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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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 inhibit their transcription, the zebrafish gastrula displays reduced expression of *hoxb1b*, the marker gene for the posterior neuroectoderm. Further analyses reveal that diminishing *znfl1s* produces the decreased expressions of *pou5f3*, whereas overexpression of *pou5f3* effectively rescues the reduced expression of *hoxb1b* in the posterior neuroectoderm. Additionally, knocking down *znfl1s* causes the reduced expression of *sall4*, a direct regulator of *pou5f3*, in the posterior neuroectoderm, and overexpression of *sall4* rescues the expression of *pou5f3* in the knockdown embryos. In contrast, knocking down either *pou5f3* or *sall4* does not affect the expressions of *znfl1s*. Taken together, our results demonstrate that zebrafish *znfl1s* control the expression of *hoxb1b* in the posterior neuroectoderm by acting upstream of *pou5f3* and *sall4*.

During vertebrate gastrulation, three definitive germ layers including ectoderm, mesoderm, and endoderm are formed, and the anterior–posterior axis of an embryo is determined. The ectoderm gives rise to epidermal ectoderm and neuroectoderm (1). The neuroectoderm itself is regionalized along the anterior–posterior axis of an embryonic body, resulting in the anterior neuroectoderm that forms forebrain and the posterior neuroectoderm that gives rise to midbrain, hindbrain, and spinal cord (2). The posterior neuroectoderm is transformed from the newly induced neuroectoderm by graded posteriorizing signals including fibroblast growth factor, Wnt, and retinoic acid (RA)[3] (3).

Transcription factors including zinc finger transcription factors (4, 5), POU domain proteins (6, 7), and homeodomain proteins (8–10) have been reported to be involved in patterning the posterior neuroectoderm in vertebrate gastrula. Zinc finger transcription factors Spalt-like (Sall) are vertebrate homologues of the *Drosophila* Spalt. In *Xenopus*, xSall4 represses the expression of *xPou5f3* to provide a permissive environment allowing 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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[3] 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, ENSDARG00000037914). The annotation of *znfl1f* is incomplete, but its annotated protein and cDNA sequences share more than 96% identity with those of *znfl1* (Ensemble, ENSDARG00000037914), 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. 1*a*). They are initially expressed at 30% epiboly (Fig. 1, *b* and *c*). The expressions are found in all the blastomeres of embryos at 50% epiboly (Fig. 1*d*), strongly distributed in the posterior neuroectoderm of embryos at 75% epiboly (Fig. 1,

Table 1

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

The sequence identities of all the other 12 paralogues are compared with *znfl1*, respectively.

Gene name	Old gene name	Genomic locus[a]	Number of exons	Number of introns	cDNA sequence identity	Protein sequence identity
					%	%
znfl1	Zinc finger-like gene 1	3:34522636–34527237:−1	2	1	100	100
znfl1b	si:dkey-103d23.5	18:5625861–5627378:−1	1	0	96	94
znfl1c	si:ch211–155k24.1	19:20559527–20564146:−1	2	1	95	93
znfl1d	si:dkey-103d23.5	9:15900132–15904731:1	2	1	95	94
znfl1e	BX004876.1	11:12715661–12717178:−1	1	0	95	93
znfl1f[b]	CABZ01054718.1	KN150451.1:13411–14916:1	1	0	98	96
znfl1g	si:ch211–168h21.3	13:45195299–45199801:1	3	2	95	94
znfl1h	si:dkey-14o18.1	19:9852492–9857150:−1	1	0	96	95
znfl1i	si:dkey-250i3.3	9:31947924–31952506:−1	4	3	95	94
znfl1j	si:dkey-210i3.1	23:17117759–17120427:1	2	1	95	94
znfl1k	si:dkeyp-11g8.3	20:34471856–34476777:1	1	0	96	95
znfl1l	si:ch211–196c10.11	8:23224357–23228957:1	2	1	96	96
znfl1m	si:ch211–152n14.4	6:19444281–19448656:−1	3	2	96	95

[a] "1" denotes forward strand, whereas "−1" denotes reverse strand.

[b] 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.

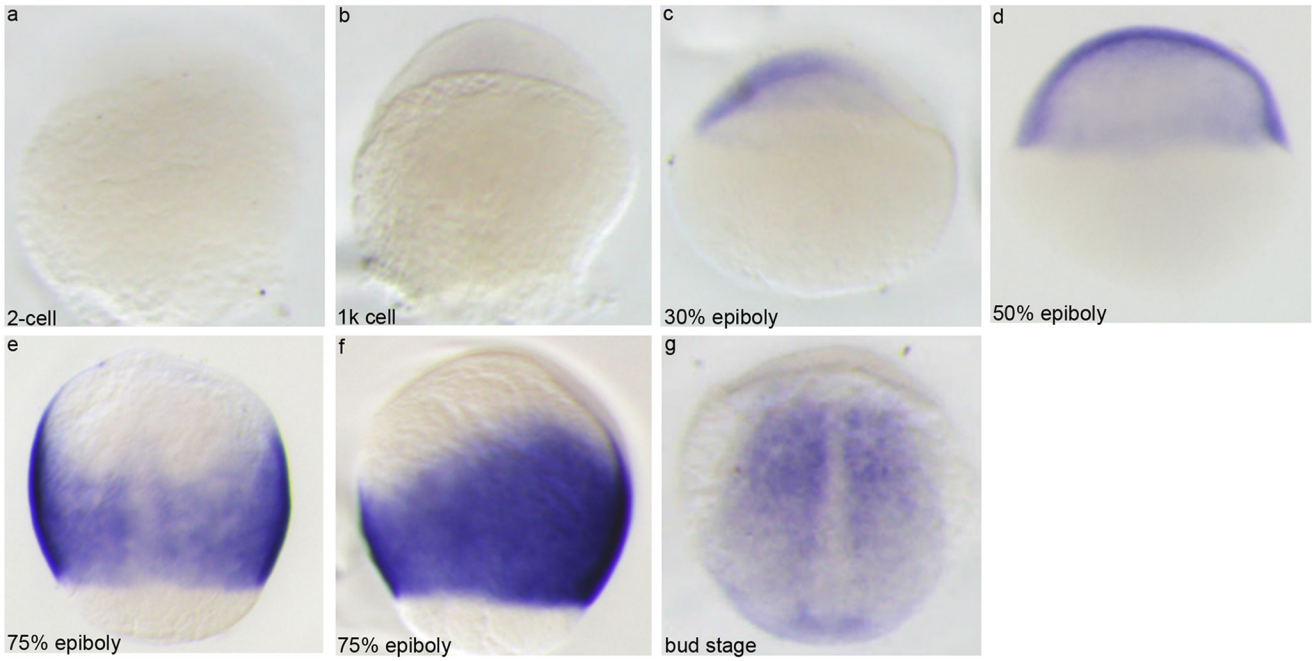

Figure 1. Zebrafish *znfl1s* were expressed in the posterior neuroectoderm of gastrula. The mRNAs of zebrafish *znfl1s* were not present in the embryos at the two-cell stage (*a*) and 1,000 (*1k*)-cell stage (*b*). They were initially detected in the embryos at 30% epiboly stage (*c*), widely expressed 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.

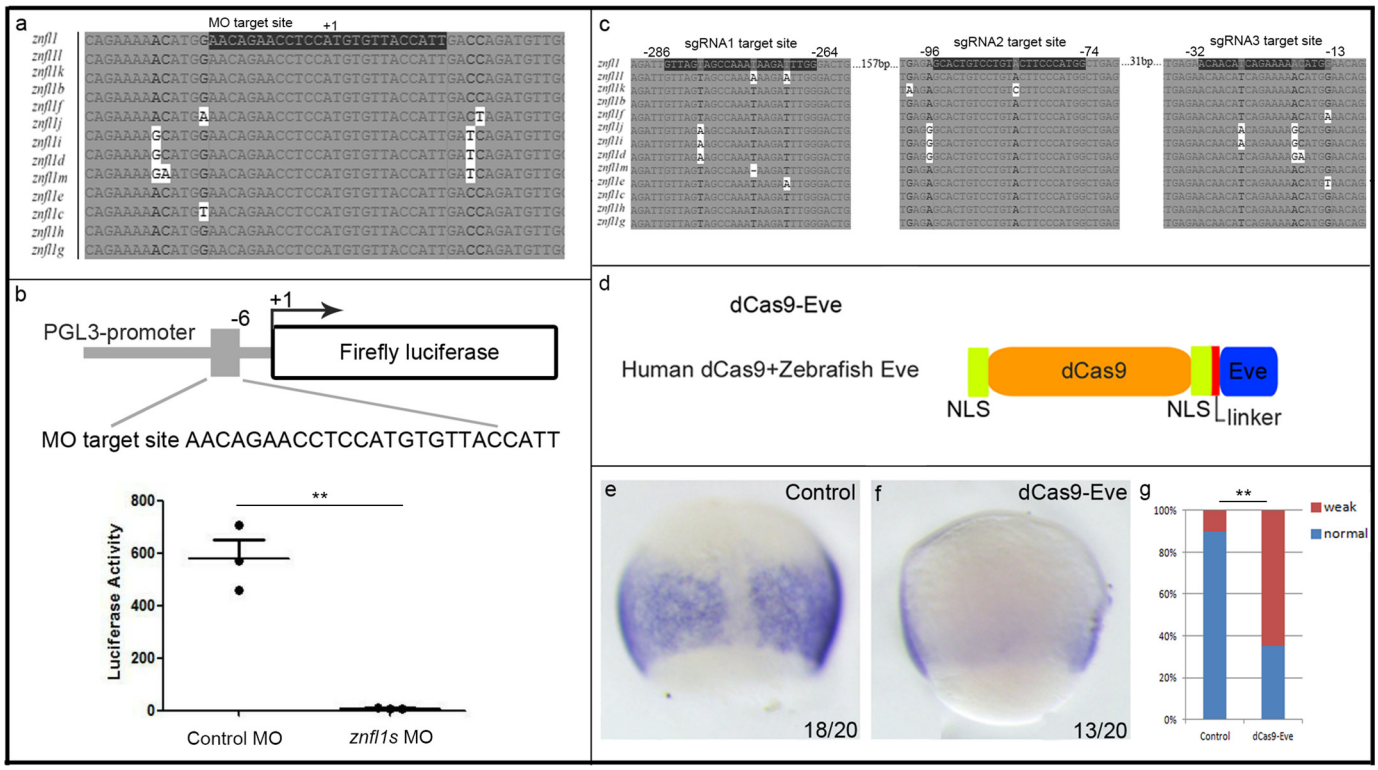

Figure 2. The expressions of *znfl1s* were effectively knocked down by morpholino and dCas9-Eve in zebrafish embryos. *a*, cDNA sequence alignment of partial cDNAs of *znfl1s*. +*1* denotes the first nucleotide of the start codon. The *letters* highlighted in *black* are the MO target sequences that are the same in all paralogues of *znfl1s*. *b*, *top*, schematic diagram showing the translation blocker of the *znfl1* MO used to inhibit the translations of the mRNAs of *znfl1s*. The *arrow* denotes the site where translation starts. *Bottom*, scatter plot showing the Dual-Luciferase reporter assay results revealing that the *znfl1* MO represses translation of *znfl1s* significantly. *c*, genomic sequence alignment of part of the 5′-flanking sequence upstream of the start codons of *znfl1s*. The *letters* highlighted in *black* are the regions presumptively recognized by the three sgRNAs, respectively. The first nucleotide of start codon is denoted as +*1*. *d*, schematic diagram showing the organization of dCas9-Eve. The DNA sequences encoding the linker and Eve is shown in Table 2. *NLS*, nuclear localization signal. *e* and *f*, results from whole-mount *in situ* hybridization showing that the expressions of *znfl1s* were significantly inhibited in the embryos microinjected with dCas9-Eve mRNA plus three sgRNAs (*f*) compared with their control embryos without any microinjection (*e*). *g*, diagram showing the statistical analysis of the data derived from *e* and *f*. *Error bars* represent S.D. **, $p < 0.01$.

e and *f*), and relatively weakly present in the adaxial mesoderm of embryos at bud stage (Fig. 1*g*). 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.

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. 2*a*), 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. 2*b*). 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. 2*b*). To confirm the knockdown results, we then developed a second method to deplete the expressions by using dCas9-Eve/sgRNAs (Fig. 2*d*) 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. 2*d* 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. 2*c*). 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

Table 2
Coding sequences for linker and zebrafish Eve of dCas9-Eve

Name	Sequences 5′–3′
Linker	AGCCCCAAGAAGAAGAGAAAGGTGGAGGCCAGCGGAGGTACGCGTTCTAGAACC
Zebrafish Eve	ATGGAAACCACAATAAAGGTTTGGTTTCAGAACCGTCGCATGAAGGACAAGAGACAGCGTCTGGCTATGACCTGGCCGCATCCTGCCGACCCCG CCTTTTACACCTATATGATGAGCCATGCTGCAGCAACGGGCAGTCTGCCCTATCCATTTCAATCTCATCTTCCCCTTCCTTACTATTCTCCAC TAAGCAGTGTGACTGCAGGTTCAGCCACTGCCACTGCGGGTCCATTCTCAAATCCCCTGCGCTCGCTGGATAGTTTTCGGGTGCTTTCGCATC CATACCCGCGACCTGAACTGCTGTGCGCCTTCAGACACCCATCACTGTACCCCAGCCCGGGTCATGGGCTTGGTCCCGGTGGAAGTCCATGCTC CTGCCTTGCTTGCCACGCTTCCAGTCAAACAAACGGGCTCCAACATAGATCCAATAATGCAGAATTCTCGTGTTCGCCCACGACCAGGACTGAG GCCTTCCTCACTTTCTCGCCAGCAGTCATCAGCAAATCATCTTCGGTGTCTTTGGACCAGAGGGAGGAAGTGCCACTAACTAGA

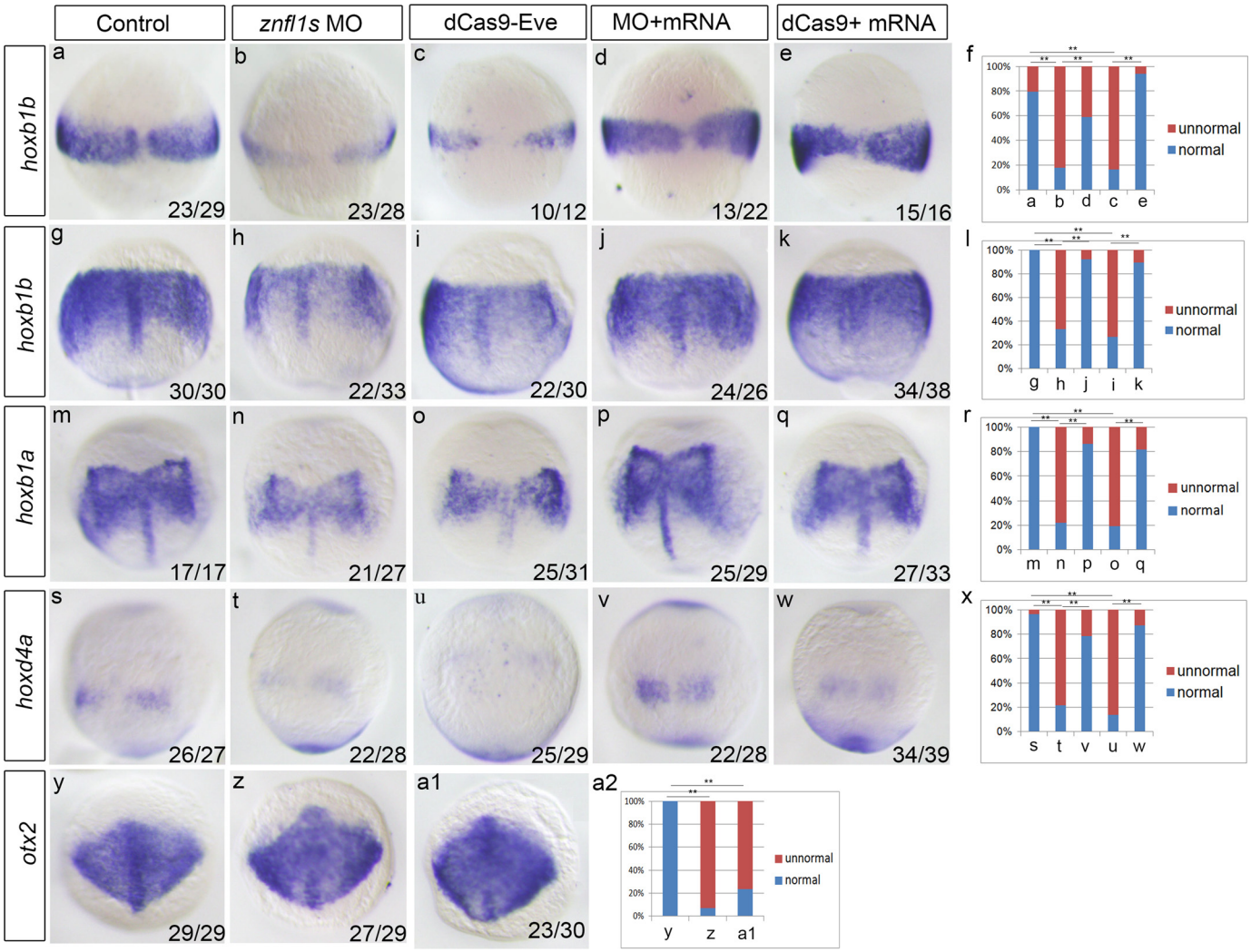

Figure 3. Knocking down *znfl1s* affects the posterior neuroectoderm development through reducing the expressions of *hoxb1b*, *hoxb1a*, and *hoxd4a* in zebrafish gastrula. Expressions of *hoxb1b*, *hoxb1a*, *hoxd4a*, and *otx2* were examined in 8- (*a–e*) and/or 10-hpf (*g–k*, *m–q*, *s–w*, *y*, *z*, 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 sgRNAs and *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. **, $p < 0.01$.

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 overexpressing *znfl1* mRNA (Fig. 3, *a*, *b*, *d*, 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, *a*, *c*, and *f*), 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* and *hoxb1a*, two marker genes for presumptive rhombomere 4 (r4) (19), and *hoxd4a*, a marker gene for presumptive r7 and r8 of hindbrain (20), in zebrafish embryos at the end of gastrulation. The results showed that the expressions *hoxb1b*, *hoxb1a*, and *hoxd4a* were all significantly decreased in the *znfl1* knockdown embryos at bud stage (Fig. 3, *g–i*, *i*, *l–o*, *r–u*, and *x*). Moreover, the decreased expressions of *hoxb1b*, *hoxb1a*, and *hoxd4a* were

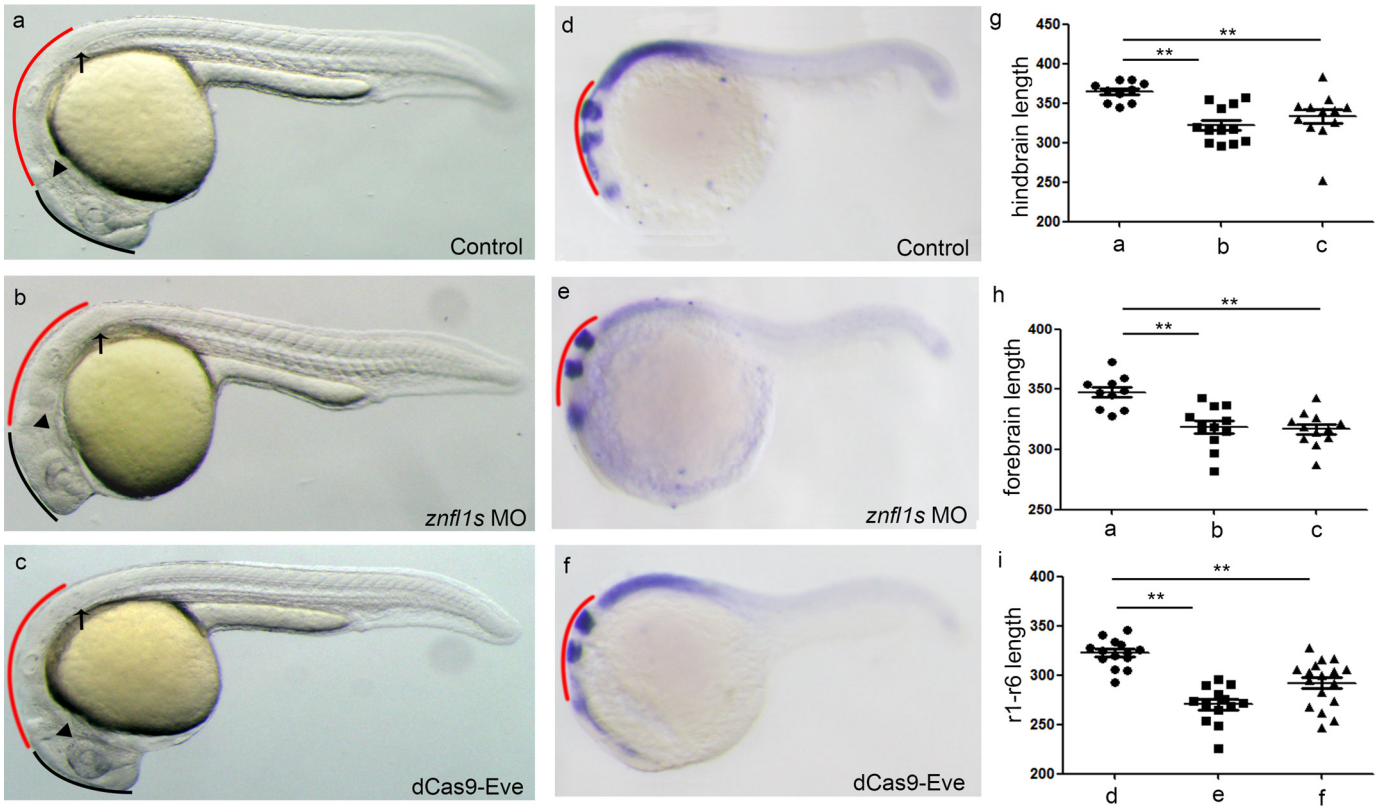

Figure 4. Knocking down *znfl1s* affects the hindbrain development. Morphological phenotypes of the 24-hpf embryos microinjected with the control MO (*a*), *znfl1* MO (*b*), and dCas9-Eve mRNA plus sgRNAs (*c*) and the 20-hpf whole-mount *in situ* hybridized embryos microinjected with the control MO (*d*), *znfl1* MO (*e*), and dCas9-Eve mRNA plus sgRNAs (*f*) were observed under a dissecting microscope. The statistical analyses of the data derived from *a–c* and *d–f* are shown in scatter plot diagrams in *g–i*, respectively. All embryos were positioned in lateral view. In *a–c*, the *red* curve shows the length of hindbrain, and the *black* curve shows the length of forebrain. The *arrow* indicates the position where the first somite starts. The *arrowhead* points to the midbrain–hindbrain boundary. *d–f*, embryos hybridized with the RNA probes detecting the expressions of *eng2a*, *egr2b*, and *hoxb4a*. The *red* curve shows the length of r1–r6. *Error bars* represent S.D. **, $p < 0.01$.

all effectively rescued by overexpression of *znfl1* in the *znfl1* knockdown embryos, respectively (Fig. 3, *g–x*). Consistently, the expression of *otx2*, the prospective anterior neural maker gene, was increased and expanded in the *znfl1* knockdown embryos (Fig. 3, *y*, *z*, *a1*, and *a2*). Furthermore, the hindbrain lengths of r1–r8 were significantly reduced (Fig. 4, *a–c* and *g*) in the *znfl1* knockdown embryos at 24 hpf. Unexpectedly, decreased lengths of forebrain were also observed in the knockdown embryos (Fig. 4, *a–c* and *h*). Consistent with the morphological observation, the lengths of r1–r6 defined by hindbrain marker genes including *eng2a* (marking the midbrain–hindbrain boundary) and *hoxb4a* (marking the anterior boundary of r7) in the *znfl1* knockdown embryos at 20 hpf (21) were significantly reduced (Fig. 4, *d–f*, and *i*). Taken together, these results strongly suggest that *znfl1s* are essential for the formation of posterior neuroectoderm.

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. 5, *a*, *b*, *d*, *e*, and *g*). 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, *o–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

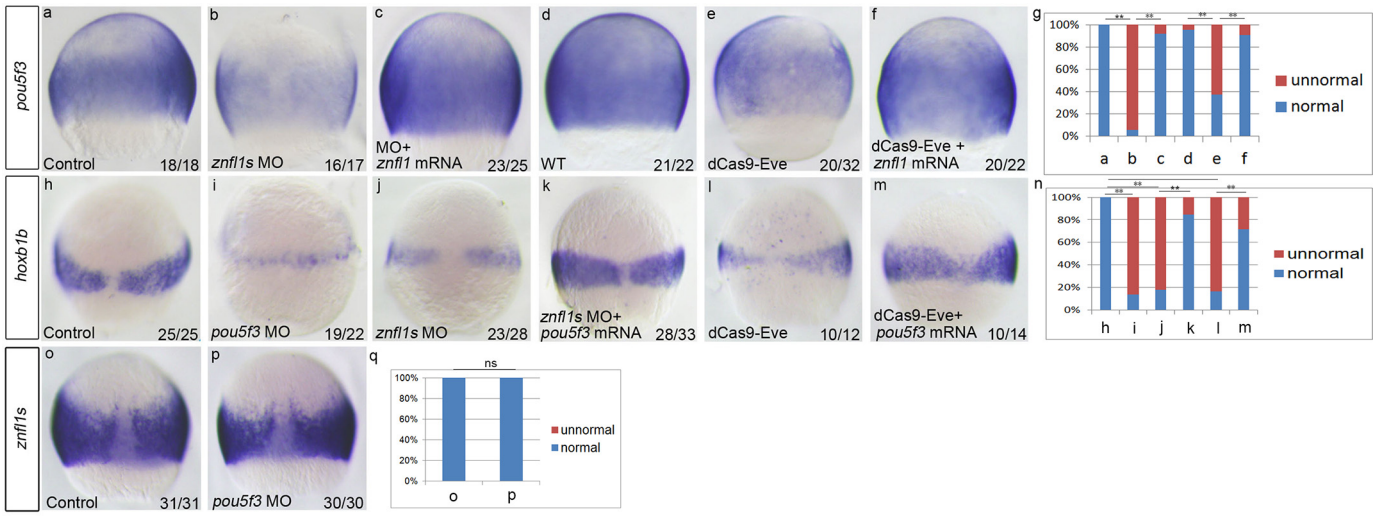

Figure 5. Zebrafish *znfl1s* control *hoxb1b* expression by working upstream of *pou5f3* in gastrula. Expressions of *pou5f3* were examined in the embryos microinjected with the control MO (*a*), *znfl1* MO (*b*), *znfl1* MO together with *znfl1* mRNA (*c*), dCas9-Eve mRNA plus sgRNAs (*e*), and dCas9-Eve mRNA plus sgRNAs and *znfl1* mRNA (*f*) and wild-type control embryos (*d*) at 75% epiboly, respectively. Expressions of *hoxb1b* were examined in 8-hpf embryos microinjected with the control MO (*h*), *pou5f3* MO (*i*), *znfl1* MO (*j*), *znfl1* MO plus *pou5f3* mRNA (*k*), dCas9-Eve mRNA plus sgRNAs (*l*), and dCas9-Eve mRNA plus sgRNA and *pou5f3* mRNA (*m*), respectively. Expressions of *znfl1s* were examined in the 8-hpf embryos microinjected with the control MO (*o*) and *pou5f3* MO (*p*), respectively. The statistical analyses of the data derived from *a*–*f*, *h*–*m*, and *o* and *p* are shown in diagrams *g*, *n*, and *q*, respectively. Embryos were positioned in lateral view (*a*–*f*) or dorsal view (*h*–*m* and *o*–*p*). **, $p < 0.01$; *ns*, no significance.

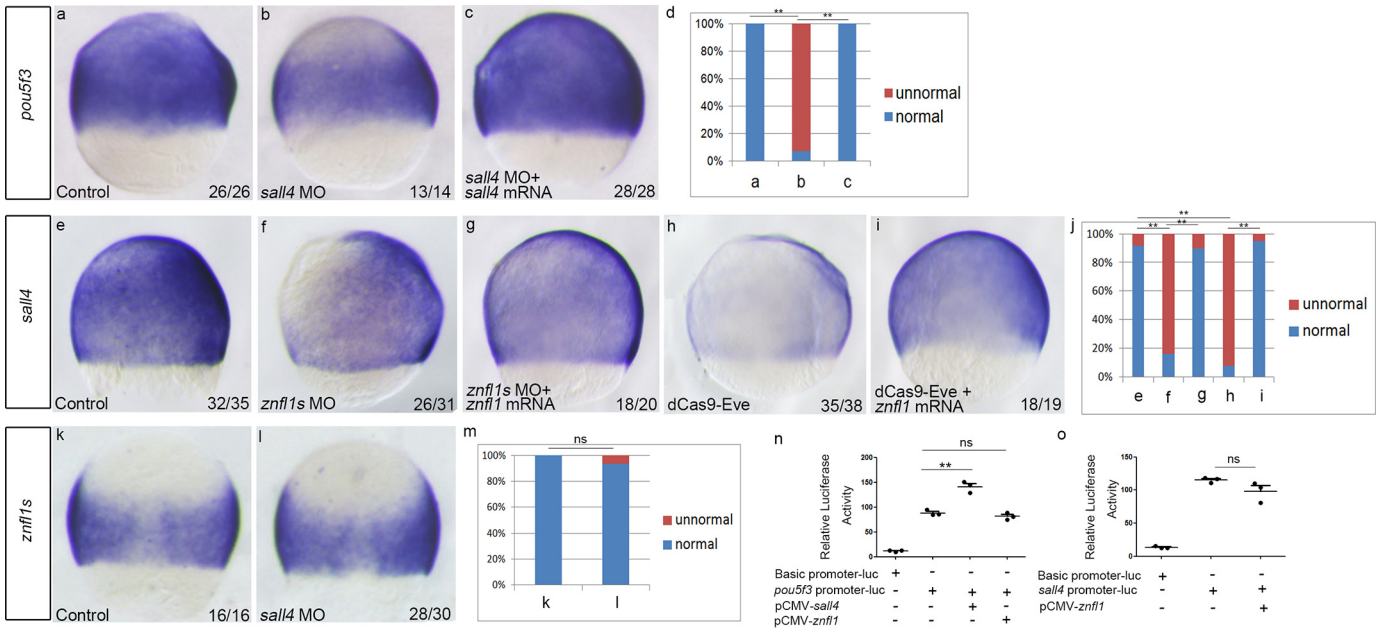

Figure 6. Zebrafish *znfl1s* control *pou5f3* expressions through *sall4* in gastrula. Expressions of *pou5f3* were examined in the embryos microinjected with the control MO (*a*), *sall4* MO (*b*), and *sall4* MO plus *sall4* mRNA (*c*), respectively. Expressions of *sall4* were examined in the embryos microinjected with the control MO (*e*), *znfl1* MO (*f*), *znfl1* MO plus *znfl1* mRNA (*g*), dCas9-Eve mRNA plus sgRNAs (*h*), and dCas9-Eve mRNA plus sgRNAs and *znfl1* mRNA (*i*), respectively. Expressions of *znfl1s* were examined in the embryos microinjected with the control MO (*k*) and *sall4* MO (*l*), respectively. Embryos were positioned in lateral view (*a*–*c* and *e*–*i*) or dorsal view (*k* and *l*), and all were examined at 8 hpf. The statistical analyses of the data derived from *a*–*c*, *e*–*i*, and *k* and *l* are shown in diagrams *d*, *j*, and *m*, respectively. Scatter plots showing that no significant change of the firefly luciferase activity driven by the *pou5f3* or *sall4* promoter was found in the cells transfected with pCMV-*znfl1* compared with control vehicle pGL3 vectors (*n* and *o*), but significant changes of the *pou5f3* promoter activity were found in the cells transfected with pCMV-*sall4* compared with control vehicle pGL3 vectors (*n*). *Error bars* represent S.D. **, $p < 0.01$; *ns*, no significance.

(12). 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-

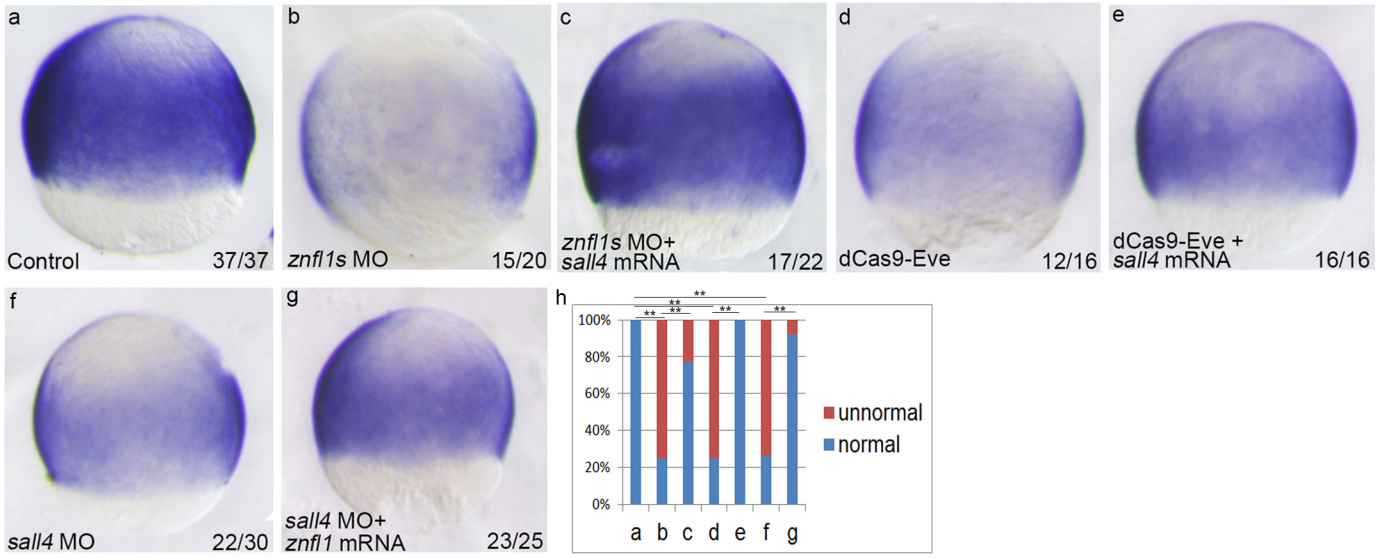

Figure 7. Zebrafish *znfl1s* control *pou5f3* expressions through other factors in addition to *sall4* in gastrula. Expressions of *pou5f3* were examined in the embryos microinjected with the control MO (*a*); *znfl1* MO (*b*), *znfl1* MO plus *sall4* mRNA (*c*), dCas9-Eve mRNA plus sgRNAs (*d*), dCas9-Eve mRNA plus sgRNAs and *sall4* mRNA (*e*), *sall4* MO (*f*), and *sall4* 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*. **, $p < 0.01$.

ity in response to Sall4. The results showed that the promoter's activity was significantly increased ($p < 0.01$) when *sall4* was overexpressed (Fig. 6*n*). However, the promoter had no response ($p > 0.05$) to the overexpression of *znfl1* (Fig. 6*n*). The results support that *pou5f3* is a direct target gene of *sall4* but not *znfl1s* during the formation of zebrafish posterior neuroectoderm.

Now that Sall4 was shown to directly regulate the expression of *pou5f3* that works downstream of *znfl1s* to pattern posterior neuroectoderm, we therefore explored whether *sall4* works downstream of *znfl1s* during the formation of posterior neuroectoderm in zebrafish gastrula. To do this, we examined the expressions of *sall4* in the *znfl1* knockdown embryos. Results from whole-mount *in situ* hybridization showed that a dramatic decrease of *sall4* expression occurred in the *znfl1* morphants (Fig. 6, *f* and *j*) and the embryos in which the expressions of *znfl1s* were inhibited by dCas9-Eve (Fig. 6, *h* and *j*), and overexpression of *znfl1* mRNA effectively rescued the decreased expression of *sall4* in the embryos microinjected with the *znfl1* MO (Fig. 6, *e*–*g* and *j*) or dCas9-Eve mRNA plus sgRNAs (Fig. 6, *e* and *h*–*j*). Furthermore, the expressions of *znfl1s* were not changed in *sall4* morphants (Fig. 6, *k*–*m*).

To investigate whether *sall4* is directly regulated by Znfl1s, we performed the Dual-Luciferase reporter assay on its promoter's activity in response to Znfl1. The results showed that the promoter of *sall4* had no response ($p > 0.05$) to the overexpression of *znfl1* (Fig. 6*o*), suggesting that *sall4* is not a direct target gene of *znfl1s.* Taken together, the results demonstrate that *sall4* works downstream of *znfl1s* to directly control the expression of *pou5f3* in zebrafish gastrula.

Next, we asked whether the loss of *sall4* 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 *sall4* was overexpressed. The results revealed that overexpression of *sall4* 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 data support the conclusion that *znfl1s* control *pou5f3* expression by directly regulating *sall4*. However, the expression of *pou5f3* was effectively rescued in *sall4* 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 *sall4*.

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 mining GenBank™ (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

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* knockdown 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-binding sites that are present in the promoter of *Pou5f3* both in mammalian embryonic stem cells and zebrafish embryos (12, 27). One explanation for the differences is that different molecules have come to assume distinct functions in different cell lineages (*e.g.* Snail-family proteins play different roles in zebrafish, *Xenopus*, and mouse) (28). Consistent with the directly regulated expression of *pou5f3* by Sall4, we demonstrate in this study that knocking down *sall4* results in the reduced expression of *pou5f3*, and the decreased expression of *pou5f3* is effectively returned to normal by overexpressing *sall4* in zebrafish *sall4* morphants (Fig. 6, *a–d*). Moreover, results of the promoter activity assay of *pou5f3* support that zebrafish *pou5f3* is directly regulated by Sall4 during the formation of posterior neuroectoderm in zebrafish gastrula (Fig. 6*n*).

Although the *znfl1* knockdown embryos display reduced expression of *sall4* in the neuroectoderm (Fig. 6, *e*, *f*, and *h*) and overexpression of *sall4* rescues the developmental defects of *pou5f3* expression in the *znfl1* knockdown embryos (Fig. 7, *a–e* and *h*), overexpression of *znfl1s* in *sall4* knockdown embryos effectively rescues the expression of *pou5f3* in neuroectoderm (Fig. 7, *f–h*). We therefore conclude that *znfl1s* regulate the expression of *pou5f3* through other factor(s) in addition to *sall4* (Fig. 7). This is consistent with the observation that the expression level of *pou5f3* in the *znfl1* morphants (Figs. 5*b* and 7*b*) is significantly lower than in *sall4* morphants (Figs. 6*b* and 7*f*).

In summary, we demonstrate in this study that Znfl1s pattern posterior neuroectoderm through regulating *hoxb1b* expression by acting upstream of *pou5f3* and *sall4* (Fig. 8). It is well known that RA signaling ensures normal posterior neuroectoderm patterning through directly regulating *hoxb1b* expression (3, 29). In this study, our results provide a new finding that other transcription factors like Znfl1s, Sall4, and Pou5f3 are involved in regulating the formation of posterior neuroectoderm through controlling *hoxb1b* expression. Future work should be performed to explore whether *znfl1s* are the direct target genes that mediate the roles of RA signaling in patterning the posterior neuroectoderm of zebrafish gastrula.

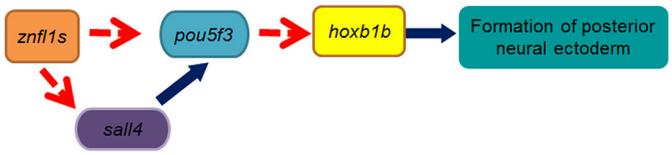

Figure 8. Working model proposed by this study. *Blue arrows* show direct regulation. *Broken red arrows* represent action upstream.

Experimental procedures

Zebrafish maintenance

Zebrafish were raised in the zebrafish facility of the Model Animal Research Center, Nanjing University, in accordance with the Institutional Animal Care and Use Committee-approved protocol. The embryos were staged as described previously (30).

Bioinformatics analysis

All sequences were retrieved from Ensemble (http://asia.ensembl.org/index.html)[4] 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*); ENSDARG00000079036 (*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).

Microinjection of morpholinos into zebrafish embryos

MOs were purchased from Gene Tools. The *znfl1* MO (AATGGTAACACATGGAGGTTCTGTT) was designed to block the translations of the mRNAs of all *znfl1s*. Zebrafish *pou5f3* and *sall4* were knocked down using the MO as described previously (31, 32); the sequences are CGCTCTCTCCGTCATCTTTCCGCTA (*pou5f3*) (32) and CGCTCCAAACTCACCATTTTCTGTC (*sall4*) (31). The sequence of control MO is CCTCTTACCTCAGTTACAATTTATA (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-

[4] Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party-hosted site.

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, AGCTTAACAGAACCTCCATGTGTTACCATTGACCAGC and CATGGCTGGTCAATGGTAACACATGGAGGTTCTGTTA, that contain the MO target sequence of *znfl1s* was first annealed and then recombined into pGL3-Basic vector (Promega) through HindIII and NcoI restriction endonuclease sites. 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 coding sequence for the putative Eve repressor domain of zebrafish Evx1 (even-skipped homeobox 1) (NM_131249.2) using as template the cDNA derived from zebrafish embryos at 24 hpf with forward primer (TCTAGAACCATGgaaaccacaataaaggtttg) and reverse primer (CTCGAGGCTAGCGAGCTCtctagttagtggcacttcct). The PCR fragment was cloned into pGEM-T (pGEMT-Eve) and sequenced to confirm its identity. After digesting pGEMT-Eve and pCS2+ with XbaI and XhoI, we then cloned the coding sequence for zebrafish Eve into pCS2+, resulting in the expression plasmid named pCS2+Eve. We next fused the coding sequences of zebrafish Eve with the partial sequence encoding the C terminus of dCas9-Eve by performing overlapping PCR. We first amplified the partial sequence encoding the C terminus of dCas9-Eve using pKS-dCas9 as template with the primers dC9KS-F1 (GAAACCGGGGAGATCGTGTG) and dC9KS-R1 (gaacgcgtacctccGCTGGCCTCCACCTTTCTCTTC) and then amplified the sequence encoding the zebrafish Eve using pCS2+Eve as template with primers dC9CS-F2 (GTGGAGGCCAGCggaggtacgcgttctagaaccatg) and dC9CSdr-R2 (agatctctcgagTCAgctagcgagctcTCTAGTTAG). Overlapping PCR was then performed using as template the mixture of the two purified PCR fragments described above with primers dC9KS-F1 and dC9CSdr-R2. After digestion with SphI and XhoI, 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 GTTAGTAGCCAAATAAGATTTGG, ACAACATCAGAAAAACATGG, and GCACTGTCCTGTACTTCCCATGG (the underlined sequences represent protospacer adjacent motifs).

To synthesize sgRNA *in vitro*, templates of sgRNA were amplified by PCR with pYSY-gRNA vector (YSY, China) using the method reported previously (35). sgRNAs 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 CTATTTTGTTGTATTGTTA (reverse) for *znfl1* (NM_194389), GAAGATCTTTACTATTCGCCCCTGAT (forward, the underlined sequences represent restriction site hereafter) and GGACTAGTGGTTTGGGAACAACTGGA (reverse) for *sall4* (NM_001080609), and GAAGATCTATGACGGAGAGAGCGCAGAGC (forward) and GGACTAGTTTAGCTGGTGAGATGACCC (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), *egr2b* (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 GACAATGAGGAGGGCTAT (forward) and AAACTGCTTGAACAGGTG (reverse) for *znfl1*, CTGAGTTCTCGCAGCGTTCT (forward) and TGGAAGTCGGCTGGCTAA (reverse) for *sall4*, CAGAGCCCAACAGCAGCAGA (forward) and GTGAGATGACCCACCAAACCAG (reverse) for *pou5f3*, TGGAACTGGGACAACAAG (forward) and AGACGAAGTGGAGGAAGC (reverse) for *hoxb1a*, and AGCTTCTTCTCGGTTGAT (forward) and GCTGTCCGAGAACGTTTG (reverse) for *hoxd4a.*

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 ATTTTGGGCTGCTAAGTT and GGGTCGTCCGAATTGATAT (for *sall4*) and TGTTACTTTGTGCCCTGCTC and CTTTCCGCTAAAAAGGTTGT (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 CTATCGATAGGTACCGAGCTCATTTTGGGCTGCTAAGTTCTATCGATAGGTACCGAGCTCGGGAAAACTCAGTCACTTC (forward) and ACTTAGATCGCAGATCTCGAGGGGTCGTCCGAATTGATAT (reverse) for *sall4* promoter and CTATCGATAGGTACCGAGCTCTGTTACTTTGTGCCCTGCTC (forward) and ACTTAGATCGCAGATCTCGAGCTTTCCGCTAAAAAGGTTGT (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 χ^2 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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