflect different insertion site of the construct in the genome and may not imply any Notch dependent regulation (the surrounding sequence can influence the activity of the reporter to some extend and different insertion sites will give different strength of expression). In order to discover the real cause of these discrepancies in the expression, we could take the non-mutated Rabbit construct and drive Notch RNAi in places where 18446 is expressed (e.g. in clones in the brain, gut, ovaries…); if its expression in these

tissues is regulated by Notch, the blue color should disappear. It would also be interesting to test the how CG18446 influences the activity of signaling pathways. These experiments could be performed using immunostaining techniques, comparing the expression of reporters of specific pathways (Fig. S2, Supplementary Data) under different conditions. In a complementary approach we could manipulate various signaling pathways and check the changes in the expression of the CG18446, to reveal which signaling pathways regulate CG18446 expression.

4.2. The role of CG18446 in general stress response

As the expression of CG18446 was noticed in fat body (Tanasić 2014), specific assays were performed in order to assess the metabolic role of CG18446. Moreover, the fat body plays an active role in the systemic immune response (Ekström & Hultmark 2016).

According to the results of oxidative stress test, CG18446 mutants males are less sensitive to increased amount of reactive oxygen species (ROS) when compared to the control (Fig. S4, Supplementary Data). Difference has also been observed in the case of CG18446 mutant females (Fig. S5, Supplementary Data), but not as considerable as in the case of males. A connection between Notch and ROS has been already reported, regarding the specification of immune cells (Small et al. 2014). Namely the increase in ROS in the lymph gland downregulates Notch, block crystal cells differentiation and allows lamellocytes proliferation. CG18446 could serve as a sensor for the levels of oxidative species, in the lymph gland or in the fat tissue, and any changes in CG18446 activity would be reflected in the "preparation" of the organism for the stress. These preparations include redirecting the energy from normal process either into immune response (Bajgar et al. 2015) or utilizing saved nutrients resources (Arrese & Soulages 2010) (depending on the type of stress). If there is no sensor suggesting changed levels of ROS (in CG18446 mutants), there is no need for energy switch and the organism uses it for regular metabolic processes. In other words, these flies (CG18446 mutants) behave as in the case of normal levels of ROS, as if no stress would be present. However, this would further suggest that wild type flies under the oxidative stress are dying due to the immune response overactivation and it was hard to find a support for this in the literature. It would be interesting to measure the survivalship of CG18446 mutants exposed to the oxidative stress after the infection; if this hypothesis is correct, these flies would not have

any energy to invest in the immune response and they would die more easily than wild type. Our observation of Hop^{tum}; CG18446 mutants encourages us that we are thinking in the proper direction, since these stocks seem very unhappy and they are not breeding well.

Additionally, we found CG18446 mutants less resistant to starvation stress (Fig. S6 and Fig, S7, Supplementary Data). These findings correspond to the previous results of the same experiments, done during the Bachelor thesis work (Tanasić 2014). CG18446 mutants possibly have troubles in accumulating enough of fat body that could be then used under energy-consuming conditions such as starvation. Or, if we view CG18446 as a general stress sensor promoting the stress resistance, it may regulate the energy usage in the fat body or relocate other energy stores.

Another condition of this kind is the cold stress. As it can be seen from results (Fig. S8 and Fig. S9, Supplementary Data), both males and females of CG18446 mutants had serious troubles to recover from cold stress, almost half of them did not succeed at all. Cold stress has been reported to influence enzymes involved in metabolism of lipids and carbohydrates (MacMillan et al. 2016). This could point to another role of CG18446 regarding the organism's capability to store nutrients. It is possible that flies lacking CG18446 have troubles to create/maintain/utilize body reserves, making the mutants more sensitive in stress conditions when energy is needed, such as starvation and cold stress.

4.3. The role of CG18446 in oogenesis

The expression of CG18446 has also been noticed in follicle cells of *Drosophila*'s ovaries (Tanasić 2014), suggesting the potential role of this gene in oogenesis. This experiment is technically much more precise repetition of the very similar one performed during the work on Bachelor thesis (Tanasić 2014). Results obtained imply the same trend. As it can be seen on the Figure S10 (Supplementary Data), CG18446 mutants are laying fewer eggs than wild type flies within the same period of time. The number of the larvae hatched compared to the total number of eggs doesn't seem to significantly differ amongst genotypes (Fig. S11, Supplementary Data). This could suggest the role of CG18446 in the production/laying of eggs but not in the hatching of larvae out of the same.

5. CONCLUSION

CG18446 is expressed in plasmatocytes and posterior signaling center under normal and infection like conditions. CG18446 mutants have lower number of circulating hemocytes and have problems in maintaining the pool of immature hemocytes in both circulating and sessile hemocytes. It is also likely that CG18446 mutants are capable of producing more lamellocytes under infection-like conditions. The open reading frame of CG18446 contains regulatory sequences and some domains are probably responsible for the regulation of the expression by Notch. CG18446 mutants are found to be less sensitive to higher levels of reactive oxygen species and more sensitive to starvation and cold stress. They are also found to lay fewer eggs.

All this together could suggest the role of CG18446 in maintaining the pool of immature hemocytes and preventing their further differentiation. This could possibly be achieved through the sensitivity of this gene to any changes in levels of oxidative species present in the organism. It is also likely that CG18446 assists in establishing and preserving fat body reserves. Finally, this gene could play a role at the onset or/and during of *Drosophila* oogenesis.

6. SUPPLEMENTARY DATA

6.1. The role of CG18446 in immune response

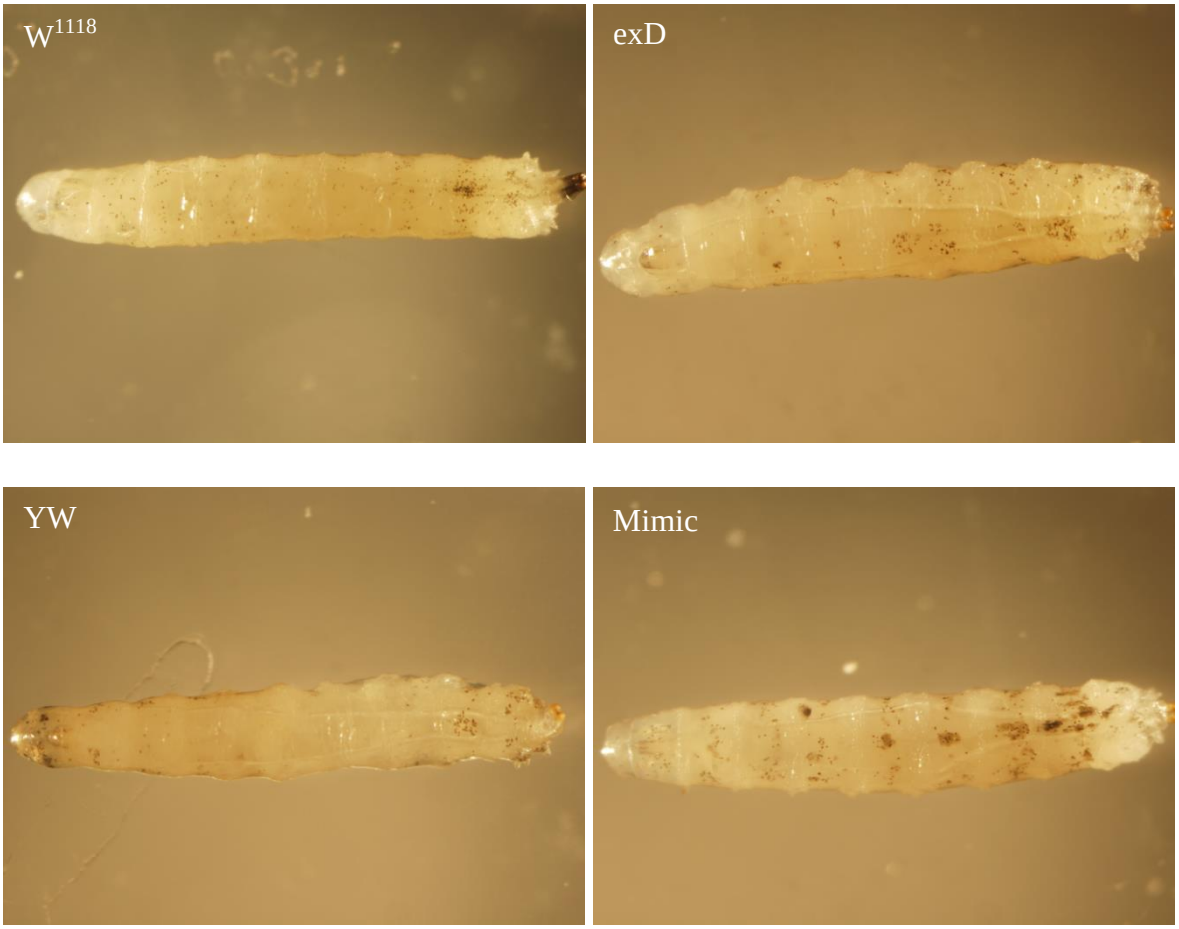


Figure S1: w^{1118} (wild type), yw (wild type), CG18446 mutant I (exD) and CG18446 mutant II (Mimic) larvae after the heat treatment at 65°C for 10 minutes. Number of larvae tested per genotype tested 20.

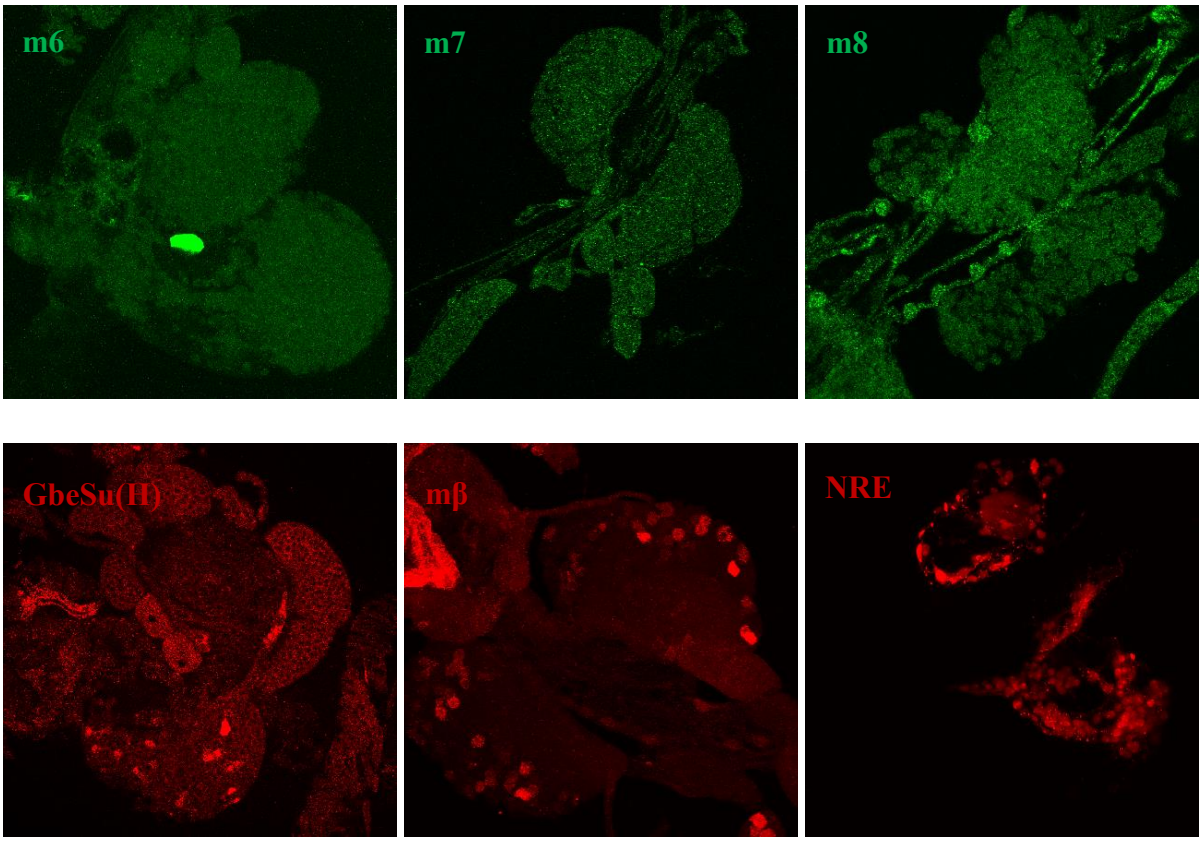


Figure S2: Notch reporters and their expression in the lymph gland. No expression observed of m6, m7 and m8 (green) and expression noticed for GbeSu(H), mβ and NRE (red).

6.2. The role of CG18446 in general stress response

6.2.1. Introduction

Despite the main focus being the role of CG18446 gene in the immune response, this gene was assayed in some metabolic tests as well. The reasoning behind is the previous observation of CG18446 expression in the fat body (Tanasić 2014). Both males and females of CCG18446 mutants were exposed to oxidative, starvation and cold stress and their survivalship was monitored. Similar experiment (starvation assay) was performed during the work on my bachelor thesis, but technical part was significantly improved this time, with the greater sample number and two replicates.

6.2.2. Materials and methods

6.2.2.1. Stress test protocol (Oxidative and starvation stress)

Flies used in stress tests were carefully staged in a following way (Linford et al. 2013):

1. 150-200 pairs of flies were placed into the collection cage
2. To allow the adaptation of flies, plates were changed once per day in the following day or two.
3. Embryos were then collected from the plate on which flies were laying eggs for 16-22 hours.
4. Embryos were gently removed from the surface of the plate and washed 3 times with PBS
5. After washings, aliquots of 32 µL of eggs were distributed into CT bottles.
6. Seeded CT bottles were placed back into the incubator until the emerging of adults (in approximately 10 days).
7. In an ideal case, leave flies to emerge and collect them over 12h period, transfer them into fresh CT bottles and let them mate for 48h before sorting them into the single sex (Bjedov et al. 2010). Nevertheless, due to high number of phenotypes tested in the first repetition of the experiment, flies were not synchronized and they started emerging at different time. This implies that 12h period collection was not possible therefore flies were left to emerge for three days. On the third day, they were transferred into the fresh CT bottle and finally assays started three days after. In other words, all flies used for both assays were 5-6 days old mated males and females. In assay 20 animals are put in a vial, with 10 vials per assay/genotype (thus 100 flies per genotype ideally).

Assays are performed in a following way:

1.1 For oxidative stress test let the flies starve in an empty vial with water-soaked filter paper (to avoid desiccation) for 6h at 25°C and then transfer them on the standard food containing oxidative agent (20mM paraquat) (Junger et al. 2003; Wu et al. 2009). This food contained two layers: lower layer not having paraquat and upper layer with paraquat. Another difference between these two layers is methylparaben, since it is put only in the bottom layer. Food is always prepared fresh, on the day of flipping flies into the vials.

1.2 For starvation stress test, transfer the flies in the vials containing 1% agar in water as a substrate (it provides more uniform moist environment for long-term studies, >12hours) (Tennessen et al. 2014).
2. Flip the flies on the fresh food every two days (Wu et al. 2009);
3. Keep all flies at 25°C, 60% humidity and 12-12 light-dark cycle;
4. According to the literature, flies are counted every 12h (Junger et al. 2003). However, in order to obtain more time points, flies are counted every 8h.

6.2.2.2. Fly food preparation (Oxidative and starvation stress)

Use the standard sugar/yeast/agar (SYA) diet for stress experiments:

Component	Percentage	Amount (g)/1l
Sugar (Glucose)	7.5	75
Yeast	1.5	15
Agar	0.45	4.5
Cornmeal	8	80

Once food is cooled down to below 60C, add 25 ml of methylparaben /1l.

Note: Paraquat should be added to the food after cooling to the 40°C (Junger et al. 2003).

6.2.2.3. Chill coma recovery

Chill coma recovery assay was performed according to the procedure modifed from the litearture (Colinet et al. 2010). Used flies were of the same age (5-6 days old). Flies were chilled in empty vials on ice for while and then distributed gently on the ice-precooled piece of aluminium.They were then transferred into 96 well plate by tweezers (one fly per well). The plate was then covered with aluminium and burried into ice. The ice box with ice and burried plate was then placed in the cold room for 16 hours. Finally, flies were softly placed on the filter paper and recovery time for each fly was noted. The recovery point was considered when the fly was stainding up on legs. Flies that were not recovering after 90 minutes were declared dead.

6.2.3. Results

Two CG18446 mutants were used in our experiments, as mentioned previously (Fig. S3).

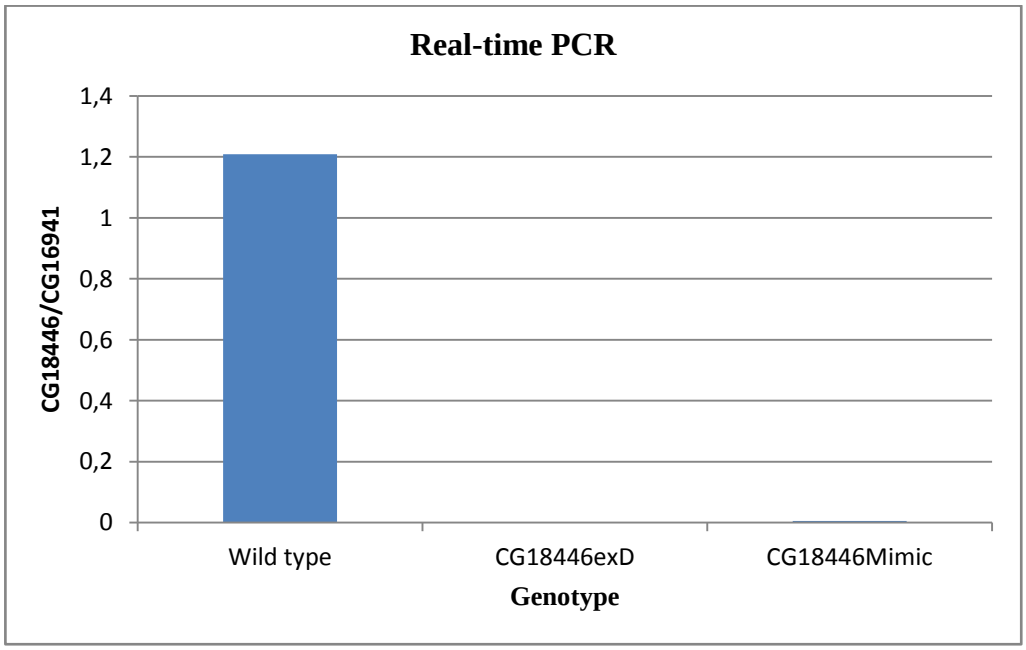


Figure S3: qPCR data for w1118 (wild type), $CG18446^{exD}$ (CG18446 mutant) and $CG18446^{Mimic}$ (CG18446 mutant) ran for CG18446 gene from genomic RNA, using primers described in 2.7. section of Materials and Methods. It is normalized to the signal for CG16941 as a positive control.

6.2.3.1. Oxidative stress

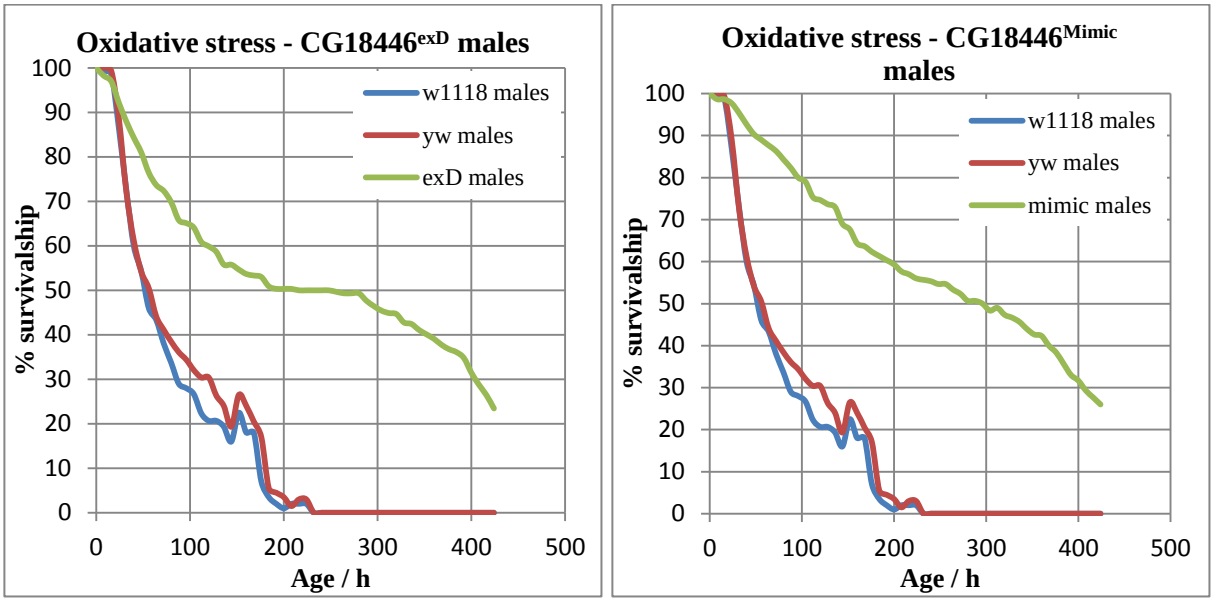


Figure S4: Oxidation stress summary for males of following genotypes: w^{1118} (wild type), yw (wild type) and $CG18446^{exD}$/$CG18446^{Mimic}$ (CG18446 mutants). Three replicates were done.

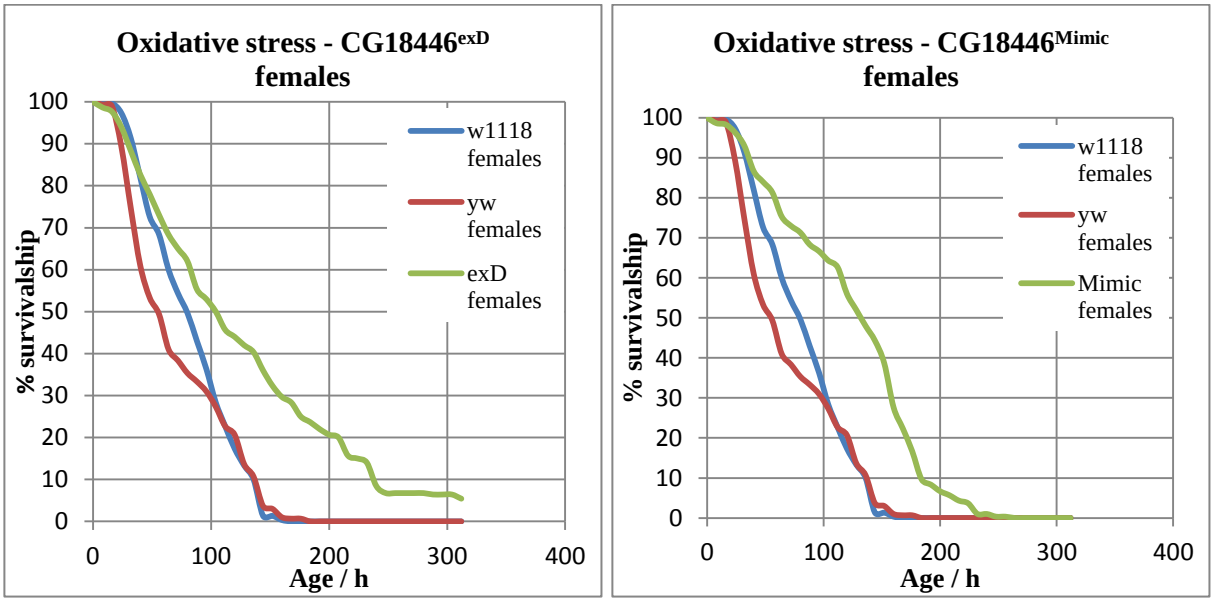


Figure S5: Oxidation stress summary for males of following genotypes: w^{1118} (wild type), yw (wild type) and $CG18446^{exD}$/$CG18446^{Mimic}$ (CG18446 mutants). Three replicates were done.

6.2.3.2. Starvation stress

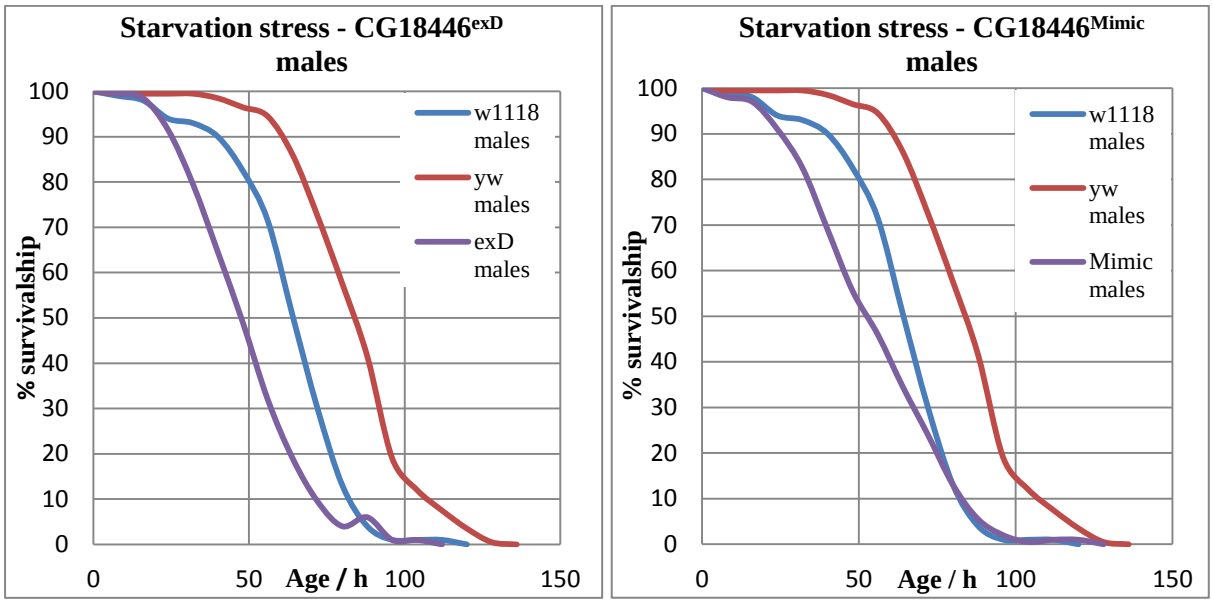


Figure S6: Starvation stress summary for males of following genotypes: w^{1118} (wild type), yw (wild type) and $CG18446^{exD}$/$CG18446^{Mimic}$ (CG18446 mutants). Two replicates were done.

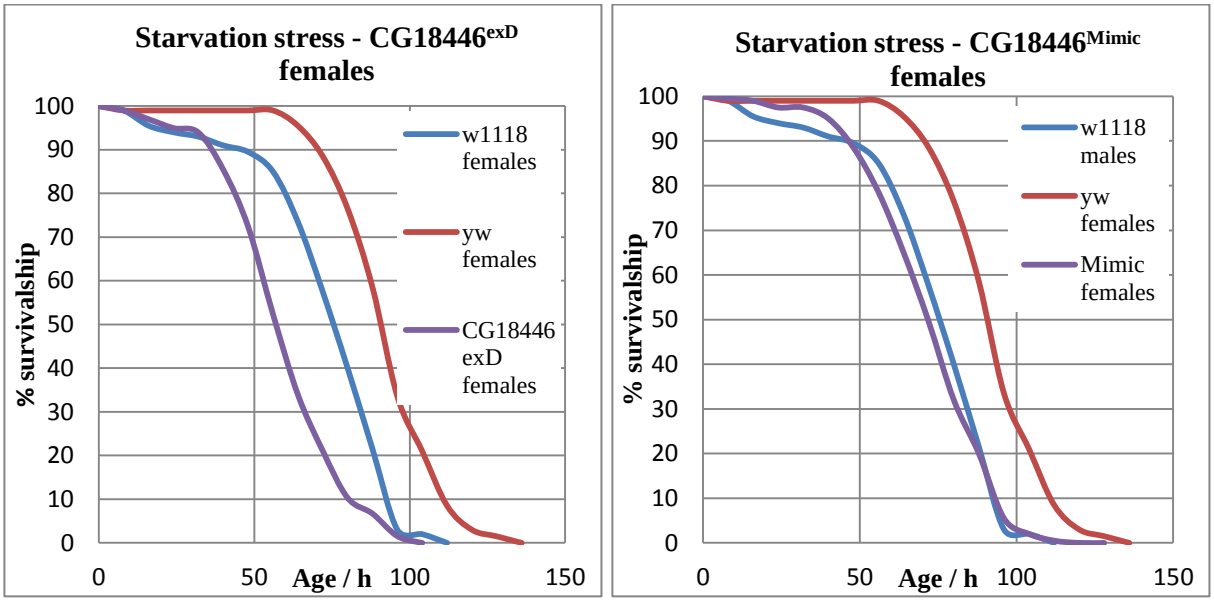
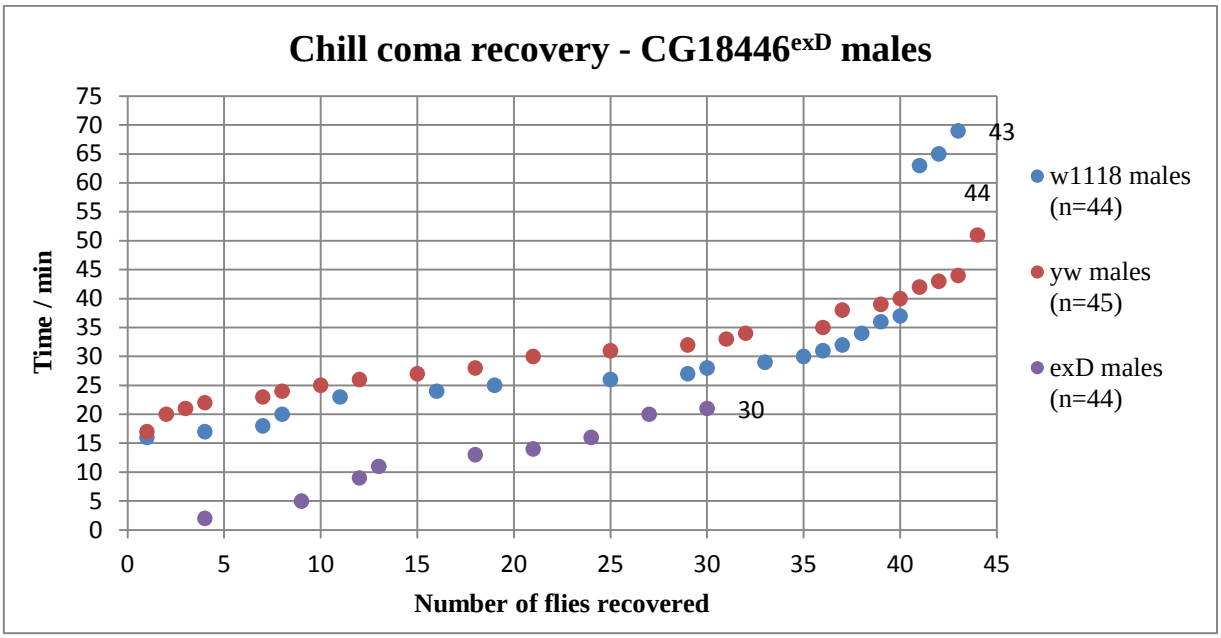


Figure S7: Starvation stress summary for females of following genotypes: w^{1118} (wild type), yw (wild type) and $CG18446^{exD}$/$CG18446^{Mimic}$ (CG18446 mutants). Two replicates were done.

6.2.3.3. Chill coma recovery



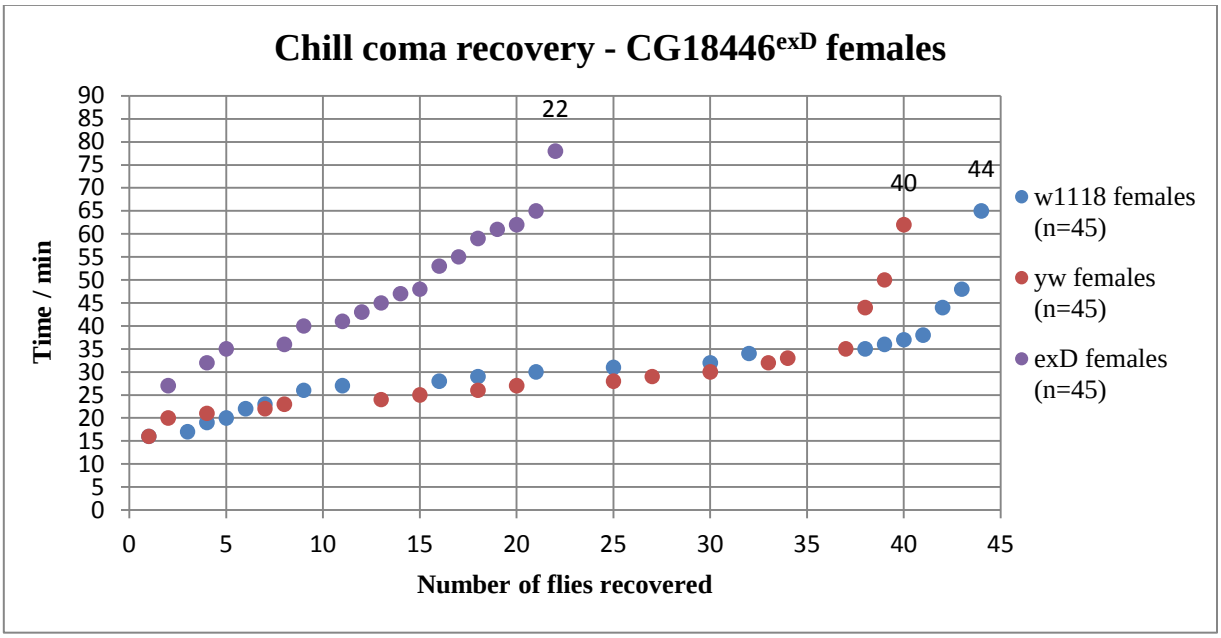


Figure S8: Results of chill coma recovery assay performed for males and females of following genotypes: w^{1118} (wild type), yw (wild type) and $CG18446^{exD}$ (CG18446 mutants). The numberd on the highest point for a specific phenotype indicates the last fly recovered.

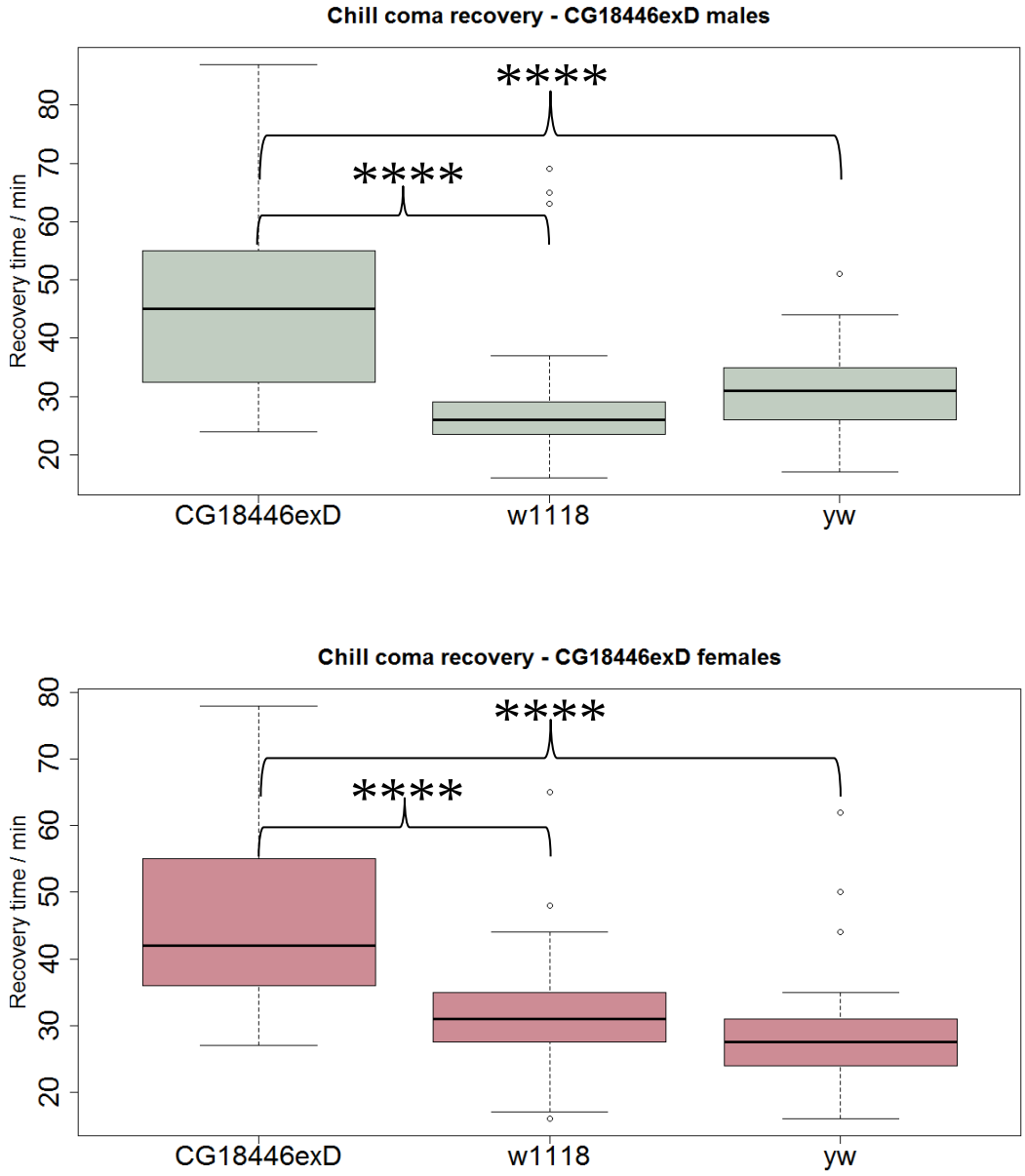


Figure S9: Results of chill coma recovery assay performed for males and females of following genotypes: w^{1118} (wild type), yw (wild type) and $CG18446^{exD}$ (CG18446 mutants). Slightly different representation than in the Fig. S7. Error bars illustrate standard deviation. In case of males, p-value when comparing w1118 and $CG18446^{exD}$ was calculated to be 0,00000129 and when comparing yw and $CG18446^{exD}$ 0,0000081. In the case of females, in comparison of w1118 and $CG18446^{exD}$ p-value = 0,0000669 and when comparing yw and $CG18446^{exD}$ p-value = 0,00000976.

6.3. The role of CG18446 in oogenesis

6.3.1. Introduction

The expression of CG18446 has been noticed in follicle cells of adult ovaries (Tanasić 2014), therefore the fecundity and fertility assays were performed to evaluate whether CG18446 plays a role in the reproduction. For these purposes, number of laid eggs as well as larvae hatched from those eggs were counted for CG18446 mutants. Similar experiment was performed during the work on my bachelor thesis, but technical part was significantly improved this time, with the greater sample number and two replicates.

6.3.2. Materials and methods

Flies were left to emerge over 24h period, after which they were left to mate for two days. Subsequently, 30 females and 15 males were put into the small cage. After two day adjusting period, juice agar plates were changed for each cage several times during 24h period and number of laid eggs was counted for each time point/genotype. After that all plates were left at 25° C overnight to allow larvae to hatch. Number of hatched larvae was subsequently counted for each time point/genotype.

6.3.3. Results

When we calculated the number of eggs laid within 24h by 30 females and 15 males we detected significantly worse fecundity in CG18446 exD flies that laid only about 50% eggs in comparison to controls (Fig. S10). The mimic flies laid significantly fewer eggs in comparison to yw control but not when compared to the w^{1118} control. Since the mimic stock is on yw background comparison to yw line is more appropriate. Therefore we can conclude that CG18446 gene is important for the fecundity of the flies. When assessing the number of larvae hatched from the laid eggs the CG18446 mutants showed again worse output and we can conclude they are less fertile. However, we cannot distinguish whether this is due to eggs or the sperm that do not develop properly. We should repeat the experiment, taking either the males of CG18446 mutants and wild type females, or vice versa, to distinguish whether the problem in fecundity is connected with the males or females of CG18446 mutant. We know that CG18446 is expressed in the somatic cells of both the ovaries and testes so both the

scenarios are possible. Taken together, we conclude that CG18446 is important for the *Drosophila* fecundity as well as for fertility.

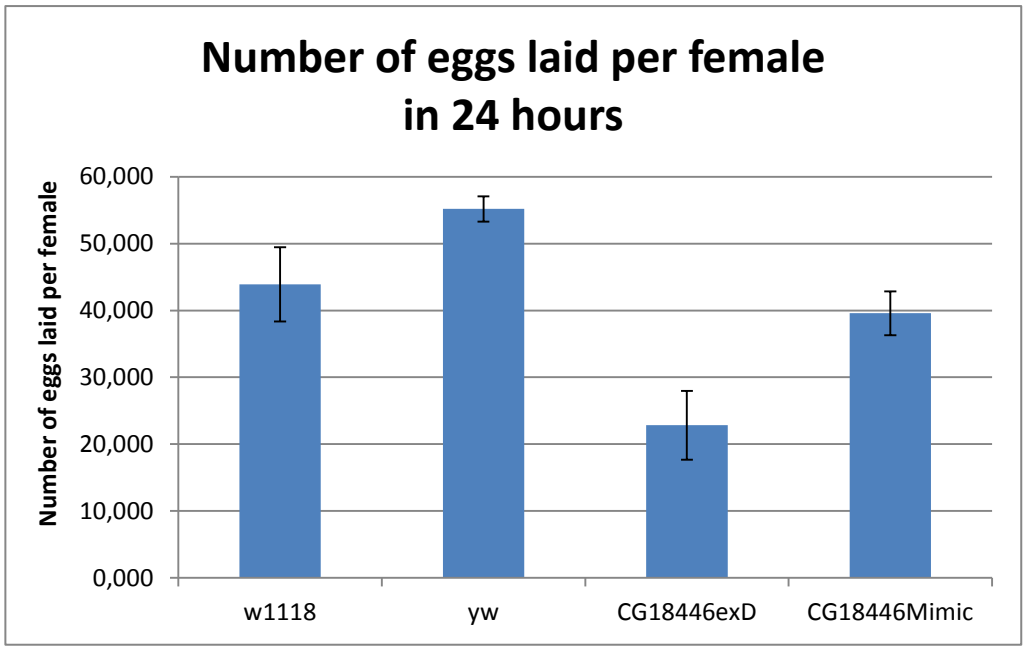


Figure S10: Number of eggs laid per female within 24 hours counted for following genotypes: w^{1118} (wild type), yw (wild type) and $CG18446^{exD}/CG18446^{Mimic}$ (CG18446 mutants). Error bars represent standard deviation and two replicates were done.

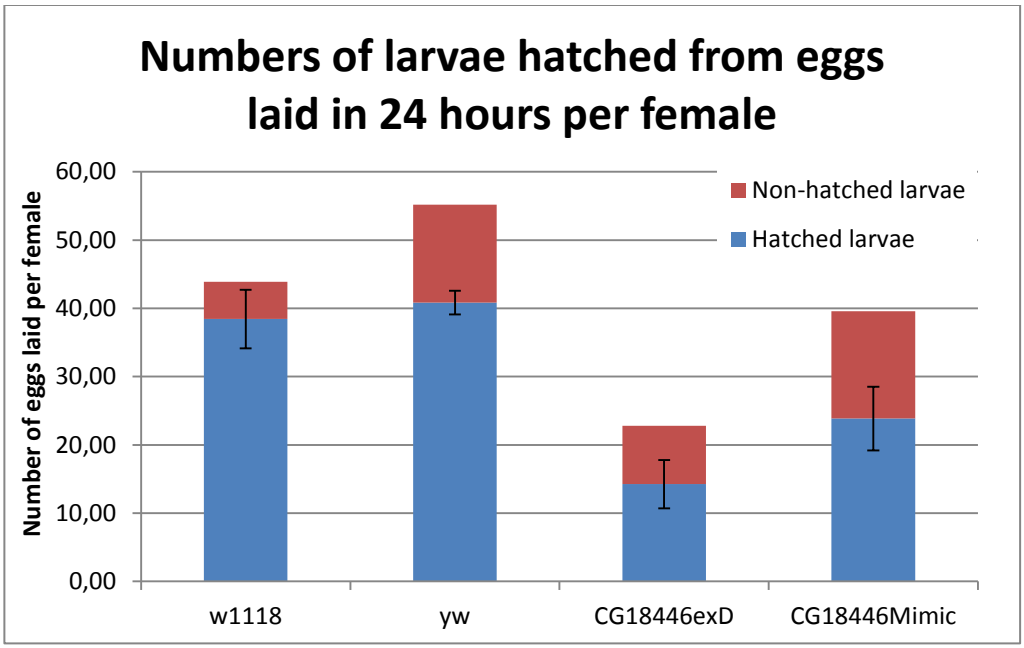


Figure S11: Number of larvae hatched from eggs laid within 24 hours counted for following genotypes: w^{1118} (wild type), yw (wild type) and $CG18446^{exD}$/$CG18446^{Mimic}$ (CG18446 mutants). Error bars represent standard deviation and two replicates were done.

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