

## Research Paper

# Eaf1 and Eaf2 mediate zebrafish dorsal-ventral axis patterning *via* suppressing Wnt/ $\beta$ -Catenin activity

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## Abstract

During early vertebrate embryogenesis, maternal Wnt/ $\beta$ -catenin signaling is thought to locally initiate expression of dorsal-specific genes. Here, *eaf1* and *eaf2* were identified as important maternal and zygotic modulators of Wnt signaling to initiate and specify ventral genes. Expression of ventral *ved*, *vent*, and *vox* was all obviously enhanced in either maternal or zygotic *eaf1/2* morphants, and in both *eaf1* heterozygous and homozygous mutants, but their expression was suppressed in embryos with over-expression of *eaf1/2*. Additionally, *eaf1/2* were revealed to suppress ventral fates in embryos *via* Wnt/ $\beta$ -catenin1/Tcf signaling, complimentary to their roles in suppressing dorsal fates *via* Wnt/ $\beta$ -catenin2 signaling. Moreover, *eaf1/2* were also revealed to obviously suppress the expression of *axin2* induced by  $\beta$ -catenin2 rather than by  $\beta$ -catenin1, and the dorsal expression of *axin2* in embryos was obviously suppressed by ectopic expression of *eaf1/2*. This study uncovers a novel dorsal-ventral patterning pathway, with *eaf1* and *eaf2* inhibiting ventral cells *via* suppressing Wnt/ $\beta$ -catenin1/Tcf signaling and inducing dorsal cells indirectly *via* suppressing  $\beta$ -catenin2-induced-*axin2* on the dorsal side of embryos.

Key words: *eaf1*, *eaf2*,  $\beta$ -catenin, *axin2*, dorsal-ventral patterning

## Introduction

Vertebrate embryos form their axes by dorsal-ventral patterning and anterior-posterior patterning. Maternal factors are required to induce zygotic expression of ventral or dorsal genes to establish dorsal-ventral polarity during the first step of axis formation. In zebrafish, the dorsal-ventral specification starts at approximately 128-cell stage when maternal  $\beta$ -catenin accumulates in the nuclei of blastomeres in the dorsal domain (1), initiating the expression of dorsal organizer genes, such as *sqt*, *boz*, and *gsc* (2, 3). Lower expression of maternal  $\beta$ -catenin2 and reduced expression of dorsal genes are observed in zebrafish *ich* mutants (2, 3), where maternal  $\beta$ -catenin2 rather than maternal  $\beta$ -catenin1 has been found to be essential for the specification of dorsal fates in early stage embryos (4, 5). Reciprocal activities are needed to induce the expression of

ventral genes. Maternal TGF- $\beta$ /Rdr, Wnt/ $\text{Ca}^{2+}$  pathways and Pou2 complementarily specify ventral *bmp2b* and *bmp4* expression (6–8). Moreover, maternal *runx2* is the only maternal determinant identified to initiate the zygotic ventral factors of *vent*, *vox*, and *ved* (9). Despite observations of nuclear accumulation of  $\beta$ -catenin protein in both dorsal and ventral cells of *axin*-depleted embryos at early blastula stage (10) as well as ubiquitous expression of both  $\beta$ -catenin genes in early stage embryos (4), it still remains unknown whether maternal Wnt/ $\beta$ -catenin signaling functions in initiation and specification of ventral cells.

After the specification of dorsal-ventral polarity, zygotic Wnt/ $\beta$ -catenin signaling is activated and required to antagonize the dorsal organizer genes for proper dorsal-ventral axis formation. Zygotic Wnt/ $\beta$ -catenin signaling is activated by Wnt ligands

after mid-blastula transition (MBT) and maintains high expression of *vox*, *ved*, and *vent* on the ventral side, thereby antagonizing expression of dorsal genes *sqt*, *boz*, and *gsc* (11). Consistently, zebrafish homozygous mutants for *wnt8* show significant expansion of shield markers and almost absent expression of ventral-lateral mesoderm markers (12). Additionally, two dominant mutants of negative regulators of Wnt/ $\beta$ -catenin signaling, *dntcf* and *tcfBD*, can also induce ventral-lateral ectopic expression of dorsal-specific genes in the marginal region (13).

*Axin2*, a target of Wnt/ $\beta$ -catenin signaling, is part of a degradation complex that functions to phosphorylate  $\beta$ -catenin and negatively regulates the signaling pathway (14–16). In *Xenopus*, embryos exhibit severe ventralization with ectopic expression of *axin* but anterior/dorsalization after depleting maternal *axin* mRNA (10).

Eaf1 and Eaf2 are originally identified as partners of ELL (Eleven-nineteen lysine-rich leukemia), a fusion protein frequently associated with myeloid leukemia, and the two genes are found to suppress prostate cancer and leukemia (19–21). In our previous studies, during zebrafish embryogenesis, both *eaf1* and *eaf2* have been revealed to mediate effective convergence and extension movements (22), form a negative feedback loop with *wnt4* (23), and function as novel factors antagonizing canonical Wnt/ $\beta$ -catenin signaling in forebrain induction (5) and as novel factors suppressing TGF- $\beta$  signaling in germ layers specification (24). We have also found dorsal-ventral axis patterning defects in *eaf* morphants (5), but how maternal and zygote *eaf1/2* act in patterning ventral cells is still unclear. In this study, maternal expression of *eaf1/2* was revealed first. Then, *eaf1* and *eaf2* were revealed to suppress expression of ventral *vent* family genes by genetically modulating the Wnt/ $\beta$ -catenin1/Tcf pathway, complementing their roles of suppressing dorsal cell fates in the Wnt/ $\beta$ -catenin2 pathway (5). *Eaf1* and *eaf2* were also revealed to modulate the dorsal-ventral polarity of *axin2* and promote the expression of dorsal genes indirectly by suppressing *axin2* expression induced by  $\beta$ -catenin2 rather than by  $\beta$ -catenin1 in the dorsal region.

## Materials and Methods

### Fish stocks

Wild-type zebrafish (*Danio rerio*) (AB) maintenance, breeding, and staging were performed as described previously (5, 22).

### Morpholino, CRISPR/Cas9, and mRNA synthesis

The translation-blocking morpholinos (ATG

targeted), *eaf1*-MO1 and *eaf2*-MO1, and the splicing morpholinos, *eaf1*-MO3 and *eaf2*-MO3, have been described previously (5, 22).  $\beta$ -catenin1 and  $\beta$ -catenin2 antisense morpholinos have also been described previously (4, 5, 25). All morpholinos are purchased from Gene Tools, LLC (Philomath, Oregon, USA), and their sequences have been reported previously (5) and are shown in Supplemental Table 1. CRISPR/Cas9 genome editing system is reported as an effective tool for gene editing in organisms (26, 27). Cas9 system was used to construct F0 fish for editing *eaf1* or *eaf2*, and the sequences of gRNA for *eaf1* or *eaf2* were reported in our current published paper (24), but only *eaf1* mutants were constructed successfully (24). Expression of *ved*, *vent*, or *vox* was tested in embryos from in-crossed *eaf1* F1 heterozygous mutants, and the represented embryos with increased expression or normal expression were genotyped by primers in Table S2.

For mRNA preparation, capped mRNAs were synthesized using the AmpliCap SP6 High Yield Message Maker Kit (Epicentre Biotechnologies, Madison, WI, USA). The plasmids for zebrafish *eaf1*, *eaf2*, *eaf1*-N, *eaf2*-N, EnR-*eaf1*-full, EnR-*eaf2*-full, and *dntcf* mRNA were prepared as described previously (5, 22). Briefly, full-length zebrafish *eaf1* and *eaf2* were sub-cloned into PSC2-EnR to form fusion protein expression vectors EnR-*eaf1*-full and EnR-*eaf2*-full for mRNA synthesis, and exons1-3 of zebrafish *eaf1* or *eaf2* were sub-cloned into PSP64 vector for synthesizing mRNAs of *eaf1*-N or *eaf2*-N. The synthesized mRNAs and morpholinos were diluted into different concentrations and injected into one-cell stage embryos as described previously (5, 22).

### Microscopy Observations

Zebrafish embryos at 30 hpf (hours post fertilization) were de-chorioned, and embryos at 5 dpf (days post fertilization) were side-viewed and photographed under light microscope (Leica M205FA, Germany). Morphological embryos from different groups were quantified using Adobe Photoshop CS5 software.

### Whole-mount in situ hybridization

Probes for *ved*, *vox*, *vent*, *axin2*, *wnt8a*, *bmp2*, and *fgf8* have been described previously (5, 22, 25). The procedure of whole mount *in situ* hybridization (WISH) was performed as described previously (5, 22, 25).

### RNA extraction and semi-quantitative RT-PCR

Total RNA was isolated from 30 whole embryos at indicated developmental stages using TRIZOL reagent (Invitrogen). Oligo-dT-primed cDNA was

synthesized using RevertAid First Strand cDNA Synthesis Kit (Fermentas, Burlington, Ontario). The primers used for amplifying zebrafish *ved*, *vox*, *vent*, *bmp2b*, *wnt8a*, and 18s (used as an internal control) were listed in Supplemental Table 2. Semi-quantitative RT-PCR (qRT-PCR) was performed as described previously (5, 24, 25). Each sample was run in triplicate and repeated at least three times. Differences were calculated by the  $\Delta\Delta C_t$  comparative quantization method using 18s as an internal control, and the data were analyzed with one-way analysis of variance (ANOVA) and *post hoc* Tukey's test (\*\* represents  $P < 0.001$ ; \* represents  $P < 0.01$ ; \* represents  $P < 0.05$ ) (24, 28, 29).

### Plasmid construction

Plasmids of HA-zeaf1, HA-zeaf2, Myc-zeaf1, Myc-zeaf2, HA-zcatenin1 and HA-zcatenin2 have been described previously (5). Plasmids of Flag-zcatenin1, Flag-zcatenin2, Myc-zcatenin1, Myc-zcatenin2, Myc-ztcf, HA-ztcf were constructed using the primers listed in Supplemental Table 3. The *axin2* promoter-driven luciferase reporter was a generous gift from Dr. Frank Costantini (Columbia University, New York, USA).

### Luciferase reporter assay

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (HyClone). Luciferase reporter assays were performed as described previously (5, 25). The luciferase activities were determined at 24-30 hours post-transfection using the Dual-luciferase Reporter Assay System (Promega). The relative light units were measured using a luminometer (Sirius, Zylux Corporation, Oak Ridge, TN). Data were normalized by pTK-Renilla luciferase and were reported as the mean  $\pm$  SEM of three independent experiments performed in triplicate. The statistical analysis (paired t-test) was performed using GraphPad Prism 5.

### Immunoprecipitation assays

For Co-immunoprecipitation (Co-IP) assays, 293T cells were transfected with the indicated plasmids. After 28-36 hours of transfection, the cells were washed with ice-cold PBS (*phosphate-buffered saline*) buffer and then lysed with modified radio-immune-precipitation (RIPA) buffer (50 mM Tris, pH 7.5; 1% Triton X-100, 0.5% deoxycholate; 10 mM EDTA; 150 mM NaCl; 50 mM NaF; 1 mM PMSF; 20  $\mu$ g/ml Aprotinin; 1 mM Na<sub>3</sub>VO<sub>4</sub>) with a protease inhibitor cocktail (Sigma). After incubation on ice for 1 hour, cell lysates were centrifuged for 15 min at 10000 g (4 °C), and supernatants were incubated with HA conjugated-agarose beads (SC-7392AC,

Santa Cruz) for 6 h or over-night at 4°C. The immune-precipitates were washed 3 times with RIPA buffer and left with about 50  $\mu$ L liquid. Immuno-precipitates (IP) and whole cell lysates (WCL) were boiled with SDS sample buffer, separated on SDS-PAGE and transferred to PVDF membrane (Millipore). For detecting the interaction abilities of Eaf1/Eaf2 with  $\beta$ -catenin1/2, cells were transfected with HA-catenin1, Myc-eaf1/2, and gradient Flag- $\beta$ -catenin2 together, or HA-catenin2, Myc-eaf1/2, and gradient Flag- $\beta$ -catenin1 together, and incubated with conditioned media from mouse L cells. For detecting the interaction abilities between Tcf and  $\beta$ -catenin1/2, cells were transfected with the indicated combination of plasmids and then incubated with conditional media. Western blot analysis was performed as described previously (5, 24) using the following antibodies: anti-Myc antibody (Santa Cruz) with a 1:2000 dilution, anti-HA antibody (Covance) with a 1:5000 dilution, anti-Flag antibody (Santa Cruz) with a 1:2000 dilution, anti- $\beta$ -actin antibody (Abcam) with a 1:2000 dilution. The blots were photographed using a FujiFilm LAS4000 mini luminescent image analyzer.

## Results

### Eaf1 and Eaf2 are maternally expressed factors

qRT-PCR was used to test *eaf1* and *eaf2* expression from 0 hpf to 24 hpf (hour post fertilization) in zebrafish embryos. Abundant maternal *eaf1* and *eaf2* (Figs.1A2 and B) transcripts were observed in embryos at 0 hpf, with their expression at high levels from 0 hpf to sphere stage, sharply reduced from 50% epiboly stage, and at relatively low levels at 24 hpf in embryos (Fig.1).

The distribution of *eaf1* and *eaf2* in embryos at cleavage stages was also detected by WISH. Maternal and ubiquitous distribution of *eaf1* was observed in embryos at 4 cell, 8 cell, and multi-cell stages (Figs.1C1-C4), similar to its ubiquitous distribution in embryos at early blastula stage (5). Additionally, abundant *eaf2* transcripts were also uniformly distributed in each cell of embryos at 4 cell to multi-cell stage (Figs.1C5-C8), but their slightly different spatial distribution was observed in the dorsal region of embryos at gastrula stage (Figs.1D1 and D2) and tail bud stage (Figs.1D3 and D4).

### Eaf1 and Eaf2 suppress the initiation of early ventral zygotic genes

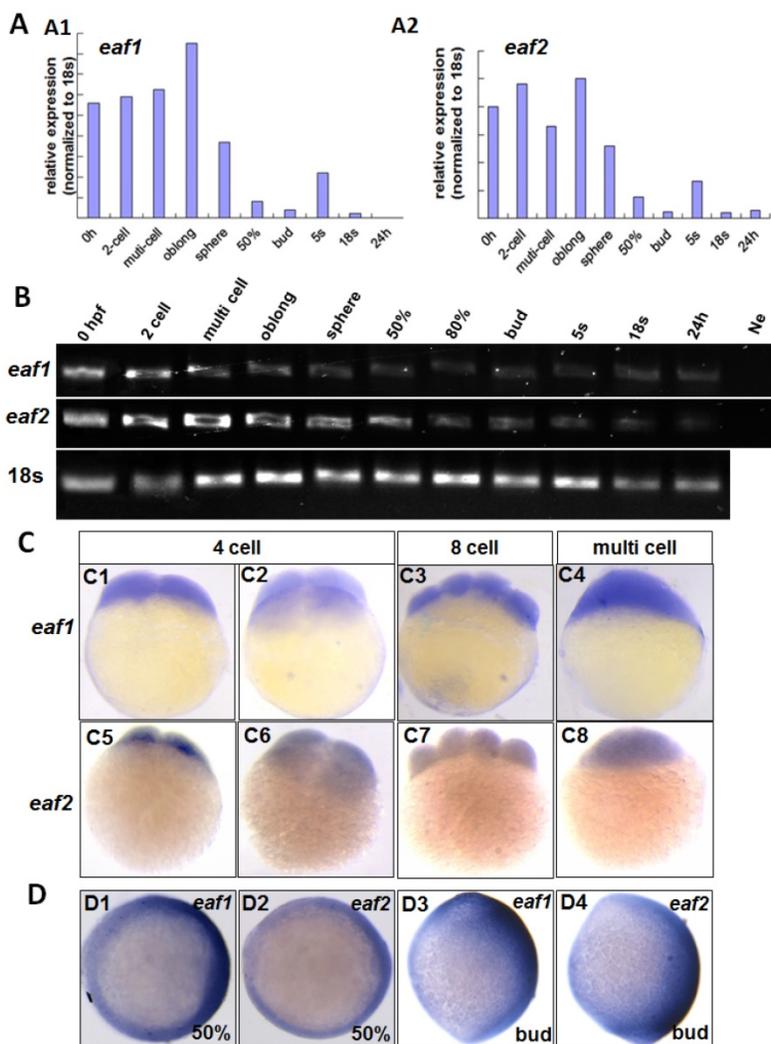
Expression of early zygotic ventral genes *ved*, *vox*, and *vent* was tested in *eaf1* or *eaf2* loss-of-function embryos first. Transcripts for ventral genes *ved* (Figs.2A1-A3, the number 12/15 in the right hand

corner in A2 indicated that 12 embryos exhibited increased *ved* expression in a total of 15 tested *eaf1*-MO1 morphants, and similar indicating numbers were used in the following panels), *vox* (Figs.2A6-A8) and *vent* (Figs.2A11-A13) were enhanced in embryos injected with either zebrafish *eaf1* (11/19 and 9/17 respectively) or *eaf2* (17/25 and 15/22 respectively) ATG morpholinos (*eaf1*-MO1 and *eaf2*-MO1, respectively) at the oblong stage (3.5 hpf to 3.7 hpf). Additionally, increased expression of *ved* was also observed in *eaf1/2* ATG morphants at 30% epiboly stage (Figs.2A14-A16) (14/15 and 16/18 respectively). To detect whether zygotic *eaf1* or *eaf2* patterned the dorsal-ventral cell fates, we targeted *eaf1* or *eaf2* with

splice-blocking MO (*eaf1*-MO3 and *eaf2*-MO3, respectively) (5, 22, 24). Consistently, the splicing morpholinos induced slightly increased expression of ventral cell markers (Figs.2A4, A5, A9, and A10). Expression of ventral genes *ved* (Figs.2A4 and A5) and *vox* (Figs.2A9 and A10) was slightly up-regulated in embryos injected with *eaf1*-MO3 (9/16 and 8/15 respectively) or *eaf2*-MO3 (11/20 and 7/13) at oblong stage.

Expression of the ventral genes in *eaf1/2* ATG or in *eaf1/2* splice morphants was further detected by qRT-PCR. Transcripts of *vox*, *ved*, and *vent* were increased significantly in *eaf1/2* ATG morphants (Fig.2B1), with 4.5-fold, 2.6-fold, and 6.4-fold up-regulation in *eaf1* ATG morphants and 4.3-fold, 4.4-fold, and 6.4-fold up-regulation in *eaf2* ATG morphants at 4 hpf (Fig.2B1). Their expression was found almost normal in embryos injected with *eaf1* splice MOs (Fig.2B2), and was up-regulated by 2-fold, 1.7-fold, and 2.2-fold in embryos injected with *eaf2* splicing MOs (Fig.2B2). Moreover, significantly increased expression of genes *vox*, *ved*, and *vent* was also observed in *eaf1/2* ATG morphants at 30% epiboly stage (Fig.2B3).

*Eaf1* mutants were constructed using CRISPR/gRNA technology, and the sequences of gRNAs for *eaf1* were reported in our current study (24). Embryos from in-crossed *eaf1* F1 heterozygous mutants (24) all exhibited wild type likeness phenotypes at both 30 hpf and 5dpf, however, they were *eaf1* homozygous mutants, heterozygous, or wild type after genotyping (we have genotyped 10 embryos from in-crossed *eaf1* F1 heterozygous mutants at 30 hpf and 5dpf respectively) (Fig.S1). Expression of *ved*, *vox*, and *vent* was tested in aforementioned embryos at both sphere stage (Fig.2C) and 30% epiboly stage (Fig.S2), and the embryos with increased expression of *ved* (Figs.2C1, C2, and Fig.S2), *vox* (Figs.2C4 and C5), or *vent* (Figs.2C7 and C8) were genotyped, and almost of them were homozygous mutants (5 bp deletion, indicated in Fig.2C12) and heterozygous (Figs.2C10 and C12) (we have genotyped nearly 50 embryos with increased *ved* expression, and the ratio of homozygous and heterozygous was nearly 1:2). However, embryos with normal expression of *ved* (Fig.2C3), *vox* (Fig.2C6), and *vent* (Fig.2C9) were all wild



**Figure 1.** Maternal expression of *eaf1* and *eaf2* was detected by qRT-PCR (A), semi quantitative PCR (B), and whole mount *in situ* hybridization (C). (A, B) Embryos from 0 hpf, 2-cell stage (2-cell), multi-cell stage, oblong stage, sphere stage, 50% epiboly stage (50%), 80% epiboly stage (80%), bud stage, 5 somite stage (5s), 18 somite stage (18s), and from 24 hours post fertilization (24 h) were used for qRT-PCR (A) and semi quantitative PCR (B). Ne: negative control. (C) Maternal and uniform distributions of *eaf1* and *eaf2* in embryos at 4 cell stage, 8-cell stage and multi-cell stage were revealed by WISH. (D) Spatial distribution of *eaf1* or *eaf2* in embryos at gastrula stage (D1, D2) and at bud stage (D3, D4), and more *eaf1* or *eaf2* was distributed in the dorsal region of embryos. C1, C3, C4, C5, C7, C8, D3, D4, lateral view, anterior to the up; C2, C6, D1, D2, animal view, dorsal to the right for D1 and D2.

type (Fig.2C11). We also injected *eaf1*-MO1 to embryos from in-crossed *eaf1* F1 heterozygous mutants, 52 embryos exhibited wild type similar phenotype in totally injected 100 in-crossed embryos. 15 injected larvae with wild type phenotype at 4 pdf were genotype, all are heterozygous or homozygous *eaf1* mutants.

Additionally, we tested the transcripts of the ventral zygotic genes in embryos with ectopic expression of different forms of *eaf* mRNA using *ved* as an indicator. Transcripts of *ved* in embryos with ectopic expression of *eaf1* or *eaf2* were reduced at both 30% epiboly (7/11 and 9/13 respectively) (Figs.3A1-A6) and 50% epiboly stage (12/19 and 13/18 respectively) (Figs.3A7-A12). The expression of *ved* was further tested in embryos with ectopic expression of *eaf*-N mRNA, a dominant negative form of the full-length *eaf1/2* (5, 24). In embryos with ectopic expression of *eaf1*-N or *eaf2*-N at blastula stage, ventral *ved* (Figs.3B1-B6) exhibited enhanced expression (15/19 and 16/21 respectively). When tested in embryos injected with EnR-*eaf1/2*-full, the *ved* expression was obviously down-regulated in embryos injected with EnR-*eaf1*-full (19/23) (Figs.3C2 and C5) or EnR-*eaf2*-full (20/25) (Figs.3C3 and C6).

### Expressions of signaling ligands in embryos with gain-of-function or loss-of-function of Eaf

Several ligands and signaling pathways are reported to regulate dorsal-ventral patterning during early zebrafish development, such as Wnt signaling, Fgf signaling, and Bmp signaling (30, 31). Therefore, we further detected the expression of those ligands in *eaf1/2* loss-of-function and *eaf1/2* gain-of-function embryos. *Wnt8a* exhibited obviously reduced expression in embryos injected with *eaf1* or *eaf2* mRNA as detected by *in situ* and qRT-PCR (Figs.4A1-A3, A7). Similarly, *bmp2b* expression was also reduced obviously in embryos with ectopic expression of *eaf1* or *eaf2* (Figs.4A4-A6, A8). However, the expression of *fgf8* was increased in embryos with ectopic expression of *eaf1* or *eaf2* (Figs.4B1-B3), and its increased expression was observed in *eaf1* or *eaf2* morphants (Figs.4B4-B9). Additionally, *wnt8a* exhibited increased expression in *eaf1* and *eaf2* morphants at both blastula and gastrula stages (Figs.4C1-C6), and *bmp2b* was also up-regulated in *eaf1/2* morphants (Figs.4C7-C9), opposite to its expression in embryos with ectopic expression of *eaf1* or *eaf2*.

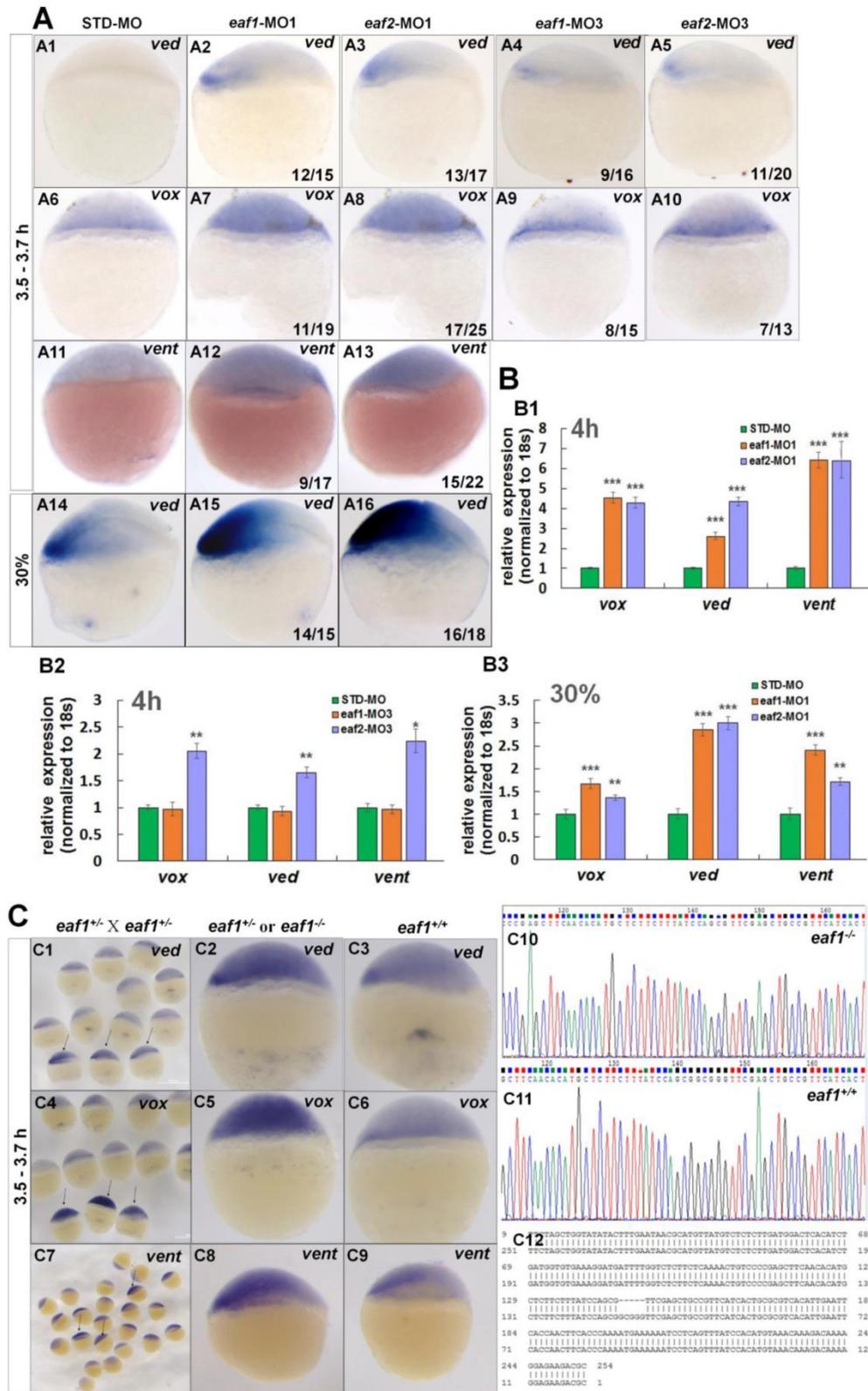
### Eaf1 and Eaf2 suppress $\beta$ -Catenin2-induced *axin2* expression

*Axin2*, a target and negative regulator of Wnt/ $\beta$ -catenin signaling (14–16), was also reported to pattern the axis during early development (10). Thus,

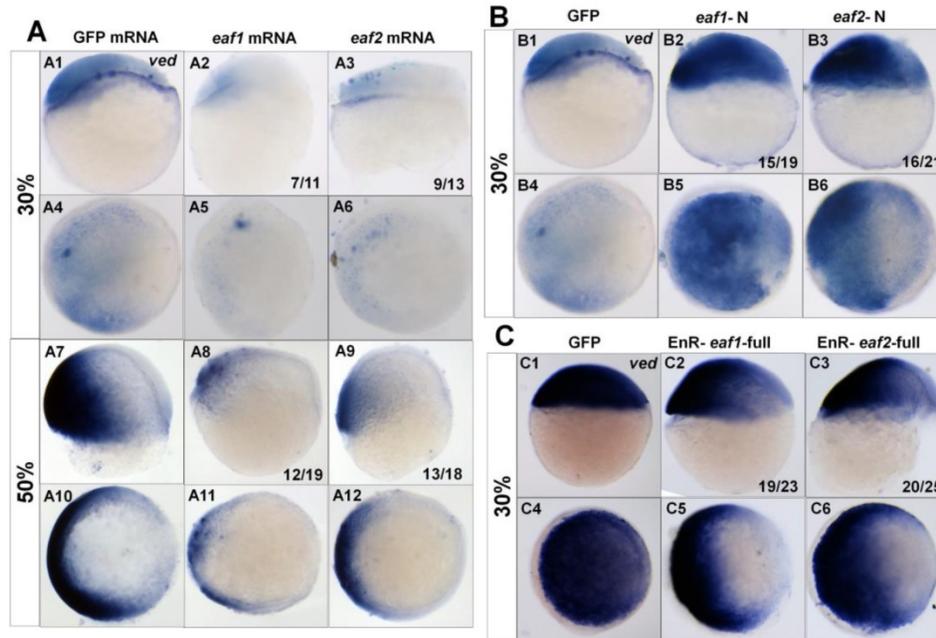
whether *eaf1* or *eaf2* could modulate *axin2* expression was further investigated in this study. Both  $\beta$ -catenin1 and  $\beta$ -catenin2 activated the *axin2* promoter in HeLa cells, but with higher inductive activity for  $\beta$ -catenin2 (Fig.5A2). Meanwhile, *eaf1* and *eaf2* only suppressed *axin2* promoter activities induced by  $\beta$ -catenin2 (Fig.5A2) but not by  $\beta$ -catenin1 (Fig.5A1). Additionally, ectopic expression of *eaf1* and *eaf2* strongly abolished *axin2* expression in the dorsal domain, but showed less suppressive effect on its expression in the ventral region of embryos (Figs.5B1-B6). *Axin2* expression was also obviously abolished by Wnt antagonists *frzb* and *dntcf* in both dorsal and ventral domains (Figs.5C1-C3), and embryos injected with *frzb* or *dntcf* exhibited obvious developmental defects (Fig.S3). We also tested the binding abilities of zebrafish Eaf1/2 proteins to zebrafish  $\beta$ -Catenin1 and  $\beta$ -Catenin2 proteins. It was revealed that Catenin1 bound more Catenin2 but not Eaf with the increased transfected Catenin2 protein in the co-transfected system (Fig.S4). In addition, it was revealed that Tcf bound more  $\beta$ -Catenin1 rather than  $\beta$ -Catenin2 by Co-IP assays in this study (data not shown).

### Eaf1 and Eaf2 suppress specification of ventral cells through maternal Wnt/ $\beta$ -catenin1/Tcf signaling

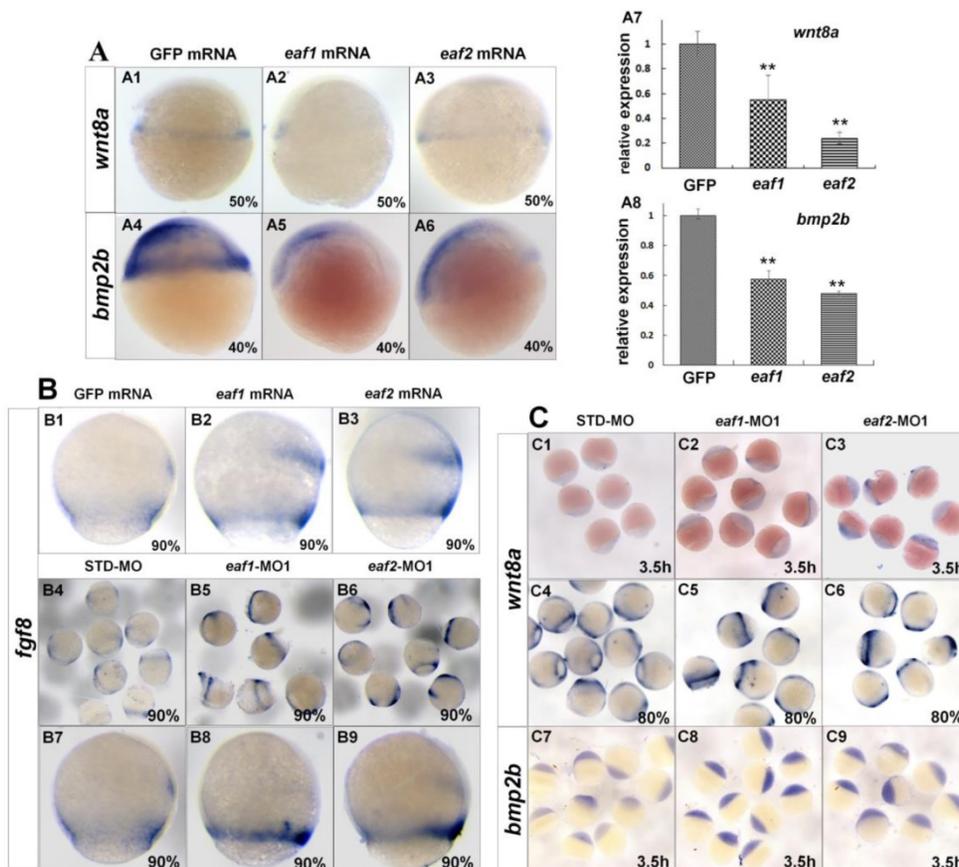
It has been reported that *vent* family genes are direct targets of Wnt8/ $\beta$ -catenin signaling and mediate the repression activities of *wnt8* on the vertebrate organizer (11). Whether  $\beta$ -catenin-MO could recover enhanced expression of ventral genes to normal level in *eaf1* or *eaf2* blastula morphants was tested by combined injection of  $\beta$ -catenin1-MO or  $\beta$ -catenin2-MO into *eaf1* or *eaf2* morphants and using the ventral gene *ved* as an indicator. In the current study, it was  $\beta$ -catenin1-MO (Figs.6A5, A6, A11, A12 and A15) rather than  $\beta$ -catenin2-MO (Figs.6A7, A8, A13, A14 and A15) that could successfully rescue *ved* expression at oblong stage (3.5 hpf) in *eaf1* or *eaf2* morphants, suggesting that *eaf1/2* might suppress *ved* expression *via*  $\beta$ -catenin1 rather than  $\beta$ -catenin2. At 30% epiboly stage,  $\beta$ -catenin1-MO nearly abolished the increased *ved* expression in embryos injected with *eaf*-ATG morpholinos (Figs.6B5, B6, B11, B12 and B15). While  $\beta$ -catenin2-MO enhanced *ved* expression in *eaf* morphants, the increased expression of *ved* was more apparent in embryos injected with *eaf*-MO and  $\beta$ -catenin2-MO together at 30% epiboly (Figs.6B7, B8, B13, B14 and B15). Additionally, *dntcf* could also recover the increased expression of ventral mesoderm genes to nearly normal level in embryos injected with *eaf*-splice-blocking morpholinos at sphere stage (Fig.7).



**Figure 2.** *Eaf1* and *eaf2* inhibited the expression of early ventral vent family genes at blastula stage. **(A)** Initial expression of *ved* (**A1-A3**), *vox* (**A6-A8**) and *vent* (**A11-A13**) was increased obviously in embryos injected with *eaf*-ATG-MO [*eaf1*-MO1 (8 ng/embryo) and *eaf2*-MO1 (8 ng/embryo)] or with *eaf*-splicing-MO (**A4, A5, A9, A10**) [*eaf1*-MO3 (8 ng/embryo) and *eaf2*-MO3 (8 ng/embryo)] at 3.5-3.7 h, and increased expression of *ved* was also observed in *eaf1/2* ATG morphants at 30% epiboly stage (**A14-A16**). **(B)** The significantly increased expression of the ventral genes in ATG morphants at 4 h (**B1**) and at 30% epiboly stage (**B3**) was revealed by qRT-PCR, and their slightly increased expression in *eaf2* splice morphants but not in *eaf1* splice morphants was revealed by qRT-PCR (**B2**). **(C)** Expression of ventral *ved* (**C1-C3**), *vox* (**C4-C6**), and *vent* (**C7-C9**) in embryos from in-crossed *eaf1* F1 heterozygous mutants, and black arrows indicated the embryos with increased expression of *ved* (**C1**), *vox* (**C4**), and *vent* (**C7**). The genotypic results for embryos with increased expression (**C2, C5, C8**) or normal expression (**C3, C6, C9**) were indicated in **C10, C11**, and **C12** respectively. *eaf1*-MO1: *eaf1* ATG morpholino; *eaf2*-MO1: *eaf2* ATG morpholino; *eaf1*-MO3: *eaf1* splicing morpholino; *eaf2*-MO3: *eaf2* splicing morpholino. **A1-A16, C1-C9**, lateral view, dorsal to the right.



**Figure 3.** Expression of *ved* in embryos with ectopic expressions of different forms of *eaf1* or *eaf2*. (A1-A12) Expression of *ved* was obviously reduced in embryos with ectopic expression of *eaf1* or *eaf2* at 30% epiboly stage (A1-A6) and at 50% epiboly stage (A7-A12). (B1-B6) *Ved* expression was obviously increased in embryos injected with *eaf1/2-N*, dominant forms of *eaf1/2*. (C1-C6) Expression of *ved* was obviously reduced in embryos injected with EnR-*eaf1/2*-full. A1-A3, A7-A9, B1-B3, C1-C3, lateral view, dorsal to the right; A4-A6, A10-A12, B4-B6, C4-C6, animal view, dorsal to the right.



**Figure 4.** Expression of ligands *wnt8a*, *bmp2b*, and *fgf8* in *eaf1* or *eaf2* gain-of-function or loss-of-function embryos. (A) *wnt8a* (A1-A3, A7) and *bmp2b* (A4-A6, A8) exhibited reduced expression in *eaf1* or *eaf2* gain-of-function embryos as detected by *in situ* and qRT-PCR. (B) Expression of *fgf8* was increased in both *eaf1/2* gain-of-function (B1-B3) and loss-of-function embryos (B4-B9). (C) Expression of *wnt8a* (C1-C6) and *bmp2b* (C7-C9) was increased in *eaf1/2* gain-of-function embryos. A1-A6, B1-B9, C1-C9, lateral view.

## Discussion

### Eaf1 and eaf2 are novel maternal suppressors of ventral cell fates

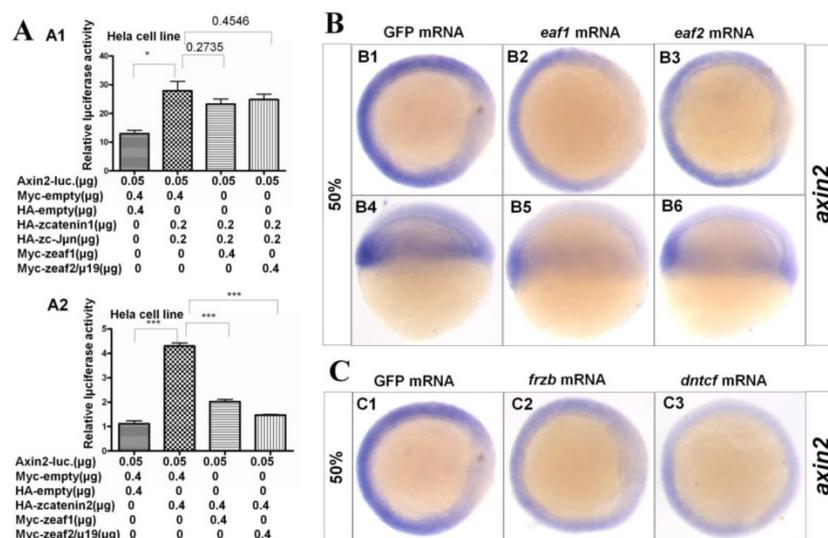
The *vent* family genes pattern the dorsal-ventral axis during embryogenesis by acting as the ventralizing transcriptional repressors (11, 32, 33). Maternal *runx2*, the only identified maternal regulator, initiates expressions of zygotic *vox*, *vent*, and *ved* at the onset of zygotic transcription (9). In this study, we unexpectedly find that ventral marker genes *ved*, *vent*, and *vox* exhibit enhanced expression in both *eaf* morphants, *eaf* homozygous or heterozygous mutant embryos, and embryos injected with dominant-negative forms of zebrafish *eaf*, *eaf1-N* or *eaf2-N*. However, their expression is almost abolished in embryos injected with mRNA of *eaf1*, *eaf2*, and *eaf* fused with EnR. All these observations suggest that *eaf1/2* act as repressors in specification of ventral cells, while exons1-3 of *eaf1* and *eaf2* act as dominant negative isoforms in dorsal-ventral patterning, similar to their roles in forebrain specification (5).

However, *eaf1* mutants at 30 hpf and 5 dpf exhibited wild type similar phenotypes. The data here is similar as some reports that embryos morphants exhibit more severe defective phenotypes compared with their mutants (34), and the data here also suggests that the genetic compensation might occur in *eaf1* mutants as previous study reported (35). Additionally, it is reported that TGF-beta factor Fascin1 mutants exhibit wild type similar phenotype at later developmental stage but reduced expression

of mesoderm and endoderm marker genes at gastrula stage although its morphants exhibit defective phenotypes at all developmental stages (36). Thus, we speculate that genetic compensation might contribute to no obviously defective phenotypes observed in *eaf1* homozygous mutants in this study. Certainly, it is possible that other unrevealed causes occurred in *eaf1* mutants or in morphants, which might also contribute to the data that *eaf1* mutants could not totally phenocopy the *eaf1* morphants in this study. Moreover, lower percentage of defective phenotype occurred in group of embryos from in-crossed *eaf1* F1 heterozygous injected with *eaf1* morpholino than group of wild type embryos injected with *eaf1* morpholino (21), and the genotypes of wild type similar embryos were all heterozygous or homozygous (data not shown). The data also suggest that genetic compensation might occur in *eaf1* homozygous or heterozygous mutants.

Uniform distribution of *eaf1* and *eaf2* is observed in embryonic cells at early cleavage stages including 4 cell, 8 cell stage, etc., and their high expression is observed in embryos from 0 hpf to sphere stage (Fig.1). Additionally, expression of ventral *vent* family genes is significantly up-regulated in *eaf1/2* ATG morphants, but slightly increased or normal in *eaf1/2* splice morphants (Fig.2B), suggesting that *eaf1* and *eaf2* might function as maternal repressors of ventral *ved*, *vent*, and *vox* at the onset of zygotic transcription. Our data implies that besides *runx2*, *eaf1* and *eaf2* might be potential maternal determinants that regulate ventral cell fate specification. However, unlike maternal *runx2* (9), *eaf1* and *eaf2* suppress, but do not induce the expression of ventral genes, and *eaf1* and *eaf2* also suppress the expression of dorsal genes at the onset of zygotic transcription (5). These differences suggest that *eaf1* and *eaf2* might act in a separate pathway to suppress the initiation of both ventral and dorsal genes.

Reduced expression of *wnt8a* and *bmp2b* is observed in embryos with ectopic expression of *eaf1* and *eaf2*, and their increased expression is observed in *eaf* morphants in this study. *Wnt8a* is not only a ligand of Wnt signaling but also acts as a ventral-lateral marker during zebrafish embryogenesis to indicate the ventral mesoderm pattern (37). BMP family genes have also been reported as important for ventral genes (38, 39). Our data implicates that *eaf1* and *eaf2*



**Figure 5.** *Eaf1* and *eaf2* obviously suppressed *axin2* expression. (A) *Eaf1* and *eaf2* suppressed *axin2* promoter activity induced by  $\beta$ -catenin2 (A2) but not by  $\beta$ -catenin1 (A1). (B) Expression of *axin2* was obviously abolished in the dorsal domain of *eaf1/2* gain-of-function embryos. (C) Expression of *axin2* was obviously abolished in both dorsal and ventral regions of embryos injected with Wnt antagonist *frzb* or *dntcf*. B1-B3, C1-C3, animal view, dorsal to the right; B4-B6, lateral view, dorsal to the right.

might be general repressors for ventral cells in dorsal-ventral axis patterning. Fgf8 signaling is revealed to act in inducing ventral and posterior cells in zebrafish (31). In this study, the expression of *fgf8* is found increased in both *eaf1/2* morphants and embryos with ectopic expression of *eaf1/2*, suggesting that *fgf8* might act parallel to *eaf1/2* in regulating specification of ventral cells and is required for neutralizing the dorsal-ventral defects in both *eaf1/2* morphants and embryos with ectopic expression of *eaf1/2*.

### **Eaf1 and eaf2 suppress the initiation of vent family genes via Wnt/ $\beta$ -catenin1/Tcf signaling**

In this study, expanded expression of *ved*, *vox*, and *vent* is revealed in *eaf* morphants at blastula stage (Fig.2), and reduced expression of *ved* is observed in embryos with ectopic expression of *eaf1* and *eaf2* at blastula stage (Fig.3). Moreover,  $\beta$ -catenin1 knock-down or ectopic expression of *dntcf* is revealed to restore *ved* expression in *eaf* morphants at blastula stage in this study (Figs.6,7), suggesting that *eaf1* and *eaf2* modulate ventral *ved* expression through antagonizing Wnt/ $\beta$ -catenin1/Tcf signaling. Previous studies have shown that maternal *XTcf3* functions as a repressor to prevent both dorsal and ventral cells from expressing dorsal genes (40, 41), and the *vent* family genes harbor  $\beta$ -catenin/Tcf binding sites in their promoters (42). Our data here suggest that *tcf* and  $\beta$ -catenin1 might suppress dorsal fates by inducing expressions of ventral *vent* genes. In the *eaf* morphants with combined injections of *dntcf* or  $\beta$ -catenin1-MO, almost no expression of *ved* was observed in several embryos (Figs.6,7), implying that *tcf* and  $\beta$ -catenin1 might act downstream of *eaf1* and *eaf2* and are essential for the induction of ventral *vent* family genes. Although we have tested the binding abilities of Eaf1/2 with Catenin1/2 by competitive Co-IP assays in this study, but it is revealed Catenin1 could bind more Catenin2 but not Eaf with the increased Catenin2 protein in the co-transfected system. However, a reasonable answer should be given for the Co-IP data in the further days.

Despite dorsal enrichment of endogenous  $\beta$ -catenin in embryos at blastula stage for the initiation of organizer genes, there is a certain amount of  $\beta$ -catenin present in the ventral region (43). We speculate that the ventrally active  $\beta$ -catenin protein might be  $\beta$ -catenin1, and its activity is suppressed by *eaf1* and *eaf2*. When *eaf1* or *eaf2* is removed by morpholino-mediated-knockdown, transcriptional activity of  $\beta$ -catenin1 is sufficient to induce expression of the ventral genes, leading to the observation of enhanced expression of *ved*, *vox*, and *vent* in the *eaf1* or

*eaf2* morphants at the blastula stage (Figs.2,6,7 and S2). Additionally, we observe the reduced transcripts of ventral gene *ved* in embryos with ectopic expression of *eaf1* or *eaf2* at the blastula stage. These observations in this study are consistent with previous studies in that Wnt/ $\beta$ -catenin target genes are abolished in blastula stage embryos with ectopic expression of Wnt inhibitors (3, 13).

This study reveals that Wnt/ $\beta$ -catenin1/Tcf signaling might initiate early zygotic expression of *vent* genes at the blastula stage. This finding compliments the role of Wnt/ $\beta$ -catenin2 signaling in the initiation of early zygotic expressions of dorsal genes (4, 5), and the induction activity of this signaling pathway in dorsal-ventral patterning is inhibited by *eaf1* and *eaf2* (Figs.6,7) (5). However, more studies need to be done to verify the finding in the present study, such as detecting active catenin proteins using catenin splicing morpholinos and detecting the distribution of nuclear catenin proteins in cells of *eaf1/2* morphants at the initiation of dorsal-ventral patterning using specific antibodies for  $\beta$ -Catenin1 or for  $\beta$ -Catenin2.

This model could also be used to explain why *ved* expression is more obviously enhanced when  $\beta$ -catenin2-MO is co-injected into *eaf1* or *eaf2* morphants.  $\beta$ -Catenin2 is essential for the expression of dorsal genes (4, 5), and co-injection of  $\beta$ -catenin2-MO into *eaf1* or *eaf2* morphants results in significantly decreased expression of dorsal genes (5). Thus, the reduction of dorsal antagonistic activities might lead to highly increased expression of ventral *ved* in *eaf* morphants co-injected with  $\beta$ -catenin2-MO (Figs. 6,7).

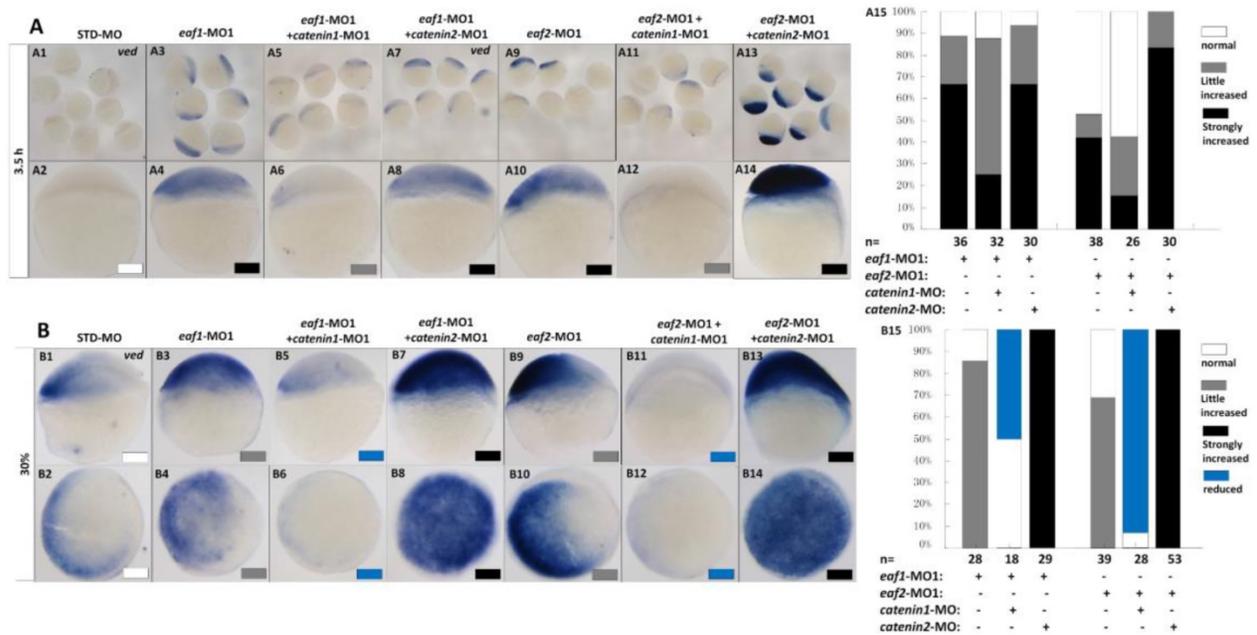
### **Eaf1 and eaf2 suppress $\beta$ -catenin2-induced axin2 expression**

In *eaf* morphants,  $\beta$ -catenin1-MO efficiently rescues expression of ventral Wnt/ $\beta$ -catenin target genes (Figs.6,7), whereas  $\beta$ -catenin2-MO rescues expression of dorsal Wnt/ $\beta$ -catenin target genes (5). Our data suggest that  $\beta$ -catenin1 and  $\beta$ -catenin2 might differentially regulate Wnt/ $\beta$ -catenin target genes, depending on the spatial distributions of those targets. Promoter assays data indicates that  $\beta$ -catenin2 activates the *axin2* promoter more effectively than  $\beta$ -catenin1 and that *eaf1* and *eaf2* can selectively suppress *axin2* expression induced by  $\beta$ -catenin2 rather than by  $\beta$ -catenin1 (Fig.5). Additionally, more transcripts of *eaf1* and *eaf2* are observed on the dorsal side of wild-type embryos (Fig.1D), with *axin2* expression almost abolished in the dorsal domain of the embryos with ectopic expression of *eaf1* or *eaf2* (Fig.5) and the dorsal organizer genes significantly expanded as detected by *in situ* and RT-PCR (5). All of

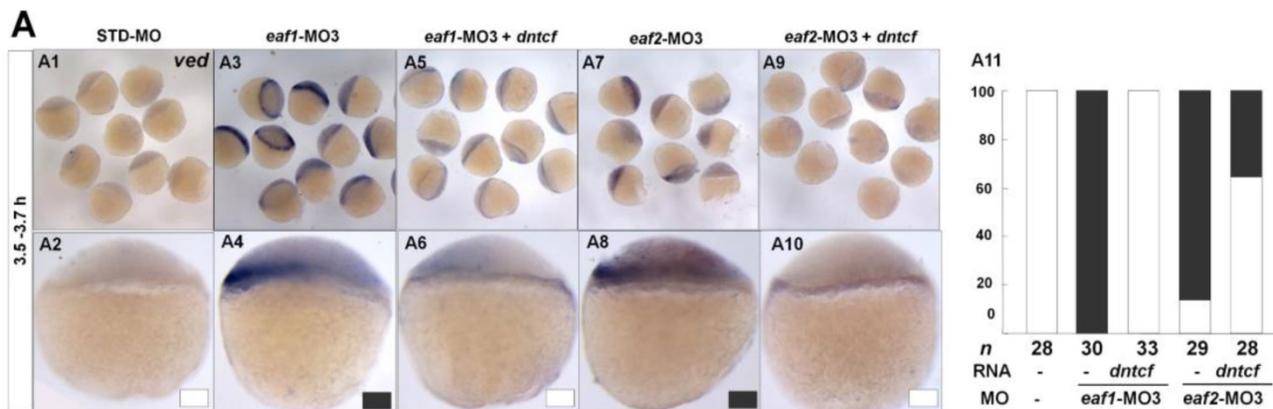
our observations suggest that *eaf1* and *eaf2* might enhance the expressions of dorsal organizer genes indirectly by suppressing  $\beta$ -catenin2-induced *axin2* expression in the dorsal domain.

A percentage of embryos have been reported to display ventralization defects, but most of them exhibit anterior/dorsal defects after ectopic expression of negative regulators of Wnt signaling, such as maternal Wnt negative regulators (25, 44–46). However, in our previous studies, almost all embryos injected with *eaf1* or *eaf2* mRNA exhibit anterior/

dorsal defects (5), which we speculate might be attributed to the distribution of more *eaf1* and *eaf2* in the dorsal region of embryos, possibly leading to their inhibition on  $\beta$ -catenin2 induced *axin2* expression. Slight changes in *axin2* expression can result in increased nuclear localization of  $\beta$ -catenin (10), thus, a slight spatial distribution and more dorsal distribution of *eaf1* and *eaf2* in the dorsal region of embryos also contributes to the almost all anterior/dorsal defects in embryos with ectopic expression of *eaf1* or *eaf2* in the present study.



**Figure 6.** *Eaf1* or *eaf2* regulated expression of ventral *ved* gene via Wnt/ $\beta$ -catenin I signaling. (A, B)  $\beta$ -catenin1-MO (8 ng/embryo) rather than  $\beta$ -catenin2-MO (8 ng/embryo) could recover enhanced *ved* expression in *eaf1* or *eaf2* morphants to normal at both oblong stage (A) and 30% epiboly stage (B). Embryos were injected with STD-MO (A1, A2, B1, B2), *eaf1*-MO1 (A3, A4, B3, B4) or *eaf2*-MO1 (A9, A10, B9, B10). Embryos were injected with either a combination of *eaf1*-MO1 and  $\beta$ -catenin1-MO (A5, A6, B5, B6), *eaf1*-MO1 and  $\beta$ -catenin2-MO (A7, A8, B7, B8), *eaf2*-MO1 and  $\beta$ -catenin1-MO (A11, A12, B11, B12), or *eaf2*-MO1 and  $\beta$ -catenin2-MO (A13, A14, B13, B14). (A15, B15) The percentage of embryos exhibiting different expression level of *ved* was scored at the sphere stage (A15) and at 30% epiboly stage (B15). White box, normal; gray box, slightly increased; black box, strongly increased; blue box, reduced. All of the injections, including MO alone or *eaf*-MO combined with catenin-MO, were performed using the same batch of embryos produced by a select number of zebrafish to eliminate error caused by embryo variation. A1-A14, B1, B3, B5, B7, B9, B11, B13, lateral view, dorsal to the right; B2, B4, B6, B8, B10, B12, B14, animal view, dorsal to the right.



**Figure 7.** *dntcf* could effectively rescue enhanced *ved* expression to normal level in *eaf1* or *eaf2* morphants. White box, normal; black box, increased. Embryos were injected with STD-MO (A1, A2), *eaf1*-MO3 (A3, A4) or *eaf2*-MO3 (A7, A8). Embryos were injected with *eaf1*-MO3 and *dntcf* mRNA (10 pg) (A5, A6) or with *eaf2*-MO3 and *dntcf* mRNA (10 pg) (A9, A10). All of the injections, including MO alone or MO combined with mRNA, were performed using the same batch of embryos produced by a select number of zebrafish to eliminate error caused by embryo variation. A1-A10, lateral view, dorsal to the right.

Our study provided a novel dorsal-ventral patterning pathway: *eaf1* and *eaf2* suppress both maternal Wnt/ $\beta$ -catenin1 signaling and Wnt/ $\beta$ -catenin2 signaling and mediate the zebrafish dorsal-ventral axis patterning by inhibiting the initiation and specification of both ventral and dorsal genes *via* differential regulation of distinct targets of  $\beta$ -catenin1 and  $\beta$ -catenin2. However, it still remains to be elucidated why  $\beta$ -catenin1 and  $\beta$ -catenin2 differentially regulate their targets depending on the dorsal or ventral distribution of those genes due to their similar and ubiquitous distribution in early stage embryos (4). A possible explanation for their differential functions is that the two catenin proteins bind to different Tcf/Lef partners. It has been reported that different Tcf/Lef genes in *Xenopus* mediate specific responses to Wnt/ $\beta$ -catenin signaling during mesoderm development (40). In this study, we observed that Tcf3 could bind more  $\beta$ -Catenin1 rather than  $\beta$ -Catenin2 in the competitive Co-IP assays. The data is consistent with the observations that catenin1-MO and *dntcf* could rescue the increased expression of *vent* family genes in *eaf* morphants.

In summary, we demonstrate the maternal expressions of *eaf1* and *eaf2* as well as their differential and spatial distribution during zebrafish embryogenesis. We also uncover the novel roles of the two factors in suppressing expressions of ventral genes *via* Wnt/ $\beta$ -catenin1/Tcf signaling. In the future, more research efforts should be focused on the roles of *eaf1/2* in cancer and during embryogenesis as well as their molecular regulation network in cells.

## Supplementary Material

Supplementary figures and tables.

<http://www.ijbs.com/v14p0705s1.pdf>

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## Competing Interests

The authors have declared that no competing interest exists.

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