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FUNCTIONAL AND MOLECULAR CHARACTERIZATION OF THE SERINE PROTEASE HOMOLOGUE CLIPA28 IN THE MELANIZATION RESPONSE OF *ANOPHELES GAMBIAE* MOSQUITOES

By
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AN ABSTRACT OF THE DISSERTATION

Layla Hussein El Moussawi for Doctor of Philosophy
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Title: Functional and Molecular Characterization of the Serine Protease Homologue CLIPA28 in the Melanization Response of *Anopheles gambiae* Mosquitoes

Anopheles gambiae mosquitoes are the main vectors of the human malaria parasite Plasmodium falciparum in sub-Saharan Africa. The innate immune system of mosquitoes is a major determinant of their vectorial capacity. Catalytic clip domain serine proteases (cSP) and their non-catalytic homologs (referred to as clip-domain serine protease homologs, cSPH) are key components of extracellular enzymatic cascades that control diverse immune responses in insects including melanization, antimicrobial peptide synthesis, and complement-mediated responses. Despite being non-catalytic, the activation of cSPHs, requires their proteolytic cleavage, yet factors that control their activation and the complexity of their interactions within these cascades remain unclear.

Here, we describe the functional and molecular characterization of a novel cSPH (CLIPA28), identified based on its co-regulated expression profile with CLIPA2, a key negative regulator of mosquito complement and melanization immune responses, in a transcriptomic database using Pearson correlation analysis. We show that CLIPA28 is required for the mosquito melanization response. Its knockdown (kd) abolished Plasmodium berghei ookinete melanization in refractory mosquito genotypes and significantly reduced hemolymph phenoloxidase activity after systemic bacterial infections. CLIPA28 kd didn't affect mosquito tolerance nor resistance to bacterial infections, but significantly compromised both in response to fungal challenge, which supports our previous published observations that melanization is required for anti-fungal defense. Biochemical analysis revealed that CLIPA28 is rapidly cleaved in the hemolymph following systemic infections. Using an RNAi-mediated gene silencing and western blot analysis, we show that the mosquito complement-like protein TEP1 and two other cSPs known to be required for melanization, SPCLIP1 and CLIPA8, are required factors for CLIPA28 cleavage, and that these three positive regulatory cSPHs are activated in a hierarchically ordered manner. Interestingly, the SPCLIP1-CLIPA8-CLIPA28 module acts upstream of CLIPA14, a key negative regulator of the melanization, suggesting that this module may be subject to negative feedback regulation by other inhibitory cSPHs, possibly to fine tune the intensity of the immune response. The abundant number of mosquito cSPHs involved in melanization and their highly ordered activation profile, suggest that cSPHs are likely to interact with cSPs at different levels within the protease network that regulates the

melanization response. We further identify SRPN2 as a master negative regulator of mosquito cSPHs, whose knockdown induced enhanced network activation, even in naïve mosquitoes, leading to a dramatic activation of both positive and negative regulatory cSPHs. In addition to revealing the complex roles of cSPHs in regulating CLIP cascades, our data should facilitate the characterization of the molecular interactions between cSP and cSPH components of CLIP cascades.

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CHAPTER I

INTRODUCTION

A. Malaria: A Global Burden

Vector borne diseases, particularly malaria, continue to be a major threat to public health worldwide. According to latest World Health Organization report (WHO, 2018) no significant progress has been made in terms of global malaria control over the past period (2015-2017), as disease estimates remain to exceed 216 million cases and 400,000 related deaths annually. In 2017, nearly half of the world population residing in 90 countries was at risk of infection; nonetheless, and despite ongoing efforts, the African Region continues to carry about 93% of the global burden, with patients of HIV/AIDs, pregnant women, and children under the age of five being the most vulnerable groups. Given the projected growth in the size of the world population by 2030, it is anticipated that more people would be living in malaria-affected countries in the near future, thus putting further strains on global health systems and national budgets worldwide. Consequently, a global technical strategy (GTS) was launched by WHO in 2015 to ensure that malaria trends remain on a downward trajectory via the continued investment in research and development of efficient intervention methods towards complete disease eradication by 2030 (WHO GTS for malaria 2015).

1. Plasmodium Infection in the Anopheles Mosquito

Malaria in humans is caused by any of the five Apicomplexan parasites of the genus *Plasmodium: P. falciparum, P. ovali, P. vivax, P. knowelsi,* and *P. malariae*, which are transmitted to humans through the bite of approximately 41 species of *Anopheles* mosquitoes [11]. In Sub-Saharan Africa, *P. falciparum* is readily vectored by *A. gambiae* and accounts for the majority of deaths in the region. Malaria parasites must complete a complex life cycle that alternates between both, the mosquito and the vertebrate host. The sporogenic phase of the parasite life cycle within its vector extends over a period of 2-3 weeks, depending on the parasite strain, upon which the mosquito becomes infective. Shortly after ingestion of a blood meal from an infected person, male and female gametocytes mature into micro- and macrogametes, respectively, in the midgut lumen of the mosquito, which eventually fuse to form a zygote. Zygotes then develop into motile ookinetes at roughly 12-24 hours post feeding. Ookinetes penetrate the newly formed peritrophic matrix and traverse the midgut epithelial wall to differentiate into oocysts beneath the basal lamina. Over the next 14-16 days, a meiotic cycle followed by multiple rounds of mitotic divisions occur within each oocyst, eventually leading to the release of thousands of haploid sporozoites into the hemocoel. Sporozoites then migrate and invade the salivary gland epithelium into the gland lumen where they further mature for a few days awaiting to be injected into the vertebrate host upon a subsequent blood meal [12] (Figure 1).

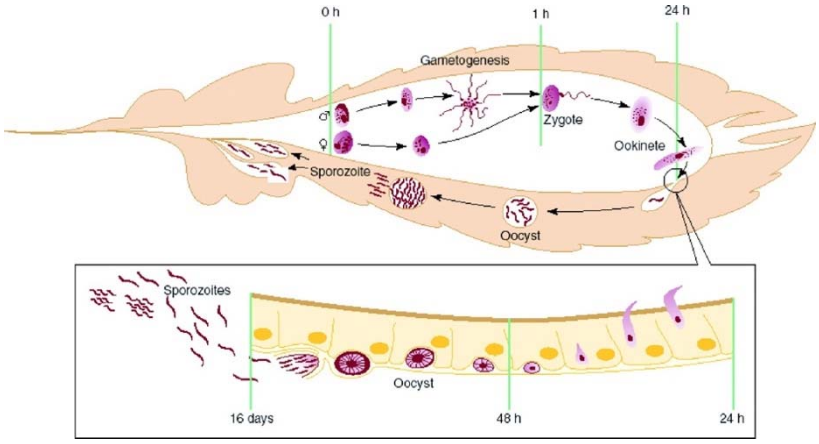


Figure 1. Schematic Representation of *Plasmdoium* (*P.berghei*) life cycle in mosquito The approximate developmental time of each stage is indicated . Transmission occurs at t=0hrs upon the ingestion of an infected blood meal. Within minutes the gametes are formed and fuse to give a zygote at 1hr. At 24hrs, ookinetes evade the midgut epithelium and differentiate into oocysts that divide and rupture approximately 2 weeks later to release thousands of sporozoites into the hemolymph. Sporozoites then invade the salivary glands awaiting to be injected into the vertebrate host. (Adapted from [2])

Susceptible Anopheles mosquitoes; however, are not completely passive to parasite development, whereby they mount efficient local and systemic immune responses against the parasite culminating in strong bottlenecks at three main developmental phases: gametocyte-to-ookinete, ookinete-to-mature oocyte, and hemocoel sporozoites –to-salivary glands sporozoites transitions. At these bottlenecks, parasites are confronted with multiple mosquito immune defenses that result in dramatic losses in their numbers, often leading to premature termination of the cycle. Nonetheless, in certain cases, a few parasites manage to escape these reactions and undergo a late amplification to complete their transmission[10] (Figure 2).

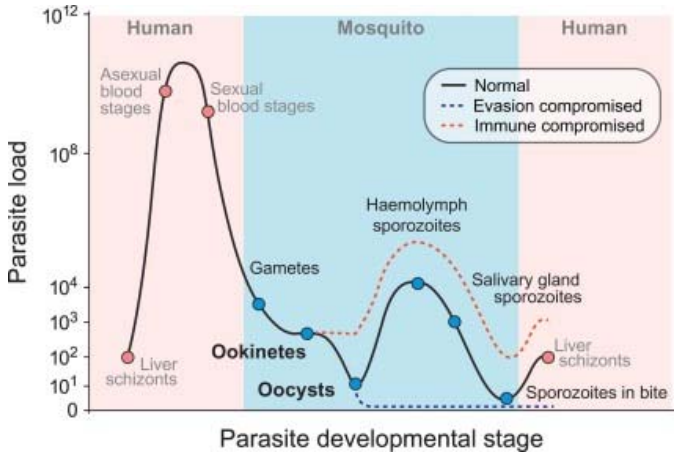


Figure 2. Plasmodium bottlenecks in *A. gambiae*. *Plasmodium* parasites are confronted with mosquito immune responses that result in major losses in their number throughout their development (Black solid line). Inactivation of mosquito immune responses increases parasite survival (Red dotted line), while disruption of parasite evasion mechanisms completely blocks transmission (Blue dotted line). Adapted from [7]

2. Vector targeted Approaches for Malaria control

Given the absence of an effective malaria vaccine[13] , and the rise in anti-malarial drug resistance among parasites [14, 15], mosquito control is currently the primary method of malaria prevention. The improvement of existing and the development of novel vector control measures that target mosquito population density and /or interfere with their ability to transmit the parasite is at the core of the WHO GTS intervention program.

a. Long Lasting Insecticidal Nets (LLINs) and Indoor Residual Spraying (IRS):

The achieved reductions in malaria incidences over the past decade has been largely attributed to the utilization of LLINs and IRS that target indoor biting and resting mosquitoes. The treatment of bed nets with insecticides, mainly pyrethroids, provides protection during sleep as mosquitoes are killed once they come in contact with the net surface [16].

Nonetheless, with the rise of pyrethroid resistance in mosquitoes, LLINs are proving to be less effective. To overcome this limitation, bed nets are now additionally coated with long-lasting chemicals, such as Piperonyl Butoide (PBO), which act as an inhibitor for certain mosquito metabolic enzymes that detoxify insecticides [17]. A comprehensive epidemiological study conducted in African regions exhibiting high mosquito insecticide resistance revealed, that the usage of Pyrethroids -PBO nets increased mosquito mortality and reduced blood feeding rates as compared to standard Pyrethroid nets, thus highlighting the importance of such synergists in improving LLINs efficacy in malaria control [18].

IRS is the application of insecticide chemicals on the interior walls and roofs of the house to kill adult mosquitoes resting on those surfaces. The use of IRS with DDT proved to be widely successful in the WHO-led malaria eradication campaign during the 1950s and 1960s, and is still implemented as one of the major vector control techniques in endemic areas (WHO, GTS for malaria; WHO implementation of indoor residual spraying for malaria control). The WHO recommended insecticides for IRS includes only four classes of chemicals: organochlorine (DDT), organophosphates, carbamates, and pyrethroids which, on the long run, restricts options for effective insecticide resistance management. In fact, a strong resistance against all approved insecticide types has been reported for *A. gambiae* mosquitoes in Mali [19] and Southern Ivory Coast in West Africa [20], and is spreading fast across other parts of the African Continent [21, 22]. The lack of novel insecticides with new modes of actions, combined with the high selective pressure on mosquito resistance [23], represent major threats to the fragile gains made in malaria control over the past years. Other challenges facing the utilization of LLINs and IRS include; the individual misuse of these interventions (bed net loss) [24], irresponsible human behavior such as staying

outdoors during the early night hours or sleeping outside without any protective measures, and change in vector behaviors, whereby some mosquito strains commonly alternate to outdoor resting and animal blood feeding for sustainability [25, 26]. While it is clear that LLINs and IRS, if properly implemented, can result in substantial reductions in malaria burdens, they are insufficient to completely block malaria transmission.

b. Paratransgenesis:

Paratransgenesis is a vector control method that involves the genetic modification of mosquito symbionts to express effector molecules against the pathogens they carry. The mosquito midgut has been a prime target for blocking parasite transmission via transgenic modification of selected members of the gut microbiota for three main reasons : (a) the most vulnerable stage (ookinete) of Plasmodium life cycle occurs in the midgut lumen, (b) the midgut compartment is shared between the microbiota and Plasmodium during early development which enhances parasite exposure to secreted effector molecules by modified bacteria, and (c) the numbers of bacteria in the midgut increases dramatically following a blood meal, proportionally increasing the output of secreted anti-Plasmodium effector molecules as well [27, 28].

Early attempts to test this strategy came about with the genetic engineering of *E. coli* laboratory strains to express two anti-Plasmodium molecules, SM1 peptide and Phospholipase A2 (PLA2) that significantly inhibited *P. berghei* development. Nonetheless, the modified *E.coli* strains survived poorly in the mosquito and the secreted proteins, in many instances, remained attached to the bacterial surfaces [29] .The subsequent use of *Pantoea agglomerans* (previously known as *E. agglomerans*) designed to

secrete five anti-parasitic proteins resulted in much more profound effects hindering the development of both *P. berghei* and *P. falciparum* up to 98% in *A.stephensi* without posing any negative impact on mosquito fitness [30]. More recently, *Serretia AS1* and *Asaia* bacterial strains with engineered antimalarial properties were found to successfully colonize *Anopheles* mosquito midguts and to spread both vertically and horizontally throughout the generations [31-33]. While still in its infancy, the use of transgenic bacteria as a tool for malaria control discloses promising perspective for the implementation of this technique in future field applications.

c. The Sterile Insect Technique (SIT)

SIT is a population suppression-based approach that relies on the mass production, sterilization, and subsequent release of infertile insects (particularly males) into the field, where sterile males will compete with wild type males and mate with virgin females to produce no progeny. Repeated releases will eventually lead to population reduction and can, under certain circumstances, result in population elimination [34]. Several methods have been proposed to achieve male sterilization in mosquitoes including: Irradiation with gamma or X-rays [35, 36], treatment with chemosterilants [37] to damage germ cells, male infection with *Wolbachia* to induce cytoplasmic incompatibility and progeny death when mating with uninfected females or females infected with a different bacterial strain [38], and transgenic manipulation of reproductive and developmental genes to confer sterility and embryonic lethality [39].

SIT has been successfully employed in the agricultural sector aiding in the eradication of various insect pests in Southern USA and Mexico [40], Libya [41], and California [42] among many other countries. Nonetheless, the effectiveness and applicability of SIT against mosquito vectors at a large-scale level is still in trial. The feasibility of SIT for malaria vector control was first demonstrated in 1974 where the isolated release of chemosterilized *A. albimanus* at EL Salvador over a period of 5 months resulted in 99 % reduction in population size [43]. When large scale attempts were performed at the Pacific coast of El Salvador, only little population suppression was achieved due to immigration of females already inseminated from wild type males into the release area despite the introduction of a barrier zone [44]. In 2004, the International Atomic Energy Agency, in collaboration with the Tropical Medicine Research Institute in Khartoum, initiated a project to develop technologies for controlling malaria with SIT in Northern of Sudan. Field cage experiments revealed the effectiveness of irradiation in inducing sterility in *A. arabiensis* males and the feasibility of their transport into the release sites with minimal fitness costs [45]. A more recent study demonstrated the ability of irradiation-sterilized *A. arabiensis* males to participate in naturally occurring swarms in Northern Sudan and compete with wild type males after their release (at a ratio of 3:1 [46]), thus providing hope for large scale field trials [47]. Before the SIT can be successfully integrated into control programs, certain technical, biological, and ecological prerequisites must be met [48, 49]. Substantial effort within the scientific community is put towards achieving this goal as SIT, due to its successful track record with other insect pest, holds much promise for vector eradication.

d. Genetically modified mosquitoes and Gene drives:

Molecular biology has been a great source for the development of genetically modified mosquitoes that aim at replacing existing susceptible populations with refractory ones. Early reports of *Anopheles* mosquito germ line modifications constituted the *piggyBac* ectopic expression of SM1 peptide and PLA2 from honeybee venom in midguts of *A. stephensi* rendering them resistant to *Plasmodium* development [50, 51]. Several proof of concept studies followed. Increased REL2 and Akt signaling conferred resistance to *P. falciparum* infections in transgenic *Anopheles* strains [52, 53]. Transposon mediated expression of single chain antibodies (scFvs) conjugated to *A. gambiae* Cecropin A antimicrobial peptide significantly reduced *P. falciparum* infection intensity and prevalence in *A. stephensi* [54]. *FREP-1* knock out resulted in profound suppression of infection with both rodent and human parasites at the oocyst and sporozoite stages in transgenic *A. gambiae* mosquitoes [55].

Despite this laboratory success, one crucial challenge for translating these findings into the field was to devise effective means to drive refractory transgenes into wild type mosquitoes without the need for long term rearing and release. For that purpose, several gene drive systems that link effector transgenes to naturally occurring "selfish genetic elements" have been proposed. These selfish elements can replicate within a host's genome and are thus inherited at higher frequencies than that predicted by Mendelian genetics until they reach fixation [56]. Among those are a class of genes known as *Homing Endonucleases* (HEs). *HE* genes code for endonucleases that cut at a specific site in the genome generating double stranded breaks within the DNA sequence. The double stranded

breaks are then repaired by either non-homologous end joining (NHEJ), an error prone process, or by homology directed repair mechanism (HDR) that leads to copying of the *HE* gene from one chromosome into the cleavage site on the homologous chromosome. If this occurs in the germline cells, then the *HE* gene has 100% chance of being inherited [57] (Figure 3). HE-based gene drive systems were shown to edit the genomes of *Anopheles* mosquitoes with high specificity [58-62]; nonetheless since their nuclease activity is intrinsically coupled with DNA target recognition, HEs that recognize new target sites were difficult to engineer. The emergence of CRISPR/Cas genome editing technology, derived from bacterial CRISPR/Cas adapted immune system, introduced a new programmable nuclease that didn't possess this constrain. In essence, CRISPR/Cas system also falls in the category of HE-drives with a similar mechanism of action. It uses a Cas9 endonuclease for cleavage that can be programmed to cut, with high precision, any sequence in the genome using a target specific guide RNA (gRNA). Since DNA target recognition (gRNA) and nuclease activity (Cas9) are distinct entities in this system , CRISPR/Cas-9 gene drives are technically much easier to design [63] and don't require protein engineering.

In their recent paper, Gantz *et al*., successfully developed CRISPR/Cas 9 system to drive the insertion of two effector genes coding for anti-*P. falciparum* antibodies in both male and female progeny at a ≥ 99.5% efficiency. For experimental convenience the authors inserted their drive construct into an eye color gene, whose knock out had a fitness cost, so it was pointed out that insertion into a neutral position is preferred for future field use [64]. Alternatively, other groups utilized a population suppression approach. Galizi *et al.,* devised a CRISPR/Cas9 sex distortion system whereby Cas9 targets and shreds X chromosome during male spermatogenesis thus producing extreme male bias among the

progeny [65]. Moreover, Hammond *et al*.[66], and Kayrou *et al*. [67], demonstrated that CRISPR/Cas9 targeted disruption of female fertility genes confers complete sterility in homozygous female and that the drive allele is able to spread rapidly reaching high prevalence within the first few generations. While promising, limitations of such nuclease-based drive systems often include emergence of drive resistance due to NHEJ- induced or *de novo* mutations at Cas9 target site, off target insertion/deletion effects related to non-specific binding of gRNA, and gene drive construct instability in different genetic backgrounds and under different environmental conditions [68-71]. It remains unclear to what extent will such constrains affect the dynamics of the driving process in field settings and what ecological consequences does it hold, nonetheless the current rate of Cas9 related research promises this technology as a highly adaptable approach for genetic alteration of vector species for disease eradication [72].

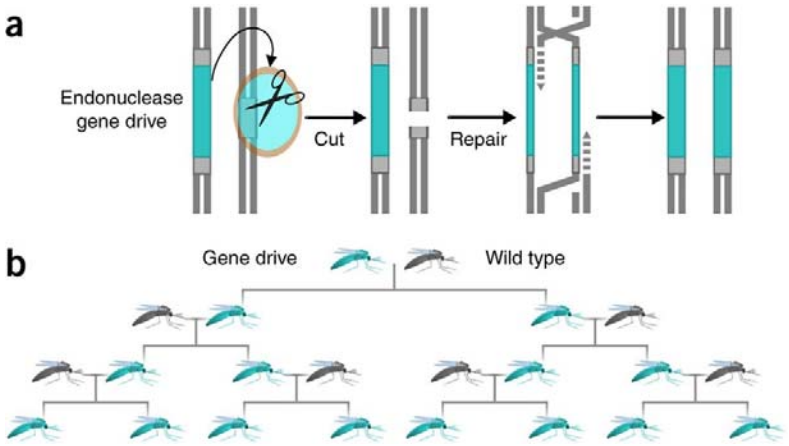


Figure 3 .Endonuclease gene drive system in Mosquitoes. (a) Endonuclease will cut at a specific site at homologous chromosome causing the cell to repair the damage by copying the endonuclease gene into the cleavage site thus converting heterozygotes into homozygotes.(b) Gene drive based systems (Blue) will enhance the odds of their inheritance (100 %) in each generation within the wild type population (Gray) (Adapted from [9])

B. Molecular and Cellular Basis of Mosquito Immunity

The realization that the mosquito's immune system is a key determinant of its vector competence, has stimulated research in mosquito immunobiology that has significantly advanced over the last 40 years. Early studies that selected for *Plasmodium* resistance in *A. gambiae* provided the first evidence for the genetic basis of mosquito refractoriness. In the isolated mosquito lines, complete refractoriness was manifested as melatonic encapsulation of invading ookinetes at the basal labyrinth of the mosquito midgut [73] ,or ookinete lysis in the cytosol of infected midgut epithelial cells [74] . Genetic mapping experiments in L3-5 melanizing mosquito strains infected with *P.berghei* [75] or *P. cynomolgi* [76, 77] , and in natural *A. gambiae-P. falciparum* isofamilies [78, 79] revealed several species-specific quantitative trait loci (QTLs) and single nucleotide polymorphisms (SNPs) [80] associated with the refractoriness phenotype. Concurrently, the sequencing of the *A. gambiae* genome [81] and the development of powerful genetic and computational tools [82] represented major milestones in the field that revolutionized our understanding of mosquito-parasite interactions. Large-scale transcriptomic analysis identified hundreds of mosquito immune genes that are differentially expressed in response to various types of stimulants including injury, blood feeding, and microbial challenge [83, 84]. *Plasmodium* invasion was shown to trigger a strong local and systemic activation of immune markers that correlated with parasite passage through mosquito tissues at various developmental stages [85, 86]. Interestingly, infection with different *Plasmodium* species at varying intensities had distinct impact on mosquito transcriptome [87, 88] and gene function [89] ,suggesting that mosquito immune responses aren't entirely universal but

have finely evolved to accommodate for different vector-pathogen genotypic interactions within the same class of pathogens [90, 91]. Nonetheless, a certain degree of overlap was also reported between mosquito anti-*Plasmodium* and other anti-microbial defense mechanisms, whereby several mosquito genes displaying anti-parasitic activity were also required for resistance against bacteria and fungi [87, 92], indicating that certain mosquito immune effector responses have a broad spectrum of activity. In this perspective, fungi and bacteria have been heavily used as model pathogens in RNAi-based studies to dissect mosquito innate immunity and have helped identify a plethora of genes that serve key functions in pathogen recognition, signal transduction and amplification, and microbial clearance.

1. Pathogen Recognition

The initiation of an immune response requires a specific interaction between insect pathogen recognition receptors (PRRs) and pathogen associated microbial patterns (PAMPs) on the surface of foreign microorganisms. The *A. gambiae* genome harbors around 150 PRR-encoding genes, the majority of which remain uncharacterized. Insect PRRs are divided into evolutionary conserved gene families that exhibit remarkable diversity, reflecting the selection pressures from different types of pathogens encountered in the insect's environment [93-95]. Among the different classes of *A. gambiae* PRRs are the Gram-negative bacteria binding proteins (GNBPs), Peptidoglycans recognition proteins (PGRPs), Fibrinogen domain proteins (FBNs), C-type lectins (CTLs), and Immunoglobulin-domain gene superfamily (IgSF)[94, 96].

a. Gram-Negative Bacteria Binding Proteins

The *A. gambiae* GNBP gene family is composed of six members belonging to two subfamilies, *GNBPA* (*GNBPA1* and *GNBPA2*) and *GNBPB* (*GNBPB1*, *GNBPB2*, *GNBPB3*, and *GNBPB4*) [96, 97]. GNBPs are typically known to recognize and bind components of bacterial and fungal cell walls [98]. All *Ag*GNBP proteins contain a conserved β-1,3-glucan binding domain and a predicted N-terminus signal peptide sequence characteristic of secretory proteins. Nevertheless, GNBPA1 possesses also a putative trans-membrane domain, suggesting that it may otherwise represent a cell-surface receptor [99]. The six gene members were reported to be immune-responsive to different pathogens, though, to varying degrees. *AgGNBPs* have been shown to be strongly induced in the carcass, midgut, abdomen, and salivary glands upon infection with bacteria (Gram –positive and Gram-negative) and *Plasmodium* parasites [85-87]. *GNBPA2, B1, B3, or B4* kd differentially compromised mosquito resistance against bacteria, and increased mosquito permissiveness to *P. berghei* or *P. falciparum* development. Among all members, GNBPB4 displayed the most profound antibacterial and anti-Plasmodium activity, suggesting that it is likely a major factor of a general defense mechanism. Similar to *Drosophila melanogaster* DGNBP1, *Ag*GNBPB4 was shown to regulate the expression of downstream effector genes common to both Toll and IMD pathways, which indicates that GNBPB4 may be mediating their activation in response to microbial challenges [98, 99].

b. Peptidoglycans Recognition Proteins

PGRPs are pattern recognition proteins that are highly conserved across organisms from insects to mammals. PGRPs recognize PGN that are unique components of bacterial cell walls but absent from eukaryotic plasma membranes [100]. Insect PGRPs are grouped into two classes based on their length: Short PGRPs (PGRP-S) are small extracellular proteins, while Long PGRPs (PGRP-L) are either intracellular or membrane-bound. The role of PGRPs in insect immunity is most studied in *D. melanogaster*. *Drosophila* has 13 PGRP genes that encode for at least 17 protein variants [97, 101].PGRP-SA and PGRP-SD, cooperate with GNBP1, to trigger the activation of Toll pathway in response to Gram-positive bacteria [102]. In this context, PGRP-SA was particularly shown to promote the cleavage of Toll-ligand Spätzle [103]. Alternatively, PGRPLC and PGRPLE were found to mediate the induction of Imd pathway in response to Gram-negative bacterial infections [104, 105].PGRPLB is an extracellular amidase that specifically binds and degrades DAP-type PGN, thus downregulating Imd signaling[106] .PGRP-SD was recently shown to strongly enhance Imd activation by antagonizing the negative effects of PGRPLB, and promoting PGN localization to PGRPLC on cell surfaces[107]. *In vitro* assays revealed that PGRPLC might also be involved in phagocytosis of Gram-negative bacteria [108] . The *A. gambiae* genome has seven *PGRP* genes; three encoding for three short PGRPs (*S1,S2,S3*) and the remaining four encoding for six long PGRPs (*LA1, LA2, LB, LC1, LC2, LC3*), with PGRPLC variants being the products of alternative splicing [96, 97]. *Ag*PGRP-LC, particularly PGRP-LC3, was shown to confer resistance to systemic bacterial challenge by triggering the expression of AMPs during early stages of infection. PGRP-LC signaling

was further implicated in controlling the proliferation of gut microbiota following blood feeding, and consequently limiting *Plasmodium* development in the mosquito midgut [109].

c. Fibrinogen Domain proteins

Members of the FBN protein family share an evolutionary conserved fibrinogen-like domain identified in invertebrates and mammals. Contrary to their function in vertebrates, the primary role of FBNs in invertebrates is defense and not blood coagulation [110]. Phylogenetic analysis in *A. gambiae* revealed that FBNs have undergone extensive gene expansion to become the largest PRR gene family with 61 putative members, resulting from the tandem duplication of the fibrinogen domain [96, 111]. Several *Anopheles* FBNs are transcriptionally regulated in response to pathogen challenge and are important determinants of infection outcome. Gene silencing of *FBN4, -5, -6, -9, -22,* or *-26* compromised mosquito survival after bacterial challenges. FBN22 and FBN39 were shown to participate in immune hemostasis and general bacterial clearance in naïve mosquitoes. FBN1 was reported to promote *Plasmodium* midgut invasion by anchoring ookinetes to the PM, while FBN6, -8,-9, and -39 were shown to synergistically antagonize oocyst development [111]. Moreover, *A. gambiae* transgenic lines overexpressing FBN9 in fat body tissues were more resistant to bacterial and *P. berghei* infections, suggesting that FBNs constitute important candidates for mosquito transgenic approaches aimed at blocking malaria transmission [112]. Immunofluorescence assays revealed that FBNs, particularly FBN9 and FBN30, associate with microbial surfaces as multimers with different affinities, which implies that FBN multimerization may be a potential strategy utilized for creating complexes with broader recognition specificities [111, 113].

d. C-Type Lectins

C-type lectins (CTLs) constitute the largest and most diverse family of carbohydrate binding proteins in animals. CTLs are either secreted or membrane bound, and interact with extracellular sugar ligands via a carbohydrate-recognition domain (CRD) in a Ca^{2+} dependent manner [114]. Majority of insect CTLs contain a single CRD; however, a few possess additional functional domains that are thought to assist in ligand recognition and improve CTL binding capacity [96, 115, 116]. The exact role of C-type lectin like domain (CTLD) proteins in invertebrate immunity is not fully understood; nonetheless, several insect CTLDs were suggested to act as PRRs mediating immune reactions such as phagocytosis, encapsulation/melanization, and hemocyte nodule formation [117].

The number of CTL genes varies greatly between insects with only 4 in *Acyrthosiphon pisum* [118] and up to 40 in *Ae.aegypti* [115].The *A. gambiae* genome contains 25 CTLD-encoding genes that are classified into different subgroups based on sequence-specific signatures[97, 115]. *In vivo* reverse genetics identified two protective *Ag*CTLs, CTL4 and CTLMA2, that promote parasite development in mosquito midgut. Silencing of either gene resulted in the significant melanization of *P. berghei* ookinetes in the basal labyrinth, thus hindering their differentiation into oocysts [119]. CTL depletion didn't; however, induce melanization of injected sephadex beads [120], suggesting that CTL4 and CTLMA2 are specifically utilized by *Plasmodium* parasite to evade mosquito immune responses. Interestingly, the kd of the same genes in *A. gambiae* infected with sympatric human *P. falciparum* field isolates didn't yield ookinete melanization except at high gametocytemia levels, indicating that CTL4 and CTLMA2 immune regulation of

Plasmodium development seems to be both species-specific and infection-intensity dependent [89, 121]. Moreover, CTL4 and CTLMA2 were shown to contribute to mosquito defense against Gram-negative bacteria. CTL4 or CTLMA2 silencing compromised mosquito survival and resistance to Gram-negative *E.coli* but not Gram-positive *S. aureus* bacteria. Biochemical analysis revealed that both CTLs circulate in the hemolymph primarily as disulfide linked heterodimers, which explains their similar roles in *Anopheles* antibacterial and anti-Plasmodium immunity [122].

In *Ae. aegypti*, functional genetic studies by RNAi identified CLSP2, a modular protein with elastase-like and CTL-domains, as a negative regulator of hemolymph melanization against *P. gallenacium* parasites [123]. Furthermore, *Aedes* CTLs ,MosGCTL1 and mosGCTL7, were shown to facilitate viral propagation in this vector by mediating viral attachment to mosquito cell surface Protein tyrosine Phosphatase-1 (mosPTP-1) and promoting its entry [124, 125] . Several other *Ae. aegypti* mosGCTL were found to function in gut homeostasis by coating commensal bacterial surfaces and protecting them from AMP mediated elimination [126].

e.PRR with Immunoglobulin Domain

The efficiency of the vertebrate immune surveillance system is partly attributed to its adaptive capacity to produce a large and diverse repertoire of antibodies, via somatic recombination of their Ig domains, capable of recognizing even the slightest variations in microbial antigens [127]. While insects don't produce antibodies, they do possess Ig-domain containing proteins with hypervariable structural features that presumably allow for high degree of interaction specificities. *A. gambiae* transcriptome identified 138 genes that

encode proteins with at least one Ig-domain, 85 of which were infection responsive. Among the selected candidates, *IRID* genes were functionally implicated in mosquito immunity. Silencing of *IRID3, IRID5*, or *IRID6* compromised mosquito survival after Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacterial challenges. Depletion of IRID3 and IRID4 proteins increased bacterial loads in hemolymph of naïve mosquitoes, suggesting that they are required for immune homeostasis. In addition, IRID4 and IRID6 strongly antagonized ookinete development in mosquito midguts, exerting an effect comparable to that observed for the very potent anti-Plasmodium factor TEP-1 [128].

Another immune-related Ig-domain containing protein in *A. gambiae* is the hypervariable Down syndrome cell adhesion molecule (Dscam). *AgDscam* gene has 101 exons that, upon exposure to different microbial elicitors, can generate over 31000 potential alternative splice forms each with distinct Ig-combinations, and consequently unique adhesion and recognition specificities [129, 130]. *AgDscam* species –specific alternative splicing against *Plasmodium* and bacteria is controlled by Imd and Toll pathways that mediate the transcriptional regulation of the splicing factors Caper and IRSF1 [131], to produce both soluble and membrane bound splice isoforms.

*Ag*Dscam was shown to be involved in the regulation of experimental and opportunistic bacterial load in the hemocoel and midgut. *AgDscam* gene silencing significantly increased mosquito susceptibility to human and rodent malaria parasites. At the cellular level, *Ag*Dscam was demonstrated to colocalize to pathogen surfaces, and mediate *in vitro* phagocytosis of bacteria, yet its antiparasitic mechanism of action remains unknown [129, 131].

2. Signal Modulation: CLIP domain serine protease cascades

a. Structure of CLIPs

Extracellular serine protease pathways have evolved in animals to mediate rapid and local responses to physiological and pathological stimuli. In arthropods, CLIP domain serine proteases (CLIPs) constitute the largest regulatory group of the chymotrypsin-like family of proteases, and function in several biological processes including embryonic development[132], blood coagulation[133, 134], and immune defenses[135]. The CLIP domain prototype was first identified in the horseshoe crab proclotting enzyme[136]. CLIP proteases are composed of one or more N-terminal disulfate-knotted clip domains connected by a linker sequence to a C-terminal serine protease domain (PD)[137]. Histidine (His), Aspartate (Asp), and Serine (Ser) amino acid residues in the active site of the PD contribute to the acyl transfer mechanism of catalysis of proteolytic CLIP proteases(cSPs). Not all CLIPs are catalytic though. Those with a mutated His-Asp-Ser triad are rendered proteolytically inactive with a protease-like domain (PLD), and are referred to as CLIP domain serine protease homologs (or cSPHs) [138-140]. CLIPs circulate in the hemolymph as zymogens that are activated by specific proteolysis in the linker region. Once cleaved; however, both the clip and protease/protease-like domains remain covalently attached, under nondenaturing conditions, by a disulfide bond that holds the two chains together [6, 135] (Figure 4).

The *A. gambiae* genome encodes a superfamily of 110 CLIPs that are classified into five clades: CLIPAs (22), CLIPBs (29), CLIPCs (12), CLIPDs (14), and CLIPEs (33).

Structural analysis revealed distinct domain organizations among these clades, whereby CLIPAs are cSPHs with CLIP-PLD assembly; CLIPBs, CLIPCs, and CLIPDs are mainly active cSPs with CLIP-PD structure; while CLIPEs have a unique domain organization of PD(or PLD)-PLD-CLIP-PLD, indicating that CLIPEs, although previously classified as non-catalytic, include putative cSPs as well [96, 141].

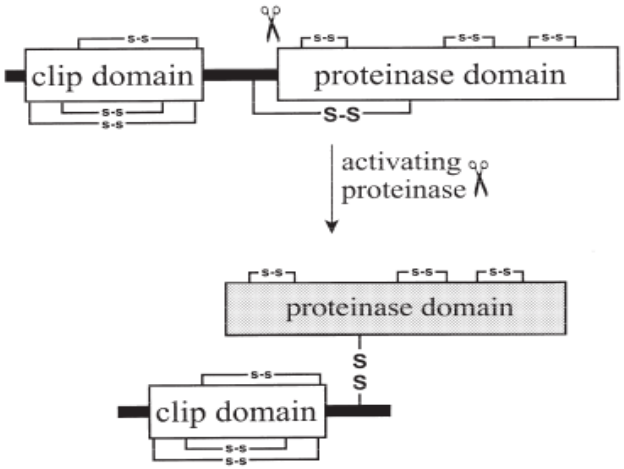


Figure 4. Structure of CLIP domain serine Proteases. CLIP domain serine proteases are composed of an N-terminal CLIP domain connected by a linker sequence to a protease or protease-like domain. CLIP domain serine proteases are secreted into the hemolymph as zymogens that are activated by proteolytic cleavage at the linker sequence. Upon activation, both domains remain attached by a disulfide bond at the linker region. Adapted from [6]

b. Organization of CLIP cascades

cSP-cSPH interactions are not fully elucidated in insects, with very few CLIPs being assembled into short cascades. Phylogenetic analysis based on the alignment of the protease domains of characterized cSPs from different model insects identified two functional clades. One clade included terminal proteases, all of which are CLIPBs, that

activate proSpatzle or prophenoloxidase (PPO), key immune effector proteins in the Toll-pathway and melanization response, respectively. Terminal CLIPBs that directly act on PPO are referred to as prophenoloxidase activating proteases (PAPs). The other clade was composed mainly of CLIPCs and represented penultimate enzymes upstream in the pathway that cleaved other CLIP substrates. As such, an evolutionary conserved protease module was proposed: specific molecular recognition triggers a self -activating non-CLIP Modular SP (ModSP) that elicits the sequential activation of CLIPCs then CLIPBs, which in turn process effector proteins or cytokines [135]

Cao et al (2018) recently devised a bioinformatics-based approach that utilizes known cSP-cSPH pathways in *D. melanogaster* and *Manduca sexta* as templates to predict hierarchical organizations of orthologous protease sets in other insects, including *A. gambiae* [1].In *Drosophila* flies, cSPs Snake and Easter sequentially activate Spätzle for establishing dorsoventral polarity during embryogenesis. According to the established framework, their respective orthologous *Ag*CLIPC2/3 and *Ag*CLIPB5 are expected to fulfill similar hierarchical functions in mosquitoes. Likewise, phylogenetic analysis based on immune cSP-cSPH modules in *M. sexta* identified a putative *A. gambiae* protease network implicated in PPO and Spatzle activation. Accordingly, it is anticipated that mosquito melanization is controlled by two distinct SP pathways: ModSP *Ag*SP217/CLIPC1/CLIPB8 or *Ag*CLIPD1/ CLIPC4-6, 9, 10/CLIPB9 in the presence of cSPHs that culminate in PPO processing in response to infection. The latter *Ag*CLIPD1/CLIPCs cascade may also mediate Toll induction by activating Spätzle processing enzyme CLIPB5 upstream of Spätzle, which reflects a potential crosstalk between Toll and melanization pathways in

mosquito antipathogen defense [1]. In support of this model, reverse genetics revealed that indeed CLIPB8 and CLIPB9 promote melanization in mosquitoes, with CLIPB9 being a putative PAP in *A. gambiae* [142-144]. Moreover, CLIPB5 was identified as a component of protease activation cascade in *Ae. aegypti* Toll antifungal response [144] (Figure 5). Given the large number of insect CLIP proteins and their potential interactions, such conceptual models are essential for inferring hypothetical relationships to guide future functional studies.

Nonetheless, the role of insect cSPHs, and their interplay with each other and other cSPs, in these cascades isn't thoroughly addressed. Conventionally, cSPHs are believed to associate with PAPs as their cofactors to ensure optimal PPO activation. In *Holotrichia diomphalia*, two cSPs (PPAF I and PPAFIII) and a protease homologue PPAFII were characterized as PO activating factors. PPAFIII was shown to cleave proPPAFII into active PPAFII, while PPAFI acts as a PAP inducing the processing of PPO into PO. In the absence of PPAFII, PPAFI generated an enzymatically inactive PO even after the subsequent addition of PPAFI to the reaction mixture, suggesting that the simultaneous presence of SPs and SPHs is necessary for proper PO processing [145-147]. Similarly, *M.sexta* SPH1 and SPH2 were shown to function synergistically to greatly enhance PPO activation by PAP1 and PAP3 [148-151]. *Anopheles* CLIPB9; however, was shown to efficiently cleave and activate *M. sexta* PPO *in vitro* without the need for an cSPH [143],which implies that the role of these cSPHs may likely extend beyond PAP modulation to serve more complex functions in immunity. RNAi functional studies in *A. gambiae* identified two cSPHs, CLIPA8 [152, 153] and SPCLIP1 [92], that are required for

the melanization of *P. berghei* ookinetes in certain mosquito genetic backgrounds. On the other hand, two other cSPHs, CLIPA2 [154] and CLIPA14 [155], function in concert to negatively regulate the melanization response; knockdown (kd) of either gene in a susceptible mosquito background triggers the massive melanization of *P. berghei* ookinetes. The substantial number of mosquito cSPHs and their contrasting RNAi phenotypes further suggests that their regulatory role in CLIP cascades is unlikely to be only restricted to the final step of PPO activation and might rather involve a multilayered control over upstream components of the CLIP cascades.

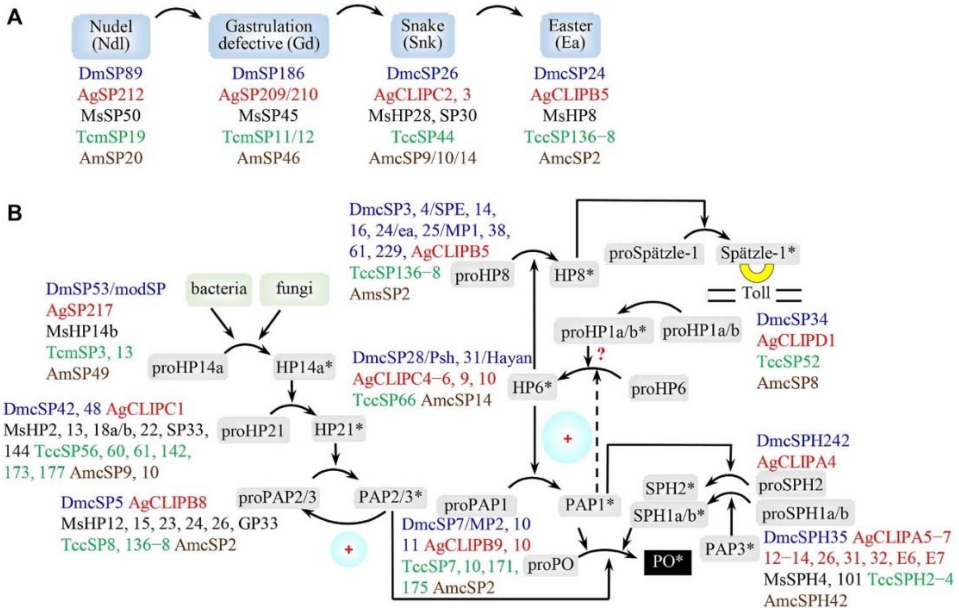


Figure 5. Predicted functions of CLIPs and other multidomain proteases based on insect pathways. (A) Components of SP cascade that establishes the dorsoventral axis in *D.melanogaster* listed as a template to predict similar pathways in other model insects that are presumably composed of the respective orthologs of the *Drosophila* SPs (B) For the same purpose, SP-SPH network that mediates PPO and Pro-Spatzle activation in *M.sexta* are presented along with orthologs or close homologs in other insects. Dm (*Drosophila melanogaster*), Ag (*Anopheles gambiae*), Ms (*Manduca Sexta*), Am (*Apis mellifera*), Tc (*Tribolium castanusm*).Circles with "+" sign represents positive feedback mechanism, and "?" sign represents a partially established step [1].

c. Regulation of Serine Protease Cascades by Serpins

Serpins are a superfamily of serine protease inhibitors that are conserved across higher eukaryotes [156]. Inhibitory serpins possess an exposed reactive center loop (RCL) near their carboxyl-terminal that acts as bait for a specific protease. The covalent binding of RCL to the protease's active site induces the cleavage of a specific RCL bond (scissile bond) that results in an irreversible conformational change in the Serpin, trapping both proteins in an inactive suicide complex[157].

The number of Serpin encoding genes varies considerably among insect species, ranging from 18 in *A. gambiae* and up to 34 in *Bombyx mori* [158, 159]. The majority of insect Serpins remain orphans with unknown endogenous protease targets. Several; however, seem to be involved in the regulation of melanization and Toll pathways. In particular, Serpins were shown to prevent spontaneous or prolonged immune activation of these reactions by binding and inhibiting several of their underlying active proteases.

Phylogenetic analysis identified SRPN 1-3 as potential PAP inhibitors in *A. gambiae*. Although RNAi-mediated silencing of SRPN 1 and -3 had no observable phenotype, silencing of SRPN-2 resulted in the spontaneous development of floating and attached melatonic masses along mosquito body [160]. Even though both *CLIPB8* and *CLIPB9* kds partially reversed the observed tissue melanization phenotype, only CLIPB9 was shown to directly interact with SRPN2, suggesting that SRPN2/CLIPB9, but not CLIPB8, functions as a putative melanization regulatory unit in *A. gambiae* [142, 143] .Incomplete reversion of *SRPN2* kd phenotype implies that SRPN2 is a multi-protease

inhibitor with additional targets to be identified. SRPN6 and SRPN10, were also shown to be functional serine protease inhibitors, but their endogenous targets remain unknown. RNAi experiments revealed that SRPN6 is directly involved in parasite killing in *A. stephensi*, but regulates downstream clearance of dead parasite in *A. gambiae*. These phenotypic discrepancies are likely attributed to the distinct functions of the respective serine proteases targeted by SRPN6 in each species [161]. Additionally, SRPN 6 was shown to mediate microbiota-immune priming against Plasmodium development in *A. stephensi* midguts [162], and to limit the number of *P. berghei* sporozoites reaching the salivary glands of *A. gambiae* [163]. Likewise, SRPN10 was identified as a strong midgut cytological invasion marker in both *A. gambiae* and *A. stephensi*. *P.berghei* infection was associated with an intense induction and nucleocytoplasmic redistribution of two SRPN10 isoforms, KRAL and RCM, in apoptotic invaded gut epithelia. In this context, and based on previous evidence [164, 165], it was speculated that SRPN10 potentially inhibits pro-apoptotic proteases to promote cytoprotection against ookinete invasion and trigger consequent cellular repair [166] .

In *Ae. aegypti*, *SRPN1, -2,* or-*3* silencing resulted in the cleavage of PPO into active PO. Melatonic pseudotumors; however, were only observed in *SRPN2* but not *SRPN1* or *3* kds. Yeast two hybrid system detected an interaction between SRPN-1, IMP-1 (CLIPB9) and IMP-2 proteases, and between SRPN-2, IMP-1 and TMP (CLIPB8). No interacting partners were detected for SRPN-3. It was further concluded that SRPN-1/IMP-1/IMP-2 module regulates immune-induced melanization, while SRPN-2/IMP-1/TMP functions in tissue melanization processes such as cuticle sclerotization and wound

healing [144].

M. sexta Serpins 9 and 3 significantly inhibited PPO activation by associating with hemolymph proteases (HPs) proHP1/HP6/HP8 and proHP1/HP2/HP6, respectively [167]. Moreover, inhibition of HP6 by Serpin5 and HP8 by Serpin-1J upstream of Spätzle blocked Toll immune induction [168, 169]. In *T. molitor*, the Spätzle Processing Enzyme (SPE) is regulated by Serpin48 and Serpin93 [170, 171], while upstream proteases are targeted by serpin40 and serpin55 [172]. In *D. melanogaster*, Spn1 and Serpin43Ac were implicated in Toll regulation, nonetheless their target proteases remain to be identified [132, 173].

3. Signal Transduction: Immune Signaling Pathways

Innate Immune signaling pathways are well characterized in *Drosophila*, and orthologous pathways, with conserved intracellular components, have been identified in several mosquito species. Toll/REL1, IMD/REL2, and JAK/STAT signaling in mosquitoes have been shown to confer resistance against an array of invading pathogens including bacteria, fungi, parasites, and viruses.

a. Toll/REL1 Pathway

While Toll pathway was initially described in *Drosophila* embryonic development [174], it was later shown to be essential for defense against Gram-positive bacteria and fungi [175]. The detection of bacterial lysine PGNs and fungal β-glucans by PGRPs (PGRP-SA and PGRP-SD) [102, 176]and GNBPs(GNBP1 and GNBP3)[176-178] triggers a self-activating Modular Serine Protease (ModSP) upstream of a serine protease cascade

(Grass, Hayan , and Spatzle Processing Enzyme) that culminates in the processing of Pro-Spätzle into active Spätzle[179]. Spätzle, in turn, binds transmembrane Toll receptor and induces intracellular signaling events that lead to the phosphorylation and degradation of the Toll inhibitor Cactus, which releases Dif/Dorsal NF-KB factors that translocate to the nucleus and initiate transcription of various effector gene , including those encoding antimicrobial peptides(AMPs) [180] .

In mosquitoes, genes of the Toll pathway are controlled by REL1, the NF-KB orthologue of Dorsal. *Ae. aegypti* genome encodes for two REL1 isoforms, REL1-A and REL1-B, that are expressed upon septic injury in larvae and adults. Ectopic expression of AaREL-1 in *Drosophila* cells or transgenic flies induced Dorsomycin AMP production and enhanced resistance against *B. bassiana* infection [181] . Moreover, depletion of the negative regulator Cactus significantly decreased Dengue viral load in *Aedes* midguts, indicating that Toll pathway is involved in anti-dengue defense [182]. In *A. gambiae*, Toll pathway has been shown to mediate hemocyte differentiation [183], and induce the expression of major antimicrobial genes in response to *P. berghei* infection. The depletion of Cactus enhanced the basal expression of *TEP-1*, *LRIM1*, *CTL4*, and *SRPN2*, and completely blocked parasite development in a REL-1 dependent manner[184]. Interestingly, Cactus silencing also mitigated *P.falciparum* infection in *A.gambiae*, but to a much lesser extent than that observed for *P.berghei*, suggesting that *A.gambiae* Toll-pathway controlled REL-1 is more potent against rodent than human *Plasmodium* infections[185].(Figure 6).

b. IMD/REL2 Pathway

The *Drosophila* IMD pathway orchestrates responses against bacteria (mainly Gram negative) and viruses. It is triggered by direct interaction of DAP-type PGNs, characteristic of the cell wall of Gram-negative bacteria and certain Gram-positive strains, with PGRP receptors of immune cells. The transmembrane receptor PGRP-LC and intracellular receptor PGRP-LE cooperatively bind DAP-PGNs and promote the recruitment of death domain containing proteins (Imd, FADD, and DREDD caspases) from the nucleus to activate a kinase complex that phosphorylates the NF-KB transcription factor Relish. Relish consists of the Rel Homology Domain (RHD) and the inhibitory ankyrin-repeat rich domain (ANK). DREDD readily cleaves the ANK domain of the phosphorylated Relish, upon which RHD –DNA binding subunit translocates into the nucleus and initiates the transcription of AMP and other target genes. Caspar acts as a negative regulator of the IMD pathway by inhibiting DREDD-mediated cleavage of Relish (reviewed in [186]) (Figure 6).

Studies in *A. gambiae* demonstrated that Gram-positive and Gram-negative bacteria are sensed by PGRPLC, which triggers a signaling pathway that activates REL2, the NF-KB orthologue of Relish, to induce the expression of major effector genes including *AMP* genes (*CEC1, CEC3, GAM1*) and other immune proteins including ,*FBN9*, *TEP-1*, *APL* , and *LRIM1* [185, 187, 188]. PGRP-LC was shown to modulate microbiota proliferation after blood feeding, and in the process boosts mosquito resistance to *Plasmodium* development [109]. *Anopheles REL2* produces two alternatively spliced transcripts: *REL2-F* that encodes full length protein consisting of both RHD and ANK

domains, and *REL2-S* that encodes the short form protein lacking the inhibitory ANK domain. As such, the REL2-S is constitutively active and is thought to confer basal immune functions, while REL2-F is localized to the cytoplasm and requires proteolytic activation. The two isoforms were shown to be differentially involved in defense against Gram-negative and Gram-positive bacteria, respectively [187]. Moreover, REL2-F was implicated in the regulation of melanization reaction against rodent malaria parasites, whereby *REL2-F* silencing caused limited but consistent melanization of *P. berghei* ookinetes in the midgut epithelium [187]. REL2 was also reported to confer protection against human malaria parasite. Transgenic *A.stephensi* mosquitoes overexpressing REL-2 in midgut and fat tissue exhibited greater resistance to *P.falciparum* compared to wild types.This Rel-2 dependent immune activation was shown to be mediated by a number of immune factors including TEP-1,APL1, and LRRD7[52]. Garver *et al*., demonstrated that Imd pathway is most potent against parasite pre-oocyst and early oocyst stages as revealed by *Caspar* silencing .Caspar/REL-2 dual depletion reversed the *Caspar* kd induced resistance and rendered mosquitoes susceptible to *P.flaciparum* development[188]. The role of Imd/REL2 in anti-Plasmodium defense was also demonstrated in *A. albimanus*, whereby depletion of Caspar significantly reduced infection levels of *P. falciparum* in these mosquitoes mirroring those observed in *A.gambiae and A. stephensi*, which suggests that Caspar silencing likely activates a conserved mechanism of *P.falciparum* inhibition in these vectors [185] .

c. JAK/STAT Pathway

The Janus kinase/signal transducers and activators of transcription (JAK-STAT)

pathway is an evolutionary conserved pathway that was first recognized in insects for its role in *Drosophila* development and antiviral immunity [189, 190]. The JAK-STAT pathway is triggered by the binding of Upd peptide ligand to the transmembrane Domeless (Dome) receptor that, in turn, dimerize and lead to the phosphorylation of the Janus kinases (JAK) associated with their intracellular domains. The activated JAK then phosphorylates the receptor dimers, which subsequently induces the phosphorylation and dimerization of STAT proteins. Lastly, activated STAT factors translocate to the nucleus to regulate the expression of target genes (reviewed in [191]) (Figure 6).

In *A. gambiae,* the ancestral *STAT-A* gene is duplicated giving rise to an additional isoform *STAT-B* that regulates the expression of *STAT-A* mRNA in adult females. STAT-A induces nitric oxide synthase (NOS) expression in response to both bacterial and parasite infections. Activation of the STAT pathway was shown to be dispensable for mosquito survival to *bacterial* challenges, but otherwise essential for mosquito resistance against Plasmodium infections. *STAT* silencing significantly enhanced *P. berghei and P. falciparum* oocyst development. Depletion of SOCs, a STAT inhibitor, increased NOS levels drastically reducing the number of early oocyst that complete maturation. Concurrently, chemical inhibition of NOS activity after oocyst formation promoted oocyst survival in infected mosquitoes [192]. Furthermore, *STAT* kd increased the number of *P.vivax* oocysts in the midguts of *A.aquasalis* [193].Taken together these data suggest that STAT-A pathway in *A*nopheles mosquitoes seems to take part in a "late-phase" anti-Plasmodium response against developing oocysts.JAK-STAT pathway was also found to play an essential role in mosquito antiviral immunity. In *Ae. aegypti*, depletion of PIAS, a

JAK-STAT inhibitor, increased resistance to *DENV* and *ZIKA* infections, suppressing viral replication in these mosquitoes [194, 195]. Moreover, Vago (antiviral cytokine) mediated activation of JAK-STAT pathway induced the expression of STAT-dependent *vir-1* antiviral gene, and restricted West Nile virus infection in *Culex* cell lines [196].

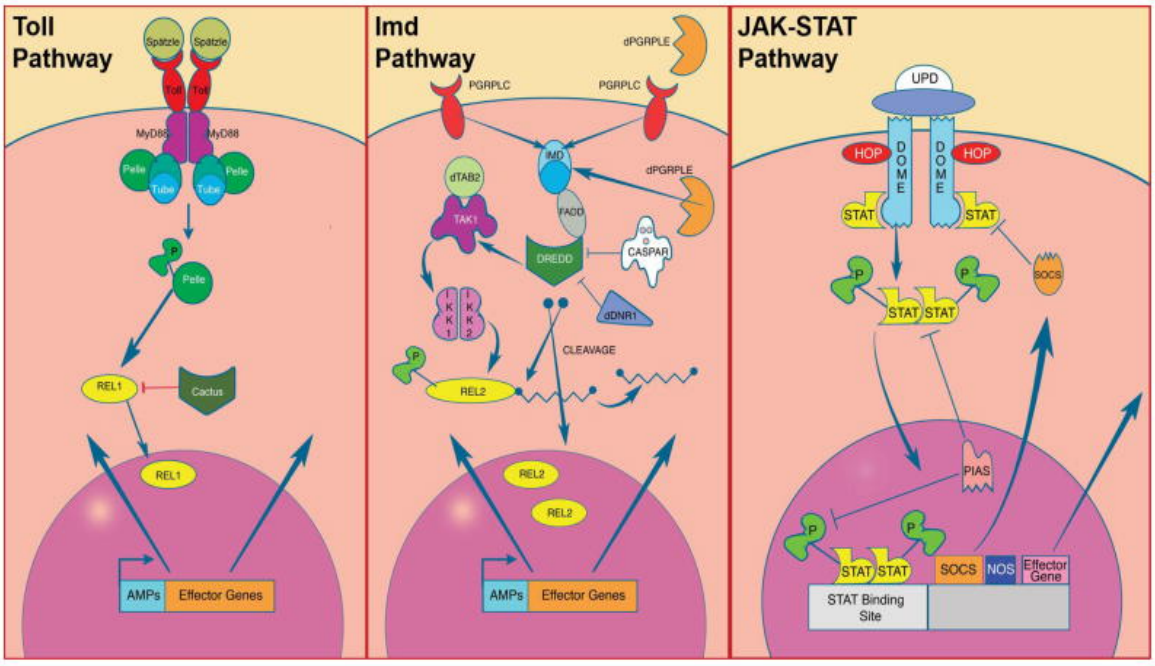


Figure 6. Immune signaling pathways in mosquitoes. Noneself recognition by mosquito PRR triggers the activation of Toll/REL1 , IMD/REL2, or JAK/STAT pathways that lead to the expression of immune effector genes [5]

4. Microbial Clearance

Pathogen killing in the hemocoel is achieved by a combination of humoral and cellular mechanisms. The humoral responses mainly encompass complement-mediated attack, melanization, and antimicrobial peptide production, while the cellular responses

include hemocyte-mediated phagocytosis and gut-mediated epithelial defenses. It is noteworthy that, despite this conceptual organization, the line between humoral and cellular immunity is blurry owed to the strong crosstalk between these two arms. Indeed, many humoral factors are produced by hemocytes. Conversely, hemocyte differentiation and immune priming is often mediated by circulating humoral components.

a. Humoral Effector Responses

i. Complement-Mediated Attack

- Mosquito complement-like proteins

Thioester-containing proteins (TEPs) are a conserved family of secreted proteins that play central roles in innate immune responses in various organisms [197] .In vertebrates, these proteins are members of the protease inhibitors α2-macroglobulin (α2-M) family and the complement system whose central component is the C3 complement factor. Three well defined activation pathways –the classical, lectin, and alternative pathways- culminate in the proteolytic processing of C3 by a transient convertase complex composed of a protease and a complement factor. C3 cleavage triggers the dissociation of a smaller fragment, C3a, and a subsequent conformational change in the remaining molecule, known as C3b, that exposes its thioester bond initiating a nucleophilic attack. C3a serves as an anaphylatoxin , while C3b acts as an opsonin that binds, via its thioester bond, to pathogen surfaces mediating their clearance by phagocytosis or cell lysis (reviewed in [198]).

The *A. gambiae* genome encodes 19 *TEP* genes, of which four pairs exhibit haplotypic features (*TEP1-TEP16, TEP5-TEP17, TEP6-TEP18, and TEP7-TEP19*) [97]. Multiple functional studies demonstrated that TEP1 is a key player in the complement -like system of *A. gambiae*. TEP1 is phylogenetically related to C3 factor but lacks its anaphylatoxin and C345C domains. TEP-1 is composed of a series of eight macroglobulin domains, CUB domain, and a thioester domain containing the β-cysteinyl-c-glutamyl reactive thioester bond .TEP1 is secreted as full length 165 KD glycoprotein (TEP1-F), and is constitutively cleaved in the hemolymph, under both naïve and septic conditions, at a protease sensitive region by a yet unknown protease/convertase to generate an 80 KD active fragment ($TEP1_{cut}$) [199]. $TEP1_{cut}$ is instantly stabilized in the circulation by a heterodimeric complex of two Leucine Rich Repeat proteins (LRR), LRIM1 and APL1C. $TEP1_{cut}$ bound to LRIM1/APLIC retains an intact thioester bond that otherwise hydrolyzes overtime and results in nonfunctional precipitation of TEP1. Indeed, knockdown of either LRR gene was shown to deplete $TEP1_{cut}$ from the hemolymph and induce its deposition on self-tissues instead of pathogen surfaces [8, 200] (Figure 7).

Two cSPHs, SPCLIP1 and CLIPA2, were found to be key regulators of the complement-like pathway in *A. gambiae*. Systemic bacterial infections were shown to deplete TEP1F from the hemolymph. *SPCLIP1* kd rescues TEP1F levels and reduces the amount of $TEP1_{cut}$ deposited on *E. coli* bioparticles, suggesting that SPCLIP1 is required for TEP1 recruitment to pathogen surfaces [92]. In contrast, *CLIPA2* kd enhances the consumption of TEP1F from the hemolymph, indicating that it is a negative regulator of TEP1 processing. *CLIPA2* silencing; however, didn't increase the amount of $TEP1_{cut}$ eluted

from *E. coli* bioparticle surfaces, likely because many were probably cleared from the circulation, thus reducing the total number of recovered TEP1 bound bioparticles [154]. Based on these data, an experimental model for the complement-like pathway in mosquitoes was proposed: the initial deposition of $TEP1_{cut}$ from the LRIM/APLIC complex onto microbial surfaces triggers the formation of a putative TEP1 convertase, which is then followed by the recruitment of additional full length TEP1 and its subsequent conversion into the active form, promoting pathogen phagocytosis, lysis, or melanization. SPCLIP1 and CLIPA2 act as the respective positive and negative regulators of the underlying convertase[7] .

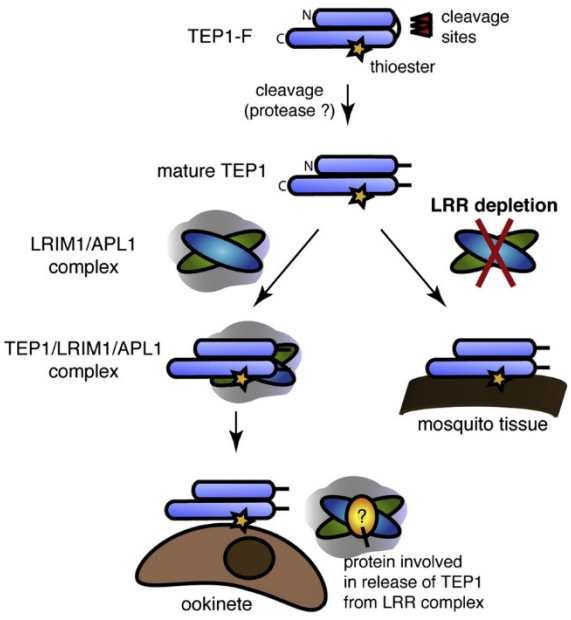


Figure 7. Model for TEP-1 function in the hemolymph. TEP-full is cleaved by yet unknown protease in the hemolymph into mature TEP-1 cut. TEP-1 cut is stabilized by an LRIM1/APL1 complex that recruit it to pathogen surfaces. In the absence of this complex, TEP-1 cut deposits on self-tissues triggering an autoimmune reaction [8]

- TEP1 role in Mosquito Immunity

TEP1 is a critical determinant of several *Anopheles* gambiae immune effector responses, including lysis, phagocytosis and melanization. TEP-1 was shown to mediate *P. berghei* killing in both susceptible (S) and refractory (R) mosquitoes by directly binding ookinete surfaces as they emerge at the basal labyrinth [201]. Silencing of *TEP1* resulted in 5-fold increase in the number of developing oocysts in S strains. In R mosquitoes, *TEP1* kd increased parasite numbers and completely abolished ookinete melanization. Accordingly, it was proposed that TEP1-mediated parasite killing involves lysis in S mosquitoes, and lysis and melanization in refractory ones. These differences in TEP1 activity were attributed to allelic polymorphisms in *TEP1* gene carried by each strain: susceptible *TEP-1s* allele in S mosquito strain, and refractory *TEP-1r* allele in R encapsulating strain. Both alleles were found to be over 90% identical with most variations lying in the hypervariable loops of TED and MG8 domains that protect the thioester bond from hydrolysis. As a result, $TEP1R_{cut}$ is rendered more stable in solution with faster binding kinetics to pathogen surfaces than that observed for $TEP1S_{cut}$ [201, 202]. Indeed, subsequent genome wide-mapping combined with allele-specific RNAi revealed that the variability in *Plasmodium* transmission among individual *A. gambiae* mosquitoes is due to polymorphisms in TEP1 gene[203, 204].Experimental parasite infections of mosquito intercrosses of *A.gambiae* strains fixed for *TEP1R* or *TEP1S* demonstrated a strong association between *TEP-1* variant and susceptibility to rodent or human parasites , whereby parasite burdens in the obtained progeny were lowest in *TEP-1*R/*R*, intermediate in *TEP-1*S/*R*, and highest in *TEP-1*S/S* mosquitoes [205]. TEP1 was reported to mediate *P. falciparum* lysis in *A.*

gambiae keele susceptible laboratory lines [185]. Moreover, *P. falciparum* isolates of different African origins (NF165 and NF166) were shown to successfully develop in susceptible mosquitoes, but appeared to suffer significant losses in their oocyst load in refractory L3-5 mosquitoes in a TEP1 dependent manner [206]. Similarly, silencing of TEP1, LRIM1, or APL1, abolished the melanization of *P. falciparum 7G8* parasites in L3-5 mosquitoes considerably reverting the refractory phenotype [207].

While TEP-1 seems to be a potent anti-Plasmodium factor, several parasite strains, however, have evolved to escape complement killing in both S and R mosquitoes. *P. falciparum* 3D7, GB4, and NF54 African parasites can readily survive in refractory L3-5 *A. gambiae* mosquitoes [73, 208]. TEP1 silencing had no effect on NF54 oocyst load in these mosquitoes, suggesting that NF54 can effectively evade complement mediated lysis [207]. In susceptible lines, immune evasion was reported for sympatric parasite-mosquito isolates that originated in the same geographic region and are thus considered evolutionary compatible. QTL mapping and functional genomics identified the highly polymorphic *P. falciparum* surface protein Pfs47 to be required for the evasion process, rendering the parasite invisible to the complement system [209]. Genetic replacement of *Pfs47* haplotypes changed parasite-mosquito compatibility by drastically decreasing the survival of parasites in their otherwise sympatric vectors [91]. Moreover, Knockout of *Pfs47* in NF45 lines significantly compromised parasite survival in refractory mosquitoes; this phenotype was reverted upon disruption of the mosquito's complement system or after genetic complementation of *Pfs47* [209]. Molecular analysis revealed that Pfs47 functions by effectively suppressing JNK signaling, and consequently epithelial nitration of ookinetes

that, in turn, escape TEP1 localization to their surfaces, and thus evade complement mediated lysis or melanization [209, 210].

The role of TEP1 in mosquito immunity further extends to defense against bacteria and fungi. TEP-1 binds to bacterial surfaces and is thought to act as an opsonin. *E. coli* and *S. aureus* incubated in media depleted of TEP-1 were less efficiently cleared by hemocyte-like cells *in vitro* [203]. Moreover, *TEP-1* silencing *in vivo* impairs bacterial phagocytosis and increases accumulation of live *E. coli* and *S. aureus* in infected mosquitoes [211]. TEP1 was also found to be essential for the melanization of both bacteria and fungi. TEP1 was shown to mediate the cleavage of CLIPA8 that is required for PO activation against septic infections, indicating that TEP1 acts upstream of this cSPH in the melanization cascade [92, 152] . Furthermore, *TEP1* kd abolished PO activity in response to systemic *E. coli* or *B. bassiana* challenges, and impaired PO recruitment to fungal hyphae, thus inhibiting their melanization [92, 212].

ii. Melanization

Melanization in arthropods plays an important role in various physiological processes such as wound healing, cuticle sclerotization, egg chorion tanning, and immunity [4, 213-215]. Melanization involves the synthesis of dark brown pigment eumelanin and its deposition on target surfaces to form a thick contiguous melanin sheath[216]. Evidence from different insect species, suggests an important role for melanization in defense against a variety of pathogens including, parasites, bacteria, fungi, and more recently viruses(reviewed in [217]).

- Biochemistry of Melanization

Melanogenesis is initiated by the hydroxylation of phenylalanine, by phenylanine 4-monooxygenase (PAH), to form the rate limiting substrate tyrosine. Tyrosinase-like phenoloxidases (POs), the rate limiting enzymes in melanogenesis, then catalyze the subsequent oxidation of tyrosine into Dopa and the highly reactive intermediate Dopaquinone. In the presence of thiol compounds, Dopaquinone is converted to cysteinyl and glutathionyl conjugates that mediate the synthesis of the cutaneous reddish pigment pheomelanin. Otherwise, Dopaquinone undergoes a spontaneous cyclization into dopachrome, which in turn is instantly decarboxylated by dopachrome conversion enzyme (DCE) to generate 5, 6-Dihyroxyindole (DHI). Following PO-mediated DHI oxidation, indole quinones polymerize and give rise to the heteropolymer eumelanin (reviewed in [218] ,[219], [220]). While DHI-eumelanin can be also derived from dopamine, produced early on upon the decarboxylation of dopa by dopa decarboxylase (DDC), this complementary pathway is more commonly associated with cuticle sclerotization rather than immune defense [221],[222] (Figure 8).

The immune-protective functions of melanization may be partly attributed to the oxidoreductive properties of melanogenic precursors that engage in various redox reactions to create a biochemically hostile environment to invaders [3, 223]. Eumelanin and quinoide intermediates deposit as cross-linking complexes on foreign nucleophilic surfaces to effectively encapsulate, immobilize, and deprive circulating pathogens from nutrients. More importantly, these eumelanotic capsules likely serve as platforms for electron transfer reactions that both generate and recruit cytotoxic reactive oxygen and nitrogen species

(RONS)[224, 225]. RONS are highly unstable electrophilic molecules that can virtually attack and destroy a large number of organic compounds. Increased levels of RONS have been associated with lipid peroxidation, altered membrane fluidity and ion transport, DNA damage, inactivation of enzymes, inhibition of protein synthesis, and ultimately pathogen cell death [226].

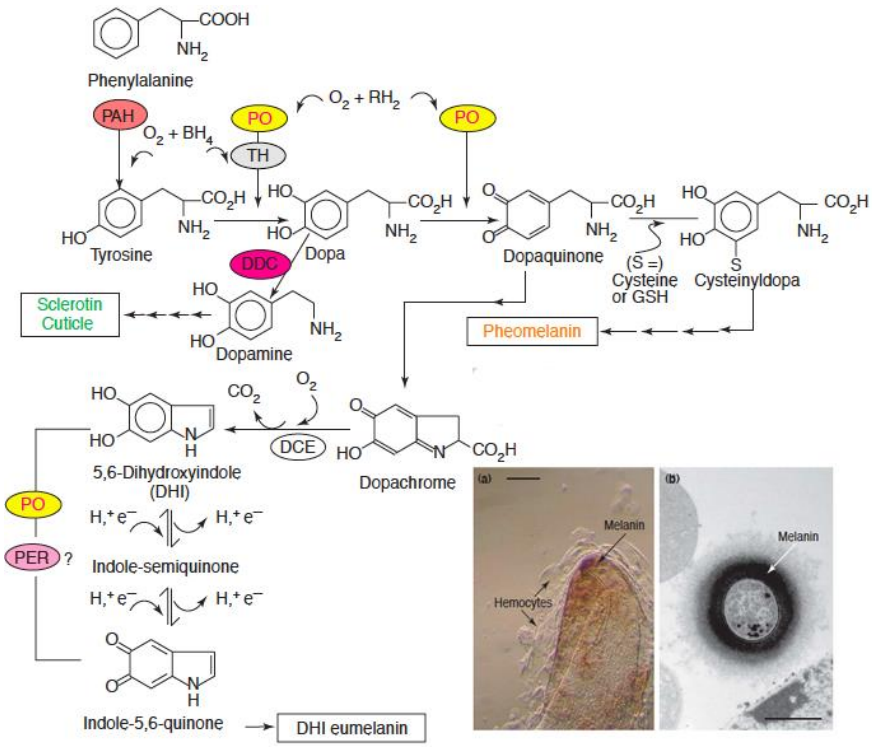


Figure 8. The biochemical pathway of melanization. The biochemical reactions of melanogenesis. (a) Cell mediated melanization of parasitoid wasp in the hemolymph of fruit fly wasp.(b) Melanization of *Enterobacter cloacae* Bacterium in the hemolymph of Aedes mosquito. Figure adapted from [4]

- Phenoloxidase activation in mosquitoes

The *A. gambiae* genome encodes nine *PPO* genes that exhibit overlapping expression profiles during development, with *PPO5* and *PPO6* being predominantly expressed in adult mosquitoes, while *PPO1-4* being mainly expressed in pre-adult stages. Other *PPOs* such as *PPO2*, *PPO3*, and *PPO9* are induced following blood feeding [97, 227-230]. Phenoloxidase is secreted as a zymogen (PPO) that is converted into active PO via CLIP-mediated proteolytic processing (Figure 9).

RNAi based functional analysis identified several *A. gambiae* CLIP proteases in the mosquito melanization response. CLIPA8 is a key molecule essential for the melatonic response against *P. berghei*, bacteria, and *B. bassiana. CLIPA8* kd abolished PO activation following systemic infections. CLIPA8 activation cleavage is controlled by TEP1 and its positive regulator SPCLIP1, suggesting that CLIPA8 is downstream of these proteins in the melanization response [152, 153, 212]. Similarly, SPCLIP1 is required for pathogen melanization; *SPCLIP1* kd blocked *P. berghei* ookinetes melanization in CTL4 silenced mosquitoes, and inhibited PO activation in response to bacterial infections [92]. In contrast, CLIPA2, CLIPA7, and CLIPA14 are inhibitory cSPHs whose silencing trigger the melanization of *P. berghei* [153, 154, 231]. *CLIPA2* kd trigger ookinete melanization by enhancing TEP1 deposition on parasite surfaces. Moreover, microscopic analysis revealed that CLIPA2 localizes to pathogen surfaces in a TEP1 dependent manner, thus implying a reciprocal interaction between both proteins [154]. Alongside CLIPA2, CLIPA14 is a newly characterized inhibitory cSPH that also requires TEP1 for its activation. *CLIPA14* kd trigger a dramatic melanotic response against *P. berghei* and an exaggerated hemolymph

PO activity in response to septic bacterial infections. More importantly, co-silencing *CLIPA2/CLIPA14* increased ookinete melanization, and enhanced PO activity to a level higher than that obtained for each of the single knockdowns alone, indicating that the two cSPHs function cooperatively to control PPO activation [231].

A set of cSPs were also shown to contribute to parasite melanization but to varying levels and depending on mosquito genetic background. Silencing of *CLIPB3*, *CLIPB4*, *CLIPB8*, *CLIPB14, CLIPB15,* or *CLIPB17* only partially abolished *P. berghei* ookinete melanization [153, 232], which indicates that CLIPBs likely function in synergistic manner with redundant roles in melanization.

The PPO cascade in *A. gambiae* is tightly controlled by SRPN2. *SRPN2* kd induced the formation of melanotic pseudotumors attached to mosquito abdomens. *SRPN2* kd also increased the number of melanized *Plasmodium* ookinetes suggesting that it may be regulating multiple cSPs involved in different melanization pathways [160]. *In vitro* assays identified CLIPB9 as a putative target for SRPN2. While both CLIPB8 and *CLIPB9* kds partially reversed SRPN2 RNAi phenotype *in vivo,* SRPN2 only inhibited CLIPB9 proteolytic activity *in vitro*, suggesting that CLIPB9 is likely a direct target for SRPN2, but its hierarchical position with respect to CLIPB8 remains to be determined [142, 143].

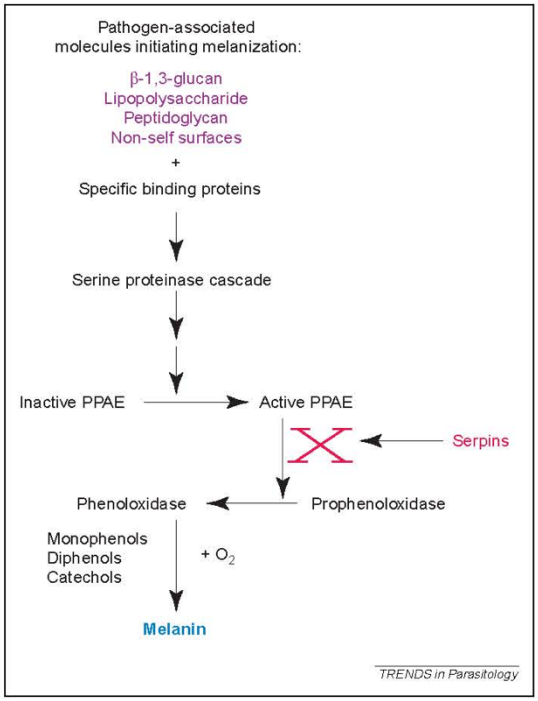


Figure 9. Phenoloxidase activation cascade. Non-self-recognition by PRRs triggers a serine protease cascade that culminates in the processing of inactive PPO into active PO and the initiation of melanogenesis. PPAE : Prophenoloxidase activating enzyme Adapted from [3]

- The contribution of the melanization response to mosquito immunity

Functional characterization of *A. gambiae* cSPHs revealed that they play a critical role in regulating mosquito susceptibility to microbial infections. CLIPA8 [153] and SPCLIP1 [92] are essential for *Plasmodium berghi* ookinete melanization, with the latter also involved in regulating TEP1 amplification on microbial surfaces. On the other hand, CLIPA2 and CLIPA14 act concertedly to negatively regulate this response [155]. Interestingly, *CLIPA8* silencing significantly compromised mosquito survival (i.e. endurance) and resistance (pathogen clearance) to infections with entomopathogenic fungus

Beauveria Bassiana [212] but not to *E.coli* or *S. aureus* [152] , suggesting that the melanization reaction is critical for antifungal but dispensable for antibacterial defense in mosquitoes, respectively. On the other hand, *CLIPA2* kd increased host endurance and resistance to fungal and bacterial challenges by enhancing TEP1 activity [154]. Surprisingly, ds*CLIPA14* despite having a similar RNAi phenotype to CLIPA2 with respect to melanization it does not seem to regulate TEP1 activity [155].

The role of melanization in immune defense seems to span viruses as well, despite the few studies available in this regard. Tamang et al., observed increased SINV titers in *Armigeres subalbatus* mosquitoes after silencing *PPO1* [233]. Moreover, *in vitro* assays revealed that Semliki Forest virus (SFV) induces PO activation in *Ae. Albopictus*-derived U4.4 cells lines. Inhibition of PO cascade in these cells by infection with recombinant SFV expressing the PO inhibitor Egf1.0, promoted viral spread among colonies. Concurrently, *Ae. aegypti* mosquitoes fed on recombinant SFV infected blood meals manifested low *in vivo* PO activity, enhanced viral replication, and compromised survival [234]. Taken together, these data highlight a potential role for mosquito melanization in defense against arboviruses while the specific nature of antiviral effector proteins remains to be determined.

The mechanisms by which melanization contributes to immune resistance remain unclear. Dudzic *et al.,* recently demonstrated a disconnect between PO mediated resistance to infection and blackening reaction in *D. melanogaster* flies. In their model, the authors revealed specific roles for two distinct serine proteases, SP7 and Hayan, in regulating either process. *SP7* null mutant flies rapidly succumbed to *S. aureus* infections, but retained high levels of blackening of the wounded cuticle and hemolymph, indicating that it is not the

blackening reaction *per se* that controls resistance. In contrast, Hayan deficient flies successfully survived *S. aureus* challenges but exhibited reduced melanin deposition at both sites. SP7 was shown to activate PPO1 only, while Hayan activates both PPO1 and PPO2. Thus, it was concluded that melanization-dependent pathogen clearance and blackening reaction in *Drosophila* are two distinct processes controlled by different pathways: SP7/PPO1 and Hayan/PPO1 and PPO2 , respectively, and that it is likely that the melanization by-products such as ROS and quinoide intermediates are the active molecules mediating immune resistance [179].

iii. Antimicrobial Peptides (AMPs)

Antimicrobial peptides constitute a diverse family of immune effector proteins that are conserved across all classes of life. AMPs are mainly cationic and amphiphilic in nature which facilitates their interaction with negatively charged pathogen surfaces. The mechanism of action of AMPs is still not fully understood, but it is believed that it mainly involves permeabilization of target membranes leading to metabolite leakage, altered membrane potential, and eventually cell lysis [235]. Antibacterial activity in insects was first observed in bacteria-immunized pupae of silk moths [236, 237], and later on in bacteria infected *D. melanogaster* [238]. Since then over 150 AMPs were identified as potent components of the insect innate immune system. In response to infection, insect AMPs are produced by fat body and hemocytes, and released systemically into the hemolymph [239, 240], or secreted locally by epithelia [241, 242]. Functional AMPs are cleaved *in vivo* from a larger precursor which contains, in addition to the mature peptide, a signal domain and a prodomain [243]. Insect AMPs can adopt different structures and

accordingly are classified into four groups: the α-helical peptides (e.g., Cecropin and Moricin), glycine-rich peptides (e.g., Attacin and Gloverin), cysteine- rich peptides (e.g., Defensin , Gambicin, and Drosomycin), and proline- rich peptides (e.g., Apidaecin, Drosocin, and Lebocin) [244, 245].

The *A. gambiae* genome encodes several AMPs including, four defensins (DEFs), four cecropins (CECs), one Gambicin (GAM), and one yet uncharacterized Attacin [97]. Studies on mosquito defensins revealed differential expression among life stages; while *DEF1* was found to be constitutively expressed in adult midgut and upregulated in hemolymph following bacterial infection [246], *DEFs 2, 3*, and *4* exhibited low level constitutive expression in adults, even under septic conditions, but showed significant increases in mRNA abundance during the larval stages [247]. DEF1 is mainly active against a broad spectrum of Gram-positive bacteria, very few Gram-negative strains, and filamentous fungi, but not yeast [246]. Moreover, disruption of the defensin gene didn't affect the development of the different stages of *P. berghei* , suggesting that defensins may not act as anti-parasitic factors [248].

The expression of three *A. gambiae* cecropin genes (*CEC A-C*) was induced *in vitro* by challenge with heat-inactivated bacteria. Additional assays revealed that cecropins, like defensins, are widely microbicidal whose activity includes yeast and Plasmodium as well [246, 249]. In *Ae. albopictus*, cecropins were shown to act against *Francisella* bacteria by significantly disrupting its cellular membrane [250].

Very little information is available about Gambicin in *A. gambiae*; nonetheless, a comprehensive study revealed that *Gambicin* expression is induced in fat body, midgut and hemocytes of mosquitoes during early and late stages of malaria infection. *In vitro* assays further showed that mature Gambicin attacks both bacterial Gram- types and fungi, but has a marginal effect on *P. berghei* ookinetes [243].

b. **Cellular Effector Responses**

i.Hemocyte mediated-defense

Hemocytes constitute the primary immune cell population in mosquitoes. It is estimated that adult *Anopheles* females harbor between 500-4000 hemocytes, but this number decreases with age [251, 252]. In general, 75% of total hemocytes occur in the circulation and 25 % occur as sessile cells attached to tissues throughout the body. The majority of sessile hemocytes are present at the abdominal wall, particularly at the periostial regions surrounding the valves of the heart which experience the heaviest hemolymph flow, and are thus strategically positioned for microbial capture [252]. In response to infection, additional hemocytes are recruited from the hemolymph to these sites to amplify pathogen phagocytosis and melanization [253].

Regardless of their distribution, mosquito hemocytes are broadly classified into three main types (granulocytes, oenocytoids and prohemocytes) based on their morphology and function. Granulocytes are the most abundant compromising 80-95% of the population, while oenocytoids and prohemocytes make up the rest. Granulocytes are polymorphic and are almost 9µm in diameter in circulation, but *ex vivo* they attach via filopodia to foreign

surfaces and extend to a diameter of 35 μm. They are highly phagocytic with a vesicle-rich cytoplasm and possess a strong acid phosphatase activity[254]. A recent study showed that granulocytes can be significantly depleted from the circulation by using cholodronate (CLD) liposomes. Flow cytometry, combined with CLD treatment and PPO6 staining, identified the presence of at least three subpopulations of granulocytes that vary in size, granularity, phagocytic potential (i.e. susceptibility to CLD) and PPO6 expression. Among these are the highly phagocytic $PPO6^{+}$, the highly phagocytic $PPO6^{-}$, and the mildly phagocytic $PPO6^{-}$ cell types [255].

Oenocytoids are spherical in shape with a 6–20 μm diameter, and don't readily spread on *ex-vivo* surfaces. They have a homogenous cytoplasm and are major producers of PPO. Prohemocytes are difficult to distinguish from oenocytoids as they are also round in morphology and non-adherent, nonetheless, prohemocytes are usually smaller (4 μm in diameter) and have a high nuclear to cytoplasm ratio than that of oenocytoids. Prohemocytes are proposed to represent the hemocyte progenitor cell lineage in mosquitoes [251, 254]; however, one study suggests that they are phagocytic and may have originated from asymmetric division of granulocytes [252].

- Hemocytes in mosquito innate immunity:

Despite being such an integral part of innate immunity, much about the hemocyte biology in mosquitoes remains unclear. Mosquito hemocytes strongly respond, both at the cellular and molecular levels, to a variety of immune stimuli. Hemocytes display a transient increase in number, size, and granularity following blood feeding [251, 256, 257] and

systemic infections [252, 258, 259]. Moreover, genome wide transcriptomic analysis of *A.gambiae* circulating hemocytes revealed unique pathogen specific signatures of gene expression in response to challenge with different bacterial species and different stages of Plasmodium infection. The majority of the differentially expressed transcripts belonged to recognized immunity related genes such as those encoding CLIP proteases, SRPNs, PPOs, TEPs, LRIM, and Imd and JNK signaling components, [260, 261]. A more recent proteomic profiling of *Anopheles* granulocytes corroborated these findings and identified dramatic, slightly overlapping, shifts in immune protein expression among these cells in response to phagocytosis, blood feeding, and *P. falciparum* challenge [262], suggesting that hemocytes ,and particularly granulocytes, significantly contribute to mosquito innate immunity.

In fact, functional studies revealed a central role for hemocytes in mosquito anti-Plasmodium response. Mosquito midgut invasion by *P. berghei* ookinetes was shown to induce hemocyte differentiation that, in turn, confers a long-lived, microbiota dependent, innate immune memory to subsequent malaria infections. Initial exposure to *Plasmodium* triggered significant increase in granulocytes in challenged mosquitoes as compared to controls. This effect was much more exacerbated in the former when both groups were subjected to another round of infection, leading to a strong reduction in live oocysts. Immune priming was eliminated in the absence of gut microbiota, suggesting that the observed enhanced immunity is likely due to an interaction of proliferating bacteria (in response to the blood meal) with midgut cells and not the persistent immune activation from the first *Plasmodium* infection [263].Moreover, challenge with different *Plasmodium*

species revealed that the strength of the priming response decreased with higher levels of compatibility between that parasite and vector [183].

The observed priming effect was later attributed to the presence of a soluble hemocyte differentiation factor (HDF), an Evokin/Lipoxin complex that is secreted into the hemolymph by gut epithelia in response to midgut injury. Transfer of cell free hemolymph from infected to naïve mosquitoes, or their direct injection with HDF, induced hemocyte differentiation and rendered mosquitos more resistant to *Plasmodium* infection. Silencing of Toll, IMD, JNK, and JAK-STAT pathway components didn't affect HDF levels; nonetheless, all but IMD were required for hemocyte differentiation in response to HDF, implying that HDF acts upstream of these pathways in the priming model [183, 264, 265] .

Once active, hemocytes attach to the basal lamina of ookinete invaded midgut epithelia [263]. Upon contact with these nitrated surfaces, hemocytes undergo apoptosis and release microvesicles (HdMv) into the basal labyrinth of the midgut. HDF injection or silencing of Toll inhibitor *Cactus* significantly enhanced HdMv release. However, the disruption of hemocyte integrity by injection of polystyrene beads into the hemolymph greatly reduced HdMv numbers and increased *Plasmodium* intensity in infected mosquitoes. While bead injection didn't affect levels of TEP1 or its active form $TEP1_{cut}$ in the hemolymph, HdMv loss decreased TEP1 binding to ookinete surfaces. As such, it was anticipated that HdMv may deliver a critical "early-phase" immunity factor that is required for TEP1 deposition on and consequent elimination of invading ookinetes [265]. Chemical depletion of granulocytes corroborated this phenotype and impaired TEP1 mediated ookinete recognition, suggesting that granulocytes are the likely source of HdMv [255].

Hemocyte differentiation was also proposed to mediate "late-phase" anti-Plasmodium immunity. STAT-A and LL3 are two transcription factors known to be involved in oocysts attrition at late stages of parasite infection [192, 266]. Smith *et al.* demonstrated that SOCS (repressor of STAT-A pathway) depletion increases granulocyte abundance in response to *Plasmodium* while LL3 silencing abolishes this effect, indicating that both STAT-A and LL3 are essential components of the hemocyte differentiation mechanism. Concurrently, loss of LL3 or STAT-A mediated hemocyte differentiation, or CLD mediated granulocytes depletion [255], abrogated oocyst clearance without affecting ookinete-oocyst transition, implying that hemocytes contribute to late phase immunity. [267].

- Phagocytosis

Phagocytosis is an evolutionarily conserved cell-mediated reaction that is based on recognition, engulfment, and intracellular destruction of invading pathogens. In the process, foreign bodies that are recognized by PRRs on phagocytic cells are internalized into a phagosome, which then fuses with a lysosome triggering the degradation of the pathogen [268] .Mosquito hemocytes, particularly granulocytes, are highly phagocytic and can initiate the reaction almost instantly after microbial exposure [269], engulfing up to hundreds of foreign particles within 24 hours of infection [270-272]. Hemocyte phagocytic activity against diverse pathogens has been widely studied [269, 272-274] . In *Culex* mosquitoes, *Candida albicans* yeast cells were readily cleared from the hemolymph by both phagocytosis and nodule formation [275]. In *A. gambiae*, granulocytes were reported to preferentially aggregate in the periosteal regions of the heart, that experience the most

hemolymph flow, where they phagocytose Gram positive (*S. aureus*, *Staphylococcus epidermidis*, *Micrococcus luteus*) and Gram negative (*E. coli*) bacteria [276]. Chemical depletion of phagocytic immune cells by mosquito treatment with CLD liposomes decreased *A. gambiae* survival to *S. aureus* and *Serratia marcescens* challenges. Moreover, the same study revealed that phagocytic hemocytes play dual roles in anti-Plasmodium immunity mediating both complement activation and PPO production against ookinetes and oocysts [255]. Direct phagocytosis of Plasmodium parasites is still controversial. Hillyer et al., observed a 2% rate of sporozoite engulfment by *A. gambiae* hemocytes, but declared it insufficient to explain the massive losses of sporozoite numbers in this vector [271]. In contrast, granulocytes were shown to heavily phagocytose *P. gallenacium* sporozoites in *Ae. aegypti*. This; however, may not necessarily reflect a definite role for phagocytosis in sporozoite clearance given that *Aedes* mosquitoes are not natural vectors for malaria parasites [272].

- Regulation of phagocytosis

Functional genetic analysis identified Dcsam, a hypervariable Ig-containing receptor, to be required for the engulfment of bacteria in *A. gambiae* [87]. Moreover, a semiquantitative phagocytic test identified additional 26 genes that are involved in the regulation of phagocytosis in this vector. These genes were ascribed to seven classes which included i) hemolymph secreted molecules , such as ,TEP-1, TEP-3, TEP-4, and LRIM1; ii) transmembrane receptors, such as the low-density lipoprotein receptor-related protein (LRP), and β2-integrin (BINT); and iii) intracellular factors , such homologs of CED2/CED5 and CED6 that mediate apoptosis and necrosis in *Caenorhabditis elegans*.

The test further placed these genes into two putative regulatory pathways: the CED5 pathway which includes TEP4, BINT receptor, and intracellular factor CED2, and the CED6 pathway that is comprised of TEP-1, TEP-3, LRIM1, and receptor LRP. Silencing the components of each pathway, independently or simultaneously, decreased the efficiency of phagocytosis of *E. coli* and *S. aureus*, and increased their proliferation in the hemolymph, but it didn't compromise mosquito survival, suggesting that loss in phagocytosis efficiency can still be rescued by other defense mechanisms [211].

ii. Gut Epithelial Immunity

Studies focusing on *Plasmodium*-mosquito gut interactions revealed that midgut traversal by ookinetes is a highly limiting step in the malaria life cycle. Initially, the distension of the mosquito midgut epithelium by a blood meal induces the cells to secrete an extracellular layer, known as the peritrophic matrix (PM), around the blood bolus. The PM is a semipermeable sheath of chitin and glycoproteins that gradually polymerizes to reach 1-20μm in thickness 24 hours post feeding [277, 278]. Rodgers *et al.*, demonstrated that the synthesis of a complete PM is dependent on gut microbiota that regulate the expression of several of its structural proteins. Antibiotic treated *A. coluzzi* mosquitoes exhibited either a complete absence of or a fragmented PM around the blood bolus as revealed from chitin-staining. Disruption of the PM increased bacterial proliferation and persistence in the gut and promoted their dissemination into the body cavity, indicating that the PM is essential for preserving gut microbiota homeostasis and preventing the seeding of a systemic infection following a blood meal [279].

Motile ookinetes anchor to PM proteins [280] and secrete chitinases and other proteases to locally disrupt and penetrate this barrier [281]. A compromised PM has been associated with increased oocyte prevalence in infected mosquitoes, suggesting that PM likely hinders ookinete invasion of midgut cells [282-285]. Nonetheless, opposing reports suggested that the PM doesn't necessarily protect against midgut traversal, and might otherwise promote parasite colonization by shielding ookinetes from collateral digestive [286] and immune damage in the gut [279].

Ookinete breaching of the PM has been shown to trigger a strong hemocyte priming effect. As proliferating bacteria leak through and come in contact with the luminal side of midgut epithelium they induce the expression of HPX7 and HPX8, enzymes that mediate the synthesis of Prostaglandin E2 (PGE2). PGE2 secretion induces HDF production which in turn attracts hemocytes to the basal surface of midgut epithelial cells and enhances their patrolling activity, thus promoting a long-lasting cellular immune response against Plasmodium infection [287].

Once at the luminal surface, ookinetes covalently adhere to and traverse the midgut epithelium via a predominantly intracellular route [288-291]. In the process, parasites inflict an irreversible chemical and physical damage to epithelial cells that become a ticking "time bomb", and ultimately undergo apoptosis. According to studies using *A. stephensi-P. berghei* infections, the "time bomb" model predicts that ookinete survival depends on their ability to escape the dying cell unharmed before the bomb detonates, and likely do so by migrating laterally across adjacent cells before emerging at the basal side [289, 291]. Invaded cells respond by mounting a strong nitration reaction, mediated by JNK signaling

and HPX2/NOX5/NOS system, that potentiates Nitric oxide (NO) toxicity in the cell and marks ookinetes for consequent attack by TEP1 [292-294] (Figure 10). Epithelial cells further prime invading ookinetes for destruction by depositing an actin-rich hood on their surface as they emerge into the subepithelial space. The hood was shown to originate from lamellipodia that extend from the invaded cell itself and healthy neighboring epithelia in an attempt to repair the midgut barrier at the site of parasite egression [289]. The *Frizzled 2* (*Fz2*) and *cell division cycle 42* (*Cdc42*) genes, together with *WASP*, a positive regulator of actin polymerization, have been implicated in the formation of this actin zone [295, 296]. The actin hood was observed on dying and dead parasites in midguts of refractory(L3-5) *A. gambiae* infected with *P. berghei* [297]. It was previously proposed that L3-5 strain clears *Plasmodium* parasites via lysis and melanization[298]. Absence of the hood in *WASP* kd L3-5 mosquitoes significantly increased the number of melanized ookinetes while allowing for sparse oocyst development, implying that the un-hooded ookinetes have escaped lysis to subsequently become melanized. In contrast, hood depletion in *CTL4* kd –melanizing *Yaoundé* mosquitoes reversed the encapsulation phenotype and allowed for ookinete survival. Microscopic analysis revealed that TEP1 staining was frequently observed on dead hooded parasites. Furthermore, silencing of *LRIM1* reduced the number of dead hooded ookinetes. These data suggest that hood formation may be linked to complement initiation on ookinete surfaces to promote parasite killing via lysis or melanization[297].

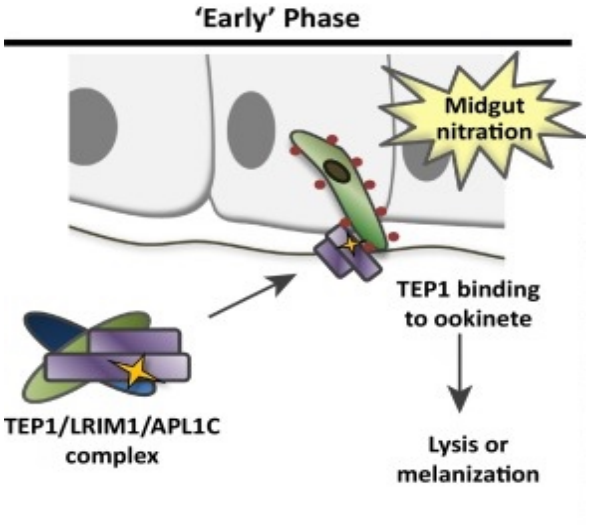


Figure 10. Early phase Epithelial Immunity. As ookinetes traverse midgut epithelium they are subjected to an early-phase nitration reaction (red dots) that tags them for subsequent attack by mosquito complement like protein TEP-1. TEP-1 deposits on the surface of nitrated ookinetes as they emerge at the basal labyrinth to induce their lysis or melanization.(Adapted from [10])

Chapter II

AIMs of the Project

Melanization is a spectacular immune response of insects that culminates in the deposition of melanin on microbial surfaces leading to their death. Despite more than half a century of investigation of this immune reaction in several insect models, important knowledge gaps remain specifically concerning the regulation of this response especially that its output is tightly regulated by complex genetic and biochemical interactions between members of structurally diverse immune gene families. Dissecting the complexity of the regulatory mechanisms operating in the context of the insect melanization response as well as other responses is essential as it may facilitate the fine manipulation of mosquito immunity to compromise their vector capacity.

Central to the modulation of the melanization response in insects are extracellular protease cascades mainly composed of CLIP-domain serine proteases (cSPs) and their non-catalytic homologs (cSPHs). Hundreds of cSPs and cSPHs have been identified in mosquitoes [299], and very few have been assembled into short cascades, nonetheless, these remain partial and non-exhaustive in most instances providing little information about the complexity of the interplay between cSPs, cSPHs, and other effector immune proteins. In the context of this thesis, we aim to provide new molecular and functional insight into CLIP cascades in *A. gambiae* melanization reaction, with particular focus on the role and organization of cSPHs.

We have previously identified a novel cSPH in *A. gambiae* whose expression profile is co-regulated with that of CLIPA2, a negative regulator of mosquito melanization and complement-like responses, in a transcriptomic database using Pearson correlation analysis (Osta MA, unpublished). Initially, we called this gene *CLIPA16*. *CLIPA16* sequence in vectorbase (www.vectorbase.org) was incomplete as two key cysteines in the clip domain were missing from the annotation. To determine the full sequence of *CLIPA16*, we amplified the whole coding sequence from *A. gambiae* G3 strain cDNA, cloned the amplicon into *pIEx-10* expression plasmid and sequenced it. The obtained sequences revealed that CLIPA16 is a true cSPH containing all 6 cysteines in the clip domain, indicating that the gene was wrongly annotated in vectorbase. In a recent publication by Cao et al. (2017) [141] which aimed to correct the annotations of *A. gambiae* SP-related gene models, CLIPA16 was annotated as CLIPA28, the term that we will use henceforth. In this project, we aim to characterize CLIPA28 at the functional and molecular levels, with particular focus on 1) Its contribution to mosquito defense against different classes of pathogens, including malaria parasites (*P.berghei*) , bacteria (*S. aureus* and *E. coli*) ,and fungi (*Beauveria Bassiana*) , and 2) Its gene interactions and hierarchical position with respect to the other cSPHs and cSPs involved in the melanization response .

Specific Aim 1: Functional genetic analysis of ClipA28 in mosquito immunity

1.1. Investigate the effect of *CLIPA28* knockdown (kd) on the melanization response to *Plasmodium* ookinetes in susceptible and melanizing mosquito genetic backgrounds

1.2. Investigate the effect of *ClipA28* kd on mosquito resistance and endurance to fungal and bacterial infections

1.3. Determine whether CLIPA28 is required to activate hemolymph phenoloxidase in response to bacterial septic infections

Specific Aim 2: Molecular characterization of CLIPA28

2.1. Determine the temporal profile of CLIPA28 proteolytic cleavage in hemolymph in response to infections with bacteria and fungi.

2.2. Identify potential CLIPA28-interacting partners in hemolymph using co-immunoprecipitation followed by mass spectrometry.

2.3. Identify the gene network (specifically cSPs and cSPHs) that controls CLIPA28 cleavage and determine its hierarchical position within this network.

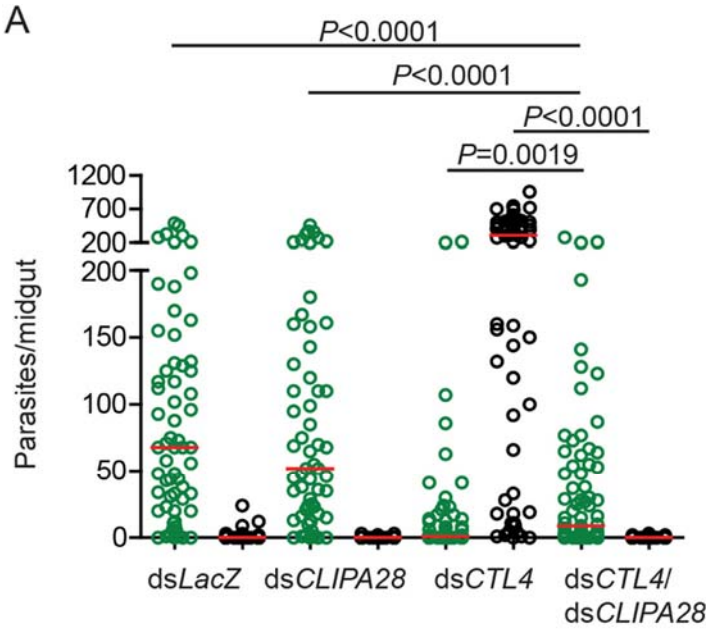
Chapter III

Results

A. CLIPA28 is a novel positive regulator of *P. berghei* ookinete melanization.

Functional studies in wildtype and refractory *A. gambiae* genetic backgrounds allowed the identification of several cSPHs that regulate the melanization of *P. berghei* ookinetes as they egress from midgut epithelial cells into the basal labyrinth. CLIPA8 [153] and SPCLIP1 [92] act as positive regulators while CLIPA2 [153, 154] and CLIPA14 [155] act as negative regulators. To identify novel cSPHs involved in the mosquito melanization response we searched for genes that are significantly co-regulated with *CLIPA2* in a developmental transcriptome data set of Expressed Sequence Tags [309]. Pearson correlation coefficient identified two CLIP clusters with similarity to CLIPA2 developmental expression greater than 0.85: CLIPC7 (PCC 0.867) and AGAP010730 (PCC 0.871). Interestingly, AGAP010730 is a cSPH that is just adjacent to CLIPA8 on chromosome 3L and shares the highest sequence homology with CLIPA8 and CLIPA9. As we were approaching completion of this work, the gene models of all *A. gambiae* serine proteases were improved and AGAP010730 was annotated as CLIPA28 [305]. Hence, we utilized the same gene name for simplicity. To determine whether CLIPA28 is involved in the mosquito melanization response, we asked first whether its knockdown (kd) modulates *P. berghei* ookinete melanization in susceptible and *CTL4* kd refractory mosquitoes [119]. To that purpose, mosquitoes injected with ds*CLIPA28* (double-stranded RNA specific to CLIPA28), ds*CTL4* and ds*CTL4*/ds*CLIPA28* mixture were infected with *P. berghei*, and

their midguts dissected at day 7 post-infection to score the numbers of live oocysts and melanized ookinetes. Mosquitoes treated with ds*LacZ* (dsRNA specific to the β-galactosidase gene) were used as control. As expected, *CTL4* silencing alone resulted in a significant decrease in number of live oocysts and a reciprocal increase in melanized ookinetes. Silencing *CLIPA28* in a wildtype background did not affect neither the number of live oocysts nor melanized ookinetes relative to control, but it abolished melanization in ds*CTL4* mosquitoes indicating that CLIPA28 is a key player in infection-induced melanization response (Figs 11 A&B).



B

Gene kd	n	Mo	P_{Mo}	%Mel	Lo	P_{Lo}
LacZ	63	0		0.9	68	
CLIPA28	59	0		0.1	52	
CTL4	63	323	<0.0001	95	1	<0.0001
CTL4/ CLIPA28	73	0		0.5	9	<0.0001

Figure 11. CLIPA28 is essential for the melanization of *P. berghei* ookinetes. (A) Scatter plots of live GFP-expressing *P. berghei* oocysts (green circles) and dead melanized ookinetes (black circles) scored in the midguts of the indicated mosquito genotypes seven days post-infection. Red lines indicate median parasite numbers. Statistical analysis for the parasite distribution was performed using the Mann-Whitney test and *P*-values less than 0.05 were considered significant. Data were pooled from four independent biological experiments. (B) Tabulated data of figure 1A showing the percentage of melanized ookinetes (% Mel) and the median numbers of melanized ookinetes (Mo) and live oocysts (Lo) per midgut. *P* values were calculated by the Mann-Whitney test.

B. CLIPA28 controls phenoloxidase activity following bacterial infections

The fact that CLIPA28 is required for parasite melanization, prompted us to test whether CLIPA28 is also required for PO activity triggered in response to systemic bacterial challenges. PO enzymatic activity was measured in the hemolymph of *dsLacZ* and *dsCLIPA28* adult female mosquitoes extracted 3 hours post infection with *S. aureus* bacteria ($O.D_{600nm}$=0.8). Extracted hemolymph was incubated with L-3,4-dihydroxiphenylalanine solution at room temperature, and absorbance (at 492 nm) was recorded at 10 minutes intervals for a total of 60 minutes. PO activity was strongly induced in infected *dsLacZ* mosquitoes as expected but significantly decreased in the hemolymph of *dsCLIPA28* mosquitoes (Fig. 12). These results suggest CLIPA28 is required for hemolymph PPO activation following bacterial infections.

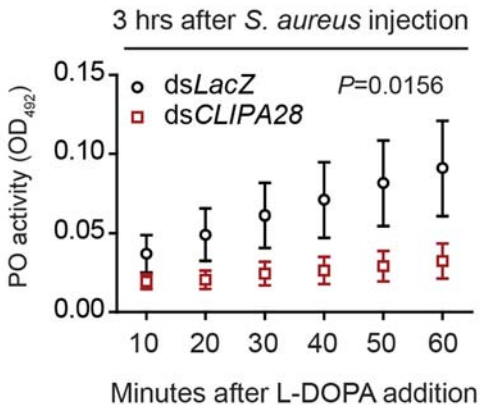


Figure 12. CLIPA28 mediates PO activation against systemic bacterial infections. Phenoloxidase (PO) enzymatic activity [detected as absorbance at OD_{492}, after conversion of L-3,4-dihydroxyphenylalanine (L-DOPA)] was measured in hemolymph extracted from ds*LacZ* (control) and ds*CLIPA28* mosquitoes at 3 hrs post-injection of live *S. aureus* (OD_{600}=0.8). The graph shows PO activity measured at 10 min intervals up to 60 min after addition of L-DOPA. Each point on the graph represents the mean calculated from seven independent biological experiments. Error bars represent standard error of the mean. In each experiment, a separate regression line was fitted for ds*CLIPA28* and ds*LacZ* control. The slopes were compared between both dsRNA treatments using the Wilcoxon signed rank test and differences were considered to be significant if $P < 0.05$.

C. CLIPA28 is required for defense against fungal but not bacterial infections

Clip protease cascades regulate multiple immune responses which include, in addition to melanization, coagulation [reviewed in [310]] and Toll pathway activation [reviewed in [132]. Since certain insect cSPs activate both the PPO and Toll pathways [311-313], investigating the potential anti-microbial functions of cSPHs remains legitimate as their regulatory roles within clip cascades might influence several immune responses not only melanization. Silencing *CLIPA28* did not influence neither mosquito tolerance nor resistance to *E. coli* (Fig 13A&D) or *S. aureus* (Fig 13B&E) infections, a result similar to that obtained previously with CLIPA8 [152]. On the other hand, *CLIPA28* kd rendered mosquitoes more susceptible to *B. bassiana* infections though to a lesser extent than that of *TEP1*, used herein as positive control (Fig 13C). Relative quantification of *B. bassiana* genomic DNA by real-time PCR revealed that fungal proliferation was significantly enhanced in *CLIPA28* and *TEP1* kd mosquitoes (Fig 13F), which confirms our previous observation that the melanization and complement responses contribute significantly to anti-fungal immunity [212].

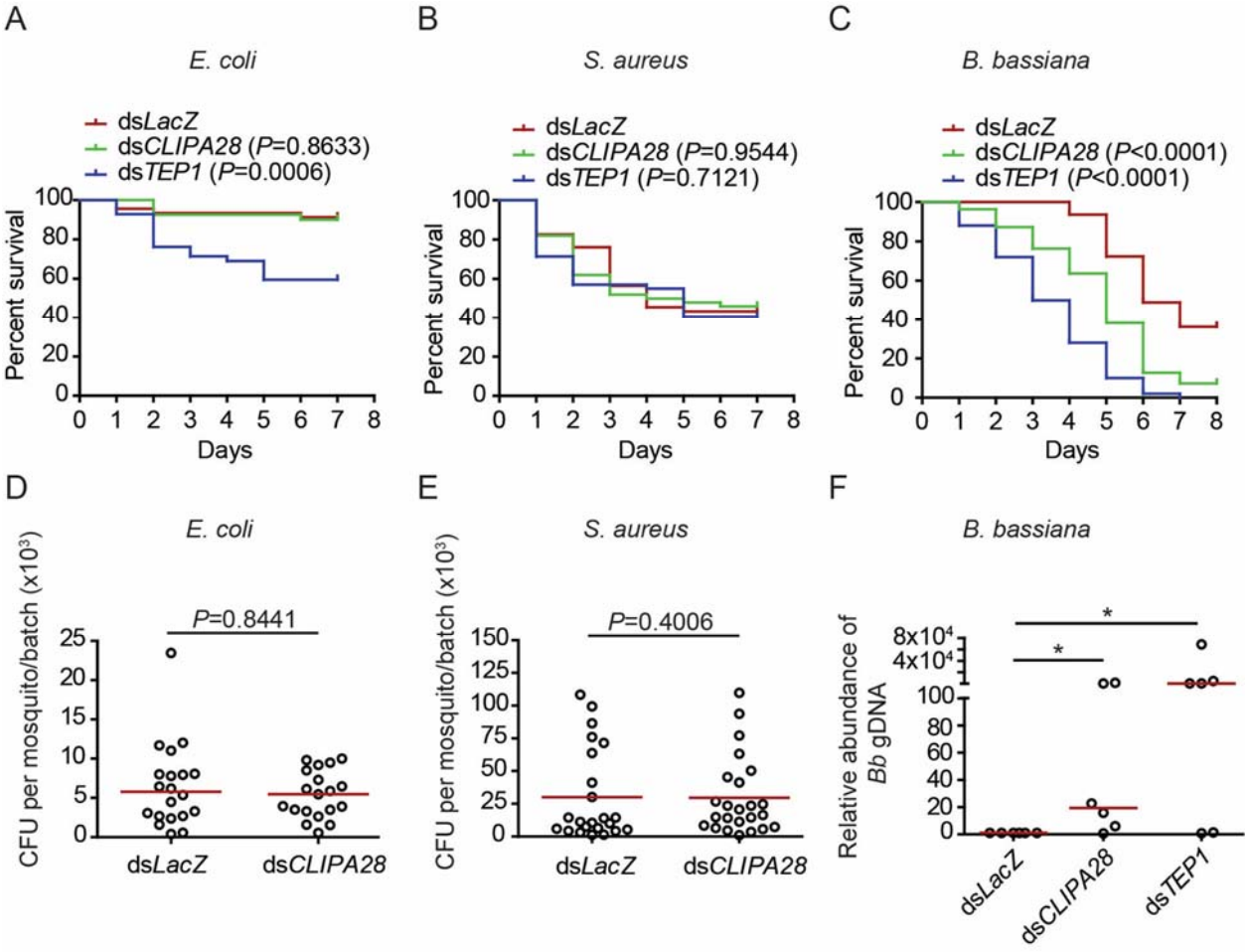


Fig 13. *CLIPA28* kd mosquitoes are susceptible to fungal but not bacterial infections. (A-C) Survival assays of the indicated mosquito genotypes following injection with (A) *E. coli* (OD_{600nm} = 0.4),d (B) *S. aureus* (OD_{600nm} = 0.4), and (C) after spraying with a *B. bassiana* suspension of $1x10^8$ spores/ml. One representative experiment is shown from three independent biological experiments. The Kaplan-Meier survival test was used to calculate the percent survival. Statistical significance of the observed differences was calculated using the Log-rank test. (D-E) Bacterial proliferation assays conducted on mosquitoes injected with (D) *E. coli* (OD_{600nm} = 0.8), and (E) *S. aureus* (OD_{600nm} = 0.4). Batches of 8 whole mosquitoes were grinded in LB medium at 48 hrs after infection, and colony forming units (CFU) were scored on LB plates supplemented with the appropriate antibiotic. Each point on the scatter plot represents the mean CFU per mosquito per batch. Statistical analysis was performed using the Mann-Whitney test. Medians (red lines) were considered significant if $P < 0.05$. Data shown are from four independent biological experiments. (F) Relative abundance of *B. bassiana* genomic DNA measured by real-time PCR in the indicated mosquito genotypes at day 4 after spore injection. Each point on the graph represents the mean relative abundance of *B. bassiana* genomic DNA in total DNA extracted from a batch of 8 mosquitoes. Data shown are from six independent biological experiments. Statistical analysis was performed using the Wilcoxon Signed Rank Test (Shapiro Wilcoxon test) and medians (red lines) were considered significant if $P < 0.05$ (marked by an asterisk).

D. A positive regulatory cSPH module exhibits a hierarchical mode of activation

1. *CLIPA28 is activated and cleaved upon bacterial and fungal infections*

Biochemical analysis in several insect species including *Manduca sexta* [148, 151, 314] and *Tenebrio molitor* [306] revealed that cSPHs require cleavage between the clip and protease domains to become functional, despite being non-catalytic. Immunoblotting of hemolymph extracted from naïve wild type mosquitoes (controls) revealed a single 48-KDa full-length CLIPA28 form (CLIPA28-F). However, septic infections with *E. coli* ($O.D_{600nm}$=0.8) and *S. aureus* ($O.D_{600nm}$=0.8) revealed an additional 37 KDa band corresponding to the cleaved non-catalytic protease-like domain of CLIPA28 (CLIPA28-C). Cleavage appeared as soon as 30 minutes and was still detected up to 12 hours post infection (Figs. 14A&B), suggesting that the processed form of CLIPA28 is stable and not rapidly cleared from the hemolymph. Similarly, injection of mosquitoes with fresh *B. basssiana fungal* spores (2000 spores/mosquito) also triggered CLIPA28 cleavage (Fig. 14C). The cleavage pattern was weak at early points but was much more pronounced at 24 hrs the time at which mycelial growth becomes apparent, triggering a more potent immune response as previously shown [212]. Hence, our data reveal that CLIPA28, like other mosquito and insect cSPHs, is quickly cleaved following septic infections.

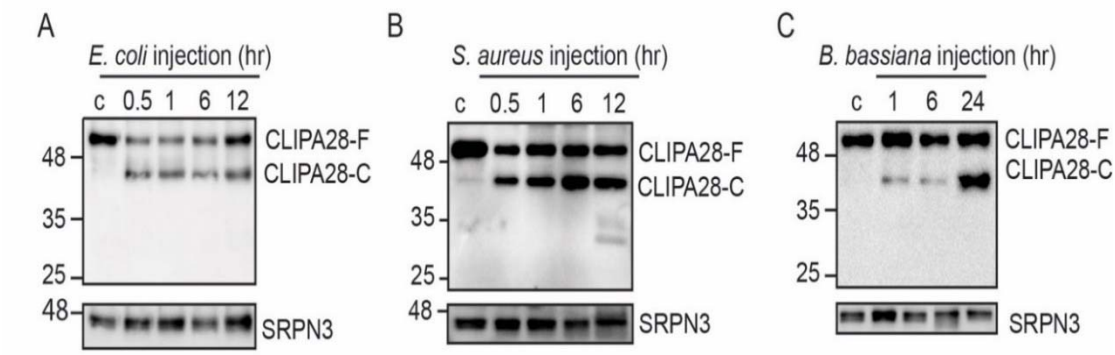


Figure 14. Cleavage of CLIPA28 by septic infections. Western blot analysis of mosquito hemolymph extracts displays full-length (CLIPA28-F) and cleaved (CLIPA28-C) forms of CLIPA28 at the indicated time points post injection with (A) *E. coli* ($O.D_{600nm}$=0.8), (B*) S. aureus* ($O.D_{600nm}$=0.8), and (C) *B. bassiana* (2000 conidia/mosquito). SRPN3 was chosen as a loading control. The figures are representative of three independent biological experiments.

2. *Mass-spectrometry (MS) identifies several immunity proteins that co-IP with CLIPA28*

We performed co-immunoprecipitation to identify immunity protein that interact with CLIPA28, specifically cSPs that might be involved in controlling its activation cleavage. Despite the fact that several *A. gambiae* cSPHs were shown to be cleaved following septic infections, the identities of the catalytic cSPs that control their cleavage remain unknown. CLIPA28 was co-IPed from the hemolymph extract of 900 *B. bassiana* naturally infected wild type mosquitoes and proteins were identified by LC/MS-MS at IGBMC (Strasbourg). Several immunity proteins appeared in the identified protein list including mainly: TEP-1; the cSPHs CLIPA2 and CLIPA14; the cSPs CLIPB4, CLIPB8, CLIPB13, CLIPB5 and CLIPD1; and the serine protease inhibitors SRPN2. Systematic functional genetic analysis was performed on candidate immunity genes to determine those that regulate CLIPA28 cleavage as detailed below.

3. *CLIPA28 acts downstream of a positive regulatory cSPH module controlled by TEP1*

TEP1, CLIPB4 and CLIPB8 identified among the proteins that co-IPed with CLIPA28 above are known to be involved in parasite melanization [153, 201]. To determine whether they control CLIPA28 cleavage, hemolymph was extracted from the indicated gene kd at 1 hr post *E. coli* or *S. aureus* infections and subjected to western blot analysis. We also included in this analysis *CLIPA8* and *SPCLIP1* kd mosquitoes despite that their corresponding proteins did not co-IP with CLIPA28; however, the key roles they play in melanization prompted their inclusion in the analysis. Whereas CLIPA28 cleavage was not affected neither by the single nor the double knockdowns of CLIPB4 and CLIPB8, it was strongly reduced after silencing CLIPA8, TEP1 or SPCLIP1 (Figs. 15A&B). The inhibition profiles were similar in *E. coli* and *S. aureus* infected mosquitoes suggesting that TEP1, CLIPA8, SPCLIP1 and CLIPA28 form a core module that is activated in response to both Gram-positive and Gram-negative bacterial infections. *CLIPA28* kd did not affect the cleavage of CLIPA8 after bacterial infections, whereas both SPCLIP1 and TEP1 dramatically reduced it (Figs. 15C&D). These data, altogether with our previous observation that SPCLIP1 is also required for CLIPA8 cleavage [92], indicate that the positive regulatory cSPHs are activated in a hierarchical manner with SPCLIP1 being upstream followed by CLIPA8 then CLIPA28(Fig. 15E).

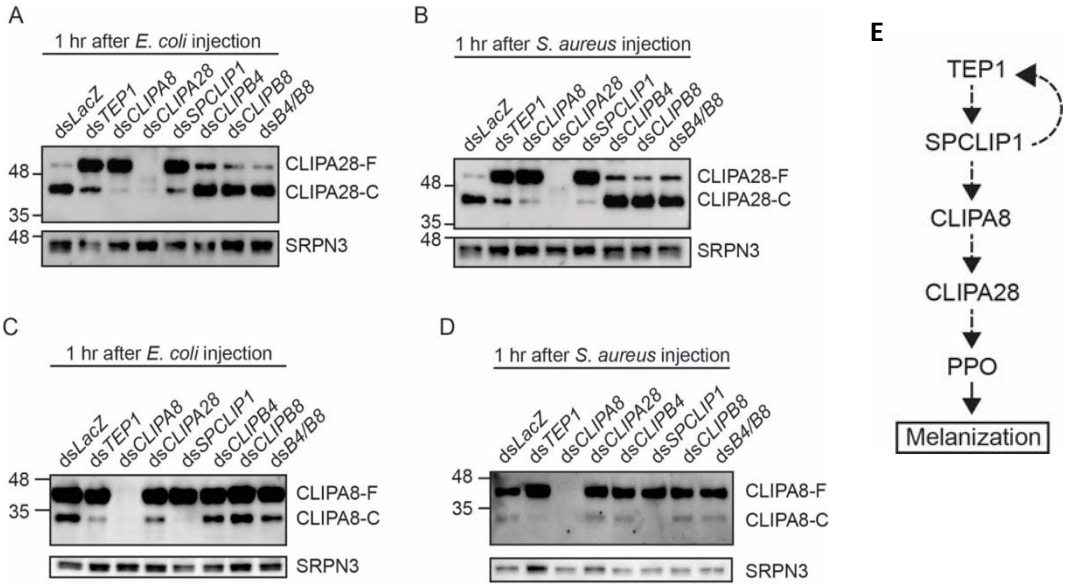


Figure 15. Molecular Regulation of CLIPA8 and CLIPA28 infection-induced cleavage. Western Blot analysis of hemolymph extracts showing the cleavage of (A-B) CLIPA28 and (C-D) CLIPA8 in the indicated gene knockdowns following challenge with *E. coli* (OD_{600}=0.8) or *S. aureus* (OD_{600}=0.8). SRPN3 was used as a loading control. Figures are representative of at least two independent biological experiments. (F) Schematic diagram of the hierarchical activation of the positive regulatory cSPHs. Dashed lines indicate that the enzymatic steps are not yet fully characterized.

Three other cSPs appeared in the list of CLIPA28 co-IPed proteins: CLIPD1, CLIPB13 and CLIPB5. CLIPB5 was not included in the analysis since previous functional genetic analysis did not reveal any role for this gene in parasite melanization [153]; whereas, silencing the first two did not affect CLIPA28 cleavage (Fig 16 and data not shown). We tested three other cSPs, CLIPB9 [143], CLIPB14 [232], CLIPB17 [153], which did not co-IP with CLIPA28 but they are known to play key roles in parasite melanization (CLIPB14 and CLIPB17) or tissue melanization induced in *SRPN2* kd (CLIPB9), yet none of them was required for CLIPA28 cleavage (Fig. 16). It is surprising

that none of the cSPs which have been shown to contribute significantly to *A. gambiae* melanization is involved in controlling the cleavage of CLIPA28 or CLIPA8. There are several plausible explanations to this observation. First, these functional cSPs might be acting downstream of the positive regulatory cSPH module constituted of SPCLIP1-CLIPA8-CLIPA28 (in order of activation). Second, a specific cSPH may be efficiently cleaved by more than one cSP. In the former case, a rigorous functional genetic screen of the remaining cSPs of the CLIPB, C and D subfamilies (55 in total [305]) should identify those involved in cSPHs activation cleavage. The latter scenario is more tricky and will require either performing double and triple kds of candidate cSPs or the use of *in vitro* reconstitution assays in which a set of candidate recombinant cSPs with engineered activation cleavage sites are used to test their ability to cleave specific recombinant cSPHs.

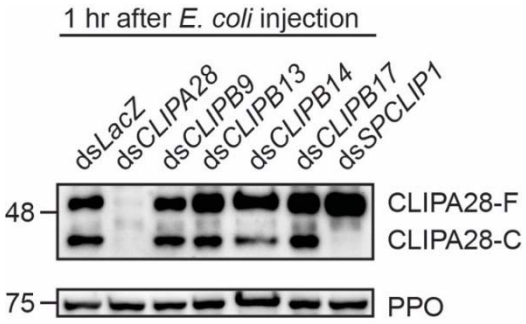


Figure 16. CLIPA28 is not cleaved by CLIPB candidates with prominent roles in melanization. Western Blot analysis of hemolymph extracted from mosquitoes silenced for the indicated genes at 1 hr post injections with *E. coli* (OD600=0.8). PPO6 was used as a loading control. Images are representative of at least two independent biological experiments.

E. The positive regulatory cSPH module and CLIPA2 regulate CLIPA14 cleavage

CLIPA14 is a negative regulator of melanization in *A. gambiae*; *CLIPA14* kd triggered a potent melanotic response against *P. berghei* ookinetes and an enhanced hemolymph PO activity following bacterial systemic infections [155]. CLIPA14 is also cleaved following septic infections in a TEP1 and SPCLIP1-dependent manner [155]. Here, we show that CLIPA14 cleavage is also dependent on CLIPA8 and CLIPA28 (Fig. 17A), indicating that CLIPA14 is downstream of the positive regulatory cSPH module composed of SPCLIP1, CLIPA8 and CLIPA28. In contrast, CLIPA14 cleavage is negatively regulated by CLIPA2; *CLIPA2* kd enhanced CLIPA14 cleavage by approximately 2.7 folds (Fig. 17A&B). This enhanced cleavage was abolished when CLIPA2 was co-silenced with either TEP1, SPCLIP1, CLIPA8 or CLIPA28 (Fig. 17A), further supporting the most downstream position of CLIPA14 among cSPHs involved in melanization. Hence, it seems that CLIPA14 is subject to multiple regulation by positive and negative regulatory cSPHs possibly reflecting the key role it plays in the melanization response, as manifested by its RNAi phenotype [155]. The placement of CLIPA14, a negative regulator, under the control of positive regulatory cSPHs is possibly a mean to fine tune the intensity of the melanization response in a way to minimize fitness cost on the host. Indeed, a deregulated melanization response due to silencing specific serpins exhibited significant fitness cost in *A. gambiae* [160] and *Drosophila* [315, 316] manifested by spontaneous tissue melanization and compromised survival.

Moreover, *CLIPA2* and *CLIPA14* single and double kds trigger an exaggerated hemolymph PO activity after septic infections [154, 155]. Here, we tested whether this

enhanced activity is associated with increased activation cleavage of the positive regulators CLIPA28 and CLIPA8. Co-silencing *CLIPA2* and *CLIPA14* triggered a significant (P=0.001) 4-fold increase in CLIPA28 cleavage, whereas the single kds did not have a significant effect (Fig.17C). In contrast, neither the single nor the double kds of *CLIPA2* and *CLIPA14* had a significant effect on CLIPA8 cleavage (Figure 17D). Our data suggest that negative feedback likely exists within the cSPH network at the level of CLIPA28 activation. Since CLIPA28 is, so far, the most downstream among the positive regulatory cSPHs, it is the likely candidate that interacts with PPO facilitating its proper cleavage by cSPs, a function well ascribed to insect cSPHs [147, 148, 151, 306]. In that case, a negative feedback loop at the level of CLIPA28 would make sense as it will directly impact the intensity of PPO activation during infection. Future *in vitro* reconstitution assays using recombinant CLIPA28 will be required to address its potential interaction with PPO and PPO-activating cSPs.

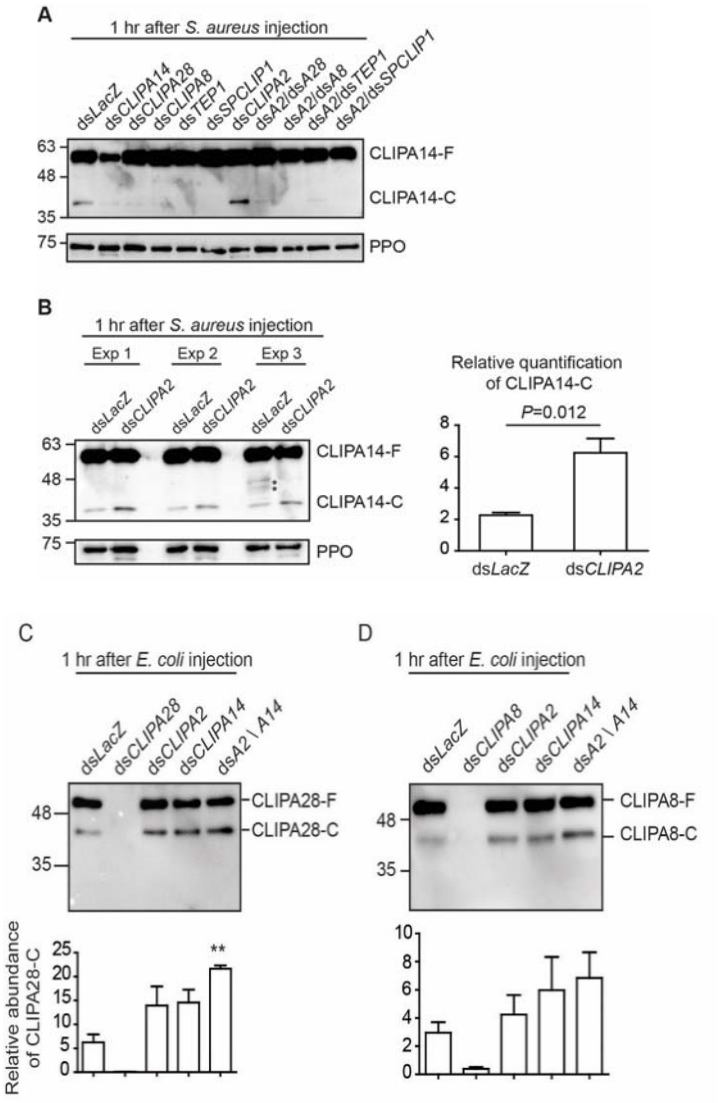


Figure 17: CLIPA14 is downstream in the cSPH module and exhibits negative feedback on CLIPA28 activation cleavage. (A) A representative western blot showing CLIPA14 cleavage in the hemolymph of the indicated gene kd mosquitoes at 1 hr after *S. aureus* (OD_{600}=0.8) injection. αPPO6 antibody was used to confirm equal loading. The image is representative of two independent biological experiments. (B) Quantification of CLIPA14-C in ds*CLIPA2* as compared to ds*LacZ* control mosquitoes from three independent western blots using Image Lab 5.0. Statistical analysis was performed using the Student's unpaired t-test and differences were considered to be significant if $P < 0.05$. (C-D) Representative western blots showing (C) CLIPA28 and (D) CLIPA8 cleavage in the hemolymph of the indicated gene kd mosquitoes at 1 hr after *E. coli* (OD_{600}=0.8) injection. Protein quantification was performed using the Bradford protein assay and equal amounts of hemolymph proteins were loaded per lane. Bands corresponding to the cleaved forms were quantified from three independent western blots using Image Lab 5.0 and data are presented in the bar graph below.

F. SRPN2 is a master negative regulator of mosquito cSPHs

SRPN2 is a key negative regulator of the mosquito melanization response; its knockdown elicits a strong melanotic response against *Plasmodium* ookinetes, and substantial tissue melanization in naïve mosquitoes with the concomitant depletion of PPO from the hemolymph, especially at later time points [160]. To determine whether SRPN2 regulates the cleavage of mosquito cSPHs, we silenced SRPN2 in female *A. gambiae* mosquitoes and monitored the cleavage profiles of CLIPA8, CLIPA28 and CLIPA14 under septic and naïve conditions. For septic infections, mosquitoes were challenged with *E. coli* at day 4 after treatment with ds*SRPN2* or ds*LacZ* (control). At 1 hr after *E. coli* challenge, *SRPN2* kd had a modest effect on the cleavage of CLIPA28 and CLIPA14 (Fig 18A&C), as manifested by a slight reduction in their full-length forms. However, *SRPN2* kd strongly increased the cleavage of CLIPA8, as shown from the enrichment of the cleaved CLIPA8-C form (Fig 18B). Interestingly, the full-length forms of these cSPHs were dramatically reduced in naïve mosquitoes at day seven after *SRPN2* kd (Figs 18A-18C) relative to control, suggesting an enhanced consumption by the exaggerated melanization response known to be elicited in this mosquito genotype, especially at late time points (beyond day 4) [160]. Despite this enhanced cSPH activation in naïve *SRPN2* kd mosquitoes, we did not observe a substantial enrichment of their cleaved forms, probably because of their sequestration in the melanotic bodies that develop in various tissues of *SRPN2* kd mosquitoes [160]. This enhanced consumption of cSPHs parallels that of PPO (Fig 18A-18C), indicating that SRPN2 is critical to keep these cSPHs inactive in naïve mosquitoes to prevent generalized PPO activation. These results also suggest that SRPN2 is a master regulator of the cSPs involved in the processing of CLIPA28, CLIPA8 and CLIPA14, and

hence acts upstream of the mosquito cSPH module that controls melanization (Fig 18D). However, so far, only CLIPB9 was shown to form inhibitory complexes with SRPN2 that are physiologically relevant [143]. Yet, *CLIPB9* kd did not affect the cleavage of CLIPA28 (Fig 16) suggesting that it might be either acting in a different branch of the melanization response or significant redundancy exists among cSPs with respect to cSPH cleavage.

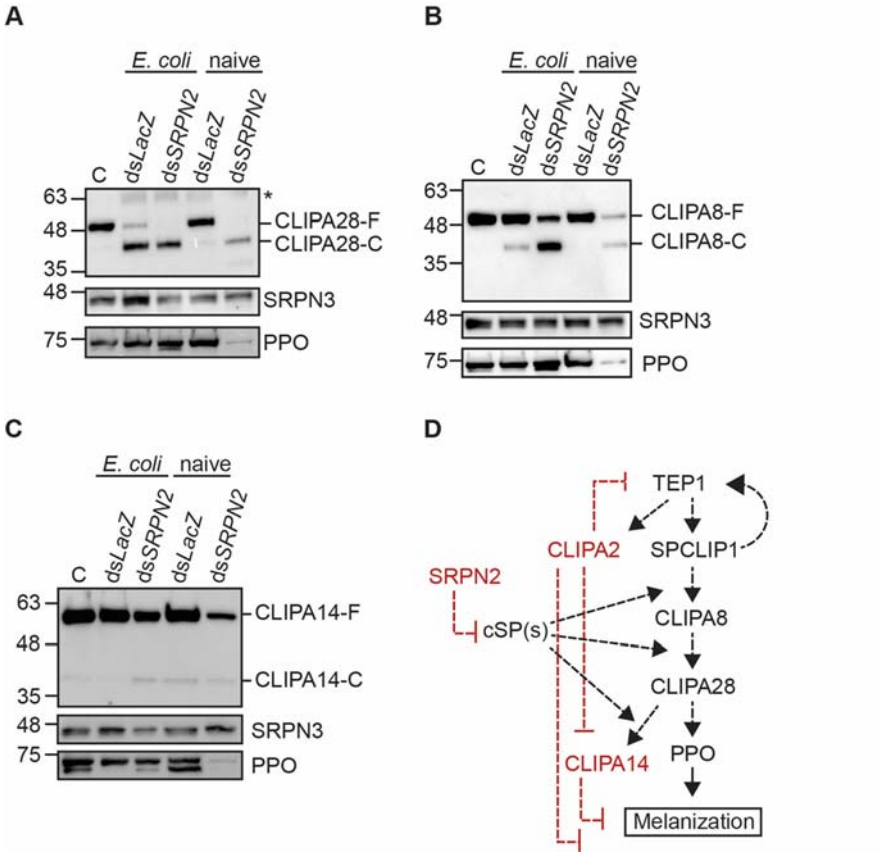


Fig 18. SRPN2 is a negative regulator of cSPH activation cleavage. (A-C) Western blots showing full-length and cleaved forms of CLIPA28, CLIPA8 and CLIPA14 after 1 hr of *E. coli* injection in mosquitoes treated for 4 days with *dsLacZ* or *dsSRPN2*, and in naïve mosquitoes at day 7 after treatment with *dsLacZ* or *dsSRPN2*. In each experiment, equal amounts of hemolymph proteins were loaded per lane. Membranes were also probed with αPPO6 to monitor PPO dynamics in *SRPN2* kd mosquitoes and αSRPN3 for loading control. (D) Schematic diagram showing the position of SRPN2 as a negative regulator of the yet unidentified cSP(s) involved in the proteolytic processing of CLIPA8, CLIPA28 and CLIPA14. Factors with negative regulatory roles in melanization are shown in red. Dashed lines indicate that the enzymatic steps are not yet fully characterized. *C*, naïve wildtype-mosquitoes. Asterisk (*) indicates non-specific bands.

Chapter IV

General Discussion

CLIP cascades play an integral part of mosquito immune responses, specifically the activation of PPO leading to melanization and Toll pathway activation leading to the synthesis of antimicrobial peptides. In the melanization response, PPO activation is regulated by complex interactions between PRRs, ModSP, cSPs, cSPHs, SRPNs as well as other classes of immune proteins that remain largely uncharacterized. Added to this complexity is the fact that all these classes of proteins belong to large gene families [96, 141]. There are several key questions that need to be addressed to enable us to draw a comprehensive scheme of the melanization response. First and foremost, what generates specificity in CLIP cascades and is there a role for the clip-domain in that process? What is the exact role of cSPHs in these cascades and at what levels (PPO, cSPs or PRRs) they mediate their regulatory function? How is PPO localized to microbial surfaces? Are all the interactions required to activate PPO taking place on microbial surfaces or some occur in the hemolymph? This thesis project focused on highlighting the role and functional organization of cSPHs in the melanization response. Studies in other model insects determined that while CLIP-SPs interact in a hierarchical manner to activate terminal PAPs, CLIP-SPHs function as cofactors for the proper cleavage and activation of PPO by a terminal cSP known as PAP (prophenoloxidase activation proteinase) [145-151] .Contrary to this classical view, RNAi-based genetic analysis in *A. gambiae* identified two cSPHs, CLIPA2 and SPCLIP1 [92, 154], as important modulators of the complement-like protein

TEP1, which according to several studies acts, so far, most upstream in the melanization response[92, 154, 231]. This argues against a role of cSPHs restricted only to the level of PPO activation. Furthermore, the identification of several other cSPHs with positive (CLIPA28,CLIPA8[152], SPCLIP1[92]) or negative (CLIPA14[231]) regulatory roles on PPO activation inform multiple roles in that process, especially that their RNAi phenotypes indicate unique non-redundant roles in *Plasmodium melanization* .

In the context of this thesis project, we identified CLIPA28 as a novel positive regulator of *A. gambiae* melanization response to *Plasmodium* ookinete stage parasites. Similar to CLIPA8 [153] and SPCLIP1 [92], *CLIPA28* kd completely abolished ookinete encapsulation in CTL4 kd melanizing mosquitoes rendering them susceptible to *P. berghei* development. Like SPCLIP1 [92] and CLIPA8 [152], CLIPA28 is also required for PPO activation following bacterial systemic infections. However, only CLIPA8, so far, was shown to be essential for PPO activation in response to fungal infection [212]. Future studies should address the roles of SPCLIP1 and CLIPA28 in this context to determine whether these three cSPHs form a core module in the CLIP network regulating the melanization response to different classes of microorganisms. Mosquito survival and bacterial proliferation assays revealed that CLIPA28 is not required for mosquito tolerance nor resistance to bacterial infections whether by Gram-negative or Gram-positive bacteria. In contrast, *CLIPA28* kd mosquitoes showed significant susceptibility to *B. bassiana* infections, which supports our previous observations that melanization is important for anti-fungal immunity[212] . However, these results do not necessarily exclude a role for melanization in anti-bacterial defense, since RNAi-mediated gene silencing is never

complete and the residual PO activity that remains in the hemolymph may still be protective against bacteria. On the other hand, fungi, which grow elaborate mycelia in mosquito tissues, may require a more potent PO activity to slow or block their growth. This might explain the different susceptibilities observed to bacterial versus fungal infections in mosquito genotypes where key positive regulatory cSPHs have been knocked down. A CRISPR/cas9 gene knockout of CLIPA28 and other positive regulatory cSPHs, that will completely deplete these proteins from the hemolymph, will be required to address their true impact on PO activity and anti-bacterial immunity. In *Drosophila*, a gene deletion approach to eliminate PPO genes revealed indeed a role for melanization in defense against Gram-positive bacteria [317]. Alternatively, we cannot exclude that the susceptibility to fungal infections in *CLIPA28* kd mosquitoes may also be due to the regulation of cSP cascades acting upstream of the mosquito Toll/Rel 1 pathway , especially that the role of Rel 1 pathway in anti-fungal immunity has been established in *A. gambiae* [318], *Ae. Aegypti* [319] and more recently *A. stephensi* [320]. However, at present it is difficult to address this point because our knowledge of Toll signaling pathway in *A. gambiae* is too fragmentary and specific gene expression signature readouts for pathway activation remain absent. It remains unclear whether CLIPA28 function requires its localization to microbial surfaces. Microscopic analysis to study the association of CLIPA28 and other cSPHs with microbial surfaces (fungi or bacteria) and the genes that regulate their recruitment will provide important insight into the spatial activation pattern of factors involved in the melanization response.

Biochemical analysis in several insect species including *Manduca sexta* [148, 151, 314] and *Tenebrio molitor* [306] revealed that cSPHs require cleavage between the clip and protease domain to become functional, despite being non-catalytic. Here, we show that CLIPA28 is also rapidly cleaved following septic infections with *E. coli* (Figure 14 A), *S. aureus* (Figure 14 B) and *B. bassiana* (Figure 14 C). In the latter, the pronounced cleavage observed at 24 h coincides with the time at which mycelial growth becomes prominent as previously described [212]. It was noted that at later time points after *S. aureus* and *B. bassiana* infections, CLIPA28 cleavage coincided with a substantial reduction in hemolymph PPO levels (data not shown), suggesting that these two classes of microbes trigger a more potent melanization response in the host than *E. coli*. RNAi-mediated gene silencing combined with western blot analysis allowed the hierarchical organization of the positive regulatory cSPHs, with SPCLIP1 being most upstream followed by CLIPA28 then CLIPA8. TEP1 is upstream of this module while the negative regulator CLIPA14 is downstream of it (Figure 19). This latter observation suggests that CLIPA14 may regulate the activation of the positive cSPHs through a negative feedback loop. CLIPA2 is another negative regulator that seems to act at the level of TEP 1 regulation. CLIPA2 kd significantly enhanced CLIPA14 cleavage in a TEP1, SPCLIP1, CLIPA8, and CLIPA28 mediated manner (Figure 17 A&B) suggesting that CLIPA2 is acting upstream of the positive cSPHs. Unexpectedly, CLIPA2 kd didn't have a significant effect on the activation cleavage of CLIPA8 nor CLIPA28. Only when both CLIPA2 and CLIPA14 were silenced, an enhanced activation of CLIPA28 was observed (Figure 17 C& D). This is in accordance with previous findings in the lab that CLIPA2 and CLIPA14 act in concert to control the melanization response [231] , and further suggest the existence of negative feedback loop

between CLIPA14 and CLIPA28. The position of CLIPA2 in this network remains unclear. It may be a negative regulator of the cSP that controls CLIPA14 cleavage, or it might indirectly control CLIPA14 cleavage by negatively regulating one or more of the cSPs that control the cleavage of the positive cSPHs. Raising antibodies that would detect the cleaved form of CLIPA2 may help address its position with respect to other cSPHs in the network. Another important question that remains to be answered is how the negative regulatory role of certain cSPHs, like CLIPA2 and CLIPA14, compares to that of serpins which are known to be key negative regulators of serine protease cascades? The fact that the knockdown of CLIPA2 or CLIPA14 triggers an exaggerated melanization response to microbial infections, clearly suggests that the role of serpins does not suffice to fine tune the intensity of that response. Serpins are suicide inhibitors of cSPs and their mode of action is well described[157] . Negative regulatory cSPHs have been, so far, only characterized in *A. gambiae*, hence there are no information from other insect species that could provide some insight into their functions. We speculate that the infection-induced cleavage of these cSPHs trigger their interaction with key cSP zymogens blocking their activation by upstream cSPs. One can think of these cSPHs as traps that reduce the amount of free cSP zymogens that can take part in the PPO activation cascades. Hence, their ole may be to fine tune the intensity of the response. The fact that CLIPA2 of CLIPA14 kd naïve (i.e. not infected) mosquitoes do not exhibit any signs of tissue melanization or enhanced hemolymph PO activity, further supports their role as fine tuners of the melanization response during infection, rather than gate keepers of clip cascades. This latter role is more ascribed to serpins whose kd in naïve mosquitoes or flies trigger extensive tissue melanization and PPO depletion [160, 315, 321].This fine tuning role will probably protect

from an exaggerated response that would lead to significant fitness costs, as has been shown for bacteria-infected CLIPA2 kd mosquitoes [154]. Future, *in vitro* reconstitution experiments using recombinant cSPs and cSPHs should help shed light on the mechanism of action of the latter.

SRPN2 is a global inhibitor of melanization in *A. gambiae*, who's kd in naïve mosquitoes elicits substantial tissue melanization. Hence it was integral to investigate whether SRPN2 regulates the activation of the cSPH module. Indeed, silencing of *SRPN2* promoted the cleavage of CLIPA8, CLIPA28, and CLIPA14 under both septic and naïve conditions, suggesting that it is a strong inhibitor of the cSP(s) underlying the activation of these cSPHs. Given the large number of *A. gambiae* cSPs, and the significant degree of functional redundancy that may exist between them with respect to cSPH cleavage, renders their identification challenging. The single and triple kds of cSPs Co-IPed with CLIPA28 (CLIPs B4, B8, and B13) or of those few with known functions in melanization (CLIPs B9, B10, B14, and B17) didn't abolish infection induced cleavage of CLIPA8 nor CLIPA28 (Figures 15&16 & data not shown). This was surprising, especially that CLIPB8 and CLIPB9 are targets for SRPN2. One possibility is that our cSPs of interest are not among the tested candidates. Hence a systematic screen of all other annotated cSP would be required to identify them. Alternatively, we may utilize a proteomic approach to identify all the cSPs that are either cleaved or consumed in response to septic infections. Newly identified candidates will then be subjected to genetic analysis as described above to test their involvement in the activation of candidate cSPHs. Another explanation of our results is that the cSPs we screened are acting downstream of the cSPH module in the network,

and this is currently under investigation, using antibodies available in hand for CLIPB8 and CLIPB10.

In conclusion, we have unprecedentedly characterized a regulatory network composed of several cSPHs, TEP1 and SRPN2 that play a central role in regulating the mosquito melanization response, providing novel insight into the functional complexity of CLIP cascades (Figure 19). The hierarchical activation of cSPHs informs a multilayered control over the cSP cascade involved in the infection-induced melanization response. cSPs and cSPHs are likely to interact at different levels and their molecular interactions are at the core of the protease network that controls melanization and possibly other immune responses including Toll pathway activation and coagulation. The substantial number of mosquito cSPHs involved in melanization and their hierarchical cleavage profile raise the hypothesis that, in addition to their classical role in directly controlling proper PPO cleavage, cSPHs might also confer specificity and order to the cleavage of cSPs, possibly as a mean to counter a random flow of information within these protease networks due to partial functional redundancies among the cSPs themselves. Evidence for such redundancy is provided by the RNAi phenotypes of certain cSPs that lead to partial reversion of ookinete melanization in refractory *A. gambiae* mosquitoes [153]. An additional layer of complexity in studying these protease networks is that their components including cSPHs, cSPs and SRPNs, and their putative target PPOs and TEPs belong to large gene families [96]. Nevertheless, analysis of the cleavage profiles and biochemical functions of cSPs and cSPHs are expected to reveal the level of redundancy and branching in the protease

network controlling melanization and provide a more comprehensive understanding of these networks that would allow fine manipulation of mosquito immunity.

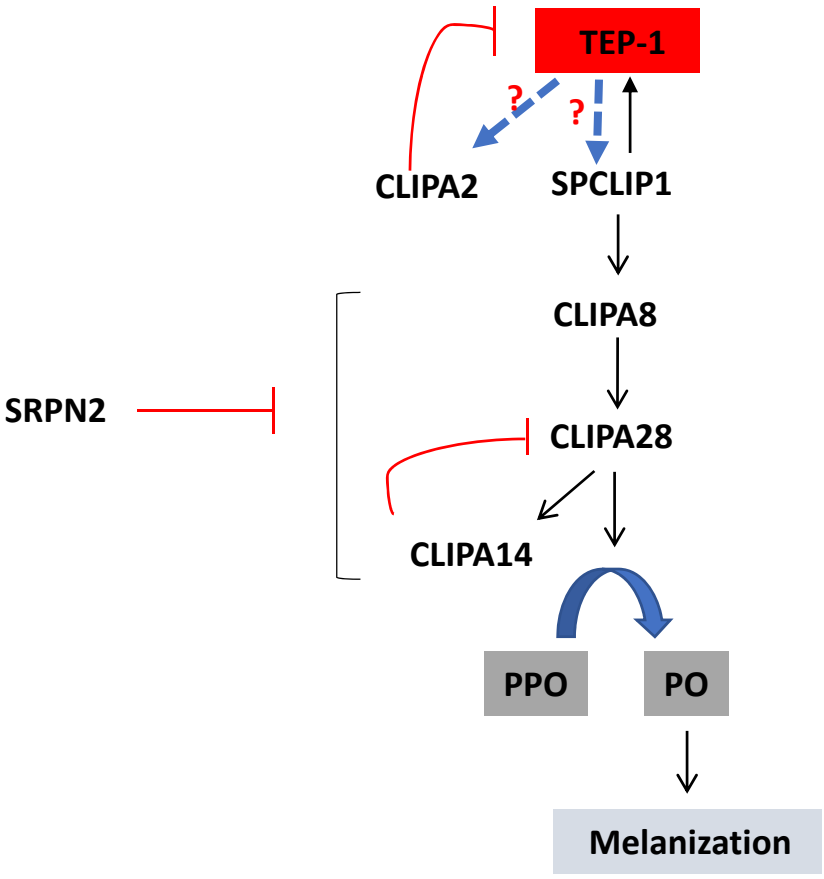


Figure 19. Schematic presentation of the hierarchical organization of the cSPH module regulating melanization in *A. gambiae*. The diagram shows the organization of the positive (SPCLIP1, CLIPA8 and CLIPA28) and negative (CLIPA2 and CLIPA14) regulatory cSPH acting downstream of TEP1 to regulate PPO activation. The positions of SPLCIP1 and CLIPA2 are still putative as their cleavage profiles have not be characterized yet. Arrows heads depict activation, while red lines indicate negative regulation. Dashed lines represent putative interactions.

Chapter V

Materials and Methods

A. Ethics Statement

This study was carried according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, USA). Animal protocol was approved by the Institutional Animal Care and Use committee IACUC of the American University of Beirut (permit number 17-10-451). The IACUC functions in compliance with the Public Health Service Policy on the Humane Care and Use of Laboratory Animals (USA), and adopts the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

B. *Anopheles gambiae* rearing

All experiments were performed with adult female *Anopheles gambiae* G3 strain mosquitoes. Mosquitoes were maintained at 27 (±1) °C and 75 (±5) % humidity with 12-hour day-night cycle. Larvae were reared in 752 cm^2 plastic pans at a density of approximately 150 larvae per pan and given tropical fish food. Freshly emerged adult mosquitoes were collected from larval pans using a vacuum collector, maintained on 10% sucrose and given BALB/c mice blood (mice were anesthetized with ketamine) for egg laying.

C. *P. berghei* infection Assay

Mosquito infections with *P. berghei* (GFP-expressing strain PbGFPCON[322]) were performed by allowing mosquitoes to feed on 5-6 week old anesthetized BALB/c mice infected with *P. berghei* at a parasitemia of 4-6 % for approximately 20 min at 20℃. Percentage of parasitemia in mice was determined by performing a blood smear stained with 1% Giemsa solution. Mosquitoes were then maintained on 10 % sucrose solution at 20℃. Mosquito midguts were dissected 7–8 days post-infection, fixed in 4% paraformaldehyde for 50 min, washed 3 times in PBS, and mounted in ProLong Gold antifade reagent (Life Technologies). The numbers of live GFP-positive oocysts (Green Floursecent) and melanized ookinetes (surrounded by dark brown capsules) per midgut were scored using a fluorescence microscope.Data were collected from at least three independent biological experiments and statistical significance was calculated using the Mann-Whitney test. Means were considered significantly different if $P<0.05$.

D. Gene Silencing by RNA interference

Double stranded RNA (dsRNA) synthesis was performed using the T7 RiboMax Express Large Scale RNA production system (Promega) according to the manufacturer's instructions and dsRNAs were purified using phenol:chloroform, precipitated using isopropanol and resuspended in nuclease free water at a concentration of 3.5 μg/μl. Primers used for dsRNA production are listed in Table 1. *In vivo* gene silencing was performed by microinjecting mosquitoes with a 69nl of a 3.5μg/μl solution of gene-specific dsRNA for single gene kd, or 138 nl of a solution containing a mixture of two dsRNAs (each at a

concentration of 3.5µg/µl) for double gene kd. Efficiency of gene silencing was measured by quantitative Real-time PCR (qRT-PCR) or western blot analysis whenever antibodies are available.

Table1: Primers used for dsRNA production

Gene	Primers used for dsRNA synthesis (T7 promoter sequence underlined)	Reference
LacZ	For: 5'-TAATACGACTCACTATAGGGAGAATCCGACGGGTTGTTACT-3' Rev: 5'-TAATACGACTCACTATAGGGCACCACGCTCATCGATAATTT-3'	[323]
TEP1 (AGAP010815)	For: 5'-TAATACGACTCACTATAGGGTTTGTGGGCCTTAAAGCGCTG-3' Rev: 5'- TAATACGACTCACTATAGGGACCACGTAACCGCTCGGTAAG-3'	[307]
CTL4 (AGAP005335)	For: 5'- TAATACGACTCACTATAGGGGTTAGCAGCATTGGGATTACCCT-3' Rev: 5'-TAATACGACTCACTATAGGGGAAGTCGCAACCCAGCTCATTGT-3'	
SPCLIP1 (AGAP028725)	For: 5'- TAATACGACTCACTATAGGGGTCACCGAACACGGCCAAC-3' Rev:5'-TAATACGACTCACTATAGGGATCGAAGCTGATCGGATCGGG-3'	[92]
CLIPA2 (AGAP011790)	For:5'-TAATACGACTCACTATAGGGATCCTAACAACGGCACACTGTGTGA-3' Rev:5'-TAATACGACTCACTATAGGGTCCTGATCGCCATGATTGGTGGTGCT-3'	[154]
CLIPA14 (AGAP011788)	For: 5'- TAATACGACTCACTATAGGGCGGCATCATCGACATCCGTGTC-3' Rev: 5'-TAATACGACTCACTATAGGGGTTGCTGTCGGCGACACGCTCCT-3'	[231]
CLIPA8 (AGAP010731)	For: 5'- TAATACGACTCACTATAGGGAACAACGAACCCGTAGAATATG-3' Rev: 5'- TAATACGACTCACTATAGGGGGTTAGCGCCTCGATACC-3'	[153]
CLIPA28 (AGAP010730)	For: 5'-TAATACGACTCACTATAGGGAGACCACCAAGGAACCGTTCCCGCAGCAA-3' Rev: 5'-TAATACGACTCACTATAGGGAGACCGCAACCGATGCCCCACGATACGAT-3'	
CLIPB4 (AGAP003250)	For: 5'- TAATACGACTCACTATAGGGAGTAGCGGTCGTGCATCAGA-3' Rev: 5'- TAATACGACTCACTATAGGGTGGCCTGCTAGAGCCAGCGT-3'	
CLIPB8 (AGAP003057)	For: 5'- TAATACGACTCACTATAGGGGTCATACCGCACCCGGAGTA-3' Rev: 5'- TAATACGACTCACTATAGGGTTCCCGTTCGACGTACGGCA-3'	
SRPN2 (AGAP006911)	For: 5'- TAATACGACTCACTATAGGGCTGGTCAATGTGATCTACTT-3' Rev: 5'- TAATACGACTCACTATAGGGATTGTTCCGAGGGTTTCAT-3'	
CLIPB9 (AGAP029769)	For: 5'- TAATACGACTCACTATAGGGAATGCACGACACCGACGAGGT-3' Rev: 5' TAATACGACTCACTATAGGGGTTTGCCCTCCTTGCGCTCA-3'	

CLIPB10 (AGAP029770)	For: 5'- TAATACGACTCACTATAGGGGAGCGTAAGGGATGAGTTCT-3' Rev: 5'- TAATACGACTCACTATAGGGCAGCACGTACCGTCCGTTGA-3'	
CLIPB13 (AGAP004855)	For: 5'- TAATACGACTCACTATAGGGTACTACCGTCGCTCCGAGTA-3' Rev: 5'- TAATACGACTCACTATAGGGTCGATGTCCGGACACAATGT-3'	
CLIPB14 (AGAP010833)	For: 5'- TAATACGACTCACTATAGGGGACTGCAAGCAGGTCAAAGGC-3' Rev: 5'- TAATACGACTCACTATAGGGTCCACGGAACATCTCCCGCT-3'	
CLIPB17 (AGAP001648)	For: 5'- TAATACGACTCACTATAGGGAGCGTGGGGAATTCCCGTGGA-3' Rev: 5'- TAATACGACTCACTATAGGGGGATCGTCCATCAGCAGCGA-3'	

E. RNA Extractions, Reverse Transcription for cDNA synthesis and qRT-PCR

Concerning qRT-PCR, in total 15 mosquitoes per experimental sample were grinded in 500μL of TRIzol reagent and nucleic acids were extracted with chloroform and treated with DNase I to remove any DNA products. Total RNA was further extracted with phenol:chloroform and precipitated with 0.7 volumes of isopropanol. RNA pellets were resuspended with nuclease free water. For cDNA synthesis, 1 μg of RNA was used in reverse using iScriptTM cDNA Synthesis kit (BioRad) according to the manufacturer's protocol. The synthesized cDNA were used as templates for qRT-PCR. SYBR® Green Quantitative RT-PCR Kit was used to perform the real-time PCR reactions. The prepared cDNA samples were diluted 20x, and were used for the real-time PCR reaction .Components for the real-time PCR were loaded into a 96-well plate, sealed properly, centrifuged at 1000g, 4°C, for 3mins, and placed in a CFx96 Systems light cycler machine (initial denaturation step: 95°C for 3mins followed by a denaturation step: 95°C for 10s repeated 39 cycles, then by an annealing step: 60°C for 30s).Real-Time Primers used to score the efficiency of gene silencing are listed in Table 2 . The ribosomal S7 gene was used as an endogenous control gene to normalize the relative mRNA expression level of

each tested gene. Relative gene expression values were calculated using the comparative C_T method after checking for the efficiency of target amplification.

Table2: Primers used in qRT-PCR

Gene	Primers used for qRT-PCR
CLIPB4	For: 5'- AGTGCTGGCGCTAGAGCTG -3' Rev: 5'- ACGAAACAGGACACTTGCC -3'
CLIPB9	For: 5'- GTCTGCCACTGACCGACTTC -3' Rev: 5'- TTTTGCTTAACCGCGCTTCG -3'
CLIPB13	For: 5'- ACCAATTTGCTTGCCGGTTAA -3' Rev: 5'- GCAGCAGGTCTGACAACGAT -3'
CLIPB14	For: 5'- GTTAGCAAGCGGTTTGTGCT -3' Rev:5'- TGAAATTCCATTCCGCAACGC -3'
CLIPB17	For:5'- TGCACGACCGAAGGCATTA -3' Rev:5'- AGCTTTTTGTGCGTGGCAC -3'

F. Survival and microbial proliferation assays

The survival of dsRNA-treated adult female mosquitoes was scored over a period of 7 days after intrathoracic injection of a suspension of ampicillin-resistant *E. coli* strain OP-50 (OD_{600}=0.4) [324] or tetracyclin-resistant *S. aureus* strain (OD_{600}=0.4) [152] in PBS, or after spraying with a suspension of *B. bassiana* (strain 80.2) containing $1x10^8$ conidia/ml in 0.05% Tween-80 prepared as follows: fungi were scraped off PD plates and collected and filtered in water suspension. The mixture is centrifuged at 4000 Rpm at room temperature for 10 minutes, the supernatant is then discarded and the pellet is washed three times with distilled water. The pellet is then suspended in 500µL of 0.05% Tween-80 solution , and a hematocytometer is used to estimate spore counts . The Kaplan-Meier survival test was used to calculate the percent survival. Statistical significance of the observed differences was calculated using the log-rank test. Experiments were repeated at least 3 times using different batches of mosquito and bacteria or fungi. For the bacterial

proliferation assays, dsRNA-treated mosquitoes were injected intrathoracically with 69nl of ampicillin-resistant *E. coli* strain OP-50 (OD_{600}=0.8) or tetracycline-resistant *S. aureus* strain (OD_{600}=0.4) for 48 h. At least 4 batches of 8 mosquitoes each per genotype were grinded in 400µl Luria Bertani (LB) Broth at 48 hrs after *E. coli* and *S. aureus* injections. Serial dilutions of mosquito homogenates were plated onto LB agar selection plates containing the appropriate antibiotic and colony-forming units (CFUs) were scored. Data shown are from four biological experiments. Statistical significance was calculated using the Mann-Whitney test. Medians were considered significantly different if $P<0.05$. The proliferation of *B. bassiana* in spore-infected mosquitoes was scored by qRT-PCR as follows. Briefly, *dsLacZ*, *dsTEP1* and *dsCLIPA28*-treated mosquitoes were injected each with approximately 30 *B. bassiana* spores in PBS. Four days later, whole mosquitoes were grinded in liquid nitrogen with a mortar and pestle to create a fine powder. The powder was collected into Eppendorf tubes and genomic DNA was extracted using the CTAB buffer (0.1M Tris-HCl, pH 8.0, 0.01M EDTA, 1.4M NaCl, 2% cetyltrimethyl ammonium bromide) as previously described [325]. QRT-PCR was performed in a CFX96 Real-Time Detection System (Bio-Rad) using the SYBR Green JumpStart™ Taq ReadyMix (Sigma-Aldrich) according to the manufacturer's instructions. *B. bassiana* primers used in qRT-PCR are the following: Bb_ITSII_F: 5'-GCC GGC CCT GAA ATG G-3'and Bb_ITSII_R: 5'- GAT TCG AGG TCA ACG TTC AGA AG-3 [326]. The *A. gambiae* ribosomal S7 gene was used as an internal control for normalization using the primers, AgS7-F: 5'-AGAACCAGCAGACCACCATC-3'and AgS7-R: 5'-GCTGCAAACTTCGGCTATTC-3' [294]. Relative gene expression values were calculated using the Comparative C_T Method after checking for the efficiency of target amplification.

G. Expression of CLIPA28 in SF9 cells and generation of CLIPA28 antibodies

The entire CLIPA28 open reading frame lacking the endogenous signal peptide and stop codon was amplified from *A. gambiae* cDNA using the following primer pair: AGAP010730 LIC-F, 5'-GACGACGACAAGATGCAAGACATTGAAGAAGAACTG-3' and AGAP010730 LIC-R, 5'-GAGGAGAAGCCCGGTTTCAATTTTATATCAAAACTCTC-3'. The underlined sequences are extensions to allow ligase-independent cloning in *pIEx10* insect cell expression plasmid (Novagen) as a fusion with an N-terminal streptavidin-tag and C-terminal His-tag according to the manufacturer's protocol. Transfection of Sf9 cells with *pIEx10-CLIPA28* and purification of recombinant CLIPA28^{HIS} was performed as previously described [154]. Purified recombinant CLIPA28^{HIS} protein was used to generate a rabbit polyclonal antibody (Eurogentec). CLIPA28 antibody was affinity purified over an AminoLink column (Pierce) containing covalently bound CLIPA28^{HIS} according to the manufacturer's protocol.

H. Western Blot analysis

Adult female *A. gambiae* mosquitoes were injected with 69 nl of *E. coli* (OD_{600}=0.8) or *S. aureus* (OD_{600}=0.8) suspension in PBS, or injected with 69 nl of *B. bassiana* suspension containing $1x10^8$ conidia/ml of water prepared as previously described. Hemolymph was generally collected from 25 mosquitoes per sample by proboscis clipping directly into protein loading dye (1x). However, for monitoring the cleavage profiles of CLIPA14, and of CLIPA28 and CLIPA8 in *SRPN2* kd, hemolymph

was extracted from 25 mosquitoes into ice-chilled PBS containing a cocktail of protease inhibitors (Roche) and protein quantification was performed using the Bradford protein assay (Fermentas) to ensure equal loading per well. Proteins were resolved by SDS-PAGE and transferred to immunoblot PVDF membrane using wet transfer (BioRad). Primary antibodies used for immunoblotting are mouse αCLIPA8 [152], rabbit αCLIPA28, rabbit αCLIPA14 [155], rabbit αPPO6 [230], rabbit αCLIPB8 [142] (kind gift from Kristin Michel), rabbit αSRPN2 [160] and rabbit αSRPN3 [152] added overnight at the following dilutions 1:30, 1:2000, 1:1000, 1:2000, 1:1000, 1:1000 and 1:1000, respectively. A lower band is sometimes observed with αPPO6 which most likely indicate cleaved form of the zymogen [230]. Following washing, blots were incubated for 1 hr with anti-mouse and anti-rabbit IgG horseradish peroxidase-conjugated secondary antibodies at dilutions of 1:6000 and 1:12000, respectively. Blots were then washed, immersed in BioRad Clarity Max western ECL substrate and imaged using ChemiDoc MP (BioRad). Band quantification was performed using Image Lab software.

I. Phenoloxidase enzymatic assay

The phenoloxidase enzymatic assay was performed 3 hrs after mosquito injections with *S. aureus* (OD_{600}=0.8) using 5-8 µg of mosquito hemolymph per reaction extracted in ice-cold 1xPBS containing EDTA-free protease inhibitor(PI) cocktail (Roche). When needed, protein quantification was done using Bradford assay (Fermantas). A total of 40 µl of hemolymph proteins in PBS/PI were mixed with 120 µl of saturated dihydroxyphenlalanine(L-DOPA) (Sigma) and loaded in a 96 well plate . The absorbance

of each sample was measured at 492 nm was at 10 minute intervals (up to 1 hr) in a MultisKan Ex microplate reader (Thermo Labsystems). The experiment was repeated three times using different batches of mosquitoes and *S. aureus* cultures.

J. Co-immunoprecipitation and mass spectrometry

Co-immunoprecipitation (coIP) reactions were performed exactly as previously described [327] using the Pierce crosslink Magnetic IP/co-IP kit. Briefly, hemolymph was collected from approximately 900 female mosquitoes by proboscis clipping at 48 hrs after mosquito spraying with *B. bassiana* conidial suspension (i.e. mimicking natural infection route) in water containing $1x10^8$ conidia/ml. Hemolymph extracts were centrifuged at 4000 g for 5 min to remove mosquito and fungal cells and incubated overnight at 4˚C with a 1:1 slurry of PBS containing agarose beads cross-linked to 4 µg of purified CLIPA28 antibody. Elution of bound proteins, precipitation with trichloroacetic acid and protein identification by mass spectrometry (IGBMC proteomic platform, Strasbourg) were performed exactly as described in [327].

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