New insights on *Drosophila* antimicrobial peptide function in host defense and beyond

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Since the first animal antimicrobial peptides (AMPs) were discovered in insects, Drosophila melanogaster has emerged as a powerful model for their characterization. Drosophila AMPs have been used extensively to monitor the activity of the Toll and Imd NF-kB pathways, but little was known of their precise functions. In this review, we summarize recent findings on the function of Drosophila AMPs not only for antimicrobial defense, but also in the gut, tumor control, and neurology. The integration of these new studies allows a new framework to be drawn that explains how AMPs can contribute simultaneously to microbe killing whilst also regulating important host cellular functions. These functions require that AMPs target not only negatively charged microbes but also aberrant host cells.

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Introduction

Antimicrobial peptides (AMPs) are small, positively charged peptides that contribute to innate defenses by targeting the negatively charged membranes of microbes [1,2]. Upon encountering microbial cell envelopes, AMPs get embedded in the hydrophobic regions of lipid membranes leading to membrane destabilization and ultimately cell death [3]. Since the first animal AMPs were discovered in silk moths [4], insects and particularly Drosophila melanogaster AMPs have commanded a great deal of attention. There are currently seven well-characterized families of inducible AMPs in D. melanogaster, including 21 AMP/AMP-like genes (Box 1). The activities of these AMPs have been determined either in vitro or deduced by comparison with homologous peptides of other insects: Drosomycin (seven genes) and Metchnikowin show antifungal activity [5,6]; Cecropins (four inducible genes) and Defensin have both antibacterial and some antifungal activities [7−10]; and Drosocin, Attacins (four genes) and Diptericins (two genes) primarily exhibit antibacterial activity [11−15]. While most of these genes are strongly induced in the fat body in response to systemic infection, many show specific patterns of expression in tissues such as the trachea, gut, ganglia, or reproductive tracts [16,17]. In the systemic response following microbial recognition, these AMPs are regulated by the Toll and Imd NF-kB signaling pathways. Accordingly, AMPs are often used as readouts to monitor the activity of these immune pathways. Beyond the well-known AMPs, there are a number of other short peptides induced upon infection whose activities await characterization. Over 15 years after their initial discovery [18], one group of peptides regulated by the Toll pathway was united as the 'Bomanins,' which share a 16-residue domain [19]. A deletion removing ten of the twelve Bomanin genes revealed that they play an essential role in defense against Gram-positive bacteria and fungi [19]. While Bomanins contribute to microbial killing in the fly hemolymph, microbicidal activity in vitro has not yet been demonstrated [20]. Owing to technical limitations now solved by CRISPR/ Cas9, it is only recently that generating loss-of-function mutants for AMP genes has become approachable. Here we summarize recent functional data on AMPs in host defense, microbiota control, and other roles beyond infection as these immune peptides have been implicated in brain function, tumor control, aging, and neurodegenerative disease. We then try to unify these findings by proposing a framework for how AMPs can work both in host defense and other physiological processes. AMPs as antimicrobials controlling pathogens Many studies have described the action of Drosophila AMPs using purified or recombinant peptides, revealing that they display potent antimicrobial activity in vitro. However it was unclear to what extent these AMPs contribute to host defense in vivo. Previously, Tzou et al. [10] combined immune-deficient mutations with overexpression of endogenous AMPs, rescuing survival in their immune deficient flies. The rescue phenotypes observed in this study were consistent with previous in vitro studies; for instance, the fly Defensin was effective in suppressing Gram-positive bacterial growth [21]. In a separate study, knockdown of AttC or DptB by RNAi resulted in increased alphavirus replication upon infection [22]. Recently, Hanson et al. [23] deleted multiple AMP families of D. melanogaster, generating various

individual and combined AMP mutants, including a strain lacking ten AMP genes. This study shows that the classic Drosophila AMPs primarily defend against Gram-negative bacteria and some fungi. Surprisingly, flies lacking these classic AMPs showed little susceptibility to Gram-positive bacterial infection, while Bomanins were essential to defense against Gram-positive bacteria and fungi. Collectively, these studies link the logical organization of Toll and Imd signaling, showing that Toll or Imd-specific microbes elicit the production of downstream effectors required to fight the classes of microbes that activate these pathways (Figure 1). This functional in vivo study also shows that groups of AMPs function in additive or synergistic fashions, complementing recent findings in vitro and in beetles [24,25]. However it also highlighted highly specific and important roles for individual AMPs in host-pathogen interactions. For instance, Diptericin alone appears to be required for defence against Providencia rettgeri infection, despite Diptericin being dispensable to defense against other Providencia species. The specific interaction between Diptericin and P. rettgeri is furthered by the observation that an amino acid polymorphism in the Diptericin A gene predicts survival to P. rettgeri [26]. Together, this suggests that Diptericins contribute to survival to P. rettgeri-like bacteria in the wild. Another example of specificity is the critical requirement of the proline-rich AMP Drosocin in defense against Enterobacter cloacae [23]. It would have been impossible to predict such unbridled specificity from in vitro approaches, highlighting that our present understanding of the precise roles for AMPs in an in vivo context is just the tip of the iceberg. AMPs in microbiota control In contrast to systemic immunity, the immune responses in epithelial surfaces such as the gut must tolerate the presence of beneficial microbes while responding to and eliminating potential pathogens. This implies a tight and specific regulation of the immune response in epithelia, carefully balancing immune activation and bacterial tolerance. In plants, hydra, other insects, and mammals, it has been proposed that the release of AMPs not only suppresses pathogens, but also shapes the microbiota by promoting colonization by beneficial microbes from the environment [27,28]. The role of AMPs in shaping the intestinal microbiota has not been characterized to the same extent in Drosophila. AMPs are mostly produced in the anterior midgut where they are thought to eliminate ingested pathogens; though AMP expression patterns in the digestive tract are complex [17,29]. Transcriptome analyses comparing the gut transcriptome of germ-free and conventionally reared flies have shown that the microbiota triggers the expression of several AMP genes in the Drosophila gut, notably Attacins AttA and AttD mostly regulated by the Imd pathway, and also Drosomycin-like 2 and 3 regulated by JAK-STAT [30,31]. As microbiota load increases upon aging, expression of AMPs increases in a compensatory manner [32,33]. The higher bacterial count in the gut of Imd-deficient flies supports the notion that intestinal AMPs control the microbiota. However, the Imd pathway has other immune functions in the gut such as regulating enterocyte shedding [34] and digestive enzymes [91], and Duox-dependent and Nox-dependent production of reactive oxygen species [35]. Peristaltic movement and acidity could also be major players in the regulation of the gut microbiota [36–38]. It has been proposed that Drosophila symbiotic microbes promote Imd signaling for the production of immune tolerance genes rather than production of antibacterial agents [39]. Accordingly, the expression of several AMP genes, but not negative regulators (e.g. PGRP-LB, PGRP-SC) is repressed in the gut by the transcription factor Caudal. In caudal mutants with high AMP expression in the gut, there is a shift in microbiota composition towards deleterious microflora [84]. This supports the notion that chronic AMP expression might actually select for AMP-resistant members of microbial communities that would lead to increased intestinal damage. Use of AMP mutant flies may help to better define the role of AMPs amongst other mechanisms in the control of microbiota. Antitumor role of AMPs In vitro studies show that some AMPs have anti-tumor activity, and these AMPs are currently the focus of translational studies to be used as a treatment in combination with cellular antitumor therapy [40]. Whether these activities apply in vivo for endogenous AMPs, and what mechanisms allow these molecules to attack aberrant host cells are debated. Two recent studies [41,42] have highlighted the antitumor effect of Drosophila AMPs. Araki et al. [41] found that several AMP genes are upregulated in Drosophila mxc$_{mbn1}$ larvae, a mutation causing hematopoietic tumors. Downregulation of Toll and Imd immune pathways exacerbated tumor growth, while overexpression of specific AMPs significantly suppressed hematopoietic organ hyperplasia. Their study reveals that some AMPs have cytotoxic effects that enhance apoptosis exclusively in the tumor cells in vivo. In another study, Parvy et al. [42], demonstrated that Defensin has potent anti-tumor activity in a disc-large (dlg) imaginal disc tumor model; Parisi et al. previously showed that humoral components of the immune system restrict dlg tumor growth [43]. In their recent study, Parvy et al. [42] show that Defensin, remotely secreted from tracheal and fat body tissues, cooperates with the Drosophila TNF-like molecule Eiger to drive tumor cell death. Interestingly, Eiger produced by macrophages provokes exposure of phosphatidylserine (PS) in tumor cells altering the charge of the outer leaflet of the plasma membrane. The addition of PS would make these tumors selectively sensitive to the action of

Defensin. Using a Defensin mutation, they further revealed that Defensin contributes to tumor cell elimination by promoting apoptosis. Parvy et al., provides one of the first in vivo demonstrations for an endogenous AMP acting as an anti-cancer agent, and describes a mechanism that explains tumor cell sensitivity to the action of AMPs [42]. Further studies should decipher whether AMPs indeed contribute to tumor elimination in more physiologically relevant contexts, and what AMP characteristics contribute to tumor elimination. Impact of AMPs on brain function and neurodegeneration The potential for AMPs to act in the normal functioning of the nervous system is implied by commonalities between AMPs and neuropeptides (reviewed extensively in Ref. Brogden et al. [44]). Amongst many immune processes, various Drosophila antimicrobial peptides may be involved in gene networks relating to memory [45]. Surprisingly, the antibacterial peptide Diptericin B (DptB) and the glucan binding like 3 gene (GNBP-like3) appear to be specifically required for long-term memory formation [46]. Importantly, the tissue of expression played a key role in memory effects: GNBP-like3 expression derived from neurons, while DptB was expressed by the perineural fat body specific to the fly head. How non-cell-autonomous DptB can affect memory formation is puzzling, but an unknown host factor may import AMPs like DptB from the hemolymph into nervous tissue. In Caenorhabditis elegans nematodes, the antimicrobial peptide NLP-29 drives neurodegeneration through binding to its cognate G-protein coupled receptor NPR-12 [47]. Finally, Toda et al. [48] recently described a Drosophila neuropeptide nemuri, with similarity to a vertebrate cathelicidin that both regulates sleep and promotes survival upon infection. Globally these studies suggest that certain AMPs could be important regulators of brain function; however, how they contribute to these processes remains an enigma. Recent evidence pertaining to neurodegenerative diseases has also implicated AMPs as causative agents. There is a growing appreciation that the Alzheimer's peptide Amyloid-ß is in fact an antimicrobial peptide [49], and that Alzheimer's disease may in part be an immune process [50]. An antimicrobial role for the Parkinson's disease protein a-synuclein has also been described, further supporting a link between neurodegenerative diseases and innate immune mechanisms [51,52]. However the precise fashions through which AMPs promote neurodegeneration remain unresolved. While functional evidence has not established AMPs as causative agents of neurodegeneration in flies, a number of studies implicate Toll and Imd NF-kB immune signaling in neurodegenerative diseases. Toll signalling molecules are involved in normal brain development [53], and suppressing Toll activity rescues neurodegeneration in Drosophila models of ALS, Amyloid-ß toxicity, and traumatic brain injury [54–56]. Meanwhile in the fly model for Ataxia-Telangiectasia (ATM), loss-of-function of ATM leads to Relish-dependent neurodegeneration [57]. Similarly, knockout of the negative regulator of Imd signalling dnr1 leads to neurodegeneration associated with a strong increase in AMP expression in the head. Blocking AMP expression by silencing Relish in glia suppresses dnr1-induced neurodegeneration [58]. An interesting recent study further showed onset of neurodegeneration in flies correlates with aging-associated increases in antimicrobial peptide expression in the head [59], and overexpression of AMPs is sufficient to promote neurodegenerative symptoms [58]. Meanwhile in human disease models using a rough-eye phenotype, knockdown of Relish and even individual AMPs can somewhat rescue eye morphology following heterologous expression of disease proteins [85]. Some studies also implicate the intestinal microbiota as a contributor to age-dependent neurodegeneration and suggest that this effect is mediated by Imd signalling [59,60]. While there is no doubt that the Imd pathway contributes to neurodegeneration and brain aging, the precise role of AMPs and other Imd-related processes remains to be investigated. It is noteworthy that components of the Imd pathway can regulate autophagy in the brain and could also contribute to Imd mediated neurodegeneration [61,62,86]. The key question is now to determine whether AMPsare passive bystanders in neuronalprocesses, or if they are active players in neuronal homeostasis. AMPs and aging Aging in humans is associated with senescence of the immune system with two symptoms: reduced ability to combat infection and a chronic activation of inflammation (aka 'inflammaging'). This is also observed in Drosophila that display an age-dependent reduction in hemocyte number and activity, and increasing lag in mounting the systemic antimicrobial response [63–65]. Importantly in the present context, an increase in antimicrobial peptide expression is a hallmark of aging in Drosophila [66,67]. It is tempting to speculate that this increase is somehow correlated with increased abundanceofbacteriain thegut.Howevera recent study showed that while the downstream components of the Imd pathway were involved in increased AMP expression with aging, Imd itself was not associated with this increased AMP expression [68]. This increased systemic activation of immune genes is seemingly derived through a separate mechanism from canonical Imd signaling, possibly through insulin signaling (e.g. FOXO), which is known to drive expression of some AMPs [69]. Age-associated increase in oxidative stress is also likely to increase the involvement of immune processes to control damaged tissues [70,71]. Supporting the involvement of AMPs in response to oxidative stress, Diptericin overexpression rescues viability in flies subjected to hyperoxia

[72]. The aging-associated increase in systemic AMP expression could contribute positively or negatively to aging, or may simply be a symptom of aging (discussed in Ref. Min and Tatar [64]). All these studies on neurodegeneration and aging converge on the notion that AMPs are beneficial in early stages of life by fighting infection, but may be deleterious in older flies. Such interactions are supported by trade offs between fitness and inducible immune defenses [73,74,87]. Conclusion: a general framework to understand the role of AMPs on host cells Functional studies have now validated the general roles for AMPs in host defense; however, a surprising observation is the high degree of specificity for some AMPs in host-pathogen interactions. Also changing is the notion that Drosophila AMPs are evolutionarily static as recent studies indicate they evolve rapidly at the sequence level under both diversifying and balancing selective pressures (Box 2 on AMP gene evolution). These findings of Darwinian selection on AMPs come at a time when functional studies are highlighting multitudinous roles for AMPs in various cellular processes beyond infection. While famous for its role in the antibacterial immune response, the Imd pathway is also involved in many processes such as cell competition, virus control, resistance to dessication, cell delamination, resistance to hyperoxia or hypoxia, autophagy, and more. The existence of AMP-deficient lines now allows us to disentangle the precise role of AMPs compared to other downstream targets of Imd in these processes. We now need a general framework to understand how microbe-killing AMPs can also target host cells. The common opinion is that AMPs as cationic molecules specifically target bacteria and fungi due to their negatively charged membranes, while eukaryotic membranes are protected by virtue of being more positively charged and by containing cholesterol. The fact that AMPs can target specific host cells such as tumor cells suggests that these eukaryotic cells undergo major changes at the membrane that render them susceptible to AMPs. Phosphotidylserine is a negatively charged lipid found in the inner membrane layer, and PS exposure is used as an 'eat-me' signal to recruit phagocytes to apoptotic cells [89,90]. The study by Parvy et al. [42] suggests that PS exposure is not just a signal for phagocytes but could make cancer cells sensitive to the action of AMPs. Thus, both cellular phagocytosis and humoral AMPs contribute to eliminate abnormal cells that are marked for elimination by changes in their membrane. PS exposure could therefore be a mechanism signaling aberrant non-self to the immune system that allows control of tumors. Brain tissues are extremely enriched in PS [75], and it is tempting to speculate that exposure of PS by neurons marks them to be targeted by both glial cells and AMPs (Figure 2). Increased immune expression, experienced following infection or injury, could lead to AMP-mediated neuron destruction culminating in neurodegeneration. Indeed, events of neurodegeneration are often increased upon infection and brain injury. It would be interesting to know if PS exposure can also modulate AMP-neuron interactionsin contexts such as memory formation. Changes in membrane composition may thus underlie the interaction of the immune system with altered self in both normal and pathologic situations. Future studies need to decipher how the immune system is activated by tumors or in the nervous system. Such studies could reveal further synergy of cellular and humoral responses to promote tumor elimination and perhaps even neurodegeneration. Far from being simple boring immune effectors, AMPs appear to be involved in physiological processes beyond expectation. Studies in Drosophila utilizing its exquisite genetics may shed light on their role, an important next step for rapid advancement considering the complexity of mammalian systems. Conflict of interest statement Nothing declared.

Acknowledgements

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Figure captions

Box 1 Summary of Drosophila AMPs

The 3D structures of antimicrobial peptides are known for some AMPs (left) [76,77]. The present summary of AMP-like genes and Bomanins (right) describes gene family members, genomic location, concentration in vivo upon immune activation, size, and gene-specific characteristics.
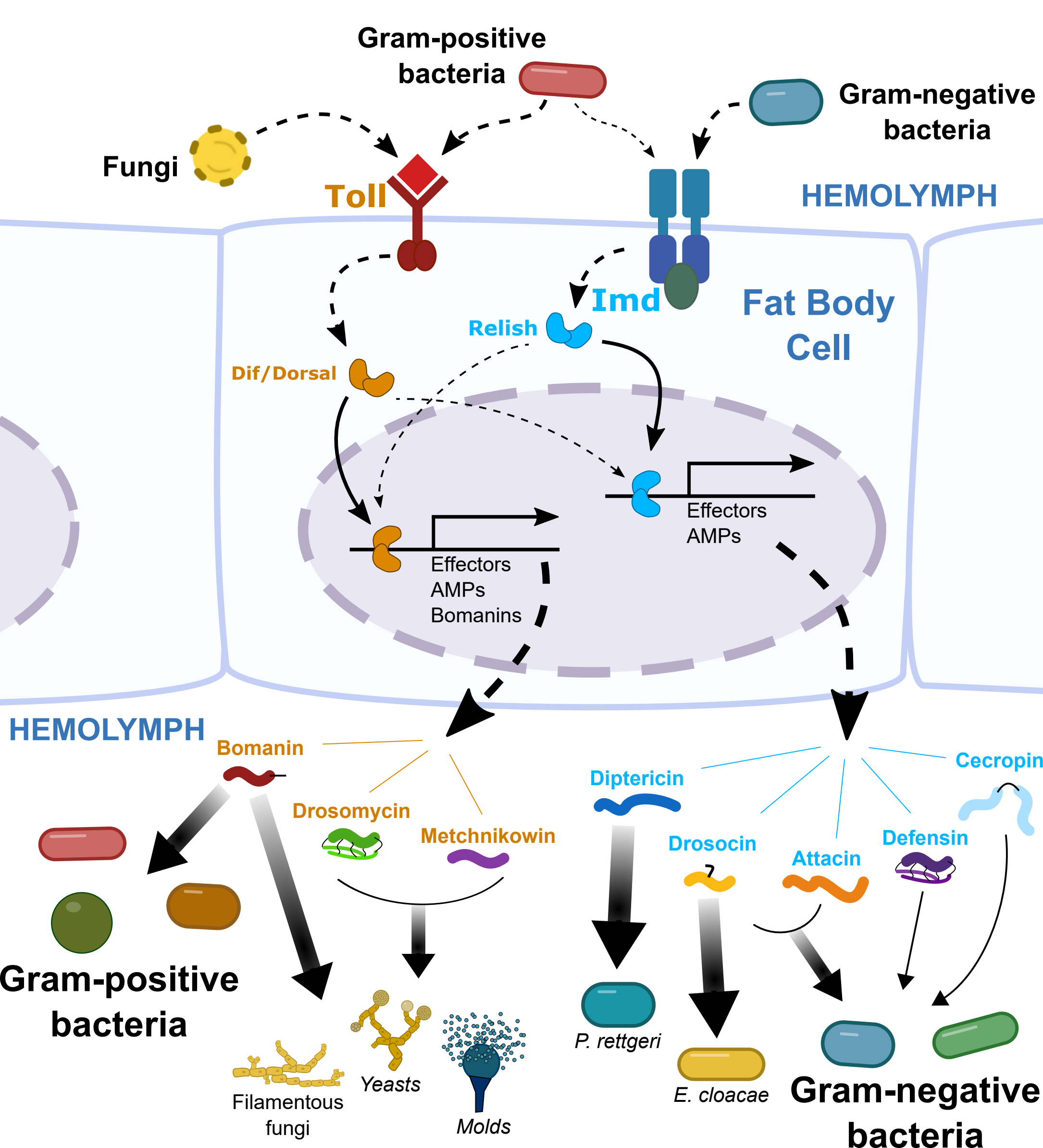

Figure 1

A simplified overview of the systemic antimicrobial response. Recognition of certain pathogen types leads to downstream production of AMPs specifically useful against those pathogen types. In most cases these effectors show broad-spectrum importance against many pathogens (e.g. Bomanin, the combined action of Drosocin, Attacin, and Diptericin). However in some instances, specific AMPs are the primary contributors to a successful defense response (Diptericin against P. rettgeri, Drosocin against E. cloacae).
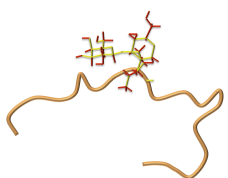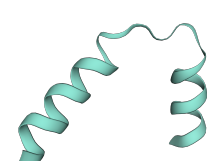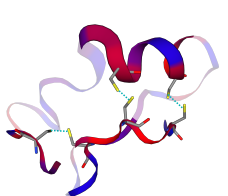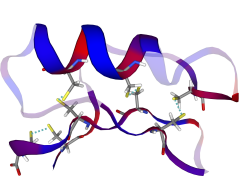

Box 2 AMP gene evolution

AMP genes show widespread copy number variation in different lineages, suggesting that duplication and gene loss play an important role in AMP adaptive evolution to pathogens [78]. However AMP duplications in Drosophila tend to largely resemble their ancestral state (e.g. Cecropins, Attacins, Bomanins), which initially suggested that AMP sequences themselves were evolutionarily static, and instead host-pathogen immune arms races played out at the level of recognition and signaling [78,81]. More recently, increased available sequence data and improved detection algorithms have established a new view of Drosophila AMP evolution that proposes AMP sequence responds to shifts in host ecology and associated pathogen pressures. Rapid evolution of Diptericin sequence has been observed within Drosophila [80], and convergent evolution towards Diptericin B-like genes has occurred in both fruit-feeding Drosophilid and Tephritid flies [88]. At the population level, balancing selection maintains polymorphisms in many AMPs [82], possibly responding to seasonal variation or other dynamic selective pressures [83]. Supporting the notion of dynamic pathogen pressures, Diptericin A null alleles are segregating in African populations [88], and balancing selection on Diptericin A maintains a Serine/Arginine polymorphism that strongly predicts susceptibility to P. rettgeri infection [26□□]. As Diptericin is seemingly the only AMP necessary for defense against this bacteria [23□□], such incredibly specific roles for individual AMPs in host defense defies previous logic that AMPs contribute in largely redundant fashions, and cements the idea that AMPs are key mediators of defense against infection. Future studies should evaluate the consequences of favored alleles on AMP activity in host defense as well as possible trade-offs involving AMPs beyond infection.
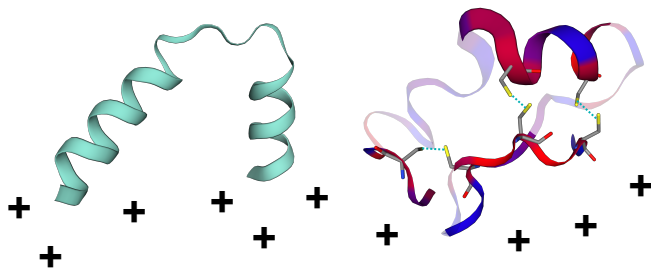

Figure 2

Model for Antimicrobial peptide activity in different contexts. AMPs are small cationic and amphipathic peptides that interfere with the negatively charged membranes of microbes (right). Because of their amphipathic nature and positive charge, AMPs can bind to the membrane and form pores or otherwise disrupt membrane integrity. Eukaryotic cells are usually insensitive to AMPs as their membranes are less negatively charged than microbes and contain cholesterol (left). Recent studies have shown that certain cancer cells expose PS at their surface, making them more negatively charged (middle). It is possible that PS exposure in various cells (and possibly other membrane changes that alter their assymetry), not only provide an 'eat-me' signal to phagocytes, but also makes them susceptible to AMPs. Thus, changes in the membrane (such as PS exposure) provide a general mechanism to signal 'abberant cells' to be targeted for elimination.

Dpt

Unknown

Att

Unknown

Dro

Cec

Def

Drs

Mtk

Unknown

AMP family	Gene	Location	[in vivo]	Size (AA)	Characteristics	Immune Expression
Diptericin	DptA	2R (55F)	0.5 µM	83	P-rich and G-rich domains, C-terminus amidated, O-glycosylated Thr^8, Asp^{52}	Imd
	DptB	2R (55F)		32, 67	Furin cleavage produces two mature peptides: one uncharacterized P-rich peptide and one G-rich with C-terminus presumably amidated	Imd, other
Attacin	AttA	2R (51C)		190	Furin cleavage produces two mature peptides: uncharacterized 9AA short peptide and Attacin G-rich peptide, C-terminus amidated	Imd, Toll, other
	AttB	2R (51C)		190	Furin cleavage produces two mature peptides: uncharacterized 9AA short peptide and Attacin G-rich peptide, C-terminus amidated	Imd, Toll, other
	AttC	2R (50A)		23, 195	Furin cleavage produces two mature peptides: MPAC (P-rich) and Attacin (G-rich), C-terminus amidated	Imd, other
	AttD	3R (90B)		115	Attacin G-rich domain, lacks a signal peptide	Imd
Drosocin	Dro	2R (51C)	40 µM	19	Furin cleavage, P-rich, O-glycosylated Thr^{11}, and also a 22AA uncharacterized C-terminal peptide	Imd, other
Cecropin	CecA1	3R (99E)	20 µM	39	Alpha-helical, C-terminus amidated	Imd
	CecA2	3R (99E)		39	Alpha-helical, C-terminus amidated	Imd
	CecB	3R (99E)		39	Alpha-helical, C-terminus amidated	Imd
	CecC	3R (99E)		39	Alpha-helical, C-terminus amidated	Imd
	Anp	3R (99E)		34	Alpha-helical	ejaculatory duct (males)
Defensin	Def	2R (46D)	1 µM	40	Furin cleavage produces two peptides: 30AA uncharacterized peptide and Defensin peptide with disulfide bonds mediated by cysteine bridges	Imd
Drosomycin	Drs	3L (63D)	100 µM	44	Disulfide bonds mediated by cysteine bridges	Toll, Imd
	Drs-like1	3L (63D)		44	Disulfide bonds mediated by cysteine bridges	
	Drs-like2	3L (63D)		50	Disulfide bonds mediated by cysteine bridges	JAK-STAT
	Drs-like3	3L (63D)		45	Disulfide bonds mediated by cysteine bridges	JAK-STAT
	Drs-like4	3L (63D)		44	Disulfide bonds mediated by cysteine bridges	
	Drs-like5	3L (63D)		44	Disulfide bonds mediated by cysteine bridges	
	Drs-like6	3L (63D)		46	Disulfide bonds mediated by cysteine bridges	
Metchnikowin	Mtk	2R (52A)	10 µM	26	P-rich	Toll, other
Bomanin *(AMP-like)*	IM1-type (6 genes)	2R (55C)	10-100 µM	16	16-residue Bomanin domain	Toll
	CG5778-type (3 genes)	2R (55C), 3R (94A)		41-97	Bomanin domain with C-terminal tail	Toll
	IM23-type (3 genes)	2R (55C), 3R (94A)		78-134	2 Bomanin domain repeats with intermediate linker	Toll