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 (tcfl7ia – 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, maternal Wnt/β-catenin signaling is essential for the induction of sgt (nrd1), 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 ventro lateral 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 Fzrb 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. 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
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 (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 and epha 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 opr (cil1 – 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 Ambitcap SP6 High Yield Message Maker Kit (Epicerin 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, opr and cdx4 were amplified from cDNA pools using the appropriate sets of primers (supplementary material Table S1). Probes for thx6 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
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. S3-B-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. S3-E-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 sgt, 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. S4Bb). 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−/− × 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 experiments...
Eafs negatively regulate Wnt/β-catenin signaling.

Eafs 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; Muhr et al., 2001). 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 nonspecific 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 50% 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-catenin-1-MO, but not β-catenin-2-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 underlying eaf1 and eaf2 antagonism of Wnt/β-catenin signaling, we used TopFlash luciferase reporter assays to monitor the transcriptional activity of β-catenin in

Embryos injected with 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).

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. S5Dm), 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. S6Ae,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.
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.

**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.
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
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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
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 or Eaf2. 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. 6. Eaf1 and Eaf2 function as novel nuclear Wnt signaling components.](image-url)
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 transcriptional activity 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 shown 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.

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**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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