Published in final edited form as:

Genesis. 2017 December ; 55(12): . doi:10.1002/dvg.23082.

Znf703, a novel target of Pax3 and Zic1, regulates hindbrain and neural crest development in *Xenopus*

Chang-Soo Hong^{(a),(b)} and Jean-Pierre Saint-Jeannet^{(b),*}

^(a)Department of Biological Sciences, College of Natural Sciences, Daegu University, Gyeongsan, Republic of Korea

^(b)Department of Basic Science & Craniofacial Biology, College of Dentistry, New York University, New York, USA

Abstract

The transcription factors Pax3 and Zic1 are critical to specify the neural plate border and to promote neural crest formation. In a microarray screen designed to identify genes regulated by Pax3 and Zic1 in *Xenopus* we isolated Znf703/Nlz1 a transcriptional repressor member of the NET protein family. At early neurula stage *znf703* is expressed in the dorsal ectoderm, spanning the neural plate and neural plate border, with an anterior boundary of expression corresponding to rhombomeres 3 and 4 (r3/r4) in the prospective hindbrain. As a bonafide target of Pax3 and Zic1, *znf703* is activated by neural plate border inducing signals, and its expression depends on Pax3 and Zic1 function in the embryo. Znf703 morpholino-mediated knockdown expanded several posterior hindbrain genes, while Znf703 overexpression completely obliterated the expression of these segmental genes, signifying that the transcriptional repressor activity of Znf703 is critical to pattern the hindbrain. Furthermore, *snai2* and *sox10* expression was severely impaired upon manipulation of Znf703 expression levels in the embryo suggesting that Znf703 participates in neural crest formation downstream of Pax3 and Zic1 in *Xenopus*.

Keywords

znf703; pax3; zic1; hindbrain; neural crest; *Xenopus*

Introduction

In vertebrate embryos, the neural crest is specified at the lateral edge of the neural plate, in a region of the ectoderm known as the neural plate border (Huang and Saint-Jeannet, 2004). As the neural plate folds into a tube, neural crest progenitors occupy the most dorsal aspect of the prospective spinal cord, while in the head region they are primarily associated with the developing midbrain, and segmental units of the hindbrain known as rhombomeres (r).

The gene regulatory network underlying neural plate border formation involves a complex interplay of transcription factors, which impart a unique molecular identity to the neural

*Correspondence: Jean-Pierre Saint-Jeannet, New York University, College of Dentistry, Department of Basic Science & Craniofacial Biology, 345 East 24th Street, New York, NY 10010 - USA, tel: 212-998-9978, jsj4@nyu.edu.

crest (Sauka-Spengler and Bronner-Fraser, 2008; Betancur et al., 2010; Simões-Costa and Bronner, 2015). Among these factors, Pax3 and Zic1 are broadly expressed at the neural plate border, and have proven to be especially critical to specify the neural crest as they are both necessary and sufficient to activate a neural crest developmental program (Monsoro-Burq et al., 2005; Sato et al., 2005; Hong and Saint-Jeannet, 2007; Gutkovich et al., 2010; Garnett et al., 2012; Milet et al., 2013).

A few years ago, using hormone inducible fusion proteins in a *Xenopus* animal cap assay, and a microarray-based approach we and others have identified several targets of Pax3 and Zic1, which significantly expanded the number of potential players in the gene regulatory network underlying neural crest development (Bae et al., 2014; Plouhinec et al., 2014). We have previously reported the expression of two of these transcriptional targets, *tfap2e* and *egr4*, and described their unique function during neural crest and hindbrain development, respectively (Hong et al., 2014; Bae et al., 2015).

Here we report the expression and function of another target of Pax3 and Zic1 in *Xenopus* ectoderm, *znf703/nlz1*, which encodes a member of the NET family of zinc finger proteins (Nakamura et al., 2004; Pereira et al., 2016). At early neurula stage *znf703* is expressed in the dorsal ectoderm, including the neural plate and neural plate border, overlapping with the expression domain of *snai2* in the neural crest and *fgf3* and *mafB* in the posterior hindbrain. The role of Znf703/Nlz1 during hindbrain development has been well documented in zebrafish where it is required for r4 specification (Runko and Sagerström, 2003; Hoyle et al., 2004; Nakamura et al., 2008). Using gain- and loss-of-function approaches we show that Znf703 has a conserved function during hindbrain patterning in *Xenopus*, moreover we show that it is also essential for the specification of neural crest progenitors downstream of Pax3 and Zic1.

Results

Xenopus laevis znf703.S

znf703.S was isolated in a screen designed to identify targets of Pax3 and Zic1, two transcription factors necessary and sufficient to specify the neural plate border in *Xenopus* (Monsoro-Burq et al., 2005; Sato et al., 2005; Hong and Saint-Jeannet, 2007). This screen was performed using hormone inducible fusion proteins (Pax3GR and Zic1GR) in a *Xenopus* animal cap assay, and DNA microarray (Bae et al., 2014). *znf703* was also isolated in a recent genome-wide study performed to identify direct transcriptional targets of the Wnt/βcatenin signaling pathway in *Xenopus* (Kjolby and Harland, 2017). Znf703 belong to the NET (for NocA/Nlz, Elbow, TLP-1) protein family, characterized by a single atypical C₂H₂ zinc finger at the C-terminal (Nakamura et al., 2004; Pereira et al., 2016). *Xenopus laevis* znf703 possesses an open reading frame encoding 531 amino acids (Fig. 1). At the amino acid level, *Xenopus laevis* Znf703.S shares 62% identity with human ZNF703 (NP_079345.1), 62% identity with mouse Znf703 (NP_001094972.1) and 59% identity with zebrafish Znf703 (NP_571897.1).

Developmental expression of *znf703*

To evaluate the expression of *znf703*, we performed whole-mount *in situ* hybridization on embryos at different stages of development. At gastrulation *znf703* is first detected around the blastopore (not shown; Hoyle et al., 2004) in a pattern resembling *wnt8* expression domain, consistent for a gene target of canonical Wnt signaling pathway (Kjolby and Harland, 2017). By stage 13/14, *znf703* is strongly expressed in the dorso-lateral ectoderm, spanning the neural plate and neural plate border, and extending ventrally in a gradient of expression. At this stage *znf703* is restricted posteriorly to the prospective hindbrain and spinal cord, and excluded from the dorsal midline (Fig 2A–D; Li et al. 2017). At later stages, *znf703* expression progressively decreases in the lateral ectoderm and remains confined dorsally to the neural crest and neural plate/tube, as well as two discrete patches of expression in the prospective midbrain (Fig 2E–J). Histological sections at the neurula stage indicate that in addition to the ectoderm, *znf703* is expressed in the lateral plate mesoderm (Fig 2G). At the tailbud stage (NF stage 25) *znf703* is diffusely expressed in the ectoderm, and slightly enriched in the hatching gland and the branchial arches (Fig 2K–M).

In order to more precisely map the anterior expression domain of *znf703*, we performed double *in situ* hybridization at stage 15 using a combination of digoxigenin-labeled RNA probes encoding genes expressed in the ectoderm, including *fgf3* (hindbrain r3/r4; Lombardo et al., 1998) *mafb/kreisler* (hindbrain r5/r6; Ishibashi and Yasuda, 2001), and *snai2* (neural crest; Mayor et al., 1995). The most lateral domain of *znf703* overlaps with *snai2* demonstrating that *znf703* is expressed in a subpopulation of neural crest progenitors (Fig 3B). However, *znf703* does not overlap with the most anterior domain of *snai2* (Fig 3B), which corresponds to the mandibular segment of the neural crest (Sadaghiani and Thiebaud, 1987). The expression of *mafb* is restricted to the posterior hindbrain (r5/r6), and lays immediately posterior to *znf703* anterior most boundary of expression, and overlaps completely with *znf703* (Fig 3C). Spatially, the anterior most boundary of *znf703* expression overlaps with *fgf3* expression domain in r3/r4 (Fig 3D). Taken together, these results indicate that *znf703* is expressed at the neural plate border, overlapping with the neural crest territory, and in the prospective spinal cord and hindbrain starting at the level of r3/r4, in a pattern highly conserved with its zebrafish ortholog (Runko et al., 2003; Hoyle et al., 2004), and suggesting an important role in both neural crest formation and hindbrain patterning.

Regulation of *znf703* expression

As a target of Pax3 and Zic1, *znf703* expression follows a pattern of activation similar to that of *snai2*; it is induced by Pax3 in animal cap explants, and this induction is enhanced by the expression of Zic1 (Fig 4A, B; Bae et al., 2014). The expression of *znf703* in the embryo is also expected to depend on Pax3 and Zic1 activity. To test this possibility we interfered with Pax3 or Zic1 function by injection of well-characterized MOs at the 2-cell stage (Monsoro-Burq et al., 2005; Sato et al., 2005; Hong and Saint-Jeannet, 2007). As previously described both MOs interfered with *snai2* expression (Hong and Saint-Jeannet, 2007). We also found that approximately 90% of the embryos injected with either pax3MO or zic1MO had a reduction of *znf703*, however unlike *snai2*, this effect was limited to the anterior domain of *znf703* expression (Fig 4C, D). These results further validate *znf703* as a genuine downstream target of Pax3 and Zic1.

We used an animal cap explant assay to further analyze the regulation of *znf703* expression by signals critical to specify the neural crest (Fig 5A; Stuhlmiller and Garcia-Castro, 2012; Bae and Saint-Jeannet, 2014). Activation of the Wnt/ β -catenin pathway in conjunction with attenuation of Bmp signaling is sufficient to induce neural crest genes (*snai2*) and repress epidermal fate (*keratin*) in naïve ectoderm (Fig 5B; Saint-Jeannet et al., 1997; LaBonne and Bronner-Fraser, 1998). As previously reported, this activity depends on Pax3 activity, as Pax3 knockdown represses *snai2* expression and promotes neural (*sox2*) fate (Fig 5B; Hong and Saint-Jeannet, 2007). We found that *znf703* was strongly upregulated by Noggin and Wnt1, similar to *snai2*, however upon Pax3 knockdown *znf703* induction appeared largely unaffected. This result reflects the dual expression of *znf703* in the neural crest and the neural plate (prospective hindbrain and spinal cord), two tissues that are differentially regulated by Wnt and Bmp signaling (Stuhlmiller and Garcia-Castro, 2012; Bae and Saint-Jeannet, 2014).

znf703 regulates neural crest formation and hindbrain patterning

To evaluate Znf703 function we performed knockdown of Znf703 protein using antisense oligonucleotides and analyzed the consequences on the expression of several hindbrain- and neural crest-specific genes at the neurula stage (NF stage14/15). A Znf703 morpholino (Znf703MO) was designed to specifically interfere with translation of *Znf703* mRNA (Fig 6A). In an *in vitro* transcription/translation assay increasing doses of Znf703MO blocked Znf703 protein production (Fig 6B). Unilateral injection of *znf703*MO (45 ng) in the animal pole region at the 2-cell stage resulted in a posterior expansion of several hindbrain markers including *erg4* (r5/r6), *hoxb1* (r4) and *fgf3* (r3/r4) (Fig 6C–E). These genes were not only expanded, their anterior boundary of expression was also shifted posteriorly. In contrast the expression of *mafb* (r5/r6) and *egr2* (r3/5) was significantly reduced/lost in morphant embryos (Fig 6F, G). It is important to point out that for *egr2* only the r5 expression domain was affected, r3 expression was mostly shifted posteriorly (Fig 6G), indicative of a loss of r4 identity in these embryos. This altered gene expression pattern in the hindbrain was associated with an expansion of the neural plate, assessed by *sox2* expression (Fig 6H), and a mild reduction of *snai2* expression (Fig 6I), while another neural crest gene, *sox10*, was more consistently and strongly affected (Fig 6J).

We also performed gain-of-function experiments to analyze the consequences of Znf703 overexpression on the same set of genes. Embryos injected with 1 ng of *znf703* mRNA exhibited a complete loss of all 5 hindbrain markers, *egr4*, *hoxb1*, *fgf3*, *mafb* and *egr2* (Fig 7A–E), consistent with the well-documented transcriptional repressor activity of *znf703* (Nakamura et al., 2008). We also observed a dramatic reduction of *snai2* and *sox10* expression at the neural plate border (Fig 7G–H), while *sox2* expression was expanded on the injected side (Fig 7F), indicating that excess Znf703 in the ectoderm is detrimental to neural crest formation.

Discussion

Here we describe the expression and function of Znf703/Nlz1, a novel target of Pax3 and Zic1 in *Xenopus* dorsal ectoderm. *znf703* is expressed in the posterior neural plate and at the

neural plate border, where it regulates both hindbrain patterning and neural crest formation, respectively. While Znf703 has been previously shown to control formation of r4 in the vertebrate hindbrain (Runko and Sagerström, 2003; Hoyle et al., 2004; Nakamura et al., 2008), its function in neural crest development is relatively novel. We propose that *znf703* is an integral part of the neural crest gene regulatory network (Sauka-Spengler and Bronner-Fraser, 2008; Betancur et al., 2010; Simões-Costa and Bronner, 2015) acting downstream of the neural plate border specifiers *pax3* and *zic1*, and upstream of neural crest specifiers such as *snai2* and *sox10*.

Znf703 belongs to the NET protein family, a group of conserved zinc finger proteins (Pereira et al., 2016). Vertebrates have two NET family members, Znf703 (also known as Nlz1) and Znf503 (also known as Nlz2), which were first described in zebrafish (Nakamura et al., 2004). *znf703* has been implicated in embryonic development, including limb formation (McGlinn et al., 2008), motoneuron specification (Ji et al., 2009) and hindbrain patterning (Runko and Sagerstrom, 2003; Hoyle et al., 2004; Nakamura et al., 2008), and it is an oncogene that has been linked to breast cancer (Sircoulomb et al., 2011; Holland et al., 2011). Family members have a Groucho-binding motif and regulate transcription through recruitment of histone deacetylases to gene promoters (Dorfman et al., 2002; Runko & Sagerstrom, 2004). Znf703/Nlz1 and Znf503/Nlz2 repress transcription both in mammalian cell lines and in developing zebrafish embryos (Nakamura et al., 2008).

The gene regulatory network orchestrating hindbrain segmentation has been extensively studied and shows a high level of conservation among vertebrates (Trainor and Krumlauf, 2000; Santagati and Rijli, 2003). Our functional studies looking at Znf703 activity in *Xenopus* hindbrain are consistent with the zebrafish studies (Runko and Sagerström, 2003; Hoyle et al., 2004; Nakamura et al., 2008), with a few differences as summarized in Table 1. In zebrafish, Znf703 is believed to control r4 specification by repressing the expression of non-r4 genes rather than inducing gene expression in r4 (Nakamura et al., 2008). Interestingly, we found that two r4 genes, *fgf3* and *hoxb1*, were strongly up-regulated in Znf703-depleted *Xenopus* embryos, and in some ways behaving like an r5 gene (*egr2*) in the zebrafish mutant (Runko and Sagerström, 2003; Hoyle et al., 2004). In gain-of-function experiments *hoxb1* and *fgf3* were down-regulated in *Xenopus* embryos, in this case following a pattern similar to that observed for r3 genes (*egr2* and *hoxa2*) upon Nzl misexpression in zebrafish embryos (Runko and Sagerström, 2003; Hoyle et al., 2004). These results indicate that while the overall blueprint of hindbrain patterning is relatively well conserved, differences are also observed in the fine regulation of rhombomere identity across species, as previously reported for another transcription factor confined to the posterior hindbrain, Egr4 (Bae et al., 2015).

Znf703 knockdown had a mild but consistent effect on the expression of the neural crest genes, *snai2* and *sox10*. Another study in frogs, using a different translation blocking MO, showed that disruption of Znf703 had no detectable effect on the expression of another neural crest gene, *foxd3*. However the dual knockdown of Znf703 and Msx1 significantly down-regulated the trunk expression of *foxd3* (Li et al., 2017). The difference in these results could be explained by a more direct dependence of *snai2/sox10* on Znf703 function as compared to *foxd3*. Znf703 misexpression resulted in a severe loss of *snai2* and *sox10*,

indicating that excess *znf703* expression is detrimental to neural crest formation in the embryo. The loss of *snai2* and *sox10* cannot be explained by a general repression of genes in the ectoderm as a result of Znf703 misexpression, since *sox2* expression was expanded in these embryos. Rather this suggests that Znf703 as an important regulator of neural plate border fate in *Xenopus*.

The similarity of the gain- and loss-of-function phenotypes on the expression of neural crest genes indicates that the levels of Znf703 in the ectoderm need to be tightly regulated to elicit neural crest development. *znf703* was recently isolated in another screen, designed to isolate factors directing lateral neuroblast specification in *C. elegans*, and this study suggested that Znf703 may function broadly across bilaterians to regulate cell fate in the ectoderm (Li et al., 2017). Consistent with this view we propose that *znf703* is a novel component of the gene regulatory network required to specify the neural crest in *Xenopus*.

Methods

Plasmid constructs

Xenopus laevis znf703.S (accession #: NM_001090979; Hoyle et al., 2004) was purchased from GE Dharmacon (pSPORT6-znf703; ThermoFisher Scientific, USA). An expression construct was generated by sub-cloning the coding region of *znf703* into pCS2+ vector (pCS2+znf703). pCS2+pax3GR and pCS2+zic1GR are hormone inducible constructs in which *pax3* or *zic1* open reading frame is fused to the hormone-binding domain of human glucocorticoid receptor (Hong and Saint-Jeannet, 2007). The activity of the fusion proteins can be regulated by addition of dexamethasone to the culture medium of whole embryos or animal cap explants (Kolm and Sive, 1995).

Xenopus embryo injections and explants culture

Embryos were staged according to Nieuwkoop and Faber (1967) and raised in 0.1X NAM (Normal Amphibian Medium; Slack and Forman, 1980). *GR*, *pax3GR*, *zic1GR* (250 pg each; Hong and Saint-Jeannet, 2007; Bae et al., 2014), *znf703* (1 ng), *noggin* (1 ng; Smith and Harland, 1992), *wnt1* (0.1 ng; Wolda et al., 1993) and β -galactosidase (0.5 ng) mRNAs were synthesized in vitro using the Message Machine kit (Ambion, Austin TX), and injected in the animal pole region of 2-cell stage embryos (NF stage 2). *znf703* (znf703MO; 45 ng; GAACAGTTCATTGGAGGAGGGATC), *pax3* (pax3MO; 60 ng; Monsoro-Burq et al., 2005) and *zic1* (zic1MO; 45 ng; Sato et al., 2005) morpholino antisense oligonucleotides (MOs) were purchased from GeneTools (Philomath, OR). The Znf703MO was designed to interfere with both copies of *znf703* (*znf703.S* and *znf703.L*), and its specificity was tested in an *in vitro* transcription/translation coupled rabbit reticulocyte lysate assay (Transcend; Promega, Madison, WI). In whole embryos MOs were injected in one blastomere at the 2-cell stage and embryos analyzed by *in situ* hybridization at stage 15. To identify the injected side, 0.5 ng of β -galactosidase mRNA was coinjected as a lineage tracer. For animal cap explant experiments, both blastomeres of 2-cell stage embryos were injected in the animal pole region, *noggin/wnt1*, *GR*, *pax3GR* or *zic1GR* mRNAs. At late blastula stage (NF stage 9), animal cap explants were dissected and cultured for 8 hours at room temperature (equivalent NF stage 13/14) in 0.5X NAM. For *GR*, *pax3GR* or *zic1GR* mRNAs injected

animal cap explants, 10 μ M Dexamethasone (Dex; Sigma-Aldrich, St. Louis, MO) was added to the culture medium to activate Pax3GR and Zic1GR. Animal explants were subsequently analyzed by qRT-PCR. For whole embryo injections and animal cap explant assays each experiment was performed on at least three independent batches of embryos.

Lineage tracing and whole-mount *in situ* hybridization

Embryos at the appropriate stage were fixed in MEMFA and successively processed for Red-Gal (Research Organics, Cleveland, OH) staining and *in situ* hybridization. Antisense DIG-labeled probes (Genius kit; Roche, Indianapolis, IN) were synthesized using template cDNA encoding *znf703* (pSPORT6-znf703), *snai2* (Mayor et al., 1995), *mafb* (Ishibashi and Yasuda, 2001), *fgf3* (Lombardo et al., 1998), *egr4* (Bae et al., 2015), *egr2* (Bradley et al., 1993), *hoxb1* (Godsave et al., 1994), *sox2* (Mizuseki et al., 1998) and *sox10* (Aoki et al., 2003). Whole-mount *in situ* hybridization was performed as previously described (Harland et al., 1991). Images were captured using an Olympus SZX9 microscope and a QImaging Micro Publisher 3.3 RTV camera.

qRT-PCR analysis

For each sample total RNAs were extracted from 10 animal explants using an RNeasy micro RNA isolation kit (Qiagen, Valencia CA) according to the manufacturer's direction. qRT-PCR was performed using primers for *snai2*, *sox2*, *keratin*, *Efla* (Hong and Saint-Jeannet, 2007) and *znf703* (F: TCCCTCCTCCAAATGAAGT; R: GTGCAGCAAGTGTCCTGTGT) on a LightCycler (Roche, Indianapolis IN) or QuantStudio 3 (Applied Biosystems; ThermoFisher Scientific, USA), as described (Hong and Saint-Jeannet, 2007).

Acknowledgments

We are grateful to Mr. Jonathan Cooney for technical help. The work was supported by grants from the National Institutes of Health to J-P S-J (R01-DE014212 and R01-DE025468).

References

- Aoki Y, Saint-Germain N, Gyda M, Magner-Fink E, Lee YH, Credidio C, Saint-Jeannet JP. Sox10 regulates the development of neural crest-derived melanocytes in *Xenopus*. *Dev Biol*. 2003; 259:19–33. [PubMed: 12812785]
- Bae, C-J., Saint-Jeannet, J-P. Induction and Specification of Neural Crest Cells: Extracellular Signals and Transcriptional Switches. In: Trainor, P., editor. *Neural Crest Cells: Evolution, Development and Disease*. Elsevier; 2014. p. 27-49.
- Bae CJ, Park BY, Lee YH, Tobias JW, Hong CS, Saint-Jeannet JP. Identification of targets of Pax3 and Zic1 in the developing neural crest. *Dev Biol*. 2014; 386:473–483. [PubMed: 24360908]
- Bae CJ, Jeong J, Saint-Jeannet JP. A novel function for Egr4 in posterior hindbrain development. *Sci Rep*. 2015; 5:7750. [PubMed: 25583070]
- Betancur P, Bronner-Fraser M, Sauka-Spengler T. Assembling neural crest regulatory circuits into a gene regulatory network. *Annu Rev Cell Dev Biol*. 2010; 26:581–603. [PubMed: 19575671]
- Bradley LC, Snape A, Bhatt S, Wilkinson DG. The structure and expression of the *Xenopus* Krox-20 gene: conserved and divergent patterns of expression in rhombomeres and neural crest. *Mech Dev*. 1993; 40:73–84. [PubMed: 8443108]

- Dorfman R, Glazer L, Weihe U, Wernet MF, Shilo BZ. Elbow and Noc define a family of zinc finger proteins controlling morphogenesis of specific tracheal branches. *Development*. 2002; 129:3585–3596. [PubMed: 12117809]
- Garnett AT, Square TA, Medeiros DM. BMP, Wnt and FGF signals are integrated through evolutionarily conserved enhancers to achieve robust expression of Pax3 and Zic genes at the zebrafish neural plate border. *Development*. 2012; 139:4220–4231. [PubMed: 23034628]
- Godsave S, Dekker EJ, Holling T, Pannese M, Boncinelli E, Durston A. Expression patterns of Hoxb genes in the *Xenopus* embryo suggest roles in anteroposterior specification of the hindbrain and in dorsoventral patterning of the mesoderm. *Dev Biol*. 1994; 166:465–476. [PubMed: 7813770]
- Gutkovich YE, Ofir R, Elkouby YM, Dibner C, Gefen A, Elias S, Frank D. *Xenopus* Meis3 protein lies at a nexus downstream to Zic1 and Pax3 proteins, regulating multiple cell-fates during early nervous system development. *Dev Biol*. 2010; 338:50–62. [PubMed: 19944089]
- Harland RM. In situ hybridization: an improved whole-mount method for *Xenopus* embryos. *Methods Cell Biol*. 1991; 36:685–695. [PubMed: 1811161]
- Holland DG, et al. ZNF703 is a common Luminal B breast cancer oncogene that differentially regulates luminal and basal progenitors in human mammary epithelium. *EMBO Mol Med*. 2011; 3:167–180. [PubMed: 21337521]
- Hong CS, Saint-Jeannet JP. The activity of Pax3 and Zic1 regulates three distinct cell fates at the neural plate border. *Mol Biol Cell*. 2007; 18:2192–2202. [PubMed: 17409353]
- Hong C-S, Devotta A, Lee Y-H, Park B-Y, Saint-Jeannet J-P. Transcription factor AP2 epsilon (Tfap2e) regulates neural crest specification in *Xenopus*. *Dev Neurobiol*. 2014; 74:894–906. [PubMed: 24616412]
- Hoyle J, Tang YP, Wuellette EL, Wardle FC, Sive H. *nlz* gene family is required for hindbrain patterning in the zebrafish. *Dev Dyn*. 2004; 229:835–846. [PubMed: 15042707]
- Huang X, Saint-Jeannet J-P. Induction of the neural crest and the opportunities of life on the edge. *Dev Biol*. 2004; 275:1–11. [PubMed: 15464568]
- Ishibashi S, Yasuda K. Distinct roles of *maf* genes during *Xenopus* lens development. *Mech Dev*. 2001; 101:155–166. [PubMed: 11231068]
- Ji SJ, Periz G, Sockanathan S. *Nolz1* is induced by retinoid signals and controls motoneuron subtype identity through distinct repressor activities. *Development*. 2009; 136:231–240. [PubMed: 19056829]
- Kjolby RAS, Harland RM. Genome-wide identification of Wnt/ β -catenin transcriptional targets during *Xenopus* gastrulation. *Dev Biol*. 2017; 426:165–175. [PubMed: 27091726]
- Kolm PJ, Sive HL. Efficient hormone-inducible protein function in *Xenopus laevis*. *Dev Biol*. 1995; 171:267–272. [PubMed: 7556904]
- LaBonne C, Bronner-Fraser M. Snail-related transcriptional repressors are required in *Xenopus* for both the induction of the neural crest and its subsequent migration. *Dev Biol*. 2000; 221:195–205. [PubMed: 10772801]
- Li Y, Zhao D, Horie T, Chen G, Bao H, Chen S, Liu W, Horie R, Liang T, Dong B, Feng Q, Tao Q, Liu X. Conserved gene regulatory module specifies lateral neural borders across bilaterians. *Proc Natl Acad Sci (U S A)*. 2017; 114:E6352–E6360. [PubMed: 28716930]
- Lombardo A, Isaacs HV, Slack JM. Expression and functions of FGF-3 in *Xenopus* development. *Int J Dev Biol*. 1998; 42:1101–1107. [PubMed: 9879707]
- Mayor R, Morgan R, Sargent MG. Induction of the prospective neural crest of *Xenopus*. *Development*. 1995; 121:767–777. [PubMed: 7720581]
- McGlinn E, et al. Expression of the NET family member *Zfp503* is regulated by hedgehog and BMP signaling in the limb. *Dev Dyn*. 2008; 237:1172–1182. [PubMed: 18351672]
- Milet C, Maczkowiak F, Roche DD, Monsoro-Burq A-H. Pax3 and Zic1 drive induction and differentiation of multipotent, migratory, and functional neural crest in *Xenopus* embryos. *Proc Natl Acad Sci USA*. 2013; 110:5528–5533. [PubMed: 23509273]
- Mizuseki K, Kishi M, Matsui M, Nakanishi S, Sasai Y. *Xenopus* Zic-related-1 and Sox-2, two factors induced by chordin, have distinct activities in the initiation of neural induction. *Development*. 1998; 125:579–587. [PubMed: 9435279]

- Monsoro-Burq AH, Wang E, Harland R. Msx1 and Pax3 cooperate to mediate FGF8 and Wnt signals during *Xenopus* neural crest induction. *Dev Cell*. 2005; 8:167–178. [PubMed: 15691759]
- Nakamura M, Runko AP, Sagerstrom CG. A novel subfamily of zinc finger genes involved in embryonic development. *J Cell Biochem*. 2004; 93:887–895.
- Nakamura M, Choe SK, Runko AP, Gardner PD, Sagerström CG. Nlz1/Znf703 acts as a repressor of transcription. *BMC Dev Bio*. 2008; 8:108. [PubMed: 19014486]
- Nieuwkoop, PD., Faber, J. Normal table of *Xenopus laevis* (Daudin). Amsterdam: North Holland Publishing Company; 1967.
- Pereira F, Duarte-Pereira S, Silva RM, Texeira da Costa L, Pereira-Castro I. Evolution of the NET (NocA, Nlz, Elbow, TLP-1) protein family in metazoans: insights from expression data and phylogenetic analysis. *Sci Rep*. 2016; 6:38383. [PubMed: 27929068]
- Plouhinec JL, Roche DD, Pegoraro C, Figueiredo AL, Maczkowiak F, Brunet LJ, Milet C, Vert JP, Pollet N, Harland RM, Monsoro-Burq AH. Pax3 and Zic1 trigger the early neural crest gene regulatory network by the direct activation of multiple key neural crest specifiers. *Dev Biol*. 2014; 386:461–472. [PubMed: 24360906]
- Runko AP, Sagerström CG. Nlz belongs to a family of zinc-finger-containing repressors and controls segmental gene expression in the zebrafish hindbrain. *Dev Biol*. 2003; 262:254–267. [PubMed: 14550789]
- Sadaghiani B, Thiebaud CH. Neural crest development in the *Xenopus laevis* embryo, studied by interspecific transplantation and scanning electron microscopy. *Dev Biol*. 1987; 124:91–110. [PubMed: 3666314]
- Saint-Jeannet J-P, He X, Varmus HE, Dawid IB. Regulation of dorsal fate in the neuraxis by Wnt-1 and Wnt-3a. *Proc Natl Acad Sci (USA)*. 1997; 94:13713–13718. [PubMed: 9391091]
- Santagati F, Rijli FM. Cranial neural crest and the building of the vertebrate head. *Nat Rev Neurosci*. 2003; 4:806–818. [PubMed: 14523380]
- Sato T, Sasai N, Sasai Y. Neural crest determination by co-activation of Pax3 and Zic1 genes in *Xenopus* ectoderm. *Development*. 2005; 132:2355–2363. [PubMed: 15843410]
- Sauka-Spengler T, Bronner-Fraser M. A gene regulatory network orchestrates neural crest formation. *Nat Rev Mol Cell Biol*. 2008; 9:557–568.
- Simões-Costa M, Bronner ME. Establishing neural crest identity: a gene regulatory recipe. *Development*. 2015; 142:242–257. [PubMed: 25564621]
- Sircoulomb F, Nicolas N, Ferrari A, Finetti P, Bekhouche I, Rousselet E, Lonigro A, Adélaïde J, Baudelet E, Esteyriès S, Wicinski J, Audebert S, Charafe-Jauffret E, Jacquemier J, Lopez M, Borg JP, Sotiriou C, Popovici C, Bertucci F, Birnbaum D, Chaffanet M, Ginestier C. ZNF703 gene amplification at 8p12 specifies luminal B breast cancer. *EMBO Mol Med*. 2011; 3:153–166. [PubMed: 21328542]
- Slack JM, Forman D. An interaction between dorsal and ventral regions of the marginal zone in early amphibian embryos. *J Embryol Exp Morphol*. 1980; 56:283–299. [PubMed: 7400747]
- Smith WC, Harland RM. Expression cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in *Xenopus* embryos. *Cell*. 1992; 70:829–840. [PubMed: 1339313]
- Stuhlmiller TJ, Garcia-Castro MI. Current perspectives of the signaling pathways directing neural crest induction. *Cell Mol Life Sci*. 2012; 69:3715–3737. [PubMed: 22547091]
- Trainor PA, Krumlauf R. Patterning the cranial neural crest: hindbrain segmentation and Hox gene plasticity. *Nat Rev Neurosci*. 2000; 2:116–124.
- Wolda SL, Moody CJ, Moon RT. Overlapping expression of Xwnt-3A and Xwnt-1 in neural tissue of *Xenopus laevis* embryos. *Dev Biol*. 1993; 155:46–57. [PubMed: 8416844]

Figure 1. Sequence comparison of Znf703 proteins across species

(A) The predicted amino acid sequences from *Xenopus laevis*, human, mouse and zebrafish *znf703* genes were aligned using ClustalW. Conserved amino acids in all four species or in at least two species are highlighted in black and grey, respectively. *Xenopus laevis* (Xl), mouse (Mm), human (Hs) and zebrafish (Dr). The zinc finger domain is underlined in red, and the position of the two Cysteines and Histidines are indicated (*). The putative Groucho binding domain is underlined in green.

Figure 2. Developmental expression of *znf703*

(A–D) By in situ hybridization at the neurula stage (NF stage 13/14) *znf703* is detected in the dorso-lateral ectoderm posteriorly, including the neural plate and the neural crest forming region, with a gap of expression at the dorsal midline. (E, F) At stages 16 through 19, *znf703* expression persists in the dorsal ectoderm where it appears to be confined to the prospective hindbrain and spinal cord. (G) Transverse section of a stage 16 embryo, dorsal to top, showing *znf703* expression in the neural plate (np), neural crest (nc), ventral non-neural ectoderm (nne) and lateral plate mesoderm (lpm). (K–M) Later in development *znf703* is diffusely expressed throughout the ectoderm, and enriched in the hatching gland (hg) and the branchial arches (ba). (A, C, E, H, K) dorsal views, anterior to top. (B, D, J, L, M) lateral views, dorsal to top, anterior to right. (F, I) frontal views, dorsal to top. The embryonic stages are indicated in the lower left corner of each panel.

Figure 3. *znf703* anterior expression domain corresponds to r3/r4

(A) At stage 15, *znf703* is detected dorsally, with a sharp anterior boundary of expression. (B–D) Double *in situ* hybridization showing the spatial relationship of *znf703* with *snai2* in the neural crest (B), *mafb* in r5/r6 (C) and *fgf3* in r3/r4 (D). Dorsal views, anterior to top. The yellow lines indicate the position of the most anterior boundary of *znf703* expression. The red asterisks indicate the position of the most anterior expression domains of *snai2*, *mafb* and *fgf3*. Insets (lower right corner) in panels (B), (C) and (D) depict single *in situ* hybridization for *snai2*, *mafb* and *fgf3*, respectively.

Figure 4. *znf703* is a true target of Pax3 and Zic1

(A–B) Fold induction of *snai2* (A) and *znf703* (B) by expression of pax3GR and zic1GR in animal cap explants (microarray data; Bae et al., 2014). (C–D) Injection in one blastomere at the 2-cell stage of MOs blocking Pax3 (pax3MO; 40 ng) or Zic1 (zic1MO; 40 ng) function resulted in a strong reduction of *snai2* expression (C), while only the most anterior expression domain of *znf703* was affected (D) at the neurula stage. The injected side is to the right as indicated by the presence of the lineage tracer (Red-Gal). The percentage of affected embryos is indicated in the upper right corner. Dorsal views, anterior to top.

Figure 5. Regulation of *znf703* expression by neural crest inducing signals

(A) mRNAs encoding *noggin* (1 ng) and *wnt1* (100 pg) were injected, alone or in combination with *pax3MO*, into both blastomeres in the animal pole region at the 2-cell stage. At the blastula stage (NF stage 9), animal cap explants were dissected, cultured for 8 hours at room temperature (equivalent stage 13/14) and analyzed by qRT-PCR. (B) In combination, Noggin and Wnt1 induce *snai2* and repress *keratin* expression. Co-injection of *pax3MO* blocks *snai2* induction by Noggin and Wnt1 and promotes neural fate (*sox2* expression). The induction of *znf703* by Noggin and Wnt1 is unaffected by injection of *pax3MO*.

Figure 6. Znf703 is required for posterior hindbrain patterning

(A) The target sequence of Znf703 morpholino antisense oligonucleotide (Znf703MO; grey box) is indicated. The position of the start codon (ATG) is indicated. (B) In vitro coupled transcription/translation reactions with plasmid encoding Znf703. Increasing amounts of Znf703MO, 10 ng (+), 100 ng (++) and 1000 ng (+++), block translation directed by *Znf703* mRNA. (C–J) Unilateral injection of *znf703*MO (45 ng) at the 2-cell stage expanded *egr4* (C), *hoxb1* (D) and *fgf3* (E) expression domains in the hindbrain at the neurula stage (NF stge 14/15). In contrast *mafb* (F) and the r5 expression domain of *egr2* were reduced or lost (G). In morphant embryos the expression domain of *sox2* was expanded (H), while the neural crest markers were mildly (*snai2*; G) or strongly (*sox10*; H) reduced. The horizontal lines (F) indicate the width of the neural plate on the control (black line) and injected (white line) sides. The injected side is to the right as indicated by the presence of the lineage tracer (Red-Gal). The percentage of affected embryos is indicated in the upper right corner. Dorsal views, anterior to top.

Figure 7. Znf703 overexpression affects hindbrain and neural crest development

(A–H) Unilateral injection of *znf703* mRNA (1 ng) at the 2 cell stage significantly reduced the expression domain of *egr4* (A), *hoxb1* (B), *fgf3* (C), *mafb* (D) and *egr2* (E), in the hindbrain at the neurula stage (NF stage 14/15). In these embryos *sox2* expression domain was expanded (F), and both neural crest markers, *snai2* (G) and *sox10* (H), were strongly reduced. The horizontal lines (F) indicate the width of the neural plate on the control (black line) and injected (white line) sides. The injected side is to the right as indicated by the presence of the lineage tracer (Red-Gal). The percentage of affected embryos is indicated in the upper right corner. Dorsal views, anterior to top.

Table 1
Comparison of hindbrain genes expression upon znf703 manipulation in fish and frog embryos.

Gene	Hindbrain expression	GOF			LOF		
		Fish (a)	Fish (b)	Frog (c)	Fish (a)	Fish (b)	Frog (c)
<i>hoxa2</i>	r2/r3	↓	↓	-	-	-	-
<i>fgf3</i>	r3/r4	-	-	↓	↓	-	↑
<i>hoxb1</i>	r4	↑	↑	↓	↓	↓	↑
<i>egr2</i>	r3/r5	↓*	↓*	↓	↑	↑	↓***
<i>malb</i>	r5/r6	U	U	↓	-	-	↓
<i>egr4</i>	r5/r6	-	-	↓	-	-	↑
<i>hnr1b</i>	r5/r6/r7	-	-	-	↑	-	-

GOF, gain-of-function; LOF, loss-of-function; r, rhombomere

(a) Runko and Sagerstom, 2003; Nakamura et al., 2008.

(b) Hoyle et al., 2004.

(c) This study.

↓ : loss/reduced expression

↑ : expanded expression

U : unaffected

- : not determined

* : only r3

*** : only r5