Formin Is Associated with Left-Right Asymmetry in the Pond Snail and the Frog

Highlights
- Animals tend to be outwardly symmetric but internally are asymmetric
- Unlike other animals, snails show inherited variation in asymmetry
- We found that both snails and frogs use a common gene to define left and right
- Asymmetry is probably an ancient and conserved property of cells and animals

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In Brief
Davison et al. have discovered a cell scaffolding protein in snails and frogs that controls body asymmetry, either the direction a snail shell coils, or whether a frog heart is placed to the left or right. Body asymmetry in animals, including humans, likely arises from a highly conserved, intrinsic asymmetry of the cell.

Accession Numbers
KU341240–KU341321
PRJEB11470
ERS1022296
ERS935814
Formin Is Associated with Left-Right Asymmetry in the Pond Snail and the Frog

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http://dx.doi.org/10.1016/j.cub.2015.12.071
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SUMMARY

While components of the pathway that establishes left-right asymmetry have been identified in diverse animals, from vertebrates to flies, it is striking that the genes involved in the first symmetry-breaking step remain wholly unknown in the most obviously chiral animals, the gastropod snails. Previous research on snails was used to show that the left-right signaling of Nodal, downstream of symmetry breaking, may be an ancestral feature of the Bilateria [1, 2]. Here, we report that a disabling mutation in one copy of a tandemly duplicated, diaphanous-related formin is perfectly associated with symmetry breaking in the pond snail. This is supported by the observation that an anti-formin drug treatment converts dextral snail embryos to a sinistral phenocopy, and in frogs, drug inhibition or overexpression by microinjection of formin has a chirality-randomizing effect in early (pre-cilia) embryos. Contrary to expectations based on existing models [3–5], we discovered asymmetric gene expression in 2- and 4-cell snail embryos, preceding morphological asymmetry. As the formin-actin filament has been shown to be part of an asymmetry-breaking switch in vitro [6, 7], together these results are consistent with the view that animals with diverse body plans may derive their asymmetries from the same intracellular chiral elements [8].

RESULTS AND DISCUSSION

Bilaterian animals are more or less symmetrical about the midline that divides left and right, but internally most organs are asymmetric in location or shape. How is symmetry broken during development if the “right” and “left” sides are essentially arbitrary? A longstanding model posits that a chiral “F molecule” is orientated relative to the anteroposterior and dorsoventral axes [9]. This asymmetric molecular reference then determines left-right (LR) differentiation at the cellular and organismal level.

Given the importance of chiral patterning in the three bilaterian superphyla, Deuterostomia, Ecdysozoa, and Lophotrochozoa, a continuing problem is a lack of knowledge of the first symmetry-breaking steps in the Lophotrochozoa, even though the first described locus that reverses the whole body structure of an animal was from the pond snail Lymnaea [10, 11]. Recently, commonalities between different species have been discovered [12–15]. For example, in both vertebrates (Deuterostomia) and snails (Lophotrochozoa), nodal and pitx encode key signaling molecules required for the establishment of LR asymmetry, suggesting that these genes may have been used in the last common ancestor of Bilateria, but lost in Ecdysozoa [1, 2, 16]. However, neither nodal nor pitx is the earliest symmetry-breaking determinant in snails, ultimately limiting a knowledge of whether this represents deep conservation or convergent use of the same genes.

Within Lophotrochozoa, snails are unique in that they exhibit genetically tractable, natural variation in chirality [17] and so may aid in understanding of the establishment and conservation of LR asymmetry. Here, we use genetics, genomics, and pharmacological inhibition to show that the Lymnaea stagnalis chirality gene is a scaffolding component of the cytoskeleton. We present strong evidence that this same molecule is one component of an early chiral cytoskeletal structure that is involved in the earliest symmetry-breaking steps across the Bilateria.

A Gene That Is Associated with Chirality in Pond Snails

The gastropod mollusc L. stagnalis is naturally variable in left-right asymmetry, outwardly visible in the chirality of the spiral shell, and under the control of a single maternally expressed locus. In L. stagnalis (Figure 1A), maternal D alleles dominantly determine a clockwise (“dextral”) twist in offspring [18, 19]. Specifically,
during the third cleavage in dextral embryos, four micromeres simultaneously emerge from four macromeres and twist clockwise ("spiral deformation"; [19]). In homozygous recessive (dd) sinistral embryos the four micromeres initially emerge neutrally, without rotation, with a later counterclockwise twist taking place during furrow ingression [19]. These differences are presaged by the orientation of metaphase-anaphase spindles. In dextral embryos, micromere spindle orientation is chiral ("spindle inclination"; [19]), while in sinistral embryos, the spindles are positioned radially, such that they do not exhibit chirality.

Previously we defined a ~0.4 Mb region of the 1 Gb L. stagnalis genome that must contain the chirality locus [18]. To identify the chirality-determining gene, we generated two new resources: a sequenced bacterial artificial chromosome (BAC) clone walk across the chirality locus interval and draft genome sequences of DD homozygote and Dd heterozygote individuals. The BAC clone walk was oriented using three-color fiber fluorescent in situ hybridization (FISH) (Figure S1). We used the offspring of a large cross to recombination breakpoint map the position, orientation, and haplotype origin (D or d) of each BAC clone relative to the original restriction associated DNA sequencing (RAD-seq) markers (rad4 and rad5) and the chirality locus D (Table S1). The chirality locus was located between markers b6 and b12, a region of 267 kb (Figure 1B). We predicted
genes across the scaffolded BAC walk assembly and mapped DD and Dd whole-genome sequencing data to the assembly to identify haplotype-specific variation.

Only six genes were in perfect linkage with D in our mapping cross, all contained on a single BAC scaffold: lysosomal pro-x carboxypeptidase (Lprcp), furry (Lfry), a tudor domain-containing protein (Ltud), a major facilitator superfamily domain-containing protein (Lmfsd), and a pair of tandemly duplicated diaphanous-related formin genes (Ldia1 and Ldia2; Figure S2A). In sinistral (dd) snails, Ldia2 was found to contain a homozygous, single base deletion in the 5’ of the coding region that causes a frameshift. The mutation was confirmed by cloning and sequencing Ldia transcripts from both sinistral and dextral snails (KU341302–KU341305). No disabling mutations were discovered in the coding sequences of the other genes, including the adjacent paralog Ldia1.

Maternal expression of genes was assessed by quantitative real-time PCR in single-cell embryos from DD, Dd, and dd mothers. Of the six candidate genes, only Ldia2 showed significant differences in expression associated with genotype (Figures 1C and S3A). Ldia2 transcripts were readily detected in dextral embryos from DD mothers. However, in sinistral embryos from dd mothers, Ldia2 transcript levels were ~0.6% that of DD embryos, whereas in embryos from heterozygous Dd mothers, they were ~50%. These differences in expression are consistent with frameshifted Ldia2sin transcripts being degraded by nonsense mediated decay.

The tandem duplication perhaps explains why a Ldia2sin mutation is not simply lethal [20–22], in that Ldia1 and Ldia2 may have overlapping roles in embryonic development, albeit with some specialized function: while we found that mRNAs for both Ldia1 and Ldia2 were present in equal quantities in the dextral 1-cell embryo, Ldia2 was ~3-fold enriched in 1-cell dextral embryos relative to somatic tissues, whereas Ldia1 was ~10-fold depleted (~30-fold difference overall).

**Asymmetric Expression of Maternal Genes Precedes Asymmetric Morphology**

Expression of candidate loci was examined in early L. stagnalis embryos using whole-mount in situ hybridization (WMISH; [23]).
The first two embryonic cleavages in *L. stagnalis* are equal, so that the four macromeres that are formed from the first two cleavages are indistinguishable [3–5], until contact between the third quartet of macromeres induces one of the macromeres to become the future D blastomere (but see 24). We expected that mRNA transcripts would initially be equally distributed between the four macromeres. Surprisingly, however, we found that *Ldia* mRNA was asymmetric at the 2-cell stage and largely confined to one macromere by the 4-cell stage (Figure 1D), with *Lfry* sometimes showing an asymmetric pattern, albeit less striking, and with more variation between individuals (Figure S3B).

While we do not know whether the *Ldia2*-positive macromere is the D blastomere, individual macromeres must have an identity as early as the two-cell stage, contrary to the expectation based on accepted models [3–5] of development in equal cleaving snails. This also further emphasizes the general point that asymmetry is determined very early and intracellulary— asymmetry of molecules precedes visible morphological asymmetry, although not necessarily in a causative manner (see [4, 25] for comparison with unequal cleaving embryos). The results also suggest an explanation for the reduced viability of sinistral embryos [20–22]—as chiral rotation of macromeres in sinistral is sometimes incomplete or error prone, inviability may be caused by a conflict in identity between individual cells. Further investigation is necessary, but the sinistral embryos seem to show more generalized, less obviously asymmetric staining, consistent with this explanation (perhaps because of reduced transport on actin microfilaments, [4]) (Figure S3B).

**Pharmacological Inhibition of Formin in Early Snail Embryos Mimics the Sinistral Phenotype**

As transgenic approaches are not yet established in *L. stagnalis*, and microinjection is usually lethal [22], we tested formin involvement in chirality using SMIFH2, an FH2 domain inhibitor [26]. SMIFH2 prevents formin nucleation and processive elongation of filamentous actin by decreasing the affinity of formin for the barbed end. Micromolar concentrations of SMIFH2 disrupt the formation of formin-dependent, but not Arp2/3 complex-dependent, actin cytoskeletal structures [26].

When 100 μM SMIFH2 was added to genetically dextral 4-cell embryos shortly after completion of the second cleavage, relatively few embryos (~30%–60%) reached third cleavage at ~80 min (Figure 2; Table S2). However, in ~25%–35% of those that did, all four micromeres emerged neutrally, with no chiral twist (SMIFH2 treated at 0 min, n = 6 experiments, 274 embryos, compared to n = 11 control experiments, 219 embryos; p < 0.001, U = 5.5, Mann-Whitney U), with up to ~45% of individual micromeres emerging neutrally (Figure 2). SMIFH2 treatment of dextral embryos thus phenocopies normal sinistral embryos. We also visualized spindles during the third cleavage of *L. stagnalis* by indirect immunofluorescence with anti-β-tubulin antibody. In line with previous findings [19], control dextral embryos showed the characteristic spindle inclination, especially in the latter stages of mitosis. In SMIFH2-treated dextral embryos, the spindles were more frequently radially symmetric, resembling untreated sinistral embryos (Figure 3).

To rule out a non-specific effect of SMIFH2, we compared the effect of another inhibitor of actin assembly, CK-666, which acts specifically on Arp2/3 complex-dependent actin patches [27] and not on formin-dependent actin cables. CK-666 also had a lethal effect when applied to genetically dextral four-cell embryos and also tended to reduce the average angle of emergence of micromeres. However, an achiral phenotype was observed in rather few CK-666-treated embryos (Figure 2; CK-666 treated 0 min, n = 3 experiments, 196 embryos, compared to n = 5 control experiments, 190 embryos; p = 0.107, U = 2.5, Mann-Whitney U). Therefore, the differential effects of SMIFH2 and CK-666 suggest that formin-mediated actin assembly may be a critical factor in determining chiral cleavage orientation between the second and third cleavages.

In SMIFH2-treated, genetically dextral embryos that continued to develop following neutral emergence of micromeres (i.e., like sinistral), the direction of the subsequent twist was dextral, rather than sinistral (6/6; Movie S1). The individual

**Figure 3. Tubulin and Actin Staining of Control and Drug-Treated Embryos**

Embryos were fixed and stained with Cy3-b-tubulin (red) and 488-phalloidin (green) to highlight the spindle microtubules and filamentous actin, respectively. DMSO-treated embryos predominantly showed spindle inclination (left image, 4-cell stage), with the micromeres usually emerging with a dextral twist (right image, 8-cell stage). A minority of SMIFH2 treated embryos had mitotic spindles that showed a radial orientation (left image), an arrangement that was not observed in DMSO control dextral embryos. In the SMIFH2 right-hand image (8-cell stage), the top middle and middle left micromeres are emerging neutrally (arrows), with the other two showing a partial rotation. Addition of SMIFH2 did not influence spindle orientation in 4-cell DMSO control or sinistral embryos, with spindles typically showing a radial orientation.
micromeres of genetic sinistrals also sometimes twisted dextrally (3% to 4% in our experiments; the fourth time-lapse in Movie S1 shows an embryo in which all four micromeres twist dextrally after neutral emergence; see also [22]). This later twist is also actin dependent [19], which suggests dextrality may be the default pathway, independent of FH2 domain function.

A Sinistral Ancestral Pond Snail?
We used genomic and transcriptomic resources, and new sequences, to explore links between diaphanous formin and chiral evolution. First, we found that the chromosomal region that contains the L. stagnalis chirality locus is deeply conserved, exhibiting synteny of dia, fry, and tud between L. stagnalis, Biomphalaria glabrata (planorb snail, sinistral cleaving), and Capitella telata (polychaete annelid, dextral cleaving). Second, while the dia duplication is also evident in Lymnaea trunculata, and so must pre-date its divergence from L. stagnalis, single-copy dia genes in B. glabrata and Physa acuta (both sinistral snails; Figure 1A) are more similar to Ldia1 than Ldia2 (Figure S2B). The B. glabrata dia gene and Ldia1 also share a non-repetitive UTR element, absent from Ldia2. Thus, Ldia2 is likely the derived paralog, which may have evolved specific function in the embryo, and for which loss leads to a sinistral phenotype.

We mapped chirality onto a new phylogeny of the Hygrophila (Figure S2C). The predominantly dextral Lymnaeidae and the sinistral Physidae cluster together, so either sinistrality evolved once, with a sinistral ancestral Lymnaeid subsequently reverting to dextral, or else, sinistrality evolved on two separate occasions (Figure 1E). Both explanations are equally parsimonious. However, only the first is consistent if the duplication was involved in enabling dextrality in the Lymnaeidae.

As no natural variation in chirality has been described in Biomphalaria or Physa, it is difficult to further test the function of dia in relation to copy number. Instead, we sampled dia orthologs in two other chirally variable snail genera, Euhadra, a Japanese land snail, and Partula, an endangered species from Polynesia (Figure 1A; [28, 29]). In both of these genera, chirality was not associated with variation in a single-copy dia that was recovered (Figure S2). Molecular understanding of chiral variation in these species is therefore likely to reveal additional components of the LR asymmetry pathway, including variants in genes that enable chiral evolution without negative pleiotropic effects upon fitness.

Formin Also Regulates LR Patterning in the Frog
A popular model of LR symmetry breaking in vertebrates relies on chiral flow of extracellular fluid during neurulation. However, this mechanism cannot be universal as many phyla lack the ciliated structures required or achieve correct LR patterning prior to the ciliated structure differentiation (reviewed in [8, 14, 15]). Inherent asymmetry in the cytoskeleton could provide an ancient, well-conserved mechanism used by vertebrate embryos at the earliest stages of development to initiate the LR pattern and instruct the entire body plan [9, 30, 31].

Figure 4. Effect of Drug Treatment and Microinjection of Overexpressed Formin on Chirality in the Frog
(A) Embryos were treated with DMSO, CK-666, or SMIFH2 at the concentrations indicated, allowed to develop, and scored for visceral organ chirality at stage 45. (B) Embryos were injected into the animal pole with mRNA encoding mouse dia1 formin and scored for visceral organ situs at stage 45. Images: Examples of organ situs for experimental microinjection with wild-type mouse dia1 mRNA. The control shows a wild-type (situs solitus) tadpole, ventral view, demonstrating the normal arrangement of the stomach (yellow arrowhead), heart apex (red arrowhead), and gall bladder (green arrowhead). Heterotaxic tadpoles (ventral view) resulting from formin overexpression show reversal of all three organs, i.e., situs inversus; the gut position and looping and gall bladder; or the heart. See also Table S3 and Figure S4.
investigated whether formin inhibition in early embryos, before the neurula ciliary flow, could affect chirality in the vertebrate model *Xenopus laevis*.

SMIFH2 and CK-666 drug treatments were carried out with *X. laevis* embryos at different stages of development and scored by measuring heterotaxia (independent organ LR inversion) in tadpoles (Figure 4; Table S3). In early embryos (stages 1–6), during which the cytoskeleton instructs LR patterning [30], treatment with 50 μM SMIFH2 had a strong effect on LR patterning (13% heterotaxia, X², p < 0.001), with a smaller but significant effect with CK-666 (7% heterotaxia, X², p < 0.001), whereas embryos treated at neurula stages (stages 19–21), when ciliary flow is present, showed a strong effect for both 50 μM SMIFH2 (12%, X², p < 0.001) and 50 μM CK-666 (16%, X², p < 0.001). Treatments spanning late blastula to gastrula (stages 8–14) had a reduced (3%–4%) but still significant effect (Table S3) on organ heterotaxia, showing that the efficacy of early treatment cannot be due to remnant drug persisting to cilia stages.

For a more specific gain-of-function test of formin function in *Xenopus*, mouse *dia1* mRNA was injected into the animal pole of frog embryos and organ *situs* assessed at stage 45. Similar to the in vitro finding that the spontaneous counterclockwise alignment of actin bundles can be reversed by overexpression of alpha-actinin [7], we found that overexpression of *dia1* resulted in a high and significant proportion of heterotaxia, whether injected 30 or 60 min post-fertilization, or in one of two or four cells (Figures 4 and S4). In addition, targeted injection into the dorsal left (DL) or ventral right (VR) blastomeres at the 4-cell stage showed that while significantly different from uninjected controls (X², p < 0.001), there is no significant difference between DL and VR in the effect on heterotaxia (n = 85 DL, 5% heterotaxia; n = 135 VR, 9% heterotaxia; p = 0.75 Student’s t-test), which would be expected if the effect were at the point of ciliary flow, as the left side of the embryo is required for ciliary flow affecting LR patterning [32].

**Formin, an F Molecule?**

The implication of a key cytoskeletal protein in LR patterning of both molluscan and vertebrate embryos is consistent with a conserved asymmetry genes. J. Molluscan Stud. 71, 192–195.

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