Induction and patterning of trunk and tail neural ectoderm by the homeobox gene *eve1* in zebrafish embryos

Carlos Cruz[a], Shingo Maegawa[b], Eric S. Weinberg[b], Stephen W. Wilson[c], Igor B. Dawid[d,1], and Tetsuhiro Kudoh[a,1]

[a]School of Biosciences, University of Exeter, Devon EX4 4SP, United Kingdom; [b]Department of Biology, University of Pennsylvania, Philadelphia, PA 19104; [c]Department of Cell and Developmental Biology, University College London, London WC1E 6BT, United Kingdom; and [d]Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892

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In vertebrates, Evx homeodomain transcription factor-encoding genes are expressed in the posterior region during embryonic development, and overexpression experiments have revealed roles in tail development in fish and frogs. We analyzed the molecular mechanisms of posterior neural development and axis formation regulated by *eve1*. We show that *eve1* is involved in establishing trunk and tail neural ectoderm by two independent mechanisms: First, *eve1* posteriorizes neural ectoderm via induction of *aldh1a2*, which encodes an enzyme that synthesizes retinoic acid; second, *eve1* is involved in neural induction in the posterior ectoderm by attenuating BMP expression. Further, *eve1* can restore trunk neural tube formation in the organizer-deficient *ichabod*$^{-/-}$ mutant. We conclude that *eve1* is crucial for the organization of the antero-posterior and dorso-ventral axis in the gastrula ectoderm and also has trunk- and tail-promoting activity.

bone morphogenic protein | neural induction | organizer | posterior development | retinoic acid

The molecular mechanisms of neural induction and patterning in chordate embryos have been extensively studied in animals such as ascidians, amphibians, fish, chick, and mouse (1, 2). Initial analyses in amphibians revealed that the dorsal organizer (Spemann's organizer) induces neural (CNS) fates in dorsal ectoderm, and subsequently vegetal marginal signals posteriorize proximal neural ectoderm to induce trunk and tail neural cell fates such as spinal cord and caudal hindbrain, whereas distant animal pole cells give rise to rostral neural tissues including the forebrain, midbrain, and part of the hindbrain (3). Molecular analyses of organizer activity have uncovered multiple molecules crucial for neural induction, including the secreted bone morphogenic protein (BMP) antagonists Chordin, Noggin, and Follistatin, leading to the conclusion that BMP inhibition is crucial for neural induction (1, 2). In addition to the BMP antagonists, FGF has an important role in neural induction in many species (4–8).

Concomitant with and subsequent to neural induction, neural ectoderm is posteriorized by the activity of several factors, among them FGF (5, 7, 9–11), Wnt (11–14). and retinoic acid (RA) (11, 15–17). RA is essential for posterior neural development in vertebrates, being required for the specification of the future hindbrain and anterior spinal cord (18, 19). In zebrafish FGF and Wnt signaling posteriorize neural ectoderm by suppressing anterior-specific gene expression independently of RA and inducing posterior genes in an RA-dependent process (11).

Some of the transcription factors acting downstream of posteriorizing signals are known and include Homeodomain proteins of the Hox cluster (6, 20), Cdx (21, 22), and Evx (7, 23) families. In the zebrafish gastrula, posterior neural ectoderm and mesoderm are marked by the expression of *eve1,* a member of the *eve/evx* family of homeobox genes that encode transcriptional repressors. *Evx* genes have been implicated in a conserved role in posterior body patterning in a variety of species, including fly, mouse, worm, frog, and zebrafish (24–26). In zebrafish, overexpression of *eve1* disrupts antero-posterior (A/P) patterning in a concentration-dependent manner, leading to loss of head structures and tail duplications and to mispatterning of posterior tissue (25). At the gastrula stage, *eve1* expression is restricted to the ventral side and is maintained by BMP signaling, a key ventralizing molecule. *Eve1* has been regarded as a ventral marker gene with a presumed role in tail development. However, *eve1* expression begins at blastula stage at around 30% epiboly, when it covers most of the margin with the exception of the presumptive organizer (27), suggesting a potentially wider role for *eve1* in posterior development. Thus it is not clear if *eve1* is involved in trunk development in addition to its accepted role in tail development. In addition, little is known about the mechanism of *eve1* function, and no loss-of-function data in fish have been reported so far. Using loss- and gain-of-function strategies, we show here that *eve1* regulates trunk and tail development. We find that *eve1* affects the formation of trunk and tail neural ectoderm via two molecular mechanisms: induction of the neural ectoderm in both trunk and tail regions at the gastrula stage, at least partly by titration of BMP levels; and posteriorization of neural ectoderm via an RA signal. Furthermore our data provide evidence that *eve1* exerts its organizing activity as a transcriptional repressor.

Results

Overexpression of *Eve1* Causes Anterior Truncation, Induces Posterior Neural Markers, and Suppresses Markers for Anterior Neural and Nonneural Tissues. To determine the role of *eve1* in A/P patterning, we overexpressed *eve1* in vivo and analyzed the expression of *otx2*, an anterior neural marker, and *hoxb1b*, a marker for prospective posterior (trunk and tail) neural tissue (7, 20, 28). Phenotypic analysis confirmed previous results (25, 29) such as truncation of head structures (75%, $n = 32$) (Fig. 1*B*), and some embryos showed more severe effects with loss of head and trunk (13%, $n = 32$) (Fig. 1*C*). The only remaining anteriorly positioned structure was the heart, which continued to beat. Consistent with the lack of anterior structures, *otx2* is suppressed in embryos injected with eve1 mRNA (94%, $n = 17$) (Fig. 1*E*), whereas *hoxb1b* expression is partially (15%) or circumferentially (85%) expanded ($n = 20$) (Fig. 1*G*).

Surprisingly, in *eve1*-injected embryos *hoxb1b* expression expanded to include the prospective epidermal domain, raising the possibility that *eve1* may have a role in neural induction in addition to its role in A/P patterning. To test this notion, we analyzed the expression of three additional neural markers, *sox3*, *zic2a*, and

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[1]To whom correspondence may be addressed. E-mail: t.kudoh@exeter.ac.uk or idawid@mail.nih.gov.

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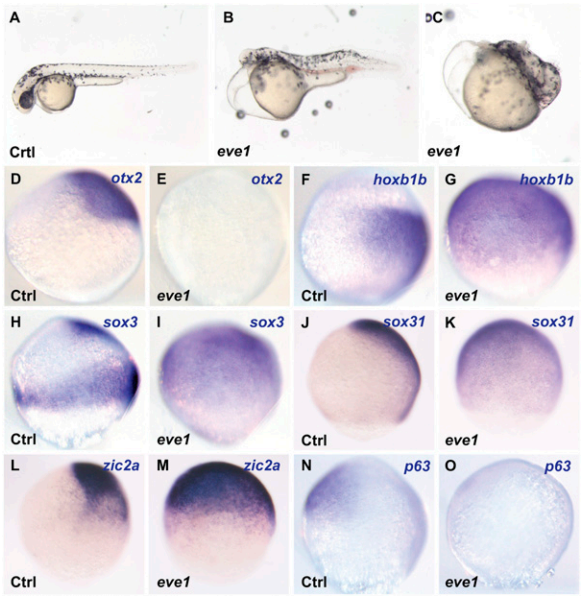

Fig. 1. *Eve1* overexpression causes anterior truncation, suppression of anterior markers, and induction of posterior markers. Zebrafish embryos were injected with *eve1* mRNA (as indicated at the bottom left corner of each panel; Ctrl, uninjected controls). (*A–C*) *Eve1* mRNA injected embryos at 48 hpf showing anterior truncation and progressive loss of posterior patterning. (*D–O*) In situ hybridization of control and *eve1* mRNA-injected embryos at 80% epiboly (lateral views, dorsal to the right, where discernible). Genes analyzed are indicated in the top right corner. Expression of the anterior gene *otx2* and epidermal gene *p63* was suppressed (*D, E*; *N, O*), whereas the expression of *hoxb1b* was expanded by *eve1* overexpression (*F, G*). Expansion also was observed for *sox3, zic2a*, and *sox31* (*H–M*).

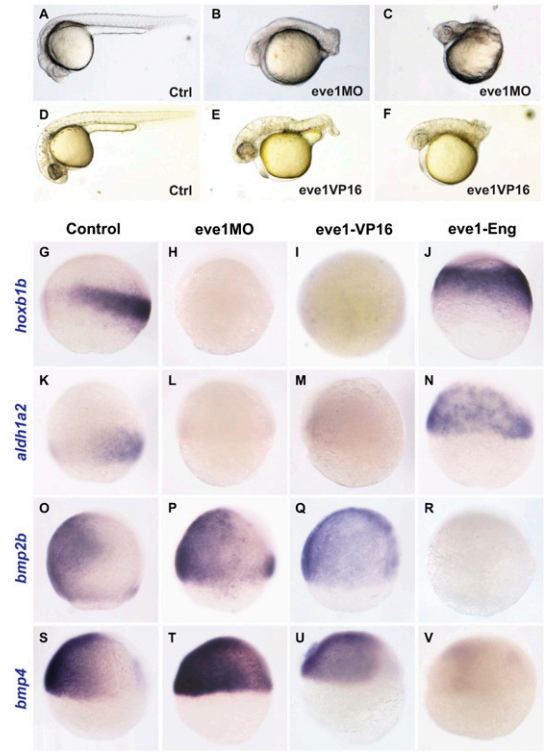

Fig. 2. *Eve1* depletion suppresses trunk and tail development, and *eve1* acts as a repressor. Zebrafish embryos were injected with eve1MO (*B, C, H, L, P, T*) or with eve1-VP16 mRNA (*E, F, I, M, Q, U*) as shown at bottom right of panels *B, C, E*, and *F* and at the top of the columns for the remainder. Embryos at 24 hpf (*A–C*) and at 28 hpf (*D–F*), show variable loss of trunk and tail tissue. (*G–V*) In situ staining of embryos at 70–80% epiboly (*G–R*) and 60% epiboly (*S–V*): lateral views, dorsal to the right (where discernible), with probes shown at the left of the rows. *Hoxb1b* and *aldh1a2* are suppressed by eve1MO (*H* and *L*) and eve1-VP16 (*I* and *M*, compare with *G* and *K*), and expression of both genes is expanded in embryos injected with eve1-Eng (*J* and *N*). Conversely, *bmp2b* and *bmp4* expression domains are expanded in embryos injected with eve1MO (*P* and *T*) and eve1-VP16 (*Q* and *U*, compare with *O* and *S*), whereas eve1-Eng suppresses expression of both BMPs (*R* and *V*).

sox31, and the epidermal marker, *p63*. In embryos injected with *eve1* mRNA, the *sox3*- (95%, $n = 21$), *zic2a*- (95%, $n = 20$), and *sox31*- (83%, $n = 18$) positive domain covers most of the embryo, including the prospective epidermal domain (Fig. 1 *I*, *K*, and *M*), with the concomitant suppression of *p63* expression (100%, $n = 24$) (Fig. 1*O*). Together, these results suggest that *eve1* acts both as a posteriorizing and a posterior neural-promoting factor.

Zebrafish *Eve1* Functions as a Repressor in Posterior Neural Development. To carry out loss-of-function analyses, we first used an *eve1* antisense morpholino (MO) directed at the intron1/exon2 acceptor splice site (eve1MO) (*Materials and Methods*). This MO led to the reduction of mature mRNA and the appearance of an alternatively spliced form of mRNA in the embryo (Fig. S1*A*). The phenotypes in embryos injected with eve1MO complement those of *eve1* overexpression, namely a loss of posterior structures with largely unaffected head structures (Fig. 2 *B* and *C*). In the most severe phenotype, most of the trunk and tail tissue is absent (Fig. 2*C* and Fig. S1*B*). Because *eve1* is thought to function as a transcriptional repressor (30–32), we reasoned that fusion of the *eve1* homeodomain (DNA binding) to the activator domain of the viral protein VP16 would generate an antimorphic construct (*Materials and Methods*). Similar to the effect of eve1MO, injection of eve-VP16 led to a variable reduction of the posterior axis (71%, $n = 28$) (Fig. 2 *E* and *F*).

We next examined the expression of *otx2* and *hoxb1b* in eve1MO-injected embryos. Consistent with the gain-of-function analysis, *hoxb1b* expression was strongly suppressed (96%, $n = 28$) (Fig. 2*H*), but we found no noticeable difference in *otx2* expression. We further examined the expression of *aldh1a2* (formerly *raldh2*), which codes for an RA synthesizing enzyme expressed in posterior paraxial mesoderm (33), as well as *meis3*, another posterior-specific neural gene (34). The expression of *aldh1a2* was much reduced (71%) or absent (29%) in eve1MO-injected embryos ($n = 24$) (Fig. 2*L*), as was the expression of *meis3* (Fig. S2*E*). To test the specificity of the eve1MO, we coinjected *eve1* mRNA and found that the expression of both *aldh1a2* (95%, $n = 21$) and *hoxb1b* (96%, $n = 28$) was restored and slightly expanded as compared with uninjected embryos (Fig. S1*C* 1–6). Furthermore, we found that epiboly defects caused by eve1MO were rescued by coinjection of *eve1* mRNA (Fig. S1*D*). These results indicate that the MO is specific for *eve1*. In further analysis of *eve1* loss of function, we found that the expression of *hoxb1b* (75%, $n = 16$) and *aldh1a2* (88%, $n = 16$) was suppressed in embryos injected with eve1-VP16 (Fig. 2 *I* and *M*).

Eve1-VP16 and eve1MO show a similar phenotype that is complementary to that of *eve1* overexpression, suggesting that *eve1* exerts its posteriorizing influence as a repressor. To explore this possibility, we fused the *eve1* homeodomain to the repressor domain of the *Drosophila* Engrailed (Eng) protein (*Materials and Methods*) (32, 35). Injection of eve1-Eng led to expansion of *hoxb1b* (70%, $n = 20$) and *aldh1a2* (56%, $n = 16$) expression (Fig. 2 *J* and *N*), phenotypes complementary to those elicited by eve1MO and eve1-VP16. Because the results of overexpression suggested that *eve1* might have a role in neural induction, we also looked at *bmp2b* and *bmp4* expression under conditions of *eve1* gain and loss of function. Injection of eve1MO expanded both *bmp2b* (83%, $n = 18$) and *bmp4* (63%, $n = 16$) expression (Fig. 2 P and *T*), as did injection of eve1-VP16 (76%, $n = 17$, and 70%, $n = 23$, respectively) (Fig. 2 *Q*

and *U*), whereas injection of eve1-Eng suppressed both *bmp2b* (74%, $n = 19$) (Fig. 2*R*) and *bmp4* (90%, $n = 20$) (Fig. 2*V*) expression. Together these data suggest that *eve1* acts as a transcriptional repressor in promoting posterior neural development.

***Eve1* Induces *Hoxb1b* Expression via RA Signaling.** Because *eve1* induces *hoxb1b* expression (Fig. 1*G*), and eve1MO injection led to loss of *aldh1a2* expression (Fig. 2*L*), we investigated whether this effect is mediated by the RA pathway by injecting *eve1* and *cyp26a1* mRNAs in different combinations and examining *hoxb1b* as well as *otx2* expression. Cyp26a1 is an RA-degrading enzyme, and overexpression of *cyp26a1* allows examination of *eve1* function when RA signaling is suppressed (11). *Otx2* expression was suppressed by injection of *eve1* mRNA alone, whereas *hoxb1b* expression was expanded (Fig. 3 *B* and *F*). However, when *eve1* and *cyp26a1* mRNAs were coinjected, both *otx2* and *hoxb1b* were suppressed (Fig. 3 *C* and *G*), suggesting that suppression of *otx2* by *eve1* is RA-independent, but expansion of *hoxb1b* is dependent on RA. As previously reported (11), *cyp26a1* injection alone had no significant effect on *otx2* expression (Fig. 3*D*) but suppressed *hoxb1b* (Fig. 3*H*). To examine further *eve1* function upstream of RA in *hoxb1b* induction, we injected *eve1* mRNA and analyzed the expression of *aldh1a2* and *cyp26a1*. *Eve1* causes the expansion of the *aldh1a2* expression domain and suppression of *cyp26a1* in anterior neural ectoderm (Fig. 3 *J* and *L*). Given that RA is a long-range signaling molecule (36), the induction of *aldh1a2* and suppression of *cyp26a1* may provide the mechanism of *hoxb1b* induction in the animal pole by overexpression of *eve1*.

To complement the *cyp26a1* and eve1MO data, we sought further confirmation that *eve1* functions upstream of *aldh1a2* and, presumably, of RA in inducing *hoxb1b*. Injection of an antisense MO directed against the *aldh1a2* gene (aldMO; *Materials and Methods*) alone resulted in a marked reduction of *hoxb1b* expression (95%, $n = 22$) (Fig. 3*N*), and this inhibition could not be rescued by coinjection of *eve1* mRNA (100%, $n = 26$) (Fig. 3*O*). A similar result was obtained with another RA-responsive gene, *meis3* (11) (Fig. S2 *A–D*). Further, there is synergism between eve1MO (2 ng/nL) and aldMO (low) action in the regulation of *hoxb1b* expression. Low concentrations of either MO alone led to a partial and variable reduction of the *hoxb1b* signal, whereas coinjection of both MOs at the same low doses led to the complete abolition of *hoxb1b* expression in most injected embryos (72%, $n = 25$); in the remainder, *hoxb1b* expression was variably reduced (Fig. 3 *P–S*). Again, we obtained similar results with *meis3* (Fig. S2 *E–H*). These data provide strong evidence that *eve1* functions upstream of RA in positively regulating the expression of *hoxb1b* and *meis3* and possibly of other RA-responsive genes as well.

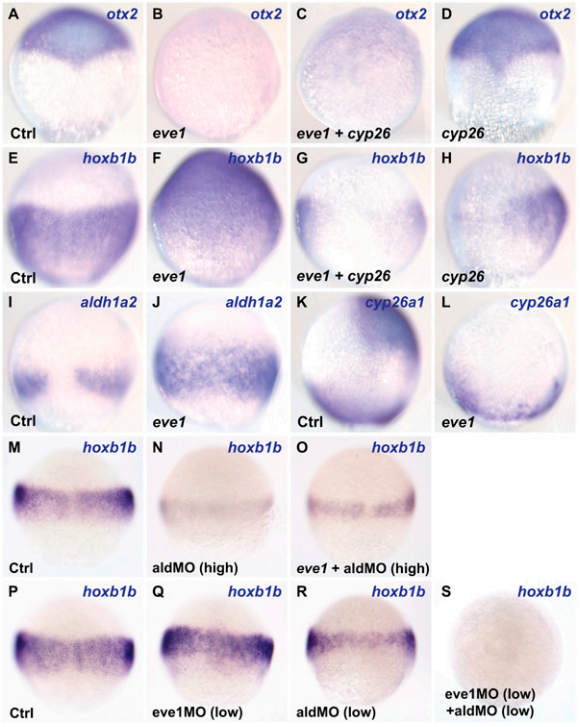

Fig. 3. *Eve1* induces *hoxb1b* expression via an *RA* signal. (*A–J* and *M–S*) Dorsal and (*K* and *L*) lateral views (where discernible, dorsal to the right) of zebrafish embryos fixed for in situ staining at 80% epiboly (*A–L*) and 60% epiboly (*M–S*) Injections are indicated at the bottom left of each panel, and genes analyzed are given at the top right. (*A–D*) Suppression of *otx2* by *eve1* does not depend on RA because it resists overexpression of the RA-metabolizing enzyme Cyp26a1. (*E–H*) *Eve1*-mediated induction of *hoxb1b* does not occur when *eve1* and *cyp26a1* mRNAs are coinjected (*G*), and *cyp26a1* injection alone suppresses *hoxb1b* expression (*H*; only one of two cells was injected in this embryo). (*I* and *J*) *Eve1* induces *aldh1a2* expression. (*K* and *L*) Anterior expression of *cyp26a1* is suppressed by *eve1* but remains unaffected at the margin. (*M–O*) Injection of high concentrations of aldMO and *eve1* mRNA (*Materials and Methods*). *Eve1* cannot rescue *hoxb1b* expression in aldMO-injected embryos (*O*). (*P–S*) Injection of low concentrations of eve1MO (2 ng/nL) and aldMO showed synergism in the suppression of *hoxb1b*.

***Eve1* Promotes Neural Ectoderm by Antagonizing BMP Expression.** Overexpression of *eve1* can suppress epidermal and induce neural marker genes (Fig. 1), suggesting that *eve1* and BMP signaling have antagonistic roles in neural versus epidermal specification in the ectoderm. We used *eve1* and *bmp2b* mRNA injection to determine whether BMP signaling can suppress *eve1*-mediated induction of the neural markers *hoxb1b* and *sox3*. When *eve1* and *bmp2b* mRNAs are coinjected, both *sox3* (100%, $n = 24$) and *hoxb1b* (96%, $n = 25$) expression is suppressed compared with control and *eve1*-injected embryos (Fig. 4 *A–F*). Because eve1MO injection leads to the variable expansion of both *bmp2b* and *bmp4* (Fig. 2 *P* and *T*), we tested for possible inhibition of *bmp4* and *bmp2b* expression in embryos overexpressing *eve1* and found that *eve1* mRNA injection inhibited *bmp4* (88%, $n = 17$) and *bmp2b* (89%, $n = 19$) expression (Fig. 4 *H* and *K*). Thus *eve1* can regulate expression levels of BMP in gastrula embryos, suggesting that BMP signaling occurs downstream of *eve1*. This suggestion is supported by the fact that injection of *bmp2b* mRNA strongly induced *bmp4* expression in the presence of exogenous *eve1* (100%, $n = 20$) (Fig. 4*I*). Likewise, injection of a low concentration of *bmp2b* RNA (7 pg/nL) together with *eve1* abolished the ability of *eve1* to induce neural markers or suppress *bmp4* (Fig. S3).

To explore further the antagonistic nature of *eve1* and BMP signaling in ectodermal fate specification, we examined their combined effects on the expression of the neural marker *sox3* and the epidermal marker *foxi.1*. At low (10 pg/nL) concentrations, neither *eve1* mRNA (100%, $n = 25$) nor bmp2bMO (100%, $n = 26$) can induce *sox3* (Fig. 4 *M* and *N*) or suppress *foxi.1* (Fig. 4 *Q* and *R*). However, when *eve1* and bmp2bMO were coinjected at low concentrations, *sox3* was expanded (95%, $n = 19$) (Fig. 4*O*), whereas *foxi.1* was suppressed (94%, $n = 17$) (Fig. 4*S*). These results support the view that *eve1* has a role in posterior neural induction via antagonism of BMP signaling.

***Eve1* Rescues Posterior Dorsal Axis and Expression of *Hoxb1b* in *Ichabod*$^{-/-}$ Mutants.** *Ichabod*$^{-/-}$ (*ich*$^{-/-}$) mutants have reduced expression of *beta-catenin 2* that leads to loss of the organizer, ventralization, and loss of head and trunk structures (37–39) (Fig. 5*A*). In such embryos, BMP expression is expanded dorsally, and this expansion is thought to account for the observed ventralization. Because *eve1* antagonizes BMP signaling and has a role in posteriorization, we injected *ich*$^{-/-}$ embryos with *eve1* mRNA to test whether *eve1* could rescue trunk and tail development. *Eve1*-injected *ich*$^{-/-}$ embryos at 24 h postfertilization (hpf) showed a

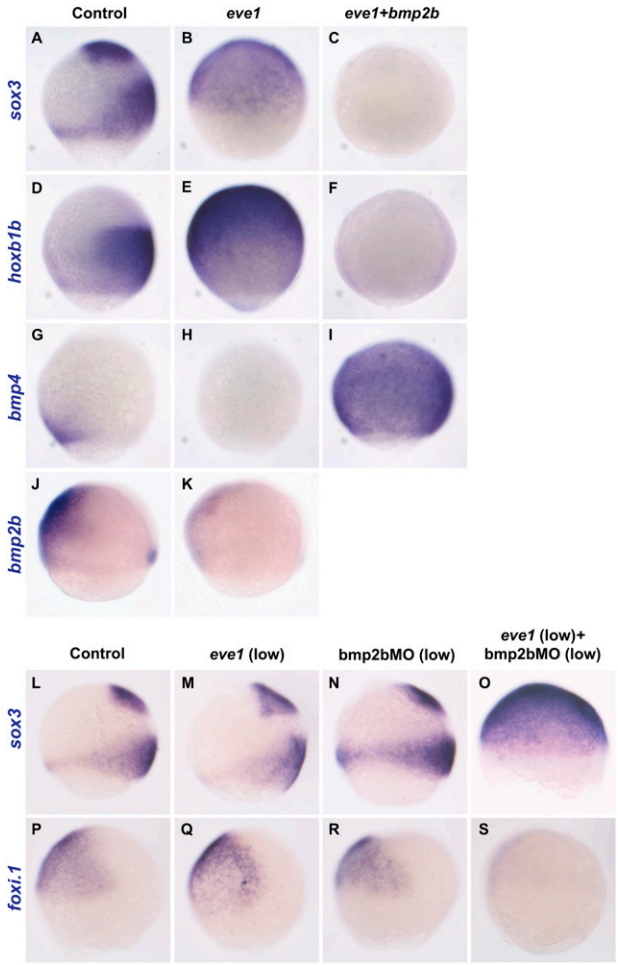

Fig. 4. Interactions between *eve1* and BMP. Lateral views (where discernible, dorsal to the right) of zebrafish embryos at 70–80% epiboly (*A–S*). Embryos were injected at the one-cell stage (*A*–K, *M*, and *Q*) or at the one– to four-cell stage (*N* and *R*). For coinjection of *eve1* mRNA and bmp2bMO, embryos first were injected with *eve1* at the one-cell stage and then were injected with bmp2bMO at the four- to eight-cell stage (*O* and *S*). Genes analyzed are indicated at the left of the rows; injections are indicated at the top of columns. *Bmp2b* suppresses neural markers *sox3* and *hoxb1b* even in the presence of *eve1* (*A–F*). *Bmp4* expression is suppressed by *eve1 (H)* but is ubiquitously induced by coinjection of *bmp2b* mRNA (*I*, compare to G). *Bmp2b* expression also is suppressed by *eve1* (*J, K*). *Eve1* and bmp2bMO synergize in ectodermal fate specification (*L–S*). Low levels of *eve1* mRNA (10 pg/nL) or bmp2bMO (100 pg/nL) injected individually do not affect *sox3* or *foxi.1* expression (*M*, *N*, *Q*, and *R*), but coinjection at the same concentrations induced *sox3* (*O*) and suppressed *foxi.1* (*S*).

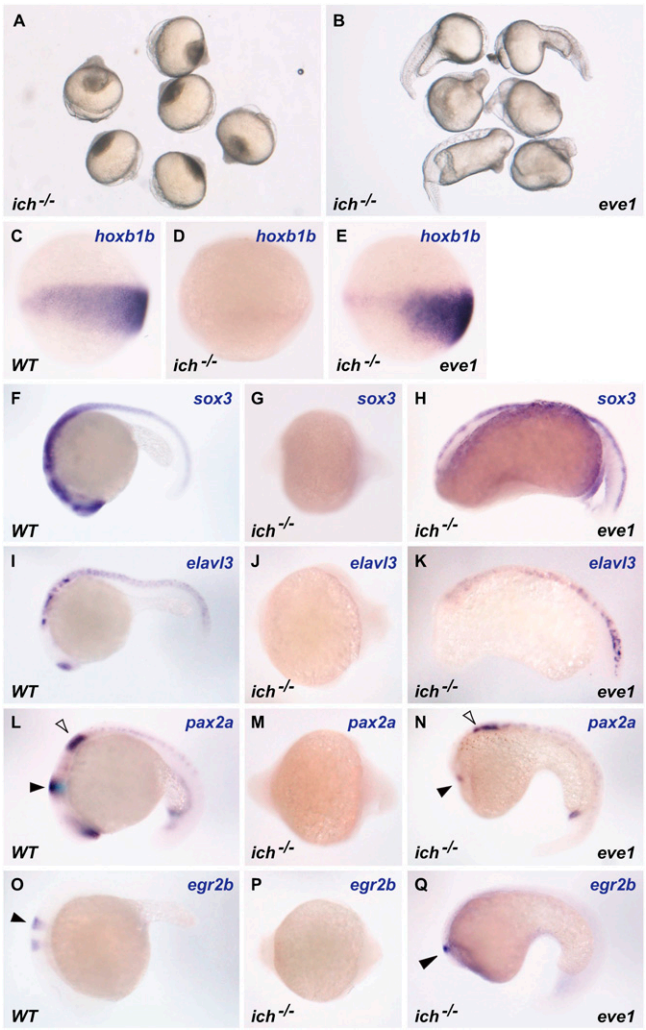

Fig. 5. *Eve1* rescues posterior neural development in $ich^{-/-}$ mutants. Homozygous $ich^{-/-}$ embryos were injected with *eve1* mRNA. Genotype is indicated at the bottom left of each panel, injections at the bottom right, and in situ probes at the top right. Uninjected $ich^{-/-}$ embryos at 24 hpf (*A*). *Eve1* mRNA injection leads to varying levels of rescue of posterior dorsal axis (*C–E*). Embryos stained for *hoxb1b* at 80% epiboly, presumed lateral view, dorsal to the right. Expression of *hoxb1b* is absent in uninjected $ich^{-/-}$ embryos (*C, D*) but is rescued by injection of *eve1* mRNA (*E*). In situ hybridization of wild-type and $ich^{-/-}$ embryos at 24 hpf (anterior to the left) (*F–Q*). Neural gene expression and posterior dorsal axis formation was partially rescued by the injection of *eve1* mRNA. Rescue of *pax2a* appears to extend to the midbrain–hindbrain boundary (*L–N*, arrowheads), whereas *egr2b* expression appears to extend to rhombomere 5 (*O–Q*, arrowheads).

partial rescue of the posterior dorsal axis in the trunk and tail (100%, $n = 38$) (Fig. 5*B*) when compared with uninjected embryos (100%, $n = 28$) (Fig. 5*A*). Expression of *hoxb1b* that is absent in $ich^{-/-}$ mutants at gastrula (Fig. 5*D*) (39) is restored in *eve1*-injected embryos (Fig. 5*E*). Expression of the neural marker *sox3* initially occurs in the trunk/tail domain of gastrula-stage $ich^{-/-}$ embryos and is reduced gradually and becomes faint by 24 hpf (Fig. 5*G*), but *sox3* expression is retained in the rescued trunk and tail neural tube in *eve1*-injected $ich^{-/-}$ embryos (Fig. 5*H*). Similarly, the neural expression domains of *elavl3* (formerly *huC*), *pax2a*, and *egr2b* (formerly *krox20*), which are variably reduced or lost in $ich^{-/-}$ embryos 24 hpf (Fig. 5 *J*, *M*, and *P*), are partially restored in the trunk and tail after *eve1* injection (penetrance = 100%) (Fig. 5 *K*, *N*, and *Q*). These data indicate that *eve1* can induce and maintain some posterior dorsal structures as well as neural gene expression in the trunk and tail of organizer-defective $ich^{-/-}$ embryos.

Discussion

***Eve1* Promotes Posterior Development as a Transcriptional Repressor.** The Evx proteins have been shown previously to function as transcriptional repressors in *Drosophila* development (31, 32, 40, 41). Our data suggest that Eve1 also functions as a transcriptional repressor in vertebrates in promoting posterior development and that eve1-VP16 acts as a dominant-negative form. Overexpression of wild-type *eve1* and eve1-VP16 results in opposite phenotypes: *Eve1* suppressed head formation, whereas eve1-VP16 suppressed trunk and tail formation, with consistent effects on the expression of marker genes. Further, inhibition of *eve1* expression by an MO

phenocopied the eve1-VP16 phenotype, whereas eve1-Eng phenocopied the effects of *eve1* overexpression on posterior neural markers and BMP expression. As a result, it is likely that the upregulation of marker genes by *eve1* is indirect. In neural induction and dorsalization, suppression of BMP by *eve1* could explain the induction of neural-specific genes, whereas in posteriorization *eve1* may well repress an as yet unidentified repressor of *aldh1a2* (Fig. 6)

***Eve1* Induces Posterior Cell Fates via Retinoic Acid.** Through gain- and loss-of-function analyses of *eve1*, we explored the mechanisms of *eve1* function in zebrafish trunk and tail development. Overexpression of *eve1* suppressed head structures and in the trunk and tail expanded neural and suppressed epidermal cell fates. These data indicate a role for *eve1* in both posteriorization and neural induction (Fig. 6). The regulation of RA levels via induction of *aldh1a2* and suppression of *cyp26a1* is necessary and sufficient for the induction of the posterior gene *hoxb1b* by *eve1*. This conclusion is supported by the observations that eve1MO inhibits *aldh1a2* expression and that *eve1* induction of *hoxb1b* and *meis3* is mediated by *aldh1a2*, because neither gene could be induced by *eve1* in aldMO-injected embryos. *Eve1* suppresses the anterior gene *otx2* via an RA-independent route, suggesting that there are two separate mechanisms for *eve1*-mediated posteriorization: RA-dependent posterior induction and RA-independent anterior suppression. This distinction may assist in creating a border between anterior (RA-negative) and posterior (RA-positive) gene-expression domains. Analogous separable mechanisms already have been observed for two other posteriorizing factors, FGF (9–11) and Wnt (13, 14, 42). Similar to the situation after reduction of RA signaling (11), but unlike the effect of FGF and Wnt (11), no posterior expansion of anterior gene expression was seen in *eve1* morphants, suggesting that suppression of anterior genes may not be a primary role of *eve1*. Because suppression of RA alone does not expand *otx2*, these results further support the idea that *eve1* posteriorizes embryos via the RA pathway. We have shown that otherwise *eve1* functions in a similar manner to FGF, RA, and Wnt posterior signaling, and, because *eve1* is induced by FGF (11, 23), it is tempting to suggest that *eve1* acts downstream of FGF in mediating posteriorization signals.

A Role for *eve1* in Neural Induction. A surprising finding was a role for *eve1* in the induction of posterior neural markers. In embryos where *eve1* is overexpressed, the expression of *sox3, sox31*, and other neural markers (Fig. 1) is expanded through the entire ectoderm, including the animal pole and presumptive epidermis. In these embryos the epidermal marker *p63* is suppressed, suggesting that prospective epidermal tissue has been respecified as neural. This conclusion is supported by the finding that *eve1* is necessary for the expression of *hoxb1b* (Fig. 2) and *meis3* (Fig. S2). In addition, *eve1* suppresses BMP expression in the gastrula embryo, but *eve1* cannot induce the expression of either *sox3* or *hoxb1b* in the presence of BMP. Thus it appears that Eve1 does not antagonize BMP signaling but rather suppresses BMP expression. As a consequence, a synergistic relationship exists between *eve1* and Bmp2bMO in the induction of *sox3* and suppression of *foxi.1*, a marker for epidermal tissue (Fig. 4). Together these data suggest

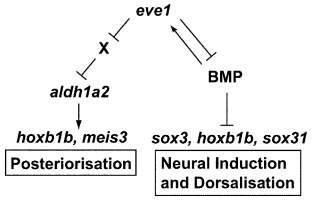

Fig. 6. Role of *eve1* in posteriorization and neural induction. See text for discussion.

that *eve1* enhances neural induction by reducing the expression of BMP in the gastrula ectoderm (Fig. 6).

Further evidence for *eve1*-mediated neural induction and maintenance comes from the experiment using ventralized *ich*$^{-/-}$ embryos. In *ich*$^{-/-}$ embryos, the expression of *hoxb1b*, *elavl3*, *pax2a*, and *egr2b* is low or absent, *sox3* is expressed only weakly, and neural tissue is greatly reduced at 24 hpf. Injection of *eve1* mRNA into these mutant embryos rescued *hoxb1b* expression and partially restored the expression of *elavl3, pax2*, and *egr2b* with a penetrance of 100%. Likewise, overexpression of *eve1* restored a posterior dorsal axis in *ich*$^{-/-}$ embryos. We suggest that *eve1* elicits these effects at least partially by a reduction of BMP expression, thereby substituting in the posterior domain for the absence of organizer-derived BMP antagonists.

Taken together the data suggest that *eve1* has dorsalizing activity (including neural induction) via regulation of BMP expression in the gastrula ectoderm. Many genes that regulate BMP expression and signaling along the dorso-ventral axis have been reported (for review, see ref. 43). For example, positive regulators of BMP, such as Bmp2b and Bmp4 in zebrafish (44) and ADMP (45) in *Xenopus*, are expressed in the organizer and may contribute to fine tuning of BMP expression and signaling. Besides secreted molecules, many transcription factors also have been shown to suppress BMP expression and to dorsalize the embryo [e.g., *hex* (46), *iro3* (47)]. Here, we propose to add *eve1* as another regulator of BMP activity that is unique in the sense that *eve1* expression is maintained by the BMP signal in the ventral side and in turn limits BMP expression (negative feedback). The variety of mechanisms regulating BMP expression levels indicates that precise control of the timing and level of BMP signaling is crucial in regulating neural versus non-neural patterning, A/P patterning, cell migration, and some aspects of gastrulation.

***Eve1* as an Effector of the Posterior Organizer.** *Eve1* has been thought to play an important role in tail development, because overexpression of *eve1* induces ectopic tail structures (25), and induction of ectopic tails by Wnt, BMP, and Nodal induces *eve1* expression (48). Although *eve1* is expressed in the prospective tail region only in the late gastrula, *eve1* expression is much wider in the blastula and early gastrula, being expressed in prospective trunk mesoderm and neural ectoderm at that stage (7, 29). *Eve1* is positively regulated by FGF (7, 23) and Wnt (49, 50), two signaling pathways that are critical for induction of both trunk and tail structures. Furthermore, it has been proposed that in both *Xenopus* and zebrafish tail formation is a continuation of trunk formation (7, 51) and that both occur as interactions between dorsal and ventral cells. Considering these ideas and our current data, we propose that *eve1* acts as a posterior organizer in regulating posterior specificity as well as dorso-ventral specificity for trunk and tail tissue. *Eve1* may function as a posterior dorsal gene in the sense that it induces caudal neural tissue. The contrasting function of *eve1* might be understood in the light of the observation that it represses BMP but enhances RA (through *aldh1a2*). Thus *eve1* would be required for posterior development (RA, and possibly other functions) but would limit the ventralizing action of BMP to facilitate formation of caudal neural tissue.

Materials and Methods

RNA Probe Synthesis and in Situ Hybridization. Probes used (except *aldh1a2*), antisense RNA probe synthesis, and in situ hybridization procedures have been previously described (34). RZPD clone IMAGp998B2417171Q1 in pExpress1 was used for synthesis of the *aldh1a2* probe.

Constructs, mRNA Synthesis, and mRNA Injection. Capped mRNAs were synthesized using the mMessage mMachine SP6 kit (Ambion) according to the manufacturer's instructions. Unless otherwise stated, the mRNA concentrations used for injections were *bmp2b*, 50 pg/nL; *eve1*, 20 pg/nL; eve1-VP16, 300 pg/nL; eve1-Eng, 300 pg/nL; and *cyp26a1*, 500 pg/nL. mRNAs were injected through the intact chorion into all blastomeres at the one- to two-cell stage. To make the

eve1-VP16 and the eve1-Eng fusion constructs, the *eve1* homeodomain was amplified by PCR (forward primer: GCCCTCGAGCAAGAATACTGCAAAGAAAGT; reverse primer: GCCTCTAGAGTGGATTTGGCCAGTGTAGAC) and subcloned into a pCS2_VP16 and pCS2_Eng vector (52).

Morpholino Analysis and Injection. *Eve1* mRNA (mildly) and eve1MO (more severely) affected epiboly movements, making the analysis of gene expression difficult in later stages in eve1MO-injected embryos; therefore in these embryos we concentrated on earlier marker analyses. The eve1MO (GeneTools LLC) corresponds to the intron1/exon2 acceptor splice site: 5′-CTGTCCTCTGCTACTGAAAAGAATA-3′. The eve1MO was injected at 5 ng/nL unless otherwise indicated. The bmp2bMO (GeneTools LLC) 5′-GCGGACCACGGCGACCATGATC-3′ targets the transcription start site; it was used at 0.1 ng/nL. The *aldh1a2* MO (Open Biosystems) (53) has the sequence 5′-GTTCAACTTCACTGGAGGTCATCGC-3′and was used at 1:2 (high) and 1:4 (low) dilutions from a 1-mM stock. In all cases, 1–2 nL of solution was injected into the yolk as close as possible to the cells of embryos at the one- to four-cell stage.

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