

# Short-Form Bomanins Mediate Humoral Immunity in *Drosophila*

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## Keywords

Antifungal activity · Antimicrobial peptides · Bomanins · *Drosophila* · Effector · Host defense · NF- $\kappa$ B · Toll receptor

## Abstract

The Bomanins (Boms) are a family of a dozen secreted peptides that mediate the innate immune response governed by the *Drosophila* Toll receptor. We recently showed that deleting a cluster of 10 *Bom* genes blocks Toll-mediated defenses against a range of fungi and gram-positive bacteria. Here, we characterize the activity of individual Bom family members. We provide evidence that the Boms overlap in function and that a single *Bom* gene encoding a mature peptide of just 16 amino acids can act largely or entirely independent of other family members to provide phenotypic rescue in vivo. We further demonstrate that the *Boms* function in *Drosophila* humoral immunity, mediating the killing of the fungal pathogen *Candida glabrata* in an in vitro assay of cell-free hemolymph. In addition, we find that the level of antifungal activity both in vivo and in vitro is linked to the level of *Bom* gene expression. Although Toll dictates expression of the antimicrobial peptides (AMPs) drosomycin and metchnikowin, we find no evidence that Boms act by modifying the expression of the mature forms of these antifungal AMPs.

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## Introduction

The innate immune system provides essential defenses against pathogens in both plants and animals, and is the sole basis for defense in all nonvertebrates. Substantial insight into this system grew out of the discovery of the Toll signaling pathway and its translation from insects to humans [1, 2]. We now have a fairly comprehensive picture of how pathogen invasion of body tissues is recognized and how an ensuing cascade of signaling triggers immune defense activation [3, 4]. In many organisms, these defenses are demonstrably essential: infection of wild-type hosts by a range of bacteria, fungi, or viruses results in minimal morbidity or mortality, whereas hosts with compromised innate immune functions succumb, often rapidly. Nevertheless, a fundamental question remains: what effector activities mediate survival in the absence of a lymphocyte-directed adaptive response?

The study of innate antimicrobial effectors began in the 1920s with the discovery of the enzyme lysozyme, which Fleming [5] found can lyse bacterial cell walls. In the 1970s, studies on insects identified activities in hemolymph that kill bacteria or fungi and increase in response to the activation of innate defenses [6, 7]. This culminated in the identification and isolation of the first 3 antimicrobial peptides (AMPs) in 1980 [8]. There was a flurry of interest in AMPs as potential therapeutic agents, and a general assumption that they represent the primary effec-

tor mechanism. However, very few mutations have been reported that link the inactivation of particular AMP genes to an immune deficiency. Moreover, in *Drosophila*, such a link was established, not for an AMP, but rather for a set of novel effectors, the *Bomanin*, or *Bom*, gene family.

*Bomanin* genes encode 3 forms of mature peptide, short, tailed, and bicipital, that are secreted into the hemolymph upon Toll pathway activation [9, 10]. Ten of the twelve defined *Bomanin* genes are tandemly arrayed in a cluster at position 55C on the *Drosophila melanogaster* second chromosome. We have demonstrated that excising this cluster disrupts immune defenses to the same extent and with the same specificity as blocking the entire Toll pathway [10]. This finding raises a number of questions regarding Bom peptides as immune effectors. Are they humoral effectors or do they promote cellular immunity? Do they complex with one another or do they act as individual peptides? Is each Bomanin specific for a different pathogen?

In this paper, we explore the effector role of Boms via a two-pronged approach. We begin by using transgenic studies to define the activity of particular *Bom* genes. We then use microbiological and biophysical assays to define the nature of Bom activity in vitro. Together, these 2 approaches reveal that the expression of individual Boms in hemolymph can provide both resistance to infection in vivo and pathogen killing in vitro.

## Materials and Methods

### *Flies and Transgenic Strains*

Flies were raised at 25 °C on standard corn-meal molasses food. The *w<sup>1118</sup>* strain was used as the wild type. *BomΔ<sup>55C</sup>* flies were described previously [10].

All transgenic constructs were based on a genomic fragment (hereafter referred to as “55C-Right”) that encodes the four 55C *Bom* genes (*Bom3*, *Bom836*, *Bom065*, and *Bom068*) remaining in the *BomΔ<sup>left</sup>* second chromosome deficiency [10]. Specifically, genomic DNA encompassing region 2R:18,387,209 to 18,391,496 was amplified and cloned into attB-pNot-CaSpeR [11]. Next, individual *Bom* ORFs were deleted using PCR SOEing [12], in such a way as to generate 4 constructs, each deleted for 3 of the 4 *Bom* genes in 55C-Right.

To generate transgenic constructs expressing a single *Bom* gene under the control of the *Bom3* promoter (*pBOM3*), the starting point was the 55C-Right construct with *Bom3* intact and the remaining *Bom* ORFs deleted. The *Bom3* ORF was then swapped out for another ORF using PCR SOEing, placing the introduced ORF directly downstream of *pBOM3*.

All constructs were introduced into the *Drosophila* genome by ΦC31-mediated transgenesis [13] at an *attP* landing site located at 86Fb (BDSC stock #24749). Stable lines established for each con-

struct carry homozygous transgenes on the third chromosome in a *BomΔ<sup>55C</sup>* background.

### *CRISPR Deletion of 55C Region*

Cloning and injections were performed using established protocols [14]. A pair of gRNAs designed to delete the region 2R:18,380,931 to 18,391,053 were cloned into pU6-BbsI-chiRNA (Addgene plasmid # 45946). Homology arms (766 bp left and 840 bp right) were cloned into pDsRed-attP (Addgene plasmid #51019). PBS-Hsp70-Cas9 (Addgene plasmid #46294) was used as the Cas9 source. Constructs were injected into *w<sup>1118</sup>* flies.

### *Microbial Culture*

*Micrococcus luteus* was cultured overnight in LB media at 37 °C, heat-killed by autoclaving for 20 min at 121 °C, and then concentrated to OD<sub>600</sub> = 300 in 20% glycerol. For infection experiments, the *Candida glabrata* strain CBS 138 [ATCC 2001] was cultured overnight in YPD media at 37 °C and concentrated to OD<sub>600</sub> = 100 in fresh PBS containing 0.01% Tween. For antimicrobial assay experiments, *C. glabrata* was grown to mid-log phase in YPD and then diluted 1:1,000 in YPD.

### *Drosophila Infection, Survival Assays, and Statistical Analyses*

Septic wounding, survival assays, and statistical analyses using GraphPad Prism were conducted, essentially as previously described [10].

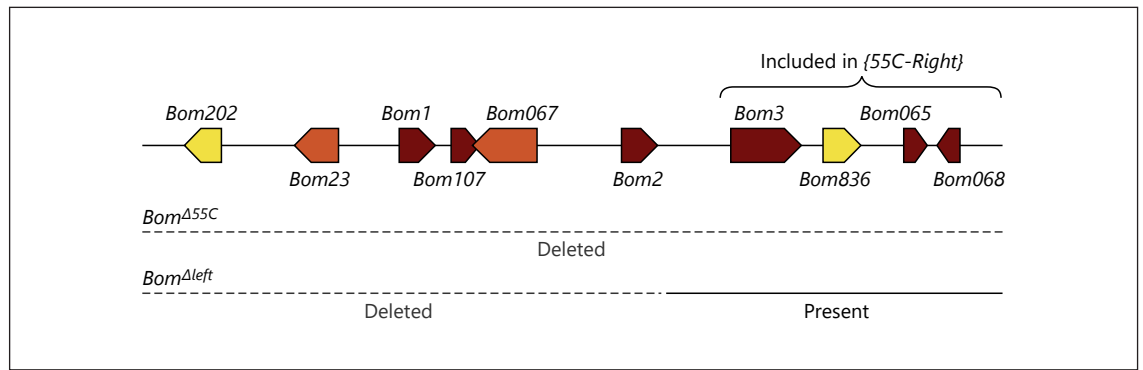
### *Bomanin Peptide Preparations*

The 16-amino acid mature forms of Bom1 (GNVIINGDCRVC-NVHG) and Bom3 (GNVIINGDCRVCNVRA) were synthesized by Lifetein and Biomatik, respectively, using a solid support resin with Fmoc and Boc chemistry. Peptides were delivered at 95% purity, confirmed by HPLC as well as mass spectrometry. Based on previously published mass spectrometry analysis [9], an intramolecular disulfide bond was introduced into both synthetic peptides and Bom1 was amidated at the carboxyl-terminus. Bom1 was dissolved in sterile water, and Bom3 was dissolved in 50% DMSO. Peptide concentrations were determined using the Qubit protein assay (Life Technologies), and were measured to be 43 μM (Bom1) and 56 μM (Bom3). These peptide solutions were introduced neat into the in vitro antimicrobial assay.

### *Hemolymph Preparation and Antimicrobial Assays*

Toll pathway activation was achieved by wounding male flies (aged 2–7 days) with heat-killed *M. luteus*. After incubating the flies at 29 °C for 24 h, cell-free hemolymph was collected from groups of 70 flies by a modification of the standard method [15]. Flies were loaded into a Zymo-Spin IC column (Zymo Research) previously washed twice with 200 μL sterile water. The flies were covered with 2-mm glass beads (Walter Stern) and centrifuged at 13,500 rpm atop a collection tube, for 20 min at 4 °C, yielding approximately 4 μL of cell-free hemolymph.

Antimicrobial assays with synthetic peptide or hemolymph were carried out as prescribed for standard antimicrobial peptide assays [16]. Two microliters of peptide, hemolymph, or a combination of the two was mixed with an equal volume of log-phase *C. glabrata* suspended at 1:1,000 in YPD. Samples were incubated either at room temperature for 1 h or on ice for 24 h. Following incubation, samples were diluted to 100 μL with YPD, spread onto YPD plates, and grown overnight at 37 °C. Yeast colonies were counted the next day.



**Fig. 1.** Organization of the *Bom* family cluster at cytogenetic map position 55C. This figure reflects a renaming of the *Bom* family members, with the previously used CG or IM prefixes replaced with the *Bom* prefix, while retaining the gene number for both DIMs (1 or 2 digits) and CGs (last 3 digits) for continuity with prior literature [9, 17]. Accordingly, the 2 remaining *Bom* genes, located outside the 55C region, are now *Bom778* and *Bom791*. For

the 10 genes in the drawing, arrowhead direction denotes gene orientation. The color indicates the form of the encoded *Bom* peptide: short (red), tailed (yellow), or bicipital (orange). The bracket at the top encompasses the *Bom* genes in the 55C-Right genomic construct and the dotted lines below illustrate the extent of the chromosomal deletions in *Bom* $\Delta^{55C}$  and *Bom* $\Delta^{left}$ .

#### Hemolymph Extraction and MALDI-TOF Analysis

After Toll activation as above, hemolymph samples for MALDI-TOF were extracted from male flies (aged 2–7 days) using glass capillaries, and transferred with a Narishige IM-300 microinjector into 2  $\mu$ L 0.1% trifluoroacetic acid (TFA)/50% acetonitrile (ACN). The samples were then mixed 1:1 with  $\alpha$ -cyano-4-hydroxycinnamic acid (HCCA) matrix (Agilent: 2037A), and 2  $\mu$ L was spotted onto a MALDI plate where sample and matrix cocrystallized in situ under a flow of warm air.

MALDI mass spectra were acquired using a Bruker Biflex IV MALDI-TOF mass spectrometer operated in either linear or reflectron mode with positive polarization. Desorption and ionization were achieved using a 337-nm laser. As an external mass calibration for linear mode spectra, equal parts of peptide calibration standard II (Bruker: 8222570) and protein calibration standard I (Bruker: 8206355) were mixed with HCCA matrix and spotted onto the same sample plate. For the calibration of reflectron mode spectra, peptide calibration standard II was mixed with HCCA matrix. For each genotype and condition, spectra from at least 5 individual flies were collected, yielding reproducible results. To identify particular peptides, the  $m/z$  values of peaks in our spectra were matched to those of the corresponding peaks in a prior study [9]. Spectra, for which representative examples are shown, were visualized using R 3.3.2, RStudio 1.0.136 and ggplot2 2.2.1.

## Results

### A Transgenic Assay Reveals the Effector Activity of a Single *Bom* Peptide

Ten *Bomanin* genes are clustered on the *D. melanogaster* second chromosome (Fig. 1). Our prior studies [10] defined 2 deletions affecting this cluster, designated 55C for its position on the polytene map. *Bom* $\Delta^{55C}$  flies, which

lack the entire 55C *Bom* cluster, fail to mount a successful immune response against pathogens normally counteracted by the Toll pathway. In contrast, flies carrying the smaller *Bom* $\Delta^{left}$  deletion are susceptible to a number of pathogens, but have wild-type resistance to a relatively weak pathogen, the yeast *C. glabrata*. Because *Bom* $\Delta^{left}$  lacks the 6 genes on the left (proximal) side of the *Bom* cluster, the 4 remaining *Bom* genes provided a useful starting point for defining the functional unit of *Bom* activity.

We generated a transgenic construct, 55C-Right, spanning the 4 intact *Bom* genes to the right of the deletion in *Bom* $\Delta^{left}$ . By generating flies carrying the 55C-Right transgene on each third chromosome and the *Bom* $\Delta^{55C}$  deletion on each second chromosome, we could recapitulate the genome of *Bom* $\Delta^{left}$ . Using 55C-Right as a template, we next generated 4 related constructs, each expressing only a single ORF from the 4 *Bom* genes originally present. We then introduced 2 copies of the wild-type or single-gene 55C-Right constructs into the *Bom* $\Delta^{55C}$  background and assayed survival after challenge with *C. glabrata* (Fig. 2).

The data in Figure 2 demonstrate that a single *Bom* gene, either *Bom3* or *Bom065*, can restore resistance to *C. glabrata* in *Bom* $\Delta^{55C}$  flies. We noted that both *Bom3* and *Bom065* encode short-form *Bom* peptides, defined as having a mature length of just 16 amino acids [10]. A third short-form *Bom* gene, *Bom068*, provided lesser but still significant rescue. Intriguingly, the resistance strength appeared to correlate with the *Bom* transcript level, as

**Table 1.** Survival after infection with *C. glabrata* correlated with total *Bom* transcript level

Transgene in <i>Bom</i> $\Delta^{55C}$	Gene: present (+) or absent (-)				Survival at 8 days <sup>a</sup>	Total transcript levels from 55C <i>Bom</i> genes <sup>b</sup>
	<i>Bom3</i>	<i>Bom836</i>	<i>Bom065</i>	<i>Bom068</i>		
{55C-Right}	+	+	+	+	96%	59
{ <i>Bom3</i> }	+	-	-	-	95%	18
{ <i>Bom836</i> }	-	+	-	-	1%	3
{ <i>Bom065</i> }	-	-	+	-	93%	24
{ <i>Bom068</i> }	-	-	-	+	25%	9
No transgene	-	-	-	-	6%	none

<sup>a</sup> See Fig. 2.

<sup>b</sup> *Bom* transcript levels from adult males (2–7 days old), quantified by real-time PCR and shown relative to *rp49*, were measured 24 h after Toll induction with heat-killed *M. luteus* (see online suppl. Table 1).

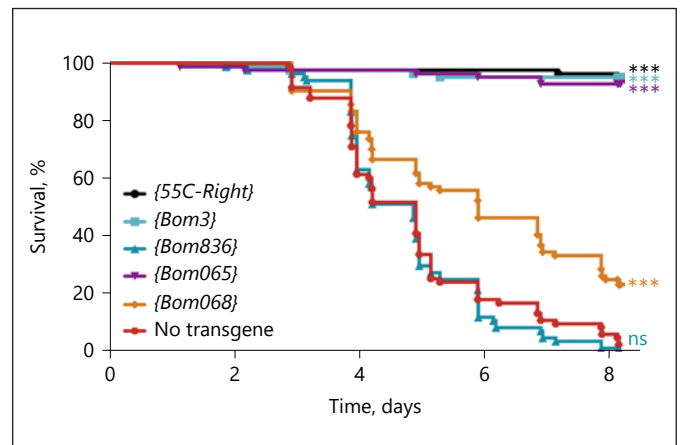
measured in Toll-induced wild-type adults (Table 1). Thus, resistance was higher for *Bom3* and *Bom065* and lower for *Bom068*.

#### Robust Expression of Short-Form Boms Conveys Resistance to *C. glabrata*

By further modifying the 55C-Right construct, we set out to compare the activity of different *Bom* forms when expressed at comparable levels. Specifically, we replaced the *Bom3* coding region with that of other *Bom* genes, i.e., we fused different *Bom* ORFs to the strongly Toll-responsive *Bom3* promoter (*pBOM3*). In this fashion, we generated 55C-Right constructs that should express a single, short, tailed, or bicipital *Bom* at the high levels normally observed for *Bom3* upon Toll pathway activation. We then assayed survival after challenge with *C. glabrata* (Fig. 3).

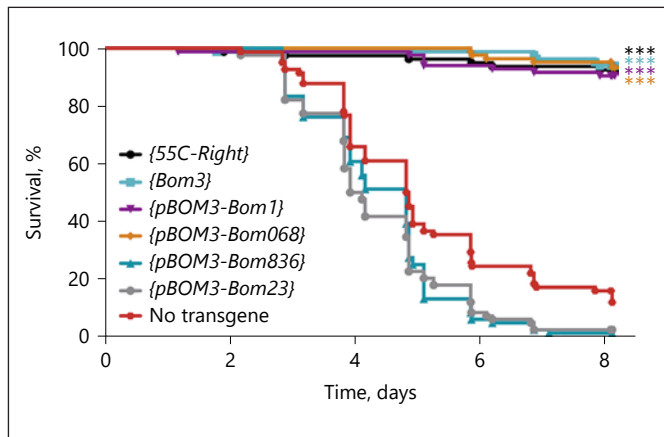
All 3 short-form *Bom* genes tested provided full resistance to *C. glabrata* when expressed under the control of *pBOM3*. This included *Bom068*, which provided considerably weaker resistance when driven by its own promoter (Fig. 2 vs. Fig. 3). These data support the hypothesis that it is the level of expression, rather than the sequence composition, of short-form Boms that determines their contribution to survival. Furthermore, we found no evidence that the short-form *Bom* peptides vary intrinsically in specificity for *C. glabrata*.

We turned next to representative examples of tailed and bicipital Bomanins. A tailed form, *Bom836*, provided no rescue of *Bom* $\Delta^{55C}$  when controlled by its endogenous promoter. Analysis of the endogenous transcript levels following Toll activation revealed far less transcript accumulation for *Bom836* than for either *Bom3* or *Bom065*



**Fig. 2.** A single *Bom* gene rescues the *Bom* $\Delta^{55C}$  immunodeficiency toward *C. glabrata*. The graph illustrates the survival of transgenic adults at indicated intervals after infection with *C. glabrata*. Each transgene encodes either the 4 *Bom* peptides of 55C-Right or a single one of these peptides, as indicated. All transgenes were present in 2 copies in a *Bom* $\Delta^{55C}$  background. Each curve represents the pooled results of 3 independent experiments involving  $\geq 25$  flies per genotype. Survival curves were compared using the Gehan-Breslow-Wilcoxon test. Significance is shown relative to the no transgene control (*Bom* $\Delta^{55C}$ ) and adjusted for multiple comparisons (\*\*\*)  $p < 0.0001$ ; ns, not significant,  $p > 0.05$ ).

(Fig. 2; Table 1). However, the *Bom836* ORF failed to generate detectable rescue even when fused to *pBOM3* (Fig. 3). Likewise, we did not observe rescue with *pBOM3*-driven expression of a bicipital form, *Bom23* (Fig. 3). Thus, neither the tailed nor the bicipital *Bom* exhibited activity against *C. glabrata*. For subsequent studies, we therefore focused on the short-form Boms, for which our



**Fig. 3.** Short-form Bom peptides mediate survival after infection with *C. glabrata*. The graph plots the survival of adults at indicated intervals after infection with *C. glabrata*. Bom transgenes were expressed under control of either their endogenous promoter or *pBOM3*. All transgenes were present in 2 copies in a *BomΔ<sup>55C</sup>* background. Data were captured and analyzed as in Figure 2.

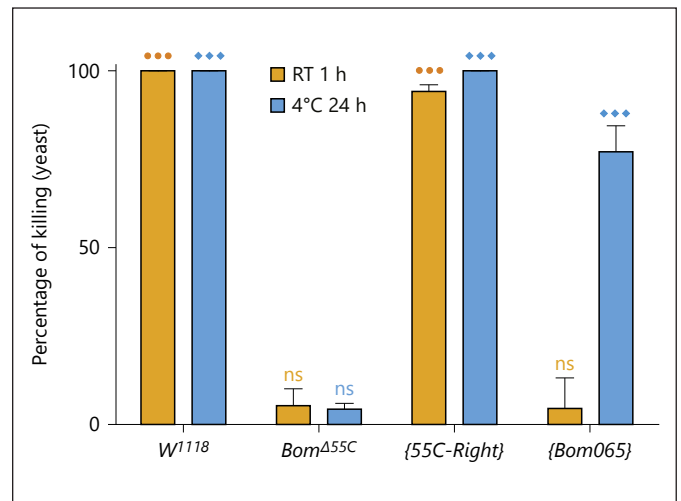
survival assay provides a robust readout in vivo for an individual gene product.

#### Short-Form Boms Mediate Cell-Free Killing of *C. glabrata*

Having found that expression of a single short-form Bom could rescue adults otherwise immunodeficient against *C. glabrata* infection, we turned our attention to in vitro studies. In initial experiments, we assayed synthetic, mature short-form Bom peptides under conditions permissive for antimicrobial activity against *C. glabrata*. No such activity was detected with the synthetic forms of mature Bom1 or Bom3 (data not shown).

In parallel, we set out to explore the in vitro activity of hemolymph, in which many of the Bom peptides are abundantly expressed upon infection. We stimulated the Toll pathway, and then collected cell-free hemolymph after 24 h and mixed it with an equal volume of mid-log *C. glabrata*. After incubation, the mixture was plated and the yeast colonies were counted the next day. Two alternative conditions were used for incubation. The first, a 1-h incubation at room temperature, was designed to mimic conditions under which infection would normally occur. The second, a 24-h incubation at 4 °C, was designed to increase assay sensitivity. Wild-type hemolymph was fungicidal under either condition, with no detectable *C. glabrata* colonies formed (Fig. 4).

When we assayed hemolymph from *BomΔ<sup>55C</sup>* adults, there was a clear and dramatic difference from the wild

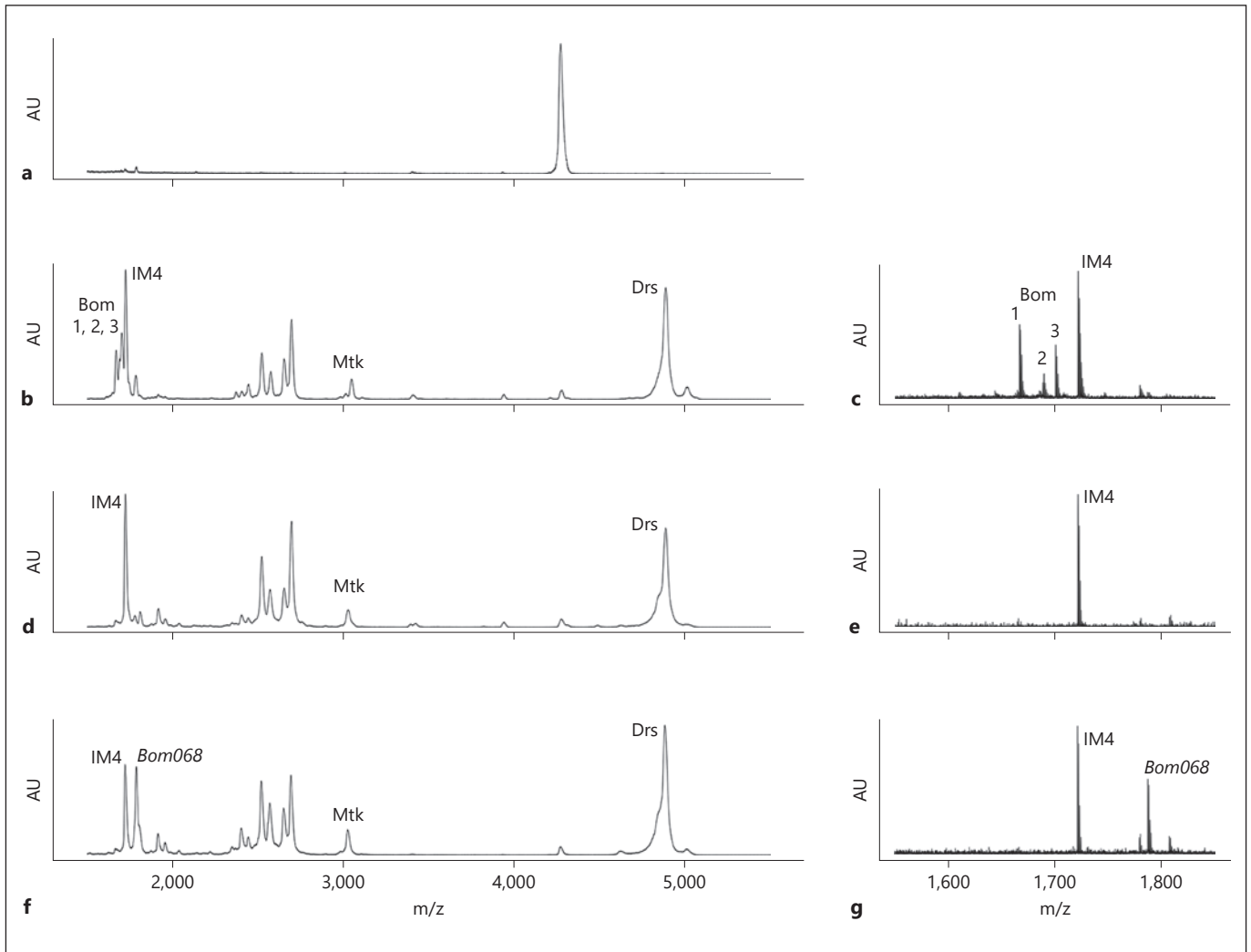


**Fig. 4.** Fungicidal activity of hemolymph is Bom-dependent. Hemolymph activity was assayed for 4 sets of flies: *w<sup>1118</sup>* (wild-type), *BomΔ<sup>55C</sup>*, and *BomΔ<sup>55C</sup>* carrying either a 55C-Right (four-gene) or *Bom065* (single-gene) construct. All flies had been pricked 1 day earlier with heat-killed *M. luteus* to induce Toll-mediated gene expression. Fungicidal activity was assayed by incubating cell-free hemolymph with *C. glabrata* for 1 h at room temperature (RT) or for 24 h at 4 °C before spreading the mixture onto a yeast (YPD) plate. Percent killing was calculated by comparing the colony count for each sample to that for yeast mixed with a *w<sup>1118</sup>* uninduced hemolymph control. Experiments were performed in triplicate; error bars represent standard error of the mean. One-way ANOVA followed by Tukey's test was performed for each incubation condition. Significance is shown relative to the *w<sup>1118</sup>* uninduced control (●●● and ◆◆◆ =  $p < 0.0001$ ; ns, not significant,  $p > 0.05$ ).

type; deleting the 55C Bom genes eliminated the fungicidal activity of hemolymph. The Bom genes thus mediate a humoral antimicrobial activity against *C. glabrata*. Furthermore, the Bom-dependent activity of hemolymph in vitro parallels the Bom-dependent resistance observed in vivo against the same pathogen.

#### Hemolymph Activity Correlates with Bom Gene Dosage

To assay the effect of gene dosage on the level of Bom-dependent hemolymph activity, we took advantage of the genetic backgrounds representing different overall levels of Bom expression. We first assayed hemolymph from *BomΔ<sup>55C</sup>*; {55C-Right} flies. Antimicrobial activity was comparable to the wild type under both conditions (Fig. 4). We next assayed hemolymph from *BomΔ<sup>55C</sup>*; {Bom065} flies, in which expression of a single Bom peptide (*Bom065*) confers *C. glabrata* resistance in the



**Fig. 5.** Analysis of hemolymph from adult *Drosophila* by MALDI-TOF MS. Hemolymph (50 nL) was collected from a single fly from each genotype and analyzed by MALDI-TOF MS using the linear (a, b, d, f) or reflectron mode (c, e, g) (see Materials and Methods). Representative MALDI spectra from  $\geq 5$  such samples are shown for hemolymph from a  $w^{1118}$  fly either uninduced (control) (a), or induced 24 h earlier by Toll pathway stimulation with heat-killed *M. luteus* (b, c), and induced flies of the following genotypes:

*Bom* $\Delta^{55C}$  (d, e) and *Bom* $\Delta^{55C}; \{pBOM3-Bom068\}$  (f, g). AU, arbitrary units. The y axis was scaled to the tallest peak within the window examined. Numbering and naming of induced peptides corresponds to published conventions [9]. As observed previously [9], a prominent peak appears in control spectra that becomes insignificant in the context of Toll induction. Drs, drosomycin; Mtk, metchnikowin.

*Bom* $\Delta^{55C}$  background (Fig. 2). In this case we found no fungicidal activity by the *Bom* $\Delta^{55C}; \{Bom065\}$  hemolymph at room temperature, but readily detectable and significant killing at 4 °C overnight (Fig. 4). Thus, the strength of antimicrobial activity in vitro, like resistance in vivo, appears to correlate with the overall level of short-form Bom expression.

#### *The Levels and Composition of Antifungal AMPs Appear Bom-Independent*

Although the Bomanins mediate antimicrobial activity, they could act upstream of known AMPs, e.g., to bring about accumulation or modification of AMP gene products. In the case of antifungal defenses, there are 2 AMPs that are robustly induced upon Toll activation and display strong antifungal activity in vitro: drosomycin (Drs) and metchnikowin (Mtk) [17–19] (a third AMP, cecro-

pin, is active against a wide range of pathogens [20], but is inactive against *C. glabrata* [21]). We have previously shown that induction of mRNA expression by Toll does not require Bom function [10]. To determine if Boms act on these AMPs at the protein level, we turned to mass spectrometry.

Uttenweiler-Joseph et al. [9] reported an approach for characterizing the peptide composition of hemolymph from a single *Drosophila* adult. Using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), they identified singly charged ions for a set of 24 immune-induced molecules (IMs). Adopting their approach, we obtained near identical spectrograms (Fig. 5a, b, compare with Fig. 3 in [9]), enabling us to identify individual peptides by their m/z ratio. Upon inducing innate immune signaling, the IMs were readily detectable, including Drs (IM19), Mtk (IM17), and 3 Boms (1, 2 and 3) in the 55C cluster (Fig. 5b, c). The m/z for an additional 55C Bom peptide, Bom23, falls outside the mass range examined.

Next, we carried out parallel experiments with hemolymph from *Bom* $\Delta^{55C}$  flies (Fig. 5d, e). As expected, no peaks were found with an m/z ratio matching that of Bom1, Bom2, or Bom3. Peaks were present, however, for both Drs and Mtk. In the case of Drs, the signal remained robust and with the same signal maximum. We noted, however, that Drs has a relatively high molecular weight, precluding detection of a minor change in m/z in our study.

For Mtk, we did observe a significant difference in peak maxima, with a shift from 3,044 m/z in the *w*<sup>1118</sup> control to 3,022 m/z in *Bom* $\Delta^{55C}$ . This shift, however, is not Bom-dependent. Rather, it reflects a difference in Mtk isoform between the 2 genetic backgrounds. There are 2 known isoforms of Mtk, encoding either a His or Arg residue at position 3 in the mature peptide. Both isoforms are active [19]. The m/z values corresponding to the 2 species exactly match those we detected here.

To verify that the Bom phenotypes do not reflect a difference in Mtk isoform, we first sequenced the *Mtk* gene in the *w*<sup>1118</sup> and *Bom* $\Delta^{55C}$  backgrounds. This analysis confirmed the isoform identification by mass spectrometry: Mtk<sup>R3</sup> in the *w*<sup>1118</sup> background and Mtk<sup>H3</sup> in the *Bom* $\Delta^{55C}$  background. Next, we used CRISPR-Cas9 to generate a *Bom* 55C deletion in the *w*<sup>1118</sup> background, eliminating the polymorphism in Mtk between the control and experimental samples. Using the newly derived *Bom* $\Delta^{55C-2}$  flies, we obtained survival curves after infection identical to those of the previously published TALEN-generated *Bom* $\Delta^{55C}$  flies (online suppl. Fig. 1; see www.

karger.com/doi/10.1159/000489831 for all online suppl. material).

Taken together, these studies reveal that deleting the *Bom* 55C cluster has no readily detectable effect on accumulation or posttranslational modification of 2 major Toll-induced antifungal peptides in hemolymph.

#### *Refined Understanding of the Bom Repertoire*

Six of the 55C Boms are not detected even in wild-type hemolymph. Why? They are unlikely to represent pseudogenes, since they are well conserved at the amino acid level and lack any obvious nonsense or missense mutations. Instead, we favor 2 explanations. First, for a Bom with robust expression at the mRNA level, the peptide is likely present but masked. For example, we noted that the calculated mass of the amidated form of Bom065 is 1,723.8 m/z. This peptide is likely to fall in the shoulder of the robust peak for IM4 (calculated mass 1,721.9 m/z) and therefore not be detected. Second, a number of Boms may have been missed simply because their level of immune-induced expression is relatively low. Here, Bom068 provides a useful example. Compared to other short-form Boms, *Bom068* mRNA accumulates to only modest levels upon Toll activation (Table 1). Consistent with this weak expression, we observed modest resistance to *C. glabrata* infection in flies expressing Bom068 from its endogenous promoter in a *Bom* $\Delta^{55C}$  background, and were unable to detect the peptide by MALDI-TOF. However, when the *Bom068* ORF was heterologously expressed under control of the strongly Toll-responsive *pBOM3* promoter, we observed wild-type resistance to *C. glabrata* infection and, furthermore, ready detection of a peak at 1,787 m/z, which matches the predicted value on MALDI-TOF for Bom068 that has undergone proteolytic processing but not amidation (Fig. 5f, g).

## Discussion

### *Individual 55C Bom Genes Mediate Antimicrobial Defense*

Transgenic analyses revealed that a single, short-form 55C *Bom* gene provides resistance to *C. glabrata* in the absence of the remaining 9 genes from the cluster. Furthermore, every short-form *Bom* gene tested displayed such activity. The highly similar structure of the short-form Boms thus appears to reflect a shared activity found in each. These data make it unlikely that all 10 genes of the cluster form a supramolecular complex or are all part of a common activity cascade.

We did not find transgenic activity for tailed or bicipital Boms. Two explanations appear very reasonable. First, these Bom forms might have specificity for particular pathogens other than *C. glabrata*. Bom activity has been observed not just for yeast, but also for representatives of hyphal fungi and gram-positive bacteria [10]. Second, the tailed and bicipital Boms might be active only in combination with other Bom family members.

*Boms* exist in a cluster across the *Drosophila* genus. Why did such a cluster arise if individual genes are active and interchangeable, at least in certain circumstances? Our results indicate that, for particularly virulent pathogens, such as *Enterococcus faecalis*, successful immune defense requires very high levels of Bomanins [10]. We hypothesize that a single transcription unit is insufficient to provide enough Bom peptide in the time frame required. According to this model, duplication provided multiple, near-identical genes that could act as a battery for defense. Several lines of evidence support this idea. First, there are multiple, strongly Toll-responsive *Bomanin* genes, each immediately downstream of a canonical TATA start site, and, almost always, a perfect or near-perfect Toll-responsive NF- $\kappa$ B element [22]. Second, the 55C-Right construct, inserted at 86Fb and lacking more than half the 55C cluster, expressed each of the 4 remaining *Bom* genes at the same level as in the full-length cluster, suggesting an absence of control elements shared across the 10 genes (online suppl. Table 1).

If duplication is driven by a need for a high overall level of expression, what should we make of the variation we observed in the sequence of Bom family members? It could provide sequence specificity for Bom action. It could also provide protection against pathogen countermeasures, varying the residues targeted by microbial enzymes or inhibitors. Lastly, variation could also be a simple consequence of duplication and drift.

It is important to note that removing the 55C *Bom* cluster drastically reduces, but does not eliminate, Bom expression. Two additional *Bomanins*, *Bom778* (tailed) and *Bom791* (bicipital), remain intact, as do 2 additional genes (*IM4*, *IM14*) that are immune-induced and encode peptides that bear sequence similarity to the Bomanins. For this reason, caution is required when drawing conclusions with regard to the complete repertoire of the Bomanin family.

#### *Bom Peptides Are Required for the Toll-Directed Humoral Defense*

Bomanins are clearly required for the humoral response, as a hemolymph preparation free from intact

cells provides Bomanin-dependent fungicidal activity in vitro. Furthermore, this activity acts rapidly, killing *C. glabrata* within an hour under normal growth conditions. An involvement in humoral immunity makes good sense, as the Bomanins are secreted from the fat body into the hemolymph [9]. Indeed, they are loaded into hemolymph at concentrations sufficiently high as to suggest a stoichiometric role in defense rather than a role as cytokines.

To date, synthetic Boms have not been active in our in vitro assays. We have no easy explanation for this. The problem is not one of concentration, since MALDI-TOF revealed that synthetic peptide concentrations were well-matched to those of the endogenously expressed forms. Furthermore, the measured mass-to-charge (*m/z*) values were also identical. Nor is the problem one of the exogenous peptides requiring a partner protein or other factor, since the synthetic Bomanins are also inactive when mixed with induced *Bom* $\Delta^{55C}$  hemolymph (unpubl. observ.). This failure to observe complementation in vitro suggests that the synthetic forms lack a conformation or stable interaction required for activity and which is normally provided in vivo prior to release into the hemolymph.

Loss-of-function mutations in multiple family members provide a powerful tool for dissecting effector function. For example, in this study, we were able to define an essential role for the *Bom* family, assess the significance of gene duplication and divergence, and define effector specificity both in vivo and in vitro. Whereas small genes of overlapping function are a poor target for traditional mutagenesis, generating mutations in a number of such genes is now both straightforward and feasible. More insights and more surprises lie ahead.

#### **Acknowledgements**

The authors would like to thank Yongxuan Su and the Molecular Mass Spectrometry Facility at UCSD for guidance in preparation and analysis of samples for mass spectrometry. We would like to acknowledge the technical assistance of Yangyang Xu and Shelief Juarez in development of the hemolymph assay. We appreciate the contributions of materials and advice from Toto Olivera and Joanna Gajewiak, and access to equipment provided by Ethan Bier and Amy Pasquinelli. We also thank Russell Doolittle, Eric Bennett, Bill McGinnis, and Scott Rifkin for helpful discussions and advice, and Emily Troemel, Matt Daugherty, Alisa Huffaker, Lianne Cohen, and Alexa Clemmons for helpful comments on the manuscript. Lastly, we express our gratitude for the enthusiasm and assistance provided by Bo Zhou, who is greatly missed.



## Disclosure Statement

The authors declare no competing financial interests.

## Funding Sources

This work was supported by National Institutes of Health (NIH. gov) grant R01 GM050545 (to S.A.W.) and NIH predoctoral training grant T32 GM008666 (to S.J.H.L.). The funders had no role in study design, data collection and analysis, the decision to publish, or preparation of the manuscript.

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