Zebrafish Znfl1 proteins control the expression of *hoxb1b* gene in the posterior neuroectoderm by acting upstream of *pou5f3* and *sall4* genes

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Received for publication, January 18, 2017, and in revised form, June 6, 2017 Published, Papers in Press, June 16, 2017, DOI 10.1074/jbc.M117.777094

Transcription factors play crucial roles in patterning posterior neuroectoderm. Previously, zinc finger transcription factor *znfl1* was reported to be expressed in the posterior neuroectoderm of zebrafish embryos. However, its roles remain unknown. Here, we report that there are 13 copies of *znfl1* in the zebrafish genome, and all the paralogues share highly identical protein sequences and cDNA sequences. When *znfl1s* are knocked down using a morpholino to inhibit their translation or dCas9-Eve to allow for additional RA signals to posteriorize the neural plate (11). Zebrafish *sall4* is a downstream target of *cdx4* but a direct upstream gene of *pou5f3* (12). POU domain transcription factor Pou5f3 is expressed in the forming mid-hindbrain boundary during organogenesis and mediates the competence to respond to Fgf8 inductive signaling in this region of zebrafish embryos (13). The zygotic *pou5f3*-null mutants (Zspg) do not form mid-hindbrain boundary (14). HOX genes are classified into 13 paralogous groups based on sequence homology and colinear expression during formation of the posterior central nervous system (15). In general, Hox1–Hox5 paralogue group genes are expressed in the hindbrain, whereas Hox4–Hox11 genes are detected in the spinal cord (16). Mice lacking *Hoxa1* exhibit defects in hindbrain segmentation, whereas *Hoxb1*-null mice do not manifest defects in early hindbrain patterning (16). In zebrafish, *hoxb1b* is the earliest gene that is expressed in the posterior neuroectoderm of gastrula (3). Zebrafish Hoxb1b shares ancestral functions with mammalian Hoxa1 and controls progenitor cell shape and oriented cell division during anterior hindbrain neural tube morphogenesis (17).

Zebrafish zinc finger-like gene 1 (*znfl1*), encoding a zinc finger transcription factor, was previously reported to be expressed in the posterior nervous system of zebrafish embryos (18). However, the functional roles of *znfl1* in the formation of posterior neuroectoderm remain unknown. In this study, we report that there are 13 copies of *znfl1* in the zebrafish genome, and all the paralogues share highly conserved protein sequences and cDNA sequences. They are

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This work was supported by the National Natural Science Foundation of China Grants 31471355 and 31271569. The authors declare that they have no conflicts of interest with the contents of this article.

The abbreviations used are: RA, retinoic acid; Sall, Spalt-like; *znfl1*, zinc finger-like gene 1; MO, morpholino; sgRNA, single-guide RNA; r, rhombomere; hpf, hours postfertilization.
zygotically expressed during embryogenesis. By knocking down znfl1s in zebrafish embryos using a morpholino (MO) to inhibit their translation or dCas9-Eve/sgRNAs to inhibit their transcription, we demonstrate that zebrafish znfl1s pattern the posterior neuroectoderm by acting upstream of pou5f3 and sall4.

**Results**

**Zebrafish has 13 paralogues of znfl1, and all are expressed in the posterior neuroectoderm of gastrula**

Performing bioinformatics analysis, we found that there are 13 copies of znfl1 in the zebrafish genome (Table 1). Although they are located in different chromosomes and have different genomic organization, 12 of the 13 paralogues of Znfl1 (except Znfl1f) share more than 93% amino acid identity among their protein sequences and exhibit more than 95% nucleotide sequence identity among their cDNAs (Ensemble, ENSDARG0000037914). The annotation of znfl1f is incomplete, but its annotated protein and cDNA sequences share more than 96% identity with those of znfl1 (Ensemble, ENSDARG0000037914), respectively (Table 1).

Because the 13 paralogues of znfl1s share high nucleotide sequence identity among their transcripts, we designed an antisense RNA probe to detect all their expressions during zebrafish early development by whole-mount in situ hybridization. As shown in Fig. 1, the mRNAs of znfl1s are not maternally detected (Fig. 1a). They are initially expressed at 30% epiboly stage (Fig. 1, b and c). The expressions are found in all the blastomeres of embryos at 50% epiboly (Fig. 1d), strongly distributed in the posterior neuroectoderm of embryos at 75% epiboly (Fig. 1e and f), and relatively weakly present in the adaxial mesoderm of embryos at bud stage (Fig. 1g). a–d and f, lateral view; e and g, dorsal view.

**Table 1**

Genomic organization of different zebrafish znfl1s and the identities of their cDNA sequences and protein sequences (%)

| Gene name | Old gene name | Genomic locus | Number of exons | Number of introns | cDNA sequence identity | Protein sequence identity |
|-----------|---------------|---------------|-----------------|------------------|-----------------------|--------------------------|
| znfl1b    | sidkey-103d23.5 | 18:5625861–5627378: | 11 0 9 6 9 3 | 96   | 94                     |
| znfl1c    | sich211–155k24.1 | 19:2055927–20564146: | 14 3 9 5 9 4 | 96   | 94                     |
| znfl1d    | sidkey-103d23.5 | 9:15900132–15904731: | 13 2 9 6 9 5 | 96   | 94                     |
| znfl1e    | BX004876.1    | 11:12715661–12717178: | 11 0 9 6 9 3 | 96   | 94                     |
| znfl1f    | CABZ01054718.1 | 13:45159239–45199801: | 13 2 9 6 9 3 | 96   | 94                     |
| znfl1g    | sich211–168h21.3 | 14:9852492–9857150: | 12 1 9 6 9 3 | 96   | 94                     |
| znfl1h    | sidkey-14o18.1 | 19:31947924–31952506: | 13 2 9 6 9 3 | 96   | 94                     |
| znfl1i    | sidkey-25033   | 9:31947924–31952506: | 13 2 9 6 9 3 | 96   | 94                     |
| znfl1j    | sidkey-21031   | 23:17117759–17120427: | 13 2 9 6 9 3 | 96   | 94                     |
| znfl1k    | sidkeyp-11g3   | 20:34471856–34476777: | 13 2 9 6 9 3 | 96   | 94                     |
| znfl1l    | sich211–196c10.11 | 8:23224357–23228957: | 13 2 9 6 9 3 | 96   | 94                     |
| znfl1m    | sich211–152m14.4 | 6:19448321–19448656: | 13 2 9 6 9 3 | 96   | 94                     |

*“1” denotes forward strand, whereas “/H11002” denotes reverse strand.

The annotation of znfl1f is incomplete. The incomplete protein sequence is 363 amino acids long, and the cDNA is 1,506 bp long. But the incomplete sequences share 98 and 96% identity with the corresponding sequences of znfl1 cDNA and protein, respectively.

**Figure 1. Zebrafish znfl1s were expressed in the posterior neuroectoderm of gastrula.** The mRNAs of zebrafish znfl1s were not present in the embryos at the two-cell stage (a) and 1,000 (1k)-cell stage (b). They were initially detected in the embryos at 30% epiboly stage (c), widely expressed in all the blastomeres of embryos at 50% epiboly (d), strongly distributed in the posterior neuroectoderm of embryos at 75% epiboly (e and f), and relatively weakly present in the adaxial mesoderm of embryos at bud stage (g). a–d and f, lateral view; e and g, dorsal view.


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**Knocking down znfl1s disrupts the formation of posterior neuroectoderm by reducing the expression of hoxb1b in zebrafish gastrula**

To explore the function of znfl1s in zebrafish early development, we developed two gene knockdown methods to inhibit the expressions of znfl1s in zebrafish embryos instead of performing knock-out due to the presence of 13 highly identical paralogues in zebrafish genome. Because all the transcripts of znfl1s share highly identical sequences around the start codon (Fig. 2a), we designed an antisense MO of znfl1s against the identical sequences to inhibit the translations of the mRNAs of all znfl1s. The specificity and efficiency of the znfl1 MO (against all znfl1s) were determined by the Dual-Luciferase reporter assay. To perform the assay, we made the transcript containing the MO target site (the same sequence in all znfl1s) fused with the coding sequence of firefly luciferase reporter gene (Fig. 2b). After 4 ng of the znfl1 MO was microinjected into zebrafish embryos with the mRNA of the reporter genes, about 90% of the translational activity of firefly luciferase reporter was blocked (Fig. 2b). To confirm the knockdown results, we then developed a second method to deplete the expressions by using dCas9-Eve/sgrNAs (Fig. 2d) to inhibit the transcription of all znfl1s. The efficiency of dCas9-Eve guided by sgRNAs in repressing the expressions of all znfl1s was examined by whole-mount in situ hybridization. To perform the test, we made dCas9-Eve by fusing dCas9 with the putative Eve repressor domain of zebrafish Evx1 (Fig. 2d and Table 2) and prepared three sgRNAs recognizing the three potential target sites that are highly identical in all the 5’-flanking sequences upstream of the start codons of znfl1s (Fig. 2c). After dCas9-Eve mRNAs and the three sgRNAs were co-microinjected into zebrafish embryos at the one-cell stage, the expressions of znfl1s were significantly reduced in the posterior neuroectoderm of gastrula (Fig. 2, e–g). These results suggest that the two methods including blocking translation by MO and repressing transcription by dCas9-Eve are effective ways to knock down the expressions of znfl1s in zebrafish embryos.

To determine whether znfl1s play crucial roles in the development of posterior neuroectoderm of zebrafish gastrula, we examined the expression of prospective posterior neural marker hoxb1b (3) in the knockdown embryos of znfl1s (referred to as the znfl1 knockdown embryos hereafter) at mid-gastrulation stage. The results showed that the morphants of

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*e and f*, and relatively weakly present in the adaxial mesoderm of embryos at bud stage (Fig. 1g). The results confirm the previous report that znfl1 is present in the posterior neuroectoderm (18) and suggest that znfl1s are involved in patterning the formation of posterior neuroectoderm in zebrafish gastrula.
znfl1s (referred to as the znfl1 morphants hereafter) exhibited a significantly reduced expression of hoxb1b in the embryos at 75% epiboly (Fig. 3, a, b, and f), and the decreased expression of hoxb1b in the znfl1 morphants was effectively rescued by over-expressing znfl1 mRNA (Fig. 3, a, c, e, and f). Consistently, the expression of hoxb1b was obviously decreased in the embryos in which the transcriptions of znfl1s were inhibited by dCas9-Eve (Fig. 3, g, h, i, and k), and overexpressing znfl1 effectively rescued the reduced expression of hoxb1b in the dCas9-Eve–microinjected embryos (Fig. 3, a, c, e, and f).

Subsequently, we determined whether the specification of posterior neuroectoderm was disrupted in the znfl1 knockdown embryos by examining the expressions of hoxb1b, hoxb1a, and hoxd4a in zebrafish gastrula. Expressions of hoxb1b, hoxb1a, and hoxd4a were examined in 8- (a–e) and/or 10-hpf (g–k, m–q, s–w, y, z, and a1) embryos microinjected with the control MO (a, g, m, s, and y), znfl1 MO (b, h, n, t, and z), dCas9-Eve mRNA plus sgRNAs (c, i, o, u, and a1), znfl1 MO plus znfl1 mRNA (d, j, p, and v), and dCas9-Eve mRNA plus znfl1 mRNA (e, k, q, and w), respectively. The statistical analyses of the data derived from a–e, g–k, m–q, s–w, and y–a1 are shown in f, l, r, x, and a2, respectively. All embryos except y, z, and a1 were positioned in dorsal view. Embryos y, z, and a1 were positioned in top view. **, p < 0.01.
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Zebrafish pou5f3 acts downstream of znfl1s to pattern the posterior neuroectoderm

Pou5f3 is a transcription factor that has been demonstrated to regulate posterior neural fates in Xenopus embryos (11). To determine whether Pou5f3 mediates the roles of znfl1s in the formation of posterior neuroectoderm of zebrafish gastrula, we first examined the expression of pou5f3 in the znfl1 knockdown embryos. The results revealed that the embryos microinjected with either the znfl1 MO or dCas9-Eve mRNA plus sgRNAs exhibited a dramatically decreased expression of pou5f3 in the posterior neuroectoderm compared with their control embryos at 75% epiboly, respectively (Fig. 3, g–x). When znfl1 was overexpressed, the decreased expressions of pou5f3 were effectively rescued in the znfl1 morphants or the embryos in which the expressions of znfl1s were inhibited by dCas9-Eve, respectively (Fig. 5, a–g). Furthermore, the expressions of znfl1s were not changed in pou5f3 morphants (Fig. 5, a–q). Taken together, the results suggest that pou5f3 works downstream of znfl1s.

We then examined the expression of hoxb1b in the pou5f3 morphants and found that it was significantly reduced (Fig. 5, i and n) like that in the znfl1 morphants (Fig. 5, j and n) and that in embryos in which the expressions of znfl1s were inhibited by dCas9-Eve (Fig. 5, l and n) compared with control embryos (Fig. 5, h and n). Moreover, the decreased expressions of hoxb1b were effectively rescued in the znfl1 morphants (Fig. 5, j, k, and n) and the embryos in which the expressions of znfl1s were inhibited by dCas9-Eve (Fig. 5, l–n) after pou5f3 mRNAs were microinjected into the two kinds of the znfl1 knockdown embryos. Taken together, these data substantiate that the function of znfl1s in posteriorizing neuroectoderm is mediated by the downstream gene pou5f3.

Zebrafish sall4 acts downstream of znfl1s and upstream of pou5f3 during its posterior neuroectoderm development

It has been demonstrated that zebrafish Sall4 can bind pou5f3 promoter to modulate the expression of pou5f3 directly...
To investigate whether this direct regulation occurs in the formation of zebrafish posterior neuroectoderm, we first checked the expression of *pou5f3* in *sall4* morphants. The results revealed that the expressions of *pou5f3* were significantly down-regulated in *sall4* morphants at 75% epiboly (Fig. 6, a, b, and d), and the decreased expression of *pou5f3* in *sall4* morphants was effectively rescued by overexpressing *sall4* (Fig. 6, a–d). The results are consistent with the previous report that Sall4 works upstream to activate *pou5f3* expression in zebrafish embryos directly (12). To further support the conclusion, we performed bioinformatics analysis on the 3.0-kbp genomic sequence upstream of the translation start site of *pou5f3* and found a presumptive Sall4-binding site with a core sequence of “ATTTGCAT” located between −558 and −551 of *pou5f3* promoter. We then cloned the 3.0-kbp genomic fragment to perform a Dual-Luciferase reporter assay on the promoter’s activ-
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![Figure 7. Zebrafish znfl1s control pou5f3 expressions through other factors in addition to sall4 in gastrula.](image)

Expression of pou5f3 were examined in the embryos microinjected with the control MO (a), znfl1 MO (b), znfl1 MO plus sal4 mRNA (c), dCas9-Eve mRNA plus sgRNAs (d), dCas9-Eve mRNA plus sgRNAs and sal4 mRNA (e), sal4 MO (f), and sal4 MO plus znfl1 mRNA (g), respectively. Embryos were positioned in lateral view, and all were examined at 8 hpf. The statistical analyses of the data derived from a–g are shown in diagram h, **, p < 0.01.

Next, we asked whether the loss of pou5f3 expression completely accounts for the decreased expression of pou5f3 caused by knocking down znfl1s in zebrafish embryos. To answer this, we examined the expression changes of pou5f3 in the znfl1 knockdown embryos in which sal4 was overexpressed. The results revealed that overexpression of sal4 effectively rescued pou5f3 expression in the znfl1 morphants (Fig. 7, a–c and h) and the embryos microinjected with dCas9-Eve (Fig. 7, a, d, e, and h). These results support the conclusion that znfl1s control pou5f3 expression by directly regulating sall4. However, the expression of pou5f3 was effectively rescued in sal4 morphants when znfl1 mRNA was overexpressed in the morphants (Fig. 7, a and f–h). The results suggest that zebrafish znfl1s play crucial roles in the formation of the posterior neuroectoderm by controlling the expression of pou5f3 through other factors besides sal4.

Discussion

Consistent with the fact that zebrafish genome underwent recent duplication, results from bioinformatics analysis reveal that it has 13 copies of znfl1, and all 13 paralogues of Znfl1 share highly identical cDNA sequences and protein sequences, although they are located in different chromosomes and have different genomic organization (Table 1 and Fig. 2, a and c). These features suggest that all the paralogues of Znfl1s should function redundantly during zebrafish early development. However, we found no gene homologues of znfl1s in other species by using GenBankTM (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The results suggest that znfl1s might be unique genes existing in the zebrafish genome.

Consistent with the previous report that znfl1 is involved in the induction of the posterior nervous system in zebrafish (18), results from whole-mount in situ hybridization reveal that znfl1s are expressed in the posterior neuroectoderm of zebrafish gastrula. To uncover the roles of Znfl1s in the formation of posterior neuroectoderm, we developed the dCas9-Eve repression method to inhibit the transcriptions of all znfl1s as reported previously (22, 23) in addition to using the MO method to inhibit their translation. Performing the two kinds of knockdown experiments, we found that they both gave the same result: the 13 znfl1s function to pattern posterior neuroectoderm through regulating the expression of hoxb1b, the
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marker gene for the formation of posterior neuroectoderm (3), in zebrafish gastrula (Fig. 3).

Zebrafish pou5f3 encodes a POU-family transcription factor (24). It is expressed both maternally and zygotically. Maternal mutant embryos of pou5f3 develop into normal fertile adult fish, although cell movements during gastrulation are slightly delayed (25). In contrast, zygotic pou5f3 mutant embryos show neural plate patterning defects, displaying extended r2 and r4; narrowed r1, r3, and r5; and altered expression of egr2b (7). Moreover, maternal-zygotic pou5f3 mutants exhibit a more severe phenotype, failure of gastrulation to proceed normally (25). In this study, we found that pou5f3 acts downstream of znfl1s to determine the formation of posterior neuroectoderm by controlling the expression of hoxb1b (Fig. 5). Because znfl1s are zygotically expressed (Fig. 1), the reduced expression of pou5f3 in the znfl1 knockdown embryos should be due to the reduced expression of zygotic pou5f3. Consistent with this observation, the defective phenotype of posterior neuroectoderm and the reduced length of hindbrain (Fig. 4) in the znfl1 knockdown embryos are more similar to zygotic pou5f3 mutants (25). This at least partially explains why the phenotype of the znfl1 knockout embryos was mimicked by microinjecting a low amount (0.1 ng) of pou5f3 MO.

Sall4 is a gene encoding a zinc finger transcription factor involved in the maintenance of embryonic stem cells (26). Although xSall4 was reported to represses xPou5f3 expression to provide a permissive environment allowing for additional Wnt/Fgf/RA signals to posteriorize the neural plate in Xenopus (11), the expression of Pou5f3 was regulated by transcription factor Sall4 through binding the core sequences of Sall4-bind-ing sites that are present in the promoter of Pou5f3 (19, 20). Recently, the expression of Pou5f3 was shown to be directly regulated by Sall4 during the formation of promoter activity assay of Pou5f3 (21). It was found that Pou5f3 is directly regulated by Sall4 through binding the core sequences of Sall4-bind-(11), the expression of Wnt/Fgf/RA signals to posteriorize the neural plate in X. laevis (24). It is expressed both maternally and zygotically. Maternal expression of Pou5f3 is effectively returned to normal by overexpressing Pou5f3 (22). We therefore conclude that Pou5f3 is regulated by Sall4 through the promoter activity assay of Pou5f3. In this study, Pou5f3 is directly regulated by Sall4 during the formation of posterior neuroectoderm in zebrafish gastrula (Fig. 6). Although znfl1s are ENSDARG00000037914, NM_194389 (znfl1); NP_001164503 (znfl1b); ENSDARG00000074668 (znfl1c); NM_001113633 (znfl1d); ENSDARG0000077877 (znfl1e); ENSDARG00000101498 (znfl1f); NM_001145701 (znfl1g); ENSDARG00000077877 (znfl1h); ENSDARG0000079126 (znfl1i); ENSDARG00000074668 (znfl1j); ENSDARG0000079036 (znfl1k); ENSDARG00000094197 (znfl1l); and ENSDARG00000074024 (znfl1m). The protein sequences or DNA sequences of the 13 Znfl1 paralogues were aligned using the software Vector NTI (Invitrogen). Promoter analysis was performed using online software (Genomatix).

**Bioinformatics analysis**

All sequences were retrieved from Ensemble (http://asia.ensembl.org/index.html) or GenBank (http://www.ncbi.nlm.nih.gov/). The ENSDARG identifiers and/or GenBank accession numbers to access the DNA and protein sequences of all znfl1s are ENSDARG00000037914, NM_194389 (znfl1); NP_001164503 (znfl1b); ENSDARG00000074359 (znfl1c); NM_001113633 (znfl1d); ENSDARG00000077719 (znfl1e); ENSDARG00000101498 (znfl1f); NM_001145701 (znfl1g); ENSDARG00000077877 (znfl1h); ENSDARG00000079126 (znfl1i); ENSDARG00000074668 (znfl1j); ENSDARG00000074024 (znfl1m). The protein sequences or DNA sequences of the 13 Znfl1 paralogues were aligned using the software Vector NTI (Invitrogen). Promoter analysis was performed using online software (Genomatix).

**Microinjection of morpholinos into zebrafish embryos**

MOs were purchased from Gene Tools. The znfl1 MO (AATGGTAACACATGGAGCTCTTGTT) was designed to block the translation of the mRNAs of all znfl1s. Zebrafish pou5f3 and sal1l4 were knocked down using the MO as described previously (31, 32); the sequences are CCGCTCTCGGTCCATTTCCCGTA (pou5f3) (32) and CGCTC-AACTCAATTTGTTGTC (sal1l4) (31). The sequence of control MO is CCTCCTACCTAGTTCAATTTATA (33).

MO was dissolved in Nanopure water and microinjected into the embryos at the one- to two-cell stage. The amount of MO microinjected into each embryo was ~1 nl of solution contain-
ing 4 ng of the *znfl1* MO, 0.1 ng of *pou5f3* MO, 4 ng of *sall4* MO, and an equal amount of control MO, respectively.

The specificity and efficiency of the *znfl1* MO were determined by the *in vitro* Dual-Luciferase reporter assay (Promega). Briefly, a pair of oligos, AGCTTAAACAGACCTTCCATGGTATTACCAATTTGGAC and CAGAAGGCTAGCGAGCTCtctagttagtggcacttcct. The fused construct consisting of MO target sequence and the coding sequence of firefly luciferase was finally inserted into pBluescript SK (Stratagene) under the control of T7 promoter. The mRNA of the fused construct was then synthesized, capped, and tailed using the mMESSAGE mMACHINE T7 Ultra kit (Ambion). 100 pg of synthesized mRNA plus 20 pg of Renilla luciferase expression vector (internal control) and 4 ng of the *znfl1* MO or control MO were microinjected into each zebrafish embryo at the one- to two-cell stage. Three pools of 20 microinjected embryos at bud stage were collected to perform the Dual-Luciferase assay following the manufacturer’s protocol as we described previously (21).

### Construction of the expression vector dCas9-Eve

The full-length coding sequence of codon-humanized Cas9 with nuclear localization signal was synthesized by BGI (China) according to the published sequences in the literature (34). The coding sequences of dCas9 were therefore made by mutating the sequences of Cas9 following the description in the literature (23). The coding sequences of dCas9 were recombined into pBluescript KS under the control of T7 promoter (pKS-dCas9).

To make dCas9-Eve that works in the zebrafish system, we first amplified the partial sequence encoding the C terminus of dCas9-Eve by performing overlapping PCR. We next fused the coding sequences of zebrafish Eve with the partial sequence encoding the C terminus of dCas9-Eve by performing overlapping PCR. Then, the overlapping PCR fragment was recombined into pKS-dCas9 to form the expression vector pKS-dCas9-Eve under the control of T7 promoter.

### Design and synthesis of sgRNAs in vitro

Three sgRNAs were prepared to recognize the 5’-flanking sequence upstream of the start codons of *znfl1s*. The template sequences of the three CRISPRs (clustered regularly interspaced short palindromic repeats) were GTTGTAGTGCGGAATATGGTAGAAGAAATTGGAAGTACGATGTCCATCATGACGACGACGTAGGTCGAGATAGG, GACGCACTGAGGATGAGGACGACGTAGGTCGAGATAGG, and GCACACACGAGCATGAGGACGACGTAGGTCGAGATAGG (the underlined sequences represent protospacer adjacent motifs).

To synthesize *sgRNA* in *vitro*, templates of *sgRNA* were amplified by PCR with pSY-sgRNA vector (YSY, China) using the method reported previously (35). *sgRNA* were synthesized using the MAXIscript *In Vitro* Transcription kit (Ambion).

### In vitro synthesis of mRNA and microinjection of RNAs into zebrafish embryos

To synthesize mRNA in *vitro*, we first cloned the full-length coding sequences of *znfl1*, *pou5f3*, and *sall4* by RT-PCR using the cDNAs derived from zebrafish embryos at 75% epiboly with primers ATGTTGGAGAATTTTAATGC (forward) and CTAATTTTTGGATATTTGTT (reverse) for *znfl1* (NM_194389), GAGATCTTTACTATTGCGCCCTCGT (forward, the underlined sequences represent restriction site hereafter) and GGACTAGTGGTTTGGGAAG (reverse) for *pou5f3* (NM_131112). The PCR products were then subcloned into pXT-7 vector under T7 promoter direction as we reported previously (33). *znfl1*, *pou5f3*, *sall4*, and dCas9-Eve mRNAs were synthesized, capped, and tailed in *vitro* using the mMESSAGE mMACHINE T7 Ultra kit. The mRNAs of *znfl1*, *pou5f3*, and *sall4* did not contain the corresponding MO target sequences. About 1 nl of 100 ng/μl *znfl1* mRNA, 100 ng/μl *sall4* mRNA, 20 ng/μl *pou5f3* mRNA, and 100 ng/μl *sgRNA* plus 250 ng/μl dCas9-Eve mRNA was microinjected into a zebrafish embryo at the one-cell stage.

### Whole-mount in situ hybridizations

**Whole-mount in situ** hybridizations were performed as we described previously (36). The templates for making the RNA probes to examine the expressions of *hoxb1b* (NM_131142), *eng2a* (NM_131044), *eg2b* (previously named *krox20*; NM_130997), *hoxb4a* (NM_131118), and *otx2* (BC115165) were prepared as we described previously (33). The cDNA templates for making antisense RNA probes of *znfl1* (NM_194389), *pou5f3* (NM_131112), *sall4* (NM_001080609), *hoxb1a* (NM_131115), and *hoxd4a* (NM_001126445) were RT-PCR-amplified fragments. The sequences of primers for cloning the probe templates were GACAATGAGGGAGTGCTTTG (forward) and AACACTTGACAGCCGCTTTG (reverse) for *znfl1*, CTGTTCTCGAGCTGATCTT (forward) and TGGAGATGTGGTGCTTGAA (reverse) for *sall4*, CAGAGCCCAACAGCAGAGAG (forward) and GTTGTAGTGCGGAATATGGTAGAAGAAATTGGAAGTACGATGTCCATCATGACGACGACGTAGGTCGAGATAGG (reverse) for *pou5f3*, TGGAGATGGAGAAGC (forward) and AGACCACTGAGGATGAGGACGACGTAGGTCGAGATAGG (reverse) for *hoxb1a*, and AGCTTCTCTCGGTTTGT (forward) and GCTGTCCGCAGAACCAAG (reverse) for *hoxd4a*.
Roles of znfl1s in patterning posterior neuroectoderm

Promoter cloning, construction of eukaryotic expression vector, and in vitro Dual-Luciferase reporter assay

2,839 bp of sall4 promoter (NM_001080609) and 3,000 bp of pou5f3 promoter (NM_131112) were cloned by PCR with the primers ATTTTTGGGTGTACATGGTT and GGGTCTC- CGAATTGATAT (for sall4) and TGTACACTTGGGCGGCCT- GTC and CTTTCCGCTAAAAGGTGT (for pou5f3) using the template genomic DNA prepared as we reported previously (37). All the PCR products were first subcloned into pGEM-T Easy vector (Promega). The amplified promoters were sequenced to confirm their identities and then recombined into pGL3-Basic luciferase reporter vector using the One Step Cloning kit (Vazyme, China) with primers CTATC-GATGTTACGAGTCTATTTGGGCGCTAATGCTCTTA- TCAGATGTTACGAGTCTGCGGAAAACCTGACGTCACT- TTC (forward) and ACTTAGATCGACAGTCTCGAG- GG1TCGTCCGAATTGATAT (reverse) for sall4 promoter and CTATCAGATGTTACGAGTCTGCGTCTTACCTTTGTGC- CCTGCTTC (forward) and ACTTAGATCGACAGTCTC- GACGCTTTCGGCTAAAGGTGT (reverse) for pou5f3 promoter. The plasmids were named pGL3-sall4 and pGL3- pou5f3, respectively. The full-length coding sequences of znfl1 and sall4 were recombined into pCMV-3Tag-7 vector (Stratagene) to form the eukaryotic expression vectors pCMV-znfl1 and pCMV-sall4, respectively.

Dual-Luciferase reporter assays were performed on 293T cells as we reported previously (38) using the Dual-Luciferase reporter assay kit (Promega). About 100 ng/μl expression vectors pCMV-znfl1, pCMV-sall4, pGL3-sall4, and pGL3-pou5f3 and 2 ng/μl Renilla luciferase expression vector were used. The assays were performed in at least three independent experiments.

Measurement of forebrain and hindbrain length

The zebrafish embryos fixed by 4% paraformaldehyde were photographed in bright field under a dissecting microscope using digital cameras. The lengths of forebrain and hindbrain were measured in laterally viewed embryos at 24 or 20 hpf with Image-Pro Plus software. The length unit was arbitrary.

Statistical analysis

Data were analyzed using the SPSS 20.0 software package (SPSS, Chicago, IL) with an independent-samples t test or χ² test between two groups. Statistical significance was defined as p < 0.05 or p < 0.01.

Author contributions—Q. Zhao contributed to the conception of the study. X. D., Jingyun L., L. H., C. G., W. J., Y. Y., Q. Zhang, and L. C performed the experiments. Q. Zhao, Jingyun L. and X. D. wrote the paper. Jun L. contributed to analysis with constructive discussions.

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