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

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Summary
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 (NocA/Nlz, Elbow, and TLP-1) 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 bona-fide 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
hindbrain, neural crest, pax3, Xenopus, zic1, znf703

1 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 & 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 crest (Betancur, Bronner-Fraser, & Sauka-Spengler, 2010; Sauka-Spengler & Bronner-Fraser, 2008; Simões-Costa & 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 (Garnett, Square, & Medeiros, 2012; Gutkovich et al., 2010; Hong & Saint-Jeannet, 2007; Milet, Maczkowiak, Roche, & Monsoro-Burq, 2013; Monsoro-Burq, Wang, & Harland, 2005; Sato, Sasai, & Sasai, 2005).

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 (Bae, Jeong, & Saint-Jeannet, 2015; Hong, Devotta, Lee, Park, & Saint-Jeannet, 2014).

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 (NocA/Nlz, Elbow, and TLP-1) family of zinc finger proteins (Nakamura, Runko, & Sagerstrom, 2004; Pereira, Duarte-Pereira, Silva, Teixeira da Costa, & Pereira-Castro, 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 (Hoyle, Tang, Wiellette, Wardle, & Sive, 2004; Nakamura,
Choe, Runko, Gardner, & Sagerström, 2008; Runko & Sagerström, 2003). 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

### 2.1 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* (Hong & Saint-Jeannet, 2007; Monsoro-Burq et al., 2005; Sato et al., 2005). 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/β-catenin signaling pathway in *Xenopus* (Kjolby & Harland, 2017). Znf703 belong to the NET protein family, characterized by a single atypical C2H2 zinc finger at the C-terminal (Nakamura et al., 2004; Pereira et al., 2016).

**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.
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 & 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 (Figure 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 (Figure 2e–j). Histological sections at the neurula stage indicate that in addition to the ectoderm, znf703 is expressed in the lateral plate mesoderm (Figure 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 (Figure 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, Isaacs, & Slack, 1998) mafb/kreisler (hindbrain r5/r6; Ishibashi & Yasuda, 2001), and snai2 (neural crest; Mayor, Morgan, & Sargent, 1995). The most lateral domain of znf703 overlaps with snai2 demonstrating that znf703 is expressed in a subpopulation of neural crest progenitors (Figure 3b). However, znf703 does not overlap with the most anterior domain of snai2 (Figure 3b), which corresponds to the mandibular segment of the neural crest (Sadagiani & 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 (Figure 3c). Spatially, the anterior most boundary of znf703 expression overlaps with fgf3 expression domain in r3/r4 (Figure 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 (Hoyle et al., 2004; Runko et al., 2003), and suggesting an important role in both neural crest formation and hindbrain patterning.

**2.3 | 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 (Figure 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 morpholino antisense oligonucleotides (MOs) at the 2-cell stage (Hong & Saint-Jeannet, 2007; Monsoro-Burq et al., 2005; Sato et al., 2005). As previously described both MOs interfered with snai2 expression (Hong & 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 (Figure 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 (Figure 5a; Bae & Saint-Jeannet, 2014; Stuhlmueller & Garcia-Castro, 2012). 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 (Figure 5b; LaBonne & Bronner-Fraser, 1998; Saint-Jeannet, He, Varmus, & Dawid, 1997). As previously reported, this activity depends on Pax3 activity, as Pax3 knockdown represses snai2 expression and promotes neural (sox2) fate (Figure 5b; Hong & 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 (Bae & Saint-Jeannet, 2014; Stuhlmiller & Garcia-Castro, 2012).

2.4 | 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 (Figure 6a). In an in vitro transcription/translation assay increasing doses of Znf703MO blocked Znf703 protein production (Figure 6b). Unilateral injection of znf703MO (45 ng) in the animal pole region at the two-cell stage resulted in a posterior expansion of several hindbrain markers including erg4 (r5/r6), hoxb1 (r4), and fgf3 (r3/r4; Figure 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 (Figure 6f,g). It is important to point out that for egr2 only the r5 expression domain was affected, r3 expression was mostly shifted posteriorly (Figure 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 (Figure 6h), and a mild reduction of snai2 expression (Figure 6i), while another neural crest gene, sox10, was more consistently and strongly affected (Figure 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 (Figure 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 (Figure 7g–h), while sox2 expression was expanded on the injected side (Figure 7f), indicating that excess Znf703 in the ectoderm is detrimental to neural crest formation.

3 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 (Hoyle et al., 2004; Nakamura et al., 2008; Runko & Sagerström, 2003), its function in neural crest development is relatively novel. We propose that znf703 is an integral part of the neural crest gene regulatory network (Betancur et al., 2010; Sauka-Spengler & Bronner-Fraser, 2008; Simões-Costa & 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, Periz, & Sockanathan, 2009) and hindbrain patterning (Hoyle et al., 2004; Nakamura et al., 2008; Runko & Sagerstrom, 2003), 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, Glazer, Weihe, Wernet, & Shilo, 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 (Santagati & Rijli, 2003; Trainor & Krumlauf, 2000). Our functional studies looking at Znf703 activity in Xenopus hindbrain are consistent with the zebrafish studies (Hoyle et al., 2004; Nakamura et al., 2008; Runko & Sagerström, 2003), 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 (Hoyle et al., 2004; Runko & Sagerström, 2003). 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 (Hoyle et al., 2004; Runko & Sagerström, 2003). 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, foxd2. 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 is 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.

**TABLE 1** Comparison of hindbrain genes expression upon znf703 manipulation in fish and frog embryos

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GOF = gain-of-function; LOF = loss-of-function; r = rhombomere.
<sup>a</sup>Nakamura et al., 2008; Runko and Sagerstom, 2003
<sup>b</sup>Hoyle et al., 2004.
<sup>c</sup>This study.

*: loss/reduced expression. #: expanded expression. U: unaffected. --: not determined. *: only r3. **: only r5.
4 | METHODS

4.1 | Plasmid constructs

* Xenopus laevis znf703.5 (accession #: NM_001090979; Hoyle et al., 2004) was purchased from GE Dharmacon (pSPORT6-znf703; Thermo-Fisher Scientific, Waltham, MA). An expression construct was generated by sub-cloning the coding region of znf703 into pcDNA3+ vector (pcDNA3+ znf703). pcDNA3+ pax3GR and pcDNA3+ 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 & 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 & Sive, 1995).

4.2 | Xenopus embryo injections and explants culture

Embryos were staged according to Nieuwkoop and Faber (1967) and raised in 0.1× normal amphibian medium (NAM; Slack & Forman, 1980). GR, pax3GR, zic1GR (250 pg each; Bae et al., 2014; Hong & Saint-Jeannet, 2007), znf703 (1 ng), noggin (1 ng; Smith & Harland, 1992), wnt1 (0.1 ng; Wolda, Moody, & Moon, 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; GAA-CAGTTCATTGAGGAGGCATC), pax3 (pax3MO: 60 ng; Monsoro-Burq et al., 2005) and zic1 (zic1MO: 45 ng; Sato et al., 2005) MOs were purchased from GeneTools (Philomath, OR). The Znf703MO was designed to interfere with both copies of znf703 (znf703S and znf703L), 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 two-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 two-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 hr at room temperature (equivalent NF Stage 13/14) in 0.5× NAM. For GR, pax3GR, or zic1GR mRNAs injected animal cap explants, 10 μM 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.

4.3 | 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 & Yasuda, 2001), fgf3 (Lombardo et al., 1998), egr4 (Bae et al., 2015), egr2 (Bradley, Snape, Bhatt, & Wilkinson, 1993), hoxd1 (Godsave et al., 1994), sox2 (Mizuseki, Kishi, Matsui, Nakaniishi, & Sasai, 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.

4.4 | Quantitative real-time polymerase chain reaction 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. Quantitative real-time polymerase chain reaction was performed using primers for snai2, sox2, keratin, Ef1a (Hong & Saint-Jeannet, 2007) and znf703 (F: TCCCTCCTCACAATGAACGT; R: GTGCAGCCAAGTGTCCTGTG) on a LightCycler (Roche, Indianapolis IN) or QuantStudio 3 (Applied Biosystems; ThermoFisher Scientific), as described (Hong & Saint-Jeannet, 2007).

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