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Toll family members bind multiple Spätzle proteins and activate antimicrobial peptide gene expression in *Drosophila*

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Running title: *Toll family member functions*

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ABSTRACT

The Toll signaling pathway in *Drosophila melanogaster* regulates several immune-related functions, including the expression of antimicrobial peptide (AMP) genes. The canonical Toll receptor (Toll-1) is activated by the cytokine Spätzle (Spz-1), but *Drosophila* encodes eight other Toll genes and five other Spz genes whose interactions with one another and associated functions are less well understood. Here, we conducted *in vitro* assays in the *Drosophila* S2 cell line with the Toll/interleukin-1 receptor (TIR) homology domains of each Toll family member to determine if they can activate a known target of Toll-1, the promoter of the antifungal peptide gene *drosomycin*. All TIR family members activated the *drosomycin* promoter, with Toll-1 and Toll-7 TIRs producing the highest activation. We found that the Toll-1 and Toll-7 ectodomains bind Spz-1, -2, and -5 and also *vesicular stomatitis* virus (VSV) virions, and that Spz-1, -2, -5, and VSV all activated the promoters of *drosomycin* and several other AMP genes in S2 cells expressing full-length Toll-1 or Toll-7. *In vivo* experiments indicated that Toll-1 and Toll-7 mutants could be systemically infected with two bacterial species (*Enterococcus faecalis* and *Pseudomonas aeruginosa*), the opportunistic fungal pathogen *Candida albicans* and VSV with different survival in adult females and males compared with wild-type fly survival. Our results suggest that all Toll family members can activate several AMP genes. Our results further indicate that Toll-1 and Toll-7 bind multiple Spz proteins and also VSV, but differentially affect adult survival after systemic infection, potentially because of sex-specific differences in Toll-1 and Toll-7 expression.

Introduction

The innate immune system of *Drosophila melanogaster* and other insects provides defense against infection by pathogenic viruses, bacteria, fungi and parasites (1). One key defense response is the production of antimicrobial peptides (AMPs), whose expression is primarily regulated by the Toll and IMD pathways (1-3).

In *Drosophila*, the Toll pathway is activated when a cytokine named Spätzle (Spz-1) binds the Toll receptor (Toll-1) (4, 5). Spz-1 is expressed and secreted into circulation as a zymogen (proSpz-1), which consists of an N-terminal pro-domain and a C-terminal cystine knot. Toll-1 structurally consists of an ectodomain containing leucine-rich repeats (LRRs), a single-pass transmembrane domain, and a cytosolic Toll/interlukin-1 receptor (TIR) homology domain (6, 7). Systemic infection by bacteria or fungi stimulates Spätzle-processing enzyme (SPE) to cleave proSpz-1 into Spz-1, which binds Toll-1 that is expressed on the surface of fat body and select other cell types (5, 8-12). Spz-1 binding promotes Toll-1 dimerization/oligomerization, which stimulates downstream signaling through the adaptor protein MyD88 (dMyD88) and kinases named Tube and Pelle. This results in phosphorylation and degradation of the IκB inhibitor Cactus (13), which enables the NF-κB transcription factors Dif and Dorsal to translocate to the nucleus and upregulate AMP genes through promoter binding (1, 14, 15).

Vertebrates encode multiple Toll-like receptors (TLRs) that exhibit similar architecture to *Drosophila* Toll-1 and regulate several immune and non-immune functions (16-19). Similarities in downstream signaling components also support shared ancestry between the Toll and TLR pathways. However vertebrate TLRs do not bind cytokines like Spz-1 but instead function as pattern recognition receptors (PRRs) that bind pathogen-associated ligands such as bacterial lipopolysaccharide (LPS), peptidoglycan (PGN), teichoic acid, flagella, CpG DNA (17, 20-22), viral single-stranded RNA, and viral double-stranded RNA (20-21).

Comparative genomic data indicate that insects also encode multiple Toll genes. *Drosophila* encodes eight other Toll family members (Toll-2 to Toll-9) in addition to Toll-1 with some evidence supporting defense functions for Toll-2 (18 wheeler, 18W), Toll-5 (Tehao), Toll-8 (Tollo) and Toll-9 (23-28). Toll-6 and Toll-7 function as neurotrophin receptors (29), although Toll-7 is also reported to recognize *vesicular stomatitis* virus (VSV) and induce antiviral autophagy (30, 31). In contrast, other results indicate that autophagy plays a minor role in hemocyte-mediated defense against VSV and does not depend on Toll-7 (32). *Drosophila* encodes 5 other Spz genes (Spz-2 to Spz-6) in addition to Spz-1, but it remains unknown whether any of these other family members bind to Toll-1 or other Toll proteins. Also unclear is whether AMP genes activated by Toll-1 are also activated by other Toll family members.

In this study, we assessed whether all or only some *Drosophila* Toll family members activate the *drosomycin* promoter, which is a known target for the canonical Toll-1 pathway (1, 14, 15). Focusing on Toll-1 and Toll-7, we also assessed binding to Spz family members and VSV, and whether each similarly or differentially effects adults after infection by different microbes. Our results indicated that the TIR domains for all Toll-family members activated the *drosomycin* promoter. We further determined that Toll-1 and Toll-7 bind multiple Spz proteins and VSV while differentially affecting adult female and male survival after systemic infection.

Results

The TIR domains of several Drosophila Toll family members activate the drosomycin promoter in S2 cells

Prior studies indicate that binding of Spz-1 to Toll-1 activates the *drosomycin* promoter as well as the promoters for select other AMP genes (1, 14, 15). To determine if other Toll family members can also activate the *Drosophila drosomycin* promoter, we conducted dual luciferase assays in S2 cells that were co-transfected with a pGL3B-*drosomycin* reporter plus pMT/BiP/V5-His that inducibly expressed the TIR domain for each *Drosophila* Toll family member as well as Toll-1 from the moth *Manduca sexta* (33). We also assessed whether any of these TIRs activated the *Drosophila diptericin* promoter, because this AMP is not activated by Toll-1 signaling but is activated by the IMD pathway (1). We first confirmed by immunoblotting that each TIR was expressed (Fig. 1A). We then conducted dual luciferase assays, which indicated that each TIR significantly activated the *drosomycin* reporter (7- to 54-fold) above an empty plasmid/pGL3B control (Fig. 1B). However, the strongest responses were elicited by the TIRs from Toll-1

(54-fold), Toll-7 (39-fold) and *M. sexta* Toll-1 (48-fold) (Fig. 1B). In contrast, no TIR significantly activated the *diptericin* promoter when compared to the empty vector control (Fig. 1B). We thus concluded that the TIR domains for each *Drosophila* Toll family member can activate the *drosomycin* promoter not the *diptericin* promoter.

The ectodomains of Toll-1 and Toll-7 interact with multiple Spz proteins

We next considered whether Toll family members interact with only one or multiple Spz proteins. For these and subsequent experiments we focused on comparing *Drosophila* Toll-1 to Toll-7 because in the preceding assays these two family members most strongly activated the *drosomycin* promoter. Previous co-immunoprecipitation (Co-IP) assays indicated that *M. sexta* Toll-1 binds the cystine knot domain of *M. sexta* Spz-1 but not full-length Spz-1 (33). We therefore used previously developed approaches (29) to first predict the cystine knot domains for the six *Drosophila* Spz family members (Fig. S1A). Alignments further indicated that Spz-1, Spz-2 and Spz-5 are more closely related to one another than to other family members with Spz-1 sharing 63% and 71% similarity with Spz-2 and Spz-5 respectively, and Spz-2 sharing 62% similarity with Spz-5 (Fig. S1B). We then cloned each cystine knot into pMT/BiP and expressed them in S2 cells as recombinant proteins with Flag epitope tags, while cloning either the ectodomains (Toll-1^{ecto}, Toll-7^{ecto}) or full-length *Drosophila* Toll-1 and Toll-7 into pMT/BiP/V5-His for expression as recombinant proteins with V5 epitope tags. Immunoblotting assays showed that each Spz cystine knot was present in both S2 cell lysates and the medium which indicated each was secreted (Fig. S2). Toll-1^{ecto} and Toll-7^{ecto} were also detected in both lysates and medium, whereas full-length Toll-1 and Toll-7 were, as expected, readily detectable in S2 cells but were not secreted due to the presence of transmembrane domains (Fig. S2). After mixing cell lysates containing each Spz cystine knot with lysates containing Toll-1^{ecto} or Toll-7^{ecto}, reciprocal Co-IP assays using anti-V5 and anti-Flag antibodies indicated that Toll-1^{ecto} bound Spz-1, Spz-2 and Spz-5 but not other Spz family members, while Toll-7^{ecto} bound Spz-1, Spz-2, Spz-5 and Spz-6 (Fig. 2).

Multiple Toll-1-Spz and Toll-7-Spz pairs activate the drosomycin promoter in S2 cells

Given evidence that Toll-1^{ecto} and Toll-7^{ecto} bound multiple Spz family members, we conducted dual-luciferase assays to determine if the same Spz family members activate the *drosomycin* promoter in S2 cells expressing full-length Toll-1 or Toll-7 plus our *drosomycin*-luciferase reporter. Results indicated that the cystine knots for Spz-1, -2 and -5 activated the *drosomycin* promoter in S2 cells expressing full-length Toll-1 492-fold, 188-fold and 122-fold, whereas other Spz family members had no significant effect on promoter activation (Fig. 3A). Spz-1, -2, and -5 also activated the *drosomycin* promoter in S2 cells expressing full-length Toll-7 98-fold, 87-fold and 83-fold (Fig. 3B). In contrast, adding the cystine knot for Spz-6 and other family members either only weakly activated or had no effect on the *drosomycin* promoter in cells expressing full-length Toll-7 (Fig. 3B). Control assays where: 1) no Spz was added to cells expressing full length Toll-1, Toll-2 and Toll-7, 2) cystine knot proteins for Spz-1, Spz-2 or Spz-5 were added to cells lacking full-length recombinant Toll-1 or Toll-7, or cells co-expressing Toll-2 and the cystine knot for Spz-1 also resulted in either little (3.5-fold to 14-fold) or no activation of the *drosomycin* promoter (Fig. 3C). Thus, with the exception of Spz-6, Spz family members that bound Toll-1^{ecto} and Toll-7^{ecto} also strongly activated the *drosomycin* promoter. In contrast, weak activation of the *drosomycin* promoter in select control assays could reflect interactions with endogenous ligands or receptors.

The ectodomains of Toll-1 and Toll-7 interact with VSV, which also activates AMP gene promoters

As previously noted, Toll-7 has previously been reported to interact with VSV, which suggested that Toll-7 could function as a PRR (30). Given that Toll-7 and Toll-1 bound largely the same Spz family members in our Co-IP assays, we asked if Toll-1 also binds VSV. Co-IP assays where we added VSV-GFP virions to culture medium containing Toll-1^{ecto} or Toll-7^{ecto}

resulted in an anti-V5 antibody plus protein G beads co-immunoprecipitating VSV virions, whereas control assays in which protein G beads alone resulted in no pull down of VSV virions, Toll-1^{ecto} or Toll-7^{ecto} (Fig. 4A). Dual luciferase assays further showed that adding VSV-GFP virions to S2 cells co-expressing full-length Toll-1 or Toll-7 and reporter plasmids significantly activated the *drosomycin* promoter as well as the promoters for two other AMP genes (*attacin*, *metchnikowin*) that are known to be activated by Toll signaling (Fig. 4B, C).

Toll-1 and Toll-7 loss of function mutants differentially affect male and female survival after infection

Real-time PCR assays indicated that transcript abundance for Toll-1 was significantly higher in 5 day old wild-type (w^{1118}) adult females than adult males while transcript abundance for Toll-7 was higher in adult males than adult females (Fig. S3A). This finding suggested to us that Toll-1 and Toll-7 could differentially affect defense in females versus males after systemic infection by different microorganisms. To assess this possibility, we compared the survival of adult female and male loss of function Toll-1 ($Tlr^{632}/Tl^{I\text{-}RXA}$ and $Tl^{I\text{-}RXA}$) and Toll-7 mutants ($Toll\text{-}7^{g1\text{-}5}/Cyo$ and $Df/Toll\text{-}7^{g1\text{-}5}$) to wild-type ($w^{1118}$) flies after infection by two species of bacteria (*Enterococcus faecalis* or *Pseudomonas aeruginosa*), the fungus *Candida albicans*, or VSV-GFP. For females, both Toll-1 mutants exhibited lower survival than wild-type flies after infection by *E. faecalis*, *C. albicans*, and VSV (Fig. 5A-D). Both Toll-7 mutants also exhibited lower survival when compared to wild-type flies after infection by VSV but did not differ from wild-type flies after infection by *E. faecalis*, *P. aeruginosa* and *C. albicans* (Fig. 5A-D). For males, we observed the reverse trend with one or both Toll-7 mutants exhibiting significantly lower survival rates than wild-type flies after infection by each microbe, whereas Toll-1 mutants exhibited lower survival rates in response to infection by *E. faecalis* and *C. albicans* but did not differ from wild-type flies in response to infection by *P. aeruginosa* (Fig. 5A-D). Overall, loss of Toll-1 adversely affected adult females more than adult males after infection by different microbes while loss of Toll-7 adversely affected adult males more than adult females.

Discussion

Toll-1 and Spz-1 were originally identified in *D. melanogaster* as components of the Toll signaling pathway that regulates dorsal-ventral patterning during embryogenesis (34). Thereafter, Toll-1, Spz-1 and Toll signaling were further implicated in regulating AMP genes and other immune-related functions (1-3). Select other Toll family members (-2, -5, -8, -9) have also been reported to affect immune defenses (24-28, 35, 36). However, no standardized platform has previously been developed that could be used to assess AMP promoter activation and ligand binding by different Toll family members.

Our approach of co-expressing the TIR domain for each Toll family member with a *drosomycin* promoter construct suggests all *Drosophila* Toll family members can activate this AMP above background levels although activation is most strongly elicited by Toll-1 and Toll-7. That no Toll family member activated the *diptericin* promoter further affirms *diptericin* is primarily if not exclusively regulated by the IMD pathway. Tauzig et al. (23) in contrast took a different approach by co-expressing the TIR domain from Toll-2, -5, -6, -7, and -8 with a truncated form of Toll-1 (Toll$^{\Delta LRR}$) to produce chimeric receptors in S2 cells. However, the only chimeric construct that activated the *drosomycin* promoter was Toll-5 (23). Given evidence that receptor dimerization/ oligomerization is required for the downstream signaling events that precede AMP gene promoter activation (1, 2), we speculate that expressing only the TIR for each Toll family member potentially enabled dimers/oligomers to more readily form, whereas the approach of producing chimeric Tolls may have resulted in only Toll-5 forming dimers/oligomers. However, our results also indicate the Toll-5 TIR more weakly activated the *drosomycin* promoter than the chimeric Toll-5 construct produced by Tauzig et al. (23), which could suggest the latter forms a more stable dimers/oligomer.

Focusing on Toll-1 and Toll-7, our Co-IP assays indicate the ectodomains of each bind Spz-1, -2 and -5 but not other family members, while also suggesting that binding of Spz-1, -2 and -5 to

full-length Toll-1 or Toll-7 results in activation of the *drosomycin* promoter. Our results also indicate that Toll-7 binds Spz-6 but surprisingly adding the cystine knot for Spz-6 to S2 cells expressing full-length Toll-7 did not activate the *drosomycin* promoter. Thus, three Spz family members (-1, -2 and -5) exhibit features consistent with functioning as cytokines that bind to Toll-1 and Toll-7, which activates downstream Toll signaling and the *drosomycin* promoter. In contrast, our results do not reveal why Spz-6 does not exhibit similar activity or what the function of this family member might be.

As previously noted, Toll-6 and Toll-7 function as neurotrophin receptors in the central nervous system with Spz-2 and Spz-5 being candidate ligands for both (29). However, Toll-7 is also reported to recognize VSV, and function as a PRR that stimulates antiviral autophagy (30, 31). Other results raise questions about both the importance of autophagy as an anti-viral immune defense and the requirement for Toll-7 (32). Nonetheless, our results corroborate that Toll-7 binds VSV while showing that Toll-1 also binds this virus. Further, infection of S2 cells expressing full-length Toll-1 and Toll-7 with VSV activated the expression of several AMP promoter constructs. Our results thus overall suggest Toll-1 and Toll-7 bind multiple Spz proteins and VSV, and that both types of ligands can activate downstream signaling that leads to AMP gene expression. Lastly, our finding that adult males and females differentially express Toll-1 and Toll-7 combined with differential sensitivity to systemic infection by VSV and other microbes suggests gender may affect the relative importance of different Toll family members in immune defense.

That Toll family members bind multiple Spz proteins raises important questions for future study regarding the inputs that regulate processing of different Spz zymogens and whether SPE or other unidentified serine proteases are involved. The function of Spz-6 is a second question of interest as is the relative importance of Toll family members binding different Spz family members versus PAMPs on microbes like VSV in regulating different immune defense responses.

Experimental procedures

Fly stocks and S2 cells

Wild-type w^{1118} flies were obtained from the laboratory of Dr. Leonard Dobens (School of Biological Sciences, University of Missouri – Kansas City, Missouri, USA). The *Toll-7*$^{g1\text{-}5}$*/Cyo* mutant line was a gift from Dr. Yashimasa Yagi (Division of Biological Science, Nagoya University, Nagoya, Japan), and the *Toll-7*$^{g1\text{-}5}$ mutant line was created by homologous recombination of an ends-in knockout system followed by hs-ICreI treatment to generate a *Toll-7* knockout line with a point mutation (37). The *Toll-7*$^{g1\text{-}5}$ line was balanced over Cyo to obtain the *Toll-7*$^{g1\text{-}5}$*/Cyo* mutant line, and heterozygotes were screened based on the existence of curled wings. The *Tlr*^{632}/*Tl*$^{I\text{-}RXA}$ and *Tl*$^{I\text{-}RXA}$/*TM6B* (*Tl*$^{I\text{-}RXA}$) Toll (also called Toll-1) mutant lines were obtained from the laboratory of Dr. Kontoyiannis (Department of Infectious Diseases, University of Texas M. D. Anderson Cancer Center, Houston, Texas, USA) (38). *Tlr*^{632}/*Tl*$^{I\text{-}RXA}$ flies were generated by crossing *Tlr*^{632}/*TM6B* and *Tl*$^{I\text{-}RXA}$/*TM6B* *Toll*-deficient flies. *Tlr*^{632} is a thermosensitive loss-of-function allele with a strong phenotype at 29°C; thus, these flies were maintained at 29°C during infection. Both the *Tlr*^{632} and *Tl*$^{I\text{-}RXA}$ mutant lines were balanced over *TM6B* and were recognized by multiple hair-type bristle in the upper lateral thorax/torso. The *Df(2R)BSC22/SM6a* line (stock # 6647) was purchased from Bloomington Stock Center (Indiana, USA); in this line, the 56D7 – 56F12 chromosome segment was deleted by exploiting hybrid element insertion (HEI) and resolution, and this line was later balanced over *SM6a* to obtain flies that can be recognized by curly wings. We generated *Df(2R)BSC22*/*Toll-7*$^{g1\text{-}5}$ (*Df*/*Toll-7*$^{g1\text{-}5}$) flies by crossing *Toll-7*$^{g1\text{-}5}$*/CyO* and *Df(2R)BSC22/SM6a* flies, which uncovers the *Toll-7* locus to obtain *Toll-7* mutants that can be screened by the presence of curly wings. All the flies were cultured on corn-meal diet (31) and transferred to fresh food at least 24 h prior to injection/infection. The *Drosophila* S2 cell line was obtained from the American Type Culture Collection (ATCC) and maintained in serum free SFX medium (Hyclone) that was supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% Gibco L-Glutamine (25030081, Thermo Fisher Scientific) (the

complete growth medium) by passaging weekly in 15 cm culture flasks (Corning).

Gene cloning

For this study, we cloned full-length Toll-1 from cDNA using total RNA from *Drosophila* adult females as the template. Full-length Toll-7 without introns was previously cloned into pAC5.1-A (37). All nine *Drosophila* Toll TIR domains and the TIR from *M. sexta* Toll-1 (33), the ectodomains of Toll-1 and Toll-7, and full-length Toll-1 and Toll-7 were amplified by PCR using the forward and reverse primers listed in Table S1 and cloned into the pMT/BiP/V5-His A vector (V413020, Invitrogen) for expression of the recombinant proteins with a V5 epitope tag at the C-terminus. Active Spz-1 to Spz-6 were generated by PCR amplifying the domains underlined in Fig. S1A using sequence specific primers (Table S1) and RNA from *Drosophila* adult females as template followed by cloning into a modified pMT/BiP A vector (33) for expression of the recombinant Spz proteins with a Flag-tag at the N-terminus. The PCR reactions were performed with the following conditions: 94°C for 3 min, 35 cycles of 94°C for 30 s, Tm-5°C for 30 s, 72°C for 30 s to 4 min, and final extension at 72°C for 10 min. The PCR products were recovered using an Agarose Gel Electrophoresis-Wizard® SV Gel and PCR Clean-Up System (A9285, Promega) and then subcloned into the T-Easy vector (A1360, Promega). Recombinant plasmid DNAs were purified using a PureYield™ Plasmid Miniprep System (A1222, Promega) according to the manufacturer's instructions and digested with respective restriction enzymes, and DNA fragments were recovered and inserted into the pMT/BiP/V5-His A or modified pMT/BiP A vector using T4 DNA ligase (M0202L, NEB). The recombinant expression plasmids were then purified and sequenced in the sequencing facility at University of Missouri – Columbia for further experiments.

Vesicular stomatitis virus stock culture

Vesicular stomatitis virus (VSV) expressing green fluorescent protein (GFP) inserted between the 3' leader and N gene (39), was obtained from the laboratory of Dr. Sean Whelan (Harvard Medical School, Boston, Massachusetts, USA) (40). VSV-GFP was cultured and maintained in HEK293 cells in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (#10082063, Invitrogen) and 1% penicillin-streptomycin solution (G6784, Sigma-Aldrich). The viral titer was measured by plaque assay using HEK293 cells (41). For infection assays with *Drosophila* S2 cells, 10,000 pfu/ml VSV-GFP was used, and for the infection assays with adult flies, 10,000 pfu of VSV-GFP (in 50 nl of PBS) were injected into each fly.

Infection assays

Drosophila adult males and females (5-7 days of age) in a batch of 20-30 flies were infected with Gram-positive *Enterococcus faecalis V583* (a gift from Dr. Michael Gilmore, Harvard Medical School, Boston, Massachusetts, USA), Gram-negative *Pseudomonas aeruginosa PA-14* (a gift from Dr. Kalai Mathee, Florida International University, Florida, USA), *Candida albicans* (a gift from Dr. Theodore White, School of Biological Sciences at the University of Missouri – Kansas City, Missouri, USA), or VSV-GFP. Briefly, overnight bacterial and fungal cultures were diluted to OD_{600} = 0.2 and OD_{600} = 0.5, respectively, washed with phosphate buffered saline (PBS, pH 7.4) and resuspended in PBS for injection. Flies were anesthetized with CO_2 (for no longer than 15 min at a time), and 50 nl of diluted *E. faecalis V583*, *P. aeruginosa PA-14*, *C. albicans*, or VSV-GFP (10,000 pfu/50 nl) was injected into each fly at the left intra-thoracic region using a Drummond nanoinjector and pulled glass capillary needles. These flies were maintained in clean bottles with fresh cornmeal diet, and the diet was changed every day throughout the course of the experiment. The flies that died within 3 h of injection were excluded from the study on the presumption of injury. Flies injected with bacteria or *C. albicans* were monitored hourly while flies injected with VSV-GFP were monitored daily by recording the number of dead males and females.

A stable S2 cell line expressing either full-length Toll-1 or Toll-7 receptor (described below) was transiently transfected with pGL3B or the pGL3B-*attacin*, pGL3B-*drosomycin* or pGL3B-*metchnikowin* AMP gene promoter individually using GenCarrier-2™ (#31-00110, Epoch Biolabs). Forty-eight hours post-

transfection, 10^6 cells were infected with 10^4 pfu/ml VSV-GFP (multiplicity of infection (MOI)=0.01) for 24 h and processed for dual luciferase assay (see below).

Transient transfection and establishment of stable S2 cell lines

Transient transfection experiments and establishment of S2 cell lines stably expressing full-length Toll-1 and Toll-7 followed previously established protocols (33). Briefly, cells were seeded overnight in complete growth medium (see above), washed with serum-free medium, and transiently transfected using GenCarrier-2™ transfection reagent according to the manufacturer's instructions. The DES®–Inducible/Secreted Kit with pCoBlast (K5130-01, Invitrogen) was used to generate stable S2 cell lines. Lines stably expressing recombinant Toll-1 and Toll-7 were selected by co-transfecting pCoBlast (Invitrogen) with the pMT/BiP/V5-His A vector, which uses the metallothionein promoter for inducible expression of the gene of interest following the addition of copper sulfate. Forty-eight hours after transfection, S2 cells were centrifuged and resuspended in the complete growth medium containing 25 μg/ml Blasticidin S hydrochloride (No.15205, Sigma-Aldrich). Resistant colonies appeared one week later.

Dual luciferase assays

Dual luciferase assays were performed as described previously (42). S2 cells were plated in 24-well culture plates (3×10^5 cells/well) overnight in the complete growth medium, washed with serum-free medium, and then transiently co-transfected with recombinant pMT/BiP/V5-His A expression plasmid (500 ng), pGL3B, pGL3B-*drosomycin* or pGL3B-*diptericin* firefly luciferase reporter plasmid (250 ng), and Renilla luciferase reporter plasmid (25 ng) (as an internal standard) (pRL-TK, Promega) with GenCarrier-2™. After overnight transfection, serum-free medium was replaced with the complete growth medium containing copper sulfate (to a final concentration of 500 μM) for protein expression, and 36 h after protein expression, the firefly luciferase and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (E1980, Promega) with a GloMax® Multi Microplate Luminometer (Promega). The relative luciferase activity (RLA) was obtained as the ratio of firefly luciferase activity to Renilla luciferase activity. The RLA obtained from S2 cells co-transfected with empty pMT/BiP/V5-His A and pGL3B (empty reporter vector) plasmids was used as the calibrator. These experiments were repeated at least three times (three independent biological samples or three independent cell cultures), and a representative set of data was used to prepare the figures.

Immunoblotting

Samples for immunoblot analysis were prepared by placing transiently or stably transfected S2 cell (5×10^6 cells/well) in six-well plates 48 h after induction of protein expression using copper sulfate (250 μM). Cell culture medium (2 ml) or S2 cells were collected. S2 cells were homogenized in 400 μl of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% NP-40, and 0.5 mM PMSF) containing protease inhibitor cocktail (P8340, Sigma-Aldrich) following a previously described protocol (43). Cell homogenates were sonicated briefly and centrifuged, and the supernatants (cell lysates) were collected. Proteins in cell culture medium (10 μl from a total volume of 2 ml) or cell lysates (10 μl from a total volume of 400 μl, equivalent to ~5×10^4 cells) were resolved on 8%, 12% or 15% SDS-PAGE gels, followed by transfer to nitrocellulose (162-0097, Bio-Rad). Blots were processed and probed with an anti-Flag M2 antibody (F-1804, Sigma-Aldrich, 1:5000 dilution) or anti-V5 antibody (V-8012, Sigma-Aldrich, 1:5000 dilution) as the primary antibodies and an alkaline phosphatase-conjugated goat anti-mouse secondary antibody (A4312, Sigma-Aldrich, 1:10,000) (43). The signal was developed using an Alkaline Phosphatase (AP)-Conjugate Color Development Kit (#170-6432, Bio-Rad).

Co-immunoprecipitation (Co-IP) Assays

S2 cell lysates (300 μl, approximately equivalent to 10^6 cells) or equivalent cell culture media containing recombinant proteins were precleared with Protein G Sepharose (50% slurry, No.17-0618-01, GE Healthcare) prior to Co-IP assays (43). Cell lysates were mixed with an anti-Flag M2 or anti-V5 antibody (final concentration

of 1 μg/μl) followed by incubation at 4°C for 10 h with gentle rocking. Protein G Sepharose (30 μl of 50% slurry) in lysis buffer was added to the protein-antibody mixture, and the resulting mixture was incubated overnight at 4°C with gentle rocking. The Sepharose beads containing immunoprecipitated proteins were collected after centrifugation, washed three times with lysis buffer, resuspended in 50 μl of 1 × SDS sample buffer, boiled at 95°C for 5 min, and used for immunoblot analysis (see above).

Co-immunoprecipitation (Co-IP) assays were also performed by collecting culture medium from S2 cells expressing Toll-1^{ecto} or Toll-7^{ecto} proteins 48 h post-transfection and mixing it with DMEM medium from VSV-GFP-infected HEK293 cells that contained VSV-GFP virions as described above. Anti-V5 antibody plus Protein G Sepharose were then added as described above. Pull-down of Toll-1^{ecto} or Toll-7^{ecto} was detected on immunoblots using anti-V5 while VSV-GFP was detected using an antibody that binds VSV glycoprotein (anti-VSV-G) [P5D4] (ab50549, Abcam, USA, 1:5000 dilution). As a control, cell culture media containing Toll-1^{ecto}, Toll-7^{ecto} or VSV-GFP virions were incubated with Protein G Sepharose beads alone followed by immunoblotting and incubation with anti-V5 or anti-VSV glycoprotein as described above.

Real-time PCR analysis

Total RNA from adult flies or S2 cells was extracted, and the expression of target genes was determined by real-time PCR as described previously (33). The flies were anesthetized on CO_2 bed, placed in 1.5-ml tubes and homogenized with disposable pestles in 1 ml of TRIzol® Reagent (T9424, Sigma-Aldrich), and the total RNA from flies and S2 cells was extracted according to the manufacturer's instructions. The RNA pellets were air-dried and resuspended in 50 μl of nuclease-free water, and the concentration of RNA was determined using a Nanodrop UV-Vis spectrophotometer (ND-1000, Thermo).

Total RNA (2 μg from each sample) was treated with RQ1 RNase-free DNase (M6101, Promega) to remove contaminated genomic DNA and then used for the synthesis of cDNAs in 25 μl reactions using Moloney murine leukemia virus (M-MLV) reverse transcriptase (M1701, Promega) and an anchor-oligo(dT)18 primer following the manufacturer's instructions. The cDNA sample (diluted 1:50) was used as the template for quantitative real-time PCR analysis. The *Drosophila* ribosomal protein 49 (*rp49*) gene was used as an internal standard to normalize the expression of target mRNA. Real-time PCR was performed in 20 μl reactions containing 10 μl of 2×SYBR® GreenER™ qPCR SuperMix Universal (No. 204141, Qiagen), 4 μl of H_2O, 4 μl of diluted cDNA template, and 1 μl (10 pmol) of each of the forward and reverse primers. The real-time PCR program was 2 min at 50°C, 10 min at 95°C, 40 cycles of 95°C for 15 s and 60°C for 1 min, and the dissociation curve analysis. The data from four replicates of each sample were analyzed with a comparative method ($2^{-\Delta\Delta CT}$) using ABI 7500 SDS software (Applied Biosystems). The baseline was automatically set by the software to maintain consistency. The cDNA sample from S2 cells transfected with empty pMT/BiP/V5-His A plasmid or wild-type flies (w^{1118}) was used as the calibrator. The expression level of target genes was calculated by the $2^{-\Delta\Delta CT}$ method (44), which provides the n-fold difference in relative expression compared with the calibrator. All the data are presented as relative mRNA expression levels, and all the experiments were repeated at least three times.

Data analysis

Three or four replicates were performed for each assay using independently prepared and collected biological samples. Data were analyzed and figures generated using the GraphPad Prism platform (GraphPad, San Diego, California, USA). Luciferase assays were analyzed by one-way ANOVA followed by a post-hoc Tukey's honest significant difference test. Survival of Toll-1 and Toll-7 loss of function mutants after systemic infection by different microbes were compared to wild-type flies by pair-wise log-rank test.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: XY and MRS designed the experiments, analyzed data, interpreted results, and wrote the manuscript; MC performed most experiments, analyzed data, interpreted results, and participated in manuscript writing; CL performed some experiments and analyzed data; ZH and YL helped perform some experiments; XL and YW participated in manuscript writing; TI provided essential reagents and assisted with data interpretation and manuscript writing.

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FOOTNOTES

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The abbreviations used are: AMP, antimicrobial peptide; Spz, spätzle; TIR, Toll/interleukin-1 receptor; VSV, *Vesicular stomatitis* virus

Figure legends

Figure 1. The TIR domains of all *D. melanogaster* Toll family members and *M. sexta* Toll-1 activate the *drosomycin* promoter. (**A**) An anti-V5 antibody detects expression of the TIRs from *M. sexta* Toll-1 and *Drosophila* Toll-1 to Toll-7 on immunoblots after cloning into the expression vector pMT/BiP/V5-His and transfection into S2 cells. Molecular mass markers are indicated to the right of each blot in kilodaltons (kDa). (**B**). Mean relative luciferase activity ± SE in extracts prepared from S2 cells co-transfected with pGL3B-*drosomycin*, pGL3B-*diptericin* or pGL3B (empty vector) plus plasmids expressing each TIR domain. Three biological replicates were generated for each treatment. For the *drosomycin* promoter, bars with different letters indicate treatments that significantly differed from one another ($p < 0.05$; one-way ANOVA followed by a post-hoc Tukey HSD test). No significant differences were detected between treatments for the *diptericin* reporter or empty vector.

Figure 2. Multiple Spz proteins bind Toll-1 and Toll-7 ectodomains. (**A, B**) Outcomes of Co-IP experiments where lysates from cells expressing Toll-1^{ecto} with a V5 epitope tag were combined with lysates from cells expressing the cystine knot domain for each Spz with a Flag epitope tag. In (**A**), adding an anti-V5 antibody plus protein G beads to the combined lysates immunoprecipitated Toll-1^{ecto} in all treatments that was detected on immunoblots using anti-V5 (upper blot), while only co-immunoprecipitating the cystine knot domains for Spz-1, -2, and -5, that were detected on immunoblots using anti-Flag (lower blot). In (**B**), the reciprocal experiment of adding anti-Flag plus protein G beads immunoprecipitated the cystine knot domains for each Spz (upper blot), while only co-immunoprecipitating Toll-1^{ecto} in the Spz-1, -2 and -5 treatments (lower blot). (**C, D**) Outcomes of Co-IP experiments where lysates from cells expressing Toll-7^{ecto} were combined with lysates from cells expressing the cystine knot domain of each Spz. In (**C**), anti-V5 plus protein G beads immunoprecipitated Toll-7^{ecto} in all treatments (upper blot) while co-immunoprecipitating the cystine knot domains for Spz-1, -2, -5, and -6 (lower blot). In (**D**), anti-Flag plus protein G beads immunoprecipitated the cystine knot domain for each Spz (upper blot) while co-immunoprecipitating Toll-7^{ecto} in the Spz-1, -2, -5 and -6 treatments (lower blot).

Figure 3. Spz-1, -2 and -5 partner with Toll-1 and Toll-7 to activate the *drosomycin* promoter. (**A**) Mean relative luciferase activity ± SE in extracts prepared from S2 cells that were co-transfected with pGL3B-*drosomycin*, pMT/BiP/V5-His A-Toll-1, and pMT/BiP-Spz-1, -2, -3, -4, -5 or -6. (**B**) Mean relative luciferase activity ± SE in in extracts prepared from S2 cells that were co-transfected with pGL3B-*drosomycin*, pMT/BiP/V5-His A-Toll-7, and pMT/BiP-Spz-1, -2, -3, -4, -5 or -6. (**C**) Mean relative luciferase activity ± SE in extracts prepared from S2 cells that were transfected with pGL3B-*drosomycin*, and pMT/BiP/V5-His-Toll-1, -2 or -7, pMT/BiP-Spz-1, -2, or -5, or pMT/BiP/V5-His A-Toll-2 plus pMT/BiP-Spz-1. Four biological replicates were generated for each treatment. For each graph, different letters above bars indicate treatments significantly differed from one another ($p < 0.05$; one-way ANOVA followed by a post-hoc HSD test).

Figure 4. VSV binds Toll-1 and Toll-7 and activates multiple AMP gene promoters. (**A**) Outcomes of Co-IP experiments where VSV virions were added to conditioned medium from S2 cells that were transfected with pMT/BiP/V5-His-Toll-1^{ecto} or -Toll-7^{ecto}. Adding anti-V5 antibody plus protein G beads immunoprecipitated Toll-1^{ecto} or Toll-7^{ecto} that were detected on immunoblots using anti-V5 (upper left blot), and VSV virions that were detected using anti-VSV-G (lower left blot). Adding protein G beads alone (negative control) resulted in no immunoprecipitation of Toll-1^{ecto}, Toll-7^{ecto}, or VSV (upper and lower right blots). (**B**) Mean relative luciferase activity ± SE in extracts prepared from VSV-infected and non-infected S2 cells expressing full length Toll-1 plus pGL3B-*attacin (att)*, -*drosomycin (drs)*, or -*metchnikowin (mtk)*. (**C**) Mean relative luciferase activity ± SE in extracts prepared from VSV-infected and non-infected S2 cell line expressing the full-length Toll-7 plus pGL3B-*attacin*, -*drosomycin*, or -*metchnikowin*. Three biological replicates were generated for each treatment. An asterisk (*) indicates

that relative luciferase activity significantly differed between VSV-infected and non-infected cells (t-test; $p < 0.001$).

Figure 5. Toll-1 and Toll-7 differentially affect the survival of adult female and male *D. melanogaster* after systemic infection by different microbes. Wild-type (w^{1118}), Toll-$7_{g1\text{-}5}$/CyO, Df/Toll-$7_{g1\text{-}5}$, $T1_{I\text{-}RXA}$ or $Tlr_{632}/Tl_{I\text{-}RXA}$ adult females (left) and males (right) were systemically infected with (**A**) *E. faecalis*, (**B**) *P. aeruginosa*, (**C**) *C. albicans* or (**D**) VSV-GFP. From 20 to 30 adults for each treatment were monitored for cumulative survival. For each sex and mutant, cumulative survival was compared to wild-type flies by log-rank test. Asterisks (*) indicate significant differences with * $p < 0.05$, ** $p < 0.01$, *** $p<0.001$ while ns indicates no significant difference was detected between a given mutant and wild-type flies.

Fig. 1

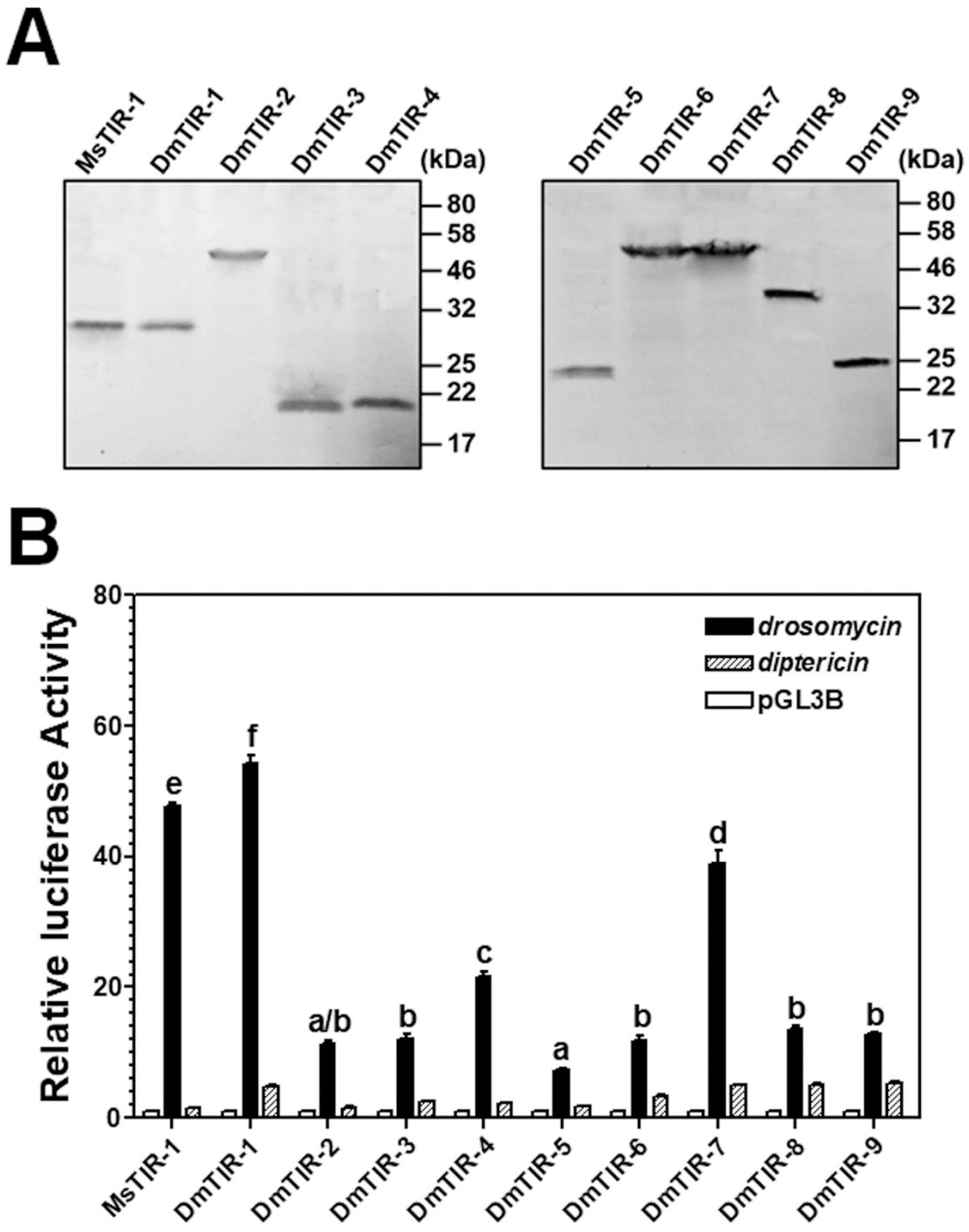


Fig. 2

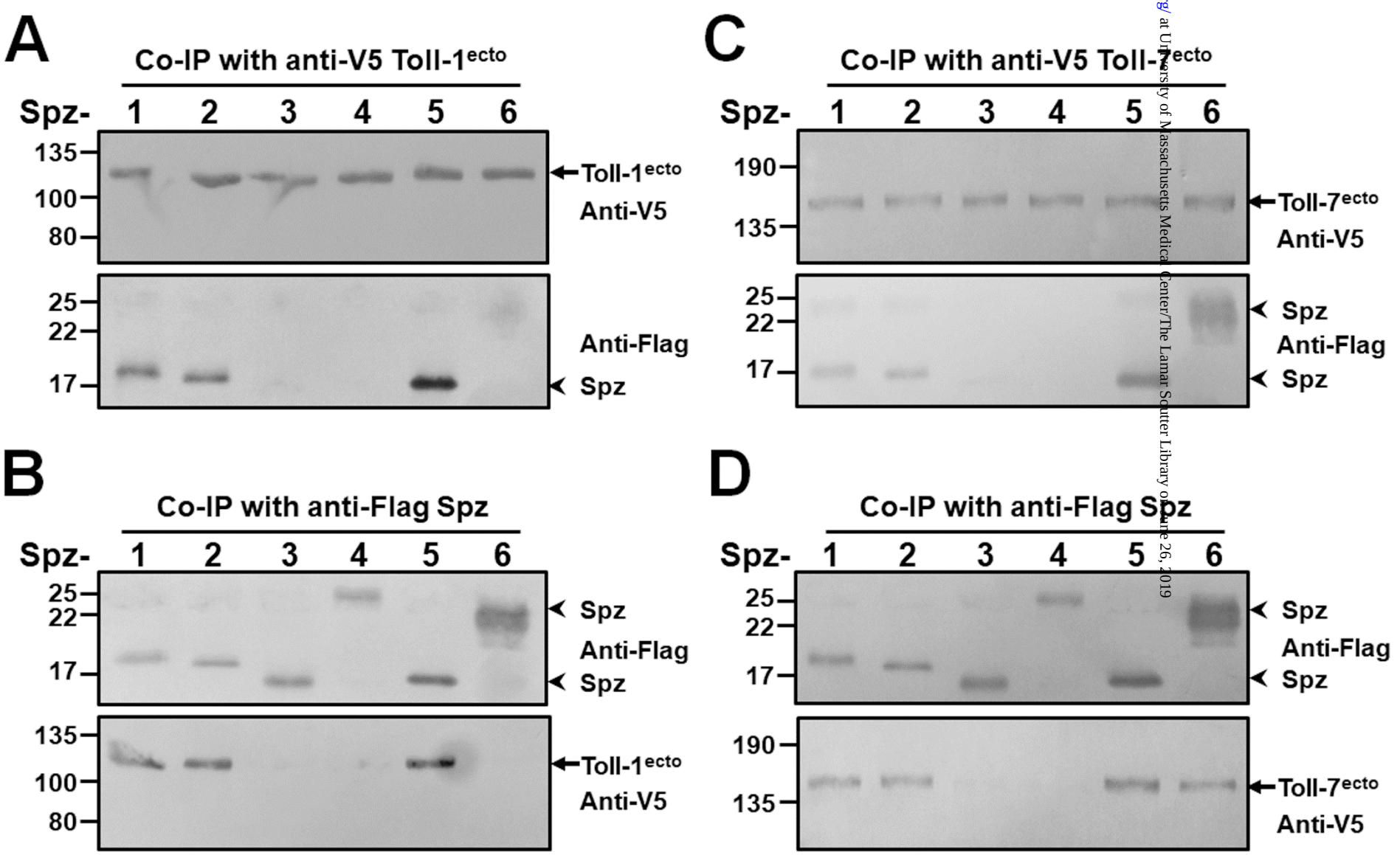


Fig. 3

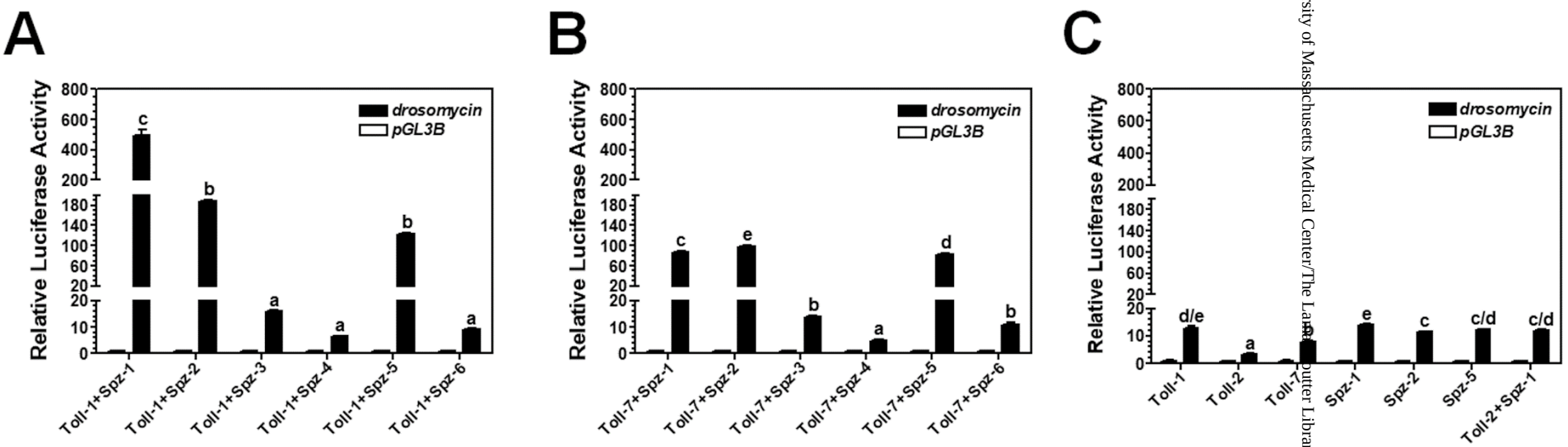


Fig. 4

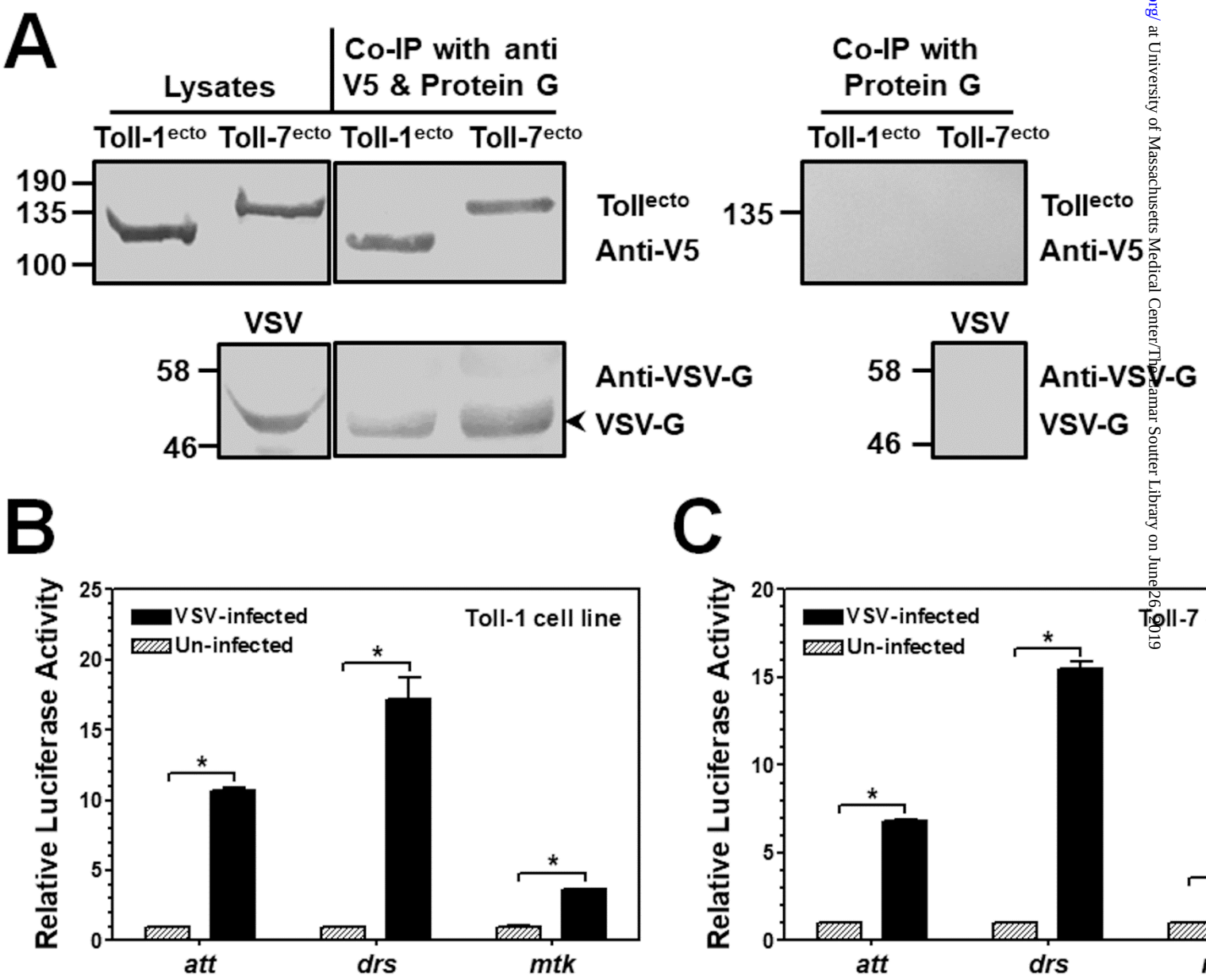
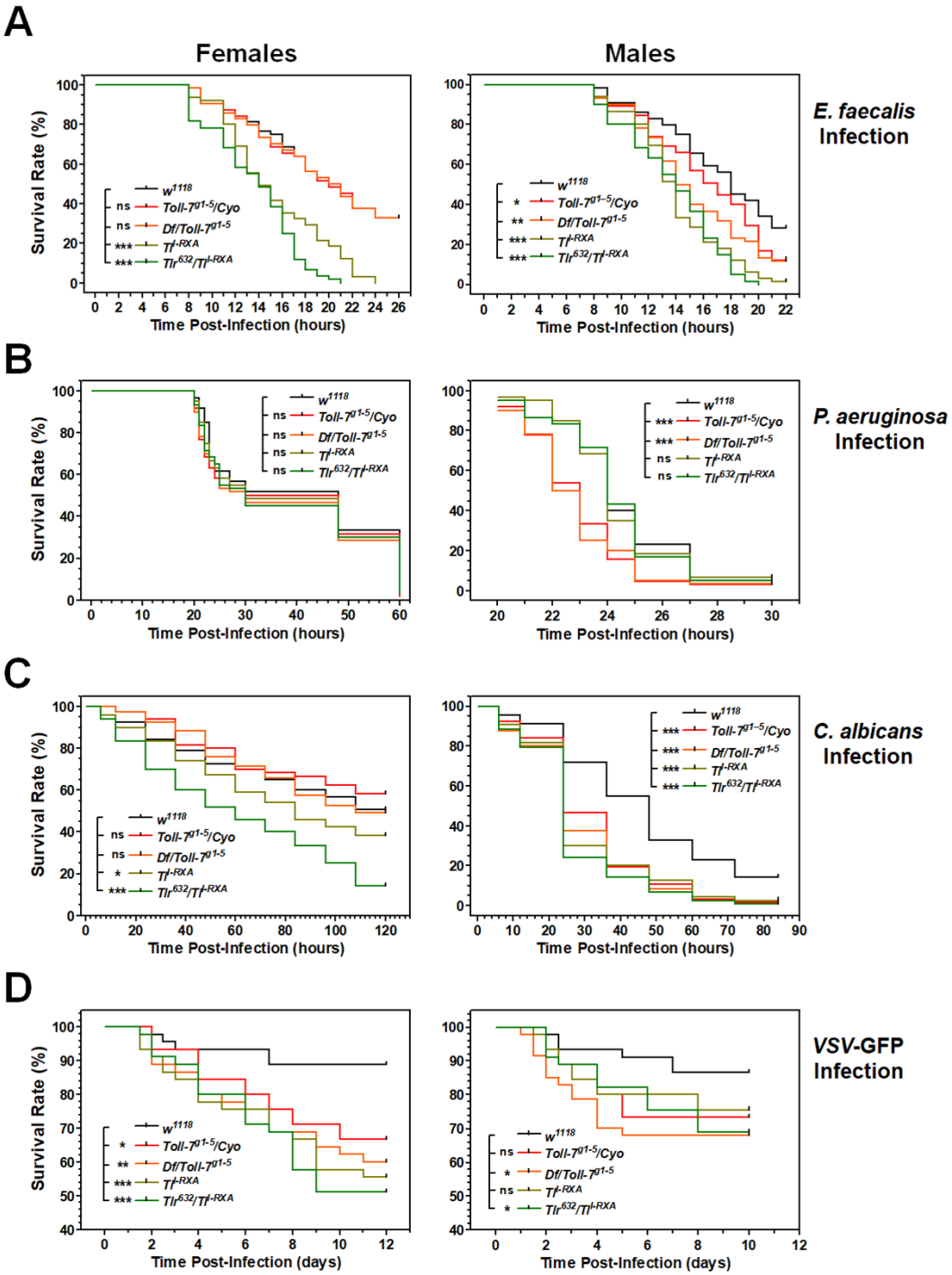


Fig. 5



Toll family members bind multiple Spätzle proteins and activate antimicrobial peptide gene expression in Drosophila

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