Published in final edited form as:

Insect Biochem Mol Biol. 2016 April ; 71: 106–115. doi:10.1016/j.ibmb.2016.02.008.

CLIPB8 is part of the prophenoloxidase activation system in *Anopheles gambiae* mosquitoes

Xin Zhang, Chunju An¹, KaraJo Sprigg², and Kristin Michel*

Division of Biology, Kansas State University, Manhattan, KS 66506, USA

Abstract

In insects and other arthropods the formation of eumelanin (melanization) is a broad spectrum and potent immune response that is used to encapsulate and kill invading pathogens. This immune response is regulated by the activation of prophenoloxidase (proPO), which is controlled by proteinase cascades and its serpin inhibitors, together forming the proPO activation system. While the molecular composition of these protease cascades are well understood in insect model systems, major knowledge gaps remain in mosquitoes. Recently, a regulatory unit of melanization in *Anopheles gambiae* was documented, comprised of the inhibitory serpin-clip-serine proteinase, CLIPB9 and its inhibitor serpin-2 (SRPN2). Partial reversion of SRPN2 phenotypes in melanotic tumor formation and adult survival by *SRPN2/CLIPB9* double knockdown suggested other target proteinases of SRPN2 in regulating melanization. Here we report that CLIPB8 supplements the SRPN2/CLIPB9 regulatory unit in controlling melanization in *An. gambiae*. As with *CLIPB9*, knockdown of *CLIPB8* partially reversed the pleiotropic phenotype induced by *SRPN2* silencing with regards to adult survival and melanotic tumor formation. Recombinant SRPN2 protein formed an SDS-stable protein complex with activated recombinant CLIPB8, however did not efficiently inhibit CLIPB8 activity *in vitro*. CLIPB8 did not directly activate proPO *in vitro* nor was it able to cleave and activate proCLIPB9. Nevertheless, epistasis analysis using RNAi placed CLIPB8 and CLIPB9 in the same pathway leading to melanization, suggesting that CLIPB8 either acts further upstream of CLIPB9 or is required for activation of a yet to be identified serine proteinase homolog. Taken together, this study identifies CLIPB8 as an additional player in proPO activation cascade and highlights the complexity of the proteinase network that regulates melanization in *An. gambiae*.

Graphical Abstract

*Corresponding author: Kristin Michel, kmichel@ksu.edu, +1-785-532-0161.

¹Current address: Department of Entomology, College of Agriculture and Biotechnology, China Agricultural University, Beijing, China.

²Current address: Medical Center, University of Kansas, Kansas City, KS 66160, USA

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

mosquito; serpin; innate immunity; host-pathogen interactions; malaria; insect biochemistry

1. Introduction

Melanization, an arthropod-specific immune response, is used against a wide variety of pathogens (Cerenius and Soderhall, 2004; Cerenius et al., 2008). Indeed, insects deposit melanin on virtually all foreign surfaces naturally or experimentally introduced into their hemocoel, including nylon, epoxy, glass, and living tissues from other insects (Grimstone et al., 1967; Lackie, 1983; Salt, 1965, 1963). As all insects, the African malaria mosquito, *Anopheles gambiae* utilizes melanization as an anti-bacterial (Binggeli et al., 2014; Hillyer et al., 2003) and anti-fungal immune response (Yassine et al., 2012) that can also kill malaria parasites (Collins et al., 1986; Michel et al., 2005). Killing of pathogens through melanization is thought to occur through nutrient starvation (Chen and Chen, 1995) as well as direct toxic effects of reaction intermediates and byproducts (Nappi et al., 2009), although experimental support in *An. gambiae* is still lacking. Successful malaria transmission is therefore only ensured if *Plasmodium* parasites can escape the melanization response. Indeed, in transmission-permitting *Plasmodium* sp./mosquito species combinations, melanized parasites are rarely observed in the field (Cohuet et al., 2006; Lambrechts et al., 2007; Riehle et al., 2006; Schwartz and Koella, 2002), as *Plasmodium* sp. have evolved either active suppression (Boete et al., 2002) or avoidance mechanisms (Michel et al., 2006; Molina-Cruz et al., 2012).

Insect eumelanin is the product of oxidative polymerization of 5,6-dihydroxyindoles (DHIs). The substrate pathway that leads from tyrosine to DHI includes a number of enzymatic and nonenzymatic reactions, causing the initial production of the *o*-diphenols dopa and dopamine and their conversion to dopachrome and dopaminechrome, which form the precursors of DHI (Nappi and Christensen, 2005; Sugumaran, 2002). The key enzyme initiating melanization is phenoloxidase (PO), a monophenoloxxygenase that catalyzes the initial hydroxylation of tyrosine to dopa as well as the oxidation of dopa and dopamine to their respective quinones (Nappi et al., 2009). PO are enzymes of about 80 kDa molecular weight that contain two binuclear copper binding sites (Hu et al., 2016). They are expressed as zymogens, called proPOs that require proteolytic cleavage at the N-terminus. While

lepidopteran insect genomes encode only two, and *D. melanogaster* encodes three proPOs, mosquito genomes encode between nine to ten proPOs, all with high sequence similarity (Bartholomay et al., 2010; Neafsey et al., 2015; Waterhouse et al., 2007), but it remains unclear if individual mosquito POs differ in their biological function. Several, but not all *An. gambiae* proPOs are upregulated by blood feeding, and expression of at least five proPOs can be altered by 20-hydroxy ecdysone (Ahmed et al., 1999; Muller et al., 1999), suggesting that temporal and physiological specification of individual PO function.

The production of eumelanin generates a number of toxic byproducts including semiquinones and reactive oxygen species (Nappi and Christensen, 2005), which ultimately can be deleterious to the insect. Overstimulation of PO activity and ultimately melanization in *An. gambiae* causes a number of deleterious effects cumulating in shortened life span (An et al., 2011; Michel et al., 2005). It is therefore not surprising that melanization is tightly controlled, most prominently through regulation of the extracellular proteolytic cleavage of proPO to PO by the proPO activation cascade. Key enzymes in this cascade are clip-domain containing serine proteinases (CLIPs) that contain one or more amino-terminal clip domain that is separated by a linker region from a carboxyl-terminal S1A family serine proteinase domain (Smith and DeLotto, 1992). CLIPs are secreted into the hemolymph as zymogens, which require proteolytic activation by cleavage within the linker region. CLIPs are found in insects and other arthropods and can be subdivided into four evolutionarily distinct clades (A–D, Waterhouse et al., 2007; Kanost and Jiang, 2015), of which members of clade A have lost their proteolytic function. These proteinases are inhibited by serine proteinase inhibitors of the serpin family, the largest family of proteinase inhibitors in metazoans. Serpins act as suicide substrate inhibitors, forming covalent, stable inhibitory complexes with their target proteinases (Gettins, 2002), including CLIPs that are required for melanization in insects (Jiang and Kanost, 2000).

Our current understanding of the molecular make-up of the proPO activation system is derived from studies in a few model organisms such as *Drosophila melanogaster* (Veillard et al., 2015), *Manduca sexta* (Kanost and Jiang, 2015), and *Tenebrio molitor* (Kan et al., 2008). A generalized view of the insect proPO activation system can be described as follows (Kanost and Jiang, 2015): The system consists of protease cascades that are triggered by the recognition of molecular patterns associated with pathogens or aberrant cells by soluble receptor molecules leading to the activation of a modular serine proteinase (MSP). MSP in turn activates a CLIPC that then activates the terminal CLIPB proteinase in this cascade, also called proPO activating proteinase (PAP) or proPO activating enzyme (PPAE). Active PAP then cleaves proPO to PO. In addition, the formation of the final active phenoloxidase complex on the foreign surface is mediated by one or more proteolytically inactive CLIPAs, which themselves require proteolytic activation in order to function. Furthermore, several protease cascades, characterized by specific PAPs and distinct upstream CLIPCs can act in parallel to regulate the melanization response in insects (An and Kanost, 2010). Under normal physiological conditions, proPO activation cascades are turned off, most prominently by a single highly conserved serpin (Park et al., 2000), referred to as Spn27A in *Drosophila melanogaster* (De Gregorio et al., 2002; Ligoxygakis et al., 2002), Serpin-3 in *Manduca sexta* (Zhu et al., 2003), and SRPN2 in mosquitoes (Bartholomay et al., 2010; Christophides et al., 2002; Waterhouse et al., 2007; Zou et al., 2010). The only experimentally confirmed

inhibitory target of these serpins in proPO activation cascades is the terminal PAP within each cascade (An et al., 2013, 2011; Zhu et al., 2003). The depletion of this serpin results in continuous activation of PO in the hemolymph, the formation of large melanotic pseudotumors, and increased mortality rates (Ligoxygakis et al., 2002; Michel et al., 2005; Zou et al., 2010).

In *An. gambiae*, CLIPB9 is currently the only known bona fide member of the proPO activation system. This proteinase functions as a PAP, directly cleaving and activating proPO *in vitro*. CLIPB9 and SRPN2 form a functional regulatory unit of a proPO activation system, as CLIPB9 is required for melanotic tumor formation in adult female *An. gambiae* that are depleted of SRPN2 and is inhibited directly by SRPN2 *in vitro* and *in vivo* (An et al., 2011). However, targeted reverse genetic screens have identified a number of additional CLIPBs required for melanization of foreign surfaces (Paskewitz et al., 2006; Volz et al., 2006). In addition, reversion of the SRPN2-depleted phenotype is incomplete after knockdown of *CLIPB9* suggesting additional proteinase cascades are required for complete activation of proPO in *An. gambiae*.

The current study was designed to test the specific hypothesis that CLIPB8 is part of the proPO activation system in *An. gambiae*. Previous studies indicate CLIPB8 is required for the ookinete melanization of the rodent malaria parasite, *Plasmodium berghei*, in *CTL4*kd *An. gambiae* mosquitoes (Volz et al., 2006) and also for melanization of Sephadex beads in *An. gambiae* hemolymph (Paskewitz et al., 2006). Phylogenetic analysis revealed that CLIPB8 is closely related to *M. sexta* PAP2 and 3 (An et al., 2010), suggesting a conserved role in mosquitoes. This is further substantiated by observations that the ortholog of *An. gambiae* *CLIPB8* in the Yellow fever mosquito, *Aedes aegypti*, binds SRPN2 *in vitro* and is required for melanotic tumor formation in this species (Zou et al., 2010). Using genetic and biochemical methodologies our laboratory established previously, we initially determined whether CLIPB8 is part of the proPO activation system that is controlled by SRPN2 in *An. gambiae*, and subsequently explored its position within this cascade and in relation to its paralog, CLIPB9.

2. Results

2.1 CLIPB8 partially reverts the SRPN2-depletion phenotype

To test whether CLIPB8 is involved in *SRPN2*kd-mediated melanotic tumor formation, we performed double knockdown (dkd) analysis of *CLIPB8* and *SRPN2* in *An. gambiae* adult females using dsRNA injection. Kd efficiencies of *CLIPB8* and *SRPN2* were assessed at the transcript levels by RT-quantitative (q)PCR in each of the treatment groups (Fig. S1). *CLIPB8* transcript levels were reduced by 90–94 % after treatment with *dsCLIPB8* or *dsCLIPB8/dsSRPN2* as compared to mosquitoes treated with only *dsGFP* or *dsSRPN2*, respectively. Transcript reduction of *SRPN2* was slightly lower at 71–73%, which is comparable to previously reported knockdown levels for this gene (An et al., 2011; Michel et al., 2005).

Kd of *CLIPB8* in a *SRPN2*-depleted genetic background decreased melanotic tumor formation caused by *SRPN2* depletion (Fig. 1A). Melanized area per abdomen of *dsSRPN2*/

dsCLIPB8-treated female mosquitoes were significantly smaller than in *SRPN2*-depleted mosquitoes (Mann-Whitney U test, $P=0.002$) (Fig. 1B). As observed previously, *dsGFP* or *dsCLIPB8* treatments alone did not result in melanotic tumors in adult mosquitoes.

In addition, *CLIPB8*kd partially reverted the accelerated daily mortality rates observed in *SRPN2*-depleted mosquitoes (Fig. 1C). Dkd of *CLIPB8* and *SRPN2* increased significantly mosquito life span as compared to *SRPN2*kd (Log Rank Test, $\chi^2 = 14.42$, $df = 1$, Bonferroni-corrected threshold of $P < 0.01$). However, mortality rates of *CLIPB8*/*SRPN2* dkd mosquitoes remained accelerated as compared to *CLIPB8*kd mosquitoes (Log Rank Test, $\chi^2 = 10.83$, $df = 1$, Bonferroni-corrected threshold of $P < 0.01$). As expected, *CLIPB8*kd did not alter mortality rates as compared to *dsGFP*-injected mosquitoes (Log Rank Test, $\chi^2 = 1.167$, $df = 1$, Bonferroni-corrected threshold of $P > 0.05$). These results identify *CLIPB8* as part of the melanization cascade in *An. gambiae* that is regulated by *SRPN2* and, if misregulated, leads to melanotic tumor formation and increased mortality.

2.2 CLIPB8 is a functional serine proteinase with trypsin-like specificity

Based on sequence analysis, CLIPB8 is a typical Clip proteinase of the subfamily B. Its single clip domain between residues 17–71 contains 25 residues between Cys-3 and Cys-4, and activation cleavage is likely to occur after lysine, both hallmarks of subfamily B clip proteinases (Jiang and Kanost, 2000). The catalytic triad required for proteinase function is conserved and is predicted to consist of residues H158, A225, and S329. Proteolytic cleavage of the proCLIPB8 zymogen required for activation of clip-serine proteinases is likely to occur between CLIP and proteinase domain with the P1 site at K112 (Paskewitz et al., 2006). To determine the mode of action of CLIPB8, we expressed and purified the full-length recombinant proCLIPB8 zymogen that includes a C-terminal His tag using a baculovirus expression system. Endogenous activating proteinases for any mosquito CLIPs still await identification, and it is unknown which proteinase activates the proCLIPB8 zymogen *in vivo*. We therefore replaced the endogenous putative activation cleavage site of proCLIPB8 with that of bovine Factor Xa, so that recombinant proCLIPB8 (proCLIPB8_{Xa}) could be cleaved and activated *in vitro*.

Based on Western blot analysis, the full length purified recombinant proCLIPB8_{Xa} has a molecular weight of around 50 kD, which is significantly larger than its calculated molecular weight of 43.4 kD, indicating that the protein is glycosylated (Fig. S2A). Incubation of the recombinant protein with bovine Factor Xa resulted in cleavage of the protein and appearance of a second band of 36-kDa on the Western blot, the predicted size of the CLIPB8_{Xa} proteinase domain (Fig. 2A). Next, we screened a number of small peptides for their ability to function as a substrate for CLIPB8. CLIPB8_{Xa} was able to cleave a range of peptide substrates with arginine at the P1 site, including IEGR, IEAR, VGR and FVR (Figs. S2B and S3), suggesting a trypsin-like substrate preference. Importantly, FVRase activity was not detected when incubated with the proCLIPB8_{Xa} zymogen, confirming that proteolytic cleavage is required to activate proteinase activity of CLIPB8 (Fig. S2B).

2.3 CLIPB8 is not a molecular target of SRPN2 inhibition

To determine whether CLIPB8 can be inhibited directly by SRPN2, we first examined whether SRPN2 can form a covalent protein complex with CLIPB8 *in vitro* by Western blot analysis. A higher molecular weight band appeared after incubation of the activated CLIPB8_{Xa} with SRPN2, indicating the formation of an inhibitory complex (Fig. 2). In the absence of active CLIPB8_{Xa}, anti-His antibodies recognized the 52-kDa CLIPB8_{Xa} zymogen and 43-kDa recombinant SRPN2 (Fig. 2A). When SRPN2 was mixed with active CLIPB8_{Xa}, a 36-kDa band corresponding to the CLIPB8 catalytic domain was detected. In addition, a new immunoreactive band at ~72-kDa, corresponding to the expected mass of the SRPN2/CLIPB8_{Xa} complex was detected. This band was also recognized by anti-SRPN2 antibodies (Fig. 2B). Analysis of tryptic peptides from the ~72-kDa band by MS/MS identified several peptides of CLIPB8 and SRPN2 (Fig. S4), confirming that SRPN2 forms a covalent complex with active CLIPB8_{Xa}. The peptides obtained for CLIPB8 covered both Clip and proteinase domain, indicating that the Clip domain remained attached to the proteinase domain upon activation by Factor Xa.

While complex formation was observed, the ratio of intensities of the complex band compared to CLIPB8_{Xa} proteinase domain and SRPN2 was low (Fig. 2), suggesting that SRPN2 may not be an efficient inhibitor of CLIPB8. We therefore analyzed the ability of SRPN2 to inhibit the FVRase activity of CLIPB8_{Xa} *in vitro*. While increasing concentrations of SRPN2 slightly dampened CLIPB8_{Xa} activity, the stoichiometry of inhibition (SI) was as high as 55 (Fig. 3). Therefore, SRPN2 is not an effective inhibitor of CLIPB8 *in vitro*, suggesting that, in contrast to CLIPB9, CLIPB8 is not a physiological target of SRPN2 *in vivo*.

2.4 CLIPB8 neither cleaves nor activates proPO

Biochemical analysis of the melanization cascade in mosquitoes is greatly enabled by the use of heterologous model systems such as *M. sexta* (Kanost and Gorman, 2008; Michel et al., 2006; Zou et al., 2010). We previously determined that CLIPB9_{Xa} functions as a PAP in *An. gambiae* (An et al., 2011), and used established assays to determine if CLIPB8 functions similarly as a terminal proteinase in the proPO activation cascade. ProPO circulates in *M. sexta* hemolymph as a heterodimer of 79-kDa PPO-p1 and 80-kDa PPO-p2 (Jiang et al., 1997). Western blot analysis of purified *M. sexta* PO with either Factor Xa or proCLIPB8_{Xa} using anti-*M. sexta* PO antibody identified two bands corresponding to the full-length proPO heterodimer at around 80-kDa (Fig. 4A), as well as a single smaller band of 56-kDa, suggesting partial proteolytic cleavage of *M. sexta* proPO. However, incubation with active CLIPB8_{Xa} did not cause an increase in PO activity above background levels. (Fig. 4B). The same bands were detected after incubation of purified *M. sexta* proPO with active CLIPB9_{Xa}. In addition, and as shown previously (An et al., 2011), a 72-kDa doublet band corresponding to mature *M. sexta* PO was detected, and PO activity increased significantly (Fig. 4A, B). As the activation cleavage sites between *An. gambiae* and *M. sexta* proPOs are conserved (Christophides et al., 2002), it is unlikely that CLIPB8 activates *An. gambiae* proPOs. Therefore, while CLIPB8 is part of the proPO activation cascade, CLIPB8 does not have proPO as its proteolytic target and consequently must act upstream of the *An. gambiae* PAP.

2.5 CLIPB8 and B9 are not proteolytic substrates of each other

Our results indicate that CLIPB8 acts upstream of a PAP in the proPO activation cascade. The only confirmed PAP in the PO activation cascade in *An. gambiae* is CLIPB9 (An et al., 2011). We therefore tested next whether CLIPB8 can function as the activating proteinase of proCLIPB9, and vice versa. To this end, we expressed and purified the wild-type forms of proCLIPB8 and proCLIPB9 in Sf9 cells, which contained the unmutated activation cleavage sites. However, we found no evidence that these two proteinases can utilize each other as substrates (Fig. 5). When activated CLIPB8_{Xa} was incubated with proCLIPB9 in equal molar ratios, the 56 kD zymogen of proCLIPB9 remained, and no additional band corresponding to the proteinase domain of CLIPB9 appeared (Fig. 5A right panel). Likewise, incubation of activated CLIPB9_{Xa} with proCLIPB8 had no effect on the 50 kD CLIPB8 zymogen (Figure 5B, right panel). These results are consistent with the conclusion that CLIPB8 is not the activating proteinase of proCLIPB9 or vice versa, and suggest that CLIPB8 either functions further upstream of CLIPB9 or may be part of a distinct proPO activation cascade that functions in parallel to CLIPB9.

2.6 CLIPB8 and B9 act within the same proPO activation cascade

Knockdown of either CLIPB8 or CLIPB9 partially reverted the SRPN2 depletion phenotypes. However, based on the biochemical data presented above, it remained unclear if these two proteinases function in the same proPO activation cascade or if they function in parallel. To test whether CLIPB8 and B9 indeed belong to the same proteolytic cascade, we set out to perform epistasis experiments using triple knockdown (tkd) of *SRPN2*, *CLIPB8* and *CLIPB9*. All dsRNA injections induced specific transcript reduction for their target transcripts, regardless if injected with one or more additional dsRNAs (Fig. S1). Furthermore, kd efficiencies remained at the same level between dkd and tkd experiments. Together, these results not only confirm efficacy of the tkd experimental design, but also reveal that neither SRPN2, CLIPB8, nor CLIPB9 influence the expression of the other two genes on a transcriptional level.

We next examined the formation of melanotic tumors in the *SRPN2/CLIPB9/CLIPB8*tkd mosquitoes compared to dkd and single kd controls. As reported previously, *CLIPB8*kd or *CLIPB9*kd in SRPN2-depleted mosquitoes significantly reduced melanotic tumor formation in adult female mosquitoes. The average area of melanization observed in *SRPN2*kd mosquitoes was reduced by nearly 50 % in either *CLIPB8*kd or *CLIPB9*kd mosquitoes in a SRPN2-depleted genetic background (Fig. 6A). However, we did not observe any significant differences in total melanized area per abdomen in the tkd mosquitoes as compared to those from *SRPN2/CLIPB9*kd or *SRPN2/CLIPB8*kd mosquitoes (Fig. 6A). These results therefore provide no evidence for additive phenotypic effects of kd of these two proteinases on SRPN2 depletion-mediated melanization, and demonstrate that CLIPB8 and CLIPB9 function in the same proteolytic cascade that activates proPO in *An. gambiae*.

Similarly, the accelerated death rates of SRPN2-depleted mosquitoes were reduced significantly in *SRPN2/CLIPB9*kd and *SRPN2/CLIPB8*kd mosquitoes. Survival curves between these dkd populations were statistically significantly different from the ds*GFP*-injected controls, but identical between the two dkd treatment groups. As observed for the

melanization phenotype, no further increase in survival in *SRPN2*kd mosquitoes was observed when transcripts of both proteinases were depleted (Fig. 6B). These results provide further evidence that kd of both *CLIPB8* and *CLIPB9* does not have an accumulative effect of the pleiotropic *SRPN2*-depletion phenotype and confirm that they function in the same pathway.

3. Discussion

Proteinase cascades, in which proteinases activate each other in a sequential order, play a central role in the mosquito immune system, regulating melanization, the Toll pathway, and most likely the complement system. To investigate the complexity anti-parasite immunity regulation in *An. gambiae*, we examined further the molecular make-up of the system that controls melanization via proteolytic proPO activation. We previously identified the first functional regulatory unit of melanization in this mosquito species, composed of *CLIPB9* and its inhibitor, *SRPN2*. This unit directly regulates cleavage of proPO and thus the production of eumelanin. In this study, we provide functional evidence that a second *CLIPB* proteinase, *CLIPB8* is required for melanization as part of the proPO activation system. In addition, we demonstrate that *CLIPB8* and *B9* function in the same proPO activation cascade, albeit hold distinct positions within this cascade.

3.1. *CLIPB8* and *B9* have distinct roles within melanization

CLIPB8 and *B9* are closely related paralogs, separated by only 2.1 kb on chromosome 2R14A (An et al., 2011), suggesting that these genes may be co-regulated (Li et al., 2013; Michalak, 2008). Indeed, their expression pattern is similar across wide variety of experimental conditions. In the *An. gambiae* expression map, which clusters the expression patterns of all annotated *An. gambiae* genes across 93 experimental conditions (Maccallum et al., 2011), *CLIPB8* and *B9*, are located in the same map region within nodes 5,16 and 3,15, respectively. Both genes have similar tissue expression profiles, with enrichment in circulating hemocytes (Pinto et al., 2009), and little to no expression in midgut or ovaries (Baker et al., 2011). Furthermore, both genes are upregulated after blood feeding (Marinotti et al., 2006), further supporting co-regulation of these two proteinases. In addition, *CLIPB8* and *B9* exhibit the highest sequence similarity among *An. gambiae* *CLIPBs*: the amino acid sequences of their proteinase domains are 40% identical (An et al., 2011). We therefore expected and experimentally confirmed that the recombinant proteins of these two proteinases have similar peptide substrate specificities *in vitro*. Finally, our RNAi data confirm that both *CLIPB8* and *B9* reduce the melanotic phenotype conferred by *SRPN2*kd, supporting our hypothesis that, just as *CLIPB9*, *CLIPB8* functions with the proPO activation system.

However, while *CLIPB8* and *B9* are similar in their expression profiles and biochemical function, we provide several independent lines of evidence that these two proteins have distinct roles *in vivo*. First, depletion of either one by RNAi in a *SRPN2*kd background causes a phenotype without affecting the other proteinase's expression level, demonstrating that these two proteinases cannot complement each other's function. Second, *CLIPB8* and *B9* have distinct physiological cleavage targets, as only *CLIPB9*, not *CLIPB8* can cleave proPO. Third, only *CLIPB9* provides an intervention point for melanization by *SRPN2*. As

we have shown previously, CLIPB9 and SRPN2 form inhibitory complexes at rates that are physiologically relevant (An et al., 2011). In contrast, inhibition of CLIPB8 amidase activity requires extremely high levels of SRPN2 that are not observed *in vivo*. Therefore, although these two proteinases function within the same proPO activation cascade, their roles are not redundant. CLIPB8 and B9 are thus an excellent example of paralogous proteins in *An. gambiae* have undergone functional diversification. This functional diversification was not driven by divergent gene expression (Hughes and Friedman, 2005), nor different cellular localization (Marques et al., 2008) or biochemical mode of action. Rather, it is likely the consequence of altered substrate recognition through mutations in the recognition domains, reminiscent of alterations recently described in transcription factors (Singh and Hannenhalli, 2008; Voordeckers et al., 2015).

3.2. CLIPB8 functions upstream of CLIPB9 within the same proPO cascade

In the generalized model of the prototypical proPO activation cascade, the modular serine proteinase MSP autoactivates in response to binding to a pattern recognition receptor (PRR) bound to microbe- or pathogen-associated patterns (Takahashi et al., 2015). Active MSP then cleaves and activates a CLIPC, which in turn activates a CLIPB. Active CLIPB then executes the terminal step in the cascade by functioning as a PAP, cleaving and activating proPO to PO (Kanost and Jiang, 2015). Based on this model and given that CLIPB8 belongs to the subfamily B of clip-domain serine proteinases, we hypothesized that CLIPB8 also acts as PAP, functioning in parallel to PAP activation activity of CLIPB9 (An et al., 2011). However, data presented in this study do not provide evidence for this hypothesis. *CLIPB8/B9*^{kd} in a SRPN2-depleted background does not further reduce melanotic tumor formation as compared to the *CLIPB8*^{kd} or *CLIPB9*^{kd} alone, demonstrating that CLIPB8 and B9 do not control PO activity in parallel. Instead, at least in the context of *SRPN2*^{kd}, CLIPB8 and B9 act within the same proPO activation cascade leading to melanotic tumor formation, likely regulating PO activity by cleavage of one or more proPOs through CLIPB9. Furthermore, CLIPB8 does not function as a PAP *in vitro*. Although we used proPO isolated from the hemolymph of *M. sexta* and not *An. gambiae*, insect proPO sequences are highly conserved and the vast majority of them share the same activation cleavage site, including those of *M. sexta* proPO1/2 and *An. gambiae* PPO1-8 (Christophides et al., 2002). Whether CLIPB8 can function as a PAP of e.g. *An. gambiae* ProPO9, which has an activation cleavage site distinct of *M. sexta* proPO1/2 and *An. gambiae* PPO1-8 (Christophides et al., 2002), warrants further investigation. However, the genetic and biochemical data detailed in this manuscript demonstrate that CLIPB8 does not function as a PAP in melanotic tumor formation *in vivo*, and must act upstream of CLIPB9.

Although belonging to the B subfamily, we nevertheless explored whether CLIPB8 functions directly upstream of CLIPB9. However, this is not the case, as CLIPB8 cannot cleave proCLIPB9 *in vitro* (or vice versa). It is nevertheless possible that CLIPB8 is further upstream in the pathway activating a yet to be identified proteinase, which is responsible for CLIPB9 activation, despite its designation in the B subfamily and the deviation from the cascade model elucidated in other insects. An alternative and more parsimonious explanation is that CLIPB8 is required for the activation of one or several proteolytically inactive CLIPAs that complement the proPO cascade. Published examples for proteolytic

activation of CLIPAs by CLIPBs exist from other insect species (Kan et al., 2008; Wang et al., 2014). A candidate for such a proteinase homolog is CLIPA8, which is required for melanization of bacteria, fungi and *P. berghei* (Schnitger et al., 2007; Volz et al., 2006; Yassine et al., 2012). Thus far, it is the only CLIPA that has been shown to be required for melanization in *An. gambiae*. Future studies will have to determine whether CLIPA8 indeed requires proteolytic activation for its function, and if this activation is mediated by CLIPB8.

3.3. The complexity of proPO activation in *An. gambiae*

The proPO activation cascade, characterized by CLIPB8 and B9, is at least partially conserved among Culicidae, as the orthologs of CLIPB8 and B9 are also required for melanotic tumor formation in *Ae. aegypti* (Zou et al., 2010). However, the regulation of proPO in mosquitoes is probably more complex, as it is likely that several proPO activation cascades exist in mosquitoes. Besides CLIPB8 and CLIPB9, several other CLIPB proteinases, including CLIPB1, 3, 4, 14, and 17 also affect murine malaria parasite and/or bead melanization (Volz et al., 2005, 2006; Paskewitz et al., 2006). While their molecular function awaits identification, it is likely that these additional CLIPBs are active proteinases contributing directly to proPO activation. In addition, reversion of the *SRPN2*kd-induced phenotype by the CLIPB9 PAP is incomplete, indicate that other PAPs also mediate melanotic tumor formation (An et al., 2011). Indeed, preliminary data indicate that at least CLIPB4 functions as a PAP *in vitro* (Zhang and Michel, unpublished). Together, these findings strongly point to the existence of several proPO activation cascades in *An. gambiae* acting in parallel.

The notion of several proPO activation cascades in *An. gambiae* is perhaps not surprising, given their existence in other distantly related insect species, including *M. sexta*. However, the complexity of the proPO activation cascade(s) is further underscored by the observation that, dependent on the initiating trigger of proPO activation, the number and identity of CLIPBs required for melanization is partially overlapping but not congruent. Melanization of Sephadex beads implanted into adult female *An. gambiae* requires at least CLIPB4 and B8, and to a lesser extend the action of CLIPB1, but not CLIPB9 nor B10 (Paskewitz et al., 2006). Melanization of *P. berghei* in the laboratory selected L35 strain of *An. gambiae* (Collins et al., 1986) is mediated by CLIPB3, 4, and 17 (Volz et al., 2006), while melanization of the same parasite in CTL4-depleted mosquito host depends only CLIPB4, 8, 14, and 17 (Volz et al., 2006). While this current study demonstrates that CLIPB8 acts upstream of CLIPB9 in tissue melanization, published data show that CLIPB8 must act upstream of a different PAP, potentially CLIPB4, for melanization of glass beads and potentially also *P. berghei* parasites (Paskewitz et al., 2006; Volz et al., 2006).

In summary, this study identifies CLIPB8 as a non-redundant member of the proPO activation system in *An. gambiae* that is required for melanotic tumor formation. CLIPB8 supplements the SRPN2/CLIPB9 regulatory unit in controlling melanization, acting upstream of CLIPB9 potentially by proteolytic cleavage of CLIPA8. Our findings suggest that proPO activation cascades in *An. gambiae* are branched and act in parallel, highlighting the complexity of the regulatory network that controls melanization in this important vector species.

4. EXPERIMENTAL PROCEDURES

4.1 Mosquito strain and rearing

The *An. gambiae* G3 strain (MRA-112) was maintained as described previously (An et al., 2011).

4.2 Recombinant serine proteinase production, purification, and antibody generation

The recombinant serine proteinase zymogens proCLIPB8, proCLIPB9, proCLIPB8_{Xa}, and proCLIPB9_{Xa} were produced in the insect Sf9 cell line using the Bac-to-Bac expression system (Life Technologies), and purified as described previously (An et al., 2011). The complete coding regions of *proCLIPB9* and *proCLIPB8* were amplified by PCR from cDNA of adult female *An. gambiae* with forward primers including a *NotI* site and reverse primers containing six codons for histidine residues followed by a stop codon and a *HindIII* site (for primer sequences see Table S1). The expression construct was assembled by inserting the PCR product digested with *NotI* and *HindIII* into the vector pFastBac1 (Life Technologies) digested with the same restriction enzymes. The resulting expression plasmids were used as template to generate the two mutant *proCLIPB9*_{Xa} and *proCLIPB8*_{Xa} expression constructs, both encoding recombinant proteins with a His-tag at the C-terminus. Briefly, the activation cleavage sites, IGAK for proCLIPB8, and IGMR of proCLIPB9, were replaced by IEGR, the cleavage site for factor Xa, using QuickChange® Multi Site-Directed Mutagenesis Kit (Stratagene) following the manufacturer's instructions. The resulting plasmids were transfected into Sf9 cells to generate recombinant baculoviruses according to the manufacturer's instructions (Life Technologies). Recombinant proteinase zymogens were expressed in Sf9 cells following the manufacturer's protocol and purified by nickel-nitrilotriacetic acid (NTA) agarose chromatography followed by ion-exchange chromatography on a Q-Sepharose column, as described previously (An et al., 2011). Fractions containing recombinant protein were examined for purity by 10% SDS-PAGE and Coomassie stain, and pooled for further use.

For the generation of antibodies against full-length *An. gambiae* CLIPB8 and B9, cDNA fragments encoding the mature zymogen without signal peptide were amplified by PCR using cDNA as template with forward primers including a *NcoI* site and six histidine codons, and reverse primers containing a stop codon and a *NotI* site. The expression construct was assembled by inserting the PCR product digested with *NcoI* and *NotI* into vector pET28a (Novagen) cut with the same restriction enzymes. Recombinant N-terminally His-tagged rCLIPB8 and rCLIPB9 were expressed in a one liter culture of *Escherichia coli* strain BL21 with 0.1 mM of isopropyl β-D-thiogalactoside for 5 h at 20°C, 250 rpm. Both, rCLIPB8 and rCLIPB9 were expressed in an insoluble form and were purified under denaturing conditions by nickel-nitrilotriacetic acid agarose affinity chromatography (Qiagen, Valencia, CA, USA). 1.5 mg aliquots of the purified rCLIPB8 and rCLIPB9 were further resolved by preparative SDS-PAGE and used for polyclonal rabbit antiserum production (Cocalico Biologicals, Reamstown, PA, USA). Antibodies were purified as described previously (An et al., 2012).

All PCR primer sequences used for generation of the recombinant proteins described above are listed in Table S1.

4.3 Recombinant serpin production and purification

Recombinant SRPN2 was expressed in *E. coli* using the pET expression system (Novagen) and purified as described previously (An et al., 2011). Briefly, the cDNA fragment encoding the full-length mature *SRPN2* was amplified with the forward primer including an *NcoI* site, a start codon, and six histidine residue codons, and the reverse primer containing a stop codon followed by a *HindIII* restriction site. The expression construct was assembled by inserting the results PCR products digested with *NcoI* and *HindIII* into the expression vector pET-28a (Novagen) digested with the enzymes. The resulting plasmids were transformed into *E. coli* BL21(DE3) strain followed by recombinant protein expression in 1 L of LB medium with 0.1 mM IPTG, at 20°C and 225 rpm for 16 h. Bacterial cells were lysed by sonication and subjected to NTA agarose chromatography followed by ion-exchange chromatography on a Q-Sepharose column. Fractions containing recombinant protein were examined for purity by 10% SDS-PAGE and Coomassie stain, and pooled for further use. All PCR primer sequences are listed in Table S1.

4.4 Activation of recombinant zymogens and amidase activity assays

The activation of rproCLIPB8_{Xa} and rproCLIPB9_{Xa} by factor Xa was performed as described previously (An et al., 2011) with some modification. Briefly, 5 µg of recombinant purified zymogen was incubated overnight with 2 µg of commercial bovine Factor Xa (New England Biolab) in a total reaction volume of 100 µl (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM CaCl₂) at 27°C. Two negative controls were set up in parallel, in which either Factor Xa or the zymogen was replaced with same volume of buffer. Cleavage of the recombinant zymogen was determined by Western blot. To examine the amidase activity following activation, 2 µl of the above reaction were transferred to 96 well flat bottomed black microplate (Corning) followed by the addition of 100 µl of 200 µM Benzoyl-FVR-AMC (Thrombin Substrate III, Fluorogenic, EMD Chemicals) in buffer (0.1 M Tris-HCl, pH 8.0, 0.1 M NaCl, 5 mM CaCl₂). Fluorescence changes were monitored immediately in a microplate fluorescence reader (Flx 800 microplate fluorescence reader, Bio-Tek Instruments, Inc.) at excitation wavelength 360 nm and emission wavelength 440 nm every 70 seconds for 20 min. One unit of amidase activity was defined as RFU/min=1. Amidase activity of the serine proteinase was defined as the activity of enzyme minus the activity of Factor Xa alone.

4.5 Serpin-proteinase complex formation and detection by Western blot and Mass spectrophotometry (MS) analysis

The potential formation of CLIPB8-SRPN2 complexes *in vitro* was monitored by Western blot. Recombinant proCLIPB8_{Xa} was activated as described in 4.4 above, mixed with purified rSRPN2 at a molar ratio of 1:10, and incubated at RT for 10 min. Reaction mixtures were then separated by 10% SDS-PAGE followed by transferring the proteins to a PVDF membrane. After blocking in 5% milk in 1x Tris-buffered saline with 0.05% Tween 20 (TBST) for 1 h, the membrane was incubated with mouse anti-His antibody (1:2000, GenScript) or rabbit anti-SRPN2 antibody [(Michel et al., 2005), 1:2000] in 1x TBST

containing 0.5% milk for 1h at RT. After three 10 min washes with 1xTBST, membranes were incubated with goat anti-mouse or anti-rabbit IgG HRP-conjugated secondary antibodies (Promega, 1:20,000 dilution) in 1xTBST containing 0.5% milk for 1h at RT. After three further 10 min washes in 1xTBST, primary antibody binding was visualized by Western Lightning Chemiluminescence Reagent Plus Kit (Perkin Elmer).

In addition, after separation by 10% SDS-PAGE, bands of interest were excised from the gel, and subjected to MS analysis. In gel trypsin digestion and MALDI-MS analysis (Bruker Daltonics Ultraflex III) were performed at Biotechnology/Proteomics Core Facility in Kansas State University. Mass spectra were searched against a SwissProt protein database and analyzed by mMass software (<http://www.mmass.org>).

4.6 Serpin inhibition assays

To examine the inhibitory effect of SRPN2 on proteinase activity, 100 ng of *in vitro* activated proteinase in a volume of 2 μ l was incubated with 4 μ l of purified rSRPN2 (at a 10:1 molar ratio serpin to active enzyme) in 20 mM Tris-HCl, pH 8.0, and 100 mM NaCl with the addition of 1 μ l BSA (2 μ g/ μ l) at room temperature (RT) for 10 min. The complete reaction was subjected to an amidase activity assay as described in 4.4 above. Substitution of rSRPN2 with 4 μ l of buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl) was used to determine 100% enzyme activity.

4.7 ProPO cleavage and in vitro PO activity assays

To examine the potential cleavage of proPO by CLIPB8 and/or CLIPB9, 40 ng of purified *M. sexta* proPO (kindly provided by M. Gorman, Kansas State University) were incubated with 250 ng of activated rCLIPB8_{Xa} or rCLIPB9_{Xa} at 37°C for 30 min. Samples were then subjected to SDS-PAGE and Western blot analysis using antibodies against *M. sexta* proPO (Jiang et al., 1997) as described previously (An et al., 2011). To determine PO activity, 1 μ g purified *M. sexta* proPO was mixed with 250 ng activated rCLIPB8_{Xa} or rCLIPB9_{Xa} at 37°C for 30 min. PO activity was measured using dopamine as a substrate as described before (Jiang et al., 2003).

4.8 RNAi and phenotypic analysis in adults

DsRNA preparation and mosquito injections were performed as described previously (An et al., 2011). Primer pairs for template amplification of ds*SRPN2*, ds*CLIPB8*, ds*CLIPB9*, and ds*GFP* are listed in Table S1. In all experiments, 1–2 day old adult female G3 mosquitoes were used for dsRNA injection, and maintained on sugar water provided *ad libitum* throughout the entire experiment (see 4.1 above). To examine potential genetic interactions between *CLIPB8*, *CLIPB9* and *SRPN2* *in vivo*, double- or triple knockdown (dKD, tKD) was performed by injection of 138 nl of solution at 1.5 μ g/ μ l for each dsRNA. For KD and dKD controls, ds*GFP* was added to keep the total dsRNA dose constant at 207 ng/mosquito between treatment and controls. Melanotic tumor formation and mortalities were recorded and analyzed as detailed in An et al., 2010. Mosquito abdomens were dissected and analyzed for melanotic tumors when mortality reached 70% in the ds*SRPN2*/ds*GFP*-injected control mosquitoes. All experiments were performed with three independent biological replicates using at least 50 mosquitoes per treatment.

4.9 Total RNA isolation, cDNA synthesis and RT-qPCR

To examine knockdown efficiency, total RNA was extracted from mosquitoes 4 days post injection using TRIzol reagent (Life Technologies). The cDNA was synthesized with the iScript cDNA synthesis kit (BioRad) using 100 ng of total RNA as template in a total reaction volume of 25 µl following the manufacturer's instructions. All qPCRs were performed in a 25 µl reaction containing 10.5 µl of 10-fold diluted cDNAs, 0.4 µM of each primer, and 1× iQ SYBR Green Supermix (BioRad) using the ABI StepOnePlus system (Applied Biosystems). AgRPS7 was used as an internal reference gene, and relative gene expression was calculated by 2^{-Ct} method. All qPCR analyses were conducted using three biological replicates for each gene, each with three technical repeated measurements.

4.10 Statistical Analyses

Statistical analyses were executed using GraphPad Prism 5.1 Software (GraphPad Software Inc.). Survival data were analyzed using Kaplan-Meier, and potential differences between treatment groups were analyzed using Log-Rank test (Mantel Cox). Melanotic tumor formation data were evaluated for normality of distribution using Shapiro-Wilk normality test; data were analyzed using (i) Mann Whitney U-test, if comparing two treatment groups, or (ii) Kruskal Wallis test for multiple treatment groups, with Dunn's Multiple Comparison post test. All enzymatic activity data were evaluated using One-Way ANOVA, with Newman-Keuls post test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Dr. M. Gorman (Kansas State University) for purified *M. sexta* proPO, Dr. D. Meekins (Kansas State University) for critical reading of the manuscript, and all members of the Michel laboratory for mosquito rearing. Thanks go to Dr. J. Tomich and B. Katz at the Kansas State Biotechnology/Proteomics Core lab for protein digestion and mass spectrometry. The following reagent was obtained through the MR4 as part of the BEI Resources Repository, NIAID, NIH: *Anopheles gambiae* G3, MRA-112, deposited by MQ Benedict. The research reported in this manuscript was supported by R01AI095842 from NIAID, NIH to K. M. KJS was supported by a KINBRE Star Trainee scholarship through the NIGMS, NIH under grant number P20GM103418. The contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH. This is Contribution 15-226-J from the Kansas Agricultural Experiment Station.

References

- An C, Budd A, Kanost MR, Michel K. Characterization of a regulatory unit that controls melanization and affects longevity of mosquitoes. *Cell Mol Life Sci.* 2011; 68:1929–1939.10.1007/s00018-010-0543-z [PubMed: 20953892]
- An C, Hiromasa Y, Zhang X, Lovell S, Zolkiewski M, Tomich JM, Michel K. Biochemical characterization of *Anopheles gambiae* SRPN6, a malaria parasite invasion marker in mosquitoes. *PLoS One.* 2012; 7:e48689.10.1371/journal.pone.0048689 [PubMed: 23152794]
- An C, Kanost MR. *Manduca sexta* serpin-5 regulates prophenoloxidase activation and the Toll signaling pathway by inhibiting hemolymph proteinase HP6. *Insect Biochem Mol Biol.* 2010; 40:683–9.10.1016/j.ibmb.2010.07.001 [PubMed: 20624461]
- An C, Zhang M, Chu Y, Zhao Z. Serine protease MP2 activates prophenoloxidase in the melanization immune response of *Drosophila melanogaster*. *PLoS One.* 2013; 8:e79533.10.1371/journal.pone.0079533 [PubMed: 24260243]

- Baker DA, Nolan T, Fischer B, Pinder A, Crisanti A, Russell S. A comprehensive gene expression atlas of sex- and tissue-specificity in the malaria vector, *Anopheles gambiae*. *BMC Genomics*. 2011; 12:Art 296.10.1186/1471-2164-12-296
- Bartholomay LC, Waterhouse RM, Mayhew GF, Campbell CL, Michel K, Zou Z, Ramirez JL, Das S, Alvarez K, Arensburger P, Bryant B, Chapman SB, Dong Y, Erickson SM, Karunaratne SH, Kokoza V, Kodira CD, Pignatelli P, Shin SW, Vanlandingham DL, Atkinson PW, Birren B, Christophides GK, Clem RJ, Hemingway J, Higgs S, Megy K, Ranson H, Zdobnov EM, Raikhel AS, Christensen BM, Dimopoulos G, Muskavitch MA. Pathogenomics of *Culex quinquefasciatus* and meta-analysis of infection responses to diverse pathogens. *Science* (80-). 2010; 330:88–90.10.1126/science.1193162
- Binggeli O, Neyer C, Poidevin M, Lemaitre B. Prophenoloxidase activation is required for survival to microbial infections in *Drosophila*. *PLoS Pathog*. 2014; 10:e1004067.10.1371/journal.ppat.1004067 [PubMed: 24788090]
- Boete C, Paul RE, Koella JC. Reduced efficacy of the immune melanization response in mosquitoes infected by malaria parasites. *Parasitology*. 2002; 125:93–98. [PubMed: 12211612]
- Cerenius L, Lee BL, Soderhall K. The proPO-system: pros and cons for its role in invertebrate immunity. *Trends Immunol*. 2008; 29:263. [PubMed: 18457993]
- Cerenius L, Soderhall K. The prophenoloxidase-activating system in invertebrates. *Immunol Rev*. 2004; 198:116–126. [PubMed: 15199959]
- Chen CC, Chen CS. *Brugia pahangi*: effects of melanization on the uptake of nutrients by microfilariae in vitro. *Exp Parasitol*. 1995; 81:72–78. [PubMed: 7628569]
- Christophides GK, Zdobnov E, Barillas-Mury C, Birney E, Blandin S, Blass C, Brey PT, Collins FH, Danielli A, Dimopoulos G, Hetru C, Hoa NT, Hoffmann JA, Kanzok SM, Letunic I, Levashina EA, Loukeris TG, Lycett G, Meister S, Michel K, Moita LF, Muller HM, Osta MA, Paskewitz SM, Reichhart JM, Rzhetsky A, Troxler L, Vernick KD, Vlachou D, Volz J, von Mering C, Xu J, Zheng L, Bork P, Kafatos FC. Immunity-related genes and gene families in *Anopheles gambiae*. *Science* (80-). 2002; 298:159–165.10.1126/science.1077136
- Cohuet A, Osta MA, Morlais I, Awono-Ambene PH, Michel K, Simard F, Christophides GK, Fontenille D, Kafatos FC. *Anopheles* and *Plasmodium*: from laboratory models to natural systems in the field. *EMBO Rep*. 2006; 7:1285–1289. [PubMed: 17099691]
- Collins FH, Sakai RK, Vernick KD, Paskewitz S, Seeley DC, Miller LH, Collins WE, Campbell CC, Gwadz RW. Genetic selection of a *Plasmodium*-refractory strain of the malaria vector *Anopheles gambiae*. *Science* (80-). 1986; 234:607–610.
- De Gregorio E, Han SJ, Lee WJ, Baek MJ, Osaki T, Kawabata S, Lee BL, Iwanaga S, Lemaitre B, Brey PT. An immune-responsive Serpin regulates the melanization cascade in *Drosophila*. *Dev Cell*. 2002; 3:581–592. [PubMed: 12408809]
- Gettins PG. Serpin structure, mechanism, and function. *Chem Rev*. 2002; 102:4751–4804. [PubMed: 12475206]
- Grimstone AV, ROTHERAM S, SALT G. An Electron-Microscope Study of Capsule Formation by Insect Blood Cells. *J Cell Sci*. 1967; 2:281–292. [PubMed: 4104129]
- Hillyer JF, Schmidt SL, Christensen BM. Hemocyte-mediated phagocytosis and melanization in the mosquito *Armigeres subalbatus* following immune challenge by bacteria. *Cell Tissue Res*. 2003; 313:117–127. [PubMed: 12838409]
- Hu Y, Wang Y, Deng J, Jiang H. The structure of a prophenoloxidase (PPO) from *Anopheles gambiae* provides new insights into the mechanism of PPO activation. *BMC Biol*. 2016; 14:2.10.1186/s12915-015-0225-2 [PubMed: 26732497]
- Hughes AL, Friedman R. Expression patterns of duplicate genes in the developing root in *Arabidopsis thaliana*. *J Mol Evol*. 2005; 60:247–56.10.1007/s00239-004-0171-z [PubMed: 15785853]
- Jiang H, Kanost MR. The clip-domain family of serine proteinases in arthropods. *Insect Biochem Mol Biol*. 2000; 30:95–105. [PubMed: 10696585]
- Jiang H, Wang Y, Ma C, Kanost MR. Subunit composition of pro-phenol oxidase from *Manduca sexta*: molecular cloning of subunit ProPO-P1. *Insect Biochem Mol Biol*. 1997; 27:835–850. [PubMed: 9474780]

- Kan H, Kim C-H, Kwon H-M, Park J-W, Roh K-B, Lee H, Park B-J, Zhang R, Zhang J, Söderhäll K, Ha N-C, Lee BL. Molecular control of phenoloxidase-induced melanin synthesis in an insect. *J Biol Chem*. 2008; 283:25316–23.10.1074/jbc.M804364200 [PubMed: 18628205]
- Kanost MR, Gorman MJ. Phenoloxidases in Insect Immunity. *Insect Immunology*. 2008;10.1016/B978-012373976-6.50006-9
- Kanost MR, Jiang H. Clip-domain serine proteases as immune factors in insect hemolymph. *Curr Opin insect Sci*. 2015; 11:47–55.10.1016/j.cois.2015.09.003 [PubMed: 26688791]
- Lackie A. Effect of substratum wettability and charge on adhesion in vitro and encapsulation in vivo by insect haemocytes. *J Cell Sci*. 1983; 63:181–190. [PubMed: 6630309]
- Lambrechts L, Morlais I, Awono-Ambene PH, Cohuet A, Simard F, Jacques JC, Bourgouin C, Koella JC. Effect of infection by *Plasmodium falciparum* on the melanization immune response of *Anopheles gambiae*. *Am J Trop Med Hyg*. 2007; 76:475–480. 76/3/475 [pii]. [PubMed: 17360870]
- Li J, Wang X, Zhang G, Githure JI, Yan G, James AA. Genome-block expression-assisted association studies discover malaria resistance genes in *Anopheles gambiae*. *Proc Natl Acad Sci U S A*. 2013; 110:20675–80.10.1073/pnas.1321024110 [PubMed: 24297936]
- Ligoxygakis P, Pelte N, Ji C, Leclerc V, Duvic B, Belvin M, Jiang H, Hoffmann JA, Reichhart JM. A serpin mutant links Toll activation to melanization in the host defence of *Drosophila*. *Embo J*. 2002; 21:6330–6337. [PubMed: 12456640]
- Maccallum RM, Redmond SN, Christophides GK. An expression map for *Anopheles gambiae*. *BMC Genomics*. 2011; 12:620.10.1186/1471-2164-12-620 [PubMed: 22185628]
- Marinotti O, Calvo E, Nguyen QK, Dissanayake S, Ribeiro JM, James AA. Genome-wide analysis of gene expression in adult *Anopheles gambiae*. *Insect Mol Biol*. 2006; 15:1–12. [PubMed: 16469063]
- Marques AC, Vinckenbosch N, Brawand D, Kaessmann H. Functional diversification of duplicate genes through subcellular adaptation of encoded proteins. *Genome Biol*. 2008; 9:R54.10.1186/gb-2008-9-3-r54 [PubMed: 18336717]
- Michalak P. Coexpression, coregulation, and cofunctionality of neighboring genes in eukaryotic genomes. *Genomics*. 2008; 91:243–8.10.1016/j.ygeno.2007.11.002 [PubMed: 18082363]
- Michel K, Budd A, Pinto S, Gibson TJ, Kafatos FC. *Anopheles gambiae* SRPN2 facilitates midgut invasion by the malaria parasite *Plasmodium berghei*. *EMBO Rep*. 2005; 6:891–897. [PubMed: 16113656]
- Michel K, Suwanchaichinda C, Morlais I, Lambrechts L, Cohuet A, Awono-Ambene PH, Simard F, Fontenille D, Kanost MR, Kafatos FC. Increased melanizing activity in *Anopheles gambiae* does not affect development of *Plasmodium falciparum*. *Proc Natl Acad Sci U S A*. 2006; 103:16858–16863.10.1073/pnas.0608033103 [PubMed: 17065316]
- Molina-Cruz A, Dejong RJ, Ortega C, Haile A, Abban E, Rodrigues J, Jaramillo-Gutierrez G, Barillas-Mury C. Some strains of *Plasmodium falciparum*, a human malaria parasite, evade the complement-like system of *Anopheles gambiae* mosquitoes. *Proc Natl Acad Sci U S A*. 2012;10.1073/pnas.1121183109
- Nappi A, Poirié M, Carton Y. The role of melanization and cytotoxic by-products in the cellular immune responses of *Drosophila* against parasitic wasps. *Adv Parasitol*. 2009; 70:99–121.10.1016/S0065-308X(09)70004-1 [PubMed: 19773068]
- Nappi AJ, Christensen BM. Melanogenesis and associated cytotoxic reactions: applications to insect innate immunity. *Insect Biochem Mol Biol*. 2005; 35:443–459. S0965-1748(05)00025-1 [pii]. 10.1016/j.ibmb.2005.01.014 [PubMed: 15804578]
- Park DS, Shin SW, Hong SD, Park HY. Immunological detection of serpin in the fall webworm, *Hyphantria cunea* and its inhibitory activity on the prophenoloxidase system. *Mol Cells*. 2000; 10:186–192. [PubMed: 10850660]
- Paskewitz SM, Andreev O, Shi L. Gene silencing of serine proteases affects melanization of Sephadex beads in *Anopheles gambiae*. *Insect Biochem Mol Biol*. 2006; 36:701–711. [PubMed: 16935219]
- Pinto SB, Lombardo F, Koutsos AC, Waterhouse RM, McKay K, An C, Ramakrishnan C, Kafatos FC, Michel K. Discovery of *Plasmodium* modulators by genome-wide analysis of circulating

- hemocytes in *Anopheles gambiae*. *Proc Natl Acad Sci U S A*. 2009; 106:21270–21275.10.1073/pnas.0909463106 [PubMed: 19940242]
- Riehle MM, Markianos K, Niare O, Xu J, Li J, Toure AM, Podiougou B, Oduol F, Diawara S, Diallo M, Coulibaly B, Ouataro A, Kruglyak L, Traore SF, Vernick KD. Natural malaria infection in *Anopheles gambiae* is regulated by a single genomic control region. *Science* (80-). 2006; 312:577–579.
- Salt G. Experimental Studies in Insect Parasitism. XIII. The Haemocytic Reaction of a Caterpillar to Eggs of its Habitual Parasite. *Proc R Soc B Biol Sci*. 1965; 162:303–318.10.1098/rspb.1965.0040
- Salt G. Experimental studies in insect parasitism—XII. The reactions of six exopterygote insects to an alien parasite. *J Insect Physiol*. 1963; 9:647–669.10.1016/0022-1910(63)90009-X
- Schnitger AK, Kafatos FC, Osta MA. The melanization reaction is not required for survival of *Anopheles gambiae* mosquitoes after bacterial infections. *J Biol Chem*. 2007
- Schwartz A, Koella JC. Melanization of plasmodium falciparum and C-25 sephadex beads by field-caught *Anopheles gambiae* (Diptera: Culicidae) from southern Tanzania. *J Med Entomol*. 2002; 39:84–88. [PubMed: 11931276]
- Singh LN, Hannehalli S. Functional diversification of paralogous transcription factors via divergence in DNA binding site motif and in expression. *PLoS One*. 2008; 3:e2345.10.1371/journal.pone.0002345 [PubMed: 18523562]
- Smith CL, DeLotto R. A common domain within the proenzyme regions of the *Drosophila* snake and easter proteins and Tachypleus proclotting enzyme defines a new subfamily of serine proteases. *Protein Sci*. 1992; 1:1225–6.10.1002/pro.5560010915 [PubMed: 1304399]
- Sugumaran M. Comparative Biochemistry of Eumelanogenesis and the Protective Roles of Phenoloxidase and Melanin in Insects. *Pigment Cell Res*. 2002; 15:2–9.10.1034/j.1600-0749.2002.00056.x [PubMed: 11837452]
- Takahashi D, Garcia BL, Kanost MR. Initiating protease with modular domains interacts with β -glucan recognition protein to trigger innate immune response in insects. *Proc Natl Acad Sci U S A*. 2015; 112:13856–61.10.1073/pnas.1517236112 [PubMed: 26504233]
- Veillard F, Troxler L, Reichhart J-M. *Drosophila melanogaster* clip-domain serine proteases: Structure, function and regulation. *Biochimie*. 2015.10.1016/j.biochi.2015.10.007
- Volz J, Muller HM, Zdanowicz A, Kafatos FC, Osta MA. A genetic module regulates the melanization response of *Anopheles* to Plasmodium. *Cell Microbiol*. 2006; 8:1392. [PubMed: 16922859]
- Voordeckers K, Pougach K, Verstrepen KJ. How do regulatory networks evolve and expand throughout evolution? *Curr. Opin Biotechnol*. 2015; 34:180–8.10.1016/j.copbio.2015.02.001
- Wang Y, Lu Z, Jiang H. *Manduca sexta* prophenoloxidase activating proteinase-3 (PAP3) stimulates melanization by activating proPAP3, proSPHs, and proPOs. *Insect Biochem Mol Biol*. 2014; 50:82–91.10.1016/j.ibmb.2014.04.005 [PubMed: 24768974]
- Waterhouse RM, Kriventseva EV, Meister S, Xi Z, Alvarez KS, Bartholomay LC, Barillas-Mury C, Bian G, Blandin S, Christensen BM, Dong Y, Jiang H, Kanost MR, Koutsos AC, Levashina EA, Li J, Ligoxygakis P, Maccallum RM, Mayhew GF, Mendes A, Michel K, Osta MA, Paskewitz S, Shin SW, Vlachou D, Wang L, Wei W, Zheng L, Zou Z, Severson DW, Raikhel AS, Kafatos FC, Dimopoulos G, Zdobnov EM, Christophides GK. Evolutionary dynamics of immune-related genes and pathways in disease-vector mosquitoes. *Science* (80-). 2007; 316:1738–1743.10.1126/science.1139862
- Yassine H, Kamareddine L, Osta MA. The mosquito melanization response is implicated in defense against the entomopathogenic fungus *Beauveria bassiana*. *PLoS Pathog*. 2012; 8:e1003029.10.1371/journal.ppat.1003029 [PubMed: 23166497]
- Zhu Y, Wang Y, Gorman MJ, Jiang H, Kanost MR. *Manduca sexta* serpin-3 regulates prophenoloxidase activation in response to infection by inhibiting prophenoloxidase-activating proteinases. *J Biol Chem*. 2003; 278:46556–46564. [PubMed: 12966082]
- Zou Z, Shin SW, Alvarez KS, Kokoza V, Raikhel AS. Distinct Melanization Pathways in the Mosquito *Aedes aegypti*. *Immunity*. 2010; 32:41–53. [PubMed: 20152169]

Highlights

- The clip-domain serine protease CLIPB8 is required for melanotic tumor formation in *Anopheles gambiae*.
- CLIPB8 is a functional serine proteinase with trypsin-like specificity.
- CLIPB8 is a non-redundant member of the prophenoloxidase (proPO) activation cascade upstream of proPO-activating proteases.
- Epistasis analyses confirm mechanistic link between melanotic tumor formation and mosquito survival.

Figure 1. *CLIPB8* knockdown partially reverts the *SRPN2* depletion phenotype

(A) Abdominal images 12d post dsRNA injection. (B) Total melanotic area per abdomen decreased significantly in *CLIPB8/SRPN2*kd mosquitoes as compared to *SRPN2*kd animals (Median with interquartile range, U-Test, $P = 0.0002$). (C) *CLIPB8/SRPN2*kd also partially decreased the mortality rate observed in *SRPN2*-depleted mosquitoes. Survival curves with different letters were statistically significantly different (Log-rank Test, $P < 0.0001$).

Insect Biochem Mol Biol. Author manuscript; available in PMC 2017 April 01.

Figure 3. SRPN2 does not inhibit CLIPB8 *in vitro*

Purified rSRPN2 and activated rCLIPB8_{Xa} were incubated at increasing molar ratios. The residual FVRase activities were plotted as mean ± S.D. (n=3) against the corresponding molar ratios of serpin and active proteinase. CLIPB8_{Xa} activity was marginally affected by the presence of SRPN2, with a theoretical Stoichiometry of inhibition (SI) of 55.

[illegible]

Figure 5. CLIPB8 and B9 do not interact directly

rCLIPB8_{Xa} does not cleave recombinant proCLIPB9 (A) or vice versa (B) as determined by Western blot analysis using anti-CLIPB8 and anti-CLIPB9-specific antibodies. (A) Recombinant proCLIPB8_{Xa} was activated by Factor Xa *in vitro* and incubated with recombinant wild-type proCLIPB9. The resulting Western blot was probed sequentially with anti-CLIPB8 and anti-CLIPB9 antibody. While anti-CLIPB8 antibody was able to detect a band corresponding to the proteinase domain of activated CLIPB8, no cleaved proteinase form was detectable by anti-CLIPB9 antibody. (B) The reciprocal experiment did not detect any cleavage product for wild-type CLIPB8 after incubation with activated CLIPB9_{Xa}. Both polyclonal antibodies were only able to detect the antigen they were raised against, and no cross reactivity to other proteins, including Factor Xa was observed. Circles, proteinase zymogen; squares, proteinase domain.

Figure 6. Analysis of the PO activation cascade by tkd of *SRPN2*, *CLIPB8* and *B9*

(A) Total melanotic area per abdomen in dkd and tkd mosquitoes was significantly reduced as compared to *SRPN2*kd animals (Median $\pm$ interquartile range, Kruskal-Wallis test, $P < 0.0001$, Dunns post test, $P < 0.05$). (B) Survival curves of dkd and tkd mosquitoes as compared to *SRPN2*kd. Data were obtained from three independent biological replicates. Survival curves with different letters were statistically significantly different (Log-rank Test, $P < 0.0001$)