LETTERS

Type ID unconventional myosin controls left–right asymmetry in *Drosophila*

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Breaking left–right symmetry in Bilateria embryos is a major event in body plan organization that leads to polarized adult morphology, directional organ looping, and heart and brain function[1–4]. However, the molecular nature of the determinant(s) responsible for the invariant orientation of the left–right axis (situs choice) remains largely unknown. Mutations producing a complete reversal of left–right asymmetry (situs inversus) are instrumental for identifying mechanisms controlling handedness, yet only one such mutation has been found in mice (inversin)[5] and snails[6,7]. Here we identify the conserved type ID unconventional myosin 31DF gene (*Myo31DF*) as a unique situs inversus locus in *Drosophila*. *Myo31DF* mutations reverse the dextral looping of genitalia, a prominent left–right marker in adult flies. Genetic mosaic analysis pinpoints the A8 segment of the genital disc as a left–right organizer and reveals an anterior–posterior compartmentalization of Myo31DF function that directs dextral development and represses a sinistral default state. As expected of a determinant, Myo31DF has a trigger-like function and is expressed symmetrically in the organizer, and its symmetrical overexpression does not impair left–right asymmetry. Thus *Myo31DF* is a dextral gene with actin-based motor activity controlling situs choice. Like mouse inversin[8], Myo31DF interacts and colocalizes with β-catenin, suggesting that situs inversus genes can direct left–right development through the adherens junction.

In wild-type males, the genital plate, to which the spermiduct is attached, undergoes a 360° clockwise (dextral) rotation when viewed from the posterior pole[9,10] (Fig. 1a). This directional looping is reminiscent of other coiling processes such as mammalian heart tube looping and snail spiral development. As in other species, one direction is dominant among the Drosophilidae, the dextral rotation, with no sinistral species reported to date[10] (Supplementary Table 1). Here we have identified an insertional mutation, *KG02246*, which shows a striking inverted phenotype when combined with deficiencies covering the 31DF genomic region. In *KG02246/Df(2L)Exel7048* or *KG02246/Df(2L)J3* males, genitalia rotation is variable, with ~60% of individuals showing sinistral rotation (Table 1). Imprecise excision of *KG02246* generated two genomic deletions, $KG02246^{1}$ and $KG02246^{2}$ (Fig. 1f), both of which presented a stronger, 100% inverted (sinistral) genitalia rotation phenotype (Fig. 1b–e and Table 1). Thus, *KG02246* alleles identify the first situs inversus mutations in *Drosophila* and provide genetic evidence for the existence of a left–right axis in this organism.

Deficiency mapping (Table 1) and molecular cloning (Fig. 1f) revealed that *KG02246* mutations target the *Myo31DF* gene (also known as *CG7438*), which encodes *Drosophila* myosin IA (ref. 11). In support of this finding, we found that inducible RNA interference (RNAi) against *Myo31DF* in the genital disc mimicked *KG02246* mutations. Furthermore, expression of wild-type Myo31DF was sufficient to fully rescue the $KG02246^{1}$ rotation phenotype (Table 1; see below). Accordingly, we renamed the situs inversus mutations $Myo31DF^{K}$ (for *KG02246*), $Myo31DF^{K1}$ (for $KG02246^{1}$) and $Myo31DF^{K2}$ (for $KG02246^{2}$).

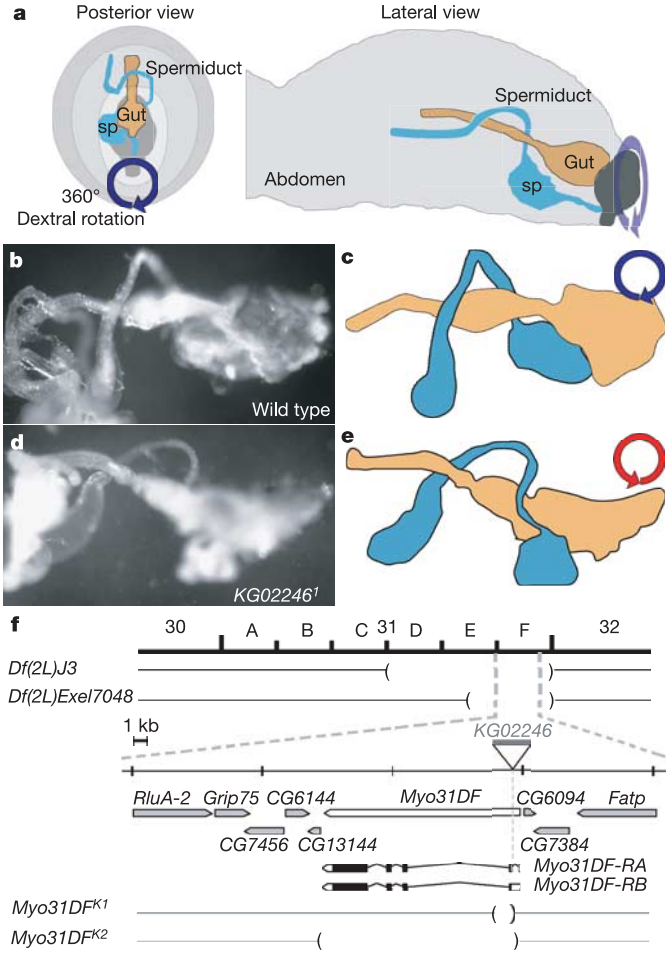

Figure 1 | *Myosin31DF* controls directional organ looping in *Drosophila*. **a**, Schematic posterior and lateral views of an adult male abdomen, showing the coiling of the spermiduct (blue) around the gut (orange) as a result of a 360° dextral rotation of the genital plate. sp, sperm pump. **b–e**, Dissected wild-type (**b**) and $Myo31DF^{K1}$ (**d**) abdomens, showing the reversal of spermiduct looping in $Myo31DF^{K1}$ flies. Panels **c** and **e** show schematic views of **b** and **d**, respectively. Colour code as in **a**. **f**, The genomic region 31E–F on chromosome 2 containing the *Myo31DF* gene.

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Drosophila Myo31DF is a conserved myosin belonging to the Myo1D family. It is known to interact with F-actin[11] and it colocalizes with actin-rich structures in different tissues (Supplementary Fig. 1a–g). After mouse inversin, *Drosophila Myo31DF* represents the second situs inversus gene to be molecularly identified. The mouse inversin gene (*Invs*) encodes a protein with ankyrin repeats and two IQ domains[12,13] that bind calmodulin, a Ca^{2+} -dependent regulatory protein[14]. Like inversin, Myo31DF contains two IQ domains essential for its function. Indeed, a Myo31DF form lacking IQ domains ($Myo31DF^{\Delta IQ}$) was unable to rescue *Myo31DF* mutations (Table 1).

To determine the function of Myo31DF in genitalia rotation, we examined its expression in the genital disc, the precursor of adult genitalia. The genital disc is composed of segments A8, A9 and A10, each with an anterior and a posterior compartment[15–18] (Fig. 2a). Immunostaining of wild-type flies with two polyclonal antibodies directed against overlapping regions of the Myo31DF tail domain, anti-Myo31DF-1P (Fig. 2) and anti-Myo31DF-3P (Supplementary Figs 2 and 3), or using a *Myo31DF–Gal4* enhancer-trap line (NP1548; Supplementary Fig. 3) revealed symmetrical expression of Myo31DF in a double chevron-like pattern restricted to the ventral domain of the male genital disc. This expression, starting in third instar larvae and remaining unchanged during this stage (Supplementary Fig. 3), was absent in $Myo31DF^{K2}$ mutant discs (Fig. 2c). Double staining with an A8-specific marker in the genital disc (*tsh–Gal4 > myr–RFP*) indicated that Myo31DF is expressed in the A8 segment (Fig. 2d), with one chevron in the posterior compartment (Fig. 2e, f) and the other in the anterior compartment. Consistently, expression of two copies of an inhibitory RNA gene ($2 \times Myo31DF^{RNAi}$) specifically in the A8 segment led to loss of Myo31DF expression (Fig. 2g) and to inverted phenotypes that mimicked *Myo31DF* mutations (Fig. 3a and Table 1). Together, these data identify the A8 segment as a left–right organizer that is required for situs choice in *Drosophila*.

To investigate the relationship between the anterior–posterior and left–right axes, we mapped the putative domain(s) of *Myo31DF* function by selectively silencing the gene in different compartments, using specific *Gal4* lines driving $2 \times Myo31DF^{RNAi}$ (Fig. 3a, Table 1 and Supplementary Fig. 4). The combinatorial removal of Myo31DF function in the anterior and/or posterior domains (summarized in Fig. 3b) led to distinct phenotypes, indicating a dual function for the Myo31DF protein in A8. First, blocking *Myo31DF* function posteriorly in A8 (using *hh–Gal4* or *en–Gal4*) resulted in a striking

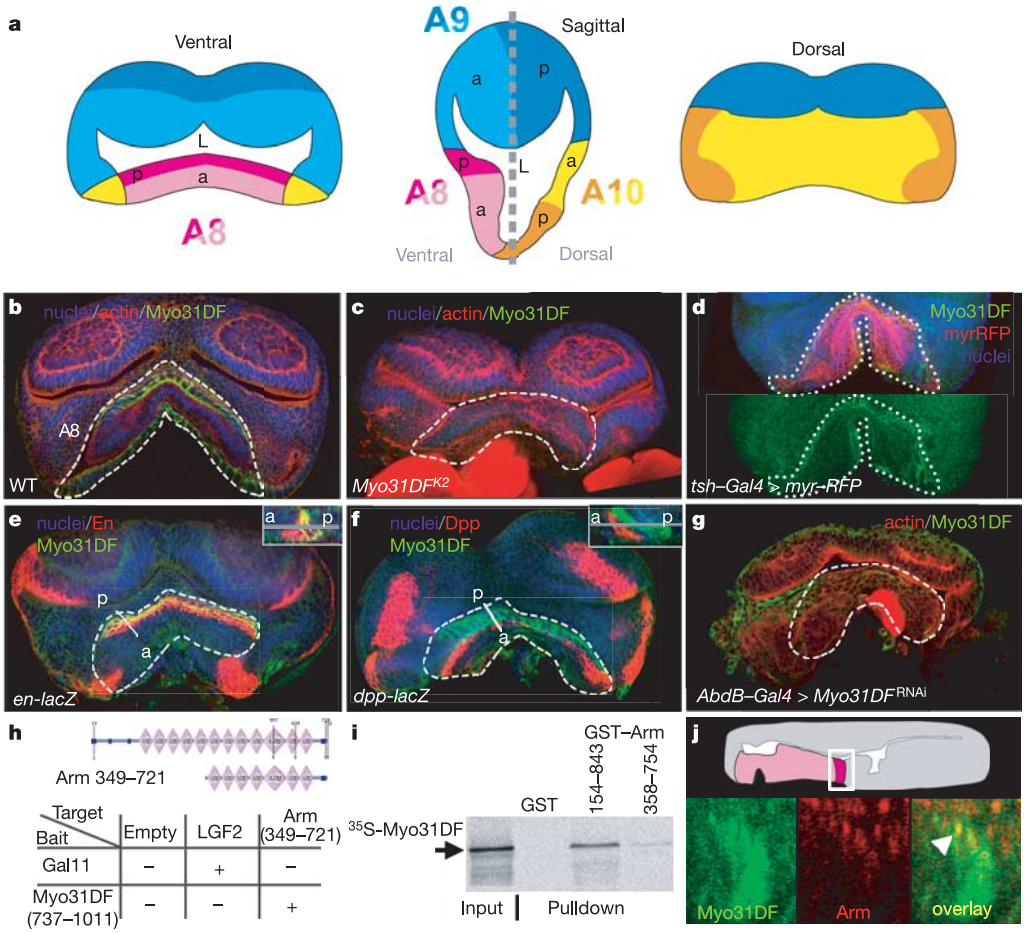

Figure 2 | Spatial expression of Myo31DF in the A8 segment of the genital disc and its interaction with Armadillo. a, Segmental organization of the genital disc showing A8, A9 and A10 segments with their anterior (a, light colours) and posterior (p, dark colours) compartments. L, lumen. **b**–**g**, Ventral views, with the A8 segment outlined (dotted line). Myo31DF expression was detected using anti-Myo31DF-1P antibodies. **b**, Endogenous Myo31DF is expressed in a double chevron-like pattern in the ventral region of the wild-type (WT) disc. **c**, Myo31DF expression is lost in the $Myo31DF^{K2}$ genital disc. **d**, Colocalization of Myo31DF with the A8-specific marker tsh. **e**, **f**, Myo31DF expression is compartmentalized in A8. En-lacZ and Dpp-lacZ are posterior and anterior markers, respectively. Bar indicates the position of the *z* section framed in the inset. Grey line in the inset is the *x*, *y* plane shown in **e** and **f**. **g**, Targeted expression of $2 \times Myo31DF^{RNAi}$ in A8 abolishes Myo31DF expression. **h**, Two-hybrid interactions identifying Armadillo (Arm, residues 349–721) as a binding partner of the Myo31DF tail domain. Gal11 and LGF2 were used as controls. **i**, GST-pulldown experiments showing the physical interaction between Myo31DF and Armadillo. **j**, Sagittal confocal section through the A8 posterior compartment (framed region in the schematic genital disc) showing colocalization of Myo31DF (green) and Armadillo (red).

non-rotated genitalia phenotype (Table 1 and Supplementary Fig. 5). This finding was confirmed in a complementary experiment using *dpp–Gal4* to rescue *Myo31DF*^{K1} in the anterior compartment (Table 1 and Fig. 3a). The absence of situs choice observed in these experiments indicates that posterior Myo31DF has an instructive role in dextral looping. Second, blocking *Myo31DF* function solely in the anterior compartment (in *dpp–Gal4* > 2 × *Myo31DF*^{RNAi} or in *Myo31DF*^{K1}/*Myo31DF*^{K1}; *hh–Gal4* > *Myo31DF* rescued males; Table 1 and Fig. 3a) led to partial dextral rotation, suggesting a permissive role for Myo31DF in this compartment. Comparing this outcome to the effect of concomitant removal of both anterior and posterior functions—the only context that led to complete reversal—indicates that the function of Myo31DF in the anterior compartment is to repress sinistral looping (compare rows 3 and 4 in Fig. 3b). These experiments demonstrate that *Myo31DF* is essential both anteriorly and posteriorly. A model illustrating the dual function of *Myo31DF* in regulating dextral development within the A8 segment is presented in Fig. 3c. In this model, A8 contains information to specify both sinistral and dextral rotation, with sinistral information being anterior and dextral information posterior. Dextral information is dominant over sinistral information, and Myo31DF function is required both posteriorly, to induce dextral development, and anteriorly, to repress sinistral development. Only in the absence of Myo31DF can sinistral development occur as a default state.

As expected of a left–right determinant with a function that precedes asymmetry, Myo31DF is expressed symmetrically in A8 (Fig. 2). In addition, as with mouse inversin[19], symmetrical overexpression does not lead to left–right defects (*AbdB–Gal4*, *tsh–Gal4* and *ptc–Gal4* lines; Table 1 and data not shown). Another predicted feature of left–right determinants is their temporally restricted, trigger-like function, which is later relayed by mechanisms acting to maintain the initial symmetry-breaking event. To test this, we carried out temperature-shift experiments using a genetically engineered temperature-sensitive *Myo31DF* allele (see Methods). Single temperature-shift experiments with 24-h (Fig. 3d) or 6-h (Fig. 3e) resolution indicated that Myo31DF function is required at day 6 of development, between 126 and 132 h. Double temperature-shift experiments allowed to determine that Myo31DF function is required for as little as 3 h within this period (Fig. 3f). These data provide high temporal resolution, allowing the temporal mapping of a left–right symmetry-breaking event and demonstrating that situs choice depends on a peak of Myo31DF function in the left–right organizer.

Cilia have emerged as important cellular structures for generating left–right asymmetry in vertebrate embryos. To address their possible contribution to invertebrate left–right determination, we stained genital discs with GT335, an antibody that labels glutamylated tubulin in cilia across species, including *Drosophila*[20]. GT335 did not detect any cilia in genital discs (data not shown). Additionally, mutations in the conserved *Rfx* gene, which controls the formation of ciliated neurons in *Drosophila*[21] and left–right asymmetry in mouse[22], did not affect genitalia rotation (data not shown). Together, these data suggest that cilia are not involved in dextral development in *Drosophila*. We propose that *Drosophila* uses primarily the actin cytoskeleton to determine left–right asymmetry. Consistently, the small GTPase Drac1 and the JNK pathway, known regulators of the actin cytoskeleton, showed specific genetic interactions with *Myo31DF* (ref. 23 and Supplementary Fig. 1h). Notably, actin is also important for situs choice in snails[24], suggesting that in invertebrates the actin cytoskeleton has a central role in left–right determination.

To start investigating how Myo31DF might act to determine left–right asymmetry, we used two-hybrid screening to identify Myo31DF interactors or cargo(es). Using the Myo31DF tail domain (amino acids 737–1011) as bait (see Methods), we found several positive clones encoding a carboxy-terminal fragment containing ARM repeats 6–12 of the Armadillo/β-catenin protein (Fig. 2h). This interaction was direct, as shown by glutathione *S*-transferase (GST)-pulldown experiments using purified proteins or S2 cell extracts (Fig. 2i and Supplementary Fig. 6a). Furthermore, endogenous Myo31DF and a functional Myo31DF–GFP (green fluorescent protein) fusion protein (Table 1 and Supplementary Fig. 6b) colocalized with Armadillo at the adherens junctions in A8 (Fig. 2j), suggesting that the two proteins can interact *in vivo*. Notably, inversin has been shown to colocalize and interact with β-catenin in vertebrate epithelial cells[8], indicating that an interaction with β-catenin is a

Table 1 | Summary of rotation phenotypes

	Dextral		No rotation 0°	Sinistral	
	Complete (+360°)	Partial (<+360°)		Partial (<−360°)	Complete (−360°)
Wild type	100	0	0	0	0
KG02246/Df(2L)J3	17	3	18	58	4
KG02246/Df(2L)Exel7048	0	5	40	47	9
Myo31DF^{K1}	0	0	0	42	58
Myo31DF^{K2}	0	0	0	23	77
UAS-2 × Myo31DF^{RNAi} crossed to*					
elav-Gal4	100	0	0	0	0
cad-Gal4	100	0	0	0	0
dpp-Gal4	53	32	8	7	0
en-Gal4	10	4	86	0	0
hh-Gal4	0	0	100	0	0
AbdB-Gal4	0	0	30	69	1
ptc-Gal4	0	0	4	57	39
tsh-Gal4	0	0	0	55	45
Rescue experiments (*Myo31DF*^{K1} background)					
ptc-Gal4 > Myo31DF†	100	0	0	0	0
ptc-Gal4 > Myo31DF-GFP	100	0	0	0	0
AbdB-Gal4 > Myo31DF	70	27	2	1	0
AbdB-Gal4 > Myo31DF-GFP	95	4	1	0	0
dpp-Gal4 > Myo31DF	0	10	82	8	0
hh-Gal4 > Myo31DF	84	16	0	0	0
ptc-Gal4 > Myo31DF$^{\Delta IQ}$†	0	0	0	89	11

Numbers represent the percentage of male flies with the indicated genitalia rotation phenotype, from wild type (360° dextral rotation) to completely inverted (360° sinistral rotation). Unless otherwise noted, all crosses were performed at 25 °C.
*Crosses performed at 30 °C.
†Cross performed at 20 °C.

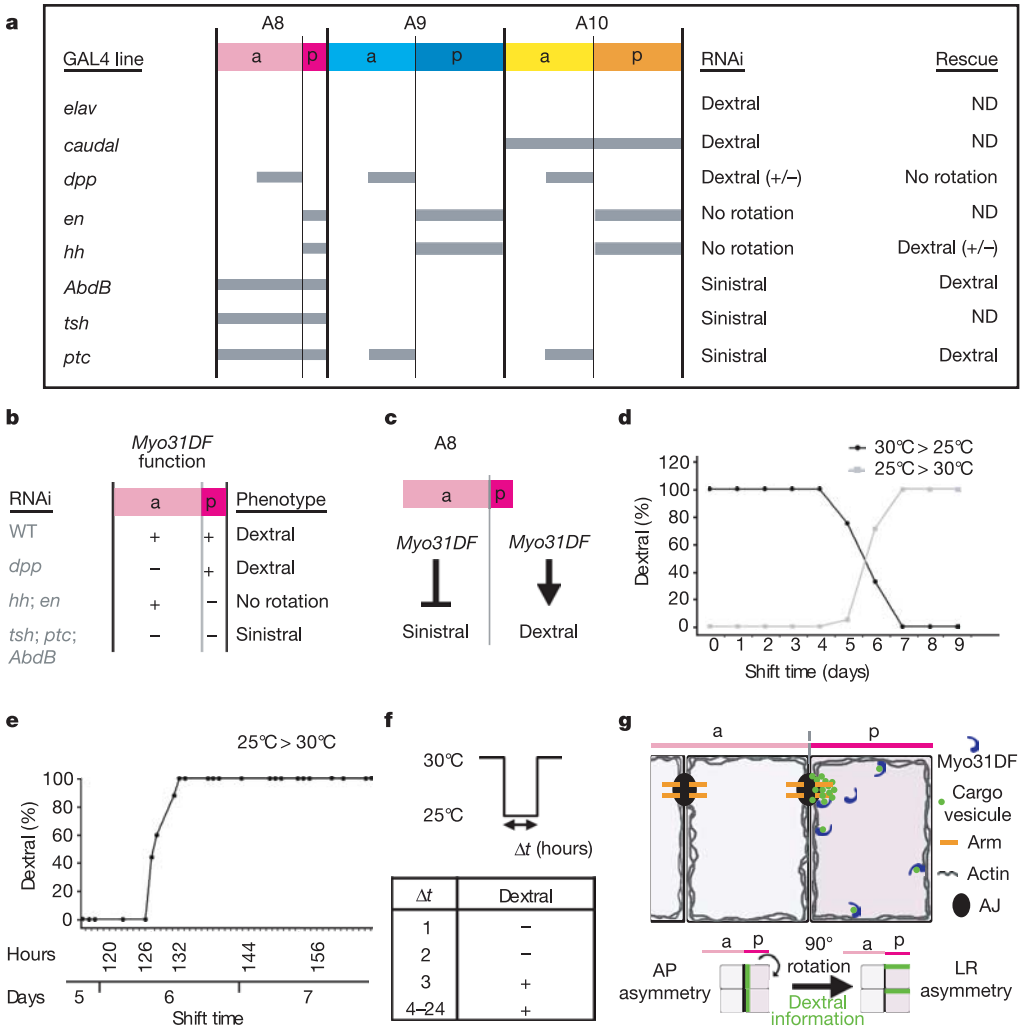

Figure 3 | Spatial and temporal organization of *Myo31DF* function in the left–right organizer. **a**, GAL4 expression domains (grey bars) and their effect on genitalia rotation upon crossing with 2 × *Myo31DF*^{RNAi} ('RNAi') or in rescue experiments using *UAS–Myo31DF* ('Rescue') (see Table 1 for details). ND, not determined. **b**, Summary of compartmental silencing of *Myo31DF* in A8. **c**, Model of compartmental *Myo31DF* function. **d**–**f**, Single (**d**, **e**) or double (**f**) temperature-shift experiments in *tub–Gal80*^{ts}; *AbdB–Gal4*, *UAS–Myo31DF*^{RNAi} male flies. **d**, Permissive (25 °C) to restrictive (30 °C) (grey) and restrictive to permissive (black) temperature-shifts. **e**, Requirement for Myo31DF function between 126–132 h in development. **f**, Double temperature-shift experiments. **g**, Proposed model of Myo31DF function at the adherens junction (AJ) (see text for details). AP, anterior–posterior; LR, left–right.

common feature of both known situs inversus genes. These results are consistent with a demonstrated role of N-cadherin in left–right asymmetry[25].

How could anterior–posterior organization of Myo31DF and interaction with Armadillo account for left–right asymmetry? The actin cable network might serve as a track for Myo31DF to deliver specific cargoes or vesicules at the adherens junction. Indeed, we found that Myo31DF interacts with dynamin (Supplementary Fig. 1i), consistent with the role of rat MyoID in vesicular transport[26]. The anterior–posterior boundary itself creates an asymmetric junction that could serve as a scaffold for Myo31DF to assemble a dextral-specific complex on the anterior side of posterior Myo31DF-expressing cells (green in Fig. 3g). Anterior–posterior asymmetry of dextral information could later be translated into left–right asymmetry through the remodelling of cell contacts (cell intercalation or rotation), as seen in other epithelia[27]. For example, 90° rotation of epithelial cells relative to the main body axis is observed in the *Drosophila* eye imaginal disc[28]. A similar, 90° planar rotation of Myo31DF-expressing cells would result in left–right orientation of the dextral junctional complex in the tissue (Fig. 3g). In this working model, a short pulse of Myo31DF activity would be essential for spatially restricting the dextral junction, as we have observed.

METHODS

Genetics. A description of genetic markers and chromosomes can be found at FlyBase (http://flybase.bio.indiana.edu). *AbdB–Gal4* was a gift from E. Sanchez-Herrero. *Myo31DF*^{K1} and *Myo31DF*^{K2} alleles were made by excision of the *KG02246* P-element using standard protocols.

DNA cloning. Constructs were made as follows. For pUASt–*Myo31DF*, the full-length *Myo31DF* cDNA (SD01662) was cut with *Eco*RI and *Xho*I and ligated into the *Eco*RI and *Xho*I sites of the transformation vector pUASt. For pUASt–*Myo31DF–GFP*, the full-length coding region of *Myo31DF* was cut with *Eco*RI and *Not*I and ligated into the *Eco*RI and *Not*I sites of the transformation vector pUASt–*GFP*. For pUASt–*Myo31DF*^{RNAi}, the *Bgl*II–*Xho*I fragment from SD01662 (nucleotides 3211–3780) was cloned into the *Bam*HI–*Xho*I sites of the transformation vector SympUASt. pUASt–*Myo31DF*$^{\Delta IQ}$ was made by cloning the *Eco*RI–*Xho*I fragment from SD01662 with deleted nucleotides 2829–2952 (residues 695–736). For each construct, several independent transgenic lines were generated and tested.

Antibodies, immunostaining and imaging. We raised antibodies against two different GST fusion proteins containing Myo31DF tail residues 728–990

(Myo31DF-1P antibody) or 823–1011 (Myo31DF-3P antibody). The sera were purified on His-tagged peptide columns and tested by western blotting to ensure specificity (Supplementary Fig. 2). We carried out immunostaining according to standard procedures, using the following antibodies and markers: purified rabbit polyclonal antibodies against Myo31DF (1:10 to 1:100 dilutions), anti-Armadillo monoclonal antibody (DSHB; 1:20 dilution); Alexa546- and Alexa488-conjugated secondary antibodies (Molecular Probes; 1:500 dilution), phalloidin-TRITC (Sigma; 0.2 $\mu g\,ml^{-1}$) and Hoeschst33258 (Sigma; 3.3 $\mu g\,ml^{-1}$).

Temperature-shift experiments. Conditional expression of Myo31DF was achieved using $Gal80^{ts}$ in *tub–Gal80*^{ts}; *AbdB–Gal4*; *UAS-2* × *Myo31DF*^{RNAi} males. At 25 °C (permissive for Gal80 and Myo31DF functions), males had a wild-type phenotype, but at 30 °C (restrictive for Gal80 and Myo31DF functions) all males had non-rotated genitalia. In single temperature-shift experiments, 1-h staged first instar larvae were grown at a given temperature and shifted once to the permissive or restrictive temperature after 24 h (Fig. 3d) or 6 h (Fig. 3e), and the phenotype of adult males was determined. In double temperature-shift experiments, 24-h staged embryos were grown at 30 °C and shifted to 25 °C for a limited period of time (1 h–24 h; Δt in Fig. 3f) before being shifted back to 30 °C until hatching. The presence of wild-type males indicated that the duration of the shift was sufficient to restore Myo31DF function.

Two-hybrid and GST-pulldown. Two-hybrid screening was performed using the Bacteriomatch II system (240065, Stratagene) according to the manufacturer's instructions. We used the Myo31DF tail domain as bait (residues 737–1011) and a *Drosophila* embryo cDNA library (982600, Stratagene) as the target. Equivalent amounts of GST, GST–Arm (residues 154–843) and GST–Arm (residues 358–754) were used to pull down *in vitro* translated, ^{35}S-labelled Myo31DF protein (TnT, Promega). After binding for 3 h at 4 °C, beads were washed four times with 1 ml lysis buffer (150 mM NaCl, 50 mM Tris pH 7.5, 0.2% Triton and protease inhibitor cocktail) and resuspended in Laemmli buffer. Samples were run on an SDS–PAGE gel and revealed using Phosphoimager.

Imaging. Images were taken on an LSM510 META confocal microscope (Zeiss) using ×25 0.80 NA and ×40 1.3 NA oil immersion objectives, and assembled using Adobe Photoshop 7.0.

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