Eaf1 and Eaf2 negatively regulate canonical Wnt/β-catenin signaling

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SUMMARY

Eaf factors play a crucial role in tumor suppression and embryogenesis. To investigate the potential mechanism of Eaf activity, we performed loss- and gain-of-function assays in zebrafish using morpholino and mRNA injections, respectively. We found that *eaf1* and *eaf2* inhibit Wnt/β-catenin signaling, thereby modulating mesodermal and neural patterning in the embryo. Moreover, ectopic expression of *eaf1* and *eaf2* in embryos and cultured cells blocked β-catenin reporter activity. By immunoprecipitation, we also observed that Eaf1 and Eaf2 bound to the Armadillo repeat region and C-terminus of β-catenin, as well as to other β-catenin transcription complex proteins, such as c-Jun, Tcf and Axin, suggesting the formation of a novel complex. In addition, the N-terminus of Eaf1 and Eaf2 bound to β-catenin and exhibited dominant-negative activity, whereas the C-terminus appeared to either harbor a suppression domain or to recruit a repressor. Both the N- and C-terminus must be intact for Eaf1 and Eaf2 suppressive activity. Lastly, we demonstrate a conservation of biological activities for Eaf family proteins across species. In summary, our evidence points to a novel role for Eaf1 and Eaf2 in inhibiting canonical Wnt/β-catenin signaling, which might form the mechanistic basis for Eaf1 and Eaf2 tumor suppressor activity.

KEY WORDS: Eaf1, Eaf2, Wnt, β-catenin, Tumor suppressor

INTRODUCTION

Eaf1 and Eaf2 were originally identified as partners of ELL (eleven-nineteen lysine-rich leukemia), a fusion protein frequently associated with myeloid leukemia (Simone et al., 2001; Simone et al., 2003). Studies suggest that Eaf2 serves as a novel tumor suppressor, especially in prostate cancer (Xiao et al., 2003; Xiao et al., 2008; Xiao et al., 2009); however, the molecular mechanisms underlying this tumor suppression activity remain largely unknown.

The Wnt/β-catenin pathway plays crucial roles in embryonic development and tissue regeneration (Klaus and Birchmeier, 2008), and dysregulation of the pathway can result in cancer. Several groups have shown that inhibition of Wnt/β-catenin signaling constitutes a common mechanism for tumor suppressor activity (Morin et al., 1997; Sadot et al., 2001; Major et al., 2007; Meani et al., 2009). Furthermore, Wnt/β-catenin signaling participates in the formation of embryonic axes and neuroectoderm in vertebrates, and perturbation of this pathway often leads to defects in neuroectodermal and mesodermal patterning. Overexpression of chick *Wnt8c* in the mouse results in anterior neuroectoderm truncation (Pöpperl et al., 1997). Consistently, mice lacking functional *Axin* or *Dkk1*, both negative regulators of Wnt signaling, exhibit anterior neuroectoderm truncation (Zeng et al., 1997; Glinka et al., 1998; Mukhopadhyay et al., 2001). In zebrafish, enhanced Wnt signaling also leads to loss of rostral neural domains. Forebrain defects and small eyes are observed in two zebrafish mutants: *masterblind* (*mbl*), which carries a mutation in *axin1* with high Wnt/β-catenin activities (Heisenberg et al., 2001), and *headless* (*hdl*), which carries a mutation abolishing the repressor function of

tcf3 (*tcf7l1a* – Zebrafish Information Network) on Wnt/β-catenin signaling (Kim et al., 2000). By contrast, zebrafish *wnt8* mutants and morphants display significant expansion of forebrain markers (Lekven et al., 2001).

In mesoderm patterning, maternal Wnt/β-catenin signaling is essential for the induction of *sqt* (*ndr1*), *boz* (*dharma*) and *gsc* during Nieuwkoop center formation, which then induces formation of the dorsal mesodermal organizer. After the mid-blastula transition, zygotic Wnt/β-catenin signaling is activated by Wnt ligands to antagonize the organizer, and is involved in ventral-posterior mesoderm and neuroectoderm induction by directly regulating ventral mesoderm *tbx6* (Szeto and Kimelman, 2004) and posterior ectoderm/mesoderm *cdx4*, respectively (Pilon et al., 2006). Zebrafish *wnt8* mutants show a significant expansion of the organizer and almost absent expression of ventrolateral mesoderm markers, similar to the phenotypes observed in *wnt8* morphants (Lekven et al., 2001).

Multiple molecules at several different points in the Wnt/β-catenin signaling pathway can modulate the output of the pathway. Extracellular proteins, including members of the Frzb or Dickkopf families, inhibit Wnt signaling by competitively binding to Wnt ligands (Leyns et al., 1997) or the LDL receptor-related protein 5/6 (Mao et al., 2001; Mao et al., 2002), respectively. By contrast, BIO, a bona fide drug that inhibits Gsk3, enhances Wnt/β-catenin signaling (Notani et al., 2010). In the cytoplasm, several proteins, including Gsk3, Apc and Axin, form a destruction complex that phosphorylates β-catenin, leading to its degradation (Barker and Clevers, 2000). As such, *apc* mutant fish have constitutively active Wnt/β-catenin signaling (Hurlstone et al., 2003; Haramis et al., 2006; North et al., 2007; Goessling et al., 2008). In the nucleus, *tcf3* is essential for forebrain formation by repressing Wnt caudal target genes (Kim et al., 2000). The dominant-negative form of Tcf3, dn-Tcf, which lacks the β-catenin-binding domain and acts as a repressor, can efficiently promote anterior brain formation (Kim et al., 2000).

Given the potential association between cancer and development, we have explored *eaf1* and *eaf2* activity in vertebrate

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DEVELOPMENT

embryogenesis, using the zebrafish model. Our previous studies demonstrate that both *eaf1* and *eaf2* mediate effective convergence and extension movements through maintaining expression of the non-canonical Wnt/β-catenin signaling ligands *wnt5* and *wnt11* (Liu et al., 2009). In addition, *eaf1* and *eaf2* regulate the expression of another non-canonical Wnt/β-catenin signaling ligand, *wnt4*, in a negative-feedback loop (Wan et al., 2010). We also observed that embryos with morpholino-mediated *eaf1* and *eaf2* knockdown display forebrain defects (Liu et al., 2009; Wan et al., 2010). Because upregulated Wnt/β-catenin signaling leads to loss of rostral neural domains of the forebrain in zebrafish (Kim et al., 2002), we investigated whether *eaf1* and *eaf2* also modulate canonical Wnt/β-catenin signaling.

In this study, we used zebrafish as an *in vivo* model together with cell culture to investigate the role of Eaf1 and Eaf2 in the regulation of Wnt/β-catenin signaling. Our results not only reveal a novel function of Eaf1 and Eaf2 during embryogenesis but also suggest a potential mechanism of Eaf1 and Eaf2 in tumor suppression.

MATERIALS AND METHODS
Fish stocks
Wild-type (AB line) zebrafish (*Danio rerio*) maintenance, breeding and staging were performed as described previously (Liu et al., 2009). *apc*mcr heterozygous mutants were kindly provided by Dr Wolfram Goessling (Brigham and Women's Hospital, Dana-Farber Cancer Institute). Genotyping for *apc* mutants was performed by sequencing the mutant allele fragment amplified from genomic DNA extracted from embryos stained by whole-mount *in situ* hybridization for *opl* (*zic1* – Zebrafish Information Network) (Hurlstone et al., 2003). Primers are listed in supplementary material Table S1. The *hs:dkk-GFP* transgenic line was kindly provided by Dr Leonard Zon (Harvard University) (North et al., 2007).

Drug exposure
Embryos developed to 50% epiboly were exposed to BIO (Sigma-Aldrich) at 5 μM.

Heat-shock modulation
Embryonic heat-shock experiments were conducted at 38°C for 20 minutes. Genotype was determined by the presence of GFP fluorescence at 3 hours post-heat shock; the non-fluorescent (wild-type) siblings were sorted and used as controls.

Morpholino (MO) and mRNA synthesis
The translation-blocking MOs (ATG targeted) Eaf1-MO1 and Eaf2-MO1 have been described previously (Liu et al., 2009). The splicing MOs Eaf1-MO3 and Eaf2-MO3 were designed based on the junction sequence between exon 1 and intron 1 of *eaf1* and *eaf2*, respectively. Eaf1-MO3 and Eaf2-MO3 specificity was validated by RT-PCR (primers listed in supplementary material Table S1). *β-catenin 1* and *β-catenin 2* antisense MOs (β-catenin1-MO and β-catenin2-MO) have been described previously (Bellipanni et al., 2006). All MOs were purchased from Gene Tools and their sequences are listed in supplementary material Table S2.

Capped mRNAs were synthesized using the Ampticap SP6 High Yield Message Maker Kit (Epicenter Biotechnologies). The plasmids for zebrafish *eaf1* and *eaf2* and human *EAF1* and *EAF2* mRNA were described previously (Liu et al., 2009). The full-length genes, exons 1-3 and exons 4-6 of zebrafish *eaf1* and *eaf2* were subcloned into PSC2+VP16 and PSC2+EnR (provided by Yonghua Sun, Institute of Hydrobiology, CAS, Wuhan, China) to form fusion protein expression vectors. In addition, exons 1-3 and exons 4-6 of zebrafish *eaf1* and *eaf2* were subcloned into PSP64 (Promega) for synthesizing mRNAs. The synthesized mRNAs were diluted to different concentrations and injected into one-cell stage embryos.

Whole-mount *in situ* hybridization
Probes for zebrafish *six3b*, *opl* and *cdx4* were amplified from cDNA pools using the appropriate sets of primers (supplementary material Table S1). Probes for *tbx6* were a generous gift from Dr Yang Wang (Institute of

Hydrobiology, Chinese Academy of Sciences, Wuhan, China). Probes for *eaf1*, *eaf2*, *gsc* and *chd* have been described previously (Liu et al., 2009).

Plasmid construction
The full-length zebrafish *β-catenin 2* (*ctnnb2*) vector was a generous gift from Dr Gianfranco Bellipanni (Temple University, Philadelphia, USA). Full-length zebrafish *β-catenin 1* (*ctnnb1*) and *c-jun* were amplified from cDNA pools using the appropriate sets of primers (supplementary material Table S1). Zebrafish *ctnnb1*, *ctnnb2*, *c-jun*, *eaf1* and *eaf2* were subcloned into pCGN-HAM (provided by William Tansey, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA), pCMV-Myc, pEGFP-N1, PM-RFP or PM vectors (Clontech). Sequences encoding the N-terminus, Armadillo repeats and C-terminus of zebrafish *ctnnb1* and *ctnnb2* were subcloned into pCGN-HAM, pCMV-Myc and PM vectors. Sequences encoding the N-terminus (exons 1-3) and C-terminus (exons 4-6) of zebrafish *eaf1* and *eaf2* were subcloned into pCMV-Myc. All plasmids were verified by sequencing. Human Myc-EAF1 and Myc-EAF2 were described previously (Zhou et al., 2009). Human HA-AXIN and HA-c-JUN were generous gifts from Dr Lin Li (Institute of Biochemistry and Cell Biology, Chinese Academy of Science, Shanghai, China). Full-length human β-catenin and *TCF4* (*TCF7L2* – Human Gene Nomenclature Committee) were amplified from cDNA pools and subcloned into pCGN-HAM.

Luciferase reporter assay
Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (HyClone). The luciferase reporter assay was performed as described previously (Zhou et al., 2009). The reporter vectors 8xTopFlash and FopFlash were generous gifts from Dr Randall Moon (University of Washington, Seattle, USA). The pER-luc vector was purchased from Stratagene. A c-Myc promoter luciferase reporter was amplified by PCR based on the human *c-MYC* promoter region described previously (Chung et al., 1986) and cloned into pGL3-Basic (Promega). Statistical analysis of luciferase assay data was performed using GraphPad Prism 5.0.

Fluorescence microscopy
Cos-7 cells were transfected with different combinations of zebrafish plasmids. After 24-30 hours, cells were directly observed under a Nikon T-2000 Eclipse inverted fluorescence microscope.

Western blots and immunoprecipitation assays
Western blots were performed as described (Zhou et al., 2009). HEK 293T cells and mouse L cells (with *Wnt3a* expression) were cultured in DMEM. Immunoprecipitation assays were performed as described (Xiao et al., 2009). The following antibodies were used in the assays: anti-Myc (Santa Cruz), anti-HA (Covance), anti-β-catenin (Santa Cruz), anti-β-catenin-ABC (Santa Cruz), anti-GAPDH (Abcam), anti-PCNA (Epitomics), anti-α-tubulin (Epitomics) and anti-H3 (Cell Signaling Technology).

Nuclear and cytoplasmic separation
Cell fractionation followed the method of Suzuki et al. (Suzuki et al., 2010) with the following modifications. Briefly, the embryos were collected in 24-well plates, Pronase E was added (1 mg/ml; Solarbio, Beijing, China) and incubated for 15-30 minutes at 37°C. The chorion was discarded and the embryos washed three times with PBS, then transferred to 1.5-ml Eppendorf tubes, 1 ml PBS added, and the embryos disrupted using a micropipette tip. The embryos were then spun for 5 minutes at 1100 rpm to separate the yolks. Embryos were collected at the gastrula stage and HEK 293T cells were harvested 18-26 hours after transfection.

RESULTS
Patterning of anterior neuroectoderm and mesoderm in zebrafish embryos requires *eaf1* and *eaf2*
In our previous studies, we observed ubiquitous expression of zebrafish *eaf1* and *eaf2* during early embryogenesis (supplementary material Fig. S1) (Liu et al., 2009). Furthermore, *eaf1* and *eaf2* morphants displayed forebrain defects (Liu et al., 2009). In addition
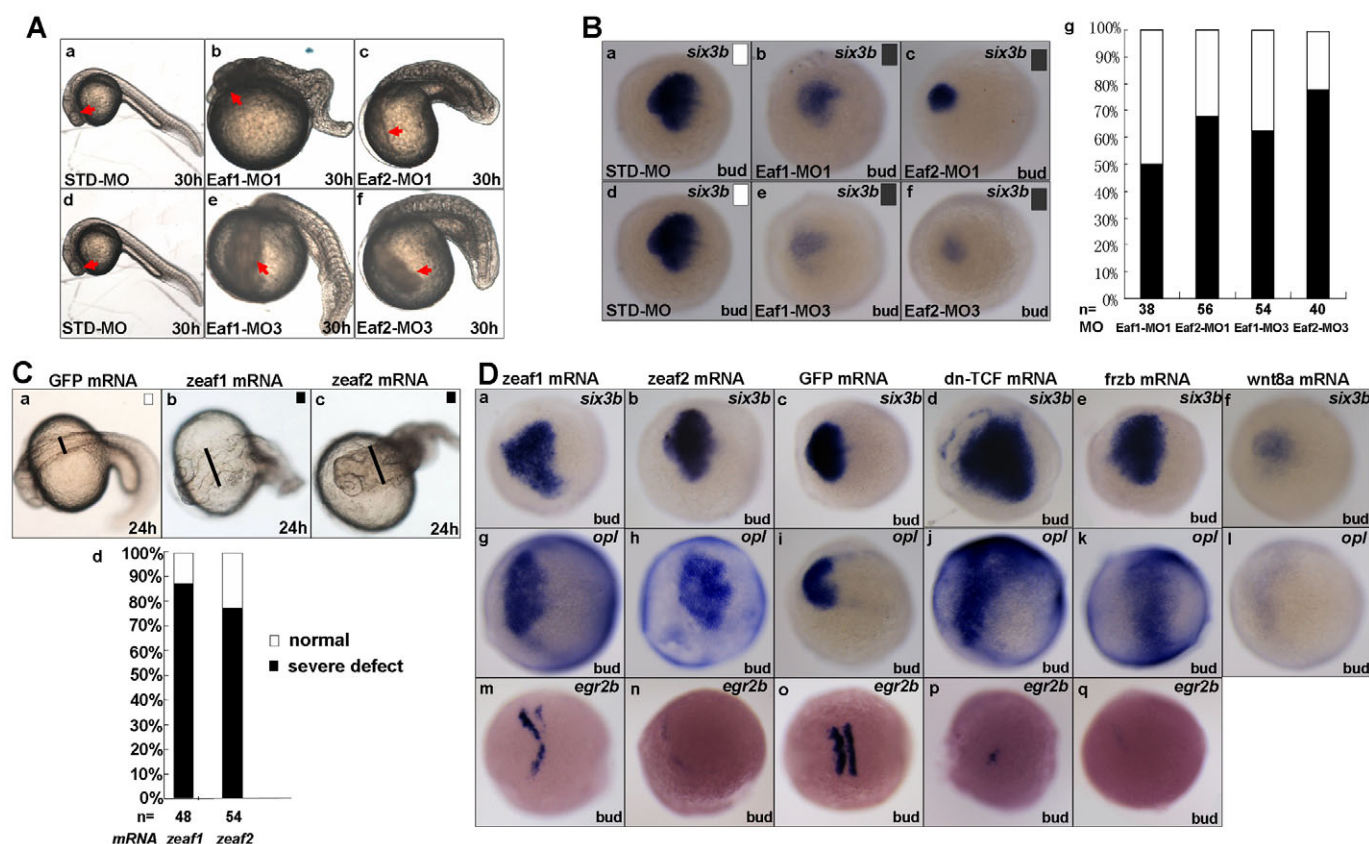
Fig. 1. Effects of *eaf1* and *eaf2* knockdown and overexpression on zebrafish embryos. (**A**) Morphology of representative embryos injected with the indicated MOs (8 ng/embryo) at 30 hpf. STD-MO, standard MO. (**B**) (a-f) *In situ* hybridization for the anterior brain marker *six3b* in the presence of the indicated MOs. (g) Scoring of *six3b* expression: black, reduced expression; white, normal expression. (**C**) (a-c) Morphology of representative embryos injected with the indicated mRNAs (200-500 pg/embryo). zeaf1 and zeaf2 refer to zebrafish *eaf1* and *eaf2*. (d) Morphology was scored as normal (white) or severely defective (black) in *eaf1*- and *eaf2*-overexpression embryos. The expanded dorsal part is indicated by the black line. (**D**) *eaf1* and *eaf2* function as forebrain inducers in embryos. Embryos injected with *eaf1* and *eaf2* mRNA displayed increased expression of *six3b* (a,b) and *opl* (g,h), similar to embryos injected with dn-*Tcf* (d,j) or *frzb* (e,k) mRNA, but in contrast to embryos injected with *wnt8a* mRNA (f,l); *egr2b* exhibited reduced expression in embryos injected with *eaf1* and *eaf2* mRNA (m,n), similar to embryos injected with dn-*Tcf* (p) or *frzb* (q) mRNA. Ba-f,Da-q, dorsal views, anterior to the left.

to the translation-blocking MOs, Eaf1-MO1 and Eaf2-MO1 (Liu et al., 2009), we included two splicing-blocking MOs, Eaf1-MO3 and Eaf2-MO3. The efficiency and specificity of the splicing-blocking MOs were confirmed by RT-PCR assays (supplementary material Fig. S2). Knockdown of *eaf1* or *eaf2* resulted in forebrain defects in embryos (Fig. 1Ab,c,e,f), consistent with our previous observations (Liu et al., 2009).

In order to further characterize the observed phenotypes, we evaluated the expression of the anterior neuroectoderm markers *six3b*, *foxg1* and *opl*, as well as the hindbrain marker *egr2b*, as a means to assess defects in neuroectoderm anterior-posterior patterning. We also evaluated the dorsal mesoderm markers *sqt*, *gsc*, *dkk1*, *frzb*, the posterior ectoderm/mesoderm marker *cdx4*, and the ventral mesoderm marker *tbx6*, to assess defects in dorsal-ventral patterning.

In the Eaf morphants at the bud stage, expression of *six3b* was dramatically reduced (Fig. 1Ba-f) and expression of *foxg1* was almost completely abolished (supplementary material Fig. S3A). Moreover, quantitative analysis indicated that at least half of the embryos with Eaf knockdown had reduced *six3b* expression (Fig. 1Bg). We also detected defects in dorsal-ventral mesoderm patterning as indicated by enhanced expression of *sqt* and *gsc*, the main direct targets of maternal Wnt/β-catenin signaling (Kelly et

al., 1995; Shimizu et al., 2000), at the blastula stage (supplementary material Fig. S3B-D), as well as increased expression of *cdx4* and *tbx6*, two zygotic Wnt direct targets (Szeto and Kimelman, 2004; Pilon et al., 2006), at the gastrula stage (supplementary material Fig. S3E-H). The observations suggested that Wnt/β-catenin activities increased in Eaf morphants.

As expected, overexpression of *eaf1* and *eaf2* by mRNA injection (200-500 pg/embryo) caused phenotypes opposite to those of Eaf morphants. Of the embryos injected with zebrafish *eaf1* or *eaf2* mRNA (Fig. 1Cb,c), 75-85% displayed obvious dorsalized phenotypes with expansion and widening of the dorsal part (Fig. 1C, black lines). Similarly, overexpression of *eaf1* or *eaf2* produced opposite effects on marker gene levels to the morphants. The expression of *six3b* and *opl* increased dramatically in embryos with ectopic expression of *eaf1* or *eaf2* (Fig. 1Da,b,g,h). These resembled embryos with ectopic expression of dn-*Tcf* (Fig. 1Dd,j) or *frzb* (Fig. 1De,k) (Kim et al., 2002; Momoi et al., 2003), but not *wnt8a* (Fig. 1Df,l) (Kelly et al., 1995; Kim et al., 2002). Injection with *eaf1* or *eaf2* mRNA reduced the expression of *egr2b* in most embryos (Fig. 1Dm,n), similar to ectopic expression of dn-*Tcf* (Fig. 1Dp) or *frzb* (Fig. 1Dq). *eaf1* or *eaf2* mRNA also significantly increased the expression of *sqt* and *gsc*, as revealed by both whole-mount *in situ* hybridization and RT-PCR analysis at the gastrula stage

(supplementary material Fig. S4A), again similar to embryos with ectopic expression of dn-*Tcf* (supplementary material Fig. S4Bb) or *frzb* (supplementary material Fig. S4Bc).

eaf1 and eaf2 function in anterior neuroectoderm and mesoderm patterning by antagonizing Wnt/β-catenin signaling

It has been reported that hyperactive Wnt/β-catenin signaling suppresses the rostral neural domains of the forebrain in zebrafish by promoting the posterior midbrain and hindbrain domains (Kim et al., 2002). Here, we observed that expression of the direct Wnt targets *sqt*, *gsc*, *cdx4* and *tbx6* increased in Eaf morphants at blastula and gastrula stages (supplementary material Fig. S3). To confirm these results, we examined the expression of *axin2*, a bona fide direct target of Wnt/β-catenin signaling at bud stage. As expected, we observed an obvious increase in *axin2* expression in the midbrain of Eaf morphants at bud stage (supplementary material Fig. S5A, red arrows). These observations suggested that Wnt/β-catenin signaling was upregulated in Eaf morphants during early embryogenesis.

The above results, which were based on detecting the expression of Wnt signaling direct target genes through loss- and gain-of-function assays, implied that *eaf1* and *eaf2* might serve as antagonists of Wnt/β-catenin signaling to affect neuroectoderm and mesoderm patterning. If this were the case, then ectopic expression of *eaf1* or *eaf2* should counteract the effects of enhanced Wnt/β-catenin signaling induced by ectopic expression of *wnt8a*. To test

this, we performed rescue experiments by monitoring *opl* expression. As expected, co-injection of *eaf1* or *eaf2* mRNA with *wnt8a* mRNA rescued *opl* expression dramatically (Fig. 2A; scored based on the expression level of *opl*). In addition, *eaf1* or *eaf2* also partially counteracted the strong inhibitory effect of BIO on *opl* expression (Fig. 2B).

We also performed rescue experiments using *apc* mutant zebrafish embryos, which have enhanced Wnt/β-catenin activity. As expected, in the offspring of $apc^{+/-} \times apc^{+/-}$, the ratio of strongly reduced to reduced to normal *opl* was close to 1:2:1, correlating with the predicted genotype ratio (Fig. 2Cb, column 1 from top to bottom). Further genotyping of these offspring not only confirmed the genotype ratio of 1:2:1 (data not shown) but also showed that the phenotypes (indicated by *opl* expression) mirrored the genotypes very well (supplementary material Fig. S5B). After injection of *eaf1* or *eaf2* mRNA, wild-type embryos ($apc^{+/+}$) exhibited increased *opl* expression, whereas the heterozygous embryos ($apc^{+/-}$) exhibited normal or reduced *opl*, and most of the homozygous embryos ($apc^{-/-}$) exhibited reduced *opl* (Fig. 2C). Therefore, ectopic expression of *eaf1* or *eaf2* could increase *opl* levels in embryos of all three genetic backgrounds (Fig. 2Cb). These results further suggest that *eaf1* and *eaf2* are involved in Wnt/β-catenin signaling and might function downstream of *apc*.

If *eaf1* and *eaf2* truly act as antagonists of Wnt/β-catenin signaling, ectopic expression of Wnt/β-catenin signaling inhibitors or knockdown of *β-catenin 1* and *2* should counteract the effects seen with Eaf knockdown. To test this, we performed rescue
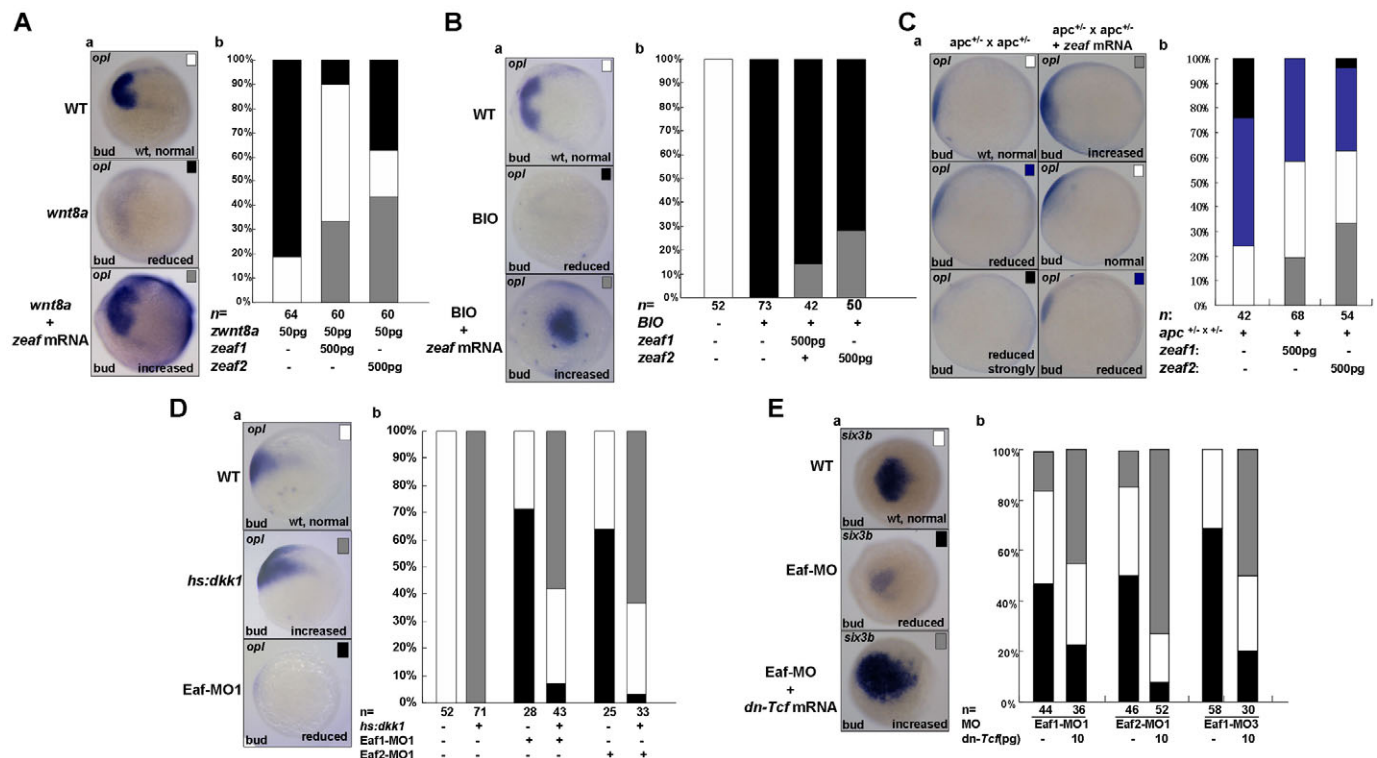

Fig. 2. Eafs antagonize Wnt/β-catenin signaling in anterior neuroectoderm patterning. (A) *eaf1* and *eaf2* counteract *wnt8a*-mediated forebrain defects. (a) *In situ* hybridization for the anterior neuroectoderm marker *opl* in wild type (WT) and in the presence of *wnt8a* mRNA alone or together with *eaf1* or *eaf2* mRNA. (b) Scoring of *opl* expression: black, reduced; white, normal; gray, expanded. **(B)** *eaf1* or *eaf2* partially counteracts the strong reduction in *opl* expression that occurs after exposure to the Gsk3 inhibitor BIO, which enhances Wnt/β-catenin signaling. *opl* expression is scored as in A. **(C)** *eaf1* and *eaf2* rescue forebrain defects in *apc* mutants. *opl* expression is scored as: black, strongly reduced; blue, reduced; white, normal; gray, increased. **(D)** Heat shock-induced expression of *dkk1* at 50% epiboly rescues forebrain defects in Eaf morphants. *opl* expression is scored as in A. **(E)** dn-*Tcf* partially rescues the anterior brain defects in Eaf morphants. Embryos were scored by *six3b* expression: black, reduced; white, normal; gray, increased. Aa,Ba,Ea, dorsal views, anterior to the left; Ca,Da, lateral views, anterior to the left.

experiments using *hs:dkk-GFP* transgenic zebrafish. Heat shock of *hs:dkk-GFP* embryos at 50% epiboly resulted in all of the embryos displaying enhanced *opl* expression (Fig. 2Db). In Eaf morphants at 50% epiboly, *dkk1* induced by heat shock efficiently rescued the reduced *opl* expression seen with Eaf knockdown (Fig. 2D). Some morphants even displayed enhanced *opl* expression (Fig. 2Db), suggesting that *dkk1* might be more efficient than Eaf in inhibiting Wnt/β-catenin signaling. Similarly, co-injection of dn-*Tcf* mRNA effectively restored *six3b* expression in Eaf morphants (Fig. 2E). Subsequently, we evaluated morphants after simultaneous knockdown of *β-catenin 1/2* and *eaf1/2*. Co-injection of β-catenin1-MO with the Eaf MOs effectively restored *six3b* expression (supplementary material Fig. S5C). Surprisingly, co-injection of β-catenin2-MO with the Eaf MOs resulted in a further reduction in *six3b* expression compared with embryos injected with the Eaf MOs alone (supplementary material Fig. S5C).

For mesoderm patterning, only β-catenin2-MO rescued *gsc* expression in Eaf1-MO morphants at 30% epiboly (supplementary material Fig. S5Da-h), whereas both β-catenin1-MO and β-catenin2-MO partially rescued *gsc* expression in Eaf1-MO morphants at the sphere stage (supplementary material Fig. S5Di-m), although β-catenin2-MO was more effective (supplementary material Fig. S5Dm).

To gain a more complete picture of *eaf1* and *eaf2* in Wnt/β-catenin signaling, we also determined whether β-catenin could modulate *eaf1/2* expression. Embryos injected with the active form of *β-catenin* mRNA displayed reduced expression of *eaf1* and *eaf2* (supplementary material Fig. S6). Similarly, embryos with ectopic *wnt8a* also displayed a decrease in *eaf1* and *eaf2* expression (supplementary material Fig. S6Ac,g). However, the expression of *eaf1* and *eaf2* remained unchanged in embryos injected with *frzb* mRNA (supplementary material Fig. S6Ad,h), implying a complicated regulatory relationship between *eaf1/2* and the Wnt/β-catenin signaling pathway.

Eaf1 and Eaf2 act as transcriptional repressors in patterning anterior neuroectoderm and mesoderm

Previous studies showed that Eaf1 and Eaf2 contain a transactivation domain in the C-terminus and that Eaf2 binds to specific nucleotides (Simone et al., 2001; Xiao et al., 2003; Xiao et al., 2006). These observations suggest that Eaf1 and Eaf2 might act as transcription factors. To explore this possibility, we made constructs by fusing full-length *eaf1* or *eaf2* with the transcriptional activator *VP16* or with the transcriptional repressor *engrailed* (*EnR*) (Gómez-Skarmeta et al., 2001; Muhr et al., 2001). We also made constructs by fusing the N-terminal sequences (encoding amino acids 1-112) of *eaf1* or *eaf2* (*eaf1*-N or *eaf2*-N) or their C-terminal sequences (encoding amino acids 113-256 for *eaf1*-C and 113-259 for *eaf2*-C) with *VP16* or *EnR* for mapping their functional domains (Fig. 3Aa).

Embryos injected with *EnR* mRNA or *VP16* mRNA displayed the same phenotype as wild-type embryos (supplementary material Fig. S7A; data not shown), indicating that VP16 or EnR alone was not functional *in vivo*, probably owing to the lack of specific DNA- or protein-binding domains. Most embryos injected with *eaf1/2-EnR* or *eaf1/2*-N-*EnR* mRNA (50 pg/embryo) displayed obviously dorsalized phenotypes (Fig. 3Ac-f; data not shown), similar to embryos injected with full-length *eaf1* or *eaf2* mRNA (Fig. 1C). By contrast, most embryos injected with *eaf1/2-VP16* or *eaf1/2*-N-*VP16* mRNA (50 pg/embryo) displayed phenotypes with obvious anterior neuroectoderm truncation (Fig. 3Ag-j; data not shown),

similar to Eaf morphants (Fig. 1A). However, embryos injected with either *eaf1/2-C-EnR* mRNA or *eaf1/2-C-VP16* mRNA (50 pg/embryo) did not exhibit any abnormal phenotypes (supplementary material Fig. S7B; data not shown), similar to embryos injected with either *EnR* or *VP16* mRNA alone (supplementary material Fig. S7A), further ruling out any non-specific effect of EnR or VP16.

In addition, embryos injected with *eaf1/2-EnR* or *eaf1/2-N-EnR* mRNA exhibited expanded *six3b* and *opl* expression (Fig. 3Ba-j) and reduced *egr2b* expression (Fig. 3Bk-o), similar to embryos injected with full-length *eaf1/2* mRNA (Fig. 1D). Embryos injected with *eaf1/2-EnR* or *eaf1/2-N-EnR* mRNA displayed enhanced expression of the dorsal-ventral patterning markers *gsc* (supplementary material Fig. S7Ca-e) and *chd* (supplementary material Fig. S7Cf-j), similar to embryos injected with full-length *eaf1* and *eaf2* mRNA (supplementary material Fig. S4Ad-f). Taken together, these phenotypes suggest that *eaf1* and *eaf2* might modulate embryogenesis by acting as transcriptional repressors rather than activators *in vivo*.

Interestingly, the phenotypes exhibited by embryos injected with *eaf1/2*-N or with full-length *eaf1/2* fused with the *EnR* transcriptional repressor were similar to those of embryos injected with full-length *eaf1/2* mRNA. This promoted us to further examine the function of the N- and C-termini of Eaf1 and Eaf2.

Both the N- and C-termini must be intact for Eaf1 and Eaf2 suppressive activity

We next investigated the function of the N- and C-termini of Eaf1 and Eaf2 by ectopic expression in embryos. Embryos injected with *eaf1*-N or *eaf2*-N mRNA (50 pg/embryo) displayed remarkable anterior neural truncation (nearly 90%; Fig. 4A), in contrast to embryos injected with full-length *eaf1* or *eaf2* mRNA (Fig. 1C). Embryos injected with *eaf1*-C or *eaf2*-C mRNA (50 pg/embryo) displayed no obvious defects (data not shown). Furthermore, *six3b* expression was reduced dramatically and that of *opl* was almost completely abolished in embryos injected with *eaf1*-N or *eaf2*-N mRNA (Fig. 4B; data not shown), whereas their expression was unchanged in embryos with ectopic expression of *eaf1*-C or *eaf2*-C (Fig. 4C; data not shown). After co-injecting embryos with full-length *eaf1* or *eaf2* mRNA and *eaf1*-N or *eaf2*-N mRNA, the reduced expression of *six3b* was rescued (Fig. 4Da,b). In addition, co-injection of *eaf1*-N-*EnR* with *eaf1*-N mRNA also restored *six3b* expression (Fig. 4Db), which further reinforced the suppressor role of Eaf1.

Next, we determined whether the N-terminus of Eaf1/2 functions as a dominant-negative form to affect neural and mesodermal patterning by upregulating Wnt/β-catenin signaling. Co-injection of dn-*Tcf* mRNA counteracted the reduced *six3b* expression that resulted from ectopic expression of *eaf1*-N or *eaf2*-N (Fig. 4D). In addition, co-injection of β-catenin1-MO, but not β-catenin2-MO, effectively rescued *six3b* expression in embryos injected with *eaf1*-N or *eaf2*-N mRNA alone (Fig. 4Dc).

Taken together, these observations suggest that the C-terminus of Eaf1/2 might function as repressor or as a repressor recruiter and that both the N-terminus and the C-terminus must remain intact for Eaf1 and Eaf2 to function as suppressors in Wnt/β-catenin signaling.

Eaf1 and Eaf2 attenuate the transcriptional activity of β-catenin

To determine the mechanisms underling *eaf1* and *eaf2* antagonism of Wnt/β-catenin signaling, we used TopFlash luciferase reporter assays to monitor the transcriptional activity of β-catenin in
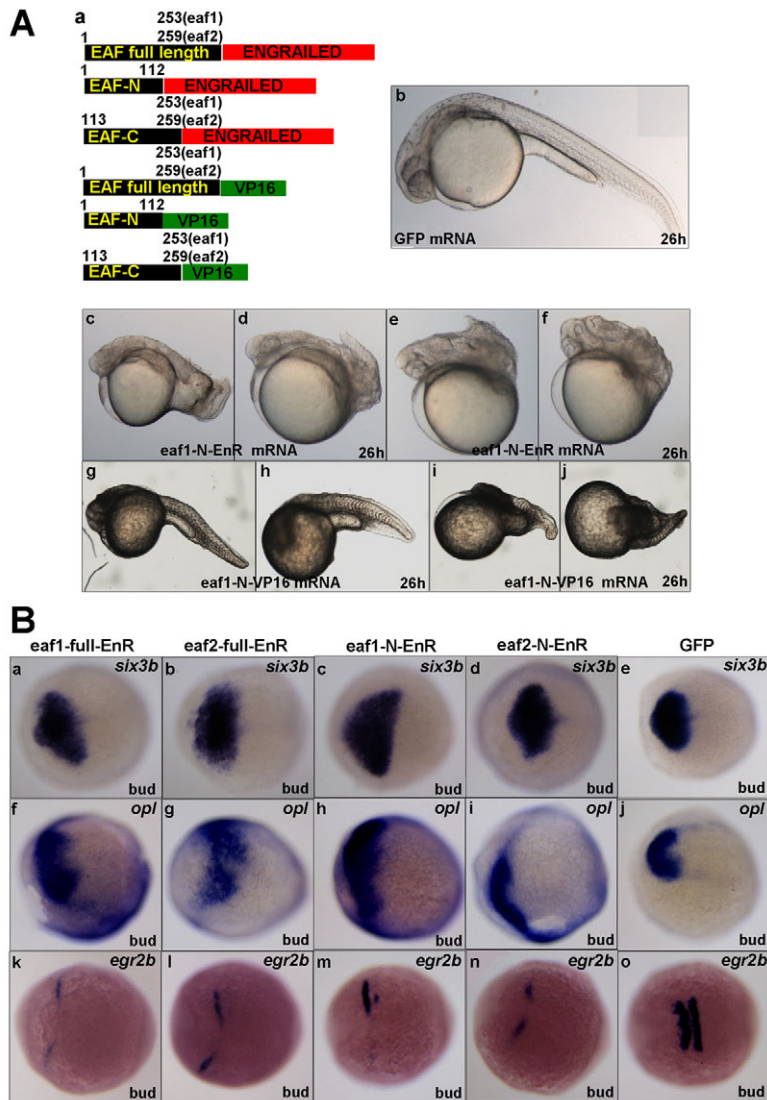
Fig. 3. Zebrafish *eaf1* and *eaf2* induce anterior neuroectoderm by acting as transcriptional repressors. (**A**) (a) mRNAs used for injection encode full-length or the N- or C-terminus of Eaf1 or Eaf2 fused with the transcriptional activator VP16 or the engrailed transcriptional repressor domain (EnR). Numbers refer to amino acid residues in Eaf1 and Eaf2. (b) Embryos injected with *GFP* mRNA at 26 hpf exhibit normal morphology. (c-f) Embryos injected with *eaf1-N-EnR* mRNA display similar morphology to embryos injected with full-length *eaf1* mRNA. (g-j) Embryos injected with *eaf1-N-VP16* mRNA display phenotypes with obvious anterior neuroectoderm truncation, similar to Eaf morphants. (**B**) *eaf1* and *eaf2* act as anterior neuroectoderm inducers by repressing transcription. *six3b* (a-e) and *opl* (f-j) displayed expanded expression in embryos injected with *eaf1/2-EnR* or *eaf1/2-N-EnR* mRNA. (k-o) *egr2b* expression was reduced in embryos injected with *eaf1/2-EnR* or *eaf1/2-N-EnR* mRNA. Ba-o, dorsal views, anterior to the left. All mRNA injections were at 50 pg/embryo.

zebrafish embryos and cell lines (Aoki et al., 1999; Playford et al., 2000). First, we injected Eaf1-MO1 alone or together with the 8xTopFlash reporter and an internal control vector, *pTK-renilla*, and then performed luciferase assays. At 5 hours post-fertilization (hpf), embryos injected with Eaf MOs exhibited obviously enhanced reporter activity as compared with control embryos injected with a standard MO (Fig. 5A). Western blots revealed that injection of Eaf MOs did not change the protein levels of either total β-catenin or active β-catenin (supplementary material Fig. S8A). In addition, to determine whether ectopic expression of *eaf1/2* mRNA in embryos could directly suppress the transactivity of β-catenin *in vivo*, we injected embryos at the one-cell stage with expression plasmids of Myc-tagged zebrafish Eaf1 (Myc-z*eaf1*), Eaf2 (Myc-z*eaf2*) or empty vector (control) together with the 8xTopFlash reporter as well as *pTK-renilla*. At a series of time points after injection (6, 8 and 10 hours), Myc-z*eaf1* or Myc-z*eaf2* dramatically suppressed 8xTopFlash activity as compared with the control vector (Fig. 5B), in contrast to what was observed in embryos with Eaf1/2 knockdown.

To further determine the role of *eaf1* and *eaf2* in Wnt/β-catenin signaling, we also evaluated the effect of ectopic Eaf expression on β-catenin transcriptional activity in mammalian cell lines. In HEK 293 cells (Fig. 5Ca,b), zebrafish *eaf1* and *eaf2* dramatically suppressed 8xTopFlash reporter activity induced by zebrafish β-catenin 1 or β-catenin 2. In addition, we found that *eaf1* and *eaf2* suppressed the endogenous β-catenin activity induced by conditioned medium containing *Wnt3a* (Fig. 5Cc). Again, zebrafish *eaf1* or *eaf2* did not affect the protein level of either *β-catenin 1* or *β-catenin 2* (supplementary material Fig. S8B).

Given that *c-Myc* represents a bona fide target of Wnt/β-catenin signaling (He et al., 1998), and that *c-Myc* is a well-defined proto-oncogene that is often upregulated in many types of cancers, we next evaluated whether *eaf1* or *eaf2* could also suppress *c-Myc* expression. Indeed, zebrafish *eaf1* or *eaf2* (Fig. 5Da,b) significantly suppressed the activity of a human *c-MYC* promoter in a dose-dependent manner. Western blots confirmed the expression of ectopic zebrafish Eaf1 and Eaf2 proteins (supplementary material Fig. S8C). Using semi-quantitative RT-PCR, we detected decreased expression of endogenous *c-MYC* mRNA 20 hours after transfection with zebrafish *eaf1* or *eaf2* (Fig. 5Dc), suggesting that they might suppress *c-Myc* expression through modulating Wnt/β-catenin signaling.

Eaf1 and Eaf2 interact with β-catenin 1 and β-catenin 2 to form a protein complex

Using co-immunoprecipitation assays, we found that zebrafish Eaf1 and Eaf2 strongly interact with zebrafish β-catenin 1 and β-catenin 2
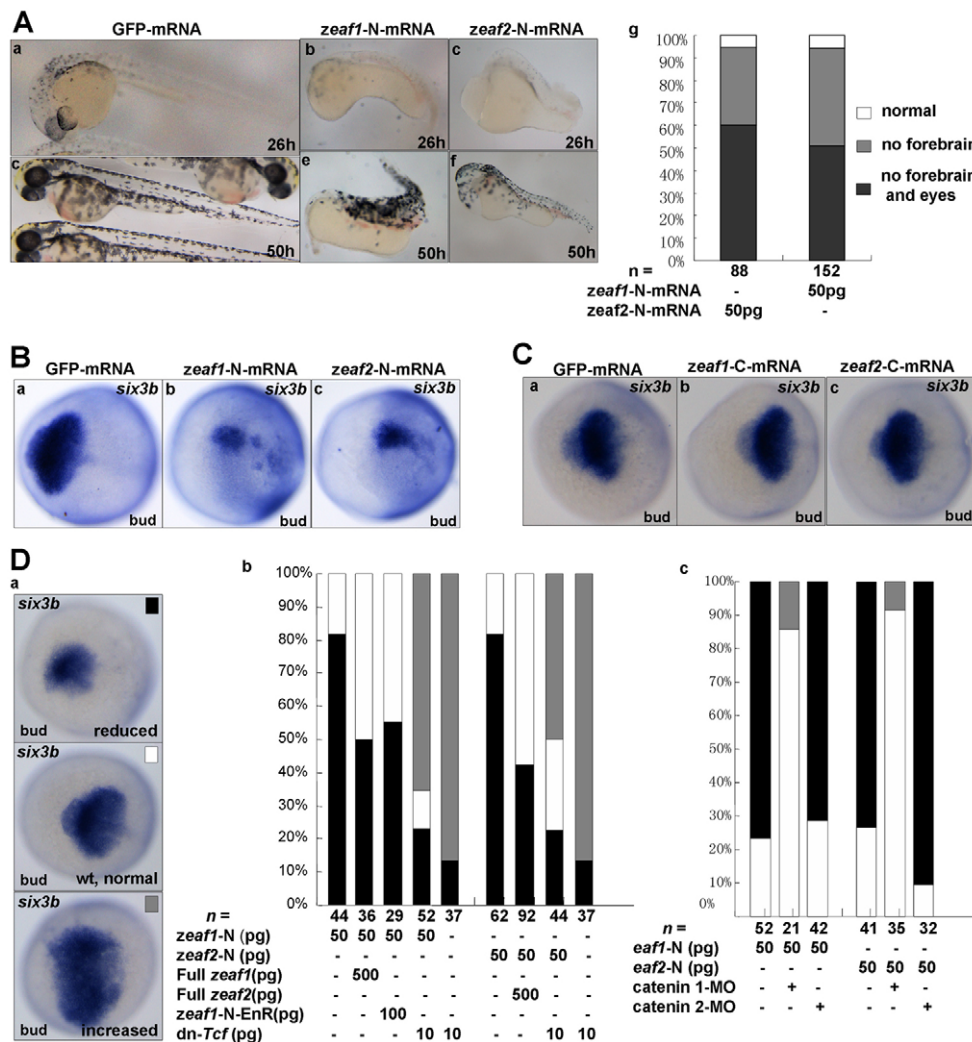
Fig. 4. The N-terminus of Eaf1 or Eaf2 acts as a dominant negative. (**A**) (a-f) Representative morphology of embryos injected with *GFP* mRNA or mRNA encoding the N-terminus of Eaf1 or Eaf2 (exons 1-3) at 26 or 50 hpf. (g) Embryos were scored morphologically at 50 hpf: white, the percentage of normal embryos; gray, embryos without forebrain; black, embryos without forebrain and eyes. (**B,C**) *six3b* expression was reduced in embryos injected with mRNA encoding the N-terminus (exons 1-3) of Eaf1 or Eaf2 (B) but was normal in embryos injected with mRNA encoding the C-terminus (exons 4-6) of Eaf1 or Eaf2 (C). (**D**) The N-terminus (exons 1-3) of Eaf1/2 suppresses forebrain formation by promoting enhanced Wnt/β-catenin activity. (a) Representative embryos showing normal, reduced and increased expression of *six3b*. (b) Embryos were scored for *six3b* expression after injection with mRNAs at the dosages indicated. (c) Abnormal expression of *six3b* in embryos injected with mRNA encoding the N-terminus (exons 1-3) of Eaf1 or Eaf2 was rescued by co-injection with β-catenin1-MO (8 ng/embryo) but not with β-catenin2-MO (8 ng/embryo). Ba-c,Ca-c,Da, dorsal views, anterior to the left. mRNA injections were at 50 pg/embryo unless stated otherwise (Db).

2 (Fig. 6B). Domain mapping indicated that Eaf1 and Eaf2 interact with the β-catenin center region that contains 12 Armadillo repeats and the C-terminus (Fig. 6D,E), but not the N-terminus (Fig. 6C). This might explain why *eaf1* and *eaf2* could also suppress the transactivity of the C-terminus as well as full-length β-catenin 1 and β-catenin 2. Furthermore, domain mapping for Eaf1 and Eaf2 revealed that the N-terminal region (amino acids 1-112; Fig. 6F-H), but not the C-terminal region (amino acids 113-253 for Eaf1 and 113-259 for Eaf2; Fig. 6I), bound to β-catenin 1/2. In addition, zebrafish Eaf1 and Eaf2 also interacted with c-Jun (Fig. 6J) (Gan et al., 2008).

To confirm the interaction between Eaf1/2, β-catenin 1/2 and c-Jun, we performed colocalization assays. Eaf1 and Eaf2 colocalized with β-catenin 1/2 and c-Jun in Cos-7 cells after transfection with GFP- or RFP-tagged proteins (supplementary material Fig. S9).

To determine whether *eaf1* or *eaf2* also suppresses β-catenin transactivity by altering the cytoplasmic-nuclear shuttling of β-catenin, we measured β-catenin levels in the cytoplasm and nucleus by western blot analysis after ectopic expression or knockdown of *eaf1/2*. Zebrafish β-catenin was found mainly in the nucleus, and altering *eaf1/2* expression did not affect the distribution of the protein (supplementary material Fig. S10). Thus, *eaf1* and *eaf2* do not affect the translocation of β-catenin between the cytoplasm and nucleus.

The suppressive effect of Eaf1 and Eaf2 on Wnt/β-catenin signaling is evolutionarily conserved across species

In our previous study, we found that zebrafish *eaf1* and *eaf2* share a high degree of identity with their human homologs *EAF1* and *EAF2*. In addition, human *EAF1/2* mRNA could efficiently rescue the phenotype associated with zebrafish *eaf1/2* morphants (Liu et al., 2009). This prompted us to explore whether the suppressive function of *eaf1* and *eaf2* on Wnt/β-catenin signaling is conserved in humans.

Using zebrafish as our *in vivo* model, we overexpressed human *EAF1/2* mRNA in embryos by mRNA injection and found that the human and zebrafish Eafs behaved in a very similar manner. At 72 hpf, the human *EAF1/2*-injected embryos exhibited an expanded forebrain and shortened bodies with either a short tail or no tail (75-85% of embryos; Fig. 7A,B), fully phenocopying the embryos injected with zebrafish *eaf1/2* mRNA. We then used marker genes for further phenotyping. As expected, injection of human *EAF1* or *EAF2* mRNA resulted in expanded expression of *six3b* (Fig. 7C) and increased expression of both *dkk1* and *frzb* (supplementary material Fig. S11A,B).

We next performed transcription assays using the TopFlash reporter. Human *EAF1* and *EAF2* significantly suppressed the reporter activity induced by ectopic expression of *β-catenin* in HEK
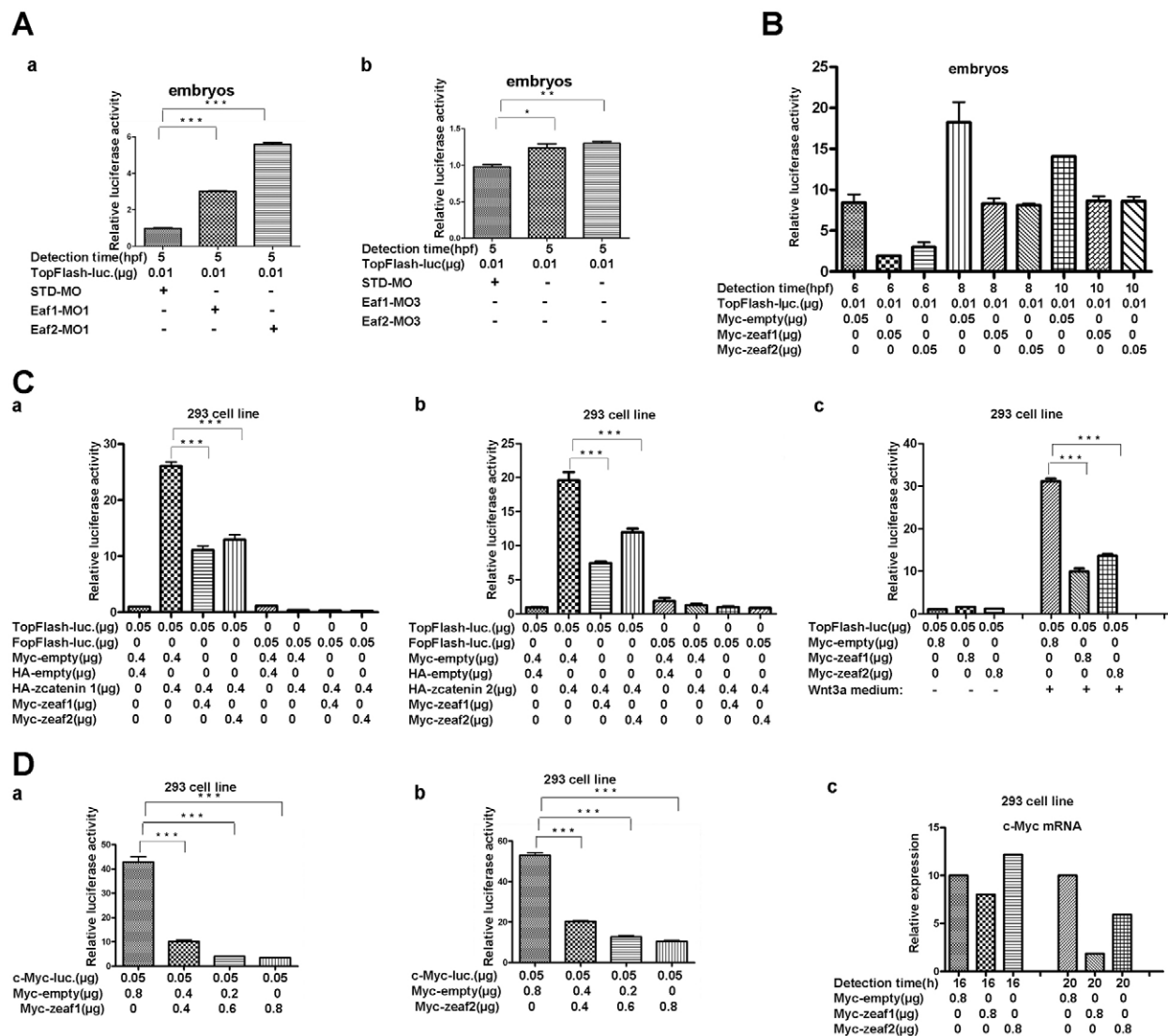
Fig. 5. *eaf1* and *eaf2* inhibit Wnt reporters. (A) Endogenous β-catenin transcriptional activity in zebrafish embryos was enhanced by knockdown of Eaf1 or Eaf2. One-cell stage embryos were injected with the plasmids indicated, together with 8xTopFlash as a reporter and *TK-renilla* as an internal control; luciferase activity was measured at 5 hpf. **(B)** Endogenous β-catenin transcriptional activity in zebrafish embryos was suppressed by ectopic expression of *eaf1* or *eaf2*. Luciferase activity was measured at 6, 8 or 10 hpf. **(C)** In the HEK 293 mammalian cell line, 8xTopFlash activity enhanced by Wnt/β-catenin was suppressed by overexpression of *eaf1* or *eaf2*. (a,b) 8xTopFlash activity enhanced by *β-catenin 1* (*zcatenin1*) (a) or *β-catenin 2* (*zcatenin2*) (b) was suppressed by both *eaf1* (zeaf1) and *eaf2* (zeaf2). (c) 8xTopFlash activity enhanced by addition of *Wnt3a*-conditioned medium was suppressed by ectopic expression of *eaf1* or *eaf2*. **(D)** The expression of the Wnt/β-catenin target gene human *c-MYC* was suppressed by *eaf1* or *eaf2*. (a,b) Dose-dependent downregulation of *c-MYC* promoter activity by *eaf1* (a) and *eaf2* (b). (c) Semi-quantitative RT-PCR analysis of endogenous human *c-MYC* expression after ectopic expression of *eaf1* or *eaf2*. Data from the luciferase reporter assays are reported as mean±s.d. of three independent experiments performed in triplicate; ***$P<0.001$, **$P<0.01$, *$P<0.05$ (Student's *t*-test).

293 cells (Fig. 7D). Similarly, human *EAF1* and *EAF2* suppressed the *c-MYC* promoter activity (Fig. 7E).

Human EAF1 and EAF2 bound to human β-catenin as well as to other complex components, including Tcf, c-Jun and Axin (Fig. 7F,G). Similar to zebrafish *eaf1/2*, human *EAF1* and *EAF2* did not affect the protein stability of ectopic β-catenin or endogenous active β-catenin (supplementary material Fig. S11C,D). The cytoplasmic-nuclear translocation of β-catenin was also unaffected by overexpression of human EAF1 or EAF2 in HEK 293 cells (data not shown), and nor were the protein levels of the other β-catenin transcriptional complex proteins Tcf, c-Jun and Axin (supplementary material Fig. S11E-G).

Together, these observations suggest that the suppressive role of Eaf1 and Eaf2 in Wnt/β-catenin signaling is evolutionarily conserved across species.

DISCUSSION
Zebrafish *eaf1* and *eaf2* are novel antagonists of Wnt/β-catenin signaling
In this report, loss-of-function of *eaf1* and *eaf2* in zebrafish embryos resulted in truncation of the anterior neuroectoderm, a similar morphology to embryos with enhanced Wnt signaling (Kelly et al., 1995; Kim et al., 2000; Kim et al., 2002). Gain-of-function of *eaf1* and *eaf2* resulted in enhanced expression of dorsal and anterior brain
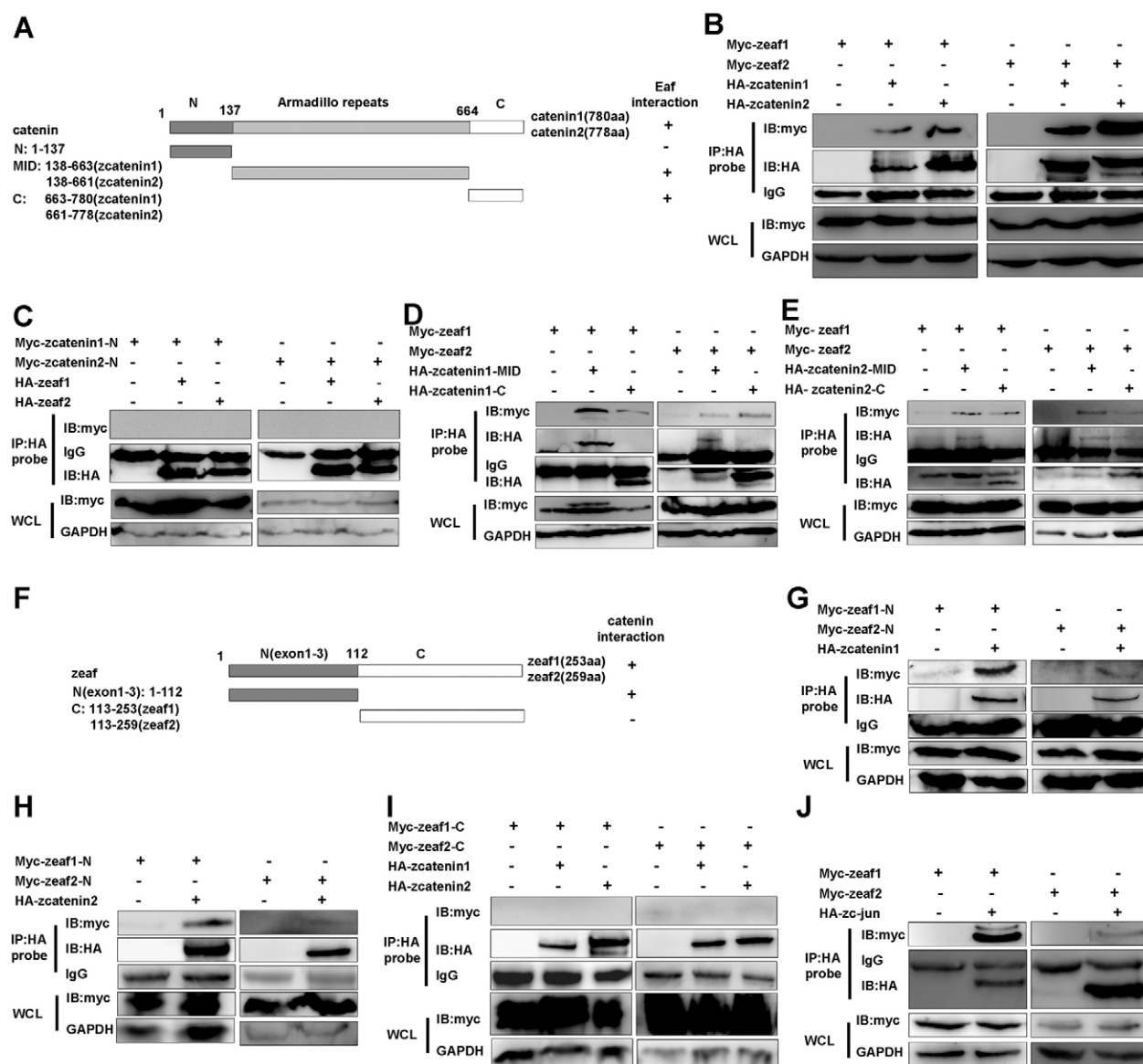
Fig. 6. Eaf1 and Eaf2 function as novel nuclear Wnt signaling components. (**A**) The domains in zebrafish β-catenin 1 and β-catenin 2 that interact with Eaf1 and Eaf2: +, interaction; –, no interaction. MID, the β-catenin center region that contains 12 Armadillo repeats. (**B**) Co-immunoprecipitation of Eaf1 or Eaf2 with full-length β-catenin 1 or β-catenin 2. (**C**) Eaf1 or Eaf2 did not interact with the N-terminus of β-catenin 1 or β-catenin 2. (**D,E**) Eaf1 or Eaf2 interacted with both the Armadillo repeats and the C-terminal transcriptional domain of β-catenin 1 (D) and β-catenin 2 (E). (**F**) The domains in Eaf1 and Eaf2 that interacted with β-catenin 1/2: N, exons 1-3 of Eaf1 or Eaf2; C, exons 4-6 of Eaf1 or Eaf2; +, interaction; –, no interaction. (**G,H**) β-catenin 1 (G) and β-catenin 2 (H) interacted with the N-terminus of Eaf1 or Eaf2. (**I**) β-catenin 1/2 did not interact with the C-terminus of Eaf1 or Eaf2. (**J**) Co-immunoprecipitation of Eaf1 or Eaf2 with full-length zebrafish c-Jun. WCL, whole cell lysate.

markers. This expression pattern mirrors that of *wnt8* morphants, *wnt8* mutants, or embryos expressing ectopic Wnt inhibitors (Lekven et al., 2001; Kim et al., 2002; Momoi et al., 2003). In addition, we observed enhanced expression of the Wnt/β-catenin maternal targets *sqt* and *gsc* at the blastula stage, the zygotic targets *cdx4* and *tbx6* at the later gastrula stage, and the direct target *axin2* in midbrain at bud stage. All these observations indicate that *eaf1* and *eaf2* are required for forebrain and mesoderm patterning by negatively regulating Wnt/β-catenin signaling.

eaf1 and *eaf2* counteracted the effects of enhanced Wnt/β-catenin signaling in *apc* mutants, as well as in embryos with ectopic expression of *wnt8* or BIO treatment. However, β-catenin1-MO and dn-*Tcf* mRNA rescued the defects in *gsc* and *six3b* expression

exhibited by Eaf morphants. This suggests that *eaf1* and *eaf2* might antagonize Wnt/β-catenin signaling downstream of *wnt8*, but upstream of, or parallel to, *β-catenin* and *tcf*.

Interestingly, β-catenin2-MO effectively rescued dorsal gene expression in Eaf morphants, but caused severe forebrain defects in the same embryos as well as in those injected with mRNA encoding the N-terminus of Eaf1/2. This is in contrast to the effect of β-catenin1-MO. This outcome is consistent with a previous report that *β-catenin 1* and *β-catenin 2* play essential but opposing roles in the formation of axis and neuroectoderm, and that *β-catenin 2* is essential for dorsal mesoderm and forebrain formation (Bellipanni et al., 2006).

In a previous study we had shown that Eafs contribute to the regulation of convergence and extension movements through non-
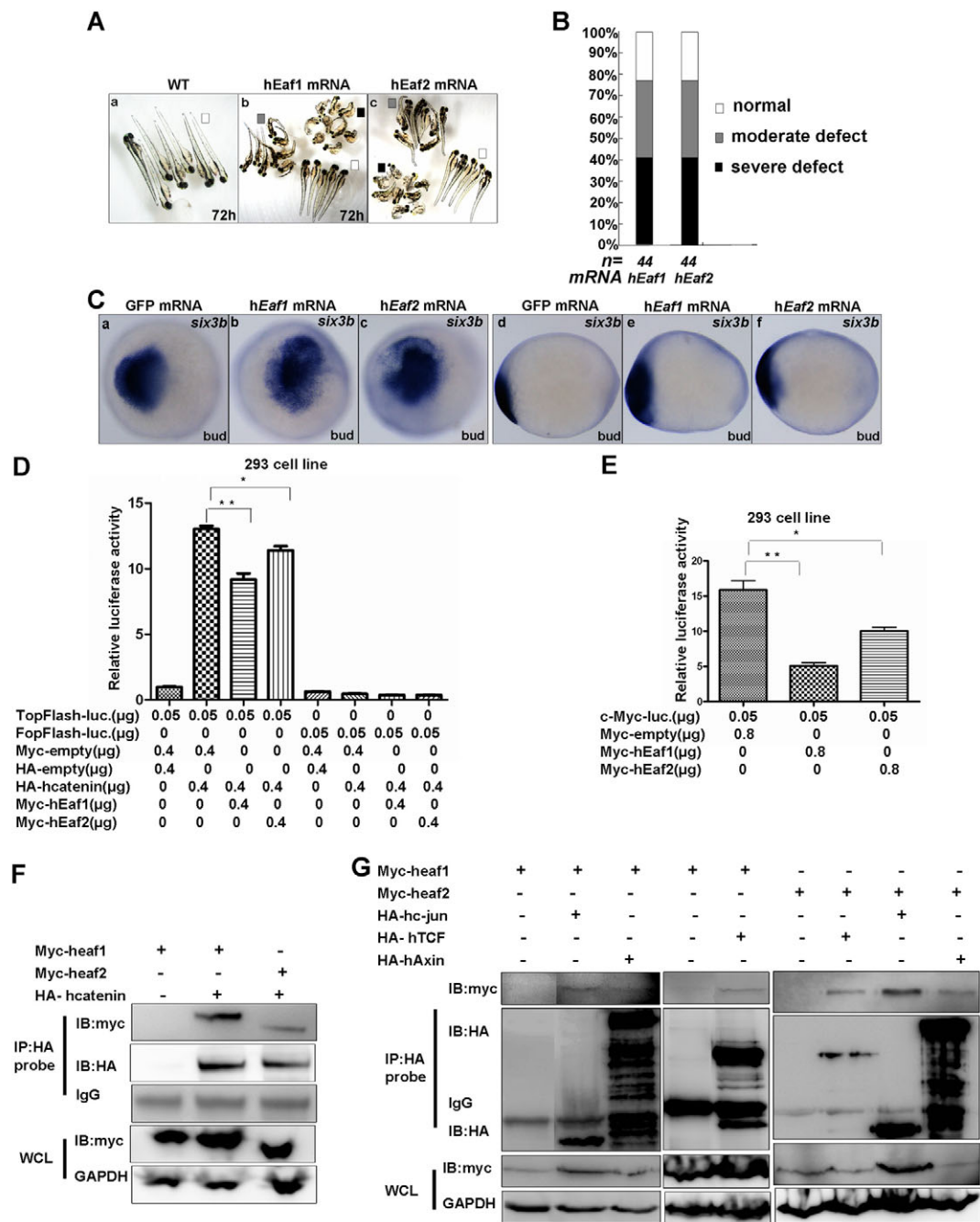
Fig. 7. Eaf1/2 activity is evolutionarily conserved from zebrafish to human. (**A**) Phenotypes of zebrafish embryos injected with human *EAF1* (hEaf1) or *EAF2* (hEaf2) mRNA at 72 hpf. (**B**) Morphology of zebrafish embryos injected with human *EAF1* or *EAF2* mRNA at 3 dpf (200-500 pg/embryo) was scored as normal (white), moderately defective (gray) or severely defective (black); embryos injected with *GFP* mRNA (200-500 pg/embryo) were used as a control. (**C**) *six3b* displayed enhanced expression in zebrafish embryos in which human *EAF1* or *EAF2* was ectopically expressed. (**D**) 8xTopFlash activity enhanced by human β-catenin was suppressed in the HEK 293 cell line by human EAF1 or EAF2. (**E**) Downregulation of *c-MYC* promoter activity by human full-length EAF1 or EAF2. Mean±s.d.; **$P<0.01$, *$P<0.05$ (Student's *t*-test). (**F**) Co-immunoprecipitation of human EAF1 or EAF2 with full-length human β-catenin. (**G**) Human EAF1 and EAF2 interact with components of the human β-catenin transcriptional complex, including TCF4, c-JUN and AXIN, *in vivo*. Ca-c, dorsal views, anterior to the left; Cd-f, lateral views, anterior to the left. WCL, whole cell lysate.

canonical signaling (Liu et al., 2009). Many factors, such as *dkk1* and *naked*, regulate both canonical and non-canonical Wnt signaling (Caneparo et al., 2007; Van Raay et al., 2007). In embryos with ectopic expression of factors that promote Wnt/β-catenin signaling, such as *wnt8a* and active *β-catenin*, Eaf expression was reduced, but remained unchanged in embryos with ectopic expression of the

Wnt/β-catenin inhibitor *frzb*. Together with the evidence that Eafs negatively regulate Wnt/β-catenin signaling, this supports the possible existence of a regulatory feedback loop between *eaf1/2* and canonical Wnt signaling.

In this study, *eaf1* and *eaf2* did not appear to function redundantly, even though they exhibit partially redundant function in regulating

convergence and extension movements (Liu et al., 2009). Regarding the similar phenotypes shown in embryos injected with each MO alone (Eaf1-MO or Eaf2-MO), the function of *eaf1* and *eaf2* might be dosage dependent.

Eaf1 and Eaf2 are novel components of the β-catenin transcriptional complex

Multiple factors serve as either antagonists or activators in Wnt/β-catenin signaling. These factors then execute their function by modulating the protein degradation, stabilization or distribution of β-catenin or of other components of the β-catenin transcriptional complex (Morin et al., 1997; Behrens et al., 1998; Gan et al., 2008). Here, we provide evidence that Eaf1 and Eaf2 not only bind to β-catenin, but also to other components of the β-catenin transcriptional complex, such as Tcf, Axin and c-Jun, which are evolutionarily conserved between zebrafish and human. However, overexpression or knockdown of Eafs did not alter the total or nuclear levels of β-catenin protein or of the other components of the β-catenin transcriptional complex, which suggests that mechanisms other than the regulation of protein stability and localization account for the suppressive role of Eaf1 and Eaf2.

β-catenin transcriptional activity is reported to emanate from its most N-terminal Arm repeat region, as well as from its C-terminal region, with the latter being the most potent transactivation domain (van de Wetering et al., 1997; Hecht et al., 1999). Eaf1/2 interacted with both the Arm repeat domain and C-terminus of β-catenin, and β-catenin interacted with the N-terminus of Eaf1/2. *In vivo*, embryos with ectopic expression of Eaf-EnR and Eaf-N-EnR show the same phenotype as embryos injected with mRNA encoding full-length *eaf1/2*. In all, these observations suggest that Eaf1/2 either act as a novel transcriptional repressor or recruit a repressor for the β-catenin transcriptional complex. Of note, Eafs bind to the same regions of β-catenin that commonly interact with other β-catenin antagonists, such as ICAT, Chibby and APC (Tago et al., 2000; Takemaru et al., 2003; Mosimann et al., 2009). This further supports the notion of a suppressive function for Eaf1 and Eaf2 in Wnt/β-catenin signaling.

Because they harbor transactivation domains in their C-terminus, Eaf1 and Eaf2 are implicated as transcriptional activators (Simone et al., 2001; Simone et al., 2003; Kong et al., 2005; Xiao et al., 2008). Here, we provided evidence that Eaf1 and Eaf2 actually function as repressors rather than activators to inhibit the transactivity of β-catenin. These results suggest that Eaf1 and Eaf2 might act to suppress transactivation of their binding partners, but directly activate the expression of their own downstream genes as transcription factors. This phenomenon has been reported for other transcription factors, such as p53. As a classic transcription factor, p53 can inhibit HIF1α transactivity by direct interaction (An et al., 1998).

Eaf1 and Eaf2 may function as tumor suppressors through antagonizing Wnt/β-catenin signaling

Compelling evidence points to a role for Wnt/β-catenin signaling in cancer (Kim et al., 2002; Moon et al., 2004; Clevers, 2006). Cancer-associated mutations result in the constitutive activation of Wnt/β-catenin signaling (Clevers, 2006). Indeed, multiple cancers, such as colon cancer, hair follicle tumors, prostate cancer and leukemia, show a high frequency of aberrant stabilization and constitutive activation of β-catenin (Morin et al., 1997; Chan et al., 1999; Jamieson et al., 2004; Lo Celso et al., 2004). In addition, transgenic mice with constitutive activation of β-catenin develop prostatic intraepithelial neoplasia (mPIN) (Yu et al., 2009; Yu et al., 2011). Eaf2 has been showed to have a tumor suppressive function

in multiple cancers (Xiao et al., 2003; Xiao et al., 2008). In this study, through both loss- and gain-of-function assays in the zebrafish model, we revealed that *eaf1* and *eaf2* antagonize Wnt/β-catenin signaling. Furthermore, we found that Eaf1 and Eaf2 dramatically suppress β-catenin transcriptional activity by interacting with β-catenin and other components of the β-catenin transcriptional complex, which is evolutionarily conserved between zebrafish and human. Although further studies in human tumor samples or mammalian models are required, we propose that the human Eaf gene family might act as tumor suppressors through inhibiting Wnt/β-catenin signaling.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.086157/-/DC1

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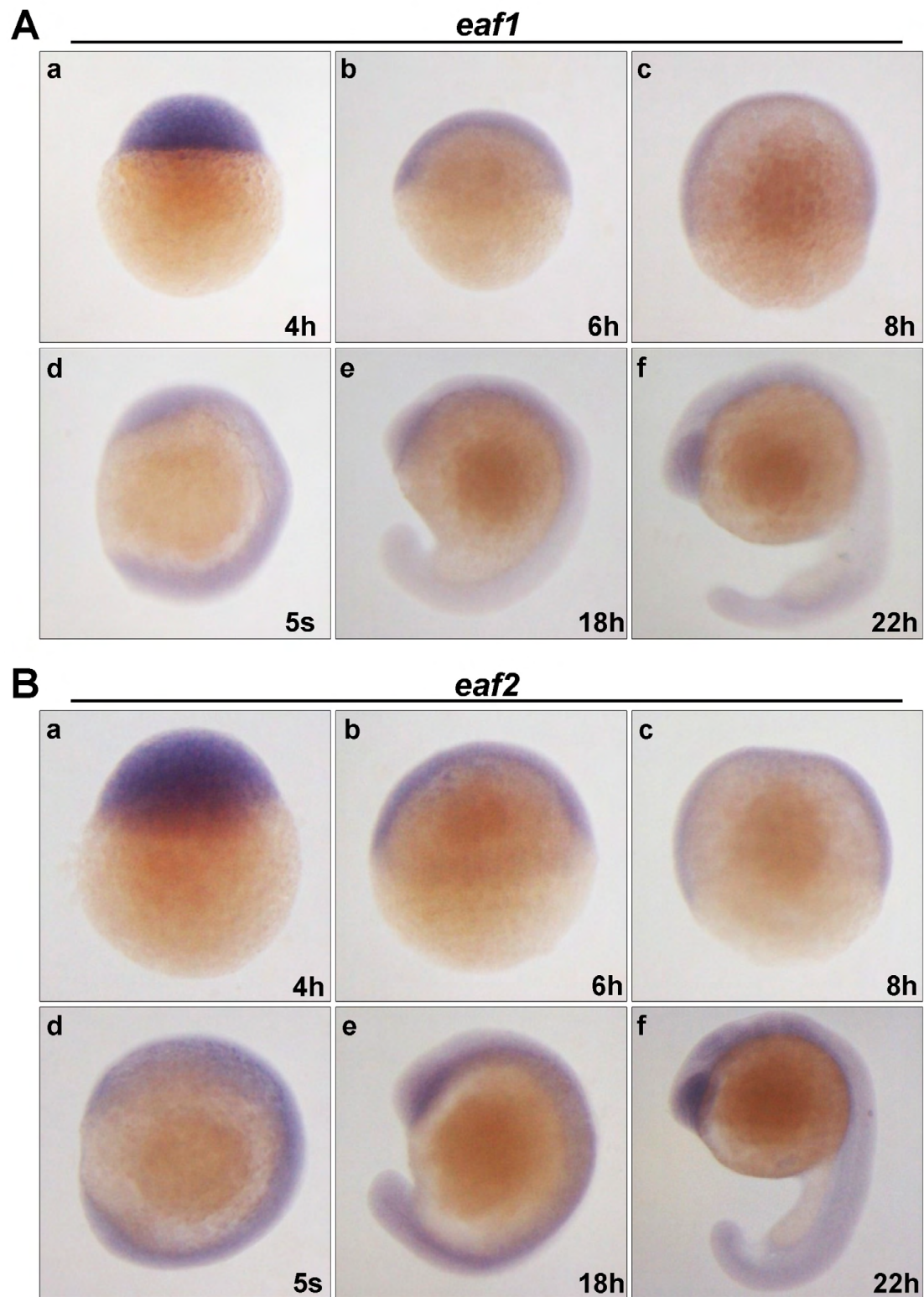
Fire S1. The expression patterns of zebrafish *eaf1* and *eaf2* during embryogenesis. (A) *eaf1* expression. **(B)** *eaf2* expression. Lateral view, dorsal to the right.
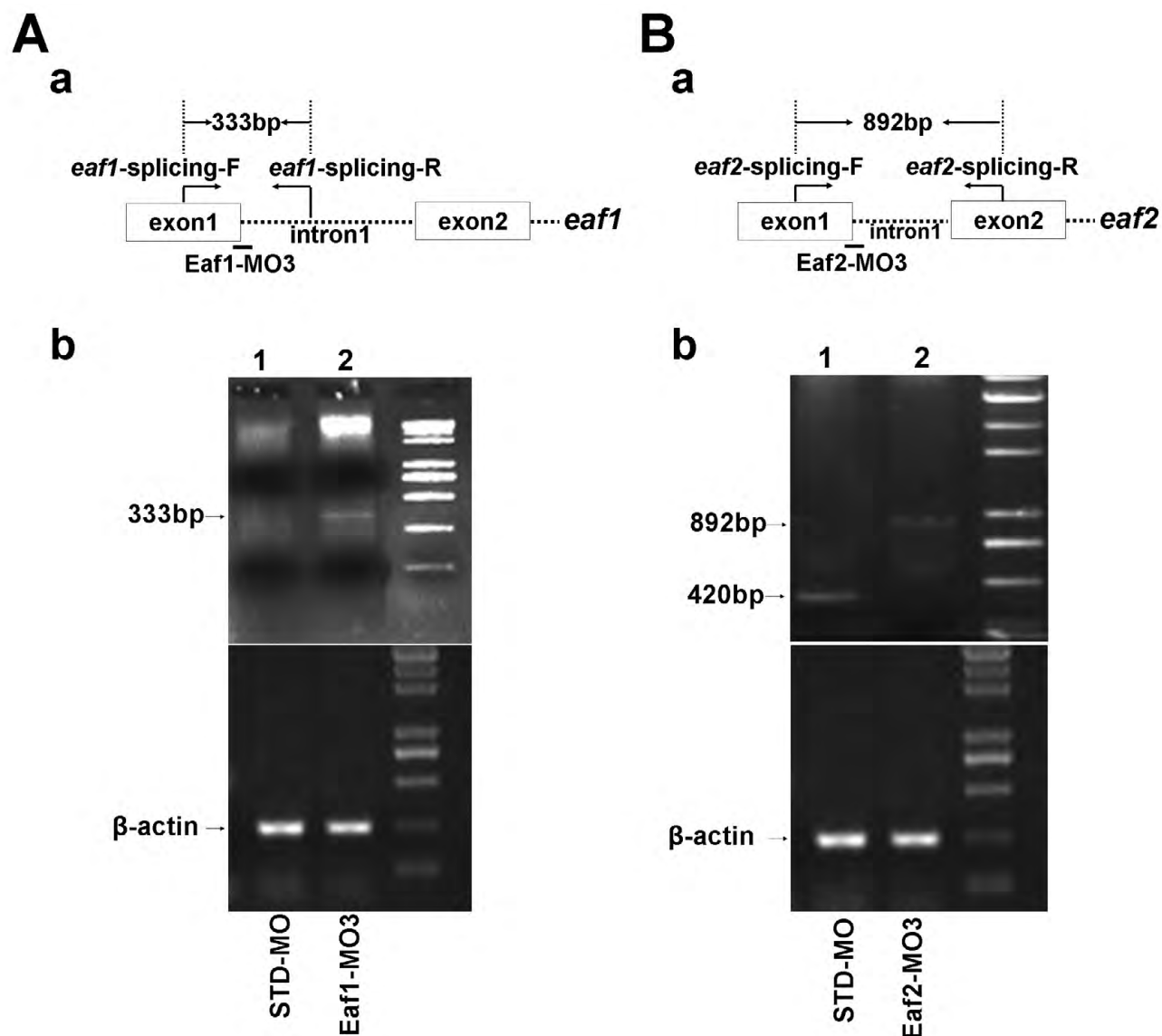
Fig. S2. Validation of Eaf1-MO3 and Eaf2-MO3. (**A**) (a) Schematic of *eaf1* exon-intron structure, Eaf1-MO3 targeting position and RT-PCR primer locations. (b) In STD-MO injection embryos, an expected 333 bp band could not be detected by RT-PCR, but in embryos with Eaf1-MO3 injection, a 333 bp band could be detected by RT-PCR. (**B**) (a) Schematic of *eaf2* exon-intron structure, Eaf2-MO3 targeting position and RT-PCR primer locations. (b) A 420 bp band was amplified from embryos injected with STD-MO by RT-PCR, but a 892 bp band was amplified from embryos injected with Eaf2-MO3, which contained intron 1 of *eaf2*. Embryos were collected at the bud stage for RNA extraction. Lane 1 is RNA from embryos injected with STD-MO, and lane 2 is RNA from embryos injected with Eaf1-MO3 (Ab) or Eaf1-MO3 (Bb).
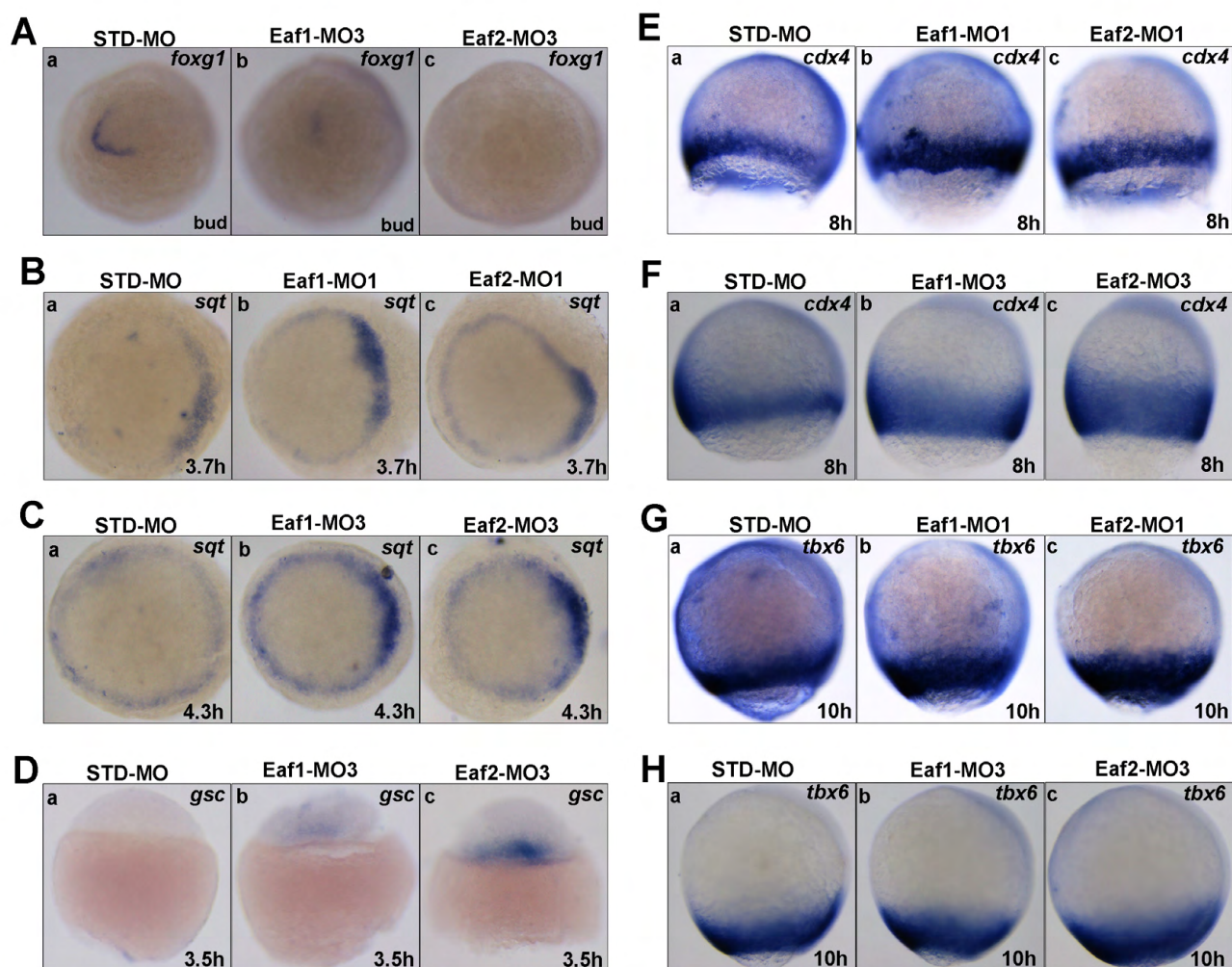
Fig. S3. Knockdown of zebrafish Eaf1 and Eaf2 causes defects in anterior neuroectoderm patterning and mesoderm patterning. (**A**) The expression of anterior neuroectoderm marker *foxg1*. (**B-D**) The expression of dorsal mesoderm marker genes *sqt* and *gsc*. (**E-H**) The expression of posterior ectoderm/mesoderm marker *cdx4* and ventral mesoderm marker *tbx6*. (A) Dorsal view, anterior to the left; (D) dorsal view; (B,C) animal view, dorsal to the right; (E-H) lateral view, dorsal to the right.
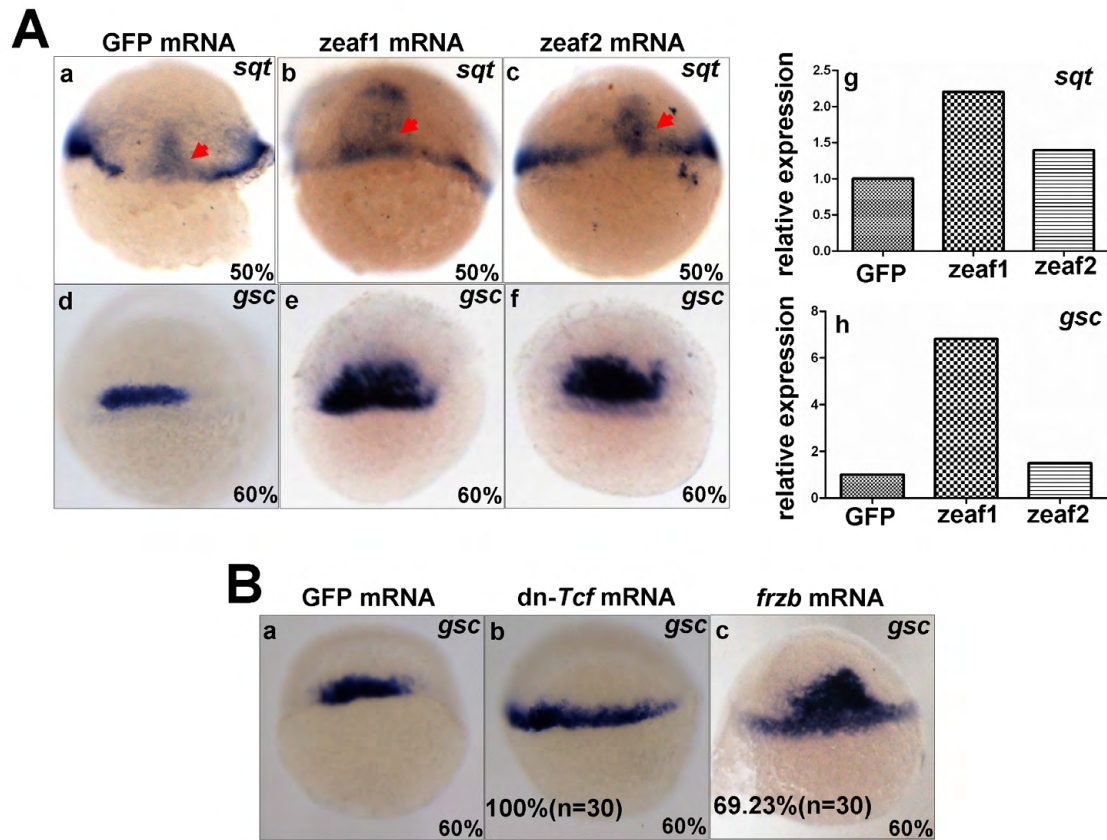
Fig. S4. Ectopic expression of zebrafish Eaf1 and Eaf2 increases the expression of *sqt* and *gsc*. (**A**) At the gastrula stage, dorsal marker genes *sqt* (a-c) and *gsc* (d-f) displayed expanded expression, particularly in the ventral domain. qRT-PCR was used to measure expression of *sqt* (g) and *gsc* (h) in these embryos. (**B**) In embryos injected with dn-*Tcf* mRNA (b) or *frzb* mRNA (c), the expression of *gsc* expanded ventral-laterally at the gastrula stage, compared with embryos injected with *GFP* mRNA (a). Dorsal views.
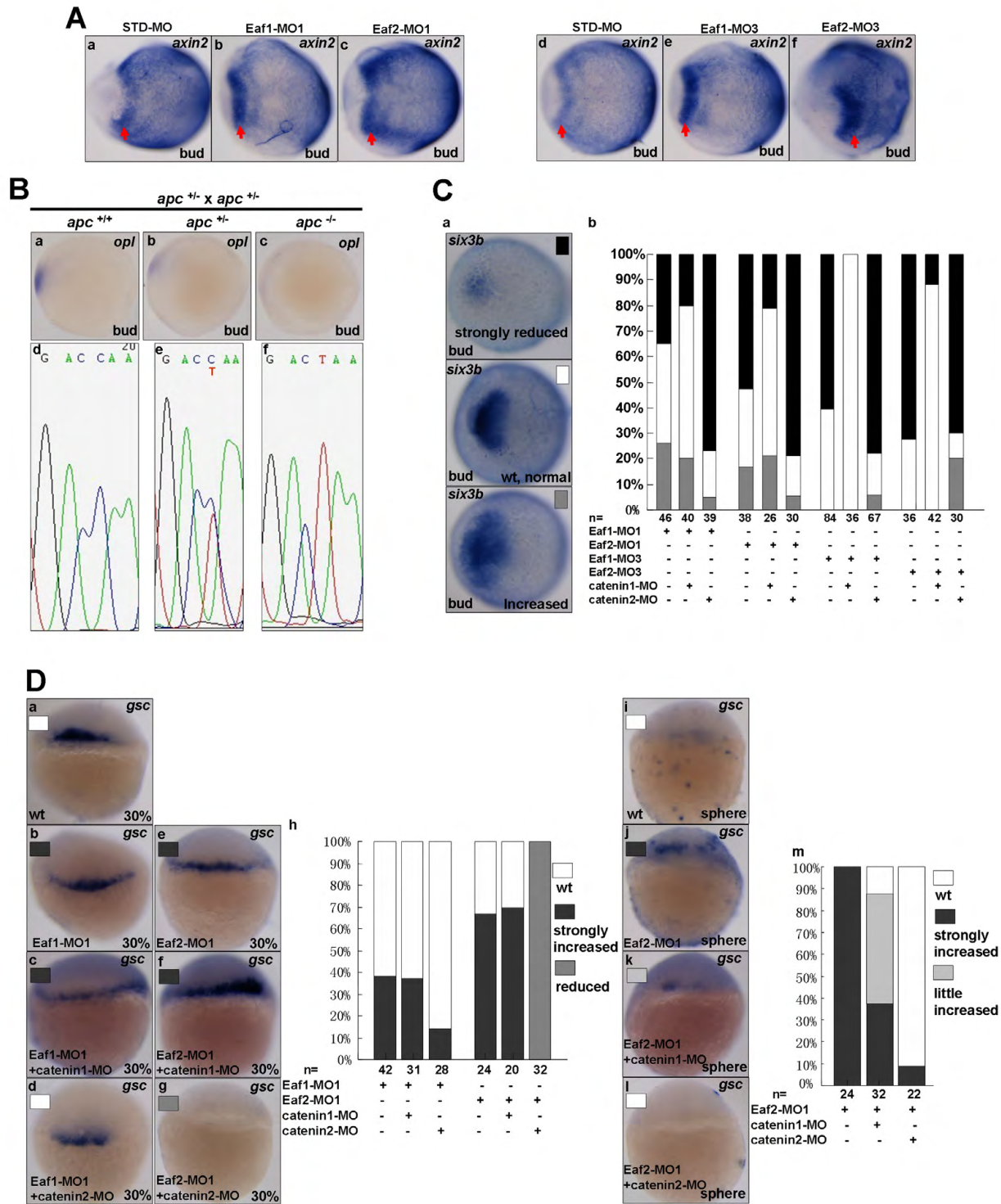
Fig. S5. *eaf1* and *eaf2* antagonize Wnt/β-catenin signaling. (A) The expression of *axin2*, a bona fide direct target of Wnt/β-catenin signaling at bud stage, was enhanced after knockdown of *eaf1* (b,e) or *eaf2* (c,f) (red arrows). (B) The offspring of *apc*^{+/−} × *apc*^{+/−} were genotyped by sequencing the mutant allele DNA fragment amplified from genomic DNA extracted from WISH-stained embryos. The embryos with normal *opl* expression contain the wild-type allele (C/C) (a,d), whereas the embryos with reduced *opl* expression contain the heterozygous allele (C/T) (b,e) and the embryos with strongly reduced *opl* expression contain the homozygous mutated allele (T/T) (c,f). (C) β-catenin1-MO, but not β-catenin2-MO, partially rescued the anterior brain defects in Eaf morphants. (D) At the 30% gastrula stage, β-catenin2-MO partially reduced the increased *gsc* expression associated with Eaf morphants (a-h); both β-catenin1-MO and β-catenin2-MO partially reduced the increased *gsc* expression of Eaf2-MO1 morphants at the sphere stage (i-m), and β-catenin2-MO was more effective (m). (A-C) Dorsal view, anterior to the left; (Da-g,i-l) dorsal view.
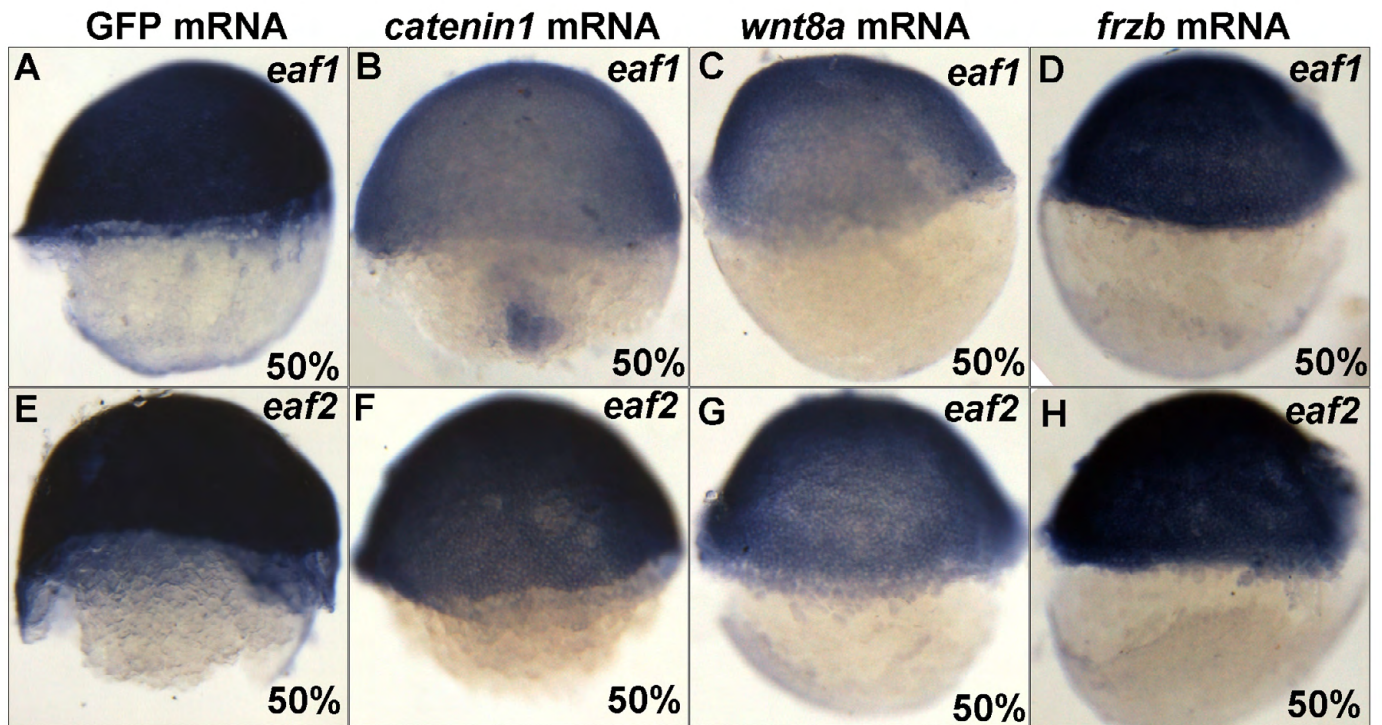
Fig. S6. The regulation of zebrafish *eaf1* and *eaf2* by Wnt/β-catenin signaling. The expression of *eaf1* and *eaf2* is downregulated in embryos with ectopic expression of active β-*catenin 1* mRNA (**B**,**F**) or *wnt8a* mRNA (**C**,**G**) as compared with embryos injected with *GFP* mRNA (**A**,**E**), but was maintained in embryos injected with *frzb* mRNA (**D**,**H**). Lateral views, dorsal to the right.
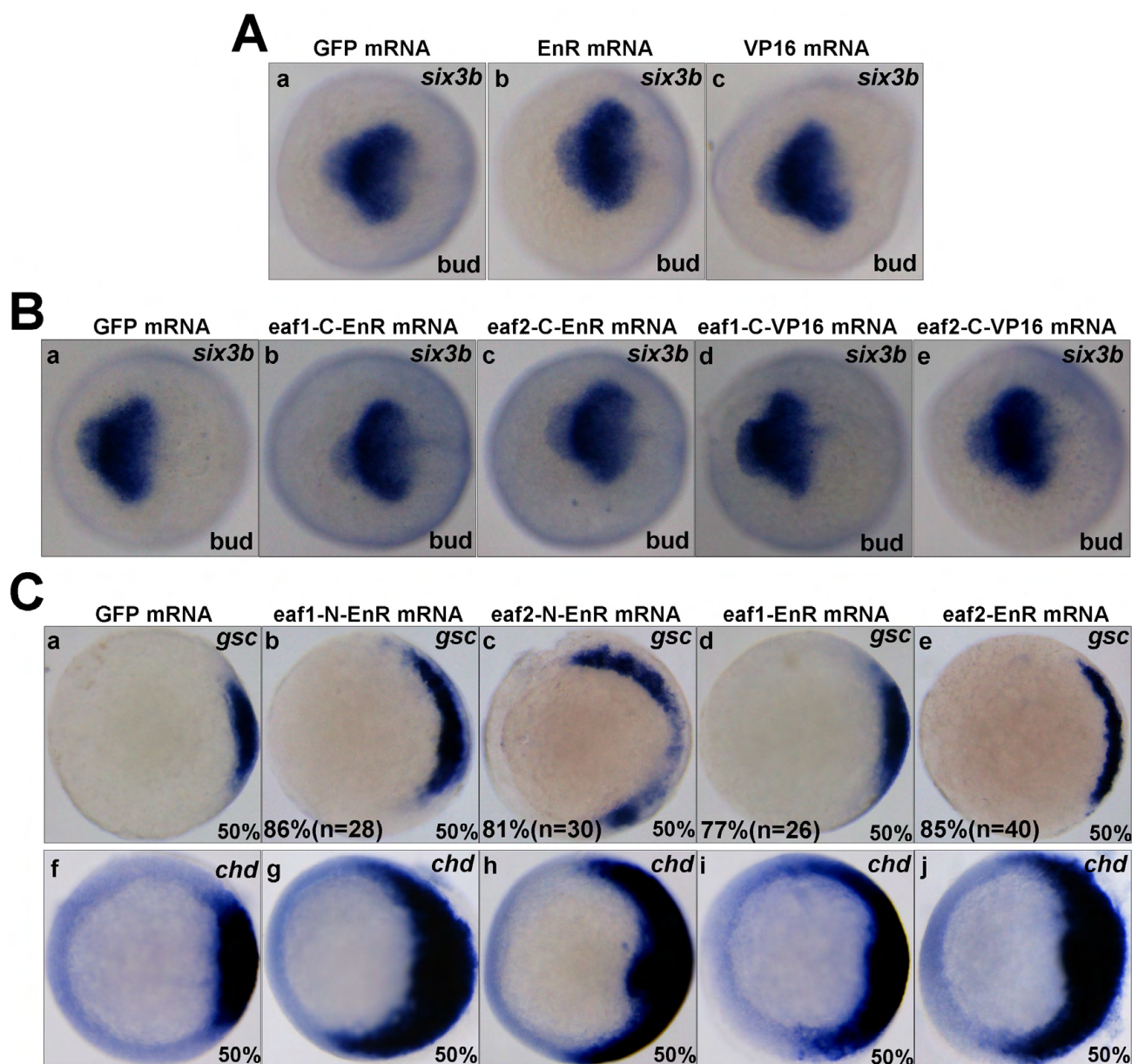
Fig. S7. VP16 and EnR are not functional *in vivo* and Eaf1/2 act as transcriptional repressors. (A,B) *Six3b* staining indicated normal patterning of anterior neural ectoderm in embryos injected with mRNA of the engrailed domain (EnR) or the VP16 domain (A), and in embryos injected with EnR or VP16 fused with the C-terminus of Eaf1 or Eaf2 (B). (**C**) In the embryos injected with *eaf1/2-EnR* or *eaf1/2-N-EnR* mRNA, the expression of the dorsal marker genes *gsc* and *chd* displayed similarly expanded patterns. (A,B) Dorsal views, anterior to the left; (C) animal views, dorsal to the right. All mRNA injections were 50-100 pg/embryo.
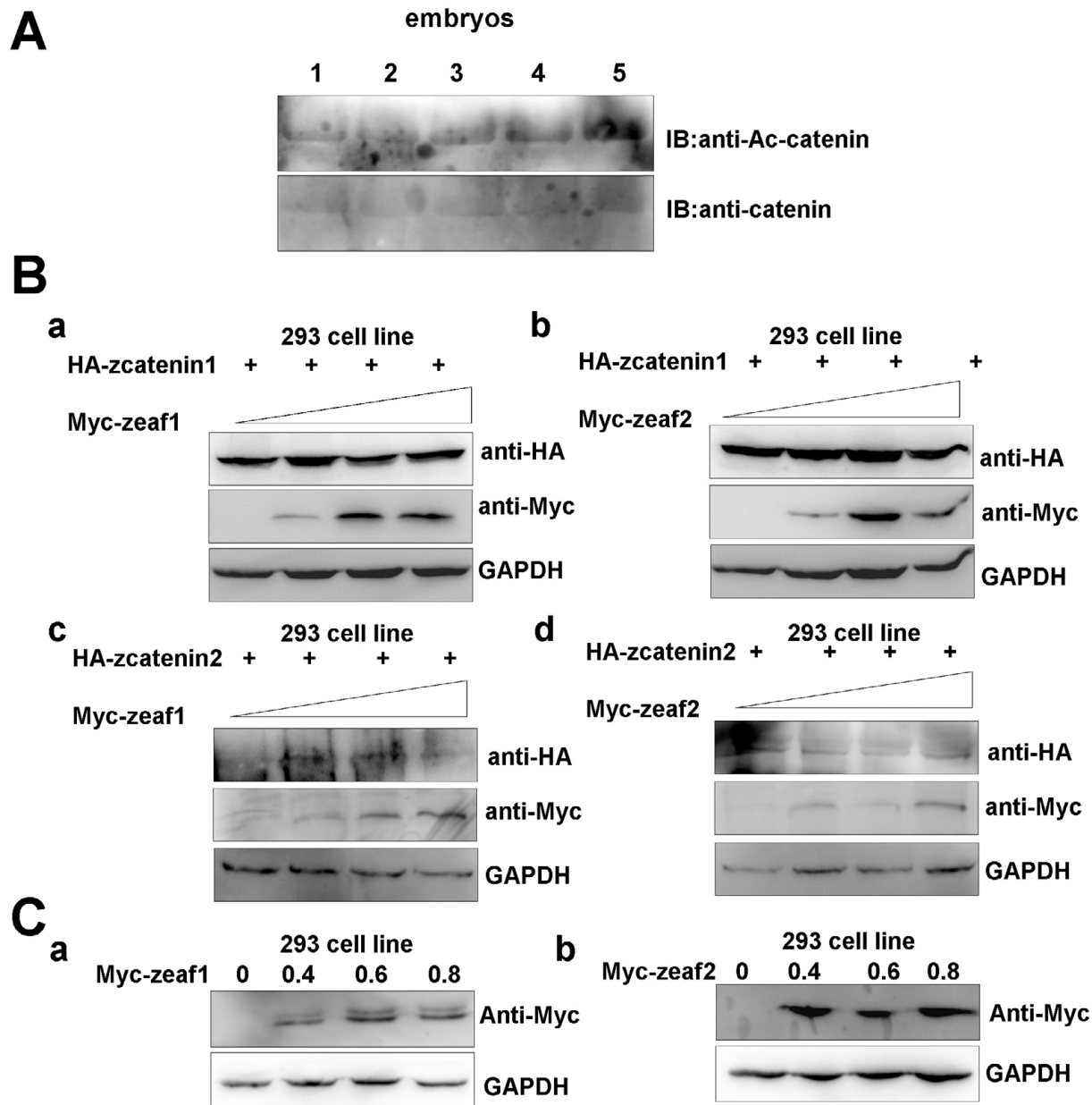
Fig. S8. Zebrafish Eaf1 or Eaf2 does not affect the protein level of either β-catenin 1 or β-catenin 2. (**A**) Western blot analysis for total β-catenin and active β-catenin protein (Ac-catenin) in embryos injected with STD-MO (lane 1), Eaf1-MO1 (lane 2), Eaf2-MO1 (lane 3), Eaf1-MO3 (lane 4) or Eaf2-MO3 (lane 5) (8 ng/per embryo). (**B**) The co-transfection of increased amounts of *eaf1* or *eaf2* together with β-*catenin 1* and β-*catenin 2* did not change the protein level of β-catenin 1 and β-catenin 2. (**C**) Western-blot analysis confirmed ectopic expression of zebrafish Eaf1 and Eaf2.
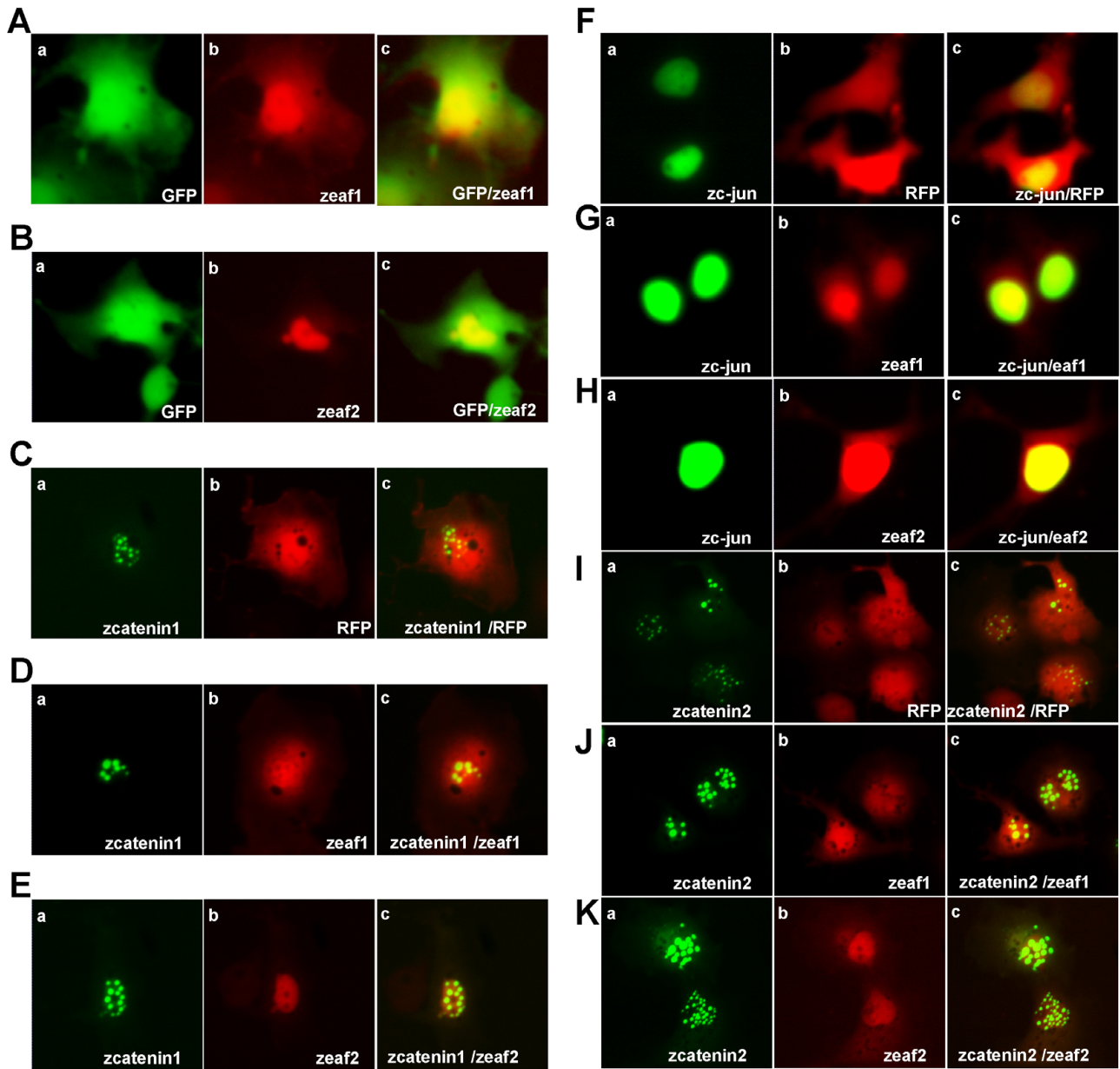
Fig. S9. Zebrafish Eaf1 or Eaf2 colocalizes with zebrafish β-catenin 1, β-catenin 2 and c-Jun. Cos-7 cells were transfected with: (**A**) empty GFP together with *eaf1*-RFP; (**B**) empty GFP together with *eaf2*-RFP; (**C**) empty RFP together with β-*catenin1*-GFP; (**D**) β-*catenin1*-GFP together with *eaf1*-RFP; (**E**) β-*catenin1*-GFP together with *eaf2*-RFP; (**F**) *c-jun*-GFP together with empty RFP; (**G**) *c-jun*-GFP together with *eaf1*-RFP; (**H**) *c-jun*-GFP together with *eaf2*-RFP; (**I**) β-*catenin2*-GFP together with empty RFP; (**J**) β-*catenin2*-GFP together with *eaf1*-RFP; (**K**) β-*catenin2*-GFP together with *eaf2*-RFP. The cells were photographed under a fluorescence microscope 24 hours after transfection.
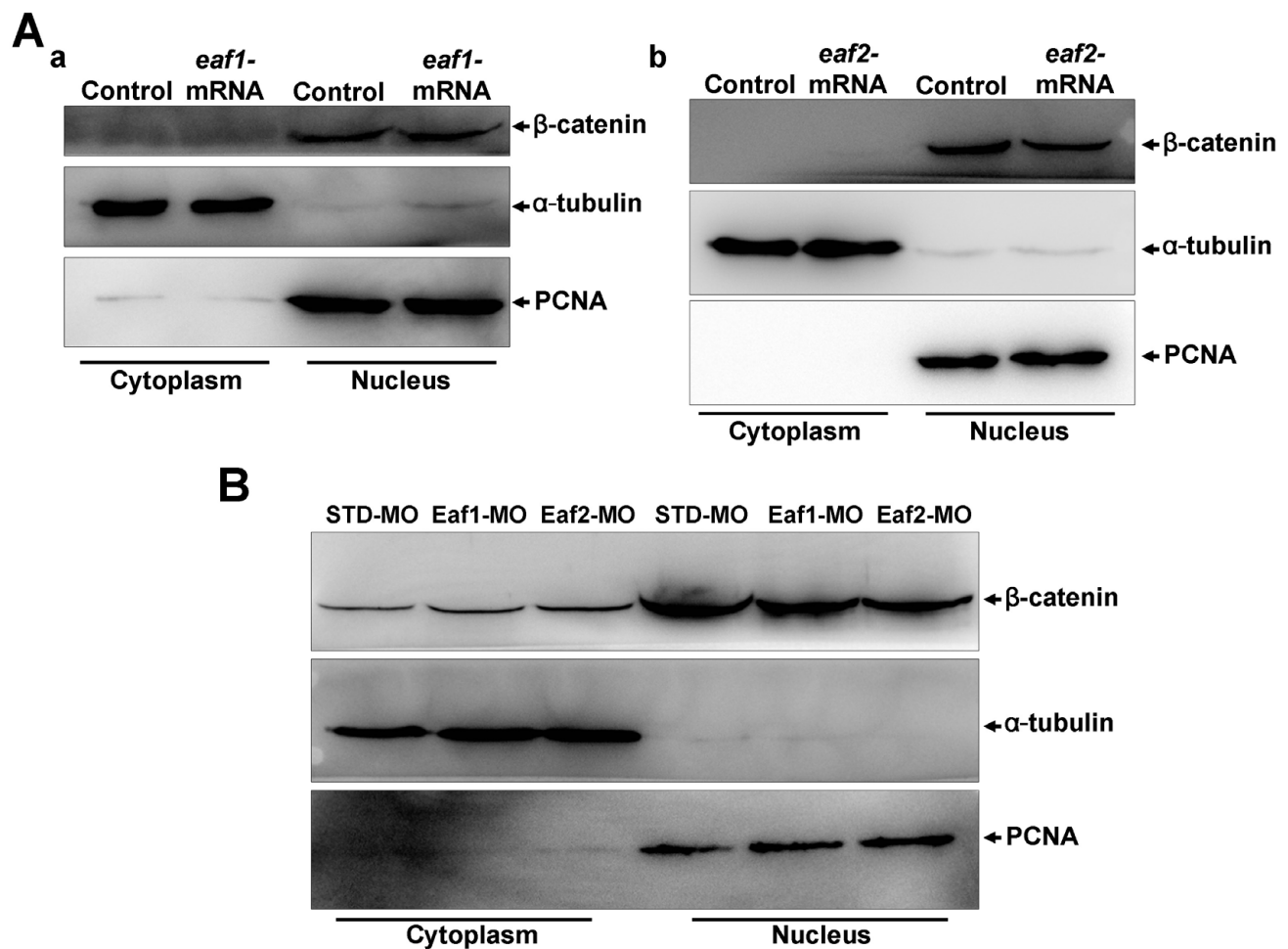
Fig. S10. Ectopic expression or knockdown of zebrafish *eaf1* or *eaf2* in embryos at the gastrula stage does not change cytoplasmic-nuclear translocation of β-**catenin.** (**A**) Ectopic expression of *eaf1* (a) or *eaf2* (b) did not change cytoplasmic-nuclear translocation of β-catenin. (**B**) Knockdown of *eaf1* or *eaf2* did not change cytoplasmic-nuclear translocation of β-catenin.
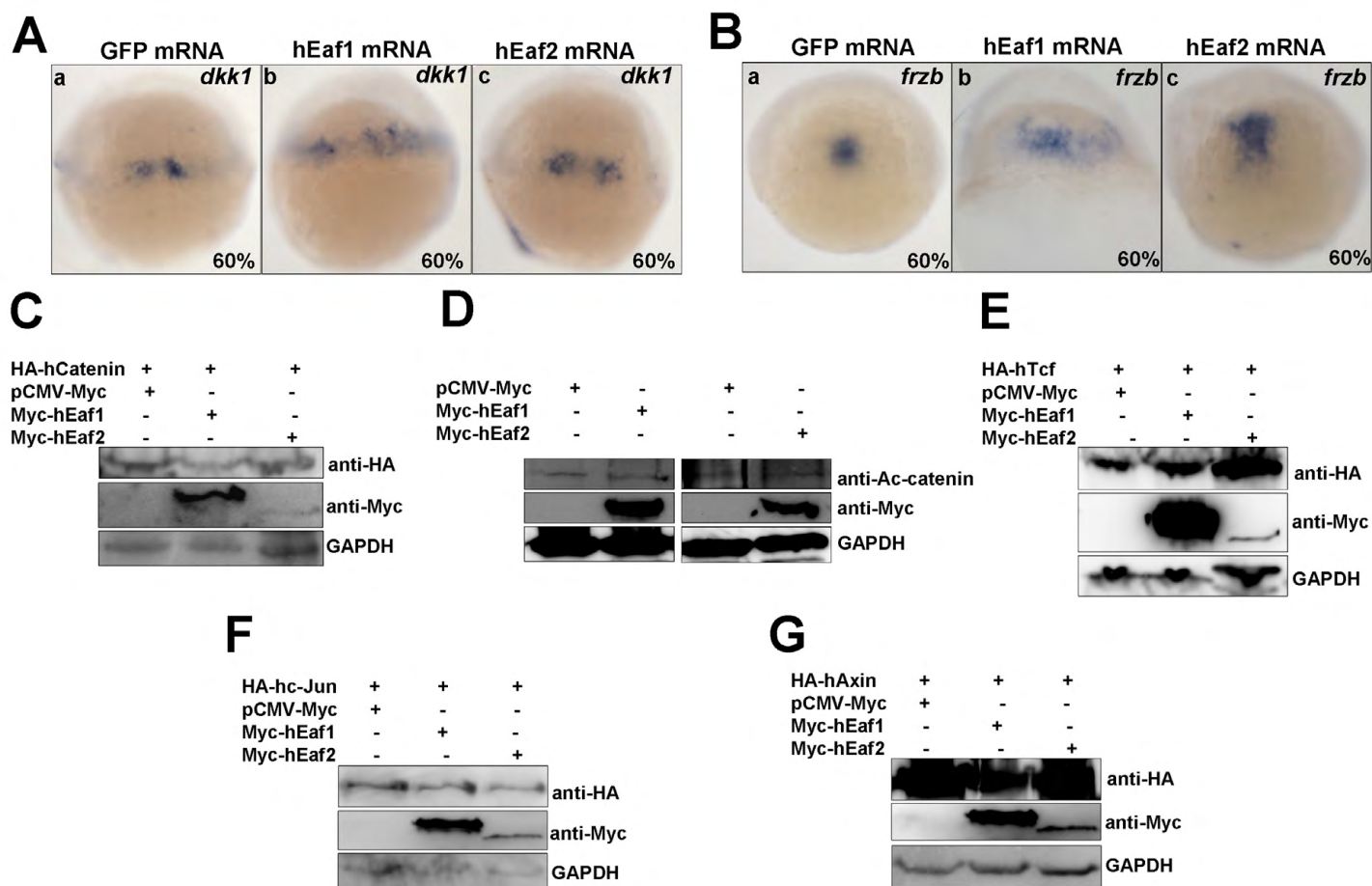
Fig. S11. Human Eaf function is evolutionary conserved in regulating Wnt/β-catenin signaling. (**A,B**) In zebrafish embryos injected with human *EAF1* or *EAF2* mRNA, *dkk1* (A) and *frzb* (B) displayed enhanced expression. (**C**) Overexpression of human *EAF1* or *EAF2* did not affect co-transfected human β-catenin stability. (**D**) Overexpression of human *EAF1* or *EAF2* did not change the endogenous active β-catenin protein level. (**E-G**) Ectopic expression of human *TCF4* (E), human *c-JUN* (F) and human *AXIN* (G) after co-transfection with human *EAF1* or *EAF2* into the HEK 293T cell line.

Table S1. Primers used for qRT-PCR, probe amplification, splice MO validation, cDNA and promoter cloning

Primer	Sequence (5′-3′)
sqtFq	GCTGGTGGTGGAAGTGAAC
sqtRq	CCTTCACACCGATAAGCGTTG
gscFq	GCAAGAGACGACACCGAAC
gscRq	TGAACCAAACCTCTACCTTCTC
18sFq	GAGAAACGGCTACCACATCC
18sRq	CACCAGACTTGCCCTCCAA
c-mycFq (human)	GGCGAACACACAACGTCTTGGA
c-mycRq (human)	CTTACGCACAAGAGTTCCGTAGC
18sFq (human)	TCAACTTTCGATGGTAGTCGCCGT
18sRq (human)	TCCTTGGATGTGGTAGCCGTTTCT
cdx4-probe-F	CGTCCATGAGGAACATACAGC
cdx4-probe-R	CAAGAGCCTCCAGCATTTCG
opl-probe-F	GGCGAAGTTACAGACAGA
opl-probe-R	GACATGACCGTATTGCTC
six3b-probe-F	TTTGGTCGTTGCCCGTAG
six3b-probe-R	CGTGATGCTGAAGCCTGT
eaf1-splicing-F	TGGTGAATTCAATGTGACGCGCAG
eaf1-splicing-R	GATTTGTTGGTGTGCGAATGTGGT
eaf2-splicing-F	AGAAGTGAGGCGTCTATTTCTGCC
eaf2-splicing-R	CTTGACCGCAATGTTGTTGCTGAG
zf-catenin2-F	ATATCTCTAGAAGGATTGACGCAACGATGGCTAG
zf-catenin2-myc-R	ATATCGCGGCCGCGTCCTTCGCTCAGCAGCTCTCTA-3
zf-catenin1-F	ATATCGAATTCGGTCTAGATGGCTACCCAGTCTGACTTGA

zf-catenin1-myc-R	ATATCGCGGCCGCTTACAGATCGGTGTCAAACCAGG
zf-c-jun-F	ATATCGAATTCGGTCTAGACCTTCTATGTCTACCAAGATGG
zf-c-jun-myc-R	ATATCGCGGCCGCGTCCTTCGGCTCTCCTCAGAAG
apc-sequencing-F	CTACCCAACTTTACCTATATCAG
apc-sequencing-R	GACTCTCAAAACTGTCAAGGG

Primers are zebrafish, except where marked for human.

Table S2. Morpholino sequences

Morpholino	Sequence (5′-3′)
Eaf1-ATG-MO (Eaf1-MO1)	GCGGCGGGTTCGAGCTGCCGTTCAT
Eaf2-ATG-MO (Eaf2-MO1)	ATGCTGTTCCATTCATTCTAATCCA
Eaf1-splice-MO (Eaf1-MO3)	GTCTCTCTTGATGGACTCACATCTG
Eaf2-splice-MO (Eaf2-MO3)	AAAAGATGCAACTTACATCGTACCG
β-catenin1-MO	CTGGGTAGCCATGATTTTCTCACAG
β-catenin2-MO	CCTTTAGCCTGAGCGACTTCCAAAC