Setdb2 restricts dorsal organizer territory and regulates left–right asymmetry through suppressing *fgf8* activity

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Dorsal organizer formation is one of the most critical steps in early embryonic development. Several genes and signaling pathways that positively regulate the dorsal organizer development have been identified; however, little is known about the factor(s) that negatively regulates the organizer formation. Here, we show that Setdb2, a SET domain-containing protein possessing potential histone H3K9 methyltransferase activity, restricts dorsal organizer development and regulates left–right asymmetry by suppressing *fibroblast growth factor 8* (*fgf8*) expression. Knockdown of Setdb2 results in a massive expansion of dorsal organizer markers *floating head* (*flh*), *goosecoid* (*gsc*), and *chordin* (*chd*), as well as a significant increase of *fgf8*, but not *fgf4* mRNAs. Consequently, disrupted midline patterning and resultant randomization of left–right asymmetry are observed in Setdb2-deficient embryos. These characteristic changes induced by Setdb2 deficiency can be nearly corrected by either overexpression of a dominant-negative *fgf* receptor or knockdown of *fgf8*, suggesting an essential role for Setdb2–Fgf8 signaling in restricting dorsal organizer territory and regulating left–right asymmetry. These results provide unique evidence that a SET domain-containing protein potentially involved in the epigenetic control negatively regulates dorsal organizer formation during early embryonic development.

set domain | histone methylation | epigenetics | embryonic development | zebrafish

Under bilateral external symmetry, vertebrates conserve a left–right asymmetry placement of internal organs and nervous system. By using mouse, chicken, *Xenopus*, and zebrafish as animal models, the developmental and molecular mechanisms of how left–right asymmetry is established during embryogenesis have been well characterized in the past few years: two embryonic structures—the node [also known as Kupffer's vesicle (KV) in zebrafish] and the midline, mainly consisting of notochord and floorplate—are considered essential for maintaining the left–right asymmetry (1, 2). Inside the KV, cilia are specifically organized to generate a counterclockwise fluid flow, by which the first laterality signal is propagated to the left lateral plate mesoderm (LPM), resulting in an asymmetric gene expression on the left side (3, 4). The *southpaw* (member of *nodal*-related subfamily of TGF-β superfamily) is the earliest known gene that is asymmetrically expressed in the left LPM at 10–12 somite stage to initiate the left–right signaling through inducing its downstream targets *lefty2* and *pitx2* in the left LPM, and *lefty1* in the axial midline that acts as a molecular barrier to prevent the left-sided signals from leaking to the other side. This signaling cascade is highly conserved during vertebrate evolution (5).

It has been shown that the KV is originated from the dorsal forerunner cells (DFCs), a group of noninvoluting cells at the leading edge of the embryonic dorsal organizer or shield, which also produces the midline/notochord (6, 7). Eighty-five years ago Spemann and Mangold originally identified the dorsal organizer in amphibian through transplanting the dorsal lip of blastopore to the ventral region of a host embryo, which led to a secondary axis formation. Since then, the dorsal organizer has been well studied in many systems including *Xenopus* and zebrafish, and it has been found that many inducers released by the dorsal organizer are essential for patterning the embryonic midline (8, 9) and that developmental defects in the midline are usually accompanied by KV abnormality and disturbed left–right asymmetry (10, 11). Genes or signaling transduction pathways that positively regulate the formation of the dorsal organizer have been identified, including homeobox genes *dharma/bozozok* and *iroquois3* and the T-box gene *eomesodermin* (12–14), as well as Wnt/β-catenin signaling transduction pathway (15). Despite these progresses, little is known about the negative regulator of dorsal organizer development during embryogenesis (8, 9).

It has been shown that epigenetic mechanisms involving histone methylation play a critical role in establishing and maintaining heritable programs of gene expression during cellular differentiation and early embryonic development (16, 17). A family of histone methyltransferases (HMT) that catalyzes histone methylations at lysine residues contains a SET domain, which was originally identified in the members of the Su(var) family, polycomb group (PcG), and trithorax group (trxG) and was named after the genes *Su(var)3–9*, *Enhancer of Zeste* [*E(z)*], and *trithorax (trx)* (18). Although SET domain-containing proteins and histone methylations have been implicated in many embryonic developmental processes, the evidence of involvement of SET domain-containing genes in regulating dorsal organizer formation and left–right asymmetry is still lacking. In this study, we report that a SET domain-containing protein Setdb2 possesses potential transcriptional repression activity through catalyzing trimethylation at histone H3 lysine 9 (H3K9me3) and negatively regulates dorsal organizer formation by suppressing the expression of *fibroblast growth factor 8* (*fgf8*), a gene that has been shown to be involved in organizer induction, cilia formation, and left–right asymmetry (19–21).

Results

Setdb2 Mediates H3K9me3 Histone Methyltransferase Activity in Vivo. The zebrafish *setdb2* (*SET domain bifurcated 2*) gene was originally identified in our large-scale sequencing database and

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genomewide survey and developmental expression mapping of zebrafish SET domain-containing genes (22, 23). Zebrafish Setdb2 protein exhibits 32% amino acid identities to mouse and human counterparts and 34% identity to *Xenopus* (Fig. S1). Two remarkably conserved regions were observed: a methyl-CpG binding domain (MBD) and a bifurcated SET domain with an adjacent pre-SET domain (Fig. 1*A* and Fig. S1). The MBD domain usually coexisting with additional domains (bromodomain, SET domain, and PHD finger) has been reported to specifically bind to the methylated CpG dinucleotides, resulting in chromatin compaction and transcriptional repression (24). The SET domain has been shown playing an essential role in regulating gene transcription through catalyzing methylation of the lysine residues of histones, with the exception of only H3 lysine 79 (23). The structural features indicate that the Setdb2 protein may function as a transcriptional regulator through its MBD and SET domains.

If Setdb2 protein was involved in the histone methylation at lysine residues, one would expect that the histone methylation levels might be affected by Setdb2 deficiency. To test this hypothesis, we first designed two morpholino oligonucleotides (MOs), MO1 and MO2, to target the 5′-UTR and the sequences surrounding ATG of *setdb2*, respectively (Fig. 1*A*). To test the knockdown efficiency and specificity of these two morpholinos, we coinjected 8 ng of either MO1, 5-mismatch MO1 control, or MO2 with 400 pg of *EGFP* mRNA reporter containing MO-targeting sequences into one-cell-stage embryos. The results indicate that both MO1 and MO2 morpholinos were able to efficiently and specifically block the translation of the *EGFP* reporter (Fig. S2).

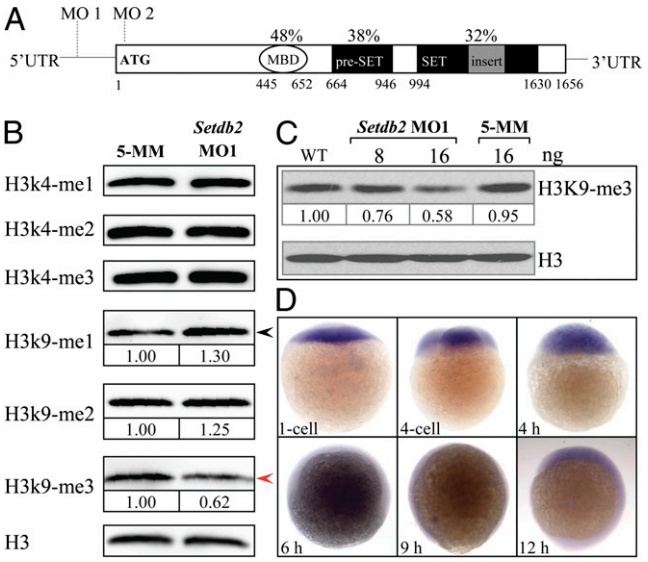

Fig. 1. Setdb2 is a maternally expressed gene in zebrafish and possesses H3K9me3 activity in vivo. (*A*) Evolutionarily conserved domain architecture of zebrafish Setdb2 protein. The Setdb2 protein is composed of a methylated DNA binding domain (MBD), a pre-SET domain, and a bifurcated SET domain. The MO1 and MO2 indicate the positions of sequences targeted by the morpholino oligonucleotides. The percentages indicate the amino acid identities between human and zebrafish Setdb2. The Arabic numbers denote the position of each domain. (*B*) Western blot analyses of histone methylation levels in the control and the Setdb2-deficient embryos at 10 hpf. Red and black arrows denote a reduction and an increase of the level of H3K9me3 and H3K9me1, respectively. (*C*) Dose-dependent reduction of H3K9me3 level in the embryos injected with indicated amounts of *setdb2* morpholino. (*D*) Spatial and temporary expression of *setdb2* during early embryonic development. The Arabic numbers at the *Bottom* denote the extent of methylation modification compared with histone H3 level by densitometry.

Next, we performed Western blot analyses to determine the levels of histone 3 methylations with a panel of antibodies against methylated H3 (mono-, di-, and trimethylations of H3K4, H3K9, and trimethylation of H3K27) in the protein lysates extracted from 10-hours postfertilization (hpf) embryos injected with 8 and 16 ng of either *setdb2* MO1 or 16 ng of 5-mismatch (5-MM) control morpholino oligonucleotides. The results showed that the level of H3K9 trimethylation was significantly reduced in a dose-dependent fashion (Fig. 1*B*, red arrow, and Fig. 1*C*), accompanied by a concomitant 1.3-fold increase in the level of H3K9 monomethylation in the Setdb2-deficient embryos (Fig. 1*B*, black arrow). No changes in the levels of other H3 methylations were detected (Fig. 1*B*). These results suggest that the Setdb2 possesses H3K9me3 activity and may function as a negative transcriptional regulator of gene expression in vivo (25).

Setdb2 mRNA Is Maternally Provided and Ubiquitously Expressed Throughout Embryogenesis. To examine the spatial and temporal expression of *setbd2* during embryogenesis, whole-mount mRNA in situ hybridization (WISH) assays were performed using digoxigenin-labeled antisense RNA probe. The *setdb2* expression was readily detected between one-cell stage and 18 hpf (Fig. 1*D* and Fig. S3*A*). By 24–120 hpf, *setdb2* mRNA expression was faintly observed (Fig. S3*A*), which is consistent with the semiquantitative RT-PCR results (Fig. S3*B*). The results suggest that Setdb2 might play a role in early embryonic development.

Knockdown of Setdb2 Randomizes Visceral and Diencephalic Asymmetry. During the processes of analyzing the molecular and morphological phenotypes in Setdb2-deficient embryos, we unexpectedly found randomization of heart laterality as evidenced by WISH assay using *cmlc2* as a probe at 30 hpf (Fig. 2 *A* and *B*). Approximately 26 and 29% of the MO1- and MO2-injected

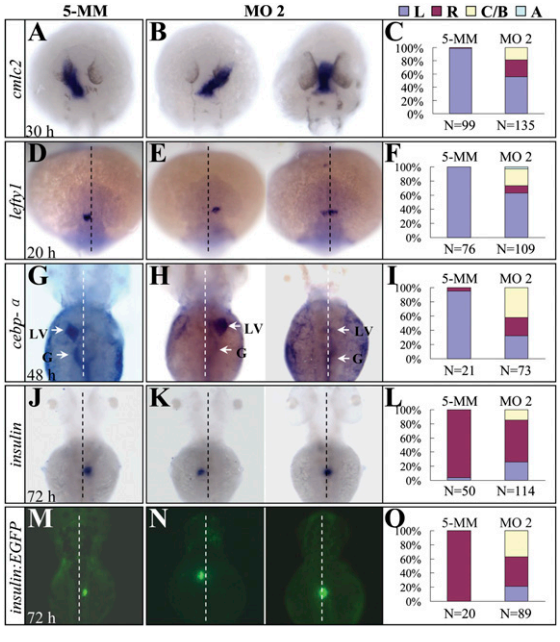

Fig. 2. Organ laterality is randomized in *setdb2*-knockdown embryos. (*A–L*) Whole-mount mRNA in situ hybridization and statistical analyses of visceral and diencephalic laterality in the 5-MM control and *setdb2* MO2-injected embryos at the indicated developmental stages with probes *cmlc2* (*A–C*), *lefty1* (*D–F*), *cebpα* (*G–I,* arrows), and *insulin* (*J–L*). (*M–O*) Determination of pancreatic laterality in 72-hpf Tg(*insulin:EGFP*) transgenic embryos injected with 5-MM control and *setdb2* MO2. Dashed lines denote the embryonic midline. LV, liver; G, gut; L, *Left* side; R, *Right* side; C/B, central or bilateral; A, absent. All embryos are dorsal views with head to the top.

embryos showed a right-sided heart tube, and 19 and 13% of MO1- and MO2-injected embryos had the heart tube positioned to the ventral midline (Fig. 2 *B* and *C* and Fig. S4*A*). In contrast, only 1.1% of embryos injected with a 5-MM control morpholino developed a right-sided heart tube (Fig. 2 *A* and *C*). Furthermore, the expression of *lefty1*, a well-known gene expressed on the left side of diencephalons at 20 hpf (26), was also randomized in the Setdb2-deficient embryos (Fig. 2 *D–F* and Fig. S4*B*). Consistent with these observations, the expression domain of *cebpa* (denotes left-sided liver and gut at 48 and 72 hpf) and *insulin* (denotes right-sided pancreas at 72 hpf) were also randomized (Fig. 2 *G–L* and Fig. S4 *C* and *D*). The abnormal left-sided location of fluorescent pancreas was also detected in the Tg(*insulin:EGFP*) transgenic embryos injected with *setdb2* MO2 (Fig. 2 *M–O* and Fig. S4*E*). These results indicated that Setdb2 had a global effect on laterality of visceral organs and nervous system.

Effects of Setdb2 Knockdown on Asymmetric Gene Expression. The earliest known molecular event to initiate the cascade of left–right asymmetry is the asymmetric expression of the nodal gene *southpaw* and its downstream targets *pitx2, lefty1*, and *lefty2* (5, 27). We examined the expression of *southpaw*, *pitx2*, and *lefty2* by WISH in the Setdb2-knockdown embryos at 20–22 hpf and found that the asymmetric expressions of all three genes were randomized (Fig. 3 *A–I*). The result suggests that *setdb2* acts upstream of *southpaw* signaling to regulates the left–right asymmetry at a very early developmental stage.

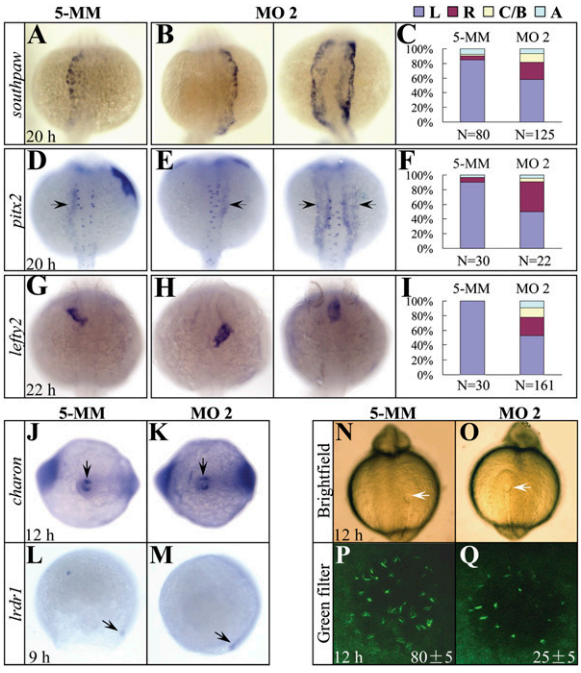

Fig. 3. The expression of asymmetric genes is randomized and cilia development in the Kupffer's vesicle is disrupted in *setdb2*-knockdown embryos. (*A–I*) Whole-mount mRNA in situ hybridization and statistical analyses of the expression of asymmetric genes *southpaw* (*A–C*), *pitx2* (*D–F*, arrows), and *lefty2* (*G–I*) in the 5-MM control and *setdb2* MO2-injected embryos at the indicated developmental stages. L, *Left* side; R, *Right* side; C/B, central or bilateral; A, absent. All embryos are dorsal views with head to the top. (*J–M*) WISH analyses of *charon* (*J* and *K*, arrows) and *lrdr1* expression (*L* and *M*, arrows) in the 5-MM control and *setdb2* MO2-injected embryos. (*N–Q*) Morphology of Kupffer's vesicle (*N* and *O*, arrows) and confocol analyses of ciliary development (*P* and *Q*, green) as revealed by immunostaining with anti-acetylated tubulin antibody. The numbers in *P* and *Q* denote the mean ± SD. Embryos are ventral views in *J, K, N*, and *O* and lateral views in *L* and *M*.

Effects of Setdb2 Knockdown on KV-Derived Cilia and Midline Development. KV is the zebrafish homologous organ of mouse and chicken node, which is a transient embryonic structure located at the end of the notochord and adjacent to the yolk cells between late gastrulation stage and somite stage (4). Previous studies have shown that abnormalities in KV development (4) or defects in the cilia formation and function within the KV disrupt left–right asymmetry (20). In *setdb2* morphants, the morphology of KV was indistinguishable from the control embryos as evidenced by appropriate expression of either *charon*, a gene expressing around KV and essential for left–right asymmetry establishment (28), or *lrdr1* that is required for ciliary motility (4) (Fig. 3 *J–O*, arrows). However, the number of cilium within the KV was significantly reduced in the *setdb2* morphants (25 ± 5 vs. 80 ± 5, n = 5/5; Fig. 3 *P* and *Q*), suggesting a reduction of the number and functional compromise of ciliated cells.

Because the precursors of ciliated cells are a group of ∼20–30 cells known as dorsal forerunner cells (DFCs), which locate at the leading edge of the developing notochord (7), and because the notochord has been known to be an important barrier for preventing the laterality signals from leaking to the other side (1, 2, 29), we performed WISH analyses with several midline markers *oep* (30), *shh* (31), and *ntl* (10) to examine the notochord and midline structures in the *setdb2*-knockdown embryos. The results showed that the midline was shorter and wider in the Setdb2-deficent embryos at 10 hpf, compared to the control embryos (Fig. S5 *A, B, E, F, I*, and *J*, n = 47/60). Interestingly, in ∼10% of Setdb2-deficent embryos, an incomplete secondary axis was observed (Fig. S5 *F* and *J*, *Right*). Furthermore, undulations of the midline were frequently observed between 18 and 28 hpf (Fig. S5 *C, D, G, H, K*, and *L*, n = 85/110). Because two midline genes *ntl* and *shh* were colocalized within the defective midline of Setdb2-deficient embryos (Fig. S6*A*; n = 18/25), and the convergence and extension (CE) movements were also inhibited in the setdb2 morphants as revealed by lateral expansion of *myoD*-positive somites (Fig. S6*B*; n = 20/28), it suggests that the abnormal expression patterns of midline genes are a consequence of defective midline development due to inappropriate CE movement.

Previous studies have shown that several signaling transduction pathways and genes are essential for midline development, CE movements, or subsequent left–right specification (11, 32). We examined the components of noncanonical WNT pathway (*wnt4/5/8/11*, *wnt11r*, *duboraya*, *dvl2*, *dvl3*, *fzd2*, *kny*, *rock2*, and *rhoA*) (32, 33), TGF-β superfamily (*bmp4*, *cyclops*, *squint*, and *smad2*) (30, 34), T-box gene family (*tbx16* and *tbxc*) (35, 36) and other reported genes involved in the regulation of left–right asymmetry such as *alk4*, *ipk1*, *pkd2*, and *pdip5* (37–40) with WISH to determine the expression levels of these genes. No detectable changes were observed between the *setdb2*-knockdown and control embryos (Fig. S6 *C–L*).

Enlargement of Dorsal Organizer in the *setdb2*-Knockdown Embryos. What developmental and molecular mechanisms might account for the abnormal midline development, decreased cilium number, and randomization of left–right asymmetry induced by Setdb2 deficiency? It has been shown that physiological midline development depends on the normal function and formation of the Spemann organizer (8, 9). We first examined the expression patterns of three well-known organizer genes *floating head* (*flh*), *goosecoid* (*gsc*), and *chordin* (*chd*) (12) at 6 hpf, and found that the expression territories of all three genes were significantly enlarged in the *setdb2*-knockdown embryos compared to the control embryos (Fig. 4 *A–F*, n = 45/60). To examine whether the earlier step of gastrulation is affected by *setdb2* deficiency, the expression pattern of two additional genes *wnt8a* and *fgf8* were analyzed by WISH at 5.3 hpf, and no detectable changes were observed (Fig. S7 *A–D*), suggesting that the expanded

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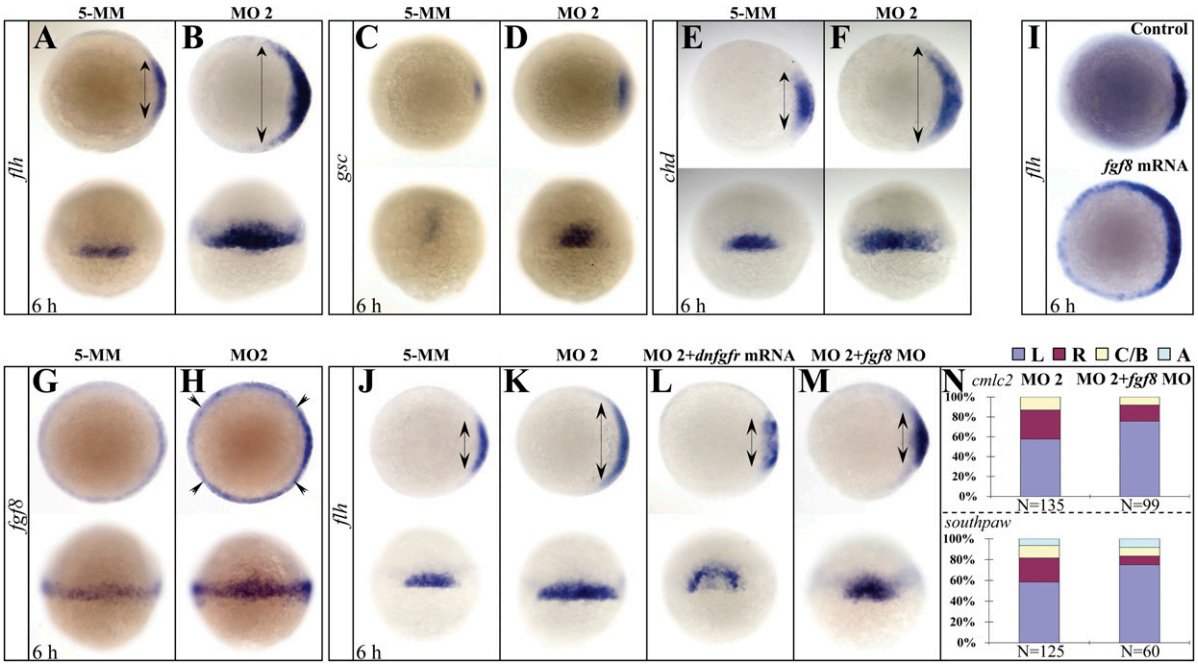

Fig. 4. Setdb2 restricts dorsal organizer formation and regulates left–right asymmetry through suppressing Fgf8 activity. (*A–F*) WISH analyses of organizer markers *flh* (*A* and *B*), *gsc* (*C* and *D*), and *chd* (*E* and *F*) in the 5-MM control and *setdb2* MO2-injected embryos at 6 hpf. Double-headed arrows denote territories of dorsal organizer. Embryos are animal views in the *Upper* panels, dorsal views in the *Bottom* panels. (*G–I*) WISH analyses of *fgf8* expression (*G* and *H*) in the 5-MM control and *setdb2* MO2-injected embryos at 6 hpf and effects of *fgf8* overexpression on the dorsal organizer development as revealed by *flh* expression (*I*). Embryos are animal views in panels *G* and *H* (*Upper*) and *I* and dorsal views in panels *G* and *H* (*Bottom*). (*J–M*) WISH analyses of *flh* in the 6-hpf embryos injected with 5-MM control (*J*), *setdb2* MO2 (*K*), *setdb2* MO2 plus dominant-negative fibroblast growth factor receptor (*dnfgfr*) mRNAs (*L*), and *setdb2* MO2 plus fgf8 morpholino oligonucleotides (*M*). (*N*) Statistic analyses of heart laterality marker *cmlc2* and asymmetric gene *southpaw* in 6-hpf embryos injected with *setdb2* MO2 and *setdb2* MO2 plus *fgf8* morpholino. Embryos are animal views in the *Upper Panels* and dorsal views in the *Lower Panels*.

dorsal organizer is not a consequence of abnormalities in the earlier stage of gastrulation. The results suggest that Setdb2 acts as a negative regulator to restrict the size of the dorsal organizer in a relatively specific manner.

To further understand the molecular mechanisms of how Setdb2 affects dorsal organizer formation, we examined the expression of several previously reported organizer-regulating genes such as *eomesodermin*, *dharma*, *iroquois3* (12–14), and members of the *fgf* family (19) at shield stage (6 hpf). Although no changes in the levels of *eomesodermin*, *dharma*, *iroquois3*, and *fgf4* expressions were observed between control and *setdb2*-knockdown embryos (Fig. S7 *E* and *F*), the levels of *fgf8* expression were significantly increased at 6 and 22 hpf upon Setdb2 deficiency (Fig. 4 *G* and *H* and Fig. S8; $n = 30/40$).

We next microinjected 300 pg of *fgf8* mRNAs into one-cell-stage embryos and found that the dorsal organizer was also significantly expanded as revealed by an enlarged *flh* expression domain, suggesting that *fgf8* may be a downstream target of *setdb2* to mediate the restrictive effects of Setdb2 on dorsal organizer development (Fig. 4*I*, $n = 15/20$). To test this hypothesis, we microinjected *setdb2* morpholino with dominant-negative *fgf receptor* (*dnfgfr*) mRNA (300 pg), which has been shown to efficiently abolish *fgf8* function (41), and found that the expanded dorsal organizer induced by Setdb2 deficiency was nearly corrected to normal size (Fig. 4 *J–L*, $n = 25/30$). Consistent with this result, knockdown of *fgf8* with a specific morpholino oligonucleotide (42) also restores the dorsal organizer to normal size in the Setdb2-deficient embryos (Fig. 4*M*, $n = 30/35$). Furthermore, the randomization of the expression of either *cmlc2* or *southpaw* was also corrected in the *setdb2–fgf8* double morphants (Fig. 4*N*). Taken together, these results suggest that the Fgf8 is a bona fide downstream target of Setdb2 to regulate the development of dorsal organizer and left–right asymmetry.

Discussion

In the past few years, epigenetic regulation has been shown to be essential for early embryonic development (16, 17). The development and formation of the dorsal organizer is one of the most critical developmental events during early embryonic development and is responsible for neural induction, mesoderm and endoderm patterning, midline and axis formation, and subsequent left–right asymmetry establishment (43, 44). Although many signaling pathways and genes have been found to be involved in the regulation of organizer development (12–15), whether or not epigenetic regulators participate in this process is largely unknown. In this study, we provided unique evidence, to our best knowledge, that the *setdb2*, an epigenetic regulator responsible for repressive H3K9me3, plays an important role in negatively regulating dorsal organizer formation and contributes to the establishment of left–right axis through suppressing *fgf8* signaling. Interestingly, *setdb1*, a paralog of *setdb2*, also possesses H3K9me3 activity and is required for peri-implantation development (23, 45). The possibility that other H3K9 methyltransferases may participate in the regulation of dorsal organizer and determination of L–R asymmetry cannot be excluded.

The establishment of left–right axis has been molecularly attributed to the asymmetric distribution of nodal signaling genes such as *southpaw*, which is required for initiating subsequent asymmetric location of downstream targets such as *lefty1/2* and *pitx2*. However, elegant studies have shown that the function and number of cilium in the developing KV during gastrulation act as an earlier physiological gatekeeper to maintain the asymmetric distribution of these nodal signaling factors through a mechanistic counterclockwise flow (4). Furthermore, several groups have convincingly shown that the formation of the dorsal organizer, which is induced by a complex interaction between signaling pathways, plays a critical role in appropriate patterning of

midline and the KV-derived cilia development (4, 7). Because loss-of-function of *setdb2* not only causes the randomizations of visceral and asymmetric gene laterality, but also results in the decreased number of cilia in the KV, disrupted midline patterning, and enlarged dorsal organizer, these results, along with previous findings, prompt us to propose that the establishment of left–right axis is a consequence not only of a highly coordinated process associated with distribution of asymmetry-initiating factors, but also a stepwise development and physiological interactions of early embryonic structures(organizer→midline→KV→cilia).

Although it has been well established that the members of *fgf* signaling (*fgf3*, *fgf4*, and *fgf8*) are essential for the development of the dorsal organizer and the establishment of left–right asymmetry (19, 46), little is known about the upstream signaling or factor that regulates the *fgf* expression at the transcriptional level through the histone methylation-mediated epigenetic mechanism. A unique finding of this study is the identification of *fgf8*, but not *fgf4*, as a downstream target of Setdb2. The facts that the Setdb2 knockdown specifically upregulates *fgf8* and that the suppression of *fgf8* function by either overexpression of dominant-negative *fgfr* mRNAs or *fgf8* knockdown nearly corrected both the expansion of the organizer and randomization of left–right asymmetry induced by Setdb2 deficiency, strongly suggest that the Setdb2 acts upstream to regulate the *fgf8* expression. Future studies are needed to clarify whether the Setdb2 is recruited to the promoter of the *fgf8* gene to directly exert its H3K9me3 and transcriptional repression activity and which transcriptional factor might mediate the proper positioning of Sedb2 to this promoter, especially given that histone methyltransferases have not been shown to directly bind promoters due to lack of sequence-specific DNA-binding domains.

Materials and Methods

Zebrafish Strains. Zebrafish stocks were maintained at 28.5 °C under standard aquaculture conditions. Embryos were staged by hours postfertilization as described previously (47). The Tg(*insulin:EGFP*) transgenic zebrafish lines were established in our laboratory.

Plasmid Constructs and RNA Probes. Full-length coding sequence for *setdb2* and fragments of *alk4*, *bmp4*, *dharma*, *dub*, *dvl2*, *dvl3*, *fzd2*, *gsc*, *ipk1*, *iroquois3*, *kny*, *pdip5*, *pkd2*, *rock2*, *smad2*, and *tbxc* were cloned into pCS2+ or pGEM– Teasy vector. The plasmids *southpaw*, *lefty1*, *lefty2*, *pitx2*, *lrdr1*, *charon*, *oep*, *shh*, *ntl*, *wnt4*, *wnt5a*, *wnt8*, *wnt11*, *wnt11-related*, *rhoA*, *cyclops*, *squint*, *tbx16*, *flh*, *chordin*, *eomesodermin*, *dnfgfr4*, and *fgf8* are generous gifts from other laboratories (*Acknowledgments*). The digoxigenin- (Dig-) or fluorescein-labeled probes were transcribed from linearized plasmids.

WISH. WISH was performed as described previously (48). The stained embryos were mounted in 4% methylcellulose and photographed using the Nikon SMZ1500 stereomicroscope.

Morpholino and mRNA Injections. Antisense MOs against either the 5′-UTR (MO1: 5′-CAGGGTTCAGGAGATTTTAATGACT-3′; 5-mismatch to MO1: 5′-CAcGGTTgAGGAcATTTaAATcACT-3′) or the start codon of the *setdb2* gene (MO2: 5′-TTGCTGTGTCGGCTTCAGTCTCCAT-3′), as well as the MO against the start codon of the *fgf8* gene (*fgf8* MO: 5′-GAGTCTCATGTTTATAGCCTCAG-TA-3′) were obtained from Gene Tools. Capped mRNAs were transcribed with mMESSAGE mMACHINE Kit (Ambion). All MOs and mRNAs were injected at the one-cell stage.

Immunofluorescence and Confocal Microscopy. Whole-mount immunofluorescence with primary antibody against mouse acetylated tubulin (1:500 dilution, Sigma) and Alexa Fluor 488-labeled secondary antibody (donkey anti-mouse, 1:500 dilution, Invitrogen) was performed as described previously (3).

The stained embryos were mounted in 1% low-melting point agarose for visualization of KV and the images were collected using a Leica TCS-LSI confocal microscope equipped with 5× objective. The 488-nm laser line was used for excitation of the Alexa Fluor 488.

Western Blot. Embryos at 10 hpf were deyolked as described previously (49). Embryos were homogenized in lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM ethylenediaminetetra-acetic acid, 10% glycerol, and 0.1% Triton X-100) containing protease inhibitor mixture and phosphatase inhibitor (Roche Diagnostics) as described previously (48). Mouse anti-histone H3 antibody (1:10,000) and a panel of antibodies against methylated histone 3 (H3K4, H3K9, and H3K27) (1:1,000) and a horseradish peroxidase-conjugated secondary antibody (1:10,000) were used. Band densities were quantified with Image-Pro Plus software.

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