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

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Received for publication 22 November 2005; revised 2 February 2006; accepted 15 March 2006
Available online 9 May 2006

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
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 hemol ectin was notable among the identified candidate clotting factors (Scherfer et al., 2004). Using RNAi, we characterized Fondu, 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. cDNA fragments corresponding to the first 500 bp of the coding sequence of the cDNA fragment corresponding to the first 500 bp of the coding sequence of the gene encodes two alternative transcripts for a protein with two isofoms 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 Fondu 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

Inspections

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

Quantitative real-time PCR (qRT-PCR)

For Dpt and Dvr 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 GGC CAA TCG CTT CTA CT-3' and antisense 5'-TGG TGG AGT AGG GGG CTT CAT G-3'), Dvr (sense 5'-CGT GAG AAC CTT TTC CAA TAT GAT G-3' and antisense 5'-TCC CAG GAC CAC CAG CAG CAT-3'), fon (sense 5'-GAT AGT AGT GTG CGG T-3' and antisense 5'-GGA AGG AGA ATG TGG 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).

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 flies, 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 Fondu 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
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 plasmatocytes and lamellocytes were detected in some hemolymph samples (data not shown).
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
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 Fondu 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 Fondu is an important protein required for clotting or clot hardening. We chose the name Fondu 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 Fondu 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 carotovara 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.](image-url)

(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 boulardi 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.

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

Acknowledgments

We thank Jacques Montagne and Akira Goto for fly stocks and our colleagues Brigitte Maroni and Mickael Poidevin for assistance. The laboratory of B.L. was funded by the Schlumberger and Bettencourt Foundation and the Agence National de la Recherche. B.L. received support from an EMBO short-term fellowship that assisted in initiating the project. This work was supported in part by grants from MITILS to R. U. The authors U. T. and M. D. received funding from the Swedish Research Council and U. T. from the Carl-Tryggers Foundation.

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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