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The Toll immune-regulated *Drosophila* protein Fondue is involved in hemolymph clotting and puparium formation

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Abstract

Clotting is critical in limiting hemolymph loss and initiating wound healing in insects as in vertebrates. It is also an important immune defense, quickly forming a secondary barrier to infection, immobilizing bacteria and thereby promoting their killing. However, hemolymph clotting is one of the least understood immune responses in insects. Here, we characterize *fondue* (*fon*; CG15825), an immune-responsive gene of *Drosophila melanogaster* that encodes an abundant hemolymph protein containing multiple repeat blocks. After knockdown of *fon* by RNAi, bead aggregation activity of larval hemolymph is strongly reduced, and wound closure is affected. *fon* is thus the second *Drosophila* gene after *hemolectin* (*hml*), for which a knockdown causes a clotting phenotype. In contrast to *hml-RNAi* larvae, clot fibers are still observed in samples from *fon-RNAi* larvae. However, clot fibers from *fon-RNAi* larvae are more ductile and longer than in wt hemolymph samples, indicating that Fondue might be involved in cross-linking of fiber proteins. In addition, *fon-RNAi* larvae exhibit melanotic tumors and constitutive expression of the antifungal peptide gene *Drosomycin* (*Drs*), while *fon-RNAi* pupae display an aberrant pupal phenotype. Altogether, our studies indicate that Fondue is a major hemolymph protein required for efficient clotting in *Drosophila*.
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Keywords: Innate immunity; Coagulation; Clotting; Homeostasis; Hemolymph; Insect; Antimicrobial peptide

Introduction

To combat infection, the fruit fly *Drosophila melanogaster* relies on both constitutive and inducible immune mechanisms. The first line of defense that prevents microbial invasion into the hemocoel is structural. It is comprised of the external cuticle, the gut peritrophic matrix, and the tracheal lining. When pathogens breach these barriers, they activate a wide range of inducible reactions. Perforation of the cuticle by injury or by microbial infection rapidly activates blood coagulation and proteolytic cascades that lead to melanization. Upon subsequent microbial or parasitic infection, a cellular immune response, mediated by different hemocyte types, is mounted and participates in pathogen clearance by phagocytosis or encapsulation (Brennan and Anderson, 2004). Antimicrobial peptides (AMPs) are synthesized as a systemic response to infection in the fat body, a functional equivalent of the mammalian liver, and secreted into the hemolymph, where they directly kill invading microorganisms (Royet et al., 2005). Genetic analyses have demonstrated that AMP genes are regulated by the Toll and the Imd pathways, which are selectively activated by different classes of microbes (Tanji and Ip, 2005). A recent DNA microarray study has shown that in addition to AMP

Abbreviations: AMP, antimicrobial peptide; *da*, *daughterless*; *Dpt*, *Diptericin*; *Drs*, *Drosomycin*; *fon*, *fondue*; *hml*, *hemolectin*; IR, inverted repeat; *ppl*, *pumpless*; RNAi, RNA interference; wt, wild type.

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genes, the Toll and Imd pathways regulate most of the genes induced upon microbial infection (De Gregorio et al., 2002).

Prior to this humoral response, coagulation acts to seal wounds and to trap microbes, blocking their entry into the hemocoel. In *Drosophila* larvae, a clot composed of fibers trapping hemocytes is rapidly generated at the site of injury. It is assumed that cross-linking enzymes including phenoloxidase and transglutaminase may be involved in hardening of clots (Theopold et al., 2004). Subsequent steps in wound closure include melanization and epithelial movements (Galko and Krasnow, 2004). While the molecular basis for hemolymph clotting has been extensively studied in horseshoe crab (Iwanaga, 2002) and crayfish (Smith, 1986; Theopold et al., 2004), little is known about the proteins involved in insect clotting. Until now, only one hemocyte-specific gene, *hml*, has been demonstrated to be required for efficient clot formation. Hemolymph from *hml-RNAi* larvae expressing a *UAS-hml-IR* construct fails to form clot fibers (Scherfer et al., 2004), consequently leading to a bleeding defect and to larger wound scabs (Goto et al., 2003). Recently, a pull-out assay has been developed to isolate clot proteins by virtue of their ability to bind to and aggregate beads, and hemolectin was notable among the identified candidate clotting factors (Scherfer et al., 2004). Using RNAi, we characterized Fondue, another clotting factor isolated by the pull-out assay. We show that *fon* is a gene coding for a novel protein with a repetitive sequence that is induced following injury. The present study demonstrates that it is required for efficient clotting.

Materials and methods

Fly stocks

Lines carrying one copy of either *fon-IR* or *daughterless-GAL4 (da-GAL4)* or wt (Oregon R) animals respectively were used as controls. RNAi transgenic fly lines of *fon* were obtained using an inducible in vivo RNAi approach. A cDNA fragment corresponding to the first 500 bp of the coding sequence of the coding sequence was amplified by PCR and inserted as an inverted repeat (*IR*) in a modified pUAST transformation vector, pUAST-R57, which includes an IR formation site consisting of paired KpnI-CpoI and XbaI-SfiI restriction sites (Leulier et al., 2002). Transformation of *Drosophila* embryos was carried out utilizing w^{1118} flies as recipients. Each experiment was repeated using two independent *UAS-RNAi* insertions. In the present study, we used larvae and adult flies carrying one copy of the *UAS-RNAi* construct combined with one copy of the *GAL4* driver. The *ppl-GAL4* driver expresses *GAL4* strongly in fat body and salivary glands of larvae (Zinke et al., 1999), while the *hml-GAL4* driver led to expression in larval hemocytes only (Goto et al., 2001). The *da-GAL4* driver expresses *GAL4* strongly and ubiquitously throughout development. A recombinant line carrying both the *da-GAL4* driver and the *hml-IR* construct was used to obtain *hml*, *fon* double *RNAi* flies. Larvae from the RNAi crosses were cultivated at 29°C except when mentioned differently.

spz^{rm7} and Rel^{E20} are null mutations affecting *spaetzle* and *Relish* respectively.

Infections

Infections and survival counts were performed as described in Tzou et al. (2002).

Quantitative real-time PCR (qRT-PCR)

For *Dpt* and *Drs* mRNA quantification from whole animals, RNA was extracted using RNA TRIzol™ (Invitrogen). cDNAs were synthesized using SuperScript II (Invitrogen), and PCR was performed using dsDNA dye SYBR Green I (Roche Diagnostics). Primer pairs for *Dpt* (sense, 5′-GCT GCG CAA TCG CTT CTA CT-3′ and antisense 5′-TGG TGG AGT GGG CTT CAT G-3′), *Drs* (sense 5′-CGT GAG AAC CTT TTC CAA TAT GAT G-3′ and antisense 5′-TCC CAG GAC CAC CAG CAT-3′), *fon* (sense 5′-GAT AGT AGT GTG CGG T-3′ and antisense 5′-GGC ACG AGA AGA TTG T-3′) and control primers for *rp49* (sense 5′-GAC GCT TCA AGG GAC AGT ATC TG-3′, and antisense 5′-AAA CGC GGT TCT GCA TGA G-3′) were utilized. SYBR Green analysis was performed on a Lightcycler (Roche). All samples were analyzed in duplicate, and the amount of mRNA detected was normalized to control *rp49* mRNA values. We used normalized data to quantify the relative levels of a given mRNA according to cycling threshold analysis (ΔCt) (as described in Pili-Floury et al., 2004).

Clotting assays

Bead aggregation assays were performed as described earlier (Scherfer et al., 2004). Draw-out reactions were carried out as stated before (Bidla et al., 2005), but the incubation time before starting the draw-out was reduced to 90 s. In order to allow incorporation of bacteria into fibers obtained during a draw-out, hemolymph samples from five larvae were allowed to coagulate for approximately 10 s before addition of bacteria (1.5 μl from an overnight culture). The draw-out was performed subsequently, and the fibers were analyzed using both phase contrast and fluorescence microscopy to visualize GFP-expressing bacteria.

Results

fon is a late immune-responsive gene regulated by the Toll pathway

Oligonucleotide microarray analysis performed on *Drosophila* adult males indicated that the previously unknown gene *CG15825* (referred to as *fon* in this paper) is induced four-fold by septic injury with a mixture of Gram-positive and Gram-negative bacteria and also induced after natural infection by the entomopathogenic fungus *Beauveria bassiana* (De Gregorio et al., 2001). The expression profile in response to septic injury shows that *fon* is a late and sustained response gene with the highest expression level at 48 h post-infection (Fig. 1A and De Gregorio et al., 2001). Quantitative real-time PCR (qRT-PCR) comparison of *fon* expression levels in wild-type (wt) flies and flies deficient for Toll (*spaetzle: spz*) or Imd (*Relish: Rel*) signaling revealed that *fon* expression is mainly controlled by the Toll pathway (Fig. 1B) as already indicated by De Gregorio et al. (2002). To examine the temporal expression profile of *fon* during *Drosophila* development, we performed a qRT-PCR analysis. *fon* mRNA was weakly expressed in embryos and both adult male and female files, while expression levels were highest in early pupae (Fig. 1C). Another microarray analysis demonstrated that *fon* is expressed in larval hemocytes (Irving et al., 2005).

The *fon* gene encodes two alternative transcripts for a protein with two isoforms of 565 and 577 amino acids length respectively (predicted molecular weights of 56.6 kDa and 58 kDa). High overall sequence conservation is observed when Fondue homologues from four *Drosophila* species are aligned (84% homology between *D. melanogaster*, *D. simulans*, and *D. yakuba*; 46% between all four species, see Supplementary Data). The protein seems to have evolved specifically in the

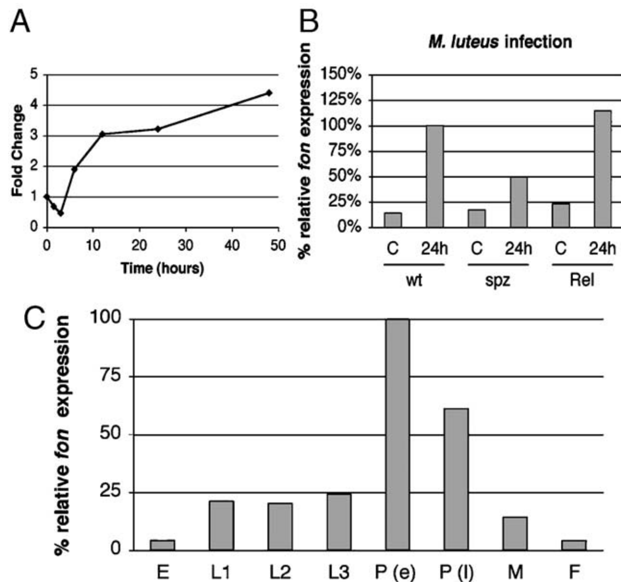
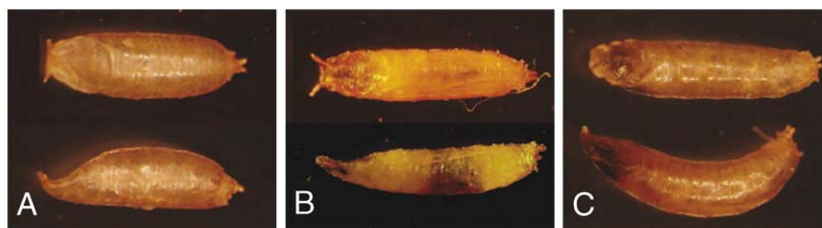


Fig. 1. Expression pattern of Fondue. (A) *fon* is an immune-responsive gene. The gene expression profile for *fon* shows an initial downregulation followed by gene induction at later time points. The data for this figure was extracted from De Gregorio et al. (2001). Wt flies were challenged with a needle dipped in a concentrated mixed bacterial culture of *E. coli* and *M. luteus*. (B) *fon* is regulated by the Toll pathway. A mutation affecting *spaetzle* (*spz*), encoding the ligand of the Toll receptor, but not a mutation in *Relish* (*Rel*), inhibited the expression of *fon* after infection of adult flies by *M. luteus* (detected by qRT-PCR), demonstrating that the gene is under the control of the Toll pathway. An increased *fon* expression was also observed after infection of flies with *E. carotovora*, although at lower levels than with *M. luteus* (data not shown). This latter induction was not abolished in *Rel* mutant flies infected with *E. carotovora*. (C) *fon* expression during development. qRT-PCR of unchallenged wt animals at different developmental stages reveals that *fon* is strongly expressed in early pupae. Results of one representative qRT-PCR are shown. E: embryos, L1–3: larvae, P (e): early pupae, P (l): late pupae, M: male flies, F: female flies.

Drosophila lineage, since homologous proteins are so far not found in other insect species including *A. gambiae*. A characteristic of the Fondue amino acid sequence is the presence of repetitive elements, consisting of similar but not identical building blocks. These repeats include numerous glycine, alanine, and serine residues. The protein has a predicted cleavable signal peptide and is thus expected to be secreted. In agreement with this, Fondue has been identified as one of the major larval hemolymph proteins (Vierstraete et al., 2003) and is strongly depleted from it during clotting (Karlsson et al., 2004).

fon-RNAi pupae exhibit an altered pupal phenotype

To analyze the role of Fondue in the *Drosophila* immune response, we used the inducible expression of *fon* double-stranded RNA in vivo. We generated transgenic flies carrying the *UAS-fon-IR* element (referred to as *fon-IR*). This construct consists of two 500-bp-long inverted repeats (IR) of the *fon* gene, separated by an intronic DNA sequence that acts as a spacer and results in a hairpin-loop shaped RNA. Two independent *UAS-fon-IR* insertions were investigated in this study (R1 and R2; R1 was utilized where not indicated otherwise). In order to activate transcription of the hairpin-encoding transgene in the progeny (referred to as *fon-RNAi* flies), these transgenic flies were crossed to flies carrying the *da-GAL4* driver expressing *GAL4* strongly and ubiquitously. We confirmed that the Fondue protein was significantly decreased in hemolymph samples from *fon-RNAi* larvae (Fig. 3B). *fon-RNAi* larvae were viable and showed no further visible phenotype except the sporadic presence of small melanotic spots. The hemocyte count in *fon-RNAi* larvae was normal, but large aggregates of plasmatocyte and lamellocytes were detected in some hemolymph samples (data not shown).



D

genotype	wt (A)	longer (B)	longer & curved (C)
fon-IR R1; *da-GAL4*	30%	24%	46%
fon-IR R2; *da-GAL4*	14%	38%	48%
fon-IR R1; *ppl-GAL4*	4%	42%	54%
fon-IR R1; +	100%	0%	0%

Fig. 2. *fon-RNAi* pupae display an abnormal shape. The pictures show pupae in dorsal (top) and lateral view (bottom), the anterior end is located to the left in all pictures. Wt pupae display an almost straight shape with a characteristic bend on the dorsal side (A). In contrast, large numbers of the *fon-RNAi* pupae were considerably elongated (B), with many of them showing a bend on the ventral side causing a banana-shaped appearance (C). All normally shaped pupae survived into adults, but no flies emerged from the "banana-shaped" pupae. Fifty young larvae were selected from each cross and transferred to a new tube to determine the penetrance of the pupal phenotype (D). The elongated shape occurred only, when *fon-IR* was expressed ubiquitously with the *da-GAL4* or specifically in the fat body with the *ppl-GAL4 (pumpless-GAL4)* driver. Control larvae carried one copy of the *fon-IR* construct in the absence of a *GAL4*-driver.

Interestingly, approximately 40% of the pupae raised at 25°C were elongated compared to wt pupae and showed an unusual banana-like shape with the dorsal part bent upwards (Fig. 2). This seemed to be connected to a defect in body retraction of late larvae prior to pupation, which attached to the vial walls with their anterior part only, while the posterior end was performing circular "searching movements" in the air. In addition, spiracle eversion seemed to be defective in most of these pupae, while the anterior end of the pupae was often heavily melanized. The penetrance of the phenotype increased at 29°C corresponding to the increased efficiency of the *UAS/GAL4* system. At this temperature, about 70 to 90% of the *fon-RNAi* pupae were longer and less compact than wt pupae, while in addition, approximately 50% of all pupae displayed the bowed shape associated with pupal lethality. It became evident during pricking experiments that the cuticles of banana-shaped pupae were more fragile than the ones of wt pupae. *fon-RNAi* adult escapers raised at 25°C or lower temperatures were viable and showed no visible difference to wt flies. The pupal phenotype was also observed when the *fon-IR* construct was expressed under the control of *ppl-GAL4*, a more restricted driver that expresses *GAL4* only in the fat body and the salivary glands, but not when using salivary gland or hemocyte-specific drivers (data not shown). This indicates that the phenotype is mainly linked to lowered *fon* expression in the fat body.

Fondue is involved in hemolymph clotting

Isolation of Fondue by the pull-out assay and its high titer in the hemolymph pointed to a possible involvement in coagulation. We tested the role of Fondue in clot formation by using the previously described bead aggregation assay (Scherfer et al., 2004). When *fon-RNAi* larvae were bled onto beads, there was no aggregation, indicating a deficient clotting reaction (Fig. 3A). This was similar to hemolymph from *hml-RNAi* larvae (Scherfer et al., 2004 and Fig. 3A). In sporadic cases, aggregation did occur but was still clearly weaker and much delayed compared to wt larvae. Interestingly, bead aggregation could be rescued by mixing hemolymph samples from *hml-RNAi* and *fon-RNAi* larvae (Fig. 3A). To clarify, if knocking down both *hml* and *fon* would lead to an even stronger clotting phenotype, we generated larvae that ubiquitously express both the *hml-IR* and *fon-IR* constructs. Double RNAi larvae did not show any additional visible phenotype. Similarly, no additive effect of the combined RNAi compared to the single RNAi lines could be observed in the bead aggregation test. Altogether, these results suggest that both proteins work in the same process, but that neither Fondue nor Hemolectin is involved in the production of the other factor.

Hemolectin is a protein exclusively released by the hemocytes. We aimed to clarify the site of production for the Fondue protein fraction involved in bead aggregation. Therefore, we expressed *fon-IR* and *hml-IR* elements with *GAL4* drivers specific of the fat body (*ppl-GAL4*) or hemocytes (*hml-GAL4*) and tested hemolymph from these crosses with the bead assay. Bead aggregation was abolished, when *hml-IR* was expressed in hemocytes, as demonstrated previously (Scherfer et al., 2004), but not after *hml-IR* expression in the fat body. In contrast, bead aggregation was strongly delayed and much weaker, when *fon-IR* was expressed in the fat body, but was only slightly affected after hemocyte expression of *fon-IR* (see Supplementary data). This experiment confirmed that Hemolectin is a protein exclusively required by the hemocytes and not the fat body, while Fondue is mainly secreted into the hemolymph by the fat body. This indicates that both hemocyte and humoral proteins contribute to clotting.

We also examined the effects of *fon-RNAi* on clotting by analyzing bead-associated protein samples (pull-out) on a

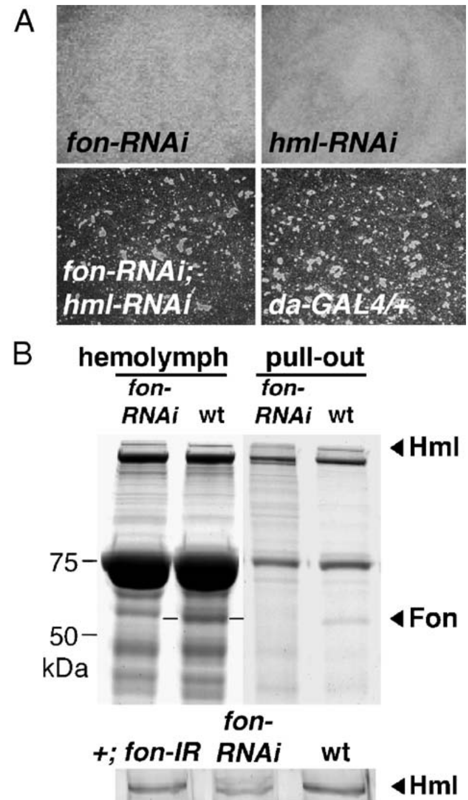


Fig. 3. Clotting impairment in *fon-RNAi* larval hemolymph. (A) Bead aggregation as an indicator of effective clotting was abolished in *fon-RNAi* larval hemolymph similar to *hml-RNAi* (Scherfer et al., 2004), which was reflected by evenly dispersed beads which did not display significant aggregation. A wt aggregation pattern of clumped beads was obtained with *da-GAL4/+* hemolymph samples and served as a control. Similarly, the wt aggregation pattern could be observed by mixing hemolymph from the two different RNAi stocks (five larvae from each *fon-RNAi* and *hml-RNAi* respectively). All shown RNAi samples were derived from crosses with *da-GAL4*. The utilized beads are 2.8 μm in diameter. B: Depletion of Fondue from the pull-out extract after RNAi. The two left lanes show the protein pattern of total hemolymph from *fon-RNAi* (*fon-IR; da-GAL4)* and wt larvae. Lanes 3 and 4 present the respective protein samples recovered in a pull-out reaction from total hemolymph. The bands corresponding to Fondue (Fon) and Hemolectin (Hml) are indicated as deduced from mass-spectrometry analysis (see Scherfer et al., 2004) and small lines specify the position of Fondue in total hemolymph samples. In *fon-RNAi* larvae, Fondue is almost completely absent from the pull-out extract, while a corresponding band of the same size also appears less abundant in complete hemolymph, confirming the efficiency of in vivo *fon-RNAi*. In the lower part of the figure, pull-out samples from *fon-RNAi* larvae as well as from *fon–IR/+* and wt control larvae were loaded on a 4% gel to reveal that in the absence of Fondue, hemolectin binding to the beads is reduced.

SDS-polyacrylamide gel (Fig. 3B). These data confirmed that Fondue is enriched in the protein fraction that binds to the beads. As expected, Fondue was not found on beads incubated with *fon-RNAi* larval hemolymph. Interestingly, the amount of bound hemolectin appeared to be reduced as well in the *fon-RNAi* samples (Fig. 3B, bottom), further suggesting that knocking down Fondue affects clotting.

Fondue inactivation leads to longer clot fibers

Since bead aggregation was defective in hemolymph samples from *fon-RNAi* larvae, we analyzed if formation of clot fibers was reduced as observed for *hml-RNAi* larvae (Scherfer et al., 2004 and Lesch et al., unpublished results). In order to analyze the physical properties of clot fibers, we performed a draw-out assay, based on the formation of fibers which can be drawn out from a hemolymph sample after coagulation (Bidla et al., 2005 and Materials and methods). In general, fibers from *fon-RNAi* hemolymph were more difficult to obtain and appeared less compact and thinner compared to normal hemolymph. In agreement with this, clot fibers from *fon-RNAi* larvae were more ductile and longer than in wt hemolymph samples (1.8 cm ± 0.38 cm in the *fon-RNAi* samples versus 0.68 ± 0.21 in the control, $P < 0.0075$ from four sets of experiments, Fig. 4A, see Materials and methods for details). Bacterial binding was observed in these fibers as in wt (Fig. 4B). Using the same draw-out assay, hemolymph from *hml-RNAi* larvae fails to form extendable fibers (Lesch et al., unpublished results).

Impaired coagulation in *hml-RNAi* flies leads to larger melanized scabs at wound sites (Goto et al., 2003). We wounded *fon-RNAi*, *hml-RNAi* and control larvae (*da-GAL4*) with a clean fine tungsten needle and compared the resulting scab sizes after 1 h. The average size of the melanized spot in *fon-RNAi* larvae was bigger than for larvae from the wt control and similar to *hml-RNAi* larvae (Fig. 4C), although there was variability between individuals. Melanization spread more widely from the wound site over the cuticle, while the central part of the wound often was insufficiently melanized. Surprisingly, in survival tests challenged *hml-RNAi* and *fon-RNAi* larvae did not show higher mortality than challenged wt (data not shown). We also wounded pupae and adult flies from all mentioned crosses but did not observe a major difference in scab size in these experiments.

Altogether, our observations indicate that Fondue is an important protein required for clotting or clot hardening. We chose the name Fondue as a reference to the elongated clotting fiber phenotype shown in Fig. 4A. In most of our assays, *fon-RNAi* animals appear to show similar clotting defects to *hml-RNAi* larvae except for the draw-out assay where they still form fibers and immobilize bacteria (Figs. 4A and B).

Constitutive Drosomycin expression in fon-RNAi larvae

We next investigated a possible role of Fondue in other immune reactions, since *fon* is regulated by the Toll pathway that controls AMP gene expression. We first assayed the susceptibility of *fon-RNAi* larvae and flies to infection with four different microorganisms: flies were challenged with a Gram-negative bacterium (*Erwinia carotovora carotovora*), a Gram-positive bacterium (*Enterococcus faecalis*) or the fungus *Candida albicans*, and naturally infected with the entomopathogenic fungus *B. bassiana*. The survival rate of *fon-RNAi* flies was significantly weaker towards Gram-negative

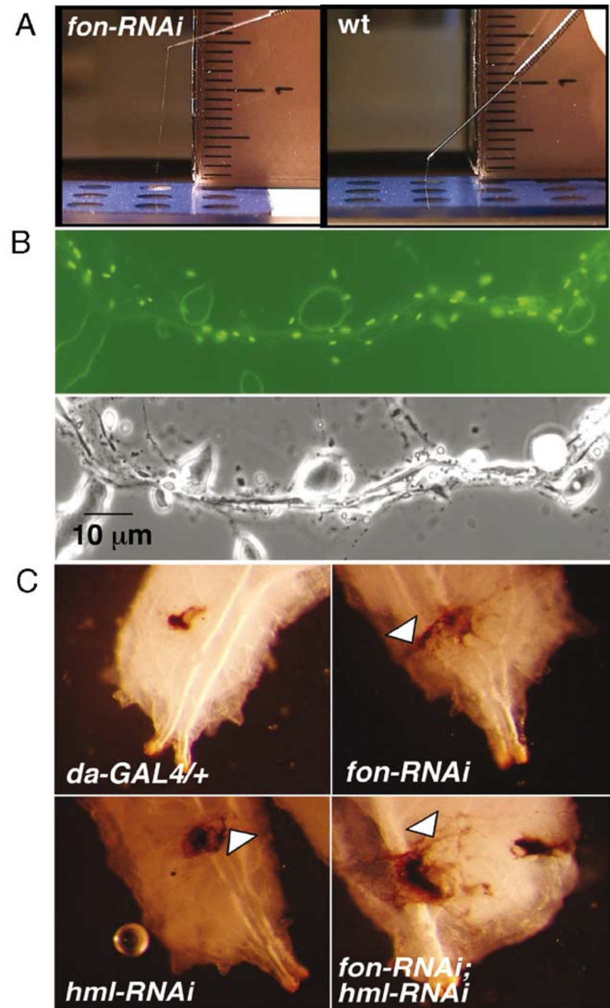


Fig. 4. Clot fibers and wound healing in *fon-RNAi* larvae. (A) Draw-out of the clot of *fon-RNAi* (*fon-IR; da-GAL4*) and wt larvae. A hemolymph sample of *fon–RNAi* larvae and wt larvae was bled and drawn out to a bundle consisting of clot fibers using a fine needle (method according to Bidla et al., 2005). The fiber length obtained from hemolymph of *fon-RNAi* larvae was significantly longer (defined by the breaking point of the fiber bundle) than the one from wt control samples. The figures were taken at the respective terminal breaking points of the clot fiber bundle. (B) Primary clot fibers from *fon-RNAi* larvae entrap bacteria. Hemolymph from *fon-RNAi* larvae was mixed with an aliquot from a culture of GFP-expressing *E. coli*, fibers were drawn out and analyzed using fluorescence (top) and phase contrast (bottom) microscopy. (C) *fon-RNAi* causes a bleeding disorder. Third instar larvae were wounded with a fine tungsten needle. Similarly as already described earlier for *hml-RNAi* (Goto et al., 2003), the spreading and subsequent melanization of hemolymph across the original wound borders was more extensive in injured *fon-RNAi* larvae than in control larvae (as indicated by arrow-heads), although the effect was slightly variable between individuals of the same genotype. No additive effect of a double RNAi for both genes was observed. Control larvae were derived from crosses between *da-GAL4* (*da-GAL4/+*) and *fon-IR* (not shown) with *w* flies.

infections but not different from wt flies in other types of infection, indicating that Fondue does not play a major role in direct pathogen killing (Fig. 5). We also parasitized *fon-RNAi* larvae with the wasp *Leptopilina boulardii* and found a lamellocyte response and melanotic spots comparable to wt and other control crosses (data not shown).

In addition, we monitored in *fon-RNAi* larvae and flies the expression of *Diptericin* (*Dpt*), an antibacterial peptide gene regulated by the Imd pathway, and *Drosomycin* (*Drs*), an antifungal peptide gene regulated mainly by the Toll cascade. The knockdown of *fon* had only a weak effect on the induction of the AMP genes *Dpt* and *Drs* in larvae or adults after septic injury with *E. carotovora* or *Micrococcus luteus* as demonstrated by qRT-PCR (Figs. 6A and B). Surprisingly, *fon-RNAi* larvae, but not adults, constitutively expressed *Drs* in absence of a challenge at high levels (Fig. 6B, left panel), similar to wt challenged larvae. In agreement with this, a *Drs-GFP* reporter construct was strongly expressed in the fat bodies of most *fon-RNAi* wandering stage larvae and all young pupae (Fig. 6C). This constitutive *Drs* expression was not observed in a *spz* mutant background (Fig. 6B, right panel). Tissue-specific expression of the *fon-IR* construct using *GAL4* drivers for fat body (*ppl-Gal4*), hemocytes (*hml-Gal4*), and gut (*cad-Gal4*) indicated that a constitutive induction of *Drs* was observed only after fat body expression of *fon-IR* (data not shown). In most larvae, high *Drs-GFP* expression was correlated with the presence of small melanotic tumors, while the location of these tumors was not restricted to the fat body.

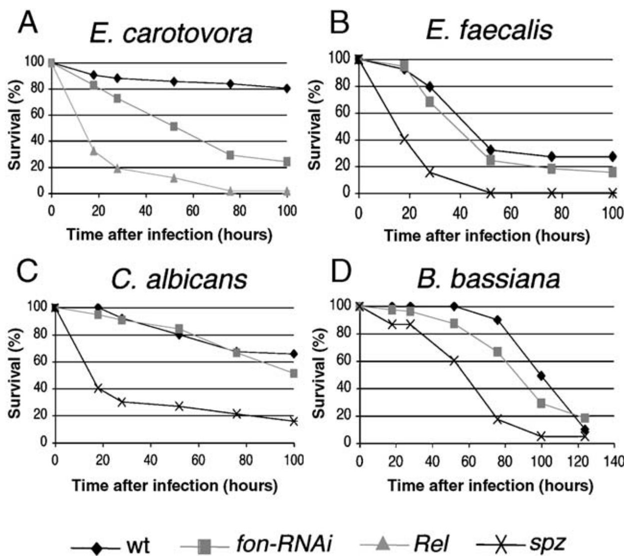


Fig. 5. Survival of *fon*-deficient flies after various microbial challenges. *fon-RNAi*, *Rel*, *spz* and wt control flies were challenged with a needle previously dipped in a concentrated pellet of an overnight culture of *Erwinia carotovora* (A), *Enterococcus faecalis* (B), *Candida albicans* (C) or naturally infected with the entomopathogenic fungus *Beauveria bassiana* (D). The optical density (OD) of the microbial pellets was OD_{600} = 200 in the case of panels A and C and OD = 30 for panel B. Survival tests were performed at 29°C with 60 flies each, and the numbers of surviving flies were counted at different time points after microbial challenge. The percentage of surviving animals was determined as an average of three independent sets of experiments. This figure shows that *fon-RNAi* flies were slightly more susceptible to Gram-negative bacterial infections, but not to infections with *E. faecalis*, *C. albicans*, or *B. bassiana*. *da-GAL4/+* flies were used as a control.

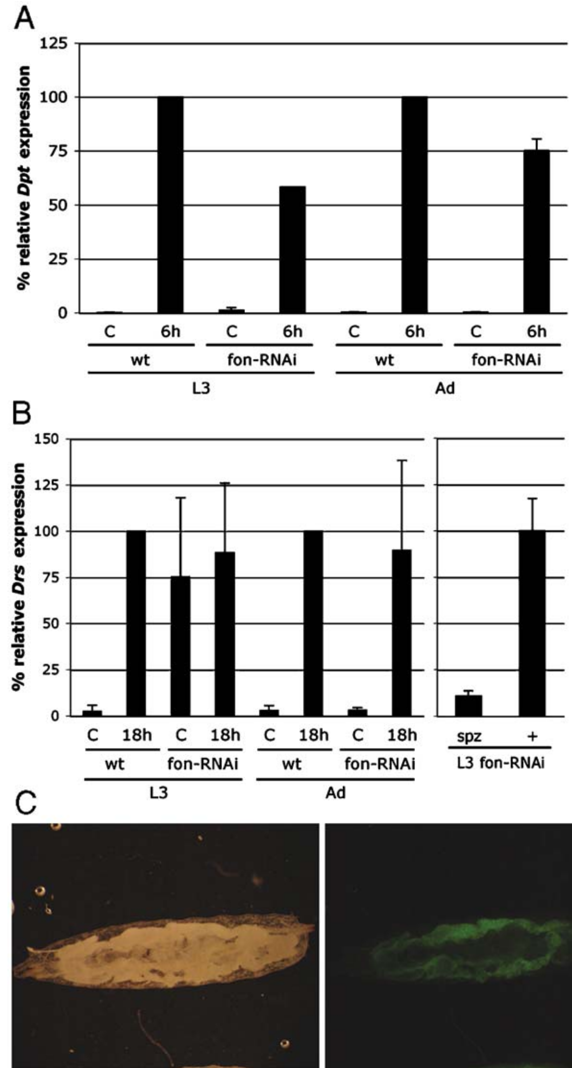


Fig. 6. Effect of *fon-RNAi* on antimicrobial peptide gene expression. (A, B) The levels of *Dpt* or *Drs* expression were monitored in wt or *fon-RNAi* larvae and flies collected after challenge with *E. carotovora* (*Dpt*) or *M. luteus* (*Drs*) respectively. The values obtained for bacteria-challenged wt animals were set as 100%. The average of at least two independent qRT-PCR experiments is shown together with the standard deviation. Expression levels of *Dpt* in challenged *fon*-RNAi animals were slightly lower than in challenged wt larvae and flies (A). Importantly, unchallenged *fon-RNAi* larvae constitutively expressed *Drs* in absence of a challenge (B, left panel). This constitutive expression was abolished in a *spz* mutant background (B, right panel). wt: *da-GAL4/+*, *fon-RNAi*: *fon-IR* 1/+; *da-GAL4/+*, *spz*: *fon-IR1/+*; *da-Gal4*, *spz/spz*, +: *fon-IR1/+*; *spz/+*; c: unchallenged control; 6, 18: hours after infection. (C) When a *Drs-GFP* reporter gene was combined with *fon-RNAi*, strong GFP fluorescence was observed in the fat bodies in absence of a challenge (right picture; on the left a phase contrast picture of the same larva). No *GFP* expression was observed in control larvae carrying either the *GFP* reporter gene or the *fon-IR* construct only (not shown).

Discussion

Recent microarray and proteomic studies have allowed the identification of many genes whose expression profiles change

in the course of an immune response. A major challenge in the current field is to identify how each of these immune-responsive genes contributes to the host defense, ultimately leading to a more complete understanding of the immune response. Genomic and proteomic approaches already indicated that *fon* is an immune-responsive gene encoding a major constitutive hemolymph protein (De Gregorio et al., 2001; Vierstraete et al., 2003). The sustained expression profile of *fon* at rather late time points after infection suggested a role in homeostasis rather than in direct pathogen killing. Since *fon* is even downregulated at earlier time points after infection, one could speculate that the late induction pattern reflects a replenishment rather than an immune reaction. Using a biochemical and genetic approach, we now demonstrate a key role of Fondue in the clotting reaction in *Drosophila*. Our study demonstrates that Fondue binds to beads, while depletion of Fondue by RNAi inhibits bead aggregation, increases ductility of drawn out strands, and leads to slightly enlarged scabs after injury. Thus, *fon* is the second gene to be identified as required for efficient clotting in *Drosophila*. Since clot fibers could still be observed in samples from *fon-RNAi* larvae in contrast to *hml-RNAi* larvae, we hypothesize that Fondue is not involved in the formation of primary clot fibers but rather in the subsequent cross-linking of these fibers. The longer clot fiber phenotype in the draw-out assay is reminiscent of the effects of phenoloxidase deficiency on the appearance of the clot (Bidla et al., 2005).

Since Fondue is a major hemolymph protein and binds to beads, it probably represents a structural component of the clot rather than a regulatory element. Some aspects of the *fon-RNAi* phenotype may be explained by a basic analysis of the primary amino acid sequence. A remarkable feature of Fondue is the presence of a large number of repeats that may influence the biophysical properties of the protein. In addition, the high occurrence of glycine and alanine and high hydrophobicity in the primary amino acid sequence of Fondue is reminiscent of other structural proteins like the silk protein fibroin (Gosline et al., 1999) and insect cuticular proteins, where these residues are thought to cause numerous internal beta-turns and a tendency to aggregate, while retaining mobility (Andersen et al., 1995). Enzymes from the transglutaminase family were shown to be crucial for final cross-linking of the clot via glutamine and lysine residues in vertebrates, crustaceans (Hall et al., 1999) and clot-hemocyte contacts in horseshoe crabs (Osaki et al., 2002). Glutamine residues are also abundant in Fondue, which was recently identified as a major substrate for the enzyme transglutaminase (Karlsson et al., 2004). Thus, it is likely that transglutaminase catalyzes at least part of the reaction strengthening the primary soft clot in insects as well.

Clotting has been hypothesized to be an integral part of the insect immune response, because it stops bleeding and inhibits pathogens from entering the body cavity through the wound (Theopold et al., 2004). Wounding of *fon-RNAi* larvae or flies did not lead to a drop in survival compared to challenged control animals, but neither was such an effect observed in *hml-RNAi* animals or for double *fon-RNAi hml-RNAi* larvae (unpublished results). It seems that impairment of clotting in vivo leads to subtle phenotypes, such as the formation of larger scabs. This suggests that other mechanisms exist to effect wound closure, including hemocyte and epithelial cell movements as well as melanization (Galko and Krasnow, 2004; Rämet et al., 2002). Even survival of *fon-RNAi* following septic injury with different microorganisms is not affected except a slightly stronger susceptibility towards Gram-negative bacteria. However, constitutive *Drs* expression in unchallenged *fon-RNAi* larvae and the moderate effect on *Dpt* expression levels in *fon-RNAi* larvae might indicate that *fon*, but not *hml*, is somehow connected to AMP regulation. We show that constitutive expression of *Drs* expression was suppressed in absence of *spz* and was linked to fat-body expression of *fon-IR*. Therefore, we hypothesize that depletion of Fondue alters certain hemolymph properties such as viscosity and pressure resulting in an abnormal activation of the proteolytic cascade upstream of the Toll receptor, which in turn would also lead to formation of melanotic tumors.

In addition, Fondue is also required during metamorphosis, as *fon-RNAi* causes lethality at the pupal stage associated with longer banana-shaped pupae. Since these longer pupae were also observed in a *spz* mutant background (data not shown), it is probably not connected to activation of the Toll pathway. This phenotype was not displayed by *hml-RNAi* pupae, suggesting that it is not directly linked to clotting in general. It is tempting to speculate that Fondue may serve a structural role in formation of the puparium and/or might lead to a secondary impairment of sclerotization events. Alternatively Fondue may contribute to particular hemolymph characteristics that are required independently during both clotting and metamorphosis. The qRT-PCR for *fon* transcripts demonstrates that this protein is strongly expressed in early pupae. This is in accordance with our suggestion that Fondue may be required for sclerotization and hardening of the pupal case. Similar elongated and curved pupal phenotypes have been observed for mutants of *Broad-Complex* and *E74* involved in ecdysone-induced early gene expression in pupae (Fletcher and Thummel, 1995), but the molecular basis underlying the pupal phenotype remains unclear. The characteristic behavior of late *fon-RNAi* larvae performing circular movements with their caudal end may point to a defect in larval attachment or body retraction at early steps of pupation.

Although coagulation is a universal response to injury, studies in arthropods such as crustaceans, insect and horseshoe crabs indicate that the molecular mechanisms underlying clot formation are not conserved. Hemolectin, a major *Drosophila* clotting factor, contains sequence elements such as von Willebrand domains that are found in vertebrate clotting proteins but are also present in homologues of different insect orders and may participate in clot formation in these species as well. In sharp contrast, Fondue is a protein that is not found outside the Drosophilidae so far. This finding indicates that even among insects the actual proteins involved in clotting and clot cross-linking may be poorly conserved, while the respective underlying aggregation mechanisms might be similar. Variability among clotting factors may in fact be the molecular basis for the large morphological variability of insect hemolymph clots, which has been known for many decades (Grégoire, 1951). In

conclusion, we propose that Fondue is a major hemolymph protein that evolved specifically as a clotting factor in *Drosophila*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.03.019.

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