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Znfl1s are essential for patterning the anterior-posterior axis of zebrafish posterior hindbrain by acting as direct target genes of retinoic acid

Jingyun Li[b], Yingmin Zhao[a], Luqingqing He[c], Yun Huang[a], Xiaojing Yang[a], Lingling Yu[a], Qingshun Zhao[c,*], Xiaohua Dong[a,*]

[a] Department of Pediatric, Jingjiang People's Hospital Affiliated to Yangzhou University, Jingjiang 214500, China
[b] Maternal and Child Health Medical Institute, Women's Hospital of Nanjing Medical University (Nanjing Maternity and Child Health Care Hospital), Nanjing, China
[c] Model Animal Research Center, Nanjing University, Nanjing 210061, China

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ABSTRACT

RA (retinoic acid) signaling is essential for the patterning the hindbrain of vertebrates. Although hundreds of potential RA targets genes are identified, the ones other than *hox* genes playing roles in patterning anterior-posterior axis of hindbrain by mediating RA signaling remains largely unknown. Previously, we reported that *znfl1s* play essential roles in the formation of posterior neuroectoderm in zebrafish embryos. Here, we revealed that *znfl1s* play a critical role in patterning the posterior axis of hindbrain by maintaining the homeostasis of RA signaling in zebrafish embryos. Knocking down *znfl1s* shortened the length of the posterior hindbrain in a similar way of reducing RA signaling in zebrafish embryos and the defective posterior hindbrain was effectively rescued by elevating RA signaling. By performing mutagenesis assays and chromatin immunoprecipitation assays on the promoter of *znfl1s*, we demonstrated that *znfl1s* are direct target genes of RA to mediate RA signaling through a functional DR1 RA response element. Taken together, our results showed that Znfl1s are essential for patterning the anterior-posterior axis development of posterior hindbrain by acting as direct target genes of RA signaling.

1. Introduction

During vertebrate early development, three axes including anterior-posterior axis, left-right axis and dorsal-ventral axis are formed. Among them, the anterior-posterior axis occurs first during vertebrae embryogenesis (Kimelman and Martin, 2012). Coincident with the formation of anterior-posterior body axis, the neural ectoderm is patterned along its anteroposterior axis. The anterior and posterior neuroectoderm patterning is initiated by posteriorizing signals at gastrula (Kudoh et al., 2002).

RA (Retinoic acid) signaling is a posteriorizing signal which acts as an instructive morphogen for specifying posterior neuroectoderm, especially for patterning rhombomeric (r) formation of hindbrain (Begemann et al., 2001; Gale et al., 1999; Gavalas, 2002; Godsave et al., 1998; Grandel et al., 2002; Hernandez et al., 2007; Maden, 2002). It's well known that RA bioavailability are tightly regulated by RA synthesizing enzymes (*aldh1a*) and RA degrading enzymes (*cyp26*) in embryogenesis (Dobbs-McAuliffe et al., 2004; Reijntjes et al., 2005). Vitamin A deficiency in amniotes and the *Aldh1a2*$^{-/-}$ mouse display hindbrain defects (Gale et al., 1999; Niederreither et al., 2002). In zebrafish, the *aldh1a2* mutant *neckless* (*nls*) or *no fin* (*nof*) exhibits anteriorized phenotypes of spinal cord and hindbrain (Begemann et al., 2001; Grandel et al., 2002). Consistently, retinoid receptor antagonist AGN193109 causes a posterior hindbrain defect in vertebrates (Yoda et al., 2003). In contrast, embryos exposed to exogenous RA could divert anterior neural tissue to a more posterior fates (Lloret-Vilaspasa et al., 2010), a similar phenotype found in *Cyp26* deficient embryos with smaller hindbrain territory and a rostrally expanded spinal cord (Hernandez et al., 2007).

RA regulates the expression of its target genes by acting as a ligand of nuclear RA receptors (RARs) that bind to the cis-element of RA response element (RARE) in the promoters of the target genes. In the absence of RA, RARs act as active repressors of gene expression by recruiting co-repressors. When they couple with RA, RARs change their conformation to function as activators by releasing the bound co-repressors and recruiting co-activators (Niederreither and Dolle, 2008). *Hox* genes are regulated to express in the developing neural tube boundaries directly regulates by RA (Dupe et al., 1997; Gould et al., 1998; Marshall et al., 1994; Morrison et al., 1996; Studer et al., 1998). Anterior-posterior patterning of the hindbrain is mainly accomplished by dynamic expressions of *hox* genes that mediate RA signaling through RAREs in the regulatory region of their promoters (Studer et al., 1994).

* Corresponding authors.
E-mail addresses: qingshun@nju.edu.cn (Q. Zhao), njfishxiaohua@163.com (X. Dong).

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Although hundreds of potential RA targets genes are identified, the ones other than *hox* genes playing roles in patterning anterior-posterior axis of hindbrain by mediating RA signaling remains largely unknown.

Previously, we reported that zebrafish *znfl1s* (zinc finger-like gene 1) have 13 copies in the zebrafish genome and they play essential roles in the formation of posterior neuroectoderm in zebrafish gastrula. In this study, we demonstrate that *znfl1s* are involved in pattering the anterior-posterior axis formation of zebrafish posterior hindbrain through mediating RA signaling directly.

2. Results

2.1. Zebrafish znfl1s are essential for patterning the anterior-posterior axis of zebrafish posterior hindbrain

Previously, we demonstrated that *znfl1s* are essential for the specification of posterior neuroectoderm in zebrafish embryos (Dong et al., 2017). Because the posterior neuroectoderm gives rise to hindbrain and spinal cord (Yoda et al., 2003), we therefore asked whether *znfl1s* participate in patterning the anterior-posterior axis formation of hindbrain. To answer this question, we knocked down *znfl1s* through morpholino method (microinjecting embryos with antisense morpholino *znfl1s* MO to inhibit their translations of *znfl1s*' mRNAs) or CRISPRi method (microinjecting the embryos with dCas9-Eve mRNAs (guided by sgRNA) to inhibit the transcriptions of *znfl1s*) as we reported previously (Dong et al., 2017). The lengths of posterior part of zebrafish hindbrain were then measured in the knockdown embryos at 11-somite. As shown in Fig. 1, the distance between r6 and s1 (marked by *egr2b* and *myod1*, respectively) of the *znfl1s* morphants was 16.1% shorter than ($p < 0.01$) that of the control embryos (138.6 ± 5.5 vs 165.3 ± 6.4) (Fig. 1A–B, D). Consistently, the lengths of r6-s1 of the CRISPRi knockdown embryos was 26.5% shorter than ($p < 0.01$) that of the control ones (119.9 ± 12.5 vs 163.1 ± 8.7) (Fig. 1F–G, I). When the *znfl1s* MO or CRISPRi knockdown embryos were co-microinjected with *znfl1* mRNA, the length of r6-s1 was recovered from 83.9% to 95.7% (138.6 ± 5.5 vs 158.2 ± 5.8) ($p < 0.01$) in *znfl1s* MO knockdown embryos (Fig. 1B–C, D), or from 73.5% to 85.3% (119.9 ± 12.5 vs 139.2 ± 9.7) ($p < 0.01$) in *znfl1s* CRISPRi knockdown embryos (Fig. 1G–H, I), respectively. However, the length between r1-r4 (marked by the *en2a* (the midbrain-hindbrain boundary marker) and *egr2b*, respectively) of *znfl1s* knockdown embryos was similar ($p > 0.05$) to that of control embryos (175.9 ± 21.7 vs 164.5 ± 9.0, or 167.9 ± 10.5 vs 166.9 ± 12.4, $p > 0.05$) (Fig. 1A–C, E, F-H, J).

To confirm the defects of posterior hindbrain, we examined the distance between r7 and s1 (marked by *mafba* and *myod1*, respectively) in *znfl1s*-depleted zebrafish embryos. The results showed that the distance between r7 and s1 of the *znfl1s* morphants was 12.9% shorter than ($p < 0.01$) that of the control embryos (67.0 ± 5.5 vs 58.3 ± 7.4) (Fig. 1K–L, N). Consistently, the length of r7-s1 in the CRISPRi knockdown embryos was 17.3% shorter than ($p < 0.01$) that of the control ones (69.0 ± 6.4 vs 57.2 ± 5.6) (Fig. 1P–Q, S). When the *znfl1s* MO or CRISPRi knockdown embryos were co-microinjected with *znfl1* mRNA, the length of r7-s1 was recovered from 87.1% to 99.9% (67.0 ± 5.5 vs 66.9 ± 7.0) ($p < 0.01$) in *znfl1s* MO knockdown embryos (Fig. 1L–N), or from 82.7% to 98.2% (69.0 ± 6.4 vs 67.8 ± 6.5) ($p < 0.01$) in *znfl1s* CRISPRi knockdown embryos (Fig. 1Q–S), respectively. However, the length between r1-r4 (marked by *en2a* and *mafba*, respectively) of *znfl1s* knockdown embryos was similar ($p > 0.05$) to that of control embryos (87.6 ± 9.2 vs 89.2 ± 8.3, or 87.8 ± 7.1 vs 88.4 ± 5.8, $p > 0.05$) (Fig. 1K–M, O, P–R, T). Taken together, the results indicated that *znfl1s* are essential for patterning the posterior but not anterior hindbrain of zebrafish embryos.

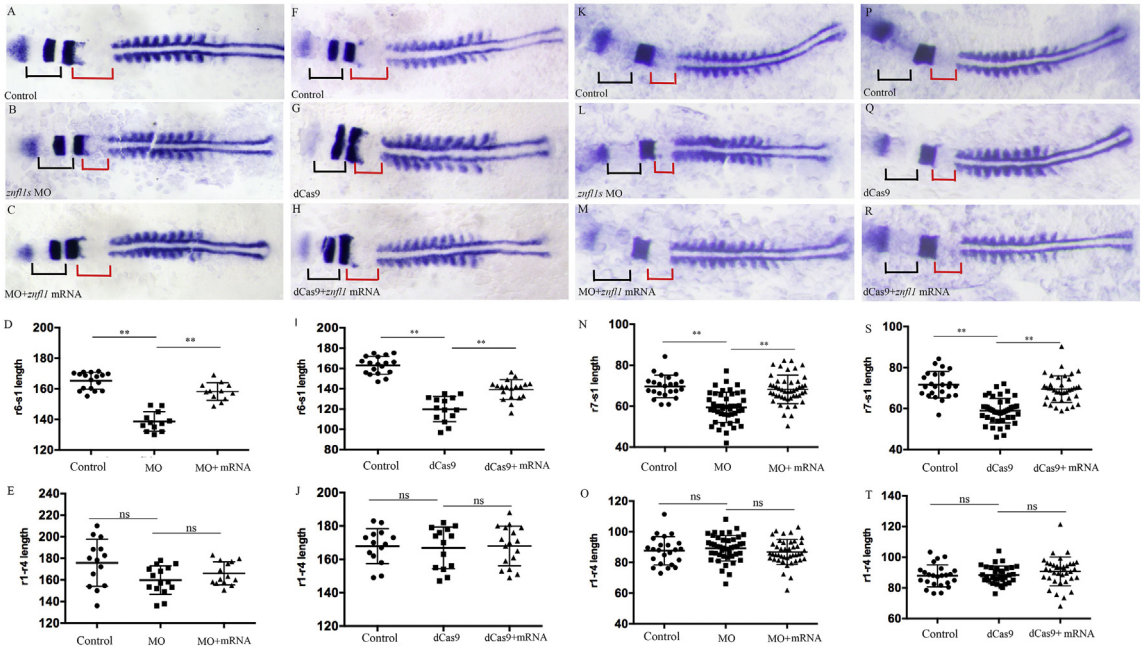

Fig. 1. Knocking down *znfl1s* disturbs the development of posterior but not anterior part of zebrafish hindbrain. Flat-mount embryos at 11–12-somites stage are shown anterior left (A–C, F–H, K–M, P–R). The expression of *en2a* marks the hindbrain-mindbrian boundary (A–C, F–H, K–M, P–R). The expression of *egr2b* marks r3/r5 territory (A–C, F–H). The expression of *mafba* marks the r5/r6 (K–M, P–R). The expression of *myod1* marks somite (A–C, F–H, K–M, P–R). At 11–12-somite stage, the lengths of r1–r4, r6–s1 and r7–s1 were measured from control embryos (A, F, K, P), *znfl1s* morphants (B, L), *znfl1s* MO plus *znfl1* mRNA overexpressed embryos (C, M), dCas9-Eve knockdown embryos (G, Q) and dCas9-Eve plus *znfl1* mRNA overexpressed embryos (H, R), respectively. The data about the lengths of r1–r4, r6–s1 or r7–s1 derived from A–C, F–H or K–M, P–R are shown in scatter plot diagrams D–E, I–J, N–O and S–T, respectively. The red line in A–C, F–H or in K–M, P–R shows the length of r6–s1 or r7–s1, respectively. The black line in A–C, F–H or in K–M, P–R shows the length of r1–r4. r, rhombmere; s1, the first somite. **: $p < 0.01$, ns: no significance.

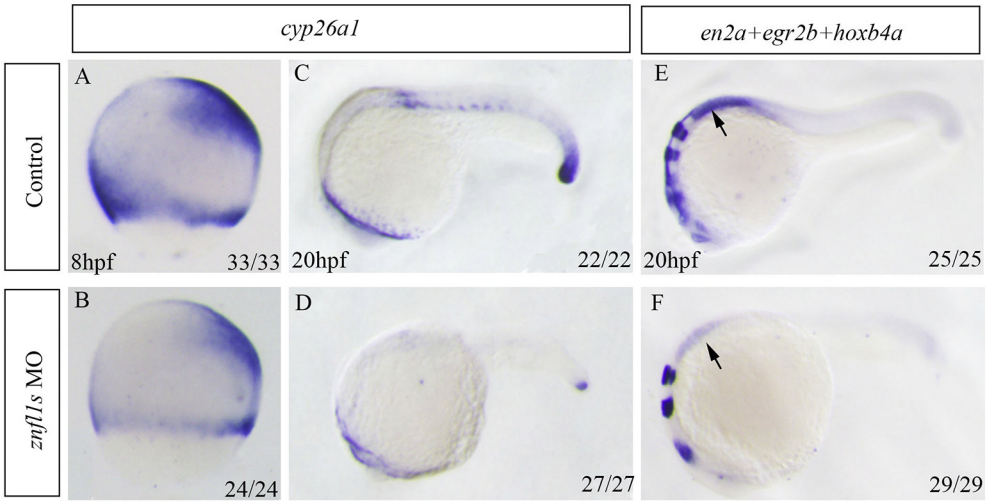

Fig. 2. Knocking down *znfl1s* reduces RA signaling in zebrafish embryos. Whole mount in situ hybridized embryos are positioned animal pole top (8 hpf, A–B) or anterior left (20 hpf, C–F). The expression of *cyp26a1* was examined in controls (A, C) and *znfl1s* morphants (B) at 8 hpf or 20 hpf (D). The expressions of *en2a*, *egr2b* and *hoxb4a* in control embryos (E) and *znfl1s* morphants (F). All embryos were positioned in lateral view. The arrow points to the expression of *hoxb4a* in E–F.

2.2. Knocking down znfl1s reduces the level of retinoic acid signaling in zebrafish embryos

RA signaling is particularly important for patterning the anterior-posterior axis of vertebrate hindbrain (Begemann et al., 2001; Hernandez et al., 2007). The phenotype of shortened lengths of r6-s1 or r7-s1 in *znfl1s* knockdown embryos were very similarly to the phenotype resulting from decreasing RA signaling (Xu et al., 2009). To figure out whether knocking down *znfl1s* disrupted posterior hindbrain pattern is due to changing RA signaling, we examined the expressions of *cyp26a1* and *hoxb4a* which are RA direct target genes, the indicators of RA homeostasis in zebrafish embryos. As shown in Fig. 2, the expression of *cyp26a1* in anterior neuroectoderm and mesoderm was significantly down-regulated in *znfl1s* morphants than the controls at 8 hpf (Fig. 2A–B). Consistent with the changes of *cyp26a1* at 8 hpf, the expression of *cyp26a1* was dramatically reduced in *znfl1s* knockdown embryos at 20 hpf (Fig. 2C–D). Similarly, the expression of *hoxb4a* was also significantly down-regulated in *znfl1s* morphants compared with those controls (Fig. 2E–F). The results suggest that knocking down *znfl1s* decreased RA signaling in zebrafish embryos.

2.3. Increasing RA signaling resumes the length of posterior hindbrain in znfl1s knockdown zebrafish embryos

Now that knocking down *znfl1s* reduces RA signaling in zebrafish embryos, we therefore asked whether increasing RA signaling could rescue the posterior hindbrain defect of the knockdown embryos. To answer the question, we compared the r6-s1 lengths of the *znfl1s* knockdown embryos with that of knockdown embryos administrated with 10 nM RA (Xu et al., 2009) at 11–12 somite stage or that of *cyp26a1* morphants. As shown in Fig. 3, the r6-s1 length of the 10 nM treated embryos is 107.1% longer ($p < 0.05$) than that of control embryos (173.5 ± 21.1 vs 162.0 ± 15.3) (Fig. 3A, C, E). Consistently, the r6-s1 length of the *cyp26a1* morphants is 115.6% longer ($p < 0.01$) than that of the control embryos (178.0 ± 17.3 vs 153.9 ± 14.2) (Fig. 3F, H, J). The results were consistent with previous studies (Hernandez et al., 2007; Xu et al., 2009). On the other hand, the r6-s1 length of *znfl1s'* morphants was 85.2% (138.1 ± 22.5 vs 162.0 ± 15.3) (Fig. 3A–B, E) or 84.3% (138.2 ± 14.2 vs 153.9 ± 14.2) (3F–G, J) shorter than that of control embryos. The shortened length was rescued from 85.2% to 101.9% (138.1 ± 22.5 vs 157.2 ± 14.9) of control embryos ($p < 0.01$) when the morphants were administrated with 10 nM RA (Fig. 3A–E), or resumed from 84.3% to 97.0% (138.2 ± 14.2 vs 156.9 ± 23.4) ($p < 0.01$) when the morphants were co-microinjected with *cyp26a1* MO (Fig. 3F–J). Taken together, the results demonstrate that the shorten posterior part of hindbrain in *znfl1s* morphants could be rescued by increasing RA signaling.

2.4. Zebrafish znfl1s are direct response to RA signaling during gastrulation

The reduced RA signaling in *znfl1s'* knockdown zebrafish embryos suggests that *znfl1s* play a role in mediating RA signaling in hindbrain patterning. We therefore asked that whether *znfl1s* mediate RA signaling directly or indirectly to pattern posterior hindbrain. To answer the question, we detected the expressions of *znfl1s* in the embryos treated with 1 μM RA or 10 μM DEAB from 0 hpf to 8 hpf. As shown in Fig. 4, the expression of *znfl1s* were strongly up-regulated in response to exogenous RA (Fig. 4B), whereas the expression of *znfl1s* were dramatically down-regulated in the DEAB treated embryos (Fig. 4C), as compared to control embryos (Fig. 4A). Consistent with the results from altering exogenously RA signaling, increasing the endogenous RA signaling by knocking down *cyp26a1* (RA degradation enzyme) slightly increased the expressions of *znfl1s* in the morphants at 8 hpf (Fig. 4G), whereas reducing the endogenous RA signaling by knocking down *aldh1a2* (RA synthesis enzyme) significantly reduced the expressions of *znfl1s* at 8 hpf (Fig. 4H), as compared to control embryos (Fig. 4F). When 10 μM CHX was co-incubated the embryos treated with 1 μM RA, *znfl1s* were still increased their expressions ectopically in the embryos at 8 hpf (Fig. 4D–E) like the ones treated with 1 μM RA alone (Fig. 4B). The results indicated that *znfl1s* mediate the RA signaling directly, or are the direct target genes of RA.

2.5. Zebrafish znfl1s' promoters contain a functional RARE to response to RA signaling

It is known that the direct target genes of RA signaling have RAREs in their promoters in response to RA (Li et al., 2012). Searching the consensus RARE (5′-PuG(G/T)TCA-3′) in the 2339 bp promoter sequence of *znfl1* (ENSDARG00000037914) using MatInspector (http://www.genomatix.de), we found that a candidate RARE site is not only present in the promoter of *znfl1* (5′-TGACCTGTGCCTC-3′), positioning from −1924 to −1911 (the 1st nucleotide of start codon was named +1), but also conserved in the promoters of all the other 12 *znfl1s* in zebrafish genome (Fig. 5A). Performing the dual luciferase reporter assays on the promoter of *znfl1* (Fig. 5B), we demonstrated that 10 nM RA could remarkably ($p < 0.01$) elevated the activity of *znfl1* promoters (Fig. 5C). When the core sequences of RARE were mutated, the promoter exhibited no response ($p > 0.05$) to 10 nM RA (Fig. 5C). The results indicate that the core sequence is critical for *znfl1s'* promoters to respond to RA signaling. To further verify the presence of functional RARE in the promoter of *znfl1s* in vivo, we performed ChIP assays on

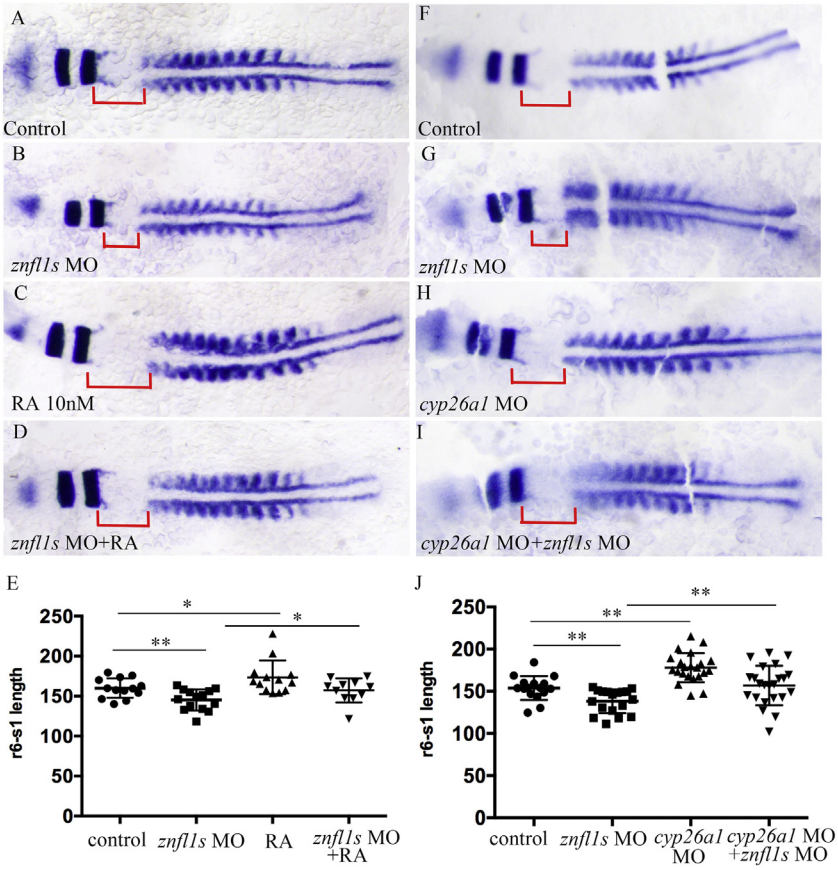

Fig. 3. Increasing RA signaling rescues the defective posterior hindbrain of *znfl1s* morphants. Flat mount embryos at 11–12-somites stage are shown anterior left (A–D, F–I). At 11–12 somite stage, the length of r6-s1 were measured from the control embryos (A, F), *znfl1s* morphants (B, G), 10 nM RA treated embryos (C), *znfl1s* MO microinjected plus RA treated embryos (D), *cyp26a1* morphants (H), *cyp26a1* MO plus *znfl1s* MO microinjected embryos (I). The data about the length of r6-s1 derived from A–D and F–I are shown in scatter diagrams E and J, respectively. The red line shows the length of r6-s1. $^{**}p < 0.01$; $^{*}p < 0.05$.

the chromatin of zebrafish embryonic cells by overexpressing Myc-tagged *RARαb* as we previously reported (Li et al., 2012). The results showed that Myc-tagged zebrafish *RARαb* was significantly ($p < 0.01$) enriched in the −1945 to −1881 region of the *znfl1* promoter but not ($p > 0.05$) in the control region (−1051 to −916) (Fig. 5D). Taken together, our results demonstrate that *znfl1s* are direct target genes of RA signaling in zebrafish embryos.

3. Discussion

Zebrafish *znfl1s* encode CCHC-type zinc finger transcription factors. Previously, we found that the hindbrain lengths of r1-r8 were significantly reduced in the *znfl1s* knockdown embryos at 24 hpf (Dong et al., 2017). In this study, we demonstrated that the *znfl1s*-depleted embryos showed defects in the posterior of hindbrain, namely shortened length of r6-s1 and r7-s1 at 11-somite stage (Fig. 1). However, the length of anterior hindbrain (r1-r4) was unchanged in *znfl1s* knockdown embryos (Fig. 1). The results suggest that the development of posterior hindbrain required *znfl1s* while the development of anterior hindbrain is independent of *znfl1s*. In other words, Znfl1s play different roles in the development of anterior hindbrain and posterior hindbrain in zebrafish embryos.

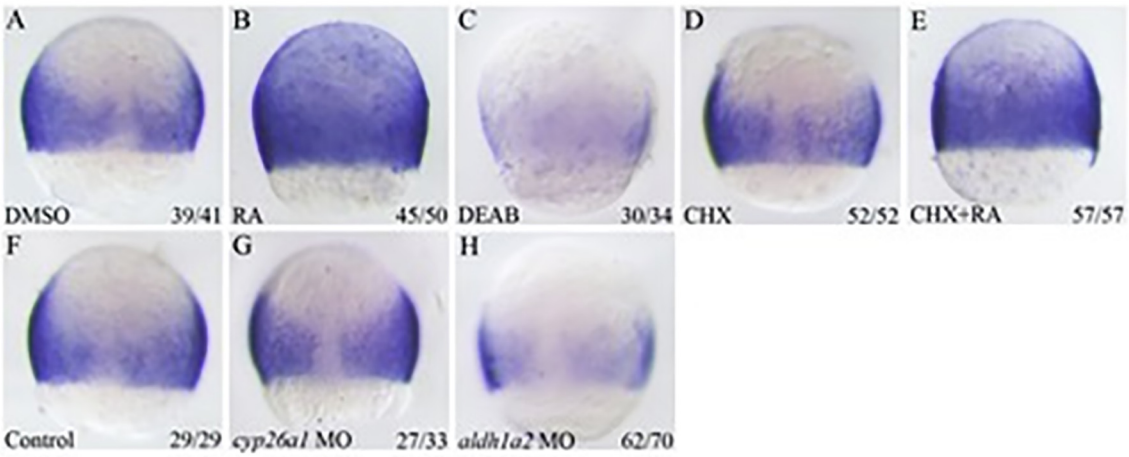

Fig. 4. Zebrafish *znfl1s* are directly regulated by RA signaling in gastrula. The expressions of *znfl1s* were present in control embryos (A), RA treated embryos (B), DEAB treated embryos (C), CHX treated embryos (D), CHX combined with RA treated embryos (E), control MO microinjected embryos (F), *cyp26a1* knocked down embryos (G) and *aldh1a2* knockdown embryos (H). All embryos were positioned in dorsal view, animal pole top. The number in the right-hand lower corner shows the number of embryos with the typical phenotype to that of total embryos examined.

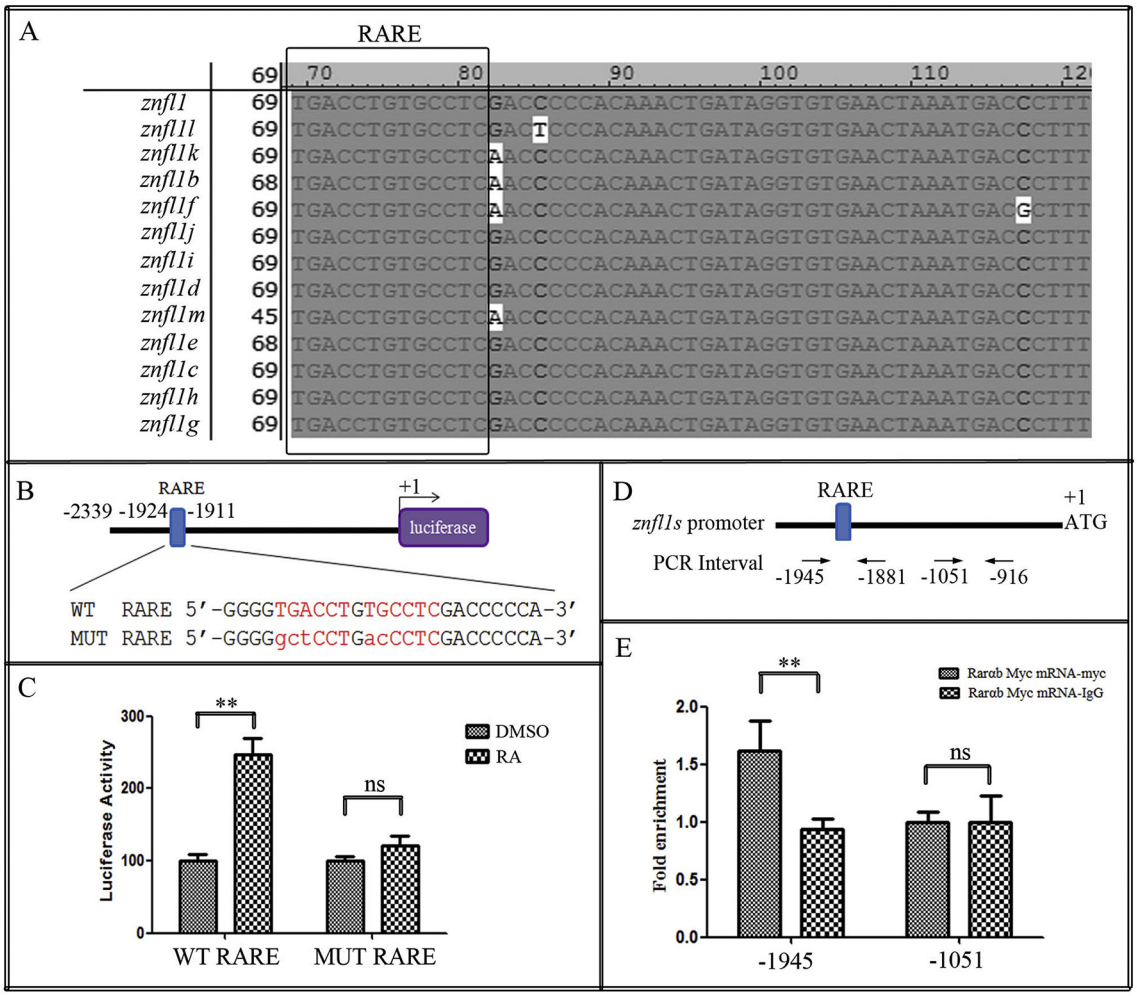

Fig. 5. Dual luciferase reporter assay and ChIP analysis show that *znfl1s* are direct target genes of RA signaling in zebrafish. (A) Sequences of RARE are conserved the same in the 13 zebrafish *znfl1s'* promoters. The core sequences of RARE are marked in frame. (B) Schematic diagram showing the different promoters of *znfl1* driving firefly luciferase reporter gene. Arrow denotes the site where translation starts. WT RARE: the promoter with wild-type RARE; MUT RARE: the promoter with mutated RARE. (C) Dual luciferase activity assay showing the RARE in *znfl1s'* promoters is required for RA inducibility. (**$p < 0.01$; ns: $p > 0.05$). (D) Schematic diagram showing the PCR fragment location for ChIP analysis. (E) ChIP assay showing the fold enrichment of zebrafish RARαb binding to *znfl1s'* RAREs.

Hindbrain is derived from the posterior neuroectoderm (Stern, 2006). Results from our previous research showed that knocking down *znfl1s* significantly reduced the posterior neuroectoderm marked by *hoxb1b* in the zebrafish gastrula (Dong et al., 2017). Zebrafish *hoxb1b* is the earliest gene that is expressed in the posterior neuroectoderm of gastrula (Kudoh et al., 2002). It shares ancestral functions with mammalian Hoxa1 and controls progenitor cell shape and oriented cell division during anterior hindbrain neural tube morphogenesis (Zigman et al., 2014). Mice lacking Hoxa1 exhibit defects in hindbrain segmentation whereas Hoxb1-null mice do not manifest defects in early hindbrain patterning (Philippidou and Dasen, 2013). Consistently, we found in this study that the expressions of *en2a* (maker for the midbrain-hindbrain boundary) and *egr2b* (marker for r3/r5) were normal in *znfl1s* knockdown embryos (Fig. 1). Therefore, the reduced posterior neuroectoderm does not mean the increased expression of forebrain markers and decreased expression of anterior hindbrain makers and the defective posterior hindbrain in *znfl1s*-depleted embryos is not necessary to be the secondary effects of the posterior neuroectoderm defects.

RA signaling plays an important role in patterning hindbrain (Gale et al., 1999; Hernandez et al., 2007) by controlling gene expressions through binding to its cognate nuclear receptors (RARs) heterodimerized with RXRs that recognize the cis-element of RAREs lying in the promoters of its direct target genes (Samarut et al., 2015). To date, hundreds of RA direct targets have been demonstrated containing RAREs on their promoter/enhancer region in response to RA signaling. Among them, several *hox* genes are reported to be involved in patterning hindbrain. However, whether others than hox genes mediating RA signaling to pattern hindbrain remains to be determined. In this study, we demonstrated the expressions of RA direct target genes such as *cyp26a1* and *hoxb4a* were significantly reduced in the *znfl1s'* knock down embryos (Fig. 2). The results indicate that *znfl1s* are involved in maintaining RA homeostasis in zebrafish embryos at early development. Actually, the shortened length of r6-s1 or r7-s1 was resumed to normal in the *znfl1s'* knockdown embryos when 10 nM exogenous RA was administrated or *cyp26a1* was knocked down (Fig. 3). Performing dual luciferase activity assay and ChIP assay on the promoter of *znfl1s* and ChIP assay, we provided evidences that RARs regulate the expression of *znfl1s* by binding the RARE core sequences in the promoters of *znfl1s* directly (Fig. 5). Taken together, our results show that *znfl1s* are involved in hindbrain pattering by acting as direct target genes. Therefore, the administrated exogenous RA resumed the defective posterior hindbrain in *znfl1s*-depleted embryos through either increasing the expressions of *znfl1s* which cannot be efficiently knocked down by *znfl1s*-MO or CRISPRi any more due to too much amount of the induced expressions or the expressions of other genes that are involved in the development of posterior hindbrain.

RAREs typically comprise hexameric direct repeats (DRs)-PuG(G/T)TCA - with interspacing of 1 bp (DR1 elements), 2 bp (DR2 elements) or 5 bp (DR5 elements) (Cunningham and Duester, 2015). Among them, DR2 and DR5 sequences but not DR1 have been identified as functional RAREs in vivo (Cunningham and Duester, 2015). For example, zebrafish *hoxd4a* has a highly conserved DR5 type RARE which is necessary for *hoxd4a* neural enhancer activity (Nolte et al., 2003) and *cyp26a1* has 3 conserved DR5 type RARE which are direct response to RA signaling (Hu et al., 2008; Li et al., 2012). *Hoxb1* promoter regional contains a DR2 RARE and a DR5 RARE which are required for response to RA signaling (Huang et al., 2002; Marshall et al., 1994; Studer et al., 1994). However, in this study, we provided evidences of promoter mutagenesis and ChIP assay demonstrating that *znfl1s'* promoter has a functional DR1 RARE that is in response to RA signal directly (FIG. 5).

In summary, we identified *znfl1s* as new RA direct target genes which participate in patterning the posterior hindbrain of zebrafish embryos.

4. Experimental procedures

4.1. Zebrafish maintenance

Zebrafish were raised in the zebrafish facility of Model Animal Research Center, Nanjing University, in accordance with IACUC-approved protocol. The embryos were maintained at 28.5 °C and staged as previously described (Kimmel et al., 1995).

4.2. Pharmaceutical treatment of zebrafish embryos

All-trans retinoic acid (RA), 4-diethylamino benzaldehyde (DEAB, inhibitor of Aldh1a), dimethyl sulfoxide (DMSO) and cycloheximide (CHX, inhibitor of protein synthesis) were purchased form Sigma (USA). Because RA is a teratogen, overdosing RA causes a lot of toxic effects on embryonic development. Therefore, we chose low concentration of RA (10 nM) to treat zebrafish embryos from 0 hpf to 14 hpf for increasing RA signaling and performing rescues experiment of defective posterior hindbrain as reported previously (Li et al., 2016; Xu et al., 2009) whereas we used 1.0 μM RA to treat embryos from 0 to 8 hpf for increasing RA signaling and 10.0 μM DEAB for reducing RA signaling to examine the response of *znfl1s* to the changes of RA signaling (Hu et al., 2008; Li et al., 2012). Additionally, 10.0 μg/ml CHX were used to treat zebrafish embryos from 7 hpf to 8 hpf for inhibiting the protein synthesis following the instructions of the manual. The final concentration of DMSO (vehicle) in each treatment and control was 0.1%.

4.3. Microinjection of morpholinos into zebrafish embryos

Morpholinos (MOs) were purchased from Gene Tools (http://www.gene-tools.com). *znfl1s* MO is to block the translation of *znfl1s* mRNA. The sequences of *znfl1s* MO were AATGGTAACACATGGAGGTTCTGTT (Dong et al., 2017). Zebrafish *aldh1a2* and *cyp26a1* were knocked down using the MO as previously described (Liang et al., 2012). The sequences of MOs are GCAGTTCAACTTCACTGGAGGTCAT (*aldh1a2* MO) and CGCGCAACTGATCGCCAAAACGAAA (*cyp26a1* MO), respectively. The sequences of control MO were CCTCTTACCTCAGTTACAATTTATA as described previously (Liang et al., 2012).

MO was dissolved in ultrapure water and microinjected into the embryos at 1–2-cell stage. The amount of MO microinjected into per embryo was about 1 nl solution containing 4 ng *znfl1s* MO, 4 ng *aldh1a2* MO or 1 ng *cyp26a1* MO and the equal amount of control MO, respectively.

4.4. In vitro synthesis of mRNA or sgRNA and microinjection of RNAs into zebrafish embryos

dCas9-Eve mRNA, *znfl1s* mRNA and sgRNA were synthesis as we previously reported (Dong et al., 2017). About 1 nl of 100 ng/μl *znfl1s* mRNA, and 100 ng/μl sgRNA plus 250 ng/μl dCas9-Eve mRNA were injected into zebrafish embryos at 1–2-cell stage.

4.5. Whole mount in situ hybridizations

Whole mount in situ hybridizations were performed as we described previously (Dong et al., 2017). The template for making RNA probe to examine the expressions of *en2a*, *egr2b*, *myod1*, *hoxb4a*, *mafba*, *cyp26a1* and *aldh1a2* were prepared as described previously (Xu et al., 2009). Flat mount in situ hybridizations were performed as previously reported (Xu et al., 2009).

4.6. Measurement of hindbrain length

The lengths of hindbrain were measured in the flat mount zebrafish embryos. ImageTool software were used for measuring the length of hindbrain. The length unit was arbitrary.

4.7. Promoter cloning and mutagenesis of znfl1 promoter

The 2339 bp of *znfl1* promoter (ENSDARG00000037914) was cloned by PCR with the forward primer (GGGAAAACTCAGTCACTTC) and the reverse primer (GTTGTTCTCAGGGTAGCTC). Briefly, the genomic DNA templates were prepared from 5 embryos randomly selected by incubating the embryos with 10 μl B solution under a program (65 °C for 30 min, 95 °C for 10 min, and 16 °C for 1 min) following the manufacturer's instruction (Nanjing YSY Biotech, China). Overlapping PCR were used to mutate the RARE in the *znfl1* promoter by primer pairs of the forward one (GGgctCCTGacCCTCGACCCCCACAAACTG ATA) and the reverse one (GAGGgtCAGGagcCCCCCCATCATATCACA TCA) (The lowercase letter denotes the mutated nucleotides). The PCR reactions were conducted with BD Advantage HF2 (Takara, Japan). The PCR conditions were 94 °C 1 min, 30 cycles of (94 °C 30 s, 58 °C 45 s, 68 °C 4 min), followed by a 68 °C 6-min extension. All the PCR products were subcloned into pGEM-T easy vector (Promega, USA). The amplified promoters (WT RARE-*znfl1s*, MUT RARE-*znfl1s*) were sequenced to confirm their identities and then recombined into pGL3 basic luciferase reporter vector (Promega, Madison, WI, USA) using One Step Cloning Kit (Vazyme, China) with primers CTATCGATAGGTACCGAGCTCGGG AAAACTCAGTCACTTC (forward) and ACTTAGATCGCAGATCTCGAG GTTGTTCTCAGGGTAGCTC (reverse).

4.8. Dual luciferase assay on the promoter's activity

Dual luciferase assays were performed on zebrafish embryos as reported previously (Li et al., 2012) using a commercial Dual Luciferase Reporter Kit (Promega, USA). About 100 ng/μl pGL3-*znfl1* (WT RARE), pGL3-*znfl1* (MUT RARE) and 2 ng/μl *Renilla* luciferase expression vector were used. Relative luciferase activity was described the fold change of each treatment to control experiment. Each treatment was repeated three times with independent microinjection experiments.

4.9. Chromatin Immunoprecipitation (ChIP) assay

ChIP assay was performed as we previously described (Li et al., 2012). Zebrafish Myc-RARαb and RXRαb were used the same as we described previously (Li et al., 2012). The IP DNA was amplified by real-time PCR with primers (CTCTGATGTGATATGATGGG and CTCAG CAGGACCCTCTTCAG) for detecting the zebrafish *znfl1s* promoter encompassing the RARE (−1945 to −1811, 135 bp), and with primers (TAATTTGGTCCATTTCCC and AAGGCTCTGTTACCTTCA) for negative

control (−1051 to −916, 136 bp). The relative enrichment of RARαb on RARE or negative control was calculated using Fold Enrichment Method (Invitrogen, USA) by normalizing the PCR signals obtained from ChIP with anti-Myc Tag antibody to the signals obtained from control ChIP with mouse IgG.

4.10. Statistical analysis

Data were analyzed using the SPSS 20.0 software package (SPSS, Chicago, IL, USA) with an independent-samples *t*-test between two groups. All values were represented as mean ± standard deviation (SD) from at least three independent experiments. Statistical significance was defined as $p < 0.05$, or $p < 0.01$.

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Conflict of interest

The authors declare that they have no competing financial interests.

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