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Maternal control of axial–paraxial mesoderm patterning via direct transcriptional repression in zebrafish

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ABSTRACT

Axial–paraxial mesoderm patterning is a special dorsal–ventral patterning event of establishing the vertebrate body plan. Though dorsal–ventral patterning has been extensively studied, the initiation of axial–paraxial mesoderm pattering remains largely unrevealed. In zebrafish, *spt* cell-autonomously regulates paraxial mesoderm specification and *flh* represses *spt* expression to promote axial mesoderm fate, but the expression domains of *spt* and *flh* initially overlap in the entire marginal zone of the embryo. Defining *spt* and *flh* territories is therefore a premise of axial–paraxial mesoderm patterning. In this study, we investigated why and how the initial expression of *flh* becomes repressed in the ventrolateral marginal cells during blastula stage. Loss- and gain-of-function experiments showed that a maternal transcription factor Vsx1 is essential for restricting *flh* expression within the dorsal margin and preserving *spt* expression and paraxial mesoderm specification in the ventrolateral margin of embryo. Chromatin immunoprecipitation and electrophoretic mobility shift assays in combination with core consensus sequence mutation analysis further revealed that Vsx1 can directly repress *flh* by binding to the proximal promoter at a specific site. Inhibiting maternal *vsx1* translation resulted in confusion of axial and paraxial mesoderm markers expression and axial–paraxial mesoderm patterning. These results demonstrated that direct transcriptional repression of the decisive axial mesoderm gene by maternal ventralizing factor is a crucial regulatory mechanism of initiating axial–paraxial mesoderm patterning in vertebrates.

Introduction

Dorsal–ventral (DV) patterning is an early development program of establishing the animal body plan. Axial–paraxial mesoderm patterning, by which the domains for generation of the notochord and flanking mesoderm are defined, is a vertebrate-specific DV patterning event. It has been confirmed that, after the establishment of DV polarity, the mesoderm is induced by Nodal signaling with low level at the ventral side and high level at the dorsal side (Green and Smith, 1990; Green et al., 1992; Gurdon et al., 1994; McDowell and Gurdon, 1999; Gurdon and Bourillot, 2001; Shen, 2007). But there is no compelling evidence supporting that a reduction of Nodal signaling in the dorsal side results in a respecification of dorsal to ventral fates (Kimelman, 2006). Experiment in zebrafish provides evidence that DV patterning of mesoderm is independent of Nodal signals (Dougan et al., 2003). A zygotic bone morphogenetic protein (Bmp) activity gradient, generated by antagonistic actions between Bmps and BMP antagonists emanated from the dorsal organizer, plays an important role in defining distinct ventrolateral fate domains along the DV axis during gastrulation (Dosch et al., 1997; Graff, 1997; Jones and Smith, 1998; Nguyen et al., 1998; Barth et al., 1999; Dale and Wardle, 1999; De Robertis et al., 2000). However, axial mesoderm is largely unaffected in Bmp pathway mutants, implicating that the zygotic Bmp activity gradient is not involved in defining the axial and paraxial mesoderm domains. In zebrafish, ventrally expressed zygotic Wnt8 activates *vox*/*vent*/*ved* gene family in cooperation with Bmp2b to repress dorsal gene expression and maintain ventrolateral identity during gastrulation (Melby et al., 2000; Imai et al., 2001; Lekven et al., 2001; Shimizu et al., 2002; Ramel and Lekven, 2004; Ramel et al., 2005). But the initial distinction between axial and non-axial domains at 30–40% epiboly was unaffected in the embryos lacking the functions of both zygotic Wnt8 and Bmp2b signaling pathways (Reim and Brand, 2006; Ramel et al., 2005). Recent study further demonstrated that zebrafish maternal Wnt8 is located at the dorsal side after fertilization and functions as a dorsal determinant during blastula stage (Lu et al., 2011). Therefore, Wnt8 and Bmp2b signaling

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pathways are unlikely involved in initiating ventral fate before gastrulation. The ventral specification of mesoderm is actively regulated by maternal ventralizing factor via suppressing maternal dorsalizing signals (Itoh and Sokol, 1999; Kuhl et al., 2000; Saneyoshi et al., 2002) or activating zygotic ventral genes, such as *bmps* and *vox*/*vent*/*ved* gene family (Goutel et al., 2000; Bauer et al., 2001; Mintzer et al., 2001; Payne et al., 2001; Kramer et al., 2002; Sidi et al., 2003; Reim and Brand, 2006; Flores et al., 2008). Among the three maternal ventralizing factors identified in zebrafish, maternal Radar and Pou2 do not influence the expression domain of dorsal organizer genes (Sidi et al., 2003; Reim and Brand, 2006), suggesting that these maternal factors are unlikely involved in defining axial and paraxial mesoderm domains. Maternal Runx2bt2 activates *vent*, *vox* and *ved* to promote non-axial mesoderm fate and can influence the distinction between axial and paraxial mesoderm domains at 50% epiboly stage (Flores et al., 2008). However, it remains unclear whether this indirect regulation is involved in initiating or maintaining the distinction between axial and paraxial mesoderm domains. Taken together, the initial regulation of axial–paraxial mesoderm patterning remains unclear.

The regulation of axial and paraxial mesoderm specification has been intensively studied in zebrafish. A transcriptional factor Spadetail (Spt) cell-autonomously regulates paraxial mesoderm specification in the ventrolateral margin of early embryo (Kimmel et al., 1989; Ho and Kane, 1990; Griffin et al., 1998; Amacher and Kimmel, 1998), and loss of Spt function can elicit the ventral expansion of the axial mesoderm domain and the absence of paraxial mesoderm marker expression (Thisse et al., 1995; Hammerschmidt et al., 1996). A homeodomain transcriptional factor Floating head (Flh) represses *spt* expression to promote axial mesoderm fate in the dorsal margin (Amacher and Kimmel, 1998; Yamamoto et al., 1998). In *flh* mutant embryos the axial mesoderm cell-autonomously converts into paraxial mesoderm (Talbot et al., 1995; Halpern et al., 1995; Melby et al., 1996; Amacher and Kimmel, 1998), although dispersed *flh* mutant cells can differentiate into notochord cells in response to notochord-promoting signals in the wild-type host embryo (Amacher and Kimmel, 1998). Interestingly, the expression domains of *spt* and *flh* initially overlap in the entire margin zone of the embryo at dome stage and are divided from 30% epiboly stage (Griffin et al., 1998; Talbot et al., 1995). Therefore, rapid repression of *flh* in the ventrolateral marginal cells from dome stage to 30% epiboly stage is essential for maintaining *spt* expression in the ventrolateral margin and a premise of axial and paraxial mesoderm patterning. Investigating how the initial expression of *flh* in the ventrolateral margin is inhibited during late blastula stage will gain an insight into the initial axial–paraxial mesoderm patterning.

A paired-like transcription factor gene *visual system homeobox-1* (*vsx1*) which encodes a protein containing homeodomain and CVC domain has been cloned in several vertebrate species (Levine and Schechter, 1993; Levine et al., 1994; Passini et al., 1998; Semina et al., 2000; Ohtoshi et al., 2001; D'Autilia et al., 2006). Vsx1 plays an important role in regulating retinal progenitor cells proliferation and differentiation, and in maintaining the function of bipolar cells in vertebrates (Héon et al., 2002; Ohtoshi et al., 2004; Valleix et al., 2006; Clark et al., 2008). Since *vsx1* transcripts were detected in zebrafish maternal mRNA pool and at early developmental stage in all the examined vertebrate species (Levine and Schechter, 1993; Levine et al., 1994; Passini et al., 1998; Semina et al., 2000; Ohtoshi et al., 2001; D'Autilia et al., 2006), it has been reasonably postulated that *vsx1* might play an important role during early embryogenesis (Ohtoshi et al., 2001). Here, we show that Vsx1 protein encoded by maternal *vsx1* mRNA can directly repress *flh* transcription to preserve *spt* expression and paraxial mesoderm specification in the ventrolateral margin of blastula embryo. In this way, the original overlapped axial and paraxial mesoderm domains are divided and the initial distinction between axial and paraxial mesoderm domains takes shape.

Results

Maternal Vsx1 is essential for normal paraxial mesoderm specification and axial–paraxial mesoderm patterning

To determine whether *vsx1* has a role in regulating early embryogenesis in zebrafish, endogenous *vsx1* was knocked down by injecting translation blocking MO (tbMO) at one cell stage. When 8 ng tbMO was injected, 89.4% of embryos ($N=152$) were arrested at the onset of gastrulation and died soon. When the dose was reduced to 4 ng, the percentage of dead embryos was reduced to 14% ($N=164$), 58% of the embryos at 24 hours post-fertilization (hpf) exhibited a morphant specific phenotype with no obvious paraxial somites but disorganized dorsal midline structures (Fig. 1D–F). When the dose was reduced to 2 ng per embryo, the ratio of abnormal embryos was very low and the abnormalities varied. Therefore, 4 ng of *vsx1* tbMO was used for subsequent experiments. The specificity of *vsx1* tbMO was verified in rescue experiment by co-injecting with *vsx1* mis-mRNA (encoding the normal Vsx1 protein but lacking the target site of the *vsx1* tbMO). Coinjection of *vsx1* mis-mRNA elicited conversion of the morphant phenotype into normal or *vsx1* overexpression phenotypes (described below) and decrease of lethality in a dose-dependent manner (Fig. 1M).

To understand how Vsx1 regulates the dorsal structure development, we examined the axial and paraxial mesoderm formation by visualizing the expression domains of an axial mesoderm marker gene *ntl* and a paraxial mesoderm marker gene *myoD*, respectively, in *vsx1* knockdown embryos at bud stage and 8–10 somite stage. Whole mount in situ hybridization showed that the formation of both axial and paraxial mesoderm was abnormal in *vsx1* tbMO injected embryos. In comparison with wild type, the *ntl* marked axial mesoderm domain in the *vsx1* knockdown embryos was expanded in width but shortened in length (Fig. 2C–F and Fig. S1E and F), while *myoD* marked paraxial mesoderm domain in the *vsx1* knockdown embryos was suppressed in various degrees with defects of convergence and somitogenesis (Fig. 2M–P and Fig. S1G and H). The suppression of paraxial mesoderm formation in *vsx1* morphants was confirmed by examining the expression of two other paraxial mesoderm marker genes *msgn1* (Yoo et al., 2003; Fior et al., 2012) and *tbx24* (Nikaido et al., 2002) at middle gastrula stage. When 4 ng *vsx1* tbMO was injected at one cell stage, the expression of *msgn1* at the ventrolateral region was significantly repressed in 61% of the embryos ($N=36$, Fig. 3, A–F), and *tbx24* at the paraxial region was significantly repressed in 69% of the embryos ($N=46$, Fig. 3G–L). These results suggest that Vsx1 is essential for promoting normal paraxial mesoderm specification and axial–paraxial mesoderm patterning during early embryogenesis.

We further examined the function of *vsx1* in regulating early embryogenesis by overexpression. When 200 pg *vsx1* mRNA was injected at one cell stage, 57.7% of the embryos ($N=156$) exhibited widely bifurcated paraxial mesoderm domains with no distinguishable dorsal midline structures in the dorsal center region at 24 hpf (Fig. 1G–I). Expression analysis of axial and paraxial mesoderm marker genes showed that, at the anterior axial region, *ntl* marked axial mesoderm specification was severely suppressed (Fig. 2G–J and Fig. S1I and J), while dispersed *myoD* marked paraxial mesoderm cells were detectable (Fig. 2Q–T). *myoD* marked paraxial mesoderm and somites were formed at the ventrolateral region but failed in converging to the normal dorsal position (Fig. 2Q–T and Fig. S1K and L). These results suggest that *vsx1* is able to repress axial mesoderm specification at early developmental stage.

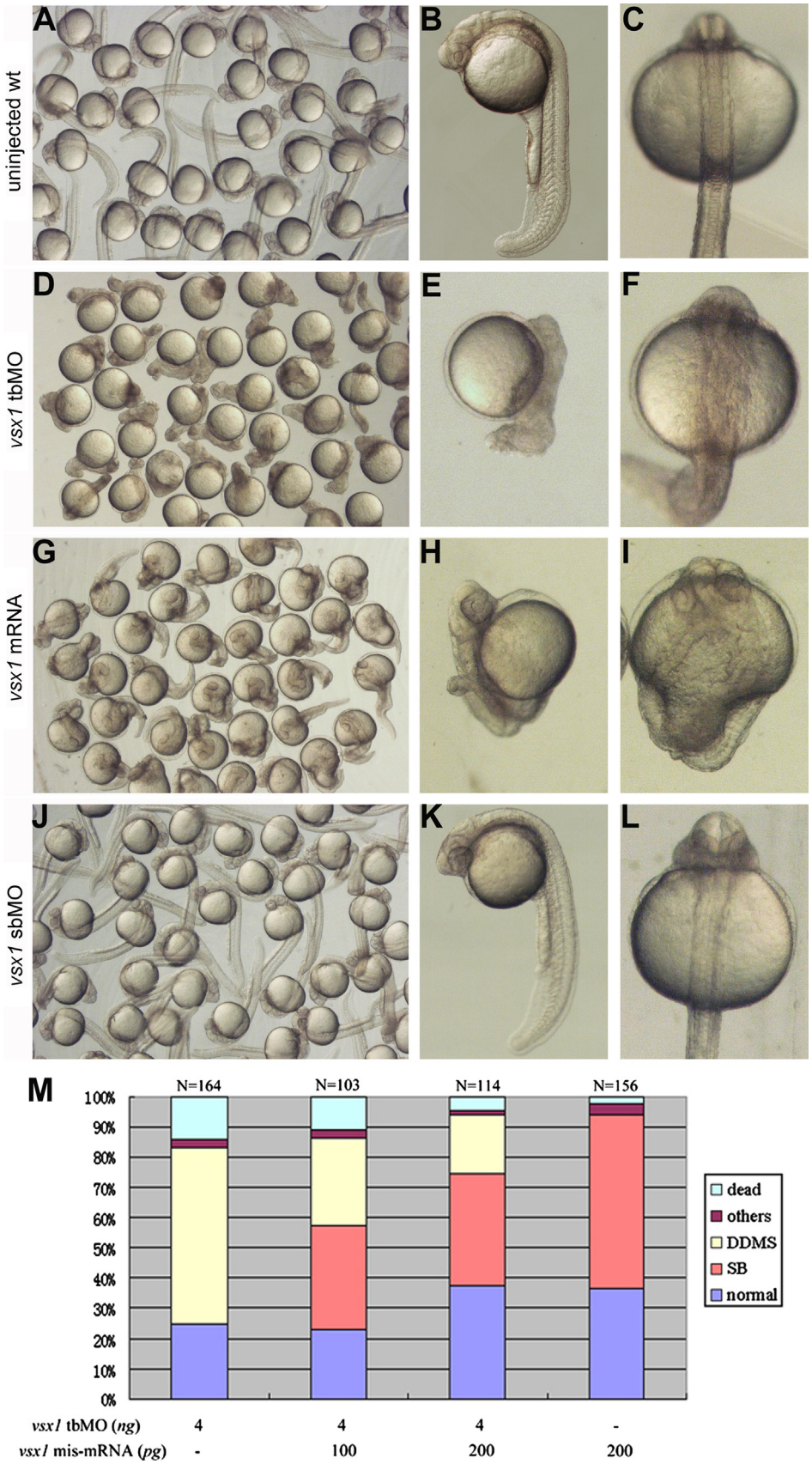

Fig. 1. Maternal Vsx1 is essential for axial–paraxial mesoderm patterning in zebrafish. (A–C) Uninjected wild-type control embryos. (D–F) *vsx1* tbMO injected embryos exhibit dorsalized phenotype with disturbed dorsal midline structure. (G–I) *vsx1* overexpression elicits inhibition of axial mesoderm specification and aberrant convergence of specified paraxial mesoderm. (J–L) Injection of *vsx1* sbMO has no detectable effect on development before 24 hpf. Phenotypes are shown at 24 hpf. (M) Proportion of different phenotypes in *vsx1* knockdown, overexpression and rescue experiments at 24 hpf. DDMS: disorganized dorsal midline structure. SB: spina bifida.

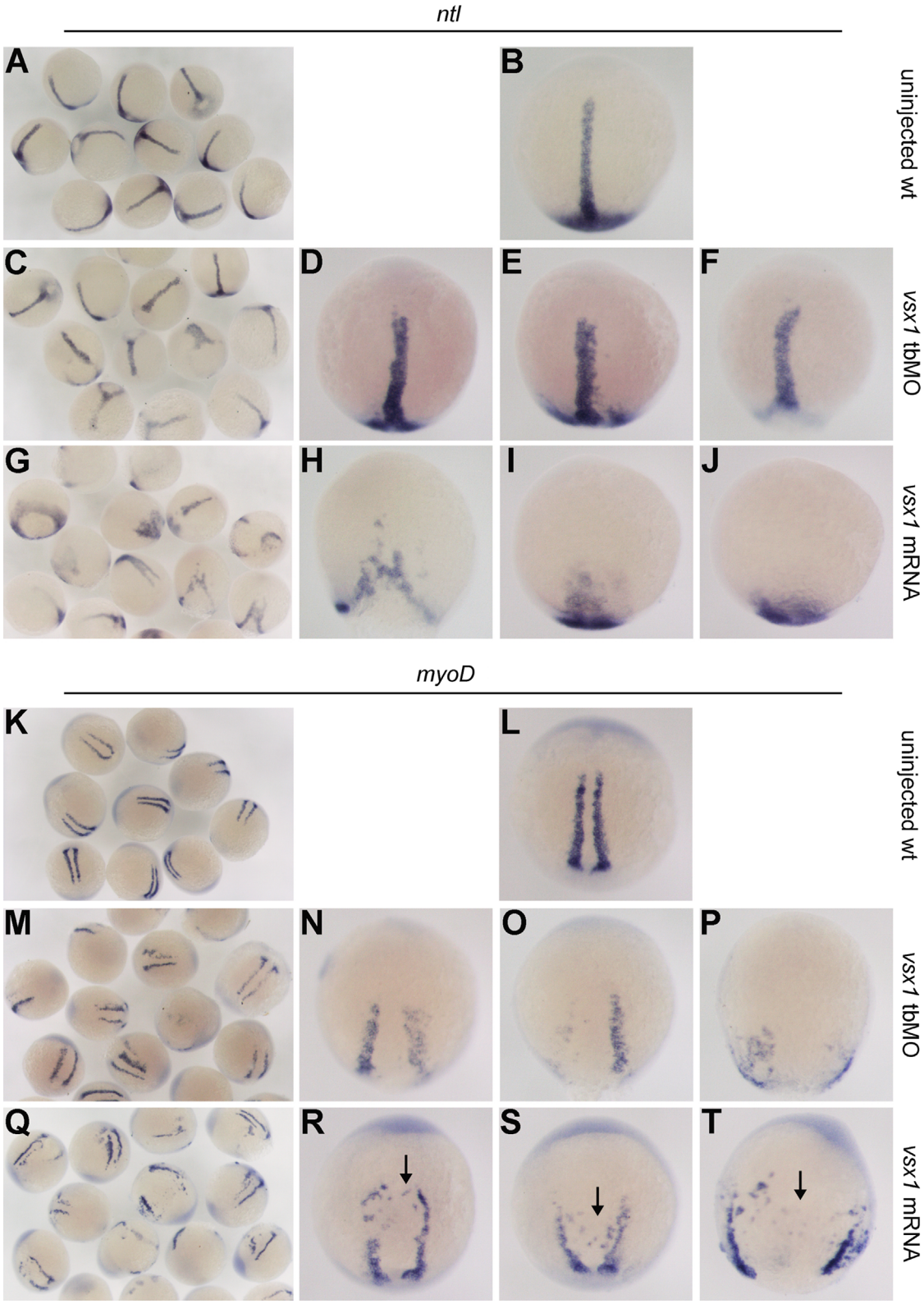

Fig. 2. Comparison of axial and paraxial mesoderm formation among wild type, maternal Vsx1 suppressed and *vsx1* overexpression embryos. (A–J) *ntl* marked axial mesoderm domain in uninjected wild-type control embryos (A and B), *vsx1* tbMO injected embryos (C–F) and *vsx1* mRNA injected embryos (G–J). (K–T) *myoD* marked paraxial mesoderm domain in uninjected wild-type control embryos (K and L), *vsx1* tbMO injected embryos (M–P) and *vsx1* mRNA injected embryos (Q–T). Note that *vsx1* overexpression inhibits the convergence of paraxial mesoderm cells but has no impact on paraxial mesoderm cell specification and somite formation. Riboprobes are indicated at the top of each group of figures. All the images of single embryo are dorsal view with animal pole towards the top.

Low level of maternal *vsx1* mRNA was detected by an RNase protection assay in zebrafish (Passini et al., 1998). Our expression profile analysis by qRT-PCR showed that the level of *vsx1* mRNA was maintained at the same from one cell stage to late blastula stage, decreased to very low during gastrulation but increased from 6-somite stage (Fig. 4A). To determine whether zygotic *vsx1*

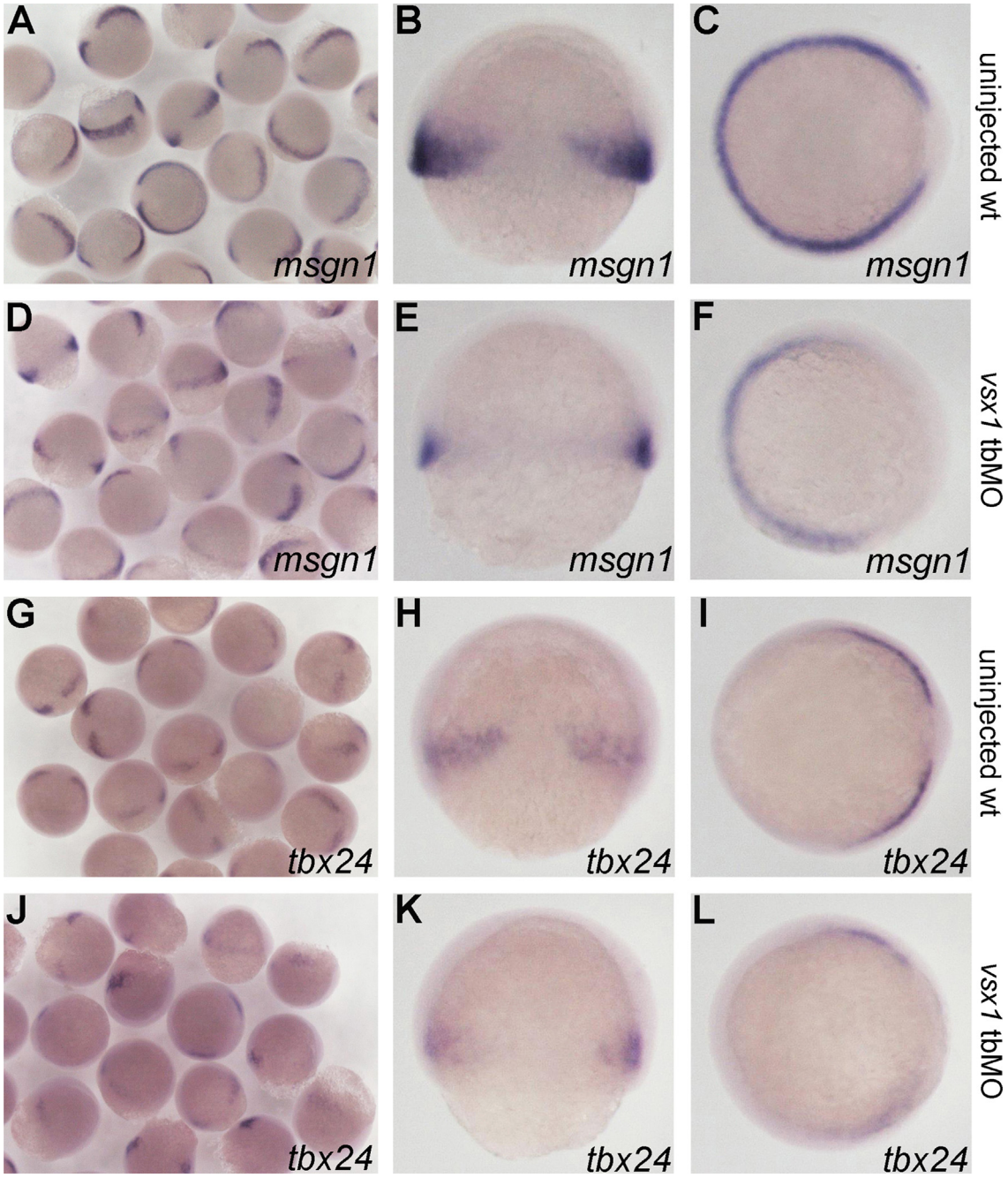

Fig. 3. Maternal Vsx1 is essential for promoting normal paraxial mesoderm specification. (A–F) comparison of *msgn1* expression between uninjected wild type control (A–C) and maternal *vsx1* tbMO injected embryos (D–F). (G–L) Comparison of *tbx24* expression between uninjected wild type control (G–I) and maternal *vsx1* tbMO injected embryos (J–L). Riboprobes are indicated at the bottom of each figure. (B, E, H and K) Dorsal view of embryos with animal pole towards the top. (C, F, I and L) Animal pole view of the embryos with dorsal towards the right.

mRNA is required for paraxial mesoderm domain patterning, a splice-blocking *vsx1* MO (sbMO), which can interfere with the splicing of newly synthesized zygotic *vsx1* mRNA but leave the maternal *vsx1* mRNA intact, was used. The embryo receiving injection of up to 15 ng *vsx1* sbMO developed a normal DV axis and no abnormality was observed until 24 hpf (Clark et al., 2008; Fig. 1J–L), indicating that zygotic *vsx1* mRNA is not involved in regulating early embryogenesis. Taken together, our results demonstrated that maternal Vsx1, rather than zygotic Vsx1, regulates axial–paraxial patterning by repressing axial mesoderm specification to preserve paraxial mesoderm specification in the ventrolateral region.

Maternal vsx1 mRNA directs translation in the ventrolaternal region at blastula stage

The expression profile (Fig. 4A) indicated that during blastula stage the amount of zygotic *vsx1* transcripts was very small (if synthesized) in comparison with that of deposited maternal *vsx1* transcripts. We next analyzed the spatial expression pattern of maternal Vsx1 at blastula stage. Whole mount in situ immunohistochemistry analysis showed that Vsx1 protein was detectable in the nuclei of most blastomeres but not in a few blastomeres on one side at 1k cell stage (Fig. 4B–D and D'). The contrast between the two regions became more striking at dome stage (Fig. 4H–J).

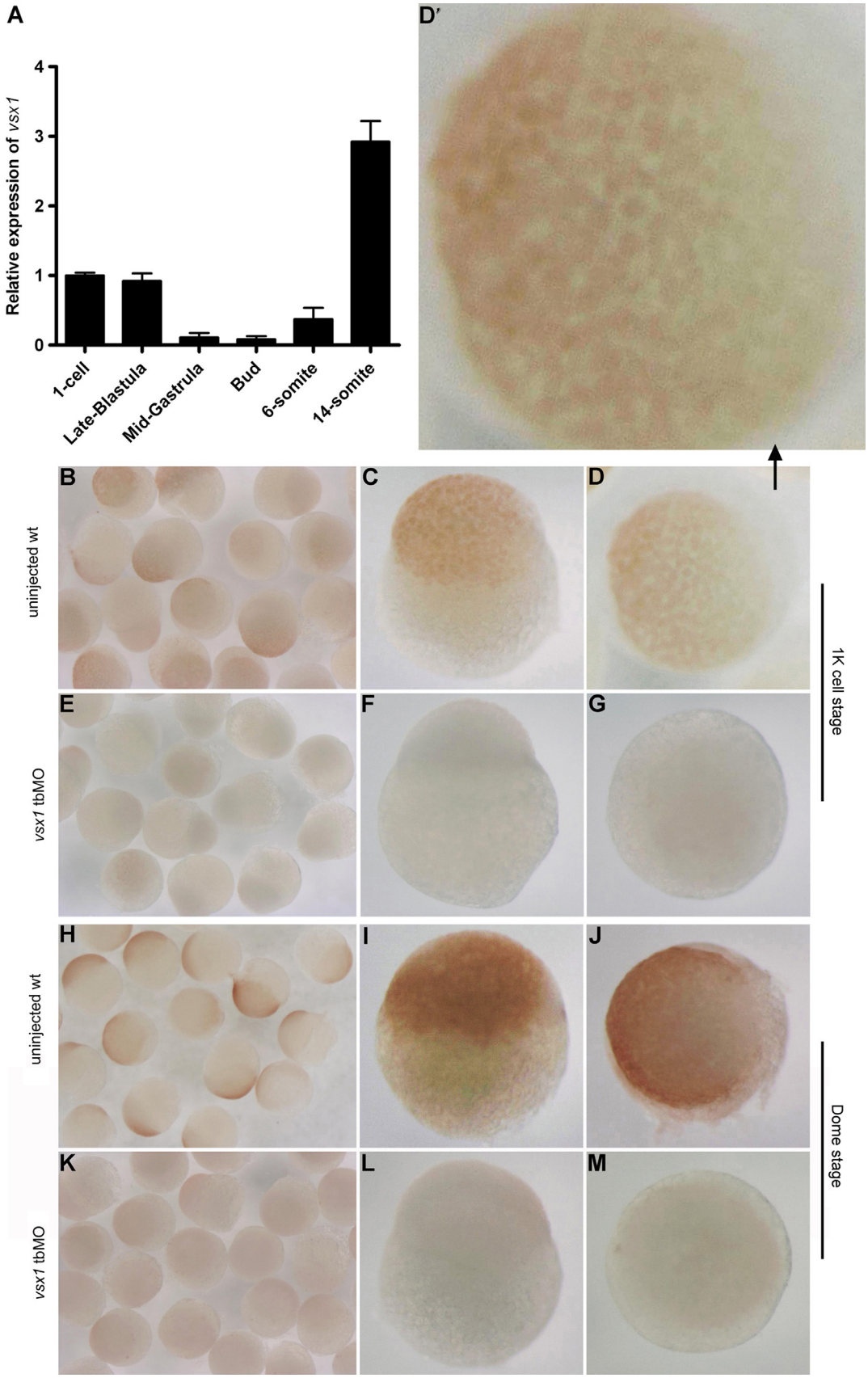

Fig. 4. Expression pattern of *vsx1* during early embryogenesis. (A) Expression profile of *vsx1* mRNA during early embryogenesis. Results are expressed as mean ± SEM. (B–M) Localization of maternal Vsx1 protein in uninjected wild type control and *vsx1* tbMO injected embryos at 1k cell (B–G) and dome stages (H–M). (C, F, I and L) Lateral view of embryos with animal pole towards the top and dorsal towards the right. (D, D', G, J and M) Animal pole view of the embryos with dorsal towards the right. D' is the magnified image of D, in which one can see that Vsx1 protein is localized in the nuclei of the blastomeres.

When 4 ng *vsx1* tbMO was injected, the Vsx1 protein was undetectable in the embryos during cleavage (Fig. 4E–G and K–M). These results suggest that maternal *vsx1* mRNA directs Vsx1 translation in the ventrolaternal region during cleavage, correlating well with that maternal Vsx1 promotes paraxial mesoderm specification in the ventrolateral side.

Vsx1 is essential for repressing flh expression in the ventrolateral margin

Because axial mesoderm regulatory gene *flh* can repress paraxial mesoderm regulatory gene *spt* and paraxial mesoderm specification (Amacher and Kimmel, 1998; Yamamoto et al., 1998), we examined whether maternal Vsx1 is able to repress *flh* expression and preserve *spt* expression during early developmental stage. Whole mount in situ hybridization analysis revealed that it is indeed the case. From 30% epiboly stage onward, the expression of *flh* was restricted within the dorsal region in the wild type embryos (Talbot et al., 1995; Fig. 5B and B', E and E') but expanded to the ventral margin in *vsx1* knockdown embryos (Fig. 5A and A', D and D'). In contrast, *flh* expression was significantly repressed in the dorsal margin of *vsx1* mRNA injected embryos (Fig. 5C and C', F and F'). Conversely, the expressions of *spt* and its downstream target gene *paraxial protocadherin* (*papc*) in the margin were dramatically suppressed in the *vsx1* knockdown embryos both at 30–40% epiboly stage (Fig. 5G and G', L and L') and during

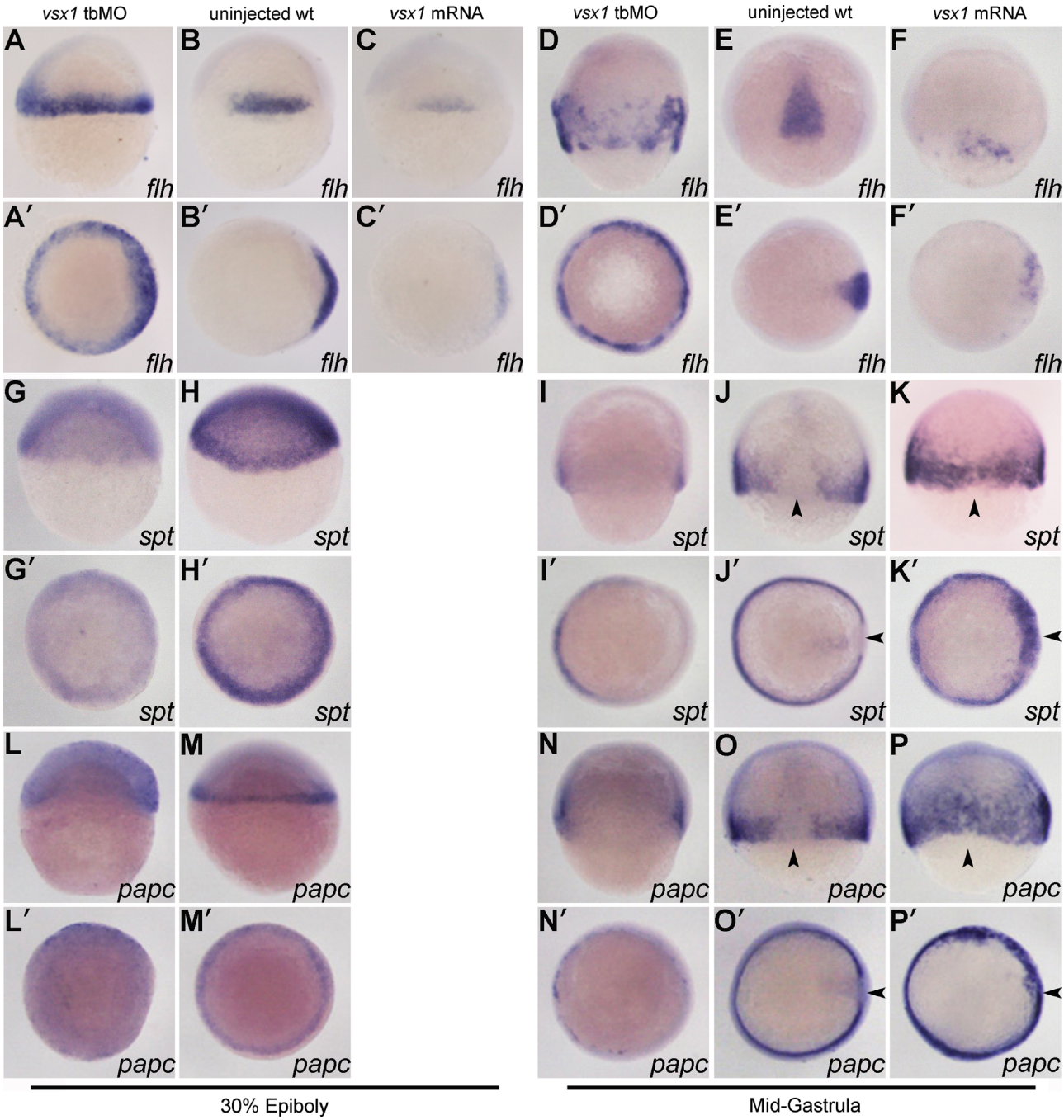

Fig. 5. Vsx1 represses *flh* to preserve *spt* and *papc* expression in the ventolateral margin. The injected reagents are indicated at the top of each column. Riboprobes are indicated at the bottom of each figure. (A–P) Dorsal view of embryos with animal pole towards the top. (A'–P') Animal pole view of the embryos with dorsal towards the right. Arrow heads indicate that the paraxial mesoderm marker is detected in the presumptive axial mesoderm region in *vsx1* overexpression embryos.

gastrulation (Fig. 5I and I', N and N'). Injection of *vsx1* mRNA resulted in expansion of *spt* and *papc* expression to the dorsal margin during gastrulation (Fig. 5K and K', P and P'), suggesting that *vsx1* overexpression elicited fate change from axial mesoderm to paraxial mesoderm in the anterior dorsal region. It is clear that maternal Vsx1 is essential for repressing *flh* ectopic expression and preserving *spt* expression in the ventrolateral margin at late blastula stage and during gastrula stage.

Since zygotic Wnt8a and Bmp2b signaling pathways are required in repressing dorsal genes expression and maintaining ventrolateral identity during gastrulation (Ramel and Lekven, 2004; Ramel et al., 2005), we next examined whether maternal Vsx1 is essential for activating the genes of the zygotic Bmp2b and Wnt8a signaling pathways. Whole mount in situ hybridization analysis showed that injection of *vsx1* tbMO has no detectable influence on the expression patterns of *wnt8a*, *bmp2b* and their targets *vent* and *vox* at middle gastrula stage (Fig. 6). Therefore, the repression of *flh* expression in the ventrolateral margin by maternal Vsx1 is unlikely mediated by zygotic Wnt8a and Bmp2b signaling pathways.

Vsx1 directly represses flh transcription

Vsx1 contains a DNA-binding homeodomain. Previous experiments identified that the potential core consensus sequence of DNA for homeodomain binding is TAATTN (Ades and Sauer, 1994; Rodrigo et al., 2004). Sequence analysis showed that there are 11 potential Vsx1 binding sites in the proximal promoter of *flh* upstream of the transcription start site (Fig. S2). To determine *vsx1* directly or indirectly represses *flh*, we first examined if Vsx1 could bind to the potential binding sites at the proximal promoter of *flh* by chromatin immunoprecipitation (ChIP) assay in normal gastrula embryos. After immunoprecipitaion with the anti-Vsx1 antibody, the binding of Vsx1 to all the 11 potential binding sites was examined by specific PCR with 5 pairs of primers (Fig. 7A). PCR amplification was only detected with the primer pair spanning the potential binding site 11 (Fig. 7B) in the *flh* promoter region 5. Sequence analysis confirmed that the PCR product was indeed identical to that of the *flh* promoter region 5. PCR products were not detected from the controls with preimmune serum or

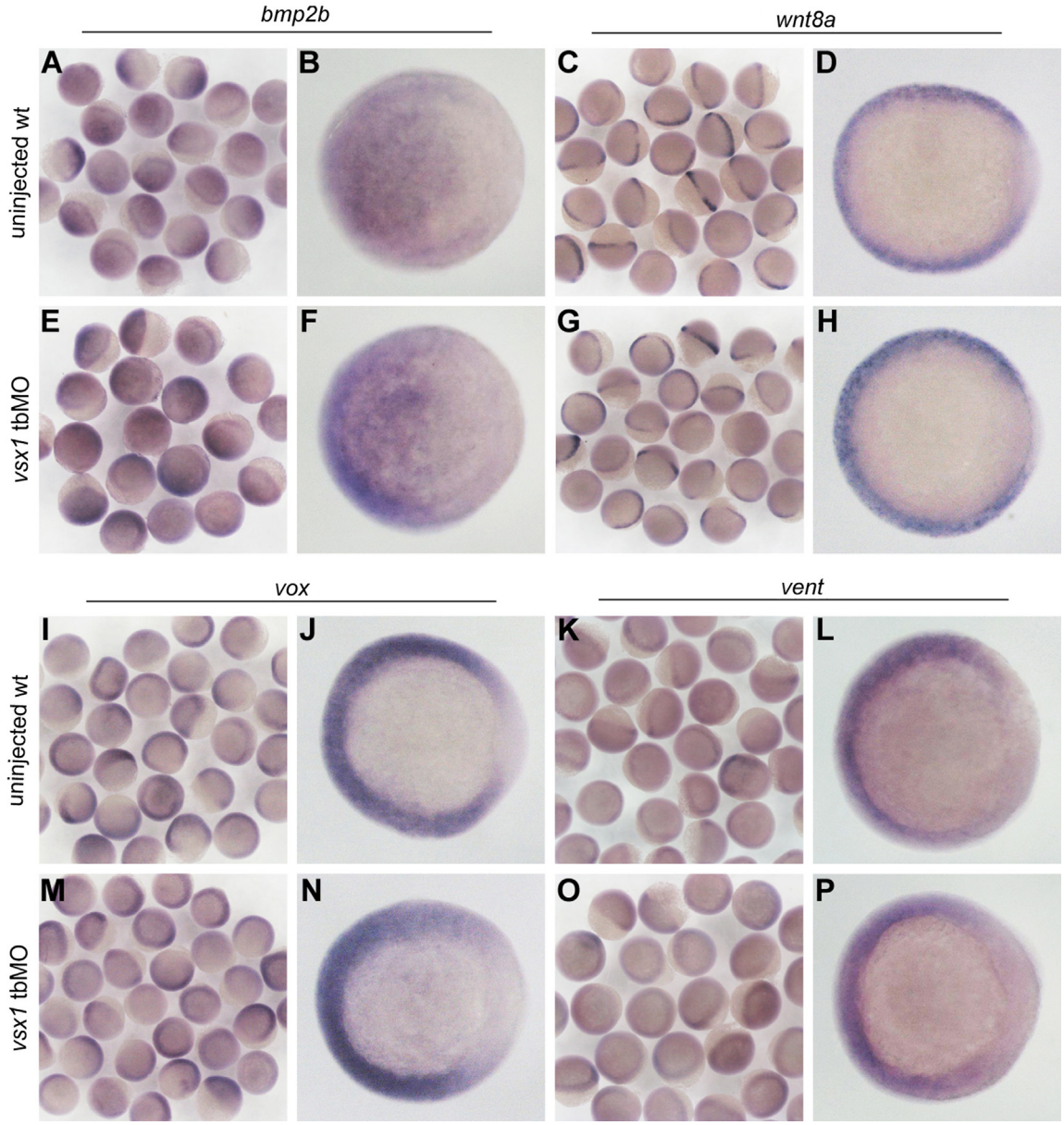

Fig. 6. Injection of *vsx1* tbMO has no obvious influence on the expression patterns of *wnt8*, *bmp2b* and their target *vent*/*vox* at shield stage. The injected reagents are indicated at the left side of the images. Riboprobes are indicated at the top of images. The images of single embryo are animal pole view with dorsal towards the right.

solution without antibody, or from the immunoprecipitation assay with primers spanning the 3rd exon region of *flh* (Fig. 7B). These results indicate that Vsx1 specifically binds to *flh* proximal promoter at the binding site 11 in normal chromatin environment.

Direct interaction between Vsx1 homeodomain and its potential binding consensus sequences at binding site 11 of *flh* promoter was tested by electrophoretic mobility shift assay (EMSA). After fusion peptide Vsx1-HD-His (containing the Vsx1 homeodomain) was incubated with the biotin-5′end-labeled probe of the *flh* promoter sequence, the binding complexes were detected with a much slower motility (Fig. 7C). The specificity of peptide–DNA binding was confirmed with unlabeled probe and mutated competitor. Dose-dependent effect was observed in competing experiment using unlabeled probe. When 500-fold of excess unlabeled probe was used prior to incubation with biotin-labeled probe, only a weak band of binding complex was detected (Fig. 7C). By contrast, no competitive effect was detected with 500-fold or more of excess unlabeled mutant competitor in which the TAATTG was converted into TCCCCG (Fig. 7C). This result indicates that Vsx1 can directly bind to *flh* promoter at the TAATTG motif near the core transcription element.

To determine whether Vsx1 can repress gene expression from the TAATTG motif of the binding site 11, we constructed different GFP reporter gene sensors driven by a 1.9k *flh* proximal promoter containing all the 11 TAATTN motifs, deleted proximal promoter containing only the TAATTG motif of the binding site 11, or a 1.9k mutant proximal promoter in which the TAATTG motif at the binding site 11 was converted to TCGATG (Fig. 7D). Transcription analysis showed that all the wild type or mutant *flh* proximal promoter fragments drove GFP expression successfully and ubiquitously after middle blastula stage (Fig. S3). However, the transcriptional level of GFP sensor driven by the 1.9k mutant *flh* proximal promoter was much higher than that

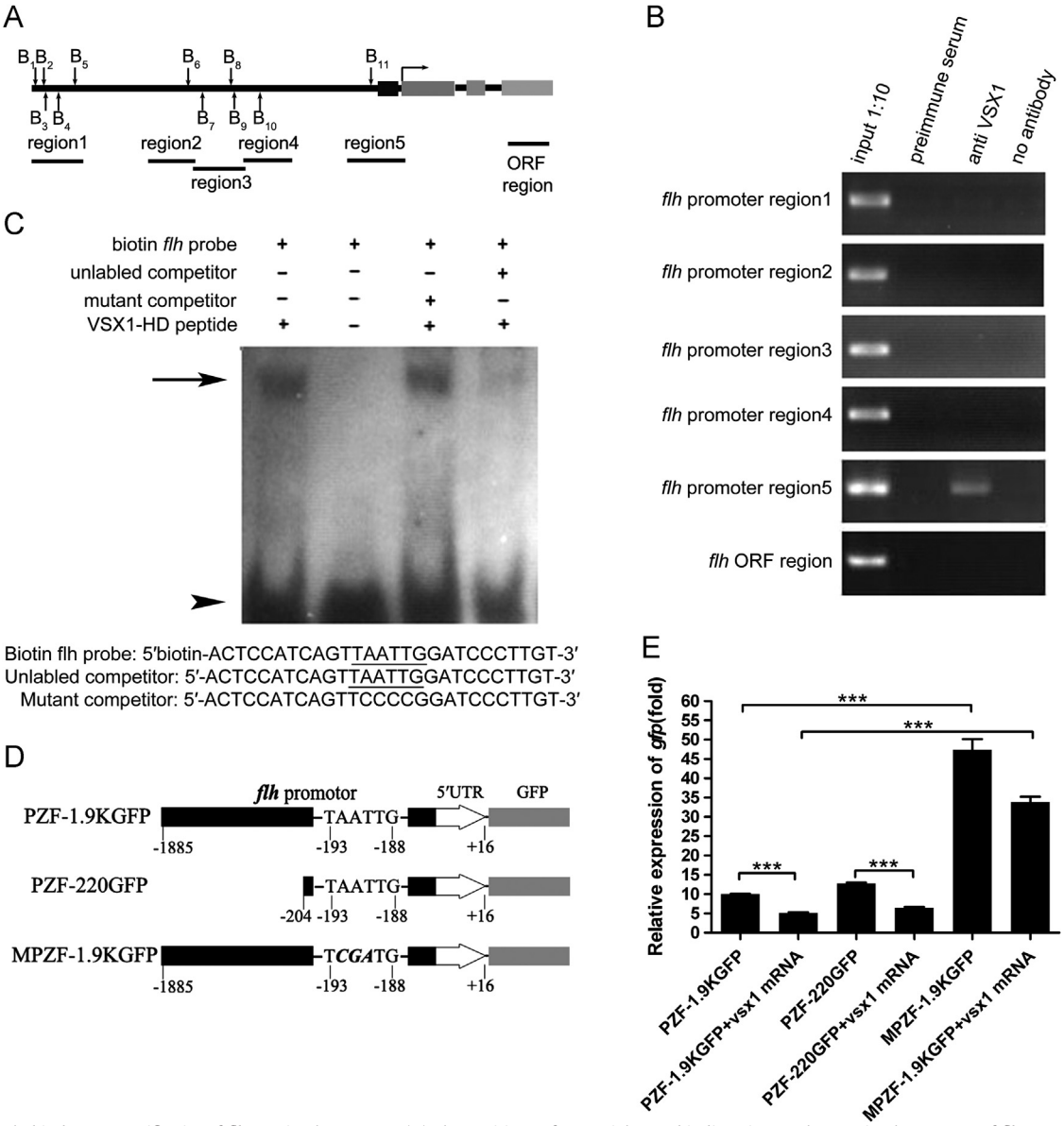

Fig. 7. Vsx1 directly binds to a specific site of *flh* proximal promoter. (A) The positions of potential Vsx1 binding sites at the proximal promoter of *flh*. B_1–B_{11} indicates the potential binding sites of Vsx1. Regions represent the examined regions in ChIP assay. (B) ChIP assay on extracts from Wild type embryos. Input is positive control with the sonicated original genomic DNA fragment. These results show the specific recruitment of Vsx1 by *flh* promoter region 5. (C) Gel electrophoretogram of EMSA of *flh* promoter region 5. Arrow and arrow head indicate the protein-bound probe and the free probe, respectively. 500-fold of excess unlabeled oligonucleotides identical to the probe and unlabeled mutant oligonucleotides were added as competitors when shown. Solution containing no Vsx1-HD polypeptide chain is the positive control. (D) Diagram of GFP reporters driven by a series of truncated or mutant fragments of the *flh* proximal promoter. (E) Vsx1 binding site 11 mediates Vsx1-dependent repression of *flh*. Results are expressed as mean ± SEM, and statistical analyses were done by unpaired *t* test. $^{***}P < 0.001$.

of the GFP sensors driven by wild type *flh* proximal promoter (Fig. 7E). When 200 pg *vsx1* mRNA was co-injected with 40 pg of each the GFP reporter sensors at one-cell stage, the level of *gfp* expression from the sensors driven by *flh* proximal promoter containing the binding site 11 was significantly decreased but from the sensor driven by the mutant kept high (Fig. 7E and Fig. S3). We noted that the expression level of GFP in the mutant reporter and *vsx1* mRNA coinjected embryos was decreased about 30% in comparison to that in mutant reporter alone injected embryo. The statistical significance of the change is $P<0.05$. Since *ntl* and *flh* are reciprocally dependent on one another in their expression (Talbot et al., 1995; Melby et al., 1997; Halpern et al., 1997), the suppression of mutant reporter by injection of *vsx1* mRNA can be explained by an indirect repression due to the suppression of endogenous *ntl*. This result established that the TAATTG motif at the binding site 11 is the sole Vsx1 binding consensus sequences for mediating Vsx1 repressing *flh* in vivo.

Vsx1 contains two amino acid motifs (FGIDKSR and FAITDLLG, Passini et al., 1998) similar to the repressor domains of Engrailed (Smith and Jaynes, 1996). To define that Vsx1 is a transcriptional repressor, we created a transcriptional repressor fusion construct mRNA *En-vsx1* by replacing the N-terminal 125 amino acids of Vsx1 with the Engrailed repressor domain (amino acids 1–298, Fan and Sokol, 1997; Fig. 8A) and a transcriptional activator fusion construct *VP16-vsx1* with the VP16 activator domain (amino acids 410–490, Sadowski et al., 1988; Fig. 8A). QRT-PCR analysis showed that *gfp* expression mediated by a 1.9k *flh* promoter is suppressed by En–Vsx1 fusion protein, whereas significantly activated by VP16–Vsx1 fusion protein (Fig. 8B). Injection of 200 pg *En–vsx1* mRNA into one-cell-stage embryos produced 58% of embryos with bifurcated axes ($N=93$) and abolished the expression of *flh* in the dorsal margin as efficiently as injection of wild type *vsx1* mRNA (Fig. 8F–H). In contrast, injection of 200 pg *VP16–vsx1* mRNA resulted in 43% of embryos ($N=71$) exhibiting a *vsx1* morphant-like phenotype and ventral expansion of *flh* expression domain (Fig. 8I–K). These results demonstrated that the function of N-terminal region of Vsx1 is similar to the Engrailed repressor domain and Vsx1 acts as a transcriptional repressor.

Discussion

To understand how the axial–paraxial mesoderm patterning is initiated, we investigated why the expression domains of paraxial mesoderm decisive gene *spt* and axial mesoderm regulatory gene *flh* initially overlap but are rapidly restricted within the ventrolateral and dorsal margin, respectively, during blastula stage. We demonstrated that maternal Vsx1 can directly repress *flh* transcription to preserve *spt* expression and paraxial mesoderm specification in the ventrolateral margin of blastula embryo. Thus, the original overlapped axial and paraxial mesoderm domains are divided and the initial distinction between axial and paraxial mesoderm domain takes shape. Inhibiting maternal *vsx1* mRNA translation resulted in confusion of axial and paraxial mesoderm markers expression and axial–paraxial mesoderm patterning. These results suggest that direct transcriptional repression of a decisive axial mesoderm gene by maternal ventralizing factor is essential for initiating axial–paraxial mesoderm patterning in vertebrates.

Initial axial–paraxial mesoderm patterning requires complex cooperation between maternal ventral and dorsal determinants

It has been well established that dorsal organizer genes can repress ventral genes. Thereby, restricting the expression of dorsal organizer genes within the normal dorsal region is also essential for normal paraxial mesoderm specification and axial–paraxial mesoderm patterning. Maternal Runx2bt2 can activate *vent*, *vox* and *ved* to restrict the expression of dorsal organizer genes (Flores et al., 2008), suggesting that it might be a maternal ventralizing factor involved in initiation of axial-paraxial mesoderm patterning. To establish a comprehensive understanding of initial axial-paraxial mesoderm patterning, it is worthy to examine whether maternal Vsx1 cooperates with maternal Runx2bt2 and cooperatively interacts to maternal dorsal determinants.

Maternal Vsx1 has a role in patterning the convergence and extension domains

Previous experiments observed that Spt can cell-autonomously regulate lateral mesoderm cell convergence to the dorsal midline (Ho and Kane, 1990; Kimmel et al., 1989) due to that *papc* is not promoted in *spt* mutant embryos (Yamamoto et al., 1998). It has been demonstrated that, during convergence, PAPC is not only an important signaling molecule of the Wnt/planar cell polarity pathway (Medina et al., 2004; Unterseher et al., 2004; Wang et al., 2008) but also a critical cell adhesion molecule essential for embryonic cell sorting and orientation migration in both *Xenopus* and zebrafish (Chen and Gumbiner, 2006; Kim et al., 1998). Therefore, *papc* expression domain depicts the convergence territory of the early gastrula embryo. Since *papc* is a downstream target of Spt and Flh is the repressor of *spt*, the patterning of *papc* expression and repression domains in the margin of embryo are the consequences of the patterning of *spt* and *flh* expression domains, respectively. Maternal Vsx1 is required for the initial definition of *flh* and *spt*/*papc* expression domains, implying that it has a role in patterning convergence and extension domains.

Indeed, *ntl* marked axial mesoderm domain was expanded in width but shortened in length in maternal *vsx1* knockdown embryos, *myoD* marked paraxial mesoderm domain failed in converging to the normal dorsal position. These phenotypes substantiate that both inhibition and misexpression of *vsx1* can result in convergence and extension defects.

There are two parallel pathways in the maintenance of paraxial mesoderm identity during gastulation

Inhibition of maternal *vsx1* translation also elicited strong ectopic expression of *flh* and concomitant repression of *spt* and *papc* in the ventrolateral margin of middle gastrula embryos (Fig. 5). Since maternal Vsx1 had been depleted in normal embryos at gastrula stage (Fig. 4A), this phenomenon suggests that maternal Vsx1 might regulate the maintenance of axial–paraxial mesoderm patterning in an indirect manner. However, maternal Vsx1 has no impact on the expression of *wnt8a*, *bmp2b*, *vox* and *vent* (Fig. 6), the genes essential for maintaining the ventral identity during gastrulation (Ramel et al., 2005). These observations indicate that there are two parallel mechanisms co-regulating the maintenance of paraxial mesoderm identity during gastrulation. Some of genes involved in paraxial mesoderm specification and differentiation are regulated by maternal Vsx1 and others are regulated by zygotic Wnt8b and Bmp2b signaling pathways. Therefore, it is impossible to fully convert the paraxial mesoderm into axial mesoderm at the trunk and tail region by inhibiting the function of maternal Vsx1 alone.

Aberrant axial–paraxial mesoderm patterning in vsx1 knockdown and overexpression embryos is due to confused gene expression and convergent extension defects

Though ectopic expression of axial gene *flh* resulted in repression of paraxial genes *spt* and *papc*, the expression of other paraxial mesoderm genes in the zygotic Wnt8a and Bmp2b signaling pathways was maintained in the ventrolateral margin

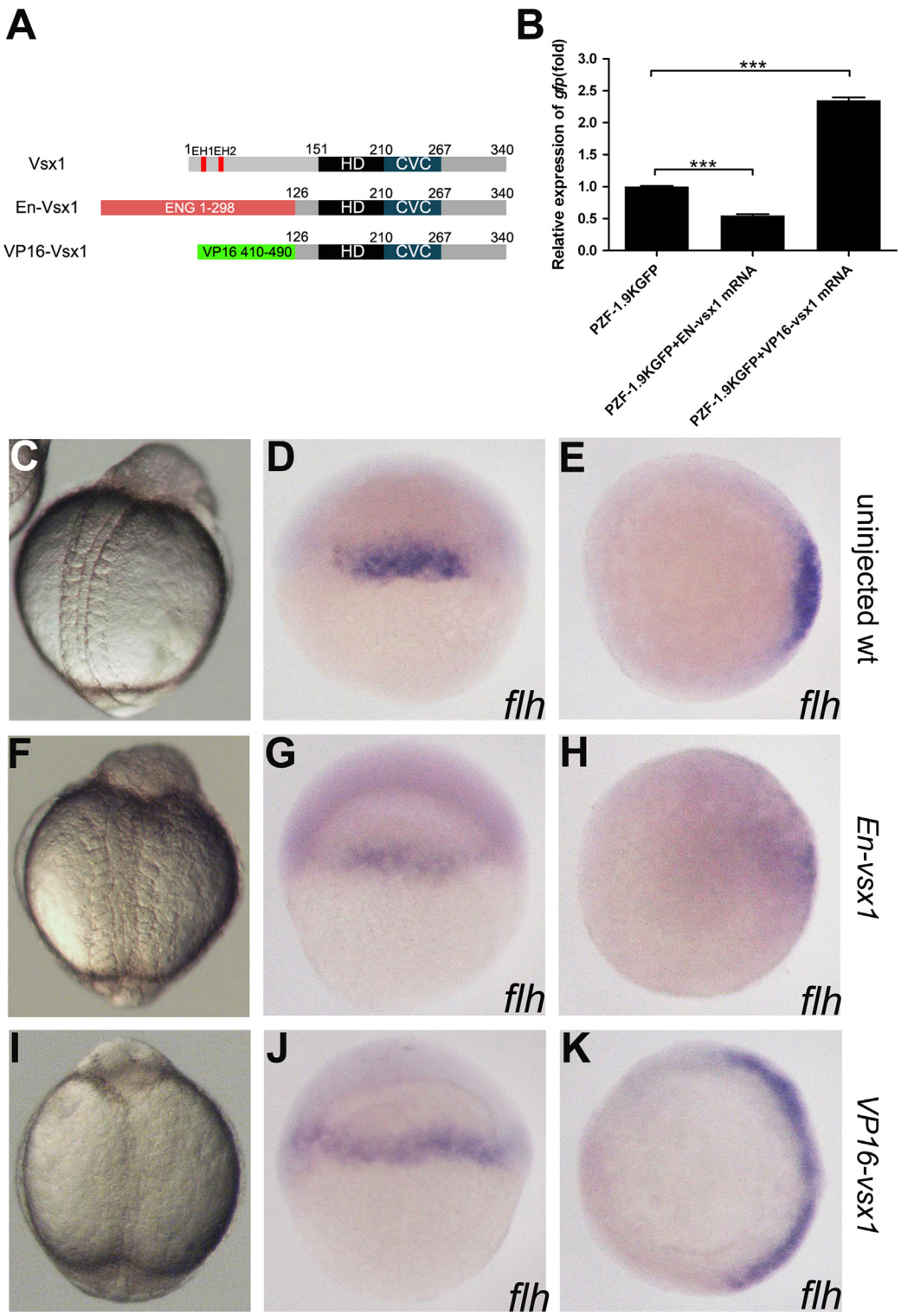

Fig. 8. Vsx1 is a transcriptional repressor. (A) Schematic representation of the Vsx1 mutants. (B) Comparison of *flh* promoter mediated *gfp* expression under the regulation of a fusion protein En–Vsx1 and a fusion protein VP16–Vsx1. Results are expressed as mean ± SEM, and statistical analyses were done by unpaired *t* test. $^{***}P < 0.001$. (F–H) En–Vsx1 fusion protein functions as a wild type Vsx1. (I–K) VP16–Vsx1 fusion protein functions as a Vsx1 antimorph. The injected reagents are indicated at the right side of the images and riboprobes are indicated at the bottom of each figure. (C, F and I) Dorsal view of phenotypes at 8–10 somite stage with head towards the top. (D, G and J) Dorsal view of embryos with animal pole towards the top. (E, H and K) Animal pole view of the embryos with dorsal towards the right. In situ hybridization of *flh* is shown at 30–40% epiboly stage.

during gastrulation. Reasonably, the suppression of paraxial mesoderm in the *vsx1* morphant cannot be simply explained by cell fate change from paraxial to axial mesoderm, but rather, by confused expression of axial and paraxial mesoderm genes and concomitant defects of convergent extension. It has been observed that the expression of a dominant negative PAPC can inhibit paraxial *myoD* expression due to convergence and extension defects (Yamamoto et al., 1998). Therefore, PAPC absence and concomitant convergent extension defects may contribute to the suppression of *myoD* expression during gastrulation in the *vsx1* morphants.

Similarly, the formation of expanded width but shortened length domain of axial *ntl* in the *vsx1* morphants also can be

explained by the confused expression of axial and paraxial mesoderm genes and concomitant convergent extension defects, rather than the cell fate change from paraxial to axial mesoderm. Previous studies have established that *ntl* and *flh* are reciprocally dependent on one another in their expression (Talbot et al., 1995; Melby et al., 1997; Halpern et al., 1997), and Spt is likely a repressor of *ntl* activity (Amacher and Kimmel, 1998). Moreover, Ntl is a possible inhibitor of cell migration of prechordal mesoderm as frog homolog Brachyury does (Kwan and Kirschner, 2003). It is possible that maternal *vsx1* knockdown may elicit *ntl* misexpression, as a consequence of the overexpression of *flh* and the loss of *spt* expression, in the paraxial mesoderm and prechordal mesoderm cells. In this case, the paraxial mesoderm cells simultaneously express markers of both axial and paraxial mesoderm (the width of *ntl* marked axial mesoderm domain appears ventrally expanded) and the migration of prechordal mesoderm cells to the anterior region is inhibited (the length of the axial *ntl* domain appears shortened). Further experiments are ongoing to test this possibility and the likely impact on early embryogenesis.

Confused expression of axial and paraxial mesoderm genes and concomitant convergent extension defects can also explain why *vsx1* overexpression embryos has widely bifurcated paraxial mesoderm domains but no distinguishable dorsal midline structures at the trunk region. In fact, it has been observed that *flh* mutant axial cells simultaneously express markers of both axial and paraxial mesoderm (Halpern et al., 1995). Therefore, by repressing *flh* expression in the axial mesoderm, injection of *vsx1* mRNA is able to disturb notochord formation and paraxial mesoderm convergent extension, but is unable to fully convert the axial mesoderm fate into paraxial mesoderm fate at the trunk and tail region.

Complex interaction between Vsx1 and binding site recognition

There are 11 potential homeodomain binding sites that contain the consensus sequence TAATTN in the analyzed proximal promoter region of *flh*. Both ChIP and mutant examinations of the binding site demonstrated that the site near the core transcription element is the sole Vsx1 binding site. This observation suggests that, beside the homeodomain and the consensus DNA binding sequence of homeodomain, other functional domains of Vsx1 and *cis*-elements of the *flh* promoter may take part in the complex interaction between the protein and DNA recognition. Of all the 11 potential binding sites at the *flh* proximal promoter, the unique structural characteristic of the actual binding site of Vsx1 is within a GC-rich region (Fig. S2). Therefore, the GC-rich region at the proximal promoter of *flh* might contain *cis*-elements for Vsx1 to select the binding site. It is interesting to investigate whether the evolutionary conserved CVC domain of Vsx1 plays a role in the binding site recognition at the GC-rich region.

Materials and methods

Animals and obtaining of embryos

Zebrafish were maintained at 28.5 °C in a 14 h/10 h light/dark cycle. Embryos were collected after fertilization and staged by morphology as described by Kimmel et al. (1995). Embryos were dechorionated with 0.25% trypsin in 1 × PBS.

RNA extraction and reverse transcription

Total RNA from zebrafish embryos or adult tissues was extracted by SV Total RNA Isolation System (Promega) and was treated with the TURBO DNA-*free*™ Kit (Ambion, USA) to remove DNA contamination. The reaction of reverse transcription (total volume of 10 μL) contained approximately 500 ng of total RNA, 0.5 μL 100 μM Random 6 mers, 0.5 μL 50 μM oligo dT Primer, 2 μL 5 × PrimeScript™ Buffer and 0.5 μL PrimeScript™ RT Enzyme Mix I using PrimeScript™ RT reagent Kit (TaKaRa, Japan). The first strand cDNA was synthesized for 15 min at 37 °C, and the RT Enzyme was inactivated at 85 °C for 5 s. The products of reverse transcription were subjected to the next PCR reactions.

Preparation of capped mRNA

According to zebrafish *vsx1* encoding sequence (GenBank accession number: BC059574.1), *vsx1* ORF was amplified by RT-PCR from zebrafish retina, using primers 5′-CAGGACGAATTCATGACGGGAAGAGAAGAAGCT-3′ and 5′-GGGCGCTCGAGTTAACTCTCATTTTCAGAATCG-3′ (the restriction enzyme sites are underlined). *vsx1* mis-ORF which has 5 synonymous mutation bases downstream of *vsx1* translation start site was generated by RT-PCR using the primers 5′-AGGACGAATTCATGACaGGcAGgGAgGAgGCa-3′ (the lowercase indicates the mutation bases) and 5′-GGGCGCTCGAGTTAACTCTCATTTTCAGAATCG-3′. Zebrafish *flh* ORF and *spt* ORF were generated by RT-PCR from embryos. Primers used for *flh* amplification were designed according to the sequence of zebrafish *flh* (GenBank accession number: NM_131055). Primers used for *spt* amplification were designed according to the sequence of zebrafish *spt* (GenBank accession number: AF077225). To generate the expression plasmids of these genes, the ORFs were inserted separately into the *EcoR* I and *Xho* I sites of an expression vector pCS107. Capped mRNAs were in vitro synthesized in the presence of cap analog using the mMESSAGE mMACHINE SP6 Kit (Ambion, USA) according to the instruction of products and purified by Quick Spin Columns (Roche, Switzerland).

Morpholinos

vsx1 Morpholino antisense oligonucleotides (MO) were designed and synthesized by Gene-tools (Philomath, OR). The sequences of *vsx1* translation blocking MO (tbMO) is TGT AGCTTCTTCTCTTCCCGTCATG and *vsx1* splice-blocking MO (sbMO) is AGCAAAGTGATTCGTACCGGAGTAA as published (Clark et al., 2008).

Generation of GFP-sensors, En–vsx1 and VP16–vsx1 fusion constructs

Wild type and mutant *flh* proximal promoter driven and mutant GFP reporter sensors were constructed by inserting different *flh* proximal promoter fragments into the pEGFP-1 plasmids between the *Sac* I and *Kpn* I sites, and were named according to the length and type of *flh* proximal fragments inserted (Fig. 7D). Fragments of *flh* proximal promoter were amplified by PCR with specific primers designed according to the zebrafish *flh* genomic sequence (GenBank accession number: BX571943.8). The *En–vsx1* construct was generated by recombinant PCR to fuse the Engrailed repressor domain (amino acids 1–298) to the N-terminal of Vsx1 and the *VP16–vsx1* construct was generated by recombinant PCR to fuse the VP16 activation domain (amino acids 410–490) to the N-terminal of Vsx1 (Fig. 8A). All the recombinants were reconfirmed by sequencing.

Microinjection

Samples were injected into the zebrafish embryos at the 1 to 2-cell stage. For co-injection, the desired samples were mixed thoroughly prior to injection. Injected embryos were maintained at 28.5 °C in tap water with antibiotics.

Whole mount in situ hybridization

The desired lengths of code sequence of genes were inserted into pBluescriptIISK plasmids. The constructed plasmids were linearized and antisense RNA probes were synthesized in vitro using 50 units of the appropriate RNA polymerase (T7 or T3) in the presence of DIG mix (Roche, Switzerland). Whole-mount in situ hybridization was carried out as described by Thisse and Thisse (2008) with minor modification.

Quantitative RT-PCR

Real-time quantitative RT-PCR was performed in a PCR Light-Cycler 480 System (Roche, Switzerland) using SYBR® Prime-Script™ RT-PCR Kit (TaKaRa, Japan) according to the recommendation of the manufacturer. *ef1α2* was employed as the internal standard. The melting curve was analyzed after amplification to identify the specific product in all PCR reactions. The threshold cycle (Ct) values of $2^{-\Delta\Delta CT}$ was calculated by qRT software provided for the LightCycler® 480 System (Roche, Switzerland). The histogram for fold comparison of different samples was generated by inputting the $2^{-\Delta\Delta CT}$ values of different samples into the GraphPad Prism4 program software (Roche, Switzerland). For each sample, the test and control reactions were run in triplicate.

Proteins expression and polyclonal antibody preparation

A polypeptide chain containing Vsx1 homeodomain (residues 132–224) and the C-terminal of zebrafish Vsx1 peptide (residue 267–340) were expressed with prokaryotic expression vector pGEX-4T-1. The purity of the two obtained protein was assessed by western blot. We prepared the mouse polyclonal antibody against zebrafish Vsx1 by injecting the purified C-terminal peptides into the mouse for 4 times according to the routine protocol. The specificity of the obtained antibody in recognizing Vsx1 protein was verified by Western blotting examination (Fig. S4).

Immunohistochemistry

Vsx1 protein was detected by immunohistochemistry. Zebrafish embryos at dome stage were fixed in 4% paraformaldehyde in PBS overnight at 4 °C, dehydrated through 25%, 50%, 75%, 100% methanol/PBST (PBS/Tween-20, 0.1%) by turns and stored in methanol at −20 °C until required (2 h to several months). At first, embryos were rehydrated by successive incubations in 75%, 50%, 25% methanol/PBST, washed twice in PBST and twice in 1% DMSO/PBST. Endogenous peroxidases were inactivated by a solution of 80% methanol with 3% H_2O_2 for 15 min at RT (room temperature). The embryos were transferred into 1% DMSO/PBST, blocked in blocking buffer (10% goat serum in 1% DMSO/PBST) overnight at 4 °C, and then immersed in polyclonal antibody against ZF-VSX1 diluted in 1:500 with blocking buffer. The goat anti-IgG (H+L) secondary antibody (cwbiotech, Lot05181013, Beijing, China) was used at a dilution of 1:1000, shaking for 2 h at room temperature. Before detection, the fresh DAB solution was made according to the manufacturer's instructions (SIGMA, D4293, USA). DAB reaction was stopped with 1% DMSO/PBST after 25 min.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed using the ChIP-IT™ Express kit (Active motif, California, USA). Wild type embryos were harvested at 90% epiboly stage and crosslinked in 1% formaldehyde, then washed in PBS for three times. Crosslinking was stopped using glycine stop-fix solution, and after washing in PBS, embryos were resolved in lysis buffer, then homogenized with Dounce homogenizer, later transferred to shearing buffer. Extract was then sonicated to produce DNA fragments between 200 and 1500 bp. After sonication, one-tenth of supernatant was removed as input DNA, the other was incubated overnight at 4 °C with 25 μl of protein G magnetic beads and respective antibodies. Beads were washed with CHIP buffer 1 and buffer 2, resuspended and incubated in Elution Buffer AM2. Cross-linking was reversed by reversed cross-linking buffer and incubating the samples at 95 °C for 15 min. Finally samples were digested with proteinase K and stopped with stop solution, thus DNA was then used in PCR immediately. A pre-denaturation of 3 min at 94 °C was followed by 30 cycles (20 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C). Primers used in amplifying different regions of *flh* proximal promoter containing potential Vsx1 binding sites (Fig. 7A) were designed according to the zebrafish *flh* genomic sequence (GenBank accession number: BX571943.8).

Electrophoretic mobility shift assay (EMSA)

Oligonucleotides were 5′end labeled with biotin. The sequences of biotin labeled probe, unlabeled wild type and mutant competitive probes are indicated in Fig. 7C. EMSA was performed according to manufacture's instruction of Lightshift Chemiluminescent EMSA Kit (Pierce, USA). Briefly, 0.8 μL expressed and purified Vsx1 homeodomain peptide was used in the binding reaction. Protein:DNA mixes were resolved on 6% non-denaturing polyacrylamide gels. After electrophoresis, DNA oligonucleotides were transferred onto nylon membrane by electroblotting and UV crosslinked. Biotin-labeled DNAs were visualized with streptavidin-bound HRP and Luminol/Enhancer chemiluminescent substrate (Pierce, USA) and chemiluminescence detected by exposure to Kodak imaging film. In competing experiments, different unlabeled probes were incubated with the purified Vsx1 homeodomain peptide ahead of the incubation with labeled probe.

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Appendix. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.11.022.

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