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Review

Left–right asymmetry in *Drosophila*

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Abstract

Seminal studies of left–right (L/R) patterning in vertebrate models have led to the discovery of roles for the nodal pathway, ion flows and cilia in this process. Although the molecular mechanisms underlying L/R asymmetries seen in protostomes are less well understood, recent work using *Drosophila melanogaster* as a novel genetic model system to study this process has identified a number of mutations affecting directional organ looping. The genetic analysis of this, the most evolutionary conserved feature of L/R patterning, revealed the existence of a L/R pathway that involves the actin cytoskeleton and an associated type I myosin. In this review, we describe this work in the context of *Drosophila* development, and discuss the implications of these results for our understanding of L/R patterning in general.

Keywords: Left–right asymmetry; *Drosophila*; Myosin I; Genitalia; Gut

Contents

1. Introduction

The asymmetric differentiation of an animal's left and right-hand sides underlies a wide diversity of forms and functions in the animal kingdom. Examples include the asymmetric positioning of single organs, the bilateral differentiation of organs like the heart, brain and lungs, the development of a specialized crusher claw in crabs and lobsters, the upward facing eyes on one side of flat fishes and the coiling of snail shells. Whether conserved mechanisms underlie these diverse left–right (L/R) asymmetries remains an open question that requires an analysis in a variety of diverse model organisms.

Three main types of L/R asymmetries can be described, each of which has a different origin: first, fluctuating asymmetry, which reflects developmental noise and results in small random differences between the two body sides; second, anti-symmetry which refers to fixed asymmetries whose determination is random and leads to a mix of the two enantiomorphs within the population, such as differentiation of a right or left crusher claw

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in lobsters [1]; and, third, stereotyped fixed L/R asymmetry (e.g., positioning of the spleen on the left side of the abdomen). In this review we focus on the latter type of L/R asymmetry, which is established during development and leads to an invariant asymmetry in the entire population, a conformation known as *situs solitus*. The abnormal establishment of L/R asymmetry can lead to *situs inversus* (reversal of the L/R axis) or *situs ambiguus* (which can be complete or partial). These defects are associated with a number of pathologies such as spontaneous abortions, the Kartagener and Ivemark syndromes, asplenia, polysplenia and congenital heart diseases [2–4].

Although an essential part of animal development, the study of L/R asymmetry only entered the molecular era in the 1990s with the discovery of the first asymmetrically expressed gene, *nodal* [5]. This seminal work has helped to reveal the process by which L/R asymmetry is established during vertebrate development. Further elegant work from several laboratories showed that the role of the *nodal* pathway in L/R determination is conserved across vertebrates, and pointed to motile cilia in the embryonic node as a conserved symmetry-breaking structure in several deuterostomes [6–13]. More recently, L/R asymmetries in the localization of ion pumps and ion flows have been observed at earlier stages of development and implicated in L/R patterning [14–21]. These results raise the interesting hypothesis that several, potentially independent symmetry-breaking events could take place during development [12].

Although this progress has been impressive, the study of L/R asymmetry in several model organisms has the potential to provide us with an evolutionary perspective. It can also reveal novel molecular mechanisms of axial patterning. More specifically, since the determination of L/R asymmetry necessarily takes place in the context of anterior–posterior (A/P) and dorsal–ventral (D/V) information (see Boxes 1 and 2), the study of L/R asymmetry establishment can provide clues as to how A/P, D/V and L/R axes interact to provide cells in a developing animal with accurate positional information. During this period however studies addressing L/R asymmetry in protostomes were largely limited to *Lymnea* snails and the nematode *Caenorhabditis elegans*.

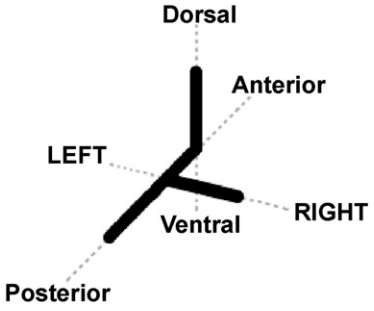
Drosophila has proven to be an invaluable model organism in which to study body patterning, but surprisingly enough it was only recently that efforts have been made to characterise the L/R axis in *Drosophila*. That L/R patterning was neglected by *Drosophila* geneticists reflects the lack of clear L/R phenotypic markers in the animal. Indeed, while vertebrates show clear stereotyped L/R asymmetries in their body plan, like side-specific positioning of organs (heart, spleen, etc.) and bilateral dimorphism (brain and lungs), similar asymmetries are not conserved in *Drosophila*, or in most other invertebrates. The *Drosophila* heart, for example, is a linear tube lining the dorsal midline, whereas in vertebrates the heart is made of highly distinct left and right chambers, each with specific vessel connections and functions. However, other L/R asymmetries are conserved between *Drosophila* and vertebrates, the most ancestral of which is likely to be the coiling of tubular organs, like the gut, which appears to serve a "packaging" function, helping to contain these long organs. The most obvious and robust L/R asymmetries in *Drosophila* organ shape are the looping of the gut in embryos, larvae and adults, as well as the looping of the testis and genitalia in adult males. These structures represent genuine L/R markers since the direction of coiling of the gut or heart tube is altered in vertebrate mutants with defects in L/R patterning. In this review, we describe the main features and the development of L/R asymmetric organs in *Drosophila*. In a second part, we discuss recent functional studies that have allowed the genetic identification of the genes regulating the *Drosophila* L/R axis. We finally discuss these results in the context of our current knowledge from other model organisms.

Box 1. Maternal versus zygotic origin of *Drosophila* L/R information

The question of the origin of the LR information, either maternal or zygotic, as well as the order into which asymmetry axes are set up during *Drosophila* development has been elegantly addressed by Hayashi et al. [94]. To reverse the embryo A/P axis without affecting egg-chamber (maternal) A/P polarity, the authors engineered embryos lacking endogenous Oskar (the posterior determinant) but expressing a chimeric construct leading to ectopic localization of *oskar* to the anterior. To restore an A/P axis in these bicaudal embryos [95], *bicoid* mRNA [96] was injected leading to the formation of head and thoracic structures posteriorly [94]. Therefore embryos develop a proper A/P axis with a reversed orientation relative to that of the mother, allowing the direct investigation of the effect of the A/P axis inversion on L/R development. A majority of these A/P reversed embryos develop a normal L/R axis with respect to the new A/P axis, indicating that L/R axis does not appear to be predetermined by maternal determinants in *Drosophila*. Supporting the idea of a zygotic definition of the L/R axis is the observation that the handedness of both embryonic and adult organs can be inverted in homozygous mutants coming from heterozygous mothers [30,59]. These results reveal a zygotic origin for the L/R asymmetry, whose orientation is defined relative to A/P axial information established earlier during development. These experiments clearly rule out the conclusions of a previous study that correlated the L/R inverted phenotype of embryos (derived from *dicephalic* (*dic*) and *wunen* (*wun*) mutant females) with A/P reversals during oogenesis [97].

Box 2. The F-molecule model

Breaking symmetry to create oriented L/R asymmetry relies on the proper orientation with respect to the two main axes, Anterior–posterior (A/P) and dorsal–ventral (D/V). Fifteen years ago, Brown and Wolpert [93] proposed an elegant theoretical hypothesis to explain how a single molecule could act as a L/R determinant. According to this model, two intrinsic properties should in principle be sufficient for a molecule to set up L/R asymmetry *de novo*: (i) to be able to orient itself relatively to the A/P and D/V axes; (ii) to be chiral. These properties are well illustrated by what was coined the F-molecule, which consists of three perpendicular branches (see figure), each representing an axis. Following alignment of two branches along the A/P and D/V axes, the chirality of the structure makes the third branch to point toward one direction (right on the figure), thus orienting the L/R axis and creating L/R asymmetry *de novo*. It must be emphasized that the F shape is not a structural model of the L/R determinant, but is rather a way to illustrate its functional properties.



1.1. Markers of L/R asymmetry in Drosophila

1.1.1. Gut

In *Drosophila*, the gut is composed of three main parts and originates from invagination of cells during the early blastoderm stage (for review, see [22–24]). The anterior-most foregut corresponds to the human oesophagus; the central midgut, to the human stomach and intestine; and the posterior-most hindgut, to the colon and rectum.

Initially a symmetrical tube, the *Drosophila* gut develops a stereotyped L/R asymmetry between stage 13 and 17 of embryogenesis (Fig. 1) [25–28]. This asymmetric looping of the embryonic gut tube first becomes apparent in the hindgut, soon after in the foregut and finally in the midgut [24,26,27]. The monolayer epithelium of the hindgut undergoes cell shape elongation and reorganization leading, by the end of stage 13, to a twist of 90° to the right of the hindgut "hook" joining the midgut. This dextral bending (when looked from the dorsal side) converts the D/V asymmetry of the ventrally expressed Dpp and dorsally expressed En into the positioning of En to the left and Dpp to the right of the embryonic hindgut [27] (Fig. 1). At stage 15, the foregut twists about 360° leftwards to form a one-step sinistral helix [27]. At stage 15, the proventriculus, which links the fore- and midgut, inclines to the left while the midgut still forms a L/R symmetric tube [29]. At early stage 16, three circular constrictions divide the midgut into four chambers aligned along the A/P axis but slightly curved along the D/V axis. Later during stage 16, the second chambers shifts to the right thus breaking L/R symmetry. Further convolutions and bends result in a fully asymmetric midgut at stage 17 (Fig. 1).

An interesting feature of the embryonic gut is the apparent independence of its parts during L/R axial patterning. Indeed, conditions have been identified that lead to reversion of the L/R axis in some structures of the gut independently of others. Thus, though spontaneous reversal of handedness is a rare event, it is striking that the frequency at which it occurs is different for each of the gut parts, ranging from 0.2% for the foregut, 0.4% for the midgut and 0.6% for the hindgut [27]. Likewise several mutations, *huckebein* (*hkb*), *brachyenteron* (*byn*), *patched* (*ptc*), *single-minded* (*sim*), *puckered* (*puc*) and *Myosin ID* (*Myo ID*) have been found to alter the L/R asymmetry of specific parts of the gut, while leaving other parts unaffected [27,29,30]. In *hkb* mutants the 90° twist of the hindgut does not occur, in *byn* mutants a randomization of the midgut convolution is observed, whereas *ptc* mutations lead to the reversion of the foregut and midgut but not the hindgut [27]. In *puc* mutants, only the proventriculus twist is changed, pointing rightward whereas midgut organization appears normal [29]. Finally depletion of *Myo ID* activity leads to full inversion of the hindgut in both the embryo and adult, and causes apparent heterotaxia of the embryonic midgut without affecting the foregut [30]. In the case of *Myo ID*, these discrepancies can be explained by the fact that *Myo ID* expression is not detected in the foregut, therefore suggesting that L/R asymmetry is *Myo ID*-independent in the foregut and *Myo ID*-dependent in the mid- and hindgut. This idea is supported by the fact that embryo-wide *Myo ID* overexpression only reverses the L/R axis in the foregut [30]. Taken together these data suggest that different mechanisms can be used to establish L/R asymmetry in different parts of a structure.

1.1.2. Genitalia

The adult epidermal structures of *Drosophila* are derived from imaginal discs, which are sac-like clusters of primordial cells. The adult terminalia, including all somatic tissues composing the genitalia and analia, originate from the genital disc. This differs from other discs in several ways. First, the genital disc is unique in being located at the ventral midline, whereas other imaginal discs are found paired on both sides of the larval body; second, it exhibits a strong sexual dimorphism; and, finally, it is a compound disc made of cells from three different embryonic segments, namely the A8, A9 and A10 (Fig. 2).

Like other imaginal discs, the genital disc forms during embryogenesis in both males and females. At around embryonic stage 13, the genital disc is made of 12–15 genital disc precursors cells (GDPCs) organized into three distinct clusters that are present ventrally at the posterior end of the embryo

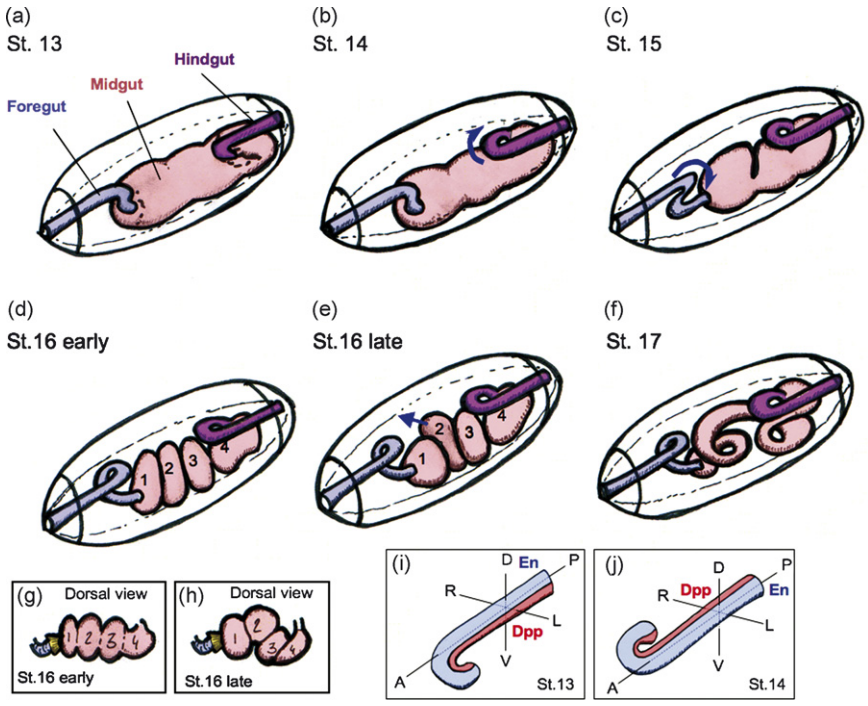


Fig. 1. L/R asymmetry of the *Drosophila* embryonic gut. The stages (St.) of embryonic gut development at which switches from L/R symmetry to L/R asymmetry occur are depicted schematically. Upon completion of a continuous digestive tube by fusion of the midgut (light pink) with the anterior foregut (light blue) and posterior hindgut (light purple) at stage 13 (a), the gut develops a stereotyped L/R asymmetry between stages 13 and 17 of embryogenesis. At stage 14, the hindgut has completed a 90° twist to the left (b). At stage 15, the foregut coils leftward forming a one-step sinistral helix (c). After stage 15, the proventriculus, found at the posterior of the foregut, inclines to the left (yellow on g and h, not depicted elsewhere for clarity). At early stage 16, three circular constrictions divide the midgut into four chambers aligned along the A/P axis but slightly offset along the D/V axis (d, dorsal view on g). During stage 16, the second of these chambers shifts to the right to break L/R symmetry (e, dorsal view on h), the acquisition of further convolutions and bends then generates the fully asymmetric midgut at stage 17 (f). (i and j) Scheme of the hindgut: At stage 13, En is detected dorsally and Dpp ventrally (i). At stage 14, after dextral bending of the hindgut, En is detected to the left and Dpp to the right (j).

[31]; two of them are symmetrically localized on both side of the midline, while the last cluster is found posterior to the others in a central position [32–34]. Later during development these cell clusters merge to form a unique genital disc located at the ventral midline.

Unlike thoracic discs, the genital disc is composed of three primordia, specified by the homeotic genes *abdominal-A* (*abd-A*), *Abdominal-B* (*Abdb*) and the homeobox-containing gene *caudal* [35], all of which are required for the correct development of the genital disc as a whole [36]. Each primordium has an anterior and posterior compartment. These can be visualised based on the complementary pattern of expression of *engrailed* (*en*) and *Cubitus interruptus* (*Ci*), markers known to be expressed in a compartment-specific manner in other discs. They represent true genetic compartments, since mitotic clones induced at the beginning of the second instar do not cross the A/P boundary [37]. It is not clear however when these compartments are established. In thoracic discs, genetic mosaic analyses have shown that the A/P boundary already exists in the blastoderm stage embryo [38–40]. This might not be the case for the genital disc for the following reasons. First, at the end of germ band extension, a stage when GDPCs are first detectable, *engrailed* expressing cells of the ventral ectoderm move dorsally leading to the juxtaposition of the anterior compartment of A8, A9 and A10. Second, in fate mapping experiments, no A/P restriction could be detected in GDPCs [41,42] and recent work showed that few GDPCs express En, which would mark them out as posterior cells of A8 [33]. Thus, it is likely that the A/P boundary is first established in the A8 segment and only later on in A9 and A10 of the genital disc.

The activity of the sex determination gene *Sxl* in females leads to the specific splicing of the gene *doublesex*, and to the production of the DsxF protein. In males, *Sxl* is inactive, leading to the production of the default DsxM protein. The analysis of gynandromorph flies (mosaic XX flies, with clones of X0 cells) showed that the female and the male genitalia are derived from two distinct primordia, A8 and A9, respectively, while the analia is derived from the same A10 primordium in both sexes [41]. In each sex, the relevant primordium (A8 in female and A9 in male) proliferates and differentiates, while the development of the other primordium (A8 in male and A9 in female) is suppressed. It was thought that repressed primordia did not contribute to adult structures. However, recent work has shown that the repressed primordium gives rise to a small eighth tergite in males and to parovaria in females [43]. These data suggest that *Dsx* plays an instructive function, regulating the A/P organizer non-autonomously to control growth of each genital primordium, and controlling the cell autonomous differentiation of each primordium in defined adult structures, together with the homeotic genes [43].

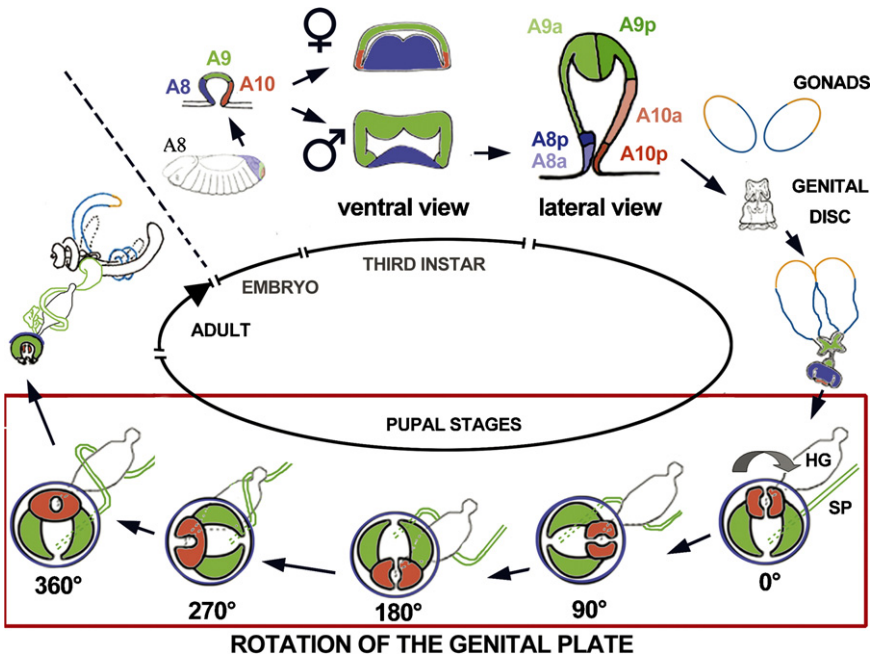


Fig. 2. Development cycle of the male genital disc. In the embryo, genital disc precursor cells belonging to the A8, A9 and A10 segments (in blue, green and red, respectively), segregate to form a sac-like structure called the genital disc. During larval stages, the genital disc undergoes a high degree of proliferation and sexual dimorphism. The DSX^M and DSX^F proteins specify male and female differentiation of the genital disc, respectively. Each segment of the genital disc is composed of a posterior and anterior compartment as illustrated by a colour code in the lateral view of a third instar larval disc. During pupal stages, the disc evaginates, exposing its apical surface to the exterior, and elongates to connect to the gonads at around 25 h after puparium formation (APF). Then the disc undergoes a 360° rotation leading to the progressive looping of the spermiduct (SP, in green) around the gut (HG, in grey). Rotation always takes place in the same direction in wild type flies and is called dextral rotation by convention.

The ventral origin of the genital disc suggests that it is likely to be organized in a manner similar to the antennal and leg discs. Indeed, *wg* and *dpp* are expressed in approximately complementary patterns at the A/P border, in the anterior compartment abutting the En expression domain. Additionally, in the genital disc their mutual repression and organising activity appears similar to that seen in other ventral discs [44,45]. Finally, mutations in several genes, including *Abd-B*, lead to the transformation of the genitalia into ventral structures like leg or antenna [46].

During metamorphosis, the genital disc evaginates exposing its apical surface as it adopts a circular shape (Fig. 2). Using En expression as a marker, it has been shown that during pupal stages (24 h after puparium formation, APF) the A8 segment forms a ring of cells surrounding the A9-A10 part of the disc [43]. During the second day after puparium formation (27–36 h APF; see [47]), the genital structures connect with the gonads (Fig. 2). Then the terminalia or genital plate undergoes a 360° clockwise or dextral rotation (when viewed from the posterior pole), leading to the coiling of the spermiduct around the gut [48] (Fig. 2). This stereotyped rotation process takes place during the second day of pupal development (25–36 h APF). It is a robust L/R marker in *Drosophila* that presents several practical advantages as an experimental system in which to study L/R patterning. First, defects in rotation can be detected directly under a dissecting microscope, which is convenient for screening purposes and for testing genetic interactions. Second, the extent of rotation can be precisely determined by simply measuring the angular positioning of the genital plate, while determining the direction of rotation requires dissection of the abdomen to determine the orientation of the spermiduct looping around the gut.

1.1.3. Testis

Drosophila melanogaster gonads develop independently of the genital disc from a group of embryonic primordial germ cells (PGC) or pole cells which migrate from the posterior pole of the blastoderm stage embryo to take up a final position on either side of the midline in segment A5 of the stage 15 embryo ([26,49]; Fig. 3). During stage 12, the PGCs remain in close contact with the somatic gonadal precursors. They then coalesce into the spherical embryonic gonad in stage 14 embryos [50,51]. Spermatogenesis starts during first instar larval stages after the stem cell niche has established at the apical pole of the ellipsoid gonads [52,53] (Fig. 3). TGFβ signalling is required for the maintenance of germline stem cells and restriction of spermatogonial proliferation [54], whereas the Jak/Stat signalling pathway contributes to stem cell self-renewal [55].

Mature spermatids first arise in the early pupae after the second meiotic division at the basal pole of the testis, indicating that spermatogenesis is complete by this stage of development [52]. Until 30 h APF, however, the shape of the testis resembles that of the ellipsoid larval testis. Then, after attachment of the vas deferens (27–36 APF) [56,57] the testis undergoes a striking morphological change. It becomes elongated, and dextral spiral coiling generates the two and a half turns seen in the adult testes

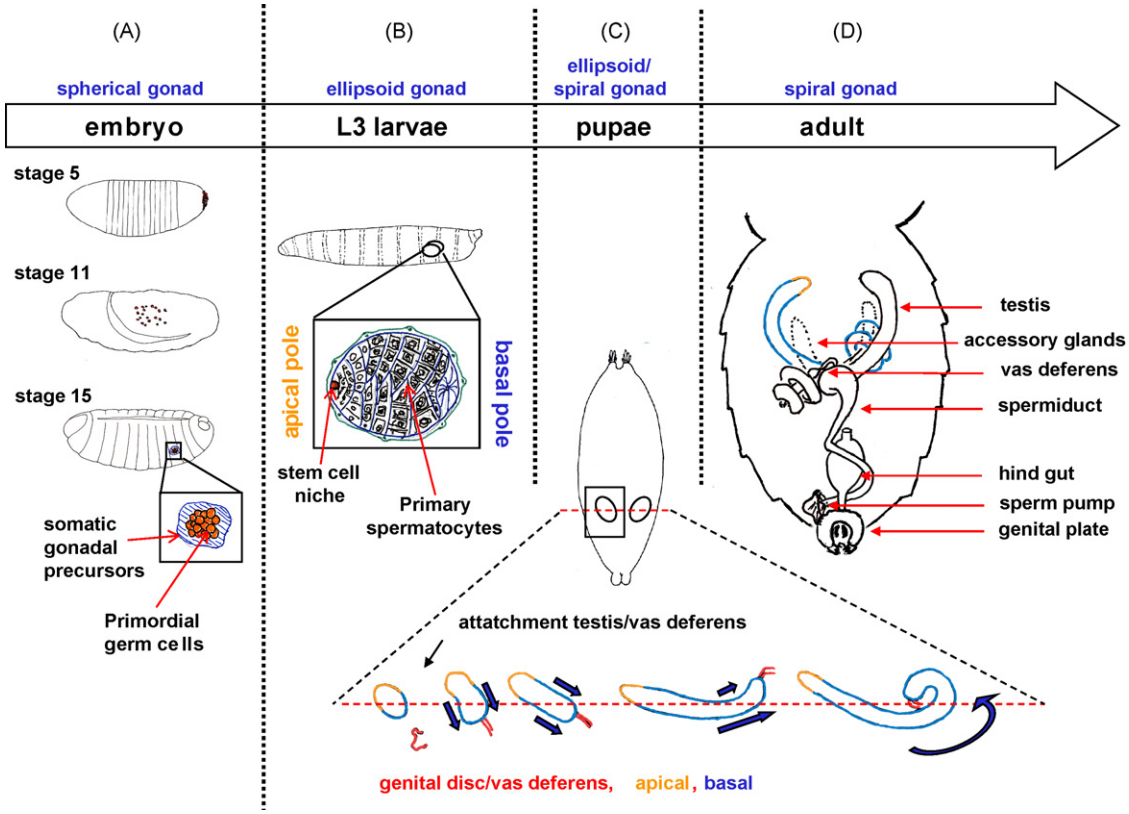


Fig. 3. Development of *Drosophila* testis. (A) Schematised migration of the primordial germ cells (PGCs) in the embryo and formation of the spherical embryonic gonads at stage 14 (adapted from [26]). (B) Ellipsoid gonads of a third instar larvae stage. The apical pole (orange) contains the stem cell niche, and the basal pole (blue) the primary spermatocytes (adapted from [98]). (C) The pupal testis undergoes elongation and coiling following attachment to the vas deferens (see enlargement), as the result of an asymmetric membrane extension of the basal part of the testis (indicated by the length of the blue arrows). (D) Adult genital structures. The spiral testis and all internal and external genital components derived from the genital disc (adapted from [58]).

[56,57] (Fig. 3). Elongation and coiling are driven by asymmetric membrane extensions at opposite sides of the basal end of the testis, which becomes attached to the vas deferens. The left testis of the early pupae elongates towards the right side, pulling the vas deferens with it, so that its spirals are situated on the right side in the adult [56–58], while the apical tip remains on the left side (Fig. 3). The reverse occurs for the right testis. Interestingly, Dobzhansky [47] observed that *Drosophila simulans* gynandromorphs testes attached to female genital ducts become spheroid and degenerate, whereas male testes that are not connected to the vas deferens remain ellipsoid shaped. This raised the intriguing hypothesis that the vas deferens might control coiling of the testis [47]. Using this observation Stern undertook experiments of intra- and interspecific testis transplantations [56,57]. He showed that an implanted donor testis always acquires the shape of the host species when attached through its basal end to the host's vas deferens. Thus the donor testis of a species with uncoiled testis would gradually take on a coiled shape characteristic of the host, while a donor testis that was normally coiled would remain uncoiled in an uncoiled host species. Stern suggested that the vas deferens determines both the coiling of the testis and its directionality, since in no case of transplantation did the donor testicle show an inverted coiling compared to the host testis when attached to the vas deferens. Stern therefore postulated the existence of a non-autonomous role for the vas deferens in providing an instructive signal that directs coiling, which can be read and interpreted by the basal pole of an otherwise naive testicle [56,57].

The positions of the adult testis in the abdomen are bilaterally asymmetric but do not represent a mirror image with respect to the midline. The apical tip of one testis lies on the opposite side of its coiling region and the according vas deferens crosses back from the basal tip to the medial ejaculatory duct (Fig. 3). The left testis (defined according to the position of its tip) is positioned anterior to the right testis and its apical end crosses ventral of apical end of the right testis. The junction between the left testis and the vas deferens points anteriorly, and conversely it points towards the posterior for the right hand one [58]. Accordingly, when viewed dorsally the left testis is coiled clockwise and the right testis counter-clockwise (although both testis exhibit clockwise (or dextral) directionality when viewed along the tip to base axis). This coiling of the adult testis has been used recently as a marker for L/R asymmetry. Hozumi et al. reported the inversion of adult testis coiling in *Myo ID* mutants [30]. Myo 1D was additionally found to be a dextral determinant in the A8 segment of the genital disc [59]. The adjacent A9 compartment gives rise to the internal and external genitalia, including the vas deferens, which in turn seems to provide

the information for testis coiling. It is thus possible that the Myo ID-dependent L/R information in A8 could be communicated to A9 and from there to the testis. How the planar signal crosses the segment border of the genital disc remains an open question.

1.1.4. The asymmetric brain

The brain has been shown to be L/R asymmetric in many vertebrate species, e.g. the asymmetry of the epithalamus [60]. In invertebrates, however, little information of this type has been generated. The prime example of invertebrate neuronal asymmetry is the bilaterally symmetric neuron pairs in *C. elegans*, which exhibit L/R asymmetric gene expression profiles and functions [61,62]. In *Drosophila* an asymmetrically localized brain structure, the Fasciclin 2 positive asymmetric body (AB), was recently described close to the mushroom bodies [63]. In 92% of wild type flies the AB is localized in the right hemisphere, whereas 8% of flies possess a symmetric brain with two ABs, which is associated with a lack of long-term memory. It remains to be seen whether this structure represents a genuine, genetically programmed L/R asymmetry, since it is not stereotyped and does not appear to depend on genes controlling body/visceral asymmetry (see below).

1.2. Mutants affecting L/R asymmetry in Drosophila

1.2.1. Role of JNK signalling in L/R asymmetry

1.2.1.1. Role of JNK signalling and apoptosis in the rotation of genitalia. The Jun N-terminal Kinase (JNK) signalling pathway is known to play a crucial role in several morphogenetic processes during development. Depending on the cellular context, it can induce cell elongation as in dorsal closure of the embryo and thorax [64–66] or can regulate apoptosis, as in imaginal discs [67,68]. Interestingly, partial genital disc rotation defects are seen in animals carrying loss of function alleles of the *Drosophila* JNKK *hemipterous* (*hep*) [65,69] and in animals over-expressing the JNK phosphatase *puckered* (*puc*) [70]. A similar phenotype is also observed for mutations in the pro-apoptotic gene *head involution defective* (*hid*) [71] and in animals in which apoptosis in the genital disc is blocked by the expression of the caspase inhibitor p35 protein [70]. This, together with the known genetic interactions between JNK and apoptotic pathways, suggests a role for the JNK pathway in the regulation of an apoptotic process that is needed for the correct rotation of the terminalia. Finally, the PVF1/PVR pathway (homologous to vertebrate PDGF/VEGF signalling) is required for proper genital rotation and for local expression of the JNK target gene *puc* [70], implying that the PVF1/PVR pathway activates the JNK pathway in the genital disc.

1.2.1.2. Role of JNK signalling in L/R asymmetry of the gut. Interestingly, a recent study has implicated JNK signalling in the laterality of the anterior midgut [29], suggesting a more general role of the JNK pathway in L/R asymmetry. Both up and down regulation of the pathway perturb asymmetric cell rearrangements in the circular visceral muscle surrounding the gut epithelium. As a consequence, a randomization of L/R asymmetry in the anterior midgut is observed. This suggests a need for precise regulation of JNK pathway activation during this process.

1.2.2. Fasciclin 2 and terpenoids

Fasciclin 2 (Fas2) is a member of the immunoglobulin superfamily, whose roles in axon growth and guidance are well studied, which was identified in a screen looking for mutations affecting the L/R asymmetric looping of the male spermiduct and genitalia. In these $Fas2^{spin}$ mutants the direction of the rotation appears normal (i.e., dextral), but the looping is incomplete (most males show a 180° rotation phenotype), indicating that genitalia rotation is affected rather than determination of the L/R axis [72]. To investigate the origin of these defects, Ádám et al. performed rescue experiments using the UAS/Gal4 system [73] to express Fas2 in a tissue-specific fashion [72]. Surprisingly, expression of the *Fas2* transgene in the central nervous system, more particularly in three pairs of neurons innervating the corpora allata in the ring gland, was sufficient to rescue the $Fas2^{spin}$ looping phenotype [72]. These results pointed toward a role for *Fas2* in juvenile hormone (JH) metabolism, since this is the major known function of the corpora allata. Interestingly, JH is related to retinoic acid (both are terpenoids), whose role in vertebrate L/R asymmetry has been well documented [74–80]. Furthermore, $Fas2^{spin}$ individuals show elevated levels of JH at pupae stage. Consistent with this, treatments with JH analogues lead to incomplete rotation while reducing JH signalling rescued $Fas2^{spin}$ rotation phenotypes [72]. Importantly, these results differentiate between establishment of the L/R axis and morphogenesis of the organs along this axis. Moreover, they draw a parallel between JH and RA that could indicate an evolutionary conservation between vertebrates and invertebrates in the determination of L/R asymmetry.

1.2.3. Single-minded

In vertebrates, midline structures play an essential role in L/R asymmetry establishment (for review see [8]). To assess whether *Drosophila* homologous structures (i.e. ventral midline cells) could also be involved in this process, Maeda and colleagues studied the role of *single-minded* (*sim*) in L/R axis establishment of the embryonic gut [81]. *sim* encodes a transcription factor containing PAS (Per-Arnt-Sim) and bHLH (basic Helix-Loop-Helix) domains. *sim* activity specifies the ventral midline, which is required, through downstream factors, for the correct development of the ventral ectoderm [82]. In *sim* mutant embryos, the handedness of the gut is affected. As for many genes affecting gut L/R asymmetry the penetrance of the *sim* mutant phenotype was different in each part of the gut. In this case, it appeared to correlate with the timing of the requirement for local *sim* activity [81]. Although it is not yet clear whether this reflects a role for proper ventral midline patterning in the establishment of the L/R axis, genes known to act downstream of *sim* in midline patterning were not required for the L/R asymmetry of the gut [81], suggesting a new specific function for *sim* in this process. Moreover, consistent with a more direct role for *sim* in the definition of the L/R axis, Sim overexpression in the hindgut was sufficient to induce a local L/R inversion.

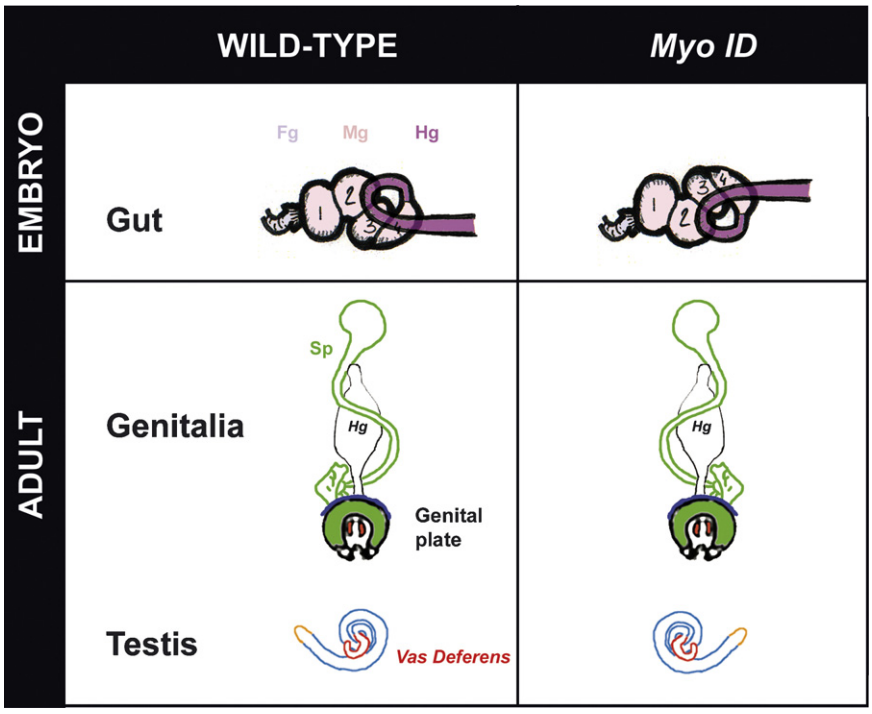


Fig. 4. L/R phenotypes in the *Myo ID* mutants. Phenotypes in wild-type (left column) and *Myo ID* mutant (right column) flies are described for the identified embryonic and adult L/R markers. In embryos, the constrictions of the midgut (light pink) as well as the bending of the hindgut (light purple) are reversed, whereas the L/R orientation of the foregut (light blue) is not affected. In adult males, both the rotation of the genital plate, which leads to the looping of the spermiduct (sp) around the gut (g), and the coiling of the testis are reversed in *Myo ID* mutant flies. Indeed these markers exhibit a dextral (clockwise) looping in wild-type flies, when viewed from the posterior for the genitalia, and from the vas deferens (vd) for the testis, but an opposite, sinistral (counter-clockwise) looping in *Myo ID* mutant flies. The colour code of the testis marker is as in Fig. 3.

1.2.4. Myosin ID

As described above, most of the genes affecting L/R patterning show poorly penetrant and variable phenotypes and are involved in other aspects of development, thus making it difficult to clearly delineate a L/R pathway in flies. However, the recent discovery of a single *Drosophila* locus whose mutations lead to a reversal of L/R markers in embryos (gut) and adults (testis and genitalia) (Fig. 4) provides strong genetic evidence for the existence of a L/R pathway. This unique locus encodes the *Drosophila* homolog of unconventional Myosin ID (Myo ID). This implicates the actin cytoskeleton in the process of L/R patterning [30,59,83].

Situs inversus is rare and the genes involved are likely to be instrumental in L/R axial determination. Until the discovery of Myo ID *situs inversus* mutants had only been identified in mice and snails. The mouse *situs inversus* gene *inversin* has been molecularly identified and shown to encode an ankyrin-repeat protein whose function during L/R asymmetry remains unclear [84–88]. In *Lymnea* snails, a single mutant dextral locus was identified that leads to inverted sinistrally coiled shells when mutated, but its molecular nature remains unknown [89–92]. In this context, the study of *Myo ID* mutants represents a beautiful paradigm to investigate the earliest steps of L/R determination and symmetry breaking in invertebrates.

The expression and function of Myo ID are L/R symmetrical and restricted to a very short time window [30,59,83]. *Myo ID* is expressed in the primordia of several L/R asymmetric organs, including the embryonic midgut and hindgut between stage 12 and 14 of embryonic development, as well as in the genital disc in third instar larvae [30,59]. To assess the tissue-specificity of *Myo ID* function in different primordia, Myo ID function was silenced using RNAi. This approach revealed that distinct regions within each primordium control the L/R patterning of different organs at different stages: Myo ID is required in the epithelium of the embryonic hindgut to determine LR asymmetry of both the midgut and hindgut, while it is required in the A8 segment of the genital disc to direct genitalia rotation [30,59].

In contrast to deuterostomes where ion and nodal flow have been implicated in symmetry breaking at the level of the whole organism, LR establishment involving *Myo ID* function appears to be set up independently in several primordia. Accordingly, the specific downregulation of *Myo ID* expression in A8 does not affect the asymmetry of the embryo gut or testes (unpublished results). Gene-specific silencing of *Myo ID* in the anterior (A8a) or posterior (A8p) compartments of the A8 L/R organizer reveals that Myo ID plays a dual role in the genitalia organizer: in A8p, Myo ID is required to direct dextral asymmetry, while in A8a it represses sinistral development [59]. These results suggest that two antagonistic activities, dextral and sinistral, act in the L/R organiser to direct normal genital rotation. In this process, the dextral activity of Myo ID appears to be dominant over the sinistral fate, which in the absence of Myo ID appears to be the default asymmetry.

Myo ID is an actin-based molecular motor comprising three main protein domains: the head, carrying the motor and catalytic activities; the neck, which binds to regulatory light chains and acts as a lever arm; and the basic tail, which is likely used to bind to cargo. As expected of a myosin, Myo ID co-localizes with filamentous actin *in vivo*. Moreover, genetic interactions with actin regulators including the Rho-family small GTPases and Moesin, an actin-binding protein, show that Myo ID requires an intact actin cytoskeleton to set up L/R asymmetry [30,59].

A two-hybrid screen identified the *Drosophila* β-catenin, Armadillo, as a direct binding partner for Myo ID, and these two proteins were shown to colocalise at adherens junctions in the A8 segment [59]. Intriguingly, the mouse Inversin protein has also been shown to physically interact with β-catenin [84,87]. These data suggest an important conserved role of the adherens junction in establishing L/R asymmetry in vertebrates and *Drosophila* [12,59,83]. It is possible that by binding to β-catenin, Myo ID could bias adherens junction remodelling to drive the asymmetrical cell intercalation that is observed during normal gut morphogenesis [29,30]. In addition to actin and β-catenin, Myo ID has been shown to interact genetically with Shibire, the *Drosophila* homolog of Dynamin, suggesting a role for trafficking in the control of L/R asymmetry establishment [59]. This is consistent with the fact that several unconventional myosins have been involved in trafficking.

It is noteworthy that two L/R asymmetric structures, the embryonic foregut and the asymmetric body in adult brain, are not reversed in *Myo ID* mutants [30] (PS & SN, unpublished results). Strikingly, however, the ectopic expression of *Myo ID* in the foregut results in an inversion, revealing the strong dextral activity of Myo ID when expressed in naive tissues. These and other data described above suggest that Myo ID functions as a dextral determinant. The intrinsic chiral activity of myosins (directional sliding on actin filament towards one end) together with the polarized activity of Myo ID along the A/P axis, make it a good candidate for an F-molecule (see Box 2). Brown and Wolpert [93] proposed that molecules of this type are required for reproducible symmetry breaking during the establishment of a L/R axis. They argued that such a protein must be able to orient itself along the reference A/P and D/V axes (illustrated by two of the branches of a three-dimensional F), while its intrinsic chirality is used to establish the perpendicular L/R axis (the third branch). If Myo ID is a true dextral determinant, the data from *Drosophila* also suggest the existence of a counteracting sinistral determinant.

A second type I unconventional myosin, Myo61DF, is present in the *Drosophila* genome, encoding the homolog of vertebrates Myosin IC (Myo IC), whose structure is very similar to Myo ID. Interestingly, overexpression of *Myo IC* in the embryonic gut leads to a *situs inversus* phenotype, mimicking a loss of *Myo ID* function [30]. These results suggest that Myo IC may antagonize Myo ID. However, silencing of *Myo IC* using targeted RNAi expression leads to a low frequency of gut inversion in embryos [30]. The apparent discrepancy in phenotypes together with the poor penetrance of the RNAi phenotype do not allow to draw firm conclusion about the role of *Myo IC* during L/R asymmetry, which will require further investigation using both the gut and genitalia markers.

2. Conclusion

The identification of Myo ID as a dextral determinant has opened up new avenues for the study of L/R asymmetry in protostomes. This work in *Drosophila* has provided new insights into the early mechanisms leading to symmetry breaking by (i) revealing the involvement of an actin-based molecular motor in body patterning and (ii) by identifying actin and adherens junctions as important cytoskeleton structures upon which L/R determinants can act to establish L/R asymmetry. There is an apparent separation between L/R symmetry breaking in protostomes and deuterostomes [12], the latter relying on microtubules and their associated motors, dynein and kinesin to make the motile chiral cilia that help to break symmetry. Whether actin could also play a role in deuterostomes, in particular during earliest steps of L/R asymmetry, remains to be investigated. Future studies both in *Drosophila* and other model organisms will help reveal how L/R asymmetry has evolved during the evolution of bilateria.

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