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Report

Drosophila Immunity: A Large-Scale In Vivo RNAi Screen Identifies Five Serine Proteases Required for Toll Activation

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Summary

Unlike mammalian Toll-like Receptors, the *Drosophila* Toll receptor does not interact directly with microbial determinants but is rather activated upon binding a cleaved form of the cytokine-like molecule Spatzle (Spz). During the immune response, Spz is thought to be processed by secreted serine proteases (SPs) present in the hemolymph that are activated by the recognition of gram-positive bacteria or fungi [1]. In the present study, we have used an in vivo RNAi strategy to inactivate 75 distinct *Drosophila SP* genes. We then screened this collection for SPs regulating the activation of the Toll pathway by gram-positive bacteria. Here, we report the identification of five novel SPs that function in an extracellular pathway linking the recognition proteins GNBP1 and PGRP-SA to Spz. Interestingly, four of these genes are also required for Toll activation by fungi, while one is specifically associated with signaling in response to gram-positive bacterial infections. These results demonstrate the existence of a common cascade of SPs upstream of Spz, integrating signals sent by various secreted recognition molecules via more specialized SPs.

Results

Despite extensive genetic screens for components of the *Drosophila* antimicrobial response, little is known about the SPs acting upstream of Spz in the regulation of the Toll pathway. In this study, we used an inducible RNAi strategy to screen for novel *SP* genes required for Toll activation by gram-positive bacteria. We generated a large collection of transgenic flies carrying inverted repeat sequences under the control of a UAS sequence for 75 distinct *SP* genes among the 200 encoded in the *Drosophila* genome [2] (see Table S1 in the Supplemental Data available with this article online). The *SP-IR* flies were individually crossed to flies carrying the *da-GAL4* driver to induce RNAi ubiquitously in the F1 progeny (referred to as *SP-RNAi*). 14 *SP-IR* insertions caused lethality when induced by *da-GAL4* and were further analyzed with the more restricted *c564-GAL4* driver that expresses GAL4 in the fat body, the major site of antimicrobial peptide production, and in hemocytes.

We assayed the susceptibility of *SP-RNAi* flies to infection with the gram-positive bacteria *Enterococcus faecalis*. In parallel, we monitored the expression of the antimicrobial peptide gene *Drosomycin* (*Drs*), a target of the Toll pathway, after infection by the gram-positive bacteria *Micrococcus luteus* by means of a *Drosomycin-lacZ* reporter gene. From this screen, we identified seven *SP-IR* strains with increased susceptibility to *E. faecalis* and at least 50% reduction in reporter gene expression as compared to infected wild-type flies (Table S1). Among these seven genes, two genes (CG6457, CG8867) were not further studied, since Blast analysis revealed that their IR constructs could inactivate other genes due to nucleotide sequence homology (Table S2). One of the SPs (CG16705) we identified in the screen was recently found to be involved in Toll activation during the immune response by means of an independent RNAi construct [3]. The latter study also provided strong evidence that this SP, named Spz processing enzyme (SPE), directly cleaves Spz, which validates our findings on this SP (see below). We focused our analysis on SPE, three *SPs* that we named *spirit*, *grass*, and *spheroide* for which the *IR* construct did not show any obvious off-target, and a pair of closely related *SP* genes, *sphinx1* and *sphinx2* (86% nucleotide identity), both inactivated by the *sphinx1-IR* construct (Figure 1A). Since we could not determine whether the *sphinx1* RNAi phenotype results from the inactivation of *sphinx1*, *sphinx2*, or both genes, we refer to this pair as *sphinx1/2*. For these five genes and one additional immune-responsive *SP* gene [4, 5], *CG6639*, for which RNAi knock-down did not lead to any phenotype in our screen, we confirmed the RNAi efficiency by showing that the levels of endogenous transcripts were strongly depleted in *SP-RNAi* flies (Figure 1B). Each *SP-IR* construct was specific and did not affect the transcripts of any other of the selected SP genes (Figure S1).

Figures 2 and 3 show that inactivation of *spirit*, *SPE*, *grass, sphinx1/2*, and *spheroide* by RNAi produced an immune-deficiency phenotype similar to Toll pathway mutants: *SP-RNAi* flies were highly susceptible to *E. faecalis* and failed to properly induce *Drs* after infection with *M. luteus*. The phenotypes were similar albeit weaker than those generated by a null mutation in *spz* or a *Dif-IR* construct targeting the gene encoding the NF-κB transcription factor activated by Toll [6]. Furthermore, the effects were specific to the five selected SPs, since *CG6639-RNAi* flies did not display any increased susceptibility or impaired *Drs* induction after challenge

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A

gene	CG number	synonym	Chromosome	precursor size
spirit	2056	c-SP33	X	393
SPE	16705	c-SP4	3R	400
spheroide	9675	SPH114	X	248
sphinx1/2	32383/2	SPH194	3L	265/251
grass	5896	c-SP1	3R	377/335

B

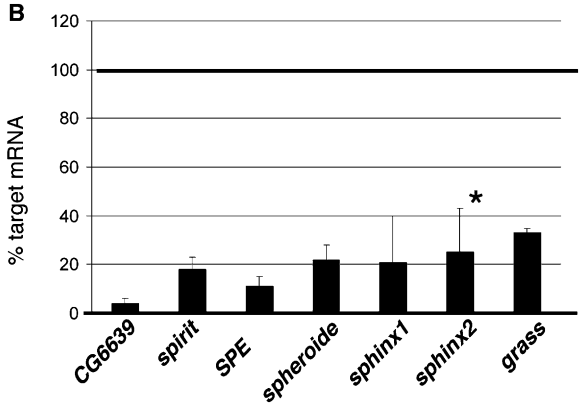

Figure 1. Synopsis of the Selected *SPs* and In Vivo Silencing by RNAi

(A) Name, CG number, synonym [2], chromosomal location, and precursor size in amino acids are given for the five selected *SPs*. Three *SPs, spirit* (for *Serine Protease Immune Response Integrator*), *SPE* (*Spatzle Processing Enzyme*), and *grass* (*Gram-positive Specific Serine protease*) encode SPs containing a Clip domain (c-SP), while the two others, *sphinx1/2* and *spheroide*, encode Serine Protease Homologs (SPHs) with an inactive catalytic site. Among the five genes, only *SPE* seems to be essential for viability, as its knockdown with *da-GAL4* driver induces pupal lethality. *SPE* was further studied with the *c564-GAL4* driver.

(B) RT-qPCR measurements confirmed that the expression of each *SP-IR* construct led to an efficient decrease in the mRNA level of the corresponding gene. The values are the percentage of SP gene expression in the corresponding *SP-RNAi* flies compared to wild-type flies except for (*) *sphinx2*, for which the bar represents the level of expression of *sphinx2*- in *sphinx1-RNAi* flies. mRNA levels were normalized to *rp49* mRNA. Total RNAs were extracted from male flies carrying a *SP-IR* element and a *da-GAL4* driver (or *c564-GAL4* for *SPE*).

with gram-positive bacteria. It has recently been shown that the Toll pathway is activated upon sensing of lysine-type peptidoglycan, a specific form of peptidoglycan found on the cell wall of most gram-positive bacteria [7]. Figure 3B shows that inactivation of *spirit*, *SPE*, *grass, sphinx1/2*, and *spheroide* genes by RNAi impaired *Drs* expression after the injection of lysine-type peptidoglycan, confirming the involvement of these SPs in the recognition of gram-positive bacteria. Importantly, the selected *SP-RNAi* flies neither induced susceptibility to gram-negative bacterial infections nor altered expression of *Dpt* after challenge by gram-negative bacteria (Figures 2 and 3). This is in agreement with the current view that the Imd pathway is not regulated by secreted SPs but by PGRP-LC, a transmembrane recognition receptor present at the surface of fat body cells [1]. The wild-type expression level of *Dpt* in *SP-RNAi* flies also indicates that the reduction of *Drs* expression in *SP-RNAi* flies is not due to a general defect of fat body metabolism, but rather results from a specific inhibition of Toll activity.

The Five SPs Function between PGRP-SA and Spz

Detection of gram-positive bacteria is mediated via the recognition of lysine-type peptidoglycan by PGRP-SA, PGRP-SD, and GNBP1 [8–11]. While PGRP-SD recognizes a specific subset of gram-positive bacterial species, GNBP1 and PGRP-SA are both required for sensing all types of gram-positive bacteria. Loss-of-function mutations in either *PGRP-SA* or *GNBP1* induce similar phenotypes of compromised survival to gram-positive bacterial infections and deficient Toll activation. Importantly, simultaneous overexpression of *GNBP1* and *PGRP-SA* triggers the Toll pathway, resulting in constitutive expression of *Drs* in the absence of an immune challenge [8]. Figure 4A shows that inactivation of any of the five selected *SPs* lowered *Drs* expression in those flies overexpressing both *GNBP1* and *PGRP-SA*, indicating that these SPs function downstream of the two recognition molecules. We also observed that *Dif-IR* but none of the selected *SP-IR* constructs blocked *Drs* expression induced by overexpression of a mature form of Spz (Spz*), which is in agreement with a function upstream of Spz (Figure 4B). However, we noticed that inactivation of *SPE* by RNAi moderately reduced the *Drs* levels induced by overexpression of Spz* (Figure 4B) but did not affect *Drs* expression stimulated by the expression of a gain-of-function allele of *Toll*, $Toll^{10b}$ (data not shown). This suggests that the presence of SPE could promote the interaction between Spz* with the Toll receptor. Altogether, these results indicate that we have identified five novel SPs that link GNBP1/PGRP-SA to Toll signaling after gram-positive bacterial infections.

Grass Is a Gram-Positive-Specific SP

Toll activation by fungal infection is independent of PGRP-SA and GNBP1 but is mediated by an extracellular cascade involving the SP Persephone (Psh) and its inhibitor, the serpin Necrotic (Nec) [12, 13]. Overexpression of *psh* or loss of *nec* is sufficient to trigger Spz-dependent activation of Toll without immune challenge. The recognition molecules involved in fungal detection and the proteases activated downstream of Psh have not yet been identified. To investigate a possible role of the five newly identified SPs in the antifungal response, we monitored the levels of *Drs* expression by RT-qPCR in *SP-RNAi* flies 24 hr after infection with the yeast *Candida albicans* or 48 hr after natural infection with the entomopathogenic fungus *Beauveria bassiana*. We also assayed the susceptibility of *SP-RNAi* flies to infections with these two microorganisms. The knockdown of *spirit, SPE, sphinx1/2*, or *spheroide* inhibited Toll activation by fungi, which is reflected by an increased susceptibility to fungal infections (Figure 2) and failure to normally express *Drs* (Figures 3C and 3D). In addition, silencing of any of these four *SPs* reduced constitutive Toll activation in flies overexpressing *psh* or a *nec-IR* construct [14] (Figures 4C and 4D). This result indicates that Psh and Nec require these four SPs to activate Toll. The observation that four distinct SPs are involved in Toll activation after both fungal and

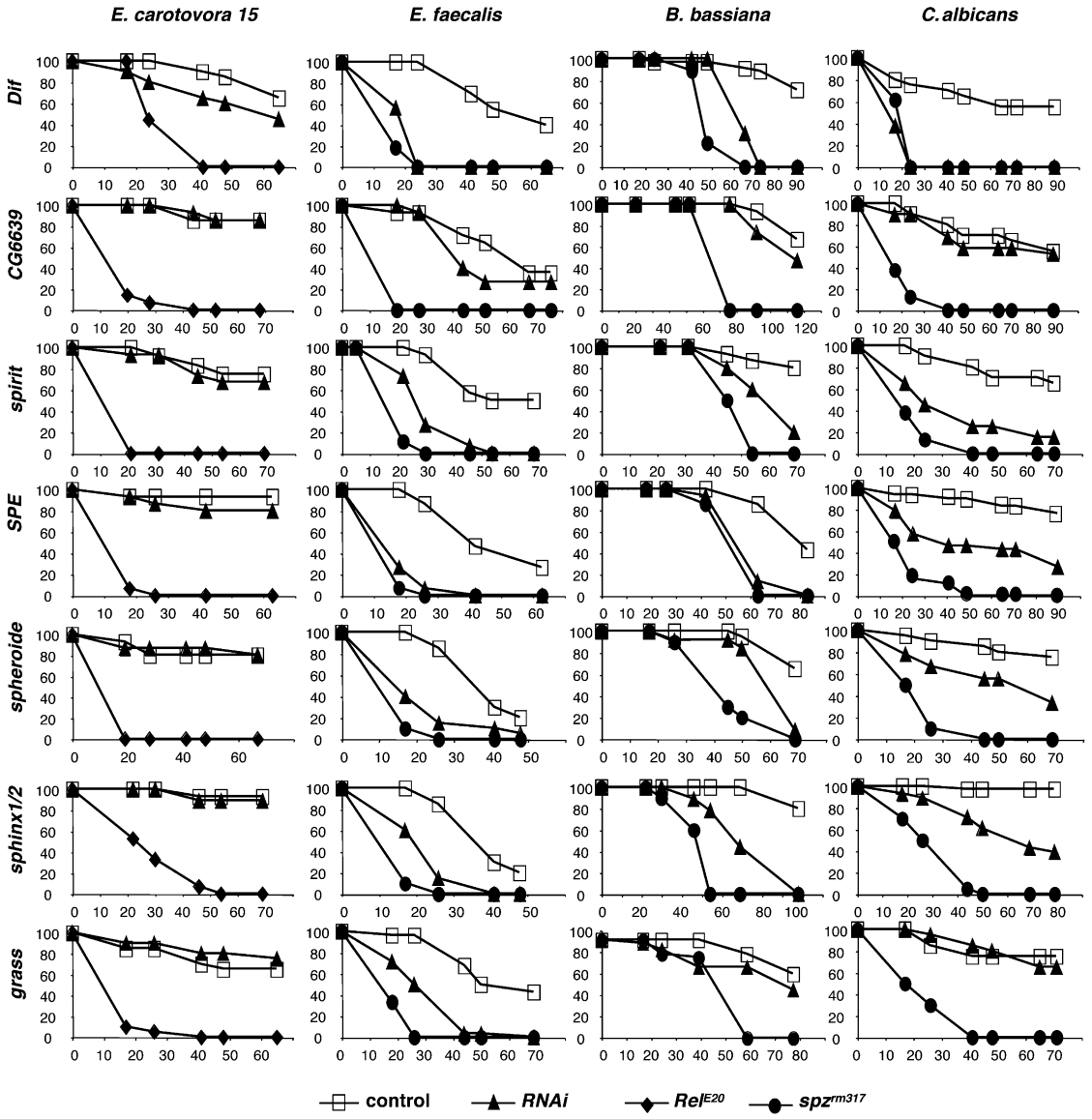

Figure 2. Five New *SP* Genes Required for Resistance to Microbial Infections

The survival rates (%) of *SP*- and *Dif-RNAi* flies infected with *E. carotovora 15* (gram-negative bacterium), *E. faecalis* (gram-positive bacterium), *B. bassiana* (filamentous fungus), and *C. albicans* (yeast) were compared to wild-type control flies and flies mutant in either the Toll pathway (spz^{rm317}) or the Imd pathway (Rel^{E20}). Inactivation of *spirit, SPE*, and *sphinx1/2* induced an increased susceptibility to gram-positive bacteria and fungi. *spheroide-RNAi* led to a similar phenotype with a less marked susceptibility to *B. bassiana*. In contrast, *grass-RNAi* flies displayed a specific immune deficiency toward gram-positive bacteria. The effect observed was not due to general susceptibility of RNAi flies, since silencing of *SP CG6639* had no effect on resistance to microbial infections and since none of the *SP-IR* lines was sensitive to *E. carotovora* injection. The X-axis represents the incubation time after infection in hours.

gram-positive bacterial infections demonstrates the existence of a common SP cascade that integrates signals from different secreted recognition molecules. In contrast, silencing of *grass* did not impair Toll activation by fungi and did not affect susceptibility to this class of microbes (Figures 2 and 3). Consistently, inactivation of this *SP* did not reduce *Drs* expression in flies overexpressing *psh* or the *nec-IR* element. These results indicate that Grass participates in the activation of Toll by gram-positive bacteria but not by fungi, demonstrating for the first time the involvement of a SP in an extracellular pathway specifically devoted to gram-positive bacterial recognition.

Discussion

Until recently, only one SP, Psh, has been shown to act upstream of Toll in response to fungi [13]. Via a large-scale RNAi screen, we have identified five novel SPs regulating Toll activity in response to gram-positive bacterial infections. Three of them, Spirit, Grass, and SPE, are functional chymotrypsin-like SPs containing a Clip domain N-terminal to the catalytic domain. The Clip domain is exclusively found in insect SPs and is believed to play a regulatory role in the sequential activation of SPs [15]. For instance, it is present in Psh as well as in Snake and Easter, two SPs that participate in the

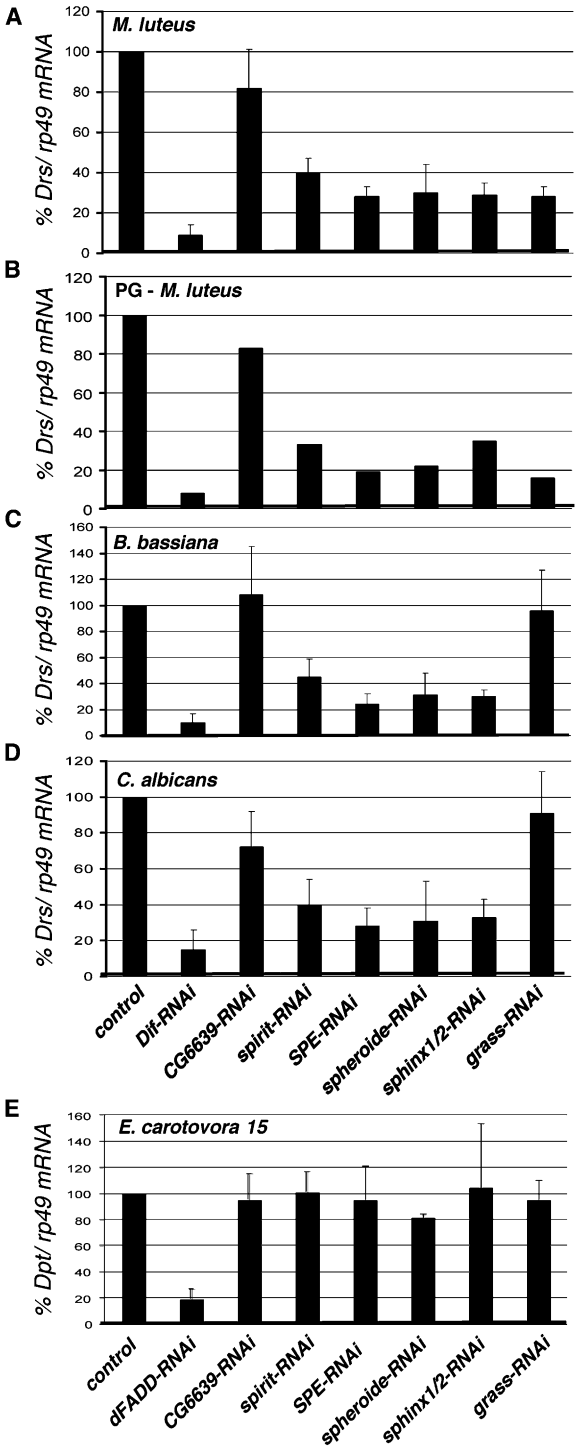

Figure 3. Analysis of Antimicrobial Peptide Gene Expression in *SP-RNAi* Flies after Different Immune Challenges

Drs (A–D) or *Dpt* (E) gene expression was monitored by RT-qPCR with total RNA extracted from wild-type control and *RNAi* males collected 24 hr after septic injury with *M. luteus* (A), 24 hr after injection of *M. luteus* peptidoglycan (B), 48 hr after natural infection with *B. bassiana* spores (C), 24 hr after septic injury with *C. albicans* (D), or 6 hr after septic injury with *E. carotovora 15* (E). RNAi extinction of *spirit*, *SPE*, *spheroide*, *sphinx1/2*, or *Dif* significantly reduced the level of *Drs* expression following *M. luteus, B. bassiana*, or *C. albicans* infections, while in *grass-RNAi* flies, *Drs* was only affected after gram-positive bacterial infection. The expression level of the antibacterial peptide gene *Dpt* after challenge with *E. carotovora* was comparable to wild-type in all *SP-RNAi* flies tested but was strongly reduced in *dFADD-RNAi* flies lacking a functional Imd pathway [24]. *CG6639-RNAi* flies showed no significant alteration in either *Drs* or *Dpt* expression. Each bar represents the mean of three to five independent experiments with standard deviation, except for the experiment in (B), which was performed only once.

processing of Spz during embryonic development [16–18]. In contrast to *spirit* and *SPE*, *grass* is required to resist gram-positive bacterial but not fungal infection, and its knock-down induces a phenotype similar to those induced by *GNBP1* or *PGRP-SA* mutations. This is in agreement with a model in which Grass is activated by GNBP1 and PGRP-SA after recognition of gram-positive peptidoglycan. Grass then transmits a signal that is integrated by a common core of downstream SPs including Spirit and SPE (see model in Figure 4E). In agreement with a position of Grass upstream in the Toll-activating cascade, we observed that the expression of an activated form of SPE and Spirit but not Grass can trigger *Drs* in S2 cells (data not shown). Further signal amplification could occur since both *spirit* and *SPE* are induced during the immune response in a Toll pathway-dependent manner [19]. Thus, this combination of controlled proteolytic SPs activation and transcriptional positive feedback would ensure an adequate response to infection.

Surprisingly, two SPs identified in our screen, Spheroide and Sphinx1/2, are unlikely to possess proteolytic activity, because their protease-like domain lacks the catalytic serine residue [2]. This class of SP homologs (SPHs) represents one quarter of *Drosophila* SP-related proteins and is thought to have regulatory functions [2, 20, 21]. Several SPHs have been shown to be involved in the proteolytic cascade that regulates the cleavage of prophenoloxidase in other insects, supporting a role of this class of SP-related proteins during the immune response [20, 21]. Since knock-downs of *spheroide* and *sphinx1/2* induces the same phenotype as that of *SPE* and *spirit*, we suggest that these two SPHs may act as adaptors or regulators of SPE and Spirit, possibly by localizing the two SPs in close proximity to Spz and/or the fat-body cell membrane to promote robust activation of the Toll receptor.

We observed that RNAi of the five SPs did not affect activation of melanization nor did it suppress lethality induced by the inactivation of *spn27A* (Figure S2, data not shown). In agreement with two recent studies [22, 23], we observed that RNAi of *SP7* (*CG3066*) reduces melanization at the wound site, confirming the implication of this SP in the prophenoloxidase cascade (data not shown). The *RNAi* of this *SP* did not affect Toll activation by gram-positive bacteria (Table S1). Collectively, these data indicate that distinct SPs mediate melanization and Toll activation, underlining the complexity of SP cascades regulating the *Drosophila* immune response. Further studies, including the generation of null mutations, are required to analyze in more detail the function of these five SPs upstream of Toll.

This study represents the first extensive in vivo RNAi screen of a large gene family in *Drosophila*. A key advantage of this strategy for functional genomic studies is that genes required during development can be inactivated in a tissue- and temporal-specific manner. It is

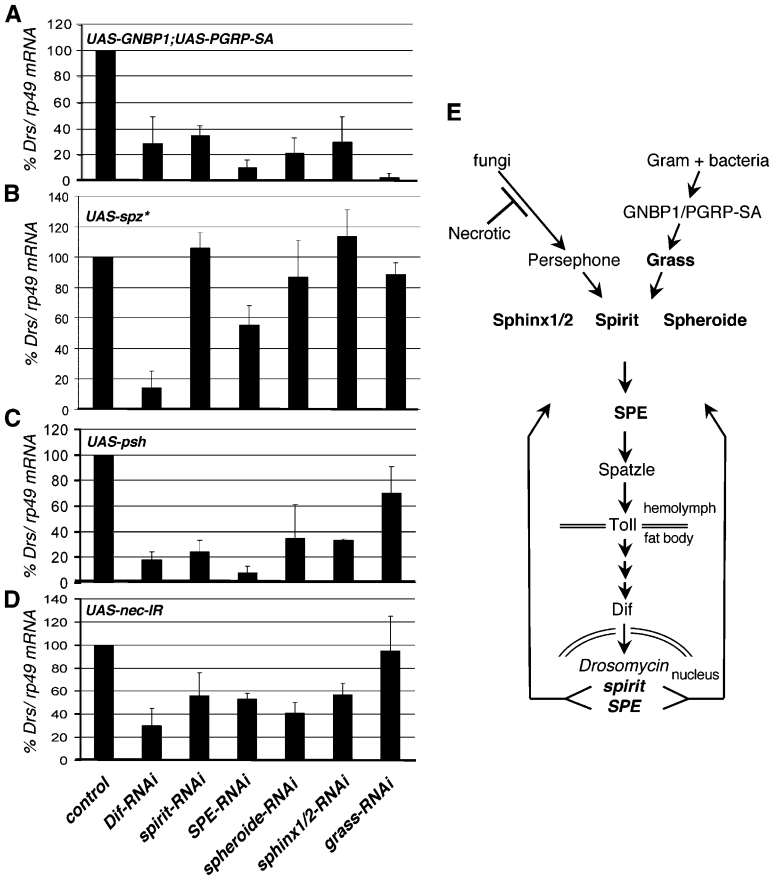

Figure 4. Epistatic Analysis of SPs Acting Upstream of Spz

(A) Overexpression of the *UAS-GNBP1* and *PGRP-SA* constructs induces the *Drs* gene in absence of infection [8]. This figure shows that *Drs* stimulation induced by overexpressing *GNBP1* and *PGRP-SA* was strongly reduced by coexpression of *Dif-IR* or the five selected *SP-IR*. The effect was more pronounced with *grass-IR*.

(B) In absence of an immune challenge, overexpression of *spz* led to constitutive *Drs* expression that was independent of *grass, spirit, SPE, spheroide*, and *sphinx1/2* but dependent on *Dif*. Overexpression of *SPE* double-stranded RNA slightly reduced *Drs* expression by Spz.

(C and D) In the absence of an immune challenge, overexpression of *UAS-psh* (C), or RNAi inactivation of *nec* (D) induced constitutive *Drs* expression that was dependent on *Dif, spirit, SPE, spheroide*, and *sphinx1/2* genes but independent of *grass*.

(E) Model for the regulation of the Toll pathway by SPs during the immune response. The Toll ligand Spz is processed by secreted SPs present in the hemolymph that are activated upon recognition of gram-positive bacteria or fungi [1]. Detection of gram-positive bacteria is mediated by two secreted proteins, PGRP-SA and GNBP1. Spz activation by fungi is controlled by an extracellular cascade involving the SP Psh and is negatively regulated by the serpin Nec. The recognition molecules involved in fungal detection have not yet been identified. The five SPs identified in this study are labeled in bold letters.

especially suitable for the analysis of genes encoding secreted proteins such as SPs that function in a non-cell-autonomous manner and therefore require in vivo studies. The *SP-RNAi* fly collection described here and made available to the scientific community should pave the way for additional biochemical studies and genetic screens to further decipher the signaling pathways acting upstream of Toll as well as to identify additional important SP functions.

Supplemental Data

Supplemental Data include two figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at http://www.current-biology.com/cgi/content/full/16/8/808/DC1/.

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