

## CORRESPONDENCE

## Formin, an opinion

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In previous work (Davison et al., 2016), we used both genetic mapping and a chemical knockdown to show that a frameshift mutation in one copy of a duplicated *formin* gene is most likely the mutation that causes changes in left-right (LR) asymmetry, or chirality, in the pond snail *Lymnaea stagnalis*. We also showed that the asymmetric morphology is preceded by asymmetric *formin* expression in snails, and that overexpressing the same gene in frogs reverses LR asymmetry.

We are therefore pleased that research by Abe and Kuroda (2019) not only corroborates our own findings, but gains definitive proof for causation. The new work puts beyond any doubt that LR asymmetry in snails originates in the cellular architecture. However, we are troubled by several errors or omissions, and also that the authors dismiss previous experimental work, by ourselves and others. These points need making because they detract from what is otherwise an important step forward, causing confusion amongst colleagues in an important area of developmental biology.

(1) The new work confuses the gene names, to the extent that several colleagues mistakenly believed that we misidentified the locus in 2016. In being the first to identify the causative gene (Davison et al., 2016; submitted in August 2015, published in February 2016), we named it *Ldia2* because it is evidently the derived version, compared with *Ldia1*. *Ldia2* is located on a long branch (evidence of rapid evolution), and *Ldia2* transcripts are enriched in the embryo relative to *Ldia1* (indicating specialized function). Despite submitting their work after ours was published (Kuroda et al., 2016; received in July 2016, published in October 2016), the Kuroda group reversed the naming of the same genes, *Lsdia1* for the mutated version and *Lsdia2* for the other copy. This fact is not mentioned at all in their new work (Abe and Kuroda, 2019). It is therefore important that these differences are made clear, and that, as in other fields, precedence should be used for describing the genes in future publications.

(2) The authors' title is that the work 'establishes the formin *Lsdia1* as the long-sought gene for snail dextral/sinistral coiling'. Notwithstanding the fact that we established the formin as the causative gene (Davison et al., 2016), and they gained definitive proof (Abe and Kuroda, 2019), all experiments prior to ~2003 were carried out in another species, *L. peregra*, for which the causative gene remains

unidentified. In this latter species, we agree that it is reasonable to suspect that formin may also be involved. This is because sinistral development in two separate isolates of *L. peregra* is pathological (Boycott et al., 1930; Freeman and Lundelius, 1982), just as in *L. stagnalis* (Davison et al., 2009; Utsuno et al., 2011). Although not cited, prior to the new work, a formin was also shown to be duplicated and associated with chiral variation and pathology in another land snail (Noda et al., 2019).

However, the genes that determine natural chiral variation in snails, without pathological effect, remain unknown. We would argue that formin is not a good candidate, mainly because of the associated pathology, but also because we ruled out one formin as causative in two snail genera, *Euhadra* and *Partula* (Davison et al., 2016).

In our opinion, there is likely no single 'long-sought gene' that flips chirality, although it is possible that there is a universal pathway that sets up an asymmetric cellular architecture in all animals and plants (e.g. via microtubules; Lobikin et al., 2012). The important question from a developmental point of view is to understand how the asymmetry is set up and amplified. The important question from an evolutionary standpoint is to understand how other species of snail can flip their chirality without apparent pathology, unlike any other animal.

- (3) Abe and Kuroda state that this is 'the first application of CRISPR/Cas9 to a mollusc'. This is repeated in a press release put out by The Company of Biologists. This is incorrect. Perry and Henry (2015) used gene CRISPR/Cas9-mediated genome modification in the mollusc *Crepidula fornicata*, albeit for transient transgenesis.
- (4) The new work shows that there are also morphological asymmetries in the first cleavage, stating that this is 'the earliest observed symmetry-breaking event linked directly to body handedness in the animal kingdom'. We agree that this is a fascinating finding but it is not unexpected. Preceding the current vogue for cellular chirality by more than a century, Conklin (1903) and later Meshcheryakov and Belousov (1975) reported that the spiral character of cleavage begins with the first division of the molluscan egg. Abe and Kuroda (2019) extend the findings to show that the morphological asymmetry of the single-cell embryo varies

within chirally variable species. Moreover, although it is rarely so evident morphologically, it is not correct that this is the only animal for which it has been shown to have such an early symmetry-breaking event. For example, the frog has a defined left and right as early as the first cell cleavage (Vandenberg et al., 2013), as do ascidians (Albrieux and Villaz, 2000).

- (5) Abe and Kuroda conclude that experiments using a chemical knockdown to corroborate the genetic results ‘do not provide any meaningful insights’, and imply that our findings of asymmetric gene expression are a technical artefact. We disagree. In their previous work, they applied the inhibitor drug very early, resulting in complete developmental arrest, and among other differences, they used an *in situ* hybridization protocol that may be less sensitive than ours (Herlitzte et al., 2018), included fixing overnight and using ten times more antibody. In our view, it is not surprising that different methods in different laboratories may produce different outcomes. To resolve the debate regarding asymmetric gene expression, an independent method, such as single cell transcriptomics and/or single molecule RNA FISH may be necessary.
- (6) It is stated in the new work (Abe and Kuroda, 2019) and in the press release that changing chirality by gene editing is likely to lead to the generation of a new species. So-called ‘single-gene speciation’ is a persistent idea, but not likely the case for these snails. It has long been known that chiral reversal per se is not sufficient to create new species (Richards et al., 2017 and references therein). This is especially the case in pond snails, in which sinistrals and dextrals are able to mate, and also because the mutation in the formin causes a ~50% reduction in egg hatch rate (supplementary figures S3 and S4 in Abe and Kuroda, 2019; see also Davison et al., 2009; Utsuno et al., 2011).

#### Competing interests

The authors declare no competing or financial interests.

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doi:10.1242/dev.187427

## CORRESPONDENCE

# Response to ‘Formin, an opinion’

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In 2016, we reported the use of genetic and positional cloning approaches to identify a frameshift mutation in one of two closely related diaphanous (formin) genes we termed *Lsdia1* and *Lsdia2* associated with left-right asymmetry in the snail *Limnaea stagnalis* (Kuroda et al., 2016). Both alleles of the *Lsdia1* gene were mutated in sinistral (but not dextral snails) suggesting, but not proving, the

gene was involved in chiral control. In the same year, Davison et al. (2016) also published a report identifying a mutation in one of the formins in sinistral snails of the same species. In new work (Abe and Kuroda, 2019), we have now used CRISPR gene editing to show decisively that *Lsdia1* is the causative gene that controls left-right coiling in *L. stagnalis*, and that it operates from the one-cell stage,

implicating internal cellular structures in early determination of left-right asymmetry.

We do not dismiss and indeed have always referenced the 2016 work of Davison and colleagues. In their Correspondence, they claim to have discovered *Ldia2* (which corresponds to our *Lsdia1*) as the causative gene for snail left-right coiling in that earlier paper, comment on the apparent reversal of gene naming in our paper (Kuroda et al., 2016) and raise several other issues relating to our most recent work (Abe and Kuroda, 2019). We believe these issues can be readily addressed and discuss each point in turn below.

### Naming of genes

Working independently and long before the 2016 Davison et al. paper appeared, we had identified the duplicated *dia* genes as of interest and arbitrarily named them *Lsdia1/Lsdia2*. We did not subsequently adopt the Davison et al. naming system, *Ldia1* (=Lsdia2) and *Ldia2* (=Lsdia1), because their published gene and inferred protein sequences are very different in key aspects from our *Lsdia1/2*. Importantly, (1) the position of their initiation codon in *Ldia2* is different from ours and consequently the critical point mutation is L62 in our LsDia1 but L19 in Davison et al.'s LDia2. Forty-three N-terminal amino acid residues are missing in Davison et al.'s sequence. (2) Furthermore, substantial sequence is missing in the FH1 region in Davison et al.'s Ldia1.

Based on our own N-terminal sequence, we made probes for our whole-mount *in situ* hybridization (WISH) work, which beautifully discriminate *Lsdia1* and *Lsdia2* mRNAs. The identity of the probe used in the Davison et al. work was not reported in their paper, but they could not discriminate the two mRNAs in WISH – they state that ‘unfortunately, it was not possible to generate a probe specific to *Ldia2*, because of cross-reactivity of the probe to *Ldia1* (sequence similarity is ~90%)’.

Using our own FH1 region sequence, we have succeeded in performing CRISPR/Cas9 gene editing. In fact, this is virtually the only place where the gene sequence is sufficiently different between *Lsdia1* and *Lsdia2* to be used as a gRNA target site.

Had we used Davison et al.'s sequence, we could not have done the high-quality WISH and CRISPR/Cas9 work. In addition to the key discrepancies, different protein lengths are reported in the data bank and in the paper's supplemental information for both LDia1 and LDia2. Correct and reliable sequence is essential. Given the significant disparities with our LsDia1/2 sequences, we decided to retain our gene naming. Our *Lsdia1* and *Lsdia2* are Davison et al.'s *Ldia2* and *Ldia1*, respectively, as we made clear in 2016 (Kuroda et al., 2016). However, we recognise that some readers might have been confused by this, and therefore provide clarification in the associated Correction (Abe and Kuroda, 2020).

### Establishing the causative gene

Contrary to statements in points 1 and 2 of the Correspondence, no one (including Davison et al., 2016 and Kuroda et al., 2016) had established the *formin* as the causative gene before our CRISPR work, as appropriately quoted in the title of the Davison et al. paper ‘*Formin is associated* with left-right asymmetry in the pond snail and the frog’.

As mentioned in the Correspondence, much of the early work was done on *L. peregra*. We have also investigated the early development of sinistral and dextral strains of this species, which show very similar cleavage patterns to those of *L. stagnalis* at the third cleavage (Shibazaki et al., 2004; Kuroda et al., 2016; unpublished work). We have previously been able to reverse the chirality not only of *L. stagnalis* but also of sinistral-only *Physa*

*acuta* by mechanical manipulation of blastomeres at the third cleavage (Kuroda et al., 2009; Abe et al., 2014). This suggests that similar handedness-determining mechanisms operate in these species (Kuroda et al., 2009; Abe et al., 2014). Evidence based on gene function assays (RNAi, morpholino or CRISPR experiments) may be needed before concluding that *formin (dia)* is not the causative gene of the chirality in land snails *Euhadra* and *Partula*. In fact, a very interesting recent study (Noda et al., 2019) also reveals the importance of paralogous *dia* genes in the chirality of the land snail *Bradybaena similaris*. Based on these considerations, we think *dia* is the important handedness-determining gene in multiple gastropods, common to *Lymnaea* species at least. We therefore believe that the title is appropriate.

### First application of CRISPR/Cas9 to a mollusc

We are aware of the work of Perry and Henry (2015) and referenced it in our 2019 paper. We consider that ‘successful gene editing’ has occurred when the germline transmission of the edited gene has been demonstrated. In the Perry and Henry paper they write ‘This study suggests that future experiments to generate transgenic specimens using the CRISPR/Cas9 technology are possible in molluscs and will aid to expand current knowledge about gene function and regulation in those systems. Based on the complexity of gene knock-in experiments, we predict that gene knockouts in *C. fornicata* may be possible with higher efficiency’. From these statements, we understood that germline transmission had yet to be achieved. We did not intend to ignore Perry's nice attempt of knock-in, and by way of clarification, we have adjusted the language in the associated Correction to our paper (Abe and Kuroda, 2020).

### The earliest observed symmetry-breaking event linked directly to body handedness in the animal kingdom

The key qualifier in our statement is ‘linked directly to body handedness’, which – in our view – means having a defined molecular link to chirality leading to body handedness. In our case, we have shown that the presence/absence of LsDia1 protein at the one-cell stage, prior to the first polar body extrusion, controls left-right coiling in *L. stagnalis*.

It is correct that Meshcheryakov and Belousov (1975) observed, by trypsin treatment, a twist at the first cleavage in the same direction to the snail coiling. They supposed that a spiral structure of the contractile ring is the basis of this twisting and subsequent coiling direction, and – based on their study of dextral *L. stagnalis* and sinistral *P. acuta* – that chirality is specific to species. We developed a modified version of Meshcheryakov's trypsin method (Abe and Kuroda, 2019) and revealed that the chirality depends on a single gene, *Lsdia1*, rather than on the species per se. The offspring of two snail lines that have exactly the same background except for the *Lsdia1* genotype show enantiomorphic cleavage patterns already at the first cleavage. Other early symmetry-breaking events observed in the frog and ascidians mentioned in Davison et al.'s Correspondence do not have a validated molecular link to body handedness. For example, in the paper by Vandenberg et al. (2013), injection of foreign genes into frog eggs at the one-cell stage caused around 50% heterotaxia, including some *situs inversus* in tadpoles. Based on these findings, the authors proposed an interesting molecular model, but this has not been validated experimentally.

We have shown that the intrinsic intracellular chirality is superseded by the intercellular interaction at the third cleavage, leading to the *nodal/Pitx* gene expressions and eventually to the organismal chirality, and all of these processes depend on the absence and the presence of functional LsDia1 protein at the

single-cell stage. We consider this an important step forwards in understanding the molecular basis of chirality at the earliest stages of development.

### Drug inhibition and WISH experiments

Here, we address briefly the concerns raised by Davison et al. regarding discrepancies between the two 2016 papers. We have reservations about studies using the anti-formin drug SMIFH2. This drug works on both *LsDia1* and *LsDia2*, and – at least in our hands – is very toxic to *L. stagnalis* embryos. We consistently see lethality at concentrations higher than 10  $\mu$ M (Kuroda et al., 2016) regardless of the timing of drug application (our unpublished results). From studies on genetically dextral embryos treated with a very high drug dose (100  $\mu$ M), Davison et al. conclude that ‘anti-formin drug treatment converts dextral snail embryos to a sinistral phenocopy’. However, arguing against this conclusion, embryos that continued to develop after the drug treatment all showed a micromere rotation at the third cleavage (which is the key determinant of snail coiling; Kuroda et al., 2009; Abe and Kuroda, 2019; Freeman and Lundelius, 1982) in which the ‘twist was dextral, rather than sinistral’ (Davison et al., 2016). Thus, we would argue that it is hard to draw definitive conclusions from these inhibitor experiments.

Secondly, we have compared Davison et al.’s WISH protocol to ours and believe that their apparent asymmetric *dia* expression may be a consequence of the ‘within-capsule’ protocol used (in which the embryo is not removed from its capsule). We used a standard, sensitive ‘outside capsule’ procedure and normal levels of antibody. Using Davison et al.’s protocol, we obtained remarkable asymmetric localization for the transcripts of housekeeping genes,  $\beta$ -actin and  $\beta$ -tubulin (unpublished results), suggesting that this may be an artifact. In any case, their probe cannot distinguish between the two formin paralogs.

### Chiral reversal and new species?

We mentioned the possible link between reversed snail coiling and evolution of new species – so-called ‘single-gene speciation’ – because it is an intriguing and much-debated concept with shifting opinion over time as to whether and how it might operate in the wild (Ueshima and Asami, 2003; Hoso et al., 2010). We agree that sinistral and dextral *Lymnaea* are able to mate, acting against the emergence of mutant sinistral populations. However, *Lymnaea* is a hermaphrodite, and through self-fertilization, could expand a sinistral population. More work is needed on the secondary factors, such as mating behaviour, geographical isolation and predation, that might facilitate or undermine reversed coiled populations.

The hatching rate of the naturally occurring sinistral strains [with a point mutation in *Lsdia1* in both alleles, (–/–)] in our lab is reasonably high, about 78%, albeit lower than the ~95% for the (+/+) strain (figure 1G in Abe and Kuroda, 2019). The low hatching rate of ~50% stated in Davison et al.’s Correspondence refers, in our

case, to the offspring of gene-edited homozygous knockout mothers. As described in our paper, this may arise because the gene editing allows the translation of the protein up to the FH1 domain, which might have some deleterious effect. Sinistral populations do exist in the wild. Thus, we do not think it is unreasonable to speculate on the possibility that reversed coiling might lead to speciation.

In summary, we appreciate the work carried out independently, and hope impartial discussion will help further understanding of snail coiling, a fascinating developmental system with possible wider relevance.

### Competing interests

The authors declare no competing or financial interests.

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doi:10.1242/dev.187435