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# Dual Activation of the *Drosophila* Toll Pathway by Two Pattern Recognition Receptors

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The Toll-dependent defense against Gram-positive bacterial infections in *Drosophila* is mediated through the peptidoglycan recognition protein SA (PGRP-SA). A mutation termed *osiris* disrupts the *Gram-negative binding protein 1* (*GNBP1*) gene and leads to compromised survival of mutant flies after Gram-positive infections, but not after fungal or Gram-negative bacterial challenge. Our results demonstrate that *GNBP1* and PGRP-SA can jointly activate the Toll pathway. The potential for a combination of distinct proteins to mediate detection of infectious nonself in the fly will refine the concept of pattern recognition in insects.

A hallmark of the host response of *Drosophila* to infection is the expression of antimicrobial peptide genes. Two intracellular signaling pathways regulate this expression. The Toll pathway is activated primarily by Gram-positive bacterial or fungal infections and, in

particular, directs the expression of the antimicrobial peptide gene *Drosomycin*. In contrast, the immune deficiency (IMD) pathway responds predominantly to infections by Gram-negative bacteria and induces expression of several antibacterial peptide genes,

including *Diptericin* (*I*). Fungal and Gram-positive infections lead to the proteolytic processing of a cytokine, the growth factor-like polypeptide Spätzle, which in its cleaved form binds directly to the transmembrane receptor Toll (2–8). This binding triggers an intracellular signaling cascade that culminates in the up-regulated transcription of multiple target genes, through the nuclear factor  $\kappa$ B-related protein Dorsal-related immune factor (DIF) (2, 9–14).

How bacterial infection leads to processing of Spätzle and subsequent activation of Toll has remained unclear. In the course of a large-scale screen designed to isolate mutants of the innate immune response of *Drosophila*, we identified a new mutation that affected the inducibility of *Drosomycin* by Gram-

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positive bacterial infection. We named the mutation *osiris* (*osi*). *Osi* flies exhibited a significant decrease in the expression of the *Drosomyacin* gene in response to several Gram-positive bacterial strains (Fig. 1A and fig. S1) (15). This was not observed in the majority of *osi* flies infected with fungi (Fig. 1C); *Diptericin* expression was normal after a challenge with Gram-negative bacteria (Fig. 1E). Although variable, data from these flies did not differ significantly from results obtained with wild-type flies (figs. S2 and S3). Survival rates of *osi* flies after injection of the Gram-positive bacteria *Enterococcus faecalis* (Fig. 1B) and *Streptococcus pyogenes* (16) were severely compromised and were similar to those of *Dif* loss-of-function mutants (6, 7). However, in contrast to these phenotypes, *osi* flies displayed levels of survival similar to those of wild-type flies in response to natural infection by the fungus *Beauveria bassiana* (Fig. 1D) and to infection by the Gram-negative bacteria *Enterobacter cloacae* (Fig. 1F) and *Erwinia carotovora* subsp. *carotovora* (16). Thus, the *osi* mutation primarily affected the defense pathway initiated against infection by Gram-positive bacteria rather than that against Gram-negative strains or fungi.

The *osi* mutation corresponds to the insertion of a modified piggyBac transposon in the coding region of the *Gram-negative binding protein 1* gene (*GNBP1*), a potential pattern recognition receptor (17). Members of the GNBP/β-(1,3)-glucan recognition proteins (βGRPs) were originally isolated from large-sized lepidopterans (18–21) (supporting online text). The *Drosophila* genome encodes three canonical members of this family. GNBP1 has been reported to bind a commercial Gram-negative bacterial lipopolysaccharide, fungal β-(1,3)-glucan, but not to bind Gram-positive bacterial peptidoglycan (17). The transposon is inserted in the second exon of *GNBP1* at nucleotide 511, which corresponds to amino acid 48 of the preprotein (or residue 29 of the predicted mature protein) (Fig. 2A). Transcripts of *GNBP1* were barely detectable (Fig. 2B), suggesting that either the transcription of the locus or the stability of the mRNA are strongly affected by the insertion. No phenotypic difference was observed when the *osi* mutation was in homozygous or hemizygous conditions, indicating that this mutation is genetically null (16). The *osi* phenotype is due to the transposon insertion in *GNBP1*, because revertant lines, in which this transposon had been excised with a piggyBac transposase, were as resistant as wild-type flies to *E. faecalis* infection (Fig. 2C). Expression in *osi* flies of a pUAS-*GNBP1* cDNA transgene driven by the ubiquitous heat-shock protein (*hsp*) 70-*GAL4* construct rescued the inducibility of *Drosomyacin* in response to Gram-positive bacterial infection, further demonstrating that the *osi* phenotype resulted from the insertion of the transposon (Fig. 2D). Be-

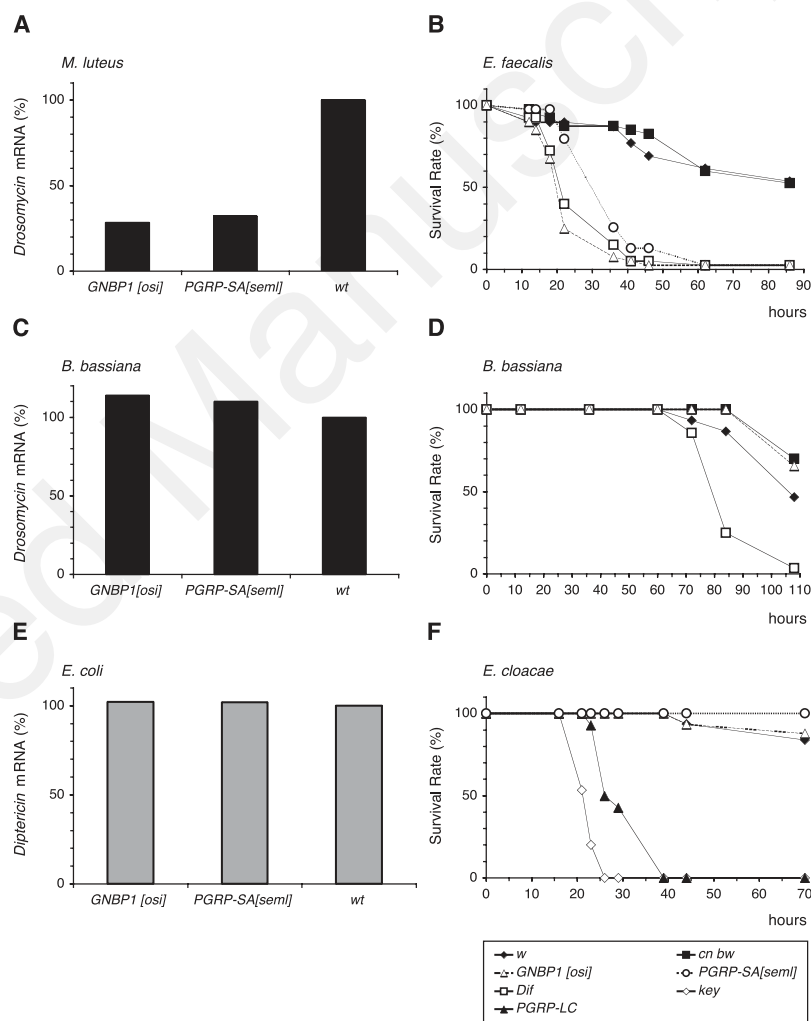
cause the *osi* mutant phenotype is due to the disruption of the *GNBP1* gene, we designated the mutation as *GNBP1<sup>osi</sup>*.

Fungal-dependent Toll activation requires the *persephone* (*psh*) gene, which encodes a serine protease and whose overexpression leads to challenge-independent Toll activation and *Drosomyacin* gene expression (5). This effect was not abolished when *psh* was overexpressed in a *GNBP1<sup>osi</sup>* genetic background (Fig. 3A), indicating that *psh*-dependent activation of Toll does not require *GNBP1*.

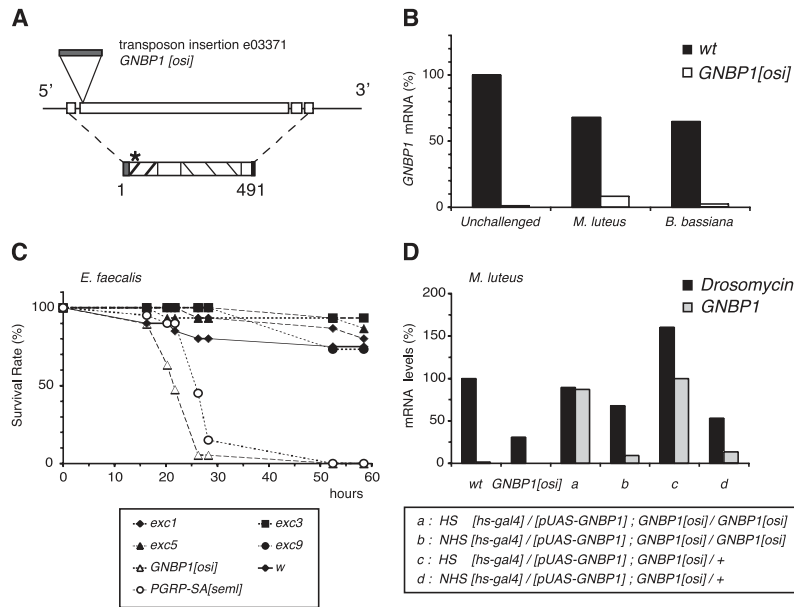
*GNBP1* encodes a 55-kD protein that has been shown in cell-culture studies to exist as

both secreted and glycosylphosphatidylinositol (GPI)-linked form in the cytoplasmic membrane (17). Transfer of hemolymph from wild-type flies to *GNBP1<sup>osi</sup>* mutants restored the inducibility of *Drosomyacin* by Gram-positive bacterial challenge (Fig. 3B), indicating that *GNBP1*-dependent activity within the hemolymph was sufficient to rescue the *GNBP1<sup>osi</sup>* mutant phenotype. The membrane-tethered form of GNBP1 appears, therefore, to be dispensable in the induction of *Drosomyacin*.

The phenotypes described here for the *GNBP1<sup>osi</sup>* mutation are very similar to those observed with the *semmelweis* (*seml*) loss-of-



**Fig. 1.** *GNBP1* is required for the host response against Gram-positive bacteria and not for that against Gram-negative bacteria or fungi. (A and C) Representative values for *Drosomyacin* mRNA steady-state levels as measured by quantitative–reverse transcription–polymerase chain reaction (Q-RT-PCR) (supporting online material). The panel shows the induction of *Drosomyacin* mRNA after an immune-challenge with (A) *Micrococcus luteus* (for 24 hours, at 20°C) or (C) *B. bassiana* (for 36 hours, at 25°C) in *GNBP1<sup>osi</sup>* (*GNBP1<sup>osi</sup>*), *PGRP-SA<sup>seml</sup>* (*PGRP-SA<sup>seml</sup>*), and wild-type (wt) control flies. The level of inducibility measured in the wild type was arbitrarily set to 100, and the measures obtained in the mutants were expressed as a percentage of this value. (B, D, and F) Survival experiments. We monitored the survival rate (expressed in percentage) after immune challenge. As expected, *Dif* mutant flies died more rapidly than wild-type (w) controls after (B) Gram-positive (*E. faecalis*, at 25°C) and (D) natural fungal (*B. bassiana*, at 29°C) infections, whereas (F) IMD pathway mutants *kenny* (*key*) (25) and *PGRP-LC<sup>ET12</sup>* (26) succumbed rapidly to *E. cloacae* (at 25°C) infection. The *Dif* and *key* mutations were originally generated in the *cn bw* genetic background, which serves as a wild-type control. (E) *Diptericin* mRNA levels 6 hours after an immune challenge at 20°C with *Escherichia coli* in *GNBP1<sup>osi</sup>*, *PGRP-SA<sup>seml</sup>*, and wild-type flies.

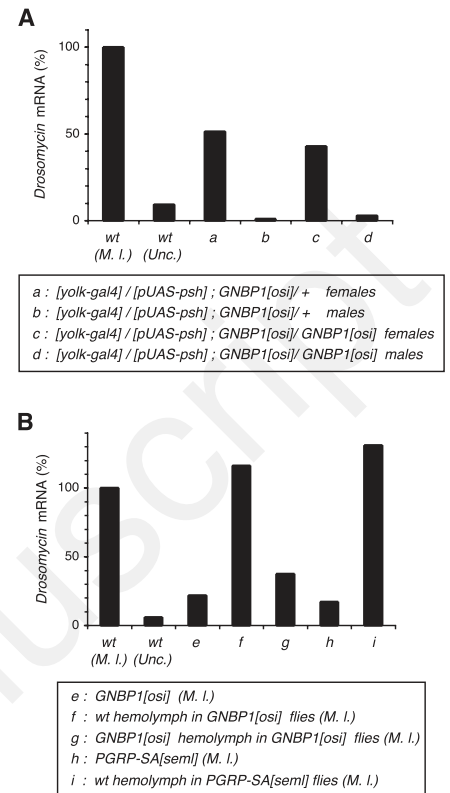


**Fig. 2.** The *osiris* insertion of a modified piggyBac transposon disrupts the *GNBP1* gene. **(A)** Scheme of the *GNBP1* region. The upper line shows the *GNBP1* genomic region with exons drawn as boxes. The *osiris* (e03371) transposon insertion is shown on top. The lower part of the panel shows the schematic structure of the *GNBP1* protein. The grey box indicates the signal sequence for amino acids 1 to 17; bold stripes show the  $\beta$ -(1,3)-glucan binding domain [amino acids 18 to 111; PFAM-B domain 4799 (27)]; thin stripes show the domain of homology to bacterial  $\beta$ -glucanases (amino acids 239 to 447, PFAM-A domain glyco-hydro16); the black box indicates the 10 C-terminal amino acids required for GPI anchoring (17). The asterisk shows the position where the open reading frame is disrupted by the insertion. **(B)** Steady-state *GNBP1* mRNA levels measured by Q-RT-PCR in wild-type and *GNBP1<sup>osi</sup>* mutant flies, either unchallenged, 24 hours after a challenge with *M. luteus*, or 48 hours after a challenge with *B. bassiana*. **(C)** *GNBP1<sup>osi</sup>* transposon excision (*exc*) strains were challenged with *E. faecalis*; w, wild-type controls. **(D)** Rescue of the *GNBP1<sup>osi</sup>* phenotype by a pUAS-*GNBP1* transgene after an immune challenge with *M. luteus*. *Drosomyacin* and *GNBP1* mRNA levels were quantified by Q-RT-PCR. Flies of the indicated genotype were submitted to a heat-shock procedure (HS). Nonheat-shocked flies (NHS) were used as a control. However, the lower yet significant expression of *GNBP1* transcripts, due to a leaky expression of the *hsp* promoter, was sufficient for the rescue of *Drosomyacin* inducibility in the (b) control flies. The *GNBP1* steady-state transcript levels measured after heat-shock [in the (c) flies] were arbitrarily set to 100%; in this case, wild-type levels reached about 1% of this value.

function mutants of a gene that encodes a member of an unrelated protein family, peptidoglycan recognition protein PGRP-SA (Figs. 1, 2C, and 3B) (6). We generated *PGRP-SA<sup>seml</sup>;GNBP1<sup>osi</sup>* double-mutant flies and found that they were not more sensitive than the single mutants in survival assays with Gram-positive bacteria. Similarly, the reduction of the levels of induced *Drosomyacin* by various Gram-positive bacterial strains was equivalent in the double and single mutants (Fig. 4, A to C). These data indicate that *GNBP1* and *PGRP-SA* are involved in a common process. Indeed, the concomitant overexpression of both genes (but not of the single genes) did lead to a significant, challenge-independent, expression of *Drosomyacin* (Fig. 4D). As expected, this expression required the *spätzle* (*spz*) gene product (Fig. 4E), demonstrating that both *GNBP1* and *PGRP-SA* act upstream of *spz*. These data provide genetic evidence to suggest that *GNBP1* and *PGRP-SA* may interact to trigger the activation of the Toll pathway. To provide biochemical support for this interaction,

we separated protein complexes from fly extracts by electrophoresis under native conditions and performed Western blot analysis for PGRP-SA and *GNBP1*. In extracts derived from wild-type flies, *GNBP1* (17) and PGRP-SA antibodies recognized a band at exactly the same position (Fig. 4F). Because both proteins have markedly different sizes and amino acid compositions, this suggested comigration of both proteins as part of a complex. In flies mutant for *GNBP1* or *PGRP-SA*, this band was either absent (*GNBP1<sup>osi</sup>*) or strongly reduced (*PGRP-SA<sup>seml</sup>*). Importantly, *seml* represents a point mutation that is assumed to affect the folding of the protein (22). Thus, both *GNBP1* and *PGRP-SA* are required for the formation of the detected band. Finally, concomitant overexpression of the *GNBP1* and *PGRP-SA* genes resulted in the appearance of an additional intense band detected by both antibodies, which may have corresponded to an activated complex.

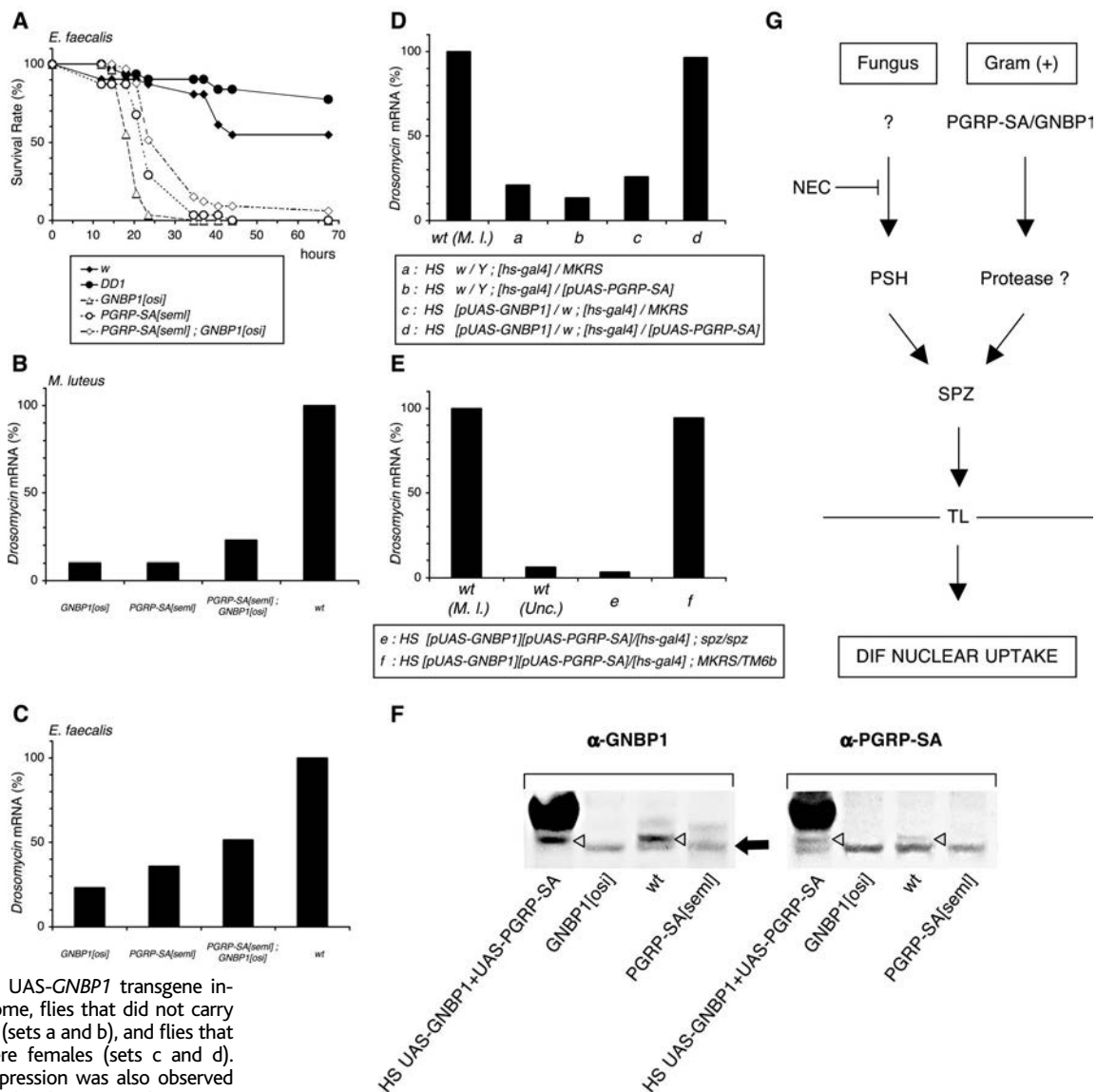
Our results demonstrate that *GNBP1* is required specifically for the Toll-dependent



**Fig. 3.** *GNBP1* function is required upstream of Toll in the blood compartment. **(A)** *GNBP1* is not epistatic to *psh*. We overexpressed the PSH protease using the GAL4/UAS system with a *yolk protein 1* promoter that drives the expression of *GAL4* specifically in the female fat body. Challenge-independent *Drosomyacin* expression was detected in females, whether (a) heterozygous or (c) homozygous for the *GNBP1<sup>osi</sup>* mutation. No expression was detected in (b) or (d) males. Challenged wild-type flies are shown. Similar results were obtained with an *hsp-GAL4* driver. M. l., *M. luteus*; Unc., unchallenged. **(B)** Hemolymph transfer experiments. The transfer of wild-type hemolymph into a *GNBP1<sup>osi</sup>* recipient (f) restores the *M. luteus* inducibility of *Drosomyacin* as determined by Q-RT-PCR. In contrast, transfer of *GNBP1<sup>osi</sup>* hemolymph into a *GNBP1<sup>osi</sup>* recipient does not significantly rescue the *GNBP1<sup>osi</sup>* phenotype (g). Results obtained with *PGRP-SA<sup>seml</sup>* (i) were similar to those previously reported (6). *Drosomyacin* transcript levels in challenged *GNBP1<sup>osi</sup>* (e) and *PGRP-SA<sup>seml</sup>* (h) mutants are shown for reference. Hemolymph collected from *PGRP-SA<sup>seml</sup>* flies could rescue the *GNBP1<sup>osi</sup>* mutant phenotype; the converse experiment, transfer of *GNBP1<sup>osi</sup>* hemolymph into *PGRP-SA<sup>seml</sup>* recipients, yielded the same result (16).

response to Gram-positive bacterial infections, but not for responses to fungi or Gram-negative bacteria (supporting online text) (Fig. 4G). Taken in conjunction with data on the *PGRP-SA<sup>seml</sup>* mutation (6), our results are compatible with a model in which the *GNBP1* and *PGRP-SA* proteins form a complex that activates a proteolytic cascade culminating in the cleavage of

**Fig. 4.** The interaction of *GNBP1* and *PGRP-SA* leads to Toll pathway activation. (A) Survival of *PGRP-SA<sup>sem1</sup>;GNBP1<sup>osi</sup>* mutant flies after *E. faecalis* infection. The survival assay was performed as in Fig. 1. w, wild-type; DD1, wild-type reference stock (15). (B and C) Expression of *Drosomycin* in *PGRP-SA<sup>sem1</sup>;GNBP1<sup>osi</sup>* double mutants 24 hours after (B) *M. luteus* or (C) *E. faecalis* challenge. (D) The concomitant overexpression of *PGRP-SA* and *GNBP1* leads to the challenge-independent expression of *Drosomycin*. *Drosomycin* steady-state transcript levels were measured as in (B) and (C). The value measured in wild-type samples (wt *M. l.*) 24 hours after an immune challenge was arbitrarily set to 100%. The GAL4/UAS system was used to overexpress *GNBP1* and/or *PGRP-SA* with an hsp-GAL4 driver. The third-chromosome balancer MKRS allows discrimination between UAS-*PGRP-SA* and the wild-type third chromosome. Because we used a UAS-*GNBP1* transgene inserted on the X chromosome, flies that did not carry this transgene were males (sets a and b), and flies that overexpressed *GNBP1* were females (sets c and d). Significant *Drosomycin* expression was also observed when the ubiquitous daughterless-GAL4 driver was used. (E) Epistatic analysis between the *GNBP1*-*PGRP-SA* and *spätzle* genes. *GNBP1* and *PGRP-SA* were overexpressed in a *spätzle* (*spz/spz*) homozygous mutant background (set e). TM6b (set f) is another balancer of the third chromosome and carries a wild-type copy of the *spz* gene. (F) Western blot of whole fly extracts that were separated by gel electrophoresis under native conditions. The blot was first incubated with an antiserum raised against a PGRP-SA peptide (right),



stripped, and then reprobed with an antibody against GNBP1 (left). The specific band recognized by both antibodies (open triangles) is absent in *GNBP1<sup>osi</sup>* and *PGRP-SA<sup>sem1</sup>* mutants (6). The lower band observed with the antibody to GNBP1 (arrow) is not specific, because it is observed in the *GNBP1<sup>osi</sup>* null mutant. (G) Scheme of Toll pathway activation by Gram-positive bacteria and fungi. NEC, necrotic serine protease inhibitor; TL, Toll receptor.

Spätzle and the activation of Toll. The x-ray structure of *Drosophila* PGRP-LB reveals the existence of a conserved surface at the back of PGRP proteins that could serve for protein-protein interactions (22). We assume that the complex would be activated on detection of microbial elicitors during infection. In this context, it may be relevant that a GNBP/βGRP from the coleopteran *Tenebrio molitor* has been reported to be associated with a serine protease (23). Together, our data imply that Toll-dependent activation by Gram-positive bacteria requires the cooperation of at least two distinct pattern recognition proteins, although the precise mechanisms of recogni-

tion and activation of these proteins during infection remain to be established. Recognition of infection in insects appears more complex than the initial model of microbial pattern recognition has postulated (24).

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