THE EMBO JOURNAL

Manuscript EMBO-2017-96580

Epigenetic regulation of the left-right asymmetry by DNA methylation

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Review timeline:

Submission date:	28 January 2017
Editorial Decision:	01 March 2017
Author's Appeal:	02 March 2017
Editor's Response:	03 March 2017
Resubmission:	25 June 2017
Editorial Decision:	28 July 2017
Revision received:	05 August 2017
Editorial Decision:	10 August 2017
Revision received:	11 August 2017
Accepted:	15 August 2017

Editor: Ieva Gailite

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 01 March 2017

Thank you for submitting your manuscript for consideration by The EMBO Journal. We have now received all three referee reports on your manuscript, which are included below for your information. I am sorry to say that, in light of these comments, we had to conclude that the study is not a sufficiently strong candidate for publication in The EMBO Journal.

As you can see, the referees in principle express interest in the proposed regulation of left-right asymmetry establishment via DNA methylation. However, all three referees raise substantive concerns regarding the experimental setup and data interpretation, and they request a range of crucial experiments with uncertain outcomes for the central message of the manuscript. Given these opinions from three trusted experts in the field, I am afraid we cannot offer further proceedings towards publication in The EMBO Journal.

Thank you in any case for the opportunity to consider this manuscript. I am sorry that I cannot communicate more positive news, but nevertheless hope that you will find our referees' comments helpful.

REFEREE REPORTS

Referee #1:

In this paper, authors have examined a role of DNA methylation in early development of zebrafish embryo, by performing MeDIP-seq and RNA-seq with wild-type and dnmt1-deficient gastrulating embryos. They found that genes involved in left-right asymmetry are preferentially affected, and in fact, dnmt1-deficient embryos exhibited laterality defects such as abnormal situs of visceral organs and bilateral expression of Spaw. Their findings would add a new player in left-right asymmetry, but current data do not sufficiently support their conclusion that the lefyt2 enhancer is a target of regulated DNA methylation for correct left-right asymmetry (see below).

Major comments

1) Authors mentioned that genes involved in left-right asymmetry are enriched in Fig. 1C. What genes, in addition to lefty2, were found to be hypo or hyper-methylated in dnmt1-deficient embryos?

2) Authors suggest that the primary defect of dnmt1-deficient embryos is hypomethylation of the lefty2 enhancer region. This would result in upregulation of lefty2, which in turn reduce the number of DFCs. However, I am not convinced that this is the main reason for left-right defects in dnmt1-deficient embryos. To test this, it is necessary to examine several (5-10) embryos for i) KV fluid flow, and ii) KV cilia motility, because L-R defects are observed only in 30~40% of dnmt1-deficient embryos. Authors would need to reconsider the mechanism upon obtaining such data.

3) Hypomethylation was detected at the nearly 20 kb upstream of lefty2 gene. Bisulfite analysis data (Fig. 4D, Fig. 6C) are convincing. Authors believe that hypomethylated regions of lefty2 are located "at the lefty2 enhancer" (page 8). Is there any evidence supporting that the enhancer is located there? If so, what is the specificity of the enhancer: active in what cell-type(s) and at what stage? How does upregulation of lefty2 result in reduction of the number of DFCs? If previous data not available, it is necessary to test if the hypomethylated regions possess enhancer activity perhaps in gastrulating zebrafish embryos.

Referee #2:

General summary:
The manuscript by Wang et al provides evidence that DNA methylation is essential for the establishment of the left-right asymmetric body plan during vertebrate development. Their data suggests that morpholino depletion of the DNA methyltransferases dnmt1 or dnmt4 in zebrafish and Xenopus leads to dorsal forerunner cell (DFC) specification defects, laterality organ malformation, and a disorder of LR patterning in zebrafish embryo. Mechanistically, the authors link these defects to hypomethylation of the lefty enhancer, leading to upregulation of lefty and repression of nodal signaling.

A link between DNA methylation and body axis is of interest, however, there are a currently a number of controls lacking from this manuscript that are necessary to support its conclusions.

Major:
1) The authors provide data indicating that abnormal heart jogging is observed in dnmt1 morphant zebrafish, but indicate that this phenotype is not recapitulated in dnmt1 mutant zebrafish. They suggest that this discrepancy is due to maternal deposition of dnmt1, which is a reasonable potential explanation. However, in the event that mutants cannot be used for analysis, minimal standards for morpholino use in zebrafish require demonstration of phenotype rescue by co-injection of morpholino resistant mRNA encoding the targeted gene. mRNA rescue of dnmt1 morpholino phenotypes has been published (Rai et al 2006), indicating that this control is feasible. It must be done for all experiments. Ideally, recapitulation of key phenotypes with a second morpholio targeting dnmt1 should also be performed.

2) Similar comments to those above apply to the use of dnmt4 and lefty morpholinos in this manuscript. mRNA rescue or corroborating mutant phenotypes are required to support all morpholino claims. Dnmt4 is only reported to be maternally deposited at low levels in the zebrafish embryo, and mutants may be viable due to the diversification of dnmt3 orthologs in zebrafish. Given the ease of genome editing in zebrafish the authors should generate and test dnmt4 mutants.

3) Previous studies have indicated that 5hmC is not detected by IF until after the tail bud stage (Almeida et al 2012) and RNA-seq data from Harvey et al 2013, suggest none of the three tets are expressed at stages through 90% epibioly. The authors suggest that there may be low levels of these genes/5hmC prior to this stage in a cell context dependent manner. However, since their data is in contrast to existing reports, Q PCR and 5hmC quantification on sorted DFCs should be performed to further demonstrate their presence in DFCs. Data indicating reduced 5hmC in DFCs in tet1/2/3 morphant embryos (or even better in mutants) should also be provided for evaluation (currently indicated as data not shown)

8) Tet1 and Tet 2 mutant zebrafish lines are viable and fertile and available (Li et al 2015). The authors should use these lines to demonstrate mitigation of the dnmt1 morphant phenotype rather than co injecting tet1/tet2 moropholinos.

Minor:
1) It would be useful for the authors to use a molecular approach such as a 5mC dot blot to quantify the extent of 5mC loss in total DNA in dnmt1 morpholino injected embryos. This will help with interpretation of results from Me-Dip experiments.

2) The experiment in Figure 1D doesn't make sense to me. Virtually all cells have 5mC. What is the significance of showing that sox17 positive DFCs have this mark?

Writing:
1) In the introduction the authors state that "In vertebrates, after fertilization, the embryo shows a lower level of DNA methylation until early cleavage stages and the methylation level increases gradually since then (Jiang et al, 2013; Potok et al, 2013; Wang et al, 2014). While this sentence is technically true, in reality methylation dynamics are very different in zebrafish and mammalian embryos. A more faithful discussion of methylation would be of use to readers.

2) Since dnmt1 is a maintenance methyltransferases and dnmt4 is a de novo methyltransferases, it is not clear why there should be synergisim in phenotype when the two morpholinos are coinjected (either should eliminate methylation). Can the authors elaborate?

3) The authors need to be more clear about what experiments are in fish and what are in frogs.

4) Loss of methylation at the lefty enhancer in morphant embryos doesn't really demonstrate that this gene is a direct target of dnmt1 in DFC specification. It seems likely, but this methylation change could be reflective of changes in transcription caused by regulation of something upstream.

Referee #3:

This manuscript uses zebrafish (and Xenopus in a few experiments) to investigate the role of the DNA methlytransferases Dnmt1 and Dnmt4 in left-right patterning. It was found that morpholinos (MO) designed to target Dnmt1 or Dnmt4 disrupt dorsal forerunner cells (DFCs) and subsequent left-right asymmetry. Further experiments show Dnmt1 MO causes upregulation of lefty2 and Dnmt4 MO causes downregulation of E-cadherin in DFCs. The identification of DNA methylation targets that impact left-right patterning is novel and of great interest. However, I have significant concerns about this paper in its current form. First, nearly all results are obtained using MO knockdowns with no controls for off-target effects. Second, the authors arrive at conclusions without considering alternative interpretations of their data. My detailed comments:

Major issues:
1. All MO experiments (fish and frog) lack confirmation of protein knockdown (IHC or western blots) and mRNA co-injection rescue data. MOs can be useful tools, but require rigorous controls

due to the well-described potential for MO off-target effects. For example, it is important to test whether co-injecting Dnmt1 mRNA with Dnmt1 MO will rescue MO phenotypes. Several groups have successfully injected mRNA into DFCs for rescue experiments (see examples: Clement, et al. Development. 138 (2), 291-302 (2011); Caron, et al. Development 139, 514-524 (2012)).

2. Instead of using rescue experiments to address specificity, the authors injected Dnmt1 MO into Dnmt1 mutants that do not show left-right patterning defects. The authors conclude that a low dose Dnmt1 MO reduces maternal expression and uncovers organ laterality defects in homozygous mutants. It would be useful to use Dnmt1 antibodies (IHC or western blots) to test this directly. It is not described how embryos were determined to be homozygous mutants or siblings. Also, only left-right asymmetry of organs (liver, pancreas) were examined in 4 day old embryos (Fig. S2). Analysis of earlier events, including the DFCs, in MO injected homozygous mutants would strengthen the argument that DFC phenotypes are specific rather than due to off-target effects. However, there is still the caveat that these are 'MO-induced phenotypes' in the mutant background. It should be clarified whether MZ-Dnmt1 mutants (lacking both maternal and zygotic Dnmt1 function) have left-right asymmetry and DFC defects.

3. No attempt was made to validate Dnmt4 MO results.

4. Dnmt1 MO was found to reduce the number of DFCs. Taking a candidate approach, it was found that Dnmt1 MO increased lefty2 expression and decreased Nodal target genes. The authors conclude from these experiments that the reduced number of DFCs is due to defects in DFC specification. However, it is possible that dysregulation of Nodal targets (or potentially many other genes) leads to reduced DFC number by affecting proliferation or apoptosis rates. DFCs are specified between sphere (4 hpf) and 50% (5.3 hpf) stages. Yet, in this study, DFCs are first analyzed at 60% epiboly stage (6.5 hpf). It would be helpful to analyze the number of sox17:GFP+ DFCs at earlier (specification) stages between 4-5 hpf to determine if there is a difference between Dnmt1 MO and control.

5. Dnmt4 MO reduces cdh1 mRNA levels, but protein levels (IHC) in DFCs should also be measured. Testing whether cdh1 mRNA injection rescue Dnmt4 MO defects in DFCs would strengthen the authors' conclusion that reduced cdh1 is responsible for the DFC clustering defect. Does Dnmt4 MO also result in upregulated lefty2? It appears lefty2 over-expression is sufficient to cause a DFC clustering defect (Fig. S4D) that is similar to cdh1 MO (Fig. 5D).

6. It would be helpful to know whether 5-AZA treatments alter lefty2 and/or cdh1 expression as observed in MO injected embryos.

Minor issues:
1. The Dnmt nomenclature used in this paper (Dnmt1-8) is simple and easy to follow, but not consistent with current gene nomenclature for this gene family found at https://zfin.org. It should be described somewhere in the paper (main text or materials and methods section) what the current zfin name is for each of the Dnmt genes tested in this study.

2. Dnmt4 MO sequence must be reported.

3. Use of English language is poor in many places, which results in confusing statements. The writing should be edited for accuracy and clarity.

Author's Appeal 02 March 2017

Thank you for your time on handling our submission and the three reviewers for their valuable comments. We appreciate that all three reviewers found our work novel and interesting in the field. In brief, they raised two major issues as follow:

1. The lefty 2 enhancer specificity issue (Reviewer 1#). We will provide more evidence to demonstrate that this hypomethylated region functions as an enhancer of lefty2 in zebrafish gastrula embryos.

2. MO issues (Reviewer 2# and 3#). We agree with both reviewers that most of our data were based on antisense MOs, largely due to the maternal effects of dnmt1 mutant, and the dnmt4 mutant was not available at the time of submission. We now have obtained a dnmt4 mutant by CRISPR/Cas9 and we are in process to generate a maternal-zygotic mutant of dnmt1 (MZ-dnmt1). In addition, we will also obtain the tet1/2 mutant zebrafish from our colleagues in China. Collectively, we will be able to address the MO issues raised by Reviewers 2# and 3# within a two-month time window.

Therefore, I cordially request a reconsideration of our submission and give us a chance to resubmit our work once we have completed all the required experiments and fully addressed the concerns by the reviewers.

Editor's Response 03 March 2017

You are welcome to submit a revised version of the manuscript if you can address the main concerns of the referees, and if the experimental outcome still upholds the main message of the manuscript. From our side, it would be also important to address the following points regarding the specific signalling pathways affecting left-right asymmetry downstream of Dnmt1/4 - see referee #1, point 2, and referee #3, point 4.

I also have to point out that in the case of a resubmission we would forward the manuscript to the same set of referees to streamline the reviewing process. Please feel free to contact me if you have any further questions.

Resubmission 25 June 2017

Response to the reviewers' comments

We are very grateful to the reviewers for their insightful comments on our manuscript. We have performed a number of critical experiments suggested to improve the quality of our manuscript, including rescue experiments, second morpholino-injection and generation of a new *dnmt4* (*dnmt3bb.1*) mutant to validate the phenotypes. The detailed point-by-point responses to reviewers' comments are shown below.

Referee #1:
In this paper, authors have examined a role of DNA methylation in early development of zebrafish embryo, by performing MeDIP-seq and RNA-seq with wild-type and dnmt1-deficient gastrulating embryos. They found that genes involved in left-right asymmetry are preferentially affected, and in fact, dnmt1-deficient embryos exhibited laterality defects such as abnormal situs of visceral organs and bilateral expression of Spaw. Their findings would add a new player in left-right asymmetry, but current data do not sufficiently support their conclusion that the lefyt2 enhancer is a target of regulated DNA methylation for correct left-right asymmetry (see below).

Major comments
1) Authors mentioned that genes involved in left-right asymmetry are enriched in Fig. 1C. What genes, in addition to lefty2, were found to be hypo or hyper-methylated in dnmt1-deficient embryos?

Response 1. We apologize that we didn't provide the complete list of genes with dysregulated methylation in the previous submission. We have now added this gene list (including some known Nodal target genes such as *cnopy*, *spw, lefty1,* and *bmp4*) in Supplemental Table 1.

2) Authors suggest that the primary defect of dnmt1-deficient embryos is hypomethylation of the lefty2 enhancer region. This would result in upregulation of lefty2, which in turn reduce the number of DFCs. However, I am not convinced that this is the main reason for left-right defects in dnmt1-deficient embryos. To test this, it is necessary to examine several (5-10) embryos for i) KV fluid flow, and ii) KV cilia motility, because L-R defects are observed only in 30~40% of dnmt1-deficient

embryos. Authors would need to reconsider the mechanism upon obtaining such data.

Response 2. We thank this reviewer for pointing out this important issue. Previous studies demonstrated that the randomized left-right asymmetry in zebrafish is caused by the disrupted fluid flow in KV and that the deficiency of DFCs also results in abnormal KV formation and ciliogenesis (Blum et al., 2014; Matsui and Bessho, 2012; Raya and Izpisua Belmonte, 2006). In our work, we have demonstrated that deficiency of *dnmt1* leads to decreased number of DFCs, thereby disrupting ciliogenesis and fluid flow afterwards. Therefore, we reasoned that the defective DFC specification is the primary reason for the left-right (LR) defects in *dnmt1*-deficient embryos. Meanwhile, to evaluate the disrupted KV fluid flow and randomized cilia motility, we examined more embryos (5 control embryos and 5 morphants) as suggested. The track diagram showed that in control embryos, beads followed the counterclockwise manner; on the contrary, beads moved randomly in morphants (see still images below and new Supplemental movie S1-2).

Of note, the defective nodal flow and the following left-right gene expression defects would lead to randomization of L-R asymmetry of internal organs including heart, liver and pancreas, therefore the observed ratio of normal vs abnormal LR asymmetry is often 50%:50%, which is different from that observed in conventional gene KO phenotypes that usually are fully penetrant, *i.e.*, 100%.

(Figures for referees not shown)

3) Hypomethylation was detected at the nearly 20 kb upstream of lefty2 gene. Bisulfite analysis data (Fig. 4D, Fig. 6C) are convincing. Authors believe that hypomethylated regions of lefty2 are located "at the lefty2 enhancer" (page 8). Is there any evidence supporting that the enhancer is located there? If so, what is the specificity of the enhancer: active in what cell-type(s) and at what stage? How does upregulation of lefty2 result in reduction of the number of DFCs? If previous data not available, it is necessary to test if the hypomethylated regions possess enhancer activity perhaps in gastrulating zebrafish embryos.

Response 3. This is a critical point. To determine the regulatory potential of this region *in vivo*, we set up a transient expression system using the EGFP reporter construct that consists of this *lefty2* enhancer and HSP70 minimal promoter (to ensure minimal activity). After one-cell stage injection, we found that the GFP expression fully recapitulates the endogenous *lefty2* expression in the anterior axial hypoblasts at gastrulation stage (See Figure 5B and below, left panel). Importantly, the *lefty2* reporter activity was further increased upon *dnmt1* knockdown at 50% epi stage (Figure 5D and below, right panel), due to hypomethylation of this *lefty2* enhancer upon *dnmt1* knockdown, strongly supporting that *lefty2* is a direct target of Dnmt1. Previous data demonstrated that the DFC number increases or decreases in response to enhanced or reduced Nodal signaling, respectively. Lefty2 is the well-known Nodal antagonist and overexpression of Lefty2 strongly decreases the DFC number through reduced Nodal signaling to prevent dorsal surface epithelial (DSE) cells transforming to DFCs (Choi et al., 2007; Oteiza et al., 2008). In this work, we showed that Nodal signaling was decreased in *dnmt1*-deficient embryos (See Fig 4C and S5E-F). Collectively, these findings indicated that Dnmt1 methylates the *lefty2* enhancer to inhibit *lefty2* expression in DFCs, and then regulates DFC specification through Nodal signaling.

(See Fig 5B and 5D in the article)

Referee #2:
General summary:
The manuscript by Wang et al provides evidence that DNA methylation is essential for the establishment of the left-right asymmetric body plan during vertebrate development. Their data

suggests that morpholino depletion of the DNA methyltransferases dnmt1 or dnmt4 in zebrafish and Xenopus leads to dorsal forerunner cell (DFC) specification defects, laterality organ malformation, and a disorder of LR patterning in zebrafish embryo. Mechanistically, the authors link these defects to hypomethylation of the lefty enhancer, leading to upregulation of lefty and repression of nodal signaling.

A link between DNA methylation and body axis is of interest, however, there are a currently a number of controls lacking from this manuscript that are necessary to support its conclusions.

Response 4. Thank you for your appreciation of and your critical comments on our work. We have performed a number of critical experiments, including rescue experiments (using modified mRNAs of *dnmt1* or of *dnmt3bb.1* for injection), *dnmt1* second morpholino-injection and generation of a new *dnmt4* mutant to validate the phenotypes as detailed in the revised manuscript and below.

Major:
1) The authors provide data indicating that abnormal heart jogging is observed in dnmt1 morphant zebrafish, but indicate that this phenotype is not recapitulated in dnmt1 mutant zebrafish. They suggest that this discrepancy is due to maternal deposition of dnmt1, which is a reasonable potential explanation. However, in the event that mutants cannot be used for analysis, minimal standards for morpholino use in zebrafish require demonstration of phenotype rescue by co-injection of morpholino resistant mRNA encoding the targeted gene. mRNA rescue of dnmt1 morpholino phenotypes has been published (Rai et al 2006), indicating that this control is feasible. It must be done for all experiments. Ideally, recapitulation of key phenotypes with a second morpholio targeting dnmt1 should also be performed.

Response 5. We thank this reviewer for pointing out this critical point. We generated *dnmt1* mis-mRNA (with the mutated atgMO target sequence without changing amino acid coding) and co-injected it with *dnmt1* MO into the 1-cell stage embryos. And the randomized organ laterality and DFC defects in *dnmt1* morphants were efficiently restored (Figure S2B; Figure S3C-D). Furthermore, a *dnmt1* splice-blocking MO was also used, and its specificity was validated by Western blotting (Figure S2C). The abnormal cardiac jogging and reduced DFCs were also found in embryos injected with *dnmt1* splice MO (Figure S2D; Figure S3B), supporting that the disrupted organ laterality was caused by *dnmt1* knockdown specifically.

2) Similar comments to those above apply to the use of dnmt4 and lefty morpholinos in this manuscript. mRNA rescue or corroborating mutant phenotypes are required to support all morpholino claims. Dnmt4 is only reported to be maternally deposited at low levels in the zebrafish embryo, and mutants may be viable due to the diversification of dnmt3 orthologs in zebrafish. Given the ease of genome editing in zebrafish the authors should generate and test dnmt4 mutants.

Response 6. We thank this reviewer for pointing out this important issue. We generated the modified *dnmt3bb.1* mRNA (*dnmt3bb.1* mis-mRNA) and this mis-mRNA injection partially restored the abnormal laterality and disrupted DFC clustering in *dnmt3bb.1* morphants (Figure S2F; Figure S6B). To further confirm these results, we generated a *dnmt3bb.1* mutant using CRISPR/Cas system. The defects of DFC clustering and organ laterality were readily observed in *dnmt3bb.1* mutant (see below and Figure S2G-H and S6A), consistent with the morphant phenotype.

(See Fig EV2G, 6C and EV2H in the article)

3) Previous studies have indicated that 5hmC is not detected by IF until after the tail bud stage (Almeida et al 2012) and RNA-seq data from Harvey et al 2013, suggest none of the three tets are expressed at stages through 90% epibioly. The authors suggest that there may be low levels of these genes/5hmC prior to this stage in a cell context dependent manner. However, since their data is in contrast to existing reports, Q PCR and 5hmC quantification on sorted DFCs should be performed to further demonstrate their presence in DFCs. Data indicating reduced 5hmC in DFCs in tet1/2/3

morphant embryos (or even better in mutants) should also be provided for evaluation (currently indicated as data not shown)

Response 7. The levels of 5hmC or *tet1/2/3* are indeed very weak at early gastrulation stages in general, however, they are detectable in the DFCs from 50% epiboly stage and onwards. We have shown this by using 5hmC immunofluorescence and qPCR with the sorted DFCs (see below, *dnmt1* as positive control). In addition, after *tet1-3* KD, the level of 5hmC in the DFCs was decreased compared to controls (see below).

However, to streamline the main story on DNA methylation on LR asymmetry and to avoid confusion, we have removed the 5hmC and Tet part in the revised manuscript.

(Figures for referees not shown)

8) Tet1 and Tet 2 mutant zebrafish lines are viable and fertile and available (Li et al 2015). The authors should use these lines to demonstrate mitigation of the dnmt1 morphant phenotype rather than co injecting tet1/tet2 moropholinos.

Response 8. Thank you for your suggestion. To streamline the main story on DNA methylation on LR asymmetry and to avoid confusion, we have removed the 5hmC and Tet part in the revised manuscript.

Minor:
1) It would be useful for the authors to use a molecular approach such as a 5mC dot blot to quantify the extent of 5mC loss in total DNA in dnmt1 morpholino injected embryos. This will help with interpretation of results from Me-Dip experiments.

Response 9: We have performed the LS-MS assay to determine the 5mC level in treated embryos, and as shown below, the 5mC levels were decreased in *dnmt1* morphants as well as in 5-AZA treated embryos, compared to controls.

(See Appendix Fig S1)

2) The experiment in Figure 1D doesn't make sense to me. Virtually all cells have 5mC. What is the significance of showing that sox17 positive DFCs have this mark?

Response 10. Thank you for your comments. We agreed that 5mC is broadly distributed during early embryogenesis in general, however, our IF and WISH results indicated that 5mC is clearly detectable while *dnmt1* and *dnmt3bb.1* are relatively enriched in the *sox17*+ DFCs during gastrulation (Figure 1D and S1D), indicating a possible and direct role of DNA methylation in this cell population.

Writing:
1) In the introduction the authors state that "In vertebrates, after fertilization, the embryo shows a lower level of DNA methylation until early cleavage stages and the methylation level increases gradually since then (Jiang et al, 2013; Potok et al, 2013; Wang et al, 2014). While this sentence is technically true, in reality methylation dynamics are very different in zebrafish and mammalian embryos. A more faithful discussion of methylation would be of use to readers.

Response 11. We have revised this part as 'In vertebrates, after fertilization, the embryo shows a relatively stable (in zebrafish) or lower level (in mice) of DNA methylation until early cleavage stages and the methylation level increases gradually since then (Jiang et al., 2013; Potok et al., 2013; Wang et al., 2014)' to avoid confusion, please see Page 3, 1st paragraph, in red.

2) Since dnmt1 is a maintenance methyltransferases and dnmt4 is a de novo methyltransferases, it is not clear why there should be synergism in phenotype when the two morpholinos are coinjected (either should eliminate methylation). Can the authors elaborate?

Response 12. Our results demonstrated that dynamic DNA methylation is essential for DFC cluster development. In particular, Dnmt1 regulates the specification of DFCs at early gastrulation, whereas Dnmt3bb.1 modulates the cohesiveness of DFCs to ensure collective migration once DFCs are formed.

3) The authors need to be more clear about what experiments are in fish and what are in frogs.

Response 13. Thank you for your suggestion. We have revised the text to clearly describe the fish and frog experiments.

4) Loss of methylation at the lefty enhancer in morphant embryos doesn't really demonstrate that this gene is a direct target of dnmt1 in DFC specification. It seems likely, but this methylation change could be reflective of changes in transcription caused by regulation of something upstream.

Response 14. Thank you for raising this critical point. To demonstrate that this hypo-methylation of lefty enhance is indeed caused by *dnmt1* deficiency directly, we cloned the enhancer region and generated a reporter construct. As shown in Figure S5E-F and below, this enhancer fully recapitulates the endogenous expression of *lefty2*. Next, we knocked down *dnmt1*, and the *lefty2* reporter activity was further increased as shown by GFP intensity at 50% epiboly stage, supporting that upregulation of *lefty2* enhancer activity is due to hypomethylation induced by loss of *dnmt1*. Please also see Response 3.

(See Fig 5B and 5D in the article)

Referee #3:

This manuscript uses zebrafish (and Xenopus in a few experiments) to investigate the role of the DNA methlytransferases Dnmt1 and Dnmt4 in left-right patterning. It was found that morpholinos (MO) designed to target Dnmt1 or Dnmt4 disrupt dorsal forerunner cells (DFCs) and subsequent left-right asymmetry. Further experiments show Dnmt1 MO causes upregulation of lefty2 and Dnmt4 MO causes downregulation of E-cadherin in DFCs. The identification of DNA methylation targets that impact left-right patterning is novel and of great interest. However, I have significant concerns about this paper in its current form. First, nearly all results are obtained using MO knockdowns with no controls for off-target effects. Second, the authors arrive at conclusions without considering alternative interpretations of their data.

Response 15. Thank you for your critical comments. We have performed a number of critical experiments, including rescue experiments (using modified mRNAs of *dnmt1* or of *dnmt3bb.1* for injection), *dnmt1* second morpholino-injection and generation of a new *dnmt4/ dnmt3bb.1* mutant to validate the phenotypes as detailed in the revised manuscript and below.

My detailed comments:
Major issues:
1. All MO experiments (fish and frog) lack confirmation of protein knockdown (IHC or western blots) and mRNA co-injection rescue data. MOs can be useful tools, but require rigorous controls due to the well-described potential for MO off-target effects. For example, it is important to test whether co-injecting Dnmt1 mRNA with Dnmt1 MO will rescue MO phenotypes. Several groups have successfully injected mRNA into DFCs for rescue experiments (see examples: Clement, et al. Development. 138 (2), 291-302 (2011); Caron, et al. Development 139, 514-524 (2012)).

Response 16. We thank this reviewer for this critical point. To test the specificity of MOs, we generated *dnmt1* and *dnmt3bb.1* mis-mRNA (with the mutated atgMO target sequence without changing amino acid coding) and co-injected them with MOs (*dnmt1* or *dnmt3bb.1*) into the 1-cell stage embryos, respectively. As expected, overexpression of mis-mRNA restored the randomized organ laterality and DFC defects in *dnmt1* or *dnmt3bb.1* morphants (Figure S2B, F; Figure S3C-D; Figure S6B). Furthermore, a *dnmt1* splice-blocking MO was also used, and its specificity was validated by Western blotting (Figure S2C). The abnormal cardiac jogging was also found in embryos injected with *dnmt1* splice MO (Figure S2D). Finally, a *dnmt4/ dnmt3bb.1* mutant was generated to further confirm the LR laterality and DFC clustering defects found in morphants (Figure S2F-H; Figure S6).

2. Instead of using rescue experiments to address specificity, the authors injected Dnmt1 MO into Dnmt1 mutants that do not show left-right patterning defects. The authors conclude that a low dose Dnmt1 MO reduces maternal expression and uncovers organ laterality defects in homozygous mutants. It would be useful to use Dnmt1 antibodies (IHC or western blots) to test this directly. It is not described how embryos were determined to be homozygous mutants or siblings. Also, only left-right asymmetry of organs (liver, pancreas) were examined in 4 day old embryos (Fig. S2). Analysis of earlier events, including the DFCs, in MO injected homozygous mutants would strengthen the argument that DFC phenotypes are specific rather than due to off-target effects. However, there is still the caveat that these are 'MO-induced phenotypes' in the mutant background. It should be clarified whether MZ-Dnmt1 mutants (lacking both maternal and zygotic Dnmt1 function) have left-right asymmetry and DFC defects.

Response 17. We thank this reviewer for these thoughtful comments.
1) We have examined the DFCs in MO-injected homozygous and sibling embryos, and found that the expression of *sox17* showed no obvious differences in DFCs among wildtype siblings, heterozygous and homozygous embryos. Then, we used a low dose of *dnmt1* MO injection into *dnmt1* homozygous mutant, which significantly reduced *sox17* expression, while it was unaltered in MO-injected wildtype siblings (see new Figure S3E).

2) We performed genomic typing PCR and HincII digestion to distinguish the *dnmt1* homozygous mutants from wildtype siblings.

(Figures for referees not shown)

3) Since the *dnmt1* mutant embryos cannot survive to adulthood, we thus generated the HSP70-driven *dnmt1*-GFP construct and injected it into *dnmt1* mutants with attempt to generate MZ-Dnmt1 mutants. However, we were not able to obtain living MZ-*dnmt1* mutants for reasons yet unknown.

3. No attempt was made to validate Dnmt4 MO results.

Response 18. We thank this reviewer for pointing out this important issue. We generated the modified *dnmt3bb.1* mRNA (*dnmt3bb.1* mis-mRNA) that partially restored the abnormal laterality and disrupted DFC clustering in *dnmt3bb.1* morphants (Figure S2F; FigureS6B). To further confirm these results, we generated a *dnmt4/dnmt3bb.1* mutant using CRISPR/Cas9 system. The defects of DFC clustering and organ laterality were readily observed in *dnmt4/dnmt3bb.1* mutant (see Figure S2G-H and S6A), consistent with the morphant phenotype.

4. Dnmt1 MO was found to reduce the number of DFCs. Taking a candidate approach, it was found that Dnmt1 MO increased lefty2 expression and decreased Nodal target genes. The authors conclude from these experiments that the reduced number of DFCs is due to defects in DFC specification. However, it is possible that dysregulation of Nodal targets (or potentially many other genes) leads to reduced DFC number by affecting proliferation or apoptosis rates. DFCs are specified between sphere (4 hpf) and 50% (5.3 hpf) stages. Yet, in this study, DFCs are first analyzed at 60% epiboly stage (6.5 hpf). It would be helpful to analyze the number of sox17:GFP+ DFCs at earlier

(specification) stages between 4-5 hpf to determine if there is a difference between Dnmt1 MO and control.

Response 19. Thank you for asking these important questions.

1) We examined our MeDIP data and found that methylation of regulatory regions of Nodal target genes such as *southpaw*, *gata6*, *ephrinB2* and *lefty1* was slightly altered but not significant, statistically. In addition, we did notice that there was more apoptosis in the DFCs of *dntm1* morphants, compared to controls; whereas there was no discernable difference on the cell proliferation in DFCs (see below and data not shown).

(See Appendix Fig S4)

2) As suggested, we analyzed the number of *sox17*:GFP+ DFCs at shield stage and found that the number of these cells was significantly decreased and we have added these important data in the revision (See Figure 3E and Figure S3D)

5. Dnmt4 MO reduces cdh1 mRNA levels, but protein levels (IHC) in DFCs should also be measured. Testing whether cdh1 mRNA injection rescue Dnmt4 MO defects in DFCs would strengthen the authors' conclusion that reduced cdh1 is responsible for the DFC clustering defect. Does Dnmt4 MO also result in upregulated lefty2? It appears lefty2 over-expression is sufficient to cause a DFC clustering defect (Fig. S4D) that is similar to cdh1 MO (Fig. 5D).

Response 20. We thank this review for these thoughtful comments.

1) We have performed immunofluorescence of Cdh1 in DFCs. The weak IF staining by using anti-Cdh1 antibody was not able to distinguish the differences between control and *dnmt4/dnmt3bb.1* morphants in our hands. In addition, although we have tried several times, but it was difficult to collect sufficient number of DFCs to perform Western blotting. Therefore, we examined the *cdh1* mRNA level, which was deceased in *dnmt4* morphants (Figure 6C).
2) We thank the review for pointing out the rescue experiments. Human *cdh1* mRNA was overexpressed in *dnmt4/dnmt3bb.1* MO-injected embryos and we found that human *cdh1* mRNA partially restored the defects of DFC clustering and organ laterality (see Fig 5G-H).
3) q-PCR results showed that the expression of *lefty2* was unaltered in *dnmt4/dnmt3bb.1*-injected embryos.

(Figures for referees not shown)

4) The main defects in *lefty2*-overexpressing embryos were the decreased number of DFCs and we have replaced the images in Figure S5C with more representative ones.

6. It would be helpful to know whether 5-AZA treatments alter lefty2 and/or cdh1 expression as observed in MO injected embryos.

Response 21. q-PCR results showed that the expression of *lefty2* was upregulated, while *cdh1* expression was decreased in 5AZA-treated embryos, consistent with the MO-injection results.

(See Appendix Fig S5C)

Minor issues:
1. The Dnmt nomenclature used in this paper (Dnmt1-8) is simple and easy to follow, but not consistent with current gene nomenclature for this gene family found at https://zfin.org. It should be described somewhere in the paper (main text or materials and methods section) what the current zfin name is for each of the Dnmt genes tested in this study.

Response 22. We have replaced *dnmt4* with *dnmt3bb.1* in the revised manuscript throughout.

2. Dnmt4 MO sequence must be reported.

Response 23. We have added this MO information into Methods in the revised manuscript. Please see Page 14, in red.

3. Use of English language is poor in many places, which results in confusing statements. The writing should be edited for accuracy and clarity.

Response 24. The revised manuscript has been edited by a professional language editor to ensure accuracy and clarity.

References

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2nd Editorial Decision 28 July 2017

Thank you for submitting a revised version of your manuscript, which has now been seen by all original referees. While they find that most of their main concerns have been addressed, they point out a few remaining issues that have to be resolved before acceptance here. Therefore I would like to invite you to submit a final revised version of the manuscript addressing the remaining concerns of all referees. In particular, please add the statistical analysis as requested by reviewers #1 and #2, and include the data presented in the point-by-point response in the manuscript (requested by reviewers #2 and #3), as this information would strengthen the manuscript.

Please let me know if you have any further questions regarding the revision. You can use the link below to upload the revised version.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to seeing the final version.

REFEREE REPORTS

Referee #1:

The authors have satisfactorily responded to three concerns that I raised previously. As far as this reviewer is concerned, the paper can be accepted after the following minor revision.

Minor comment
1) Please provide the number of the embryos examined in Fig. 5B, and 5D. Activity of a transgene can vary in this kind of assay. To compare the activity of two constructs, we need to have multiple transgenic embryos.

Referee #2:

The revised manuscript by Wang et al is much strengthened compared to the original, however there are still some significant concerns.

The authors now provide important mRNA rescue controls for morpholino experiments, however in some cases the rescue appears mild and no numbers are provided to demonstrate that the rescues are statically significant. Such statistics are essential given the importance of these controls. Assuming the data are significant they should be moved into the main figures of the paper as they represent essential controls, and are in no way supplemental.

In addition to this major concern, there are also some additional points of concern.

First, the authors have removed tet data from the results section, but still make references to this data in the abstract, introduction and conclusion.

Second, the authors have converted Dnmt4 to the Dnmt3bb.1 nomenclature, but have not carried this nomenclature through when referring to other Dnmt3 family members

Third, the authors should include some discussion of how their work in the context of the recent publication TET-mediated DNA demethylation controls gastrulation by regulating Lefty-Nodal signaling
Hai-Qiang Dai, Bang-An Wang, Lu Yang, Jia-Jia Chen, Guo-Chun Zhu, Mei-Ling Sun, Hao Ge, Rui Wang, Deborah L. Chapman, Fuchou Tang, Xin Sun & Guo-Liang Xu

Finally, the authors appear to provide some data in answer to reviewer comments (for instance LS-MS assay of methylation) that they don't provide in the text. Data used to convince reviewers should also be provided to readers at least as supplemental.

Referee #3:

The authors have made significant additions and revisions that address my concerns and have strengthened this manuscript. I find the increased levels of apoptosis in DFCs (see response 19) quite interesting and an important finding. This suggests mechanistically that Dnmt1 is regulating DFC specification and/or survival. These data, along with DFC proliferation results, should be included in the paper. In addition, the genotyping methodology (response 17) should be included in the materials and methods. Finally, the English writing is still confusing in many places, particularly in the introduction section. I have pasted below some sentences I suggest should be edited for accuracy and clarity.

Summary section:
1. "DNA methylation is a major epigenetic modification; however, the precise role of this modification during development of complex organisms is not well understood."

2. "Mechanistically, enhancer hypomethylation of lefty2 gene induced by the dnmt1 deficiency upregulates lefty2 expression, which consequently represses Nodal signaling in zebrafish."

3. "Importantly, additional knockdown of ten-eleven translocation family of methylcytosine dioxygenases (tet1-3) leads to a partial restoration of LR defects in dnmt1-deficient embryos." [These data have been removed.]

Introduction:
1. "... methylation level increases gradually since then."

2. "Concurrently, various development processes start after the cleavage stage (Kimelman, 2006)."

3. "This left-right (LR) asymmetry is first established by symmetry breaking, and then followed by laterality organizer formation, i.e., the node in mammals and the Kupffer's vesicle (KV) in zebrafish." [it is generally thought that the laterality organizer is involved in (upstream of) symmetry breaking, not downstream.]

4. "The DFC cluster starts to appear adjacent to the embryonic shield, then migrates to the vegetal pole and forms a rosette-shaped cluster, finally differentiates into ciliated epithelial cells of KV."

5. "Mechanistically, loss-of-dnmt1-induced hypomethylation of lefty2 enhancer increases lefty2 expression, which in turn inhibits Nodal signaling, therefore leading to impaired DFC specification and loss of LR asymmetry."

6. "In addition, Dnmt3bb.1 modulates cadherin 1 (cdh1)-mediated DFC clustering to determine LR determination."

7. "Importantly, additional knockdown of tet1-3 leads to a partial restoration of LR defects in dnmt1-deficient embryos." [These data have been removed.]

Results:
1. "Consistently, cdh1 knockdown specifically in DFCs also caused disrupted actin filament, disaggregation of DFCs as well as the randomized heart positioning based on expression patterns of sox17 and foxj1a in DFCs and the cmlc2:GFP fluorescence imaging, respectively (Figure 6D-F), indicating that Dnmt3bb.1 regulates the cell adhesion of DFCs via cdh1."

Discussion:
1. "Moreover, additional knockdown of tet1-3 leads to a partial restoration of LR defects in dnmt1-deficient embryos."

2nd Revision - authors' response 05 August 2017

Response to the editor's and reviewers' comments
We are very grateful to the reviewers for their insightful comments on our manuscript. We have made all the necessary changes to improve the quality of our manuscript, including English editing, figure reorganization and embryo number statistics. The detailed point-by-point responses to reviewers' comments are shown below.

Comments from Editor: Dr Ieva Gailite
Thank you for submitting a revised version of your manuscript, which has now been seen by all original referees. While they find that most of their main concerns have been addressed, they point out a few remaining issues that have to be resolved before acceptance here. Therefore I would like to invite you to submit a final revised version of the manuscript addressing the remaining concerns of all referees. In particular, please add the statistical analysis as requested by reviewers #1 and #2, and include the data presented in the point-by-point response in the manuscript (requested by reviewers #2 and #3), as this information would strengthen the manuscript.

Response to general comments by the editor: We have added the statistical analysis as requested by Reviewers #1 and #2, please see Response 1 and 2. We have also included the original data in the Response into the revised manuscript as requested by Reviewers #2 and #3.

Please also address the following editorial issues:
1. Please submit up to five keywords.
2. Please rename the "Disclosures" section into "Conflict of Interest".
3. Please ensure that the figures fit into a portrait-oriented page while remaining readable.
4. Figure 7 is not referred to in the text.
5. Please add scale bars to immunofluorescence images.
6. Please update the reference style according to our guidelines (where there are more than 20 authors on a paper, the first 20 should be listed, followed by 'et al.'):
http://emboj.embopress.org/authorguide#referencesformat
7. The manuscript currently contains only supplemental figures. We can accommodate up to five typeset EV figures, which would render the data more easily accessible for online readers. You might consider transforming up to five of the Appendix figures into EV figures. Please see our author guidelines on details about the content and preparation of Expanded View material (http://emboj.embopress.org/authorguide#expandedview).
8. Please zip movie legends with each individual movie and update movie nomenclature to Movie EV1, EV2 etc as described in the author guidelines.

Finally, papers published in The EMBO Journal include a 'Synopsis' to further enhance discoverability. Synopses are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short introductory paragraph followed by 2-5 one-sentence bullet points that summarise the paper. Please send us your suggestions for bullet points and a synopsis image. This image should provide a rapid overview of the question addressed in the study, but still needs to be kept fairly modest, since the image size cannot exceed 550x400 pixels.

Response to specific comments by the editor: We have provided all the required information in the revised manuscript, including key words, figure reorganization, scale bars, reference style, movie legends and the Synopsis.

Response to referees

Referee #1:

The authors have satisfactorily responded to three concerns that I raised previously. As far as this reviewer is concerned, the paper can be accepted after the following minor revision.

Minor comment
1) Please provide the number of the embryos examined in Fig. 5B, and 5D. Activity of a transgene can vary in this kind of assay. To compare the activity of two constructs, we need to have multiple transgenic embryos.

Response 1. We thank this reviewer for pointing out this issue and we have added the number of embryos in Fig 5B and 5D.

Referee #2:

The revised manuscript by Wang et al is much strengthened compared to the original, however there are still some significant concerns.

The authors now provide important mRNA rescue controls for morpholino experiments, however in some cases the rescue appears mild and no numbers are provided to demonstrate that the rescues are statically significant. Such statistics are essential given the importance of these controls. Assuming the data are significant they should be moved into the main figures of the paper as they represent essential controls, and are in no way supplemental.

Response 2. We have provided all the embryo numbers in the rescue data and moved them into the main figures (See Figure 3F,G; Figure 6C)

In addition to this major concern, there are also some additional points of concern.

First, the authors have removed tet data from the results section, but still make references to this data in the abstract, introduction and conclusion.

Response 3. We apologize for this mistake. We have now removed these sentences in the revised main text.

Second, the authors have converted Dnmt4 to the Dnmt3bb.1 nomenclature, but have not carried this nomenclature through when referring to other Dnmt3 family members

Response 4. We have revised the names of other Dnmt3 members in the revised manuscript accordingly.

Third, the authors should include some discussion of how their work in the context of the recent publication TET-mediated DNA demethylation controls gastrulation by regulating Lefty-Nodal signaling
Hai-Qiang Dai, Bang-An Wang, Lu Yang, Jia-Jia Chen, Guo-Chun Zhu, Mei-Ling Sun, Hao Ge, Rui Wang, Deborah L. Chapman, Fuchou Tang, Xin Sun & Guo-Liang Xu

Response 5. We thank this reviewer for pointing out this important issue. We have discussed the similarities and differences between these two studies in the "Discussion" as well as shown below.

A recent study reported that Tet-mediated DNA demethylation modulates Lefty-Nodal signaling during gastrulation and Dnmt3a/b act antagonistically to regulate the DNA methylation level of Lefty genes in this process (Dai *et al*, 2016). In our work, we reveal that DNA methytransferase1, Dnmt1, directly regulates *lefty2* expression to maintain the balanced Nodal signaling during DFC specification, which is an important pattern specification process during gastrulation, whereas another methyltransferase, Dnmt3bb.1 can target *cdh1*-mediated collective DFC migration at the onset of LR determination. Therefore, our work in zebrafish and the work by Dai et al in mice together reveal that dynamic DNA methylation and demethylation are crucial to modulating key signaling pathway during early development from different aspects.

Finally, the authors appear to provide some data in answer to reviewer comments (for instance LS-MS assay of methylation) that they don't provide in the text. Data used to convince reviewers should also be provided to readers at least as supplemental.

Response 6. We have added ELISA data into Appendix Figure S1 and added TUNEL and pH3 data into Appendix Figure S4 in the revised manuscript.

Referee #3:

The authors have made significant additions and revisions that address my concerns and have strengthened this manuscript. I find the increased levels of apoptosis in DFCs (see response 19) quite interesting and an important finding. This suggests mechanistically that Dnmt1 is regulating DFC specification and/or survival. These data, along with DFC proliferation results, should be included in the paper. In addition, the genotyping methodology (response 17) should be included in the materials and methods. Finally, the English writing is still confusing in many places, particularly in the introduction section. I have pasted below some sentences I suggest should be edited for accuracy and clarity.

Response 7. We thank this reviewer for these suggestions. We have added the TUNEL and pH3 data into the revised manuscript (Appendix Figure S4) and added the genotyping method into Materials and Methods. We apologize for the confusing English writing and this revised manuscript has been professionally edited to ensure accuracy and clarity.

Summary section:
1. "DNA methylation is a major epigenetic modification; however, the precise role of this modification during development of complex organisms is not well understood."

Response 8. We have revised this sentence as "DNA methylation is a major epigenetic modification; however, the precise role of DNA methylation in vertebrate development is still not fully understood".

2. "Mechanistically, enhancer hypomethylation of lefty2 gene induced by the dnmt1 deficiency upregulates lefty2 expression, which consequently represses Nodal signaling in zebrafish."

Response 9. We have revised this sentence as "Mechanistically, hypomethylation of the *lefty2* gene enhancer caused by loss-of-*dnmt1* can promote *lefty2* expression, which consequently represses Nodal signaling in zebrafish embryos".

3. "Importantly, additional knockdown of ten-eleven translocation family of methylcytosine dioxygenases (tet1-3) leads to a partial restoration of LR defects in dnmt1-deficient embryos." [These data have been removed.]

Response 10. We have deleted this sentence in the revised version.

Introduction:
1. "... methylation level increases gradually since then."

Response 11. We have revised this sentence as "…then the overall DNA methylation level gradually increases until gastrulation".

2. "Concurrently, various development processes start after the cleavage stage (Kimelman, 2006)."

Response 12. We have revised this sentence as "Various development processes including gastrulation and organogenesis, occur after the cleavage stages".

3. "This left-right (LR) asymmetry is first established by symmetry breaking, and then followed by laterality organizer formation, i.e., the node in mammals and the Kupffer's vesicle (KV) in zebrafish." [it is generally thought that the laterality organizer is involved in (upstream of) symmetry breaking, not downstream.]

Response 13. We thank this reviewer for this comment. As shown in literature (Kawakami *et al*, 2005; Levin *et al*, 2002; Matsui *et al*, 2012), in zebrafish embryos, the initial symmetry breaking may occur at cleavage stages, then an organizer region called Kupffer's vesicle (KV) forms by early somitogenesis. To reflect the notion from the literature and the point by this reviewer, we have revised this sentence as "This left-right (LR) asymmetry is first established likely by symmetry breaking…".

4. "The DFC cluster starts to appear adjacent to the embryonic shield, then migrates to the vegetal pole and forms a rosette-shaped cluster, finally differentiates into ciliated epithelial cells of KV."

Response 14. We have revised this sentence as "The DFC cluster first appears adjacent to the embryonic shield, then migrates to the vegetal pole and forms a rosette-shaped structure, finally differentiates into ciliated epithelial cells of KV".

5. "Mechanistically, loss-of-dnmt1-induced hypomethylation of lefty2 enhancer increases lefty2 expression, which in turn inhibits Nodal signaling, therefore leading to impaired DFC specification and loss of LR asymmetry."

Response 15. We have revised this sentence as "Mechanistically, hypomethylation of the *lefty2* gene enhancer caused by loss-of-*dnmt1* can promote *lefty2* expression, which in turn inhibits Nodal signaling, therefore leading to impaired DFC specification and loss of LR asymmetry".

6. "In addition, Dnmt3bb.1 modulates cadherin 1 (cdh1)-mediated DFC clustering to determine LR determination."

Response 16. We have revised this sentence as "In addition, Dnmt3bb.1 is required for cadherin 1 (cdh1)-mediated DFC clustering to ensure proper LR determination".

7. "Importantly, additional knockdown of tet1-3 leads to a partial restoration of LR defects in dnmt1-deficient embryos." [These data have been removed.]

Response 17. This sentence has been removed.

Results:
1. "Consistently, cdh1 knockdown specifically in DFCs also caused disrupted actin filament, disaggregation of DFCs as well as the randomized heart positioning based on expression patterns of sox17 and foxj1a in DFCs and the cmlc2:GFP fluorescence imaging, respectively (Figure 6D-F), indicating that Dnmt3bb.1 regulates the cell adhesion of DFCs via cdh1."

Response 18. We have revised this sentence as "Consistently, cdh1 knockdown specifically in DFCs also caused defects including disrupted actin filament, disaggregation of DFCs as well as the randomized heart positioning, based on expression patterns of sox17 and foxj1a in DFCs and the cmlc2:GFP fluorescence imaging, respectively (Fig 6D-F). Together, these results indicate that Dnmt3bb.1 regulates the cell adhesion of DFCs most likely via cdh1".

Discussion:
1. "Moreover, additional knockdown of tet1-3 leads to a partial restoration of LR defects in dnmt1-deficient embryos."

Response 19. This sentence has been removed.

Dai HQ, Wang BA, Yang L, Chen JJ, Zhu GC, Sun ML, Ge H, Wang R, Chapman DL, Tang F, Sun X, Xu GL (2016) TET-mediated DNA demethylation controls gastrulation by regulating Lefty-Nodal signalling. ***Nature*** **538:** 528-532

Kawakami Y, Raya A, Raya RM, Rodriguez-Esteban C, Izpisua Belmonte JC (2005) Retinoic acid signalling links left-right asymmetric patterning and bilaterally symmetric somitogenesis in the zebrafish embryo. ***Nature*** **435:** 165-171

Levin M, Thorlin T, Robinson KR, Nogi T, Mercola M (2002) Asymmetries in H+/K+-ATPase and cell membrane potentials comprise a very early step in left-right patterning. ***Cell*** **111:** 77-89

Matsui T, Bessho Y (2012) Left-right asymmetry in zebrafish. ***Cell Mol Life Sci*** **69:** 3069-3077

3rd Editorial Decision 10 August 2017

Thank you for submitting a revised version of your manuscript. I'm sorry to trouble you again, but unfortunately there remain a couple of editorial issues with the revised version that have to be fixed before we can process the manuscript further.
- Please include data on dnmt3bb.1 mutant laterality defects (Response 6C from previous response letter) and lefty2 and cdh1 expression after 5AZA treatment (Response 21) in the manuscript.

3rd Revision - authors' response 11 August 2017

Authors made requested editorial changes.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Feng Liu
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2017-96580R

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No statistical methods were used to predetermine sample size.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	No statistical methods were used to predetermine sample size
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data were excluded from the analyses.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	The experiments were not randomized.
For animal studies, include a statement about randomization even if no randomization was used.	The experiments were not randomized.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	The investigators were not blinded to allocation during experiments and outcome assessment.
4.b. For animal studies, include a statement about blinding even if no blinding was done	The investigators were not blinded to allocation during experiments and outcome assessment.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, Student's t-test was used for statistical comparisons and data are shown as mean ± s.d..
Is there an estimate of variation within each group of data?	Student's t-test was used for statistical comparisons and data are shown as mean ± s.d..
Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

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http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	anti-5Mc, Active Motif, 39649 anti-acetylated tubulin, Sigma, T6451 anti-Dnmt1, Santa Cruz Biotechnology, sc-20701
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Zebrafish strain including AB, Tg(sox17:eGFP), Tg(fabp10:dsRed, ela3l:GFP)gz12; Tg(ins:dsRed)m1081, dnmt1s872 and Tg(cmlc2:GFP) transgenic lines
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	This study was approved by the Ethical Review Committee of Institute of Zoology, Chinese Academy of Sciences, China.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We confirm compliance for this study.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The GEO accession number for the MeDIP and RNA-seq data in this study is GSE93927.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	