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The retinoic-like juvenile hormone controls the looping of left-right asymmetric organs in *Drosophila*

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SUMMARY

In vertebrate development, the establishment of left-right asymmetry is essential for sidedness and the directional looping of organs like the heart. Both the *nodal* pathway and retinoic acid play major and conserved regulatory roles in these processes. We carried out a novel screen in *Drosophila* to identify mutants that specifically affect the looping of left-right asymmetric organs. We report the isolation of *spin*, a novel mutant in which the looping of the genitalia and spermiduct are incomplete; under-rotation of the genitalia indicates that *spin* controls looping morphogenesis but not direction, thus uncoupling left-right asymmetry and looping morphogenesis. *spin* is a novel, rotation-specific allele of the *fasciclin2* (*Fas2*) gene, which encodes a cell-adhesion protein involved in several aspects of neurogenesis. In *spin* mutants, the synapses connecting specific neurosecretory cells to the corpora allata are affected. The corpus allatum is part of the ring gland and is involved in the control of juvenile hormone titers during development. Our genetic and pharmacological results indicate that *Fas2^{spin}* rotation defects are linked to an abnormal endocrine function and an elevated level of juvenile hormone. As juvenile hormone is an insect sesquiterpenoid related to retinoic acid, these results establish a new genetic model for studying organ looping and demonstrate an evolutionarily conserved role for terpenoids in this process.

Key words: Juvenile hormone, Retinoic acid, Left-right asymmetry; Fasciclin 2, Genitalia, Organ looping

INTRODUCTION

The establishment of the two major axes, anteroposterior (AP) and dorsoventral (DV), is central to the patterning of the body plan. This orthogonal, two-coordinate system results in an apparent bilateral symmetry of the body shape in many organisms. In addition to the AP and DV axes, animals have developed a third axis allowing differentiation of the left and right body sides. Left-right (LR) asymmetry arises during development of internal organs, leading to side-specific organ morphology (e.g. lung, brain) or location (e.g. heart, spleen, liver) (for a review, see Burdine and Schier, 2000; Capdevila et al., 2000; Hamada et al., 2002; Mercola and Levin, 2001; Wright, 2001). Another important consequence of LR asymmetry is the directional looping of organs along the AP axis. For example, in normal humans (situs solitus) the heart undergoes a dextral looping leading to its pointing toward the left side. The heart is the organ most sensitive to defects in LR asymmetry and organ looping, which can lead to severe and frequent life-threatening abnormalities (1% of liveborns show heart abnormalities) (Harvey, 1998; Kathiriya and Srivastava, 2000). Thus, it is important to develop models in order to analyze how LR asymmetry and looping morphogenesis are established, and how these two processes interact during embryogenesis.

Given the harmful consequences of heterotaxia (Kosaki and Casey, 1998), it is crucial for animals to genetically control LR asymmetry in order to avoid organ randomization. A breakthrough in the molecular analysis of LR asymmetry was the discovery of side-specific expression of *nodal*, a BMP family molecule, in chick embryos (Levin et al., 1995). The study of *nodal* homologs in other vertebrate embryos (Collignon et al., 1996; Lowe et al., 1996) indicates that nodal is a central and evolutionarily conserved regulator of LR asymmetry (Burdine and Schier, 2000; Capdevila et al., 2000; Mercola and Levin, 2001; Wright, 2001). In addition to *nodal*, retinoic acid (RA), a terpenoid compound, has been shown to participate in LR asymmetry in all vertebrates examined so far (Niederreither et al., 2001; Tsukui et al., 1999; Wasiak and Lohnes, 1999). Indeed, either an excess or a reduction of RA activity can lead to LR asymmetry defects (Tsukui et al., 1999). Interestingly, RA-induced LR defects are associated with abnormal expression of the asymmetric markers *nodal* or *pitx2*, indicating that RA interacts with the *nodal* pathway and is an important conserved component of the LR program in vertebrates.

Although there is considerable information about how LR asymmetry is established, very little is known about the mechanisms and molecules controlling looping morphogenesis of LR asymmetric organs. It is important to note that in the absence of LR determination, organs still loop but in a random direction. This indicates that looping morphogenesis lies downstream of the LR pathway and produces a stereotyped directional looping based on interpretation of positional information. One clear candidate in the looping process in vertebrates is RA. Indeed, depending on the species, mode of administration and stage, exogenous RA can induce partial heart looping in mouse and *Xenopus* (Chazaud et al., 1999; Drysdale et al., 1997; Iulianella and Lohnes, 2002; Niederreither et al., 2001; Tsukui et al., 1999; Wasiak and Lohnes, 1999; Zile et al., 2000). Furthermore, loss of the *Raldh2* gene, which is essential to convert vitamin A into active RA, leads to an incomplete looping of the heart in mouse (Niederreither et al., 2001). These data thus indicate that RA has a dual role, being required both in the establishment of LR asymmetry upstream of *nodal* and in the downstream morphogenetic control of looping per se. Whether the mechanisms controlling and coupling LR asymmetry and organ looping are conserved is unknown.

In contrast to the prominent sidedness found in vertebrates, LR asymmetry in invertebrates is much less pronounced (Hobert et al., 2002). For example, the *Drosophila* heart is a symmetrical structure lying at the dorsal midline and running along the AP axis. There are a few stereotyped LR markers in the fly gut, but no mutations so far have been isolated that specifically affect any of these (Hayashi and Murakami, 2001; Lengyel and Iwaki, 2002; Ligoxygakis et al., 2001). In order to establish a genetic model of LR asymmetry and organ looping in *Drosophila*, we have screened for mutations affecting the asymmetric looping of the spermiduct and genitalia in adult flies (Fig. 1A,B). We use this process as a model to dissect the genetic pathways involved in the looping of LR asymmetric organs.

We have identified a looping-specific mutation in *Drosophila*, which we call *spin*. In the *spin* mutant, direction is normal but looping is incomplete, indicating that this gene controls looping morphogenesis downstream of LR determination. Molecular cloning shows that *spin* is a novel *Fas2* allele; in *spin* mutants a specific subset of neurosecretory cells innervating the corpora allata is defective. The corpus allatum is a specialized structure of the ring gland, the major endocrine gland in *Drosophila*, that is involved in the secretion of juvenile hormone (JH), one of the main insect growth regulators. Our pharmacological and genetic data indicate that *spin* males have an abnormal level of JH during the pupal stage, and that this is responsible for genitalia and spermiduct rotation defects. As the sesquiterpenoid JH is functionally related to the terpenoid RA (Harmon et al., 1995), which plays an important role in organ looping and LR asymmetry in vertebrates, these results indicate that an evolutionary conserved terpenoid pathway controls asymmetric organ morphogenesis in animals.

MATERIALS AND METHODS

Drosophila stocks and genetics

The original *spin* mutation was generated in a P-element mutagenesis screen on the X chromosome using a P{GawB} transposon (Bourbon et al., 2002) (S.N., unpublished). Cloning of *spin* was carried out by plasmid rescue and sequencing of the insertion site was done using an automated ABI DNA sequencer. The P-element in *spin* was remobilized using an external source of P transposase in *y w spin*$^{P\{Gal4;\ w+\}}$*/Y; Δ2-3 transposase/+* males; excisions were selected through the loss of the w^+ eye marker and balanced using classical approaches and chromosomes (see FlyBase at http://flybase.bio.indiana.edu/).

Fas2^{e86}, *Fas2*^{EB112} and UAS-*Fas2* were kind gifts of C. Goodman (Grenningloh et al., 1991). *Fas2*^{rd1} was a gift of R. Davis (Cheng et al., 2001). UAS-synaptobrevin-GFP flies were provided by M. Ramaswami (Estes et al., 2000). *Met* flies were kindly provided by T. Wilson. Transgenic lines expressing GAL4 in different subsets of neurons (Aug21, Feb78, Feb170, Feb211, Kurs21) were kindly provided by T. Siegmund (Siegmund and Korge, 2001). All other stocks can be found at FlyBase (http://flybase.bio.indiana.edu/).

Rescue experiments

Short egg collections (2 hours) were raised on rich medium at 25°C and subjected to a 1 hour heat-shock at 37°C. Only one heat-shock per life cycle was applied. After HS treatment, flies were grown at 25°C until eclosion, and the extent of rotation rescue was scored in adult males. The genotype of the heat-shocked males is *Fas2*^{spinR5}/Y; *UAS-Fas2*/*HS-GAL4*.

Fas2-expressing clones, marked with GFP, were generated using the 'flip-out' technique (Struhl and Basler, 1993). Larvae with the genotype *Fas2*^{spinR5}*/Y*; *act>y+>GAL4, UAS-EGFP/UAS-Fas2; hs-FLP/+* were heat-shocked at 37°C for 1 hour.

Histology

Third instar larvae were dissected, inverted inside-out with forceps and antibody stained using standard protocols. After staining, the ring glands were dissected and mounted. The anti-Fas2 (Developmental Studies Hybridoma Bank) primary antibody was used at 1:10; Secondary antibodies used in this study are anti-mouse-FITC (1:400; Molecular Probes), anti-mouse CY3 (1:400; Molecular Probes), anti-rabbit-CY3 or FITC (1:400; Molecular Probes). Phalloidin-TRITC was used at 1:2000 (Molecular Probes). Confocal images were taken using Leica TCS-NT or TCS-SP1 confocal microscopes. Images were processed using Photoshop 6.0 (Adobe).

For scanning electron microscopy (SEM) imaging, adult flies were critical-point dried and coated with 25 nm gold using standard methods.

Topical application of pyriproxyfen

A JH analog, pyriproxyfen {2-[1-methyl-2-(4-phenoxyphenoxy)-ethoxy]-pyridine} stock solution (10 mg/l in acetonitrile from the laboratory of Dr Ehrenstorfer-Schafers, Germany) was diluted with acetone. White prepupae were collected and 0.25 μl of acetone containing the desired amount of pyriproxyfen was applied to each pupa on the dorsal side, as described by Riddiford and Ashburner (Riddiford and Ashburner, 1991).

RESULTS

The rotation of male genitalia represents a LR asymmetry and organ looping model in *Drosophila*

The rotation of genitalia takes place during metamorphosis; at this stage, the distal part of the male reproductive apparatus, the genital plate, undergoes a stereotyped 360° clockwise rotation, inducing the spermiduct to loop around the gut in a clockwise direction (see Fig. 1A,B) (Gleichauf, 1936). The rotation of genitalia can thus be compared with the oriented (dextral or sinistral) looping of internal organs in vertebrates, such as gut and heart. These indeed represent specific LR

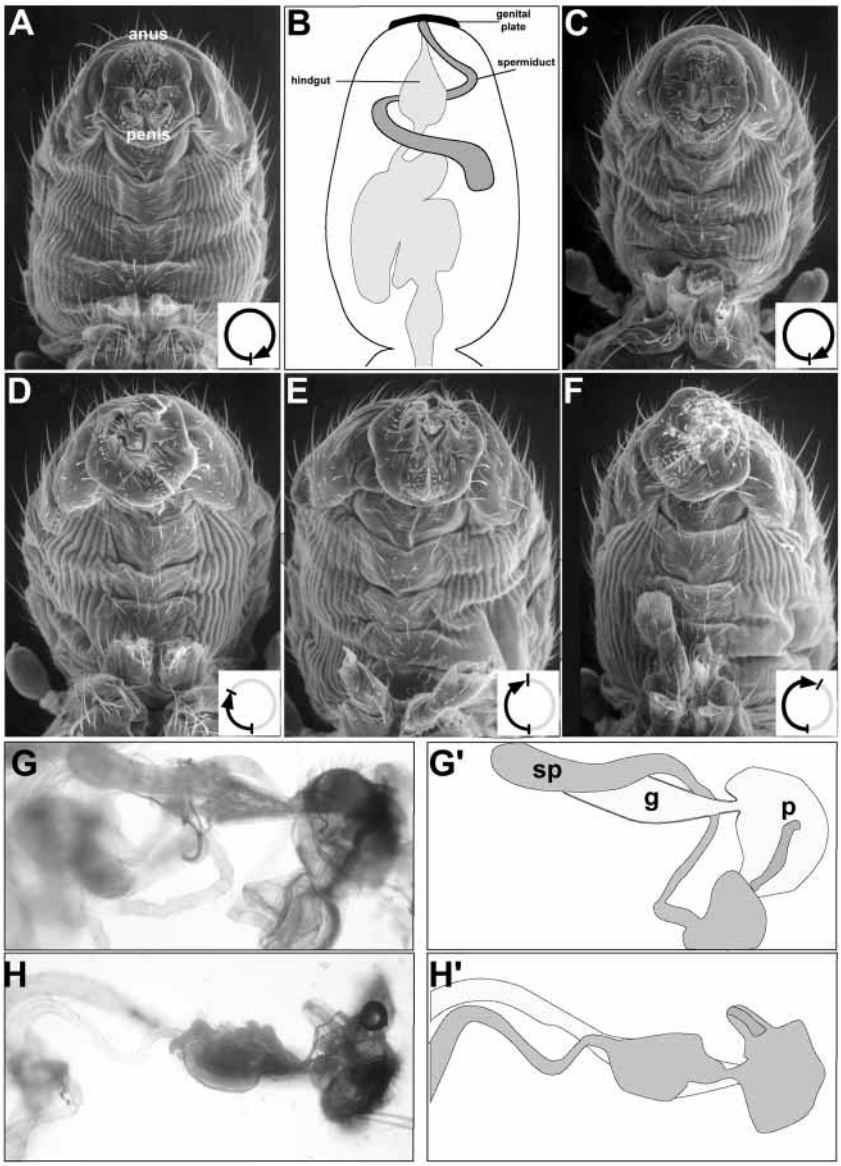

Fig. 1. Rotation of genitalia and spermiduct looping in wild-type and *spin* males. (A) Scanning electron micrograph (SEM) of a wild-type male abdomen (ventral view; posterior is upwards), showing the position of the anus and penis. The direction and extent of genitalia rotation is schematized by a looping arrow (bottom right). (B) Schematic view of a wild-type abdomen showing the internal looping of the spermiduct around the gut, after clockwise rotation of the genital plate. (C) SEM of a $spin^{GAL4}$/Y; UAS-*Fas2* male showing complete rescue of rotation. (D-F) SEM of representative *spin* males showing varying degrees of rotation. (G,G′) Dissected wild-type abdomen and its schematic representation showing the rightward looping of the spermiduct. (H,H′) Dissected *spin* male (similar to E), and its schematic representation showing an under-rotation phenotype. sp, spermiduct; g, gut; p, penis.

asymmetry markers, as mutations that affect organ positioning also perturb the direction of organ looping (see Introduction). Interestingly, mutations have been previously isolated that resulted in counterclockwise rotation of genitalia both in *Drosophila melanogaster* and *Musca domestica* (Milani, 1955; Milani, 1956).

The reversible nature of looping genitalia indicates that this structure represents a suitable LR marker that can be used as a novel genetic model to study both LR asymmetry and looping morphogenesis in *Drosophila*.

spin affects organ looping

To initiate a genetic characterization of LR asymmetry and organ looping in flies, we screened for viable mutations showing defective genitalia rotation (G.Á. and S.N., unpublished). We focus on a novel viable P-element mutation, *spin*, for which all the adult males show a characteristic mis-rotation of genitalia and are sterile. In *spin* males, the extent of rotation varies from ~30° to 320°, with a large proportion of males (84%; *n*=195) having their genital plate in a position corresponding to rotation of 135-225°. Fig. 1 shows some characteristic examples of *spin* genital plates. Because external mis-rotation does not allow discrimination between under-, hyper- or counter-rotation of the genitalia, males of different phenotypes were dissected and the looping of their spermiduct analyzed. All dissected males showed a clear under-rotation phenotype (Fig. 1G,H), indicating that *spin* is required for the genital plate and spermiduct to undergo complete looping, but has no role in directionality. Dissection of several hundred wild-type males did not reveal any rotation defect (data not shown), indicating the robustness of this process in normal males.

spin is a novel, looping specific, *fasciclin 2* allele

After remobilization of the P-element insert in *spin* (see Materials and Methods), three different w^- populations were found. In the major class (51/73; 70%), flies reverted to a fully wild-type phenotype. The two other classes included lethal alleles (18/73; 25%) and viable alleles (4/73; 5%), which retained their original *spin*-like phenotype. Altogether, these results indicate that the P{GAL4} transposon present in *spin* is responsible for the looping phenotypes and is inserted in or close to an essential gene.

The P-element in *spin* is inserted in the 5′ UTR of the *fasciclin 2* (*Fas2*) gene (Fig. 2A; see Materials and Methods) (Goodman et al., 1997; Grenningloh et al., 1991), suggesting that *spin* is a novel *Fas2* allele (hereafter referred to as $Fas2^{spin}$). This conclusion is supported by the following points. First, the expression of the Fas2 protein in the original $Fas2^{spin}$ allele and in a lethal revertant ($Fas2^{spinRM1}$) is strongly reduced or absent in embryos, respectively (Fig. 2B-E). Significantly, in $Fas2^{spin}$ third instar larvae, the expression of the Fas2 protein in eye imaginal discs or in whole brain extracts is also strongly reduced (Fig. 2F,G; data not shown). Second, we show that expression of a *UAS-Fas2* transgene under the control of the original $spin^{P\{GAL4\}}$ line can fully rescue the rotation and sterility phenotypes (Fig. 1C).

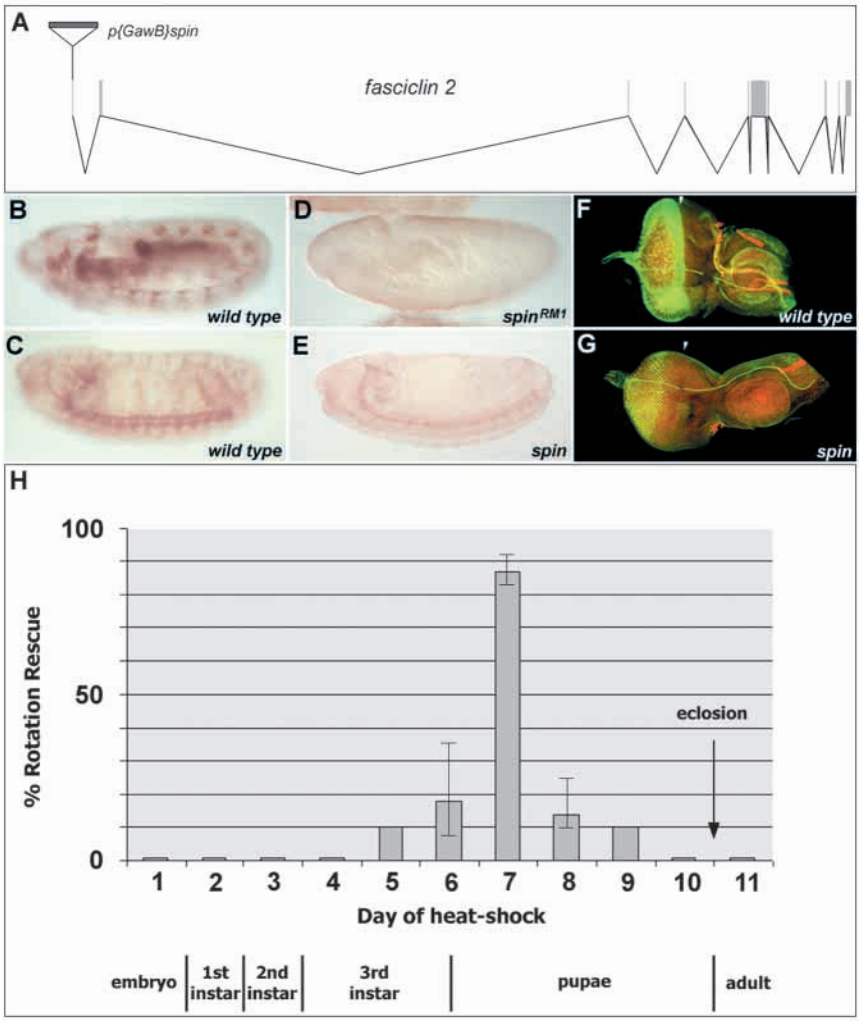

Fig. 2. *spin* is a novel, rotation-specific *Fas2* allele. Schematic view of the *Fas2* gene and the $Fas2^{spin}$ p{GawB} P-element insertion (A). Expression of Fas2 in stage 9 (B) and 14 (C) wild-type embryos. In a $Fas2^{spin}$ lethal revertant allele ($Fas2^{spinRM1}$), Fas2 expression is lost (D), while Fas2 expression in the viable $Fas2^{spin}$ allele is strongly reduced (E), when compared with wild type (compare D with B and E with C). Expression of Fas2 (green) in a wild-type eye imaginal discs starts anterior to the morphogenetic furrow (F, arrowhead), while it is strongly reduced in a $Fas2^{spin}$ eye imaginal disc (G). (H) Histogram showing the percentage of males with normal genitalia, after expression of *Fas2* using a *UAS-Fas2* transgene under the control of a heat-inducible GAL4 line. For each time-point, the phenotype of the external genitalia has been determined and the extent of rotation rescue plotted. Heat-shocked males have the following genotype: $Fas2^{spinR5}$*/Y; UAS-Fas2/HS-GAL4.* $Fas2^{spinR5}$ is a $Fas2^{spin}$ excision allele that retains the original $Fas2^{spin}$ rotation phenotype but lacks GAL4 activity.

Because genitalia rotation defects have not been described for any previously characterized *Fas2* allele, we wondered whether other viable *Fas2* alleles would show rotation defects. Interestingly, we found that $Fas2^{e86}$ (Grenningloh et al., 1991) and $Fas2^{rd1}$ (Cheng et al., 2001) alleles displayed ~5% and ~20% genitalia rotation defects, respectively (data not shown).

Taken together, these results indicate that *spin* is a novel viable, looping-specific *Fas2* allele. $Fas2^{spin}$ is the first fully penetrant defective looping mutant to be described, and thus represents an important tool to study organ looping in *Drosophila*.

Stage requirement for *Fas2* function in organ looping

Rotation of genitalia was reported by Gleichauf (Gleichauf, 1936) to take place in 2- to 3-day-old pupae over a period of 24 hours. To establish the temporal requirement for *Fas2* function in genitalia rotation, we used a heat-inducible GAL4 line to express *Fas2* under the UAS promoter. In this experiment, we rescued the $Fas2^{spinR5}$ allele, a viable $Fas2^{spin}$ excision allele retaining the rotation phenotypes but lacking the GAL4 activity associated with the original $Fas2^{spin}$ allele (data not shown). Short egg collections were subjected to a single one hour heat-shock (HS) at 37°C. Adult males were then analyzed and the extent of genitalia rotation rescue determined. As shown in Fig. 2H, a single 1 hour HS at day 7 of development is sufficient to rescue genitalia rotation. Indeed, flies that received a HS at day 7 of development showed a very high degree of rescue (up to 90%), while HS applied either before or after this period had little or no effect (Fig. 2H). Control males which have not been heat-shocked show no rescue (data not shown). These results indicate that *Fas2* is required during a limited period of time during pupal development for rotation to take place normally. The timing of the rescue is consistent with the previously described period of rotation (Gleichauf, 1936).

$Fas2^{spin}$ affects the corpora allata synapses

In order to identify the tissue(s) and cells that require *Fas2* function for genitalia rotation, we used the GAL4-UAS system (Brand and Perrimon, 1993) to drive tissue-specific expression of a *UAS-Fas2* transgene in $Fas2^{spinR5}$ males. Because *Fas2* is required in many aspects of neuronal development (Brunner and O'Kane, 1997; Goodman et al., 1997), and is expressed mostly in neurons, we first asked whether *Fas2* function was required in the nervous system for rotation. Surprisingly, we found that the *elav-GAL4* line, which drives expression specifically in the CNS during development, is able to rescue $Fas2^{spinR5}$ rotation defects fully (Fig. 4A). This result prompted us to examine in detail the expression pattern of Fas2 protein in the brain and to look for potential nervous system phenotypes in $Fas2^{spin}$. Our analysis uncovers a previously unknown function of *Fas2* in the ring gland (RG). The RG is a composite neuroendocrine organ made of three different specialized regions: the prothoracic gland (PT), the corpora cardiaca (CC) and the corpora allata (CA; Fig. 3B). The CC probably plays a role in the regulation of blood sugar levels in larvae through adipokinetic hormones (Noyes et al., 1995;

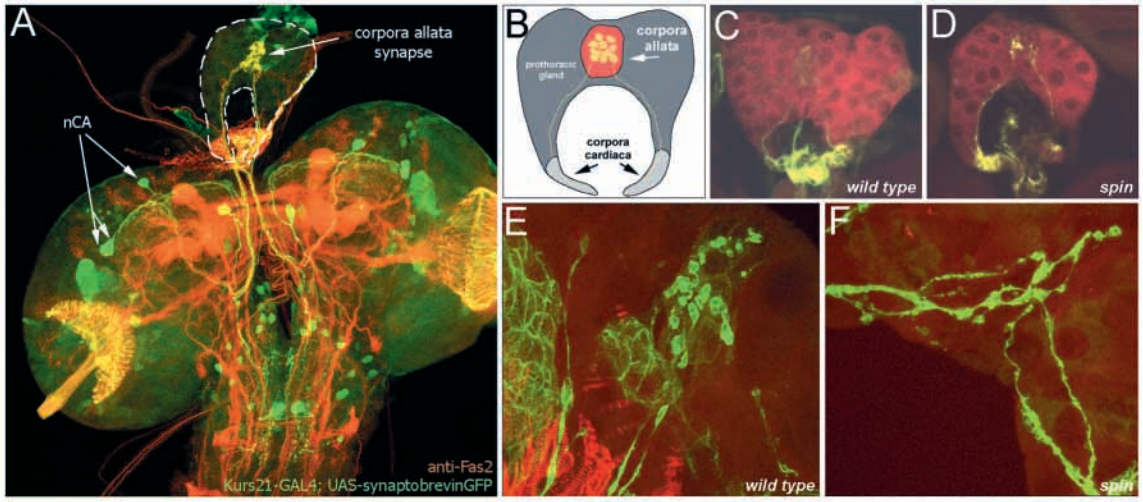

Fig. 3. Ring gland defects in *Fas2*^{spin}. (A) Third instar larval brain and ring gland (outlined) stained using the *Kurs21-GAL4; UAS-synaptobrevinGFP* (green) line and anti-Fas2 (red). nCA, neurons innervating the corpora allata and expressing GAL4. (B) The ring gland with its three different regions (corpora cardiaca, corpora allata and prothoracic gland). The axon processes of the neurosecretory cells controlling juvenile hormone synthesis are shown in yellow. These neurons express Fas2 in the axon processes and project into the corpora allata. (C,D) Anti-Fas2 staining of a wild-type (C) or *spin* (D) ring gland (green). Phalloidin-TRITC is in red. (E,F) Close-up of the corpora allata synapse from a wild-type (E) or *Fas2*^{spin} (F) ring gland.

Rulifson et al., 2002). The PT and CA are specialized cells responsible for the secretion of the two primary insect hormones ecdysone and juvenile hormone (JH), respectively (Riddiford, 1993). Interestingly, Fas2 expression is restricted to the CC and to specific axonal processes innervating the CA (Fig. 3A,C). These neurosecretory cells (nCA; see Fig. 3A) control JH level and can be easily identified using a specific GAL4 line expressed in all CA neurons (Kurs21-GAL4) (Siegmund and Korge, 2001). As Kurs21-GAL4 driven GFP expression and the anti-Fas2 staining overlap precisely, we conclude that Fas2 is expressed in all nCA terminals (Fig. 3A). In *Fas2*^{spin}, the overall morphology of the CA synapse is abnormal (Fig. 3C,D), showing fused terminal boutons and a reduced number of presynaptic nerve terminals (compare Fig. 3E,F). This result is consistent with the previous finding that the bouton number is reduced in the neuromuscular junction in strong hypomorph *Fas2* flies (Stewart et al., 1996).

Both the expression pattern of Fas2 in the nCA and the abnormal synapses in *Fas2*^{spin} mutants suggest that Fas2 may be required for JH synthesis, which in turn may be important for appropriate genitalia and spermiduct looping during metamorphosis.

Role of CA neurosecretory cells in organ looping

In order to demonstrate a direct link between *Fas2*, the CA, and genitalia rotation, we used the UAS-GAL4 system to express *Fas2* in specific subsets of neurons innervating the RG. In a recent study, Siegmund and Korge (Siegmund and Korge, 2001) identified and mapped the few neurons innervating the RG in *Drosophila*. The PT is innervated by two neurons from each brain hemisphere, whereas the CA is innervated by three neurons (Fig. 3A) (Siegmund and Korge, 2001). Importantly, neurons innervating the PT and CA are different and map to distinct regions of the brain. We used a collection of GAL4 lines expressed in different populations of neurons innervating the RG (Fig. 4) (Siegmund and Korge, 2001) to induce neuron-

A

GAL4 line	Genitalia Rotation	Expression
elavGal4	+++	all neurons
Kurs21	+++	nCA (strong)
Feb78	+/-	nCA (weak)
Feb170	+++ or -	nCA (variegated)
Feb211	-	nPT
Aug21	-	CA

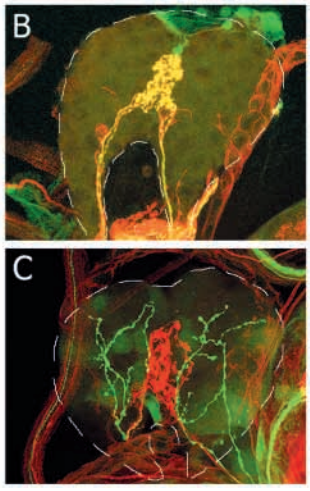

Fig. 4. Expression of *Fas2* in neurons innervating the corpora allata rescues *Fas2*^{spin} rotation defects. (A) Summary of rescue experiments using lines expressing GAL4 in different subsets of neurons in the brain. Rescued males had the following genotype: *Fas2*^{spinR5}*/Y; UAS-Fas2; GAL4*. +++, full rescue; +, partial rescue; –, no rescue. (B) Ring gland expressing a synaptobrevin-GFP fusion protein (green; to mark terminal boutons) in neurons innervating the corpora allata (nCA), using the *Kurs21*-GAL4 line. The ring gland (outlined) is double stained with anti-Fas2 (red). (C) Ring gland expressing EGFP (green) in neurons innervating the prothoracic gland (nPT), using the *Feb211*-GAL4 line. The ring gland (outlined) is double stained with anti-Fas2 (red).

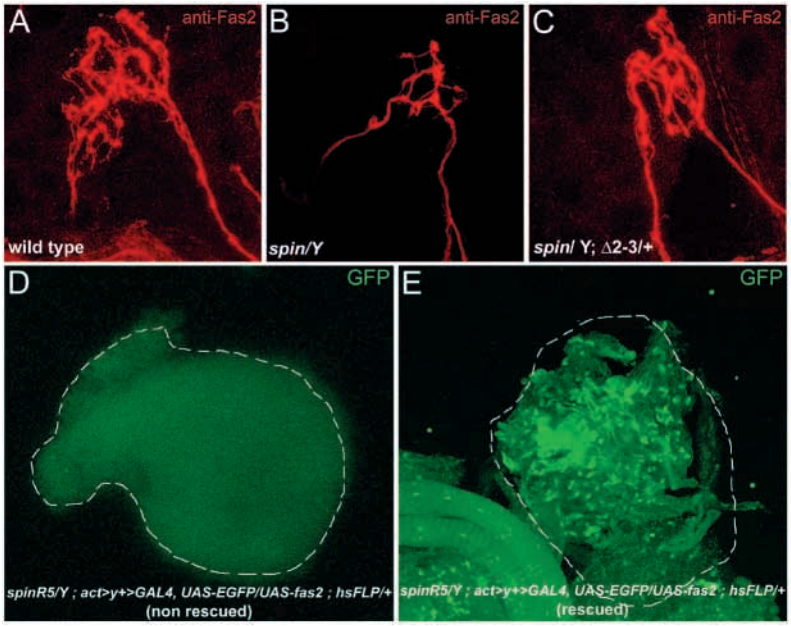

Fig. 5. *Fas2* controls genitalia rotation cell non-autonomously. Morphology of the corpora allata nerve terminals in a wild-type (A), $Fas2^{spin}$ (B) and mosaic $Fas2^{spinR5}/Y$; Δ2-3/+ (C) males. The number of terminal boutons is reduced in $Fas2^{spinR5}$, and is restored in mosaic males. (D,E) Phenotype of adult ring glands from non-rescued (D) or rescued (E) males, after generation of random, 'flip-out' clones expressing Fas2 (not shown) and EGFP (green). Only rescued males express GFP and Fas2 in the ring gland.

specific expression of *Fas2* in $Fas2^{spinR5}$ mutants. When GAL4 is expressed strongly in the neurons innervating the CA (nCA) using the *Kurs21-GAL4* line (Fig. 4A,B), the rotation of genitalia is completely rescued, just as observed with an *elav-GAL4* driver. Using another line in which GAL4 is only weakly expressed in CA neurons (Feb78), we found that the rotation, although only weakly and partially rescued, exceeds that seen in control $Fas2^{spinR5}$ males. Using another *nCA-GAL4* line (Feb170) in which GAL4 shows variegated expression (i.e. some larvae express GAL4 in nCA, while in others the expression is absent) (Siegmund and Korge, 2001), rescue is either complete or absent, respectively (Fig. 4A). By contrast, when GAL4 is expressed in the neurons innervating the prothoracic gland (nPT; Feb211; Fig. 4A,C) or in the CA itself (Aug21), no rescue was observed. These results show that *Fas2* is required in the nCA for normal genitalia rotation. In addition, they indicate that the rotation defects associated with $Fas2^{spinR5}$, and probably also with other viable *Fas2* alleles, are linked to a defective neuroendocrine function leading to abnormal synthesis of JH during pupal development (Fig. 2H).

$Fas2^{spin}$ controls looping non-autonomously

Our data support a model in which *Fas2*-expressing nCA neurons control JH titers which in turn remotely control the rotation of the genital plate (Fig. 7A). This model has two main predictions: first, if JH is a mediator of *Fas2* function during rotation, then $Fas2^{spin}$ should function cell non-autonomously; second, JH itself should have the potential to perturb rotation when its level is altered experimentally.

The cell non-autonomy of $Fas2^{spin}$ is already manifest in the previous rescue experiments using GAL4 lines expressed in subsets of neurons in the brain (Fig. 4). If Fas2 is cell non-autonomous for rotation, then it is expected that mosaic animals, in which some cells are mutant while others are wild type, should be rescued. Interestingly, we found that almost all (367/368; 99.7%) transposase-induced mosaic males ($Fas2^{spin}$/Y; Δ2-3 transposase/+) have normal, fully rotated genitalia. Consistent with a complete rescue of the external genitalia rotation phenotype, these mosaic males showed normal looping and fertility (data not shown). Importantly, the comparison of the morphology of the CA synapse in wild type, $Fas2^{spin}$ and mosaic males ($Fas2^{spin}$/Y; Δ2-3 transposase/+) indicates that all rescued mosaic males had restored a normal CA synapse (Fig. 5A-C).

In further support of the non-autonomy and nCA origin of *spin* phenotypes, we performed a different mosaic experiment in which expression of a UAS-*Fas2* transgene is induced randomly using the 'flip-out' technique (Struhl and Basler, 1993). In this experiment, random clones of cells expressing a wild-type *Fas2* transgene in a $Fas2^{spinR5}$ background are generated and positively marked with GFP (see Materials and Methods). After clone induction, the developed adult males are sorted depending on their genitalia phenotypes. Only two different categories were found, corresponding to males that either are completely rescued or not rescued at all. Males from each class were dissected and analyzed for GFP (hence *Fas2*) expression in the RG. During metamorphosis, the RG migrates posteriorly toward the proventriculus and the prothoracic gland degenerates (Dai and Gilbert, 1991). In adults, the RG is thus made only of CA and CC cells. We found that none of the males (n=5) in which genitalia is not rescued show any GFP expression in the adult RG, while all (n=7) dissected males showing full rescue of genitalia rotation had GFP expressed in the RG (Fig. 5D,E). Altogether, our mosaic analysis indicate a non-autonomous function of *Fas2* during organ looping.

Pyriproxyfen, a JH agonist, induces looping defects

The second prediction of our model is that JH, which is proposed to control rotation, should induce looping defects when its level is modified during pupal development. Interestingly, it has been shown that the JH analogs methoprene and pyriproxyfen produce rotation defects at low doses, after topical application to white pre-pupae (Riddiford and Ashburner, 1991). Higher doses of these JH analogs induce abdominal defects and lethality. Because the extent and direction of rotation in the previous study were not determined, we applied varying doses of pyriproxyfen to white pre-pupae and monitored the effects on genitalia rotation. Application of half-lethal doses of pyriproxyfen (0.25 pM/pupae) to wild-type pupae induces rotation defects that are very similar to $Fas2^{spin}$ phenotypes. Indeed, dissection of the posterior abdomen of unhatched adults (pharate adults) indicates that pyriproxyfen-treated males have an under-rotated phenotype, with some males showing a complete absence of rotation (Fig. 6A). At higher doses (0.4 pM/pupae), the treatment is lethal; as animals die as pharate adults, their morphology can be analyzed.

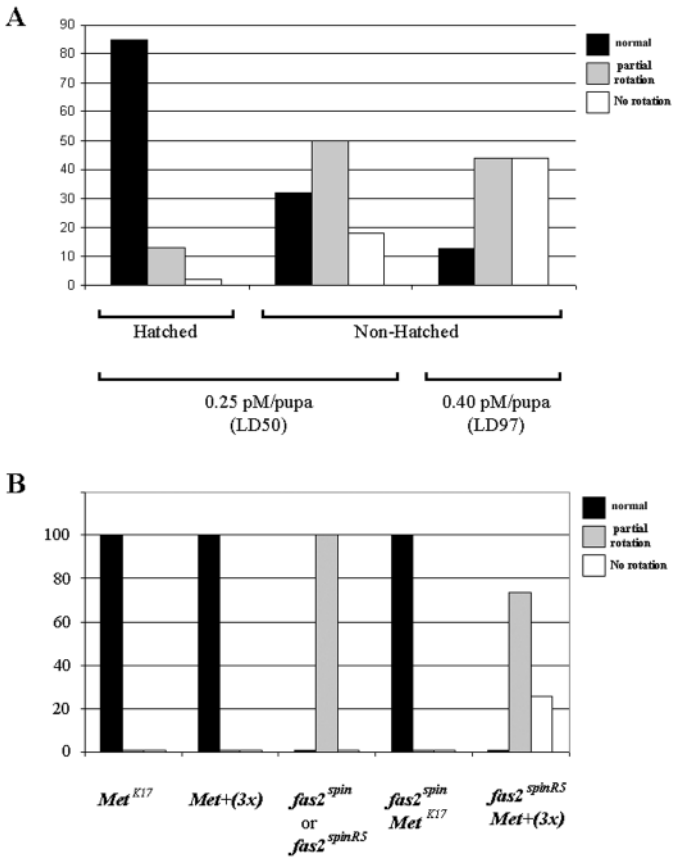

Fig. 6. Effects of JH levels on *Fas2*^{spin} and genitalia rotation. (A) Phenotype of adult or pharate adult male genitalia after topical application of pyriproxyfen (a JH analog) at the white pupae stage. The use of a half-lethal dose of pyriproxyfen (LD_{50}) induces mis-rotation phenotypes in both the hatched and non-hatched populations (16% and 68%, respectively). In the non-hatched flies, the partial and no rotation phenotypes were four and six times higher than in the hatched population, respectively. At the lethal dose of pyriproxyfen (97% lethality; LD_{97}) the pharate adult males showed a higher proportion of partial (44%) and non rotated (44%) genitalia with only 12% showing a wild-type phenotype. (B) Genetic interaction between *Fas2*^{spin} and the *Methoprene-tolerant* (*Met*) [*Rst(1)JH*] gene. The loss of *Met* function (*Met*^{K17}) completely suppresses the *Fas2*^{spin} rotation defects, while two extra copies of the *Met* gene aggravated the extent of genitalia rotation in *Fas2*^{spinR5} flies. In *Fas2*^{spinR5} males, the rotation of the genitalia stopped most frequently (51%) between 180° and 270°, and the non rotated phenotype was not observed. The *Fas2*^{spinR5}/Y; *Met*$^{+}$(3×) males died as pharate adults and the extent of rotation was dramatically reduced. In most cases (64%), rotation stopped between 0° and 90°, whereas 26% of the males showed non rotated genitalia.

Dissection reveals a shift toward a no rotation phenotype, with a larger proportion of males having their genital plate in its initial position. Thus, increasing the level of a JH analog produces looping defects ranging from partial to a complete loss of circumrotation.

Because pyriproxyfen mimics *spin* phenotypes in a dose-dependent manner, we conclude from these experiments that *Fas2*^{spin} pupae may have an elevated level of JH. To support this conclusion further, we modified the dosage of the *Methoprene-tolerant* gene (*Met*), which encodes a (bHLH)-PAS family of transcriptional regulators (Ashok et al., 1998). Flies that are mutant for *Met* are more resistant to JH analogs, indicating that the *Met* gene participates in JH signal transduction. Conversely, elevated expression of the *Met*$^{+}$ gene results in flies with higher susceptibility to JH analogs (Ashok et al., 1998). If JH is elevated in *Fas2*^{spin}, then increasing the dose of the *Met* gene should enhance *Fas2*^{spin} genitalia rotation defects, while mutations in *Met* should suppress them. We tested the effect of *Met*^{K17}, a P element-induced amorphic *Met* mutation, on *Fas2*^{spin} rotation defects. Strikingly, the loss of *Met* function completely rescues the *spin* rotation defects, indicating that *spin* and *Met* genetically interact and antagonize each other during this process (Fig. 6B). Conversely, the increase of the dose of the *Met+* gene in a *Fas2*^{spinR5} background results in lethality at the pharate adult stage, confirming the strong interaction between the two genes. Dissection of the genital plates reveals exacerbation of rotation defects, with ~25% having a complete lack of rotation of genitalia (Fig. 6B). This phenotype is very similar to the phenotype of pupae treated with high doses of pyriproxyfen (Fig. 6A). Altogether, our results indicate that *Fas2*^{spin} males have an elevated level of JH causing genitalia rotation defects. This allatotropic (promotion of JH synthesis) phenotype of *Fas2*^{spin} is consistent with studies in several insects indicating a role of the CA nerves in negatively controlling levels of JH during metamorphosis.

DISCUSSION

In this study, we identified and characterized the first looping specific mutant in *Drosophila*, allowing uncoupling of the process of LR determination from that of looping morphogenesis. The molecular and genetic analysis of *Fas2*^{spin} reveals a link between organ looping and the retinoic-like juvenile hormone (Fig. 7A). Because of the chemical similarity between JH and RA (see below), these data provide a parallel between vertebrate and invertebrate asymmetric organ looping (Fig. 7B). Our study also indicates that *Drosophila* is a promising genetic model for the study of LR asymmetry and organ looping.

Conserved role of terpenoids in organ looping

We show that asymmetric organ looping in *Fas2*^{spin} males is impaired and is due to an abnormal endocrine activity of the ring gland during the pupal stage. The effects on genitalia and spermiduct are mediated by an excess of JH, which then modifies looping morphogenesis downstream of LR determination (Fig. 7A). How do these results relate to vertebrate organ looping and LR asymmetry? The fact that JH affects looping morphogenesis in *Drosophila* suggests an important evolutionary conservation of the role of terpenoids in this process, downstream of LR determination. Like the retinoid hormones, JH is synthesized from the common isoprenoid precursor farnesyl diphosphate via the mevalonate biosynthetic pathway (Harmon et al., 1995). Furthermore, JH is a sesquiterpenoid that is chemically related to the vertebrate terpene group, represented by retinoic acid (Jones and Sharp, 1997). The common terpenoid nature of JH and RA has thus led to the proposal that these molecules may bind a common family of nuclear hormone receptors that might play similar

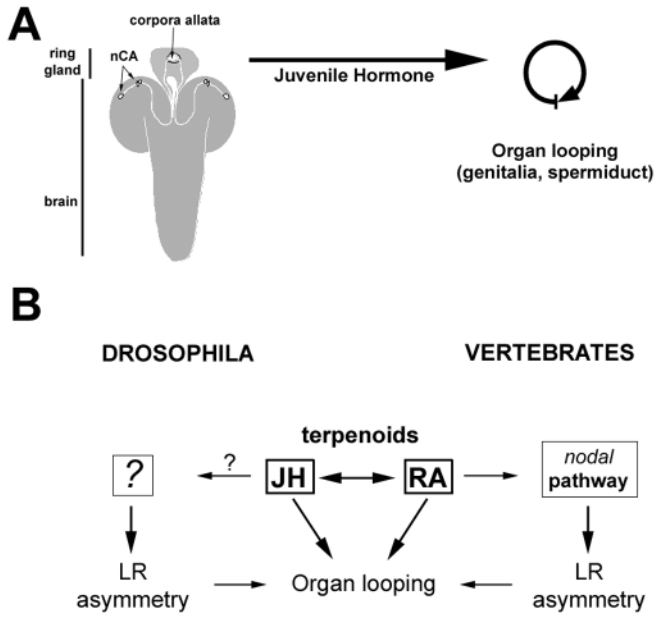

Fig. 7. Model for *Fas2* and JH action in *Drosophila* organ looping. (A) Fas2 is specifically expressed and required in neurosecretory cells innervating the corpora allata, to control JH titers. JH is released into the circulating hemolymph and reaches the genital disc to control its rotation non-autonomously. (B) Parallels between the vertebrate and the *Drosophila* pathways controlling asymmetric organ looping. In vertebrates, two conserved pathways have been implicated in LR asymmetry, the retinoic acid (RA) and the *nodal* pathways. RA plays a dual role as it is also involved in organ looping. In *Drosophila*, mutants exist that can lead to a reversion of genitalia rotation (Milani, 1955; Milani, 1956) (G.Á. and S.N., unpublished), suggesting that they are involved in LR asymmetry (collectively represented by a question mark). The analysis of *Fas2*^{spin} shows a role for JH, a RA analog, in the control of organ looping and suggests an evolutionary conserved function of terpenoids in the control of asymmetric organ looping.

functions in different organisms (Moore, 1990). In this respect, it is important to note that the JH analog methoprene, the topical application of which leads to genitalia rotation defects (Riddiford and Ashburner, 1991), can specifically bind and activate the RXR receptor in mammalian cultured cells (Harmon et al., 1995). RA signal transduction in vertebrates requires the binding to and the activation of heterodimers composed of RAR and RXR nuclear receptors isoforms. Interestingly, the only insect homolog of vertebrate RXR is encoded by the *Drosophila ultraspiracle* (*usp*) gene, which has been shown to bind to JH in vitro (Jones and Sharp, 1997; Jones et al., 2001). Altogether, these data thus suggest that JH and RA have a related activity.

In addition to sharing common chemical features, both RA and JH, when present in excess, have strikingly similar effects on organ looping. In conditions of excess RA, a series of heart defects has been observed, including reversal of symmetry or incomplete looping. In *Xenopus*, the heart tube fails to loop after continuous exposure to low doses of RA (Drysdale et al., 1997), and incomplete looping of the heart is also observed in mice treated with RA over a long period (Chazaud et al., 1999). It is important to note that the effects of excess RA on heart looping are dose sensitive and stage specific, as are the effects of JH analogs (methoprene and pyriproxyfen) on the looping of genitalia in flies (Fig. 6A) (Riddiford and Ashburner, 1991).

In addition to blocking organ looping, excess RA can also induce a reversal of LR asymmetry in several vertebrate models (see Introduction). Such a reversal of asymmetry has not been observed after topical application of pyriproxyfen in *Drosophila* (Fig. 6). This apparent discrepancy may be explained by species- and/or stage-specific responsiveness to excess terpenoids, as is found among vertebrates for RA. Another possibility is that JH in flies may have a function restricted to organ looping, not sharing the dual role of RA seen in vertebrates (Fig. 7B).

The chemical, and, as shown in this study, the functional and phenotypic similarities associated with JH and RA in flies and vertebrates, respectively, show that terpenoids play an evolutionarily conserved role in handed looping (Fig. 7B).

LR asymmetry and organ looping in *Drosophila*

In *Drosophila*, genetic control of the establishment of the two major body axes has been well described. LR asymmetry has attracted little interest and thus remained an elusive process for several reasons. First, there are only few and mostly transient (i.e. present during embryonic stages only) LR organs (Hayashi and Murakami, 2001; Lengyel and Iwaki, 2002; Ligoxygakis et al., 2001), leading to the view that flies may not represent a good model to study LR axis like vertebrates. In the case of genitalia rotation, only one dedicated study has been published (Gleichauf, 1936), and, yet, this or another LR process have not been clearly validated as candidate LR markers. Second, to our knowledge no mutations have been isolated so far that showed a fully penetrant and rotation-specific defect. Though some studies have reported rotation defects in specific allelic combinations, the rotation phenotypes are poorly penetrant and are associated with other developmental defects (e.g. Abbott and Lengyel, 1991; Holland et al., 1997; Santamaria and Randsholt, 1995; Yip et al., 1997).

In this study, we developed an approach to identify genes that are involved in two main processes underlying directional organ looping: the determination of directionality (LR asymmetry) and looping morphogenesis. Interestingly, our work reveals the existence of a zygotic control for genitalia LR asymmetry that is apparently distinct from the maternal control of LR development during embryogenesis (Hayashi and Murakami, 2001; Lengyel and Iwaki, 2002; Ligoxygakis et al., 2001). Additional work will be necessary to understand the mechanisms underlying asymmetric development in different tissues and developmental stages. Together with previously described mutations (Milani, 1955; Milani, 1956) (see Introduction) and our recent identification of a situs inversus mutation leading to a complete reversion of genitalia rotation (counterclockwise) (G.Á. and S.N., unpublished), these data suggest that just like in vertebrates, the earliest steps of symmetry breaking are accessible to genetic analysis in flies. (Mochizuki et al., 1998; Morgan et al., 1998; Yokoyama et al., 1993). Altogether, our results indicate that *Drosophila* has all the basic elements to make it a genetic model to study organ looping in the context of LR asymmetry. In this respect, it is interesting to note that asymmetric organ looping, rather than asymmetric organ localization, is a more general and common LR marker among animals. For example in vertebrates, the first appearance of LR asymmetry is indicated by embryo turning and heart tube looping. Furthermore, organ looping can

also be observed in plants through the helical growth of stalks and stems (Thitamadee et al., 2002).

We have shown a novel parallel between the programs underlying *Drosophila* and vertebrate asymmetric organ looping. Is this parallel more general? In order to address this question, future work will have to be focused on the identification of new genes involved in genitalia rotation in *Drosophila*, using genetic screens and reverse genetic approaches. One major goal of future work in *Drosophila* will be the identification of asymmetrically expressed genes and/or proteins. The fact that no such gene has been identified so far may be due to the lack of appropriate data on the developmental aspects of LR asymmetry in *Drosophila*. In this respect, our study now allows identification of the male genital disc (Sanchez and Guerrero, 2001) as a clear candidate tissue for looking at asymmetrically expressed molecular markers. The use of *Drosophila* and the comparative analysis of the LR asymmetry programs in vertebrates and invertebrates will help provide insights into the molecular mechanisms that underlie the question of symmetry breaking in animals.

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REFERENCES

Abbott, M. K. and Lengyel, J. A. (1991). Embryonic head involution and rotation of male terminalia require the Drosophila locus head involution defective. *Genetics* **129**, 783-789.

Ashok, M., Turner, C. and Wilson, T. G. (1998). Insect juvenile hormone resistance gene homology with the bHLH-PAS family of transcriptional regulators. *Proc. Natl. Acad. Sci. USA* **95**, 2761-2766.

Bourbon, H. M., Gonzy-Treboul, G., Peronnet, F., Alin, M. F., Ardourel, C., Benassayag, C., Cribbs, D., Deutsch, J., Ferrer, P., Haenlin, M. et al. (2002). A P-insertion screen identifying novel X-linked essential genes in Drosophila. *Mech. Dev.* **110**, 71-83.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Brunner, A. and O'Kane, C. J. (1997). The fascination of the Drosophila NMJ. *Trends Genet.* **13**, 85-87.

Burdine, R. D. and Schier, A. F. (2000). Conserved and divergent mechanisms in left-right axis formation. *Genes Dev.* **14**, 763-776.

Capdevila, J., Vogan, K. J., Tabin, C. J. and Izpisua Belmonte, J. C. (2000). Mechanisms of left-right determination in vertebrates. *Cell* **101**, 9-21.

Chazaud, C., Chambon, P. and Dolle, P. (1999). Retinoic acid is required in the mouse embryo for left-right asymmetry determination and heart morphogenesis. *Development* **126**, 2589-2596.

Cheng, Y., Endo, K., Wu, K., Rodan, A. R., Heberlein, U. and Davis, R. L. (2001). Drosophila fasciclinII is required for the formation of odor memories and for normal sensitivity to alcohol. *Cell* **105**, 757-768.

Collignon, J., Varlet, I. and Robertson, E. J. (1996). Relationship between asymmetric nodal expression and the direction of embryonic turning. *Nature* **381**, 155-158.

Dai, J. D. and Gilbert, L. I. (1991). Metamorphosis of the corpus allatum and degeneration of the prothoracic glands during the larval-pupal-adult transformation of Drosophila melanogaster: a cytophysiological analysis of the ring gland. *Dev. Biol.* **144**, 309-326.

Drysdale, T. A., Patterson, K. D., Saha, M. and Krieg, P. A. (1997). Retinoic acid can block differentiation of the myocardium after heart specification. *Dev. Biol.* **188**, 205-215.

Estes, P. S., Ho, G. L., Narayanan, R. and Ramaswami, M. (2000). Synaptic localization and restricted diffusion of a Drosophila neuronal synaptobrevin–green fluorescent protein chimera in vivo. *J. Neurogenet.* **13**, 233-255.

Gleichauf, R. (1936). Anatomie und variabilität des geschlechtsapparates von Drosophila melanogaster (Meigen). *Z. Wiss. Zool.* **148**, 1-66.

Goodman, C. S., Davis, G. W. and Zito, K. (1997). The many faces of fasciclin II: genetic analysis reveals multiple roles for a cell adhesion molecule during the generation of neuronal specificity. *Cold Spring Harb. Symp. Quant. Biol.* **62**, 479-491.

Grenningloh, G., Rehm, E. J. and Goodman, C. S. (1991). Genetic analysis of growth cone guidance in Drosophila: fasciclin II functions as a neuronal recognition molecule. *Cell* **67**, 45-57.

Hamada, H., Meno, C., Watanabe, D. and Saijoh, Y. (2002). Establishment of vertebrate left-right asymmetry. *Nat. Rev. Genet.* **3**, 103-113.

Harmon, M. A., Boehm, M. F., Heyman, R. A. and Mangelsdorf, D. J. (1995). Activation of mammalian retinoid X receptors by the insect growth regulator methoprene. *Proc. Natl. Acad. Sci. USA* **92**, 6157-6160.

Harvey, R. P. (1998). Cardiac looping – an uneasy deal with laterality. *Semin. Cell Dev. Biol.* **9**, 101-108.

Hayashi, T. and Murakami, R. (2001). Left-right asymmetry in Drosophila melanogaster gut development. *Dev. Growth Differ.* **43**, 239-246.

Hobert, O., Johnston, R. J. and Chang, S. (2002). Left-right asymmetry in the nervous system: the Caenorhabditis elegans model. *Nat. Rev. Neurosci.* **3**, 629-640.

Holland, P. M., Suzanne, M., Campbell, J. S., Noselli, S. and Cooper, J. A. (1997). MKK7 is a stress-activated mitogen-activated protein kinase kinase functionally related to hemipterous. *J. Biol. Chem.* **272**, 24994-24998.

Iulianella, A. and Lohnes, D. (2002). Chimeric analysis of retinoic acid receptor function during cardiac looping. *Dev. Biol.* **247**, 62-75.

Jones, G. and Sharp, P. A. (1997). Ultraspiracle: an invertebrate nuclear receptor for juvenile hormones. *Proc. Natl. Acad. Sci. USA* **94**, 13499-13503.

Jones, G., Wozniak, M., Chu, Y., Dhar, S. and Jones, D. (2001). Juvenile hormone III-dependent conformational changes of the nuclear receptor ultraspiracle. *Insect Biochem. Mol. Biol.* **32**, 33-49.

Kathiriya, I. S. and Srivastava, D. (2000). Left-right asymmetry and cardiac looping: implications for cardiac development and congenital heart disease. *Am. J. Med. Genet.* **97**, 271-279.

Kosaki, K. and Casey, B. (1998). Genetics of human left-right axis malformations. *Semin. Cell Dev. Biol.* **9**, 89-99.

Lengyel, J. A. and Iwaki, D. D. (2002). It takes guts: the Drosophila hindgut as a model system for organogenesis. *Dev. Biol.* **243**, 1-19.

Levin, M., Johnson, R. L., Stern, C. D., Kuehn, M. and Tabin, C. (1995). A molecular pathway determining left-right asymmetry in chick embryogenesis. *Cell* **82**, 803-814.

Ligoxygakis, P., Strigini, M. and Averof, M. (2001). Specification of left-right asymmetry in the embryonic gut of Drosophila. *Development* **128**, 1171-1174.

Lowe, L. A., Supp, D. M., Sampath, K., Yokoyama, T., Wright, C. V., Potter, S. S., Overbeek, P. and Kuehn, M. R. (1996). Conserved left-right asymmetry of nodal expression and alterations in murine situs inversus. *Nature* **381**, 158-161.

Mercola, M. and Levin, M. (2001). Left-right asymmetry determination in vertebrates. *Annu. Rev. Cell Dev. Biol.* **17**, 779-805.

Milani, R. (1955). Effect of natural selection on the full expression of the gene countercoiled (cc). *Dros. Inf. Serv.* **29**, 139.

Milani, R. (1956). Countercoiled genitalia in D. melanogaster. *Dros. Inf. Serv.* **30**, 137.

Mochizuki, T., Saijoh, Y., Tsuchiya, K., Shirayoshi, Y., Takai, S., Taya, C., Yonekawa, H., Yamada, K., Nihei, H., Nakatsuji, N. et al. (1998). Cloning of inv, a gene that controls left/right asymmetry and kidney development. *Nature* **395**, 177-181.

Moore, D. D. (1990). Diversity and unity in the nuclear hormone receptors: a terpenoid receptor superfamily. *New Biol.* **2**, 100-105.

Morgan, D., Turnpenny, L., Goodship, J., Dai, W., Majumder, K., Matthews, L., Gardner, A., Schuster, G., Vien, L., Harrison, W. et al. (1998). Inversin, a novel gene in the vertebrate left-right axis pathway, is partially deleted in the inv mouse. *Nat. Genet.* **20**, 149-156.

Niederreither, K., Vermot, J., Messaddeq, N., Schuhbaur, B., Chambon,

P. and Dolle, P. (2001). Embryonic retinoic acid synthesis is essential for heart morphogenesis in the mouse. *Development* **128**, 1019-1031.

Noyes, B. E., Katz, F. N. and Schaffer, M. H. (1995). Identification and expression of the Drosophila adipokinetic hormone gene. *Mol. Cell Endocrinol.* **109**, 133-141.

Riddiford, L. M. (1993). Hormones and *Drosophila* development. In *The Development of* Drosophila melanogaster. Vol. 2 (ed. M. Bate and A. Martinez Arias), pp. 899-939. New York: Cold Spring Harbor Laboratory Press.

Riddiford, L. M. and Ashburner, M. (1991). Effects of juvenile hormone mimics on larval development and metamorphosis of Drosophila melanogaster. *Gen. Comp. Endocrinol.* **82**, 172-183.

Rulifson, E. J., Kim, S. K. and Nusse, R. (2002). Ablation of insulin-producing neurons in flies: growth and diabetic phenotypes. *Science* **296**, 1118-1120.

Sanchez, L. and Guerrero, I. (2001). The development of the Drosophila genital disc. *BioEssays* **23**, 698-707.

Santamaria, P. and Randsholt, N. B. (1995). Characterization of a region of the X chromosome of Drosophila including multi sex combs (mxc), a Polycomb group gene which also functions as a tumour suppressor. *Mol. Gen. Genet.* **246**, 282-290.

Siegmund, T. and Korge, G. (2001). Innervation of the ring gland of Drosophila melanogaster. *J. Comp. Neurol.* **431**, 481-491.

Stewart, B. A., Schuster, C. M., Goodman, C. S. and Atwood, H. L. (1996). Homeostasis of synaptic transmission in Drosophila with genetically altered nerve terminal morphology. *J. Neurosci.* **16**, 3877-3886.

Struhl, G. and Basler, K. (1993). Organizing activity of wingless protein in Drosophila. *Cell* **72**, 527-540.

Thitamadee, S., Tuchihara, K. and Hashimoto, T. (2002). Microtubule basis for left-handed helical growth in Arabidopsis. *Nature* **417**, 193-196.

Tsukui, T., Capdevila, J., Tamura, K., Ruiz-Lozano, P., Rodriguez-Esteban, C., Yonei-Tamura, S., Magallon, J., Chandraratna, R. A., Chien, K., Blumberg, B. et al. (1999). Multiple left-right asymmetry defects in $Shh^{-/-}$ mutant mice unveil a convergence of the shh and retinoic acid pathways in the control of Lefty-1. *Proc. Natl. Acad. Sci. USA* **96**, 11376-11381.

Wasiak, S. and Lohnes, D. (1999). Retinoic acid affects left-right patterning. *Dev. Biol.* **215**, 332-342.

Wright, C. V. E. (2001). Mechanisms of left-right asymmetry: What's right and what's left? *Dev. Cell* **1**, 179-186.

Yip, M. L., Lamka, M. L. and Lipshitz, H. D. (1997). Control of germ-band retraction in Drosophila by the zinc-finger protein HINDSIGHT. *Development* **124**, 2129-2141.

Yokoyama, T., Copeland, N. G., Jenkins, N. A., Montgomery, C. A., Elder, F. F. and Overbeek, P. A. (1993). Reversal of left-right asymmetry: a situs inversus mutation. *Science* **260**, 679-682.

Zile, M. H., Kostetskii, I., Yuan, S., Kostetskaia, E., St Amand, T. R., Chen, Y. and Jiang, W. (2000). Retinoid signaling is required to complete the vertebrate cardiac left/right asymmetry pathway. *Dev. Biol.* **223**, 323-338.