MONTANA STATE UNIVERSITY LIBRARY

Neural crest development in Xenopus requires Protocadherin 7 at the lateral neural crest border

Authors: Dana Rashid, Paul Puettmann, Ethan Roy, & Roger S. Bradley

NOTICE: this is the author's version of a work that was accepted for publication in Mechanisms of Development. Changes resulting from the publishing process, such as peer review, editing, corrections, structural formatting, and other quality control mechanisms may not be reflected in this document. Changes may have been made to this work since it was submitted for publication. A definitive version was subsequently published in Mechanisms of Development, volume 149, February 2018, DOI# 10.1016/j.mod.2018.01.002

Rashid, Dana, Paul Puettmann, Ethan Roy, and Roger S. Bradley, "Neural crest development in Xenopus requires Protocadherin 7 at the lateral neural crest border." Mechanisms of development 149 (February 2018): 41-52. DOI: 10.1016/j.mod.2018.01.002.

Made available through Montana State University's ScholarWorks
scholarworks.montana.edu

Neural crest development in Xenopus requires Protocadherin 7 at the lateral neural crest border

Dana Rashid, Paul Puettmann, Ethan Roy, Roger S. Bradley *

Department of Cell Biology and Neuroscience, Montana State University, Bozeman, MT 59717, United States

A B S T R A C T

In vertebrates, the neural crest is a unique population of pluripotent cells whose development is dependent on signaling from neighboring tissues. Cadherin family members, including protocadherins, are emerging as major players in neural crest development, largely through their roles in cell adhesion and sorting in embryonic tissues. Here, we show that Protocadherin 7 (Pcdh7), previously shown to function in sensorial layer integrity and neural tube closure in Xenopus, is also involved in neural crest specification and survival. Pcdh7 expression partly overlaps the neural crest domain at the lateral neural crest border. Pcdh7 knockdown in embryos does not alter neural crest induction; however, neural crest specification markers, including Snail2 and Sox9, are lost, due to apoptosis of the neural crest starting after stage 13. Pcdh7 knockdown also results in downregulation of Wnt11b; both of which are co-expressed in the sensorial layer lateral to the neural crest, suggestive of a role for Wnt11b in the neural crest apoptosis. Confirming this role, apoptosis, Snail2 expression and the developmental fate of the neural crest can be partially rescued by ectopic expression of Wnt11b. These results indicate that Pcdh7 plays an important role in maintaining the sensorial layer at the lateral neural crest border, which is necessary for the secretion of survival factors, including Wnt11b.

1. Introduction

The neural crest is a distinct population of cells, unique to vertebrates, that is responsible for generating a diverse number of adult derivatives. Neural crest cells are induced early during embryonic development, originating from the border of the neural plate and epidermal ectoderm. During primary neurulation, this border ectoderm becomes incorporated into the neural folds and eventually ends up in the dorsal neural tube. Prior to, or just after, neural tube closure, depending on the species, the neural crest cells undergo an epithelial to mesenchymal transition (EMT) and delaminate from the neural ectoderm. The cells then undergo collective cell migration along well-defined routes, eventually ceasing migration and differentiating into multiple cell types that contribute to a wide variety of embryonic tissues (Le Douarin and Kalcheim, 1999; Mayor and Theveneau, 2013; Theveneau and Mayor, 2012). For example, the cranial neural crest emanates from the anterior neural tube and gives rise to the cranial ganglia and cartilage and bones of the skull and face, while the trunk neural crest originates from the posterior neural tube and forms most of the peripheral nervous system (PNS), including the dorsal root ganglia, sympathetic ganglia and Schwann cells, in addition to chromaffin cells of the adrenal medulla and melanocytes in the skin.

The contribution of the neural/epidermal border cells to neural crest formation is well established and involves a precisely regulated gene and signaling network. Secreted signals from neighboring tissues are required for neural crest induction and differentiation. These secreted factors include Wnts, from the neural and epidermal ectoderm, BMPs from the epidermal ectoderm and FGF family members from the underlying mesoderm (Klymkowsky et al., 2010; Milet and Monsoro-Burq, 2012; Rogers et al., 2012; Sauka-Spengler and Bronner-Fraser, 2008; Schille and Schambony, 2017; Stuhlmiller and Garcia-Castro, 2012). Together, these factors induce the formation of the neural crest domain, a broad progenitor region that expresses distinct molecular markers, such as Pax3, c-myc and Zic1 (Meulemans and Bronner-Fraser, 2004; Pegoraro and Monsoro-Burq, 2013). These transcription factors, in combination with continued secreted signals from adjacent tissues, activate the expression of a new set of transcription factors that function as neural crest specifiers, including Snail2, Twist, Foxd3, Sox9 and AP-2 (Milet et al., 2013; Plouhinec et al., 2014; Shi et al., 2011). Neural crest specifier genes then elicit the subsequent EMT and migration of the neural crest.

Cell-cell adhesion, as mediated by members of the cadherin superfamily, also plays important roles in neural crest development. The zinc-finger transcription factors Snail2 and Twist are potent inducers of EMT in epithelial cells and regulate expression of several cadherins in the neural crest (Batle et al., 2000; Bolos et al., 2003; Taneyhill et al., 2007; Tien et al., 2015). Disrupting cadherin expression in the neural crest, either by gain of function or loss of function, alters neural

crest EMT, migration and differentiation, confirming that regulated cadherin-mediated cell adhesion is essential for proper neural crest development (Taneyhill and Schiffmacher, 2017).

In Xenopus embryos, the ectoderm is unique in that it consists of two cell layers, an outer epidermal layer and an inner sensorial layer. The neural crest emanates from the sensorial layer, where it juxtaposes the neural ectoderm, prior to neural tube closure, as opposed to delaminating from the dorsal neural tube as occurs in other vertebrates. The cadherin family member Protocadherin 7 (Pcdh7, also known as NF-protocadherin) is highly expressed in the ectodermal inner layer, lateral to the neural plate, and is required for proper histogenesis of this layer (Bradley et al., 1998). This expression pattern suggests a role for Pcdh7 in neural crest formation in embryos. To investigate this potential role, we disrupted Pcdh7 expression in early embryos and analyzed the effect on the neural crest. When Pcdh7 is inhibited lateral to the neural plate, neural crest cells are still induced; however, they subsequently undergo apoptosis, resulting in the loss of specification markers. Neural crest cell death is due, at least in part, to loss of Wnt11b from the inner layer cells at the neural/epidermal border and ectopic expression of Wnt11b partially rescues neural crest apoptosis. In combination with our previous studies demonstrating that Pcdh7 is essential for proper epidermal ectoderm cohesion (Bradley et al., 1998), these results indicate that Pcdh7 is necessary for Wnt11b expression from the ectoderm, which is required for neural crest survival and specification.

2. Results

2.1. Xenopus Pcdh7 is required for expression of neural crest specification markers

Our previous results demonstrated that Pcdh7 is required for both proper formation of the ectodermal sensorial layer and for neural tube closure (Bradley et al., 1998; Rashid et al., 2006). While Pcdh7 is generally expressed in the sensorial layer, highest expression occurs cranially, lateral to the neural plate during neurulation, suggestive of an interaction with cranial neural crest cells, which also originate from the sensorial layer lateral to the neural plate. To investigate the role Pcdh7 plays in cranial neural crest development, we first sought to clarify where Pcdh7 is expressed relative to the presumptive neural crest. Accordingly, stage 17 embryos were assayed by in situ hybridization for Pcdh7 and two neural crest specification markers, Snail2 and Sox9 (Mayor et al., 1995; Spokony et al., 2002). As shown in Fig. 1, Pcdh7 is expressed in the ectoderm, lateral to the neural plate, as well as at the tips of the neural folds, but is not expressed in the neural plate proper, as previously described (Bradley et al., 1998). Snail2 is also expressed lateral to the neural plate, but extends more medially, with strongest staining in the cranial neural crest. Sections through embryos stained for Pcdh7 or Snail2 confirm that both genes are expressed within the inner or sensorial layer of the ectoderm (Fig. 1F, G). To investigate further the relationship between Pcdh7 and the presumptive neural crest, embryos were subjected to double in situ hybridization for both Pcdh7 and Snail2 or Sox9. As observed in whole mount, Pcdh7 is expressed lateral to, and partially overlaps, Snail2 and Sox9 along the lateral border of the neural crest (Fig. 1C–E). Furthermore, sections through double in situ embryos confirm that, while Snail2 and Sox9 are primarily expressed more dorsally than Pcdh7, there is a subset of sensorial layer cells that express both genes (Fig. 1H, I). In sum, in situ hybridization results reveal that Pcdh7 expression juxtaposes and partially overlaps neural crest markers Snail2 and Sox9 in the inner sensorial layer, at the lateral border region of the cranial neural crest.

The overlap of Pcdh7 and neural crest specification markers suggests that Pcdh7 may play a role in neural crest development. Therefore, we asked whether loss of Pcdh7 from the sensorial layer disrupts the formation or differentiation of the neural crest. To disrupt Pcdh7 we utilized either a Pcdh7 morpholino (Pcdh7MO) or a Pcdh7 dominant negative construct (Pcdh7ΔE), both previously shown to inhibit specifically Pcdh7 expression in embryos (Bradley et al., 1998; Heggem and Bradley, 2003; Rashid et al., 2006; Piper et al., 2008). Embryos were injected at the 4-cell stage into a single dorsal blastomere with Pcdh7MO, Pcdh7ΔE, or a control morpholino (CMO), along with LacZ mRNA as a tracer, and allowed to develop until stages 15–17, then analyzed by in situ hybridization for proper expression of neural plate, neural crest induction or neural crest specification markers. Results reveal that inhibiting Pcdh7 expression in the border region has no effect on induction of the neural plate or the initial induction of the neural crest, as assayed at stage 15 for expression of the neural plate markers Sox2 and Sox15 (SoxD) (Mizuseki et al., 1998a; Mizuseki et al., 1998b), the neural crest induction markers Pax3, Zic1, c-myc or the placodal marker Six1 (Pandur and Moody, 2000). In contrast, neural crest specification markers, including Sox9, Snail2 and Twist are all downregulated on the injected side at stage 17/18 when Pcdh7MO or Pcdh7ΔE is targeted to the neural border region. In comparison, embryos injected with the CMO or LacZ alone showed no change in the expression of either neural induction or specification markers on the injected side (Fig. 2 and Supplementary Table I). These results indicate that Pcdh7 is not involved in the early events in inducing the neural crest, but is required for later events during neural crest specification.

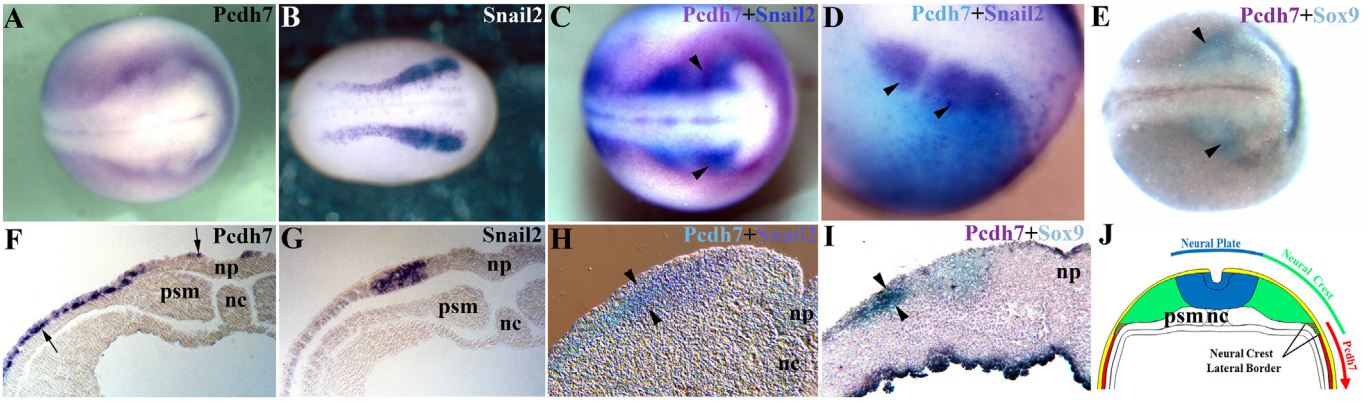


Fig. 1. Pcdh7 is expressed in a subset of neural crest cells. Embryos at stage 17/18 were subjected to in situ hybridization for Pcdh7 (A, F), or the neural crest marker Snail2 (B, G) or double in situ for Pcdh7 and Snail2 (C, D, H) or the neural crest marker Sox9 (E, I). Embryos are viewed in whole mount (A–E) or in cross-section (F—I). Pcdh7 is expressed in the sensorial layer of the ectoderm and at the tips of the neural folds (A, arrows in F), with highest expression along the lateral border of the neural crest. Snail2 is expressed in the neural crest, which derives from the sensorial layer (B, G). Embryos subjected to double in situ for Pcdh7 and Snail 2 or Sox9 (C, D, E) reveal overlap along the lateral border of the neural crest (arrowheads), which is confirmed in cross section (H, I). Anterior is the right in A–E. Sections F—I are anterior, through the prospective hindbrain region. A summary diagram of the domains of the neural plate, neural crest and Pcdh7 is shown (J). Abbreviations: np, neural plate; nc, notochord; psm, presomitic mesoderm.

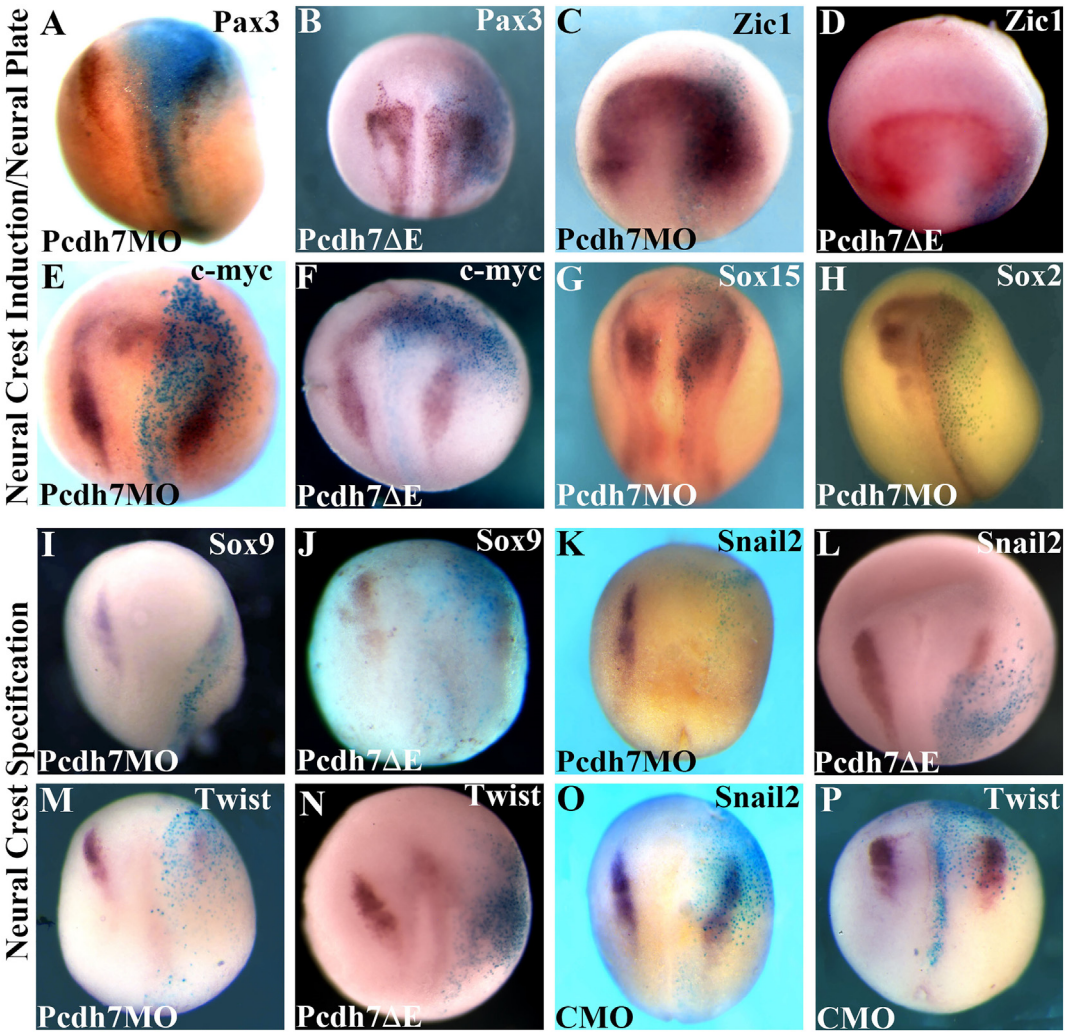


Fig. 2. Pcdh7 knockdown disrupts expression of neural crest specification markers, but not neural crest induction or neural plate markers. Embryos were injected with Pcdh7MO, Pcdh7ΔE or CMO, together with LacZ mRNA, fixed at stage 15–17, stained for β-galactosidase, and subjected to in situ hybridization for neural markers. Neither Pcdh7MO nor Pcdh7ΔE has an effect on expression of the induction markers Pax3 (A, B), Zic1 (C,D) or c-myc (E, F), nor on the neural plate markers Sox15 and Sox2 (G, H). In contrast, Pcdh7MO- or Pcdh7ΔE-injected embryos exhibit reduced expression of the specification markers Sox9 (I, J), Snail2 (K, L) and Twist (M, N). Control (CMO)-injected embryos show no change in specification markers Snail2 (O) or Twist (P). Anterior is towards the top of the photos and the injected side of the embryos is to the right.

2.2. Loss of Pcdh7 results in neural crest cell apoptosis

To determine why neural crest specification markers were downregulated in Pcdh7 knockdowns, we next asked whether disrupting Pcdh7 alters neural crest proliferation or cell death. To test this, embryos were injected with Pcdh7MO or Pcdh7ΔE and fixed at stage 17/18, then sections were immunostained for either phosphorylated histone H3 (pH 3), to detect cells in mitosis, or for activated Caspase 3, to detect apoptotic cells. The numbers of labeled cells were then quantified on the injected side. A comparison of pH 3 staining in Pcdh7MO- versus CMO-injected embryos reveals no significant difference (Supplementary Fig. 1). In contrast, embryos injected with Pcdh7MO or Pcdh7ΔE exhibit a significant increase in Caspase 3 immunostaining in the neural crest region on the injected side (Fig. 3). In comparison, very little apoptosis is observed in the neural crest on the uninjected side or in CMO-injected embryos. To distinguish between background staining, only cells in which both the nuclei and cytoplasm stained positive for Caspase-3 were counted. To verify that the observed apoptosis is specific to Pcdh7 knockdown and not due to nonspecific morpholino toxicity, we then sought to rescue the Pcdh7MO-induced cell death by co-injecting mRNA encoding full length Pcdh7. As shown in Fig. 3B, ectopic expression of Pcdh7 does rescue the Pcdh7MO-induced apoptosis. In contrast, ectopic expression of full length Pcdh7 by itself has no effect on neural crest survival. Thus, knockdown of Pcdh7, via either the Pcdh7MO or Pcdh7ΔE, results in a loss of neural crest cells via apoptosis, verifying that Pcdh7 plays an important role during neural crest specification.

As Pcdh7 knockdown altered expression of neural specification but not induction markers, we hypothesized that loss of neural crest cells by apoptosis would coincide with the timing of neural crest specification events. Accordingly, apoptosis was evaluated in Pcdh7MO-injected embryos progressively between stages 11 and 17. Quantifying the number of Caspase-positive neural crest cells revealed that apoptosis is not observed at stage 11. At stage 13, the Pcdh7MO-injected embryos exhibit, on average, one Caspase-positive apoptotic cell per section on the injected side, which is not significantly different than the uninjected side. At stage 15, Pcdh7MO-injected embryos exhibit an average of 15 Caspase-positive cells per section, with the cells undergoing clear morphological signs of cell death, including irregular borders and fragmented nuclei, and this increases to an average of 29 Caspase-positive cells per section by stage 17 (Fig. 4). In contrast, significantly less apoptosis is observed on the uninjected side of Pcdh7 morphants at stages 15 and 17. Importantly, the apoptosis observed in Pcdh7 morphants is confined to the region of the prospective neural crest: few apoptotic cells are observed outside of the neural crest region, either in the neural plate, epidermal ectoderm or the mesoderm, in

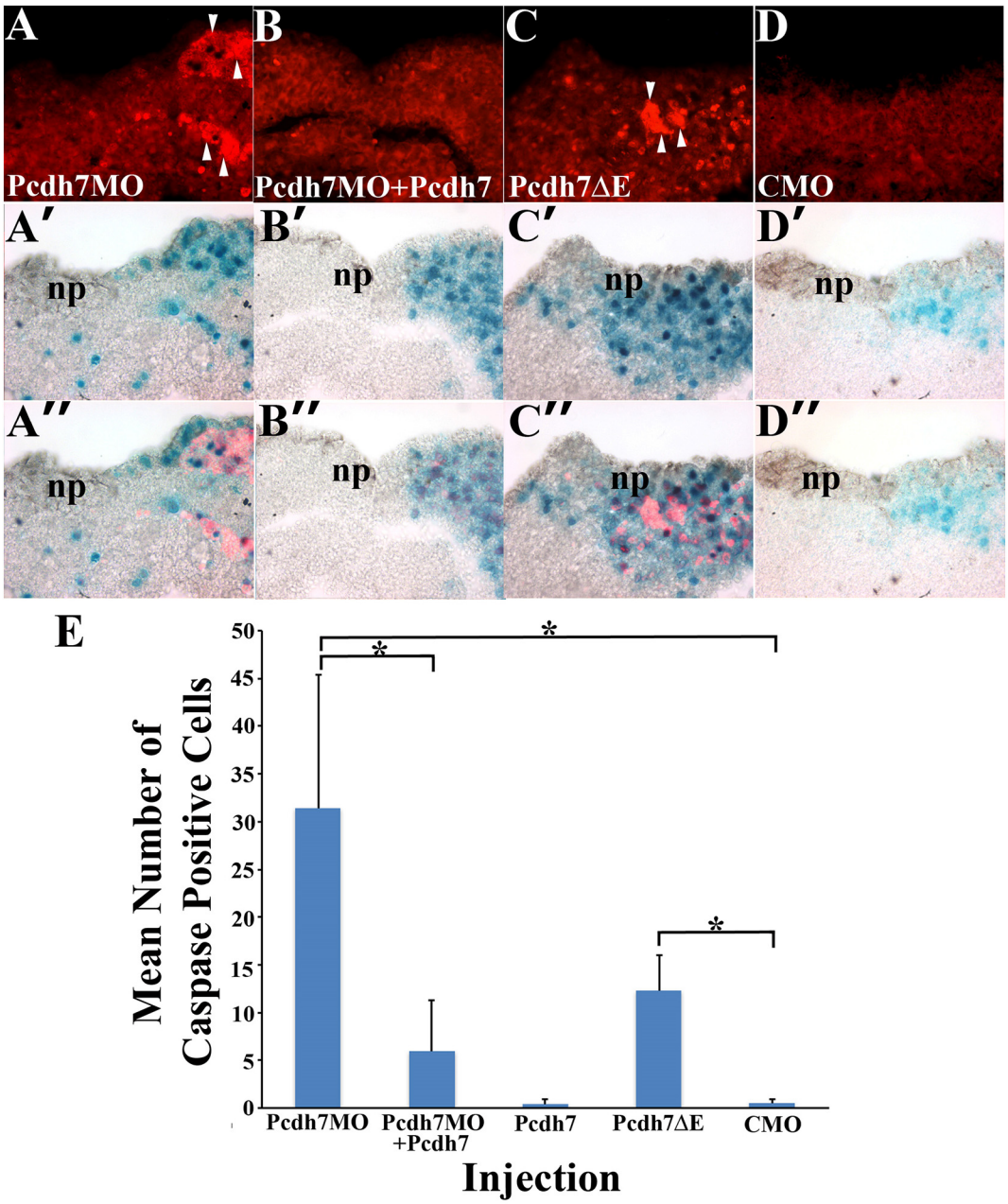


Fig. 3. Disrupting Pcdh7 results in cranial neural crest apoptosis. Embryos were injected with Pcdh7MO or the dominant-negative Pcdh7ΔE mRNA, together with LacZ mRNA, fixed at stage 17, stained for β-galactosidase, and immunostained for activated Caspase 3 to detect dying cells. Both Pcdh7MO (panel A) and Pcdh7ΔE (panel C) result in apoptosis of cranial neural crest cells (arrowheads), as compared to CMO-injected embryos (panel D). The specificity of the Pcdh7MO-induced neural crest is demonstrated by rescuing apoptosis with co-injected Pcdh7 mRNA (panel B). A, B, C, D show caspase immunofluorescence; A′B′C′D′ show the β-galactosidase staining; while A″, B″, C″, D″ are the corresponding overlays. The injected side of the embryo is to the right. Results of at least 6 embryos are quantified in E. * indicates a statistical significant difference ($p < 0.001$).

concordance with our previous studies (Heggem and Bradley, 2003). While apoptotic cells are often observed adjacent to the epidermal ectoderm or mesoderm, these appear to be dying cells that were extruded from the neural crest. Temporally, the apoptosis we observe is after neural crest induction and coincides with the expression of neural crest specification markers (Pegoraro and Monsoro-Burq, 2013), consistent with the in situ hybridization results (Fig. 2). In sum, the ability to rescue Pcdh7MO morphants by expressing full length Pcdh7, together with the fact that the Pcdh7ΔE dominant negative also causes neural crest apoptosis, strongly suggests that neural crest apoptosis is caused by the loss of Pcdh7 expression or function at the neural border. This apoptosis likely results in the loss of the neural crest specification markers observed. Taken together, these results signify an important role for Pcdh7 to promote cell survival during neural crest specification.

2.3. Pcdh7 is required for a survival signal from the sensorial layer

As our previous results established that loss of Pcdh7 disrupts the sensorial layer, but does not result in apoptosis in the non-neural ectoderm (Heggem and Bradley, 2003), the question arises as to why the loss of Pcdh7 results in apoptosis of neural crest cells, which also derive from the sensorial layer. As secreted signals from the non-neural ectoderm are required for proper formation of the neural crest, we sought to determine if loss of Pcdh7 in the sensorial layer alters a secreted factor that comes from the lateral ectoderm, at the neural crest border

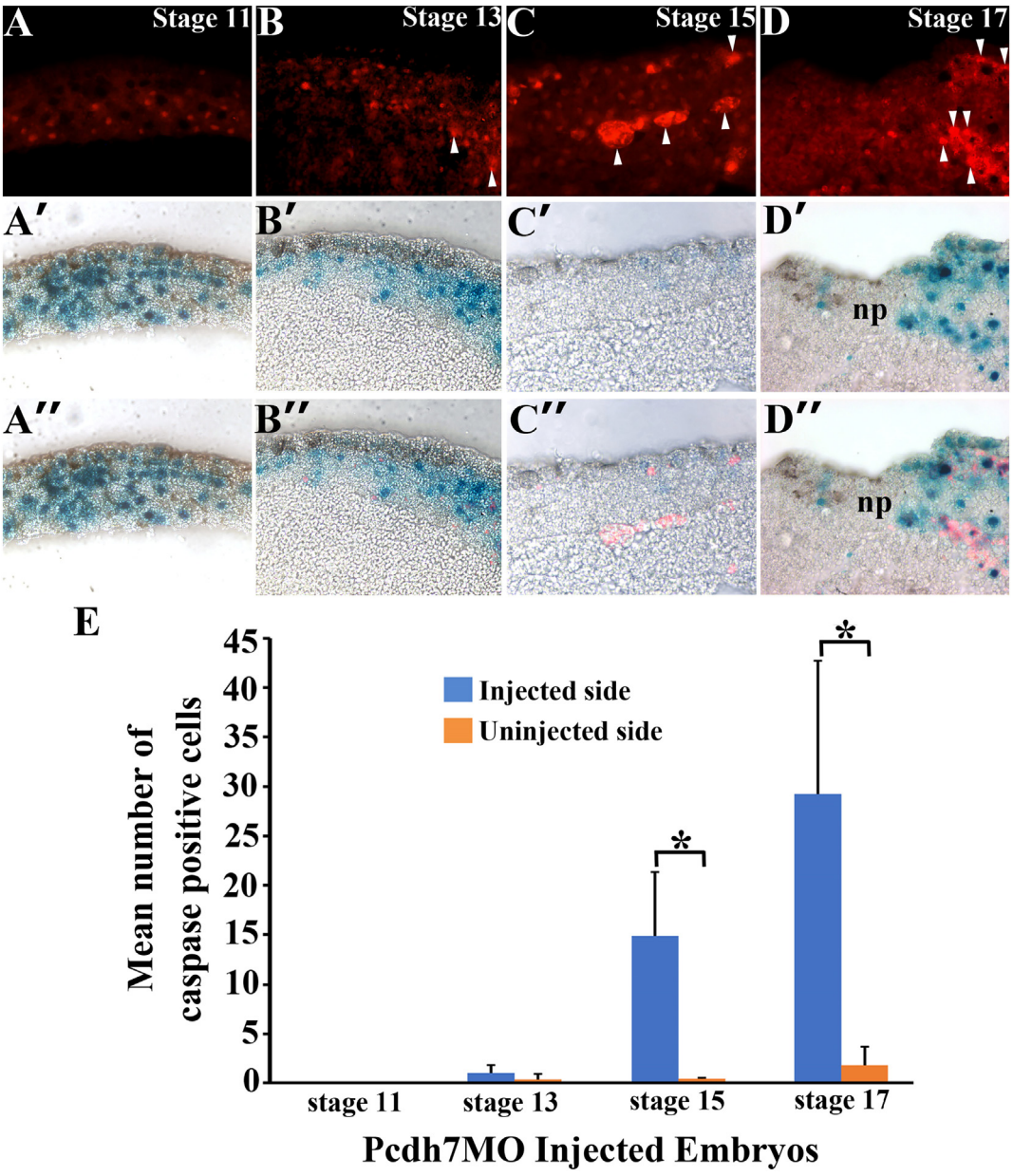


Fig. 4. Pcdh7MO-induced neural crest apoptosis begins at stage 13 and is evident by stage 15. Embryos were injected with Pcdh7MO, together with LacZ mRNA, fixed at stages 11–17, stained for β-galactosidase, and immunostained for activated Caspase 3. At stage 11 (panel A), mainly background staining is observed. By stage 13 (panel B), embryos exhibit staining for activated Caspase 3, that includes both nuclei and cell bodies (arrowheads). At stage 15 (panel C) and continuing through stage 17 (panel D), the neural crest exhibits strong Caspase 3 staining throughout the cells on the Pcdh7MO-injected side, indicating that the cell death caused by Pcdh7MO coincides with the timing of neural crest specification. A, B, C, D show caspase immunofluorescence; A′B′C′D′ show the β-galactosidase staining; while A″, B″, C″, D″ are the corresponding overlays. The injected side of the embryo is to the right. Results of at least 6 embryos are quantified in F. * indicates a statistical significant difference between the injected and uninjected sides at each stage ($p < 0.001$). Abbreviations: np, neural plate.

region that normally expresses Pcdh7. To address this question, we first verified that knockdown of Pcdh7 does indeed disrupt the sensorial layer at the lateral border of the neural crest. Therefore, we examined injected embryos for proper expression of the nuclear protein p63, which marks sensorial layer cells in Xenopus and is required for epithelial development (Koster et al., 2004; Lu et al., 2001). As shown in Fig. 5A and B, immunostaining for p63 reveals that both Pcdh7MO- and Pcdh7ΔE-injected embryos lose expression of p63 in the sensorial layer on the injected side, suggesting that the inner layer is disrupted lateral to the neural plate. To confirm that the inner layer is disrupted, we also immunostained embryos for β-catenin, a component of adherens junctions. On the uninjected side of the embryos, β-catenin localizes to the cell membranes of the sensorial layer at the lateral neural crest border, consistent with participation in cadherin-mediated cell-cell adhesion. In contrast, the Pcdh7ΔE-injected side of the embryos exhibits reduced β-catenin staining at the cell membranes, indicating that the sensorial layer cell junctions are disrupted (Fig. 5C, D). Furthermore, the outer epidermal layer appears thicker, with an increase in pigmented cells, as well as disorganized. This is confirmed by in situ hybridization for epidermal keratin, which demonstrates that the ectoderm in Pcdh7ΔE-injected embryos is thicker and disordered. Thus, these results verify that loss of Pcdh7 at the lateral neural crest border disrupts the ectodermal sensorial layer; furthermore, loss of Pcdh7 may result in the mis-sorting of sensorial layer cells to the outer layer.

Next, to elucidate whether disrupting the sensorial layer, via Pcdh7 knockdown, also alters the expression of a required secreted signal from the ectoderm, we examined the expression of Wnt11b, which was reported to be expressed in the non-neural ectoderm

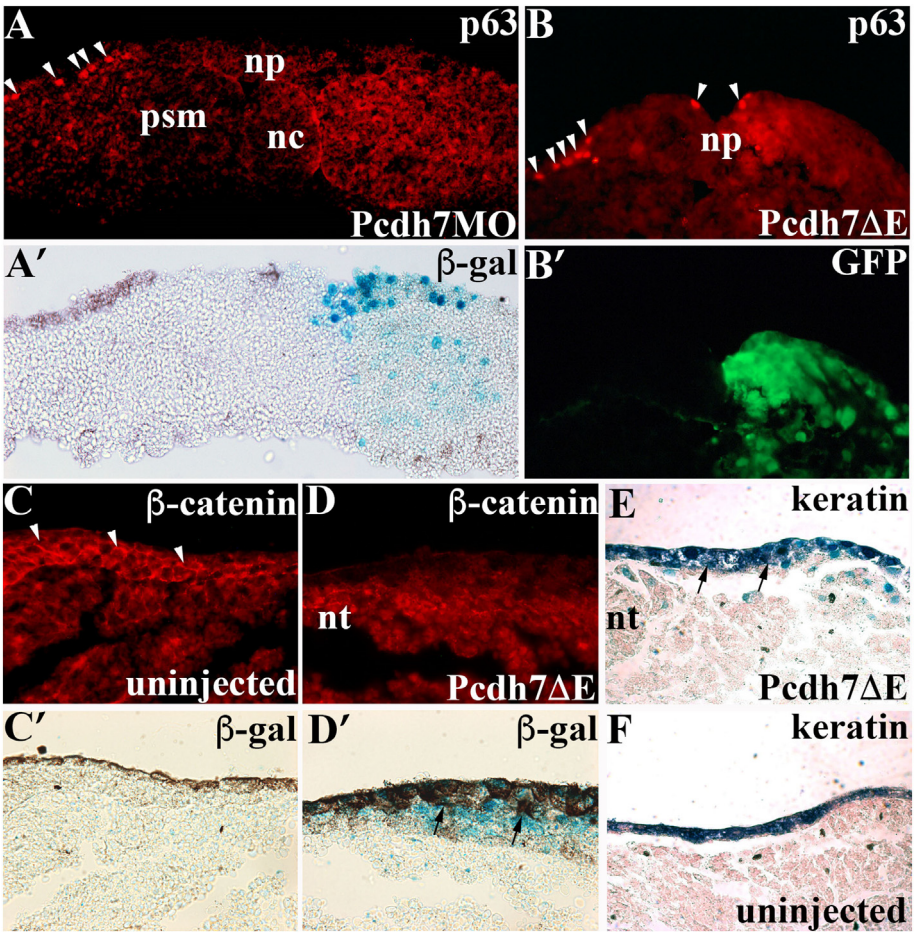


Fig. 5. Pcdh7 knockdown disrupts the sensorial layer. Embryos were injected with Pcdh7MO, together with LacZ mRNA (A, A′), or the dominant-negative Pcdh7ΔE, together with GFP mRNA (B, B′), then fixed, sectioned and immunostained for the sensorial layer marker p63. While p63 is observed in sensorial layer nuclei on the uninjected side of the embryos (arrowheads in A and B), disrupting Pcdh7 via either morpholino or dominant-negative results in loss of sensorial layer cells on the injected side. In C and D, embryos were injected with Pcdh7ΔE and LacZ mRNA, then fixed, sectioned and immunostained for β-catenin, which localizes to the cell membranes on the uninjected side of the embryo (arrowheads in C); in contrast, the injected side exhibits reduced β-catenin staining (D), and an increase in the pigmented outer layer (arrows in D′). To confirm that the ectoderm is disrupted, Pcdh7ΔE-injected embryos were subjected to in situ hybridization for epidermal keratin at stage 20 (E, F). The injected side of the embryos reveals a thicker, disorganized ectoderm (arrows in E) as compared to the uninjected side (F). Abbreviations: np, neural plate; nc, notochord; psm, presomitic mesoderm; nt, neural tube.

and required for neural crest differentiation (De Calisto et al., 2005). As revealed by in situ hybridization, Wnt11b is expressed in the sensorial layer at the neural crest border; furthermore, double in situ hybridization reveals that the cells expressing Wnt11b co-express Pcdh7 (Fig. 6A, B). Given the overlap of Pcdh7 and Wnt11b, we then asked whether Pcdh7 is required for expresssion of Wnt11b. Results indicate that in Pcdh7MO morphants, Wnt11b expression is reduced on the injected side, as compared to CMO-injected embryos (Fig. 6C, D). Thus, Pcdh7 is required for proper differentiation of the sensorial layer and for expression of Wnt11b at the lateral neural crest border. This suggests that loss of Wnt11b in Pcdh7 morphants contributes to neural crest apoptosis. To examine this directly, we asked whether the neural crest apoptosis in Pcdh7MO morphants is rescued by ectopically expressing Wnt11b. As compared to control embryos injected with the Pcdh7MO and LacZ mRNA, embryos co-injected with Pcdh7MO plus Wnt11b mRNA exhibit a substantial decrease in the number of apoptotic cells on the injected side (Fig. 7). These results indicate that loss of Wnt11b in the sensorial layer in Pcdh7MO-injected embryos is at least partially responsible for the neural crest apoptosis we observe.

As Pcdh7 knockdown results in apoptosis and consequently reduction in Snail2 expression, we next asked whether ectopic expression of Snail 2 is sufficient to prevent the Pcdh7MO-induced apoptosis of the cranial neural crest. To test this possibility, embryos were co-injected with the Pcdh7MO and Snail2 mRNA and assayed for Caspase-positive apoptotic cells. Indeed, ectopic expression of Snail2 was sufficient to prevent neural crest apoptosis in Pcdh7 morphants, as analyzed at stage 17. Snail2 has previously been implicated in the intrinsic apoptosis pathway, leading to the regulation of Bax/Bcl-2 apoptotic proteins (Tribulo et al., 2004; Zhang et al., 2006; Zhang and Klymkowsky, 2009). Therefore, to test whether loss of Pcdh7 caused the neural crest cells to die via the intrinsic apoptotic pathway, and to confirm the role of Snail2 in this pathway, we asked whether ectopic expression of the anti-apoptotic protein Xenopus Bcl2l1 (Bcl-xL) could also rescue Pcdh7MO-induced neural crest apoptosis. Results indicate that co-injecting Pcdh7MO and Bcl2l1 also rescues apoptosis of the prospective neural crest. In contrast, ectopic expression of Wnt11b, Snail2 or Bcl2l1 alone has no obvious effect on cranial neural crest apoptosis. Altogether, these results suggest that one role of Wnt11b may be to maintain Snail2 expression in the neural crest, without which the cells undergo apoptosis.

2.4. Wnt11b rescues Snail2 expression and cranial neural crest cell fate

To determine if Wnt11b is involved in maintaining Snail2 expression, we next asked whether Wnt11b rescues Snail2 expression in Pcdh7MO-injected embryos. Therefore, embryos were co-injected with Pcdh7MO and Wnt11 mRNA, along with LacZ mRNA, and assayed

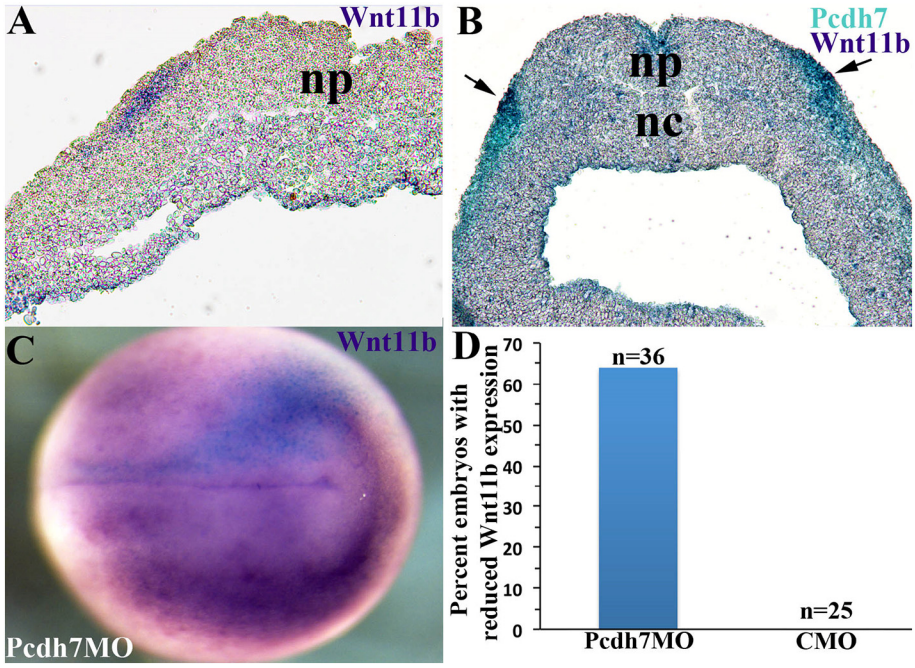


Fig. 6. Wnt11b is disrupted in Pcdh7MO-injected embryos. Embryos were analyzed by in situ hybridization for Wnt11b (A) or double in situ hybridization for Pcdh7 and Wnt11b (B), demonstrating that Wnt11b and Pcdh7 expression in the sensorial layer overlap at the neural crest border (arrows in B). To examine the effect of Pcdh7 knockdown on Wnt11b expression, embryos were injected with Pcdh7MO, together with LacZ mRNA, fixed at stage 17/18, stained for β-galactosidase, and subjected to in situ hybridization for Wnt11b (C). Results demonstrate that Pcdh7MO-injected embryos exhibit reduced Wnt11b expression on the injected side (top of photo in C), as compared to CMO-injected embryos (D).

for Snail2 expression by in situ hybridization. Results indicate that co-injection of Pcdh7MO and Wnt11b mRNA not only rescues apoptosis, but also rescues Snail2 expression. Similarly, co-injection of Pcdh7MO and Bcl2l1 mRNA also rescues Snail2 expression in embryos, confirming that Snail2 functions in the intrinsic apoptosis pathway (Fig. 8 and Supplementary Table II).

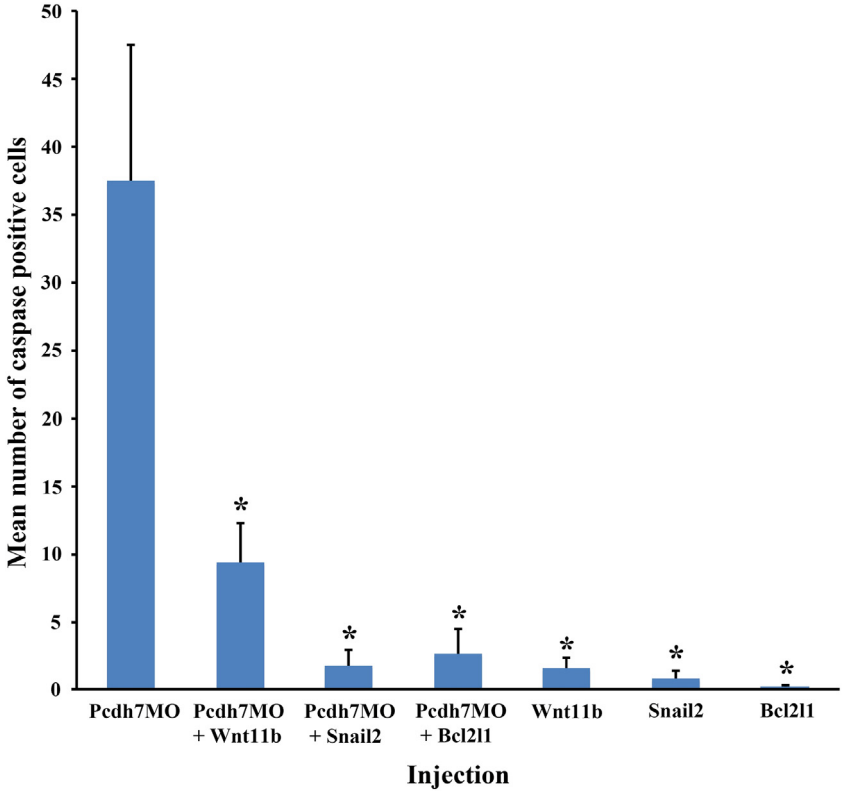


Fig. 7. Ectopic expression of Wnt11b, Snail2 or Bcl2l1 rescues Pcdh7MO-induced neural crest apoptosis. Embryos were co-injected with Pcdh7MO and mRNA encoding Wnt11b, Snail2 or Bcl2l1, together with LacZ mRNA, then fixed at stage 17, stained for β-galactosidase, and immunostained for activated Caspase 3. The mean number of caspase positive cells was determined for at least 6 embryos for each injection, demonstrating that Pcdh7-induced apoptosis is rescuable by co-injecting Wnt11b, Snail2 or Bcl2l1 mRNAs. Injecting Wnt11b, Snail2 or Bcl2l1 mRNA alone results in few apoptotic neural crest cells. * indicates statistical significant difference ($p < 0.001$) as compared to Pcdh7MO-injected embryos.

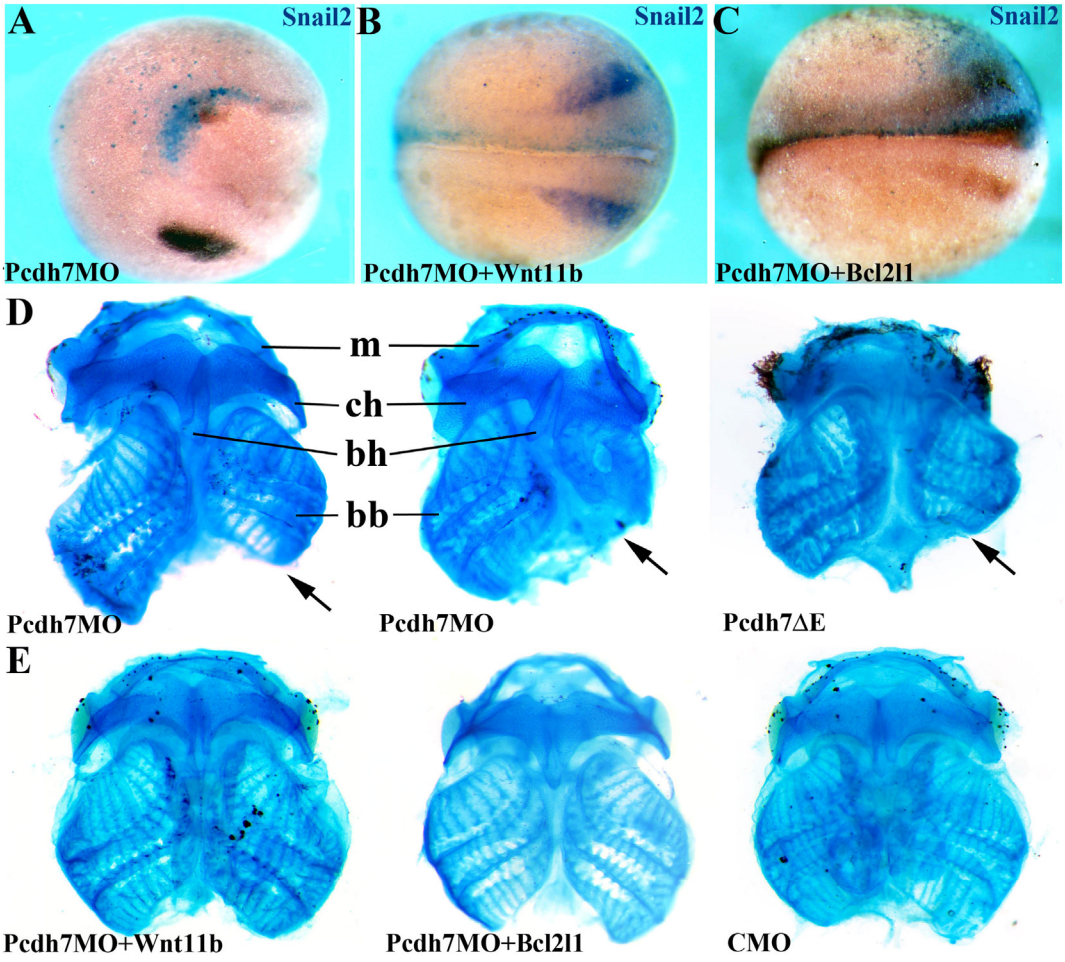


Fig. 8. Ectopic expression of Wnt11b or Bcl2l1 rescues Snail2 expression and cranial cartilage in Pcdh7MO-injected embryos. A–C) Embryos were co-injected with Pcdh7MO and mRNA encoding Wnt11b or Bcl2l1, together with LacZ mRNA, fixed at stage 17, stained for β-galactosidase, and analyzed by in situ hybridization for Snail2. Pcdh7MO (A) causes reduced Snail2 expression, which is rescued by either Wnt11b (B) or Bcl2l1 (C). The injected side of the embryos is up and anterior is to the right in A–C. D) Pcdh7 knockdown results in cranial cartilage defects. Embryos were injected with Pcdh7MO, together with GFP mRNA, sorted for GFP expression in the head, fixed at stage 46 and stained for cranial cartilage with Alcian Blue. Pcdh7MO-injected embryos exhibit defects in the branchial basket (bb) cartilage that range mild (arrow, left panel), to moderate (arrow, middle panel). Similarly, Pcdh7ΔE -injected embryos (arrow, right panel), exhibit bb defects, confirming the specificity of Pcdh7 knockdown. E) The Pcdh7MO-induced cranial cartilage defects are rescued by ectopic expression of Wnt11b (left panel) or Bcl2l1 (middle panel). In comparison, CMO-injected embryos exhibit no cranial cartilage defects (right panel). The injected side of the embryo is to the right in D and E. Abbreviations: bb, branchial basket; bh, basihyobranchial cartilage; ch, ceratohyal cartilage; m, Meckel's cartilage.

While the above results demonstrate that Wnt11b can rescue both apoptosis and Snail2 expression in Pcdh7MO-injected embryos, we next asked whether specification, and the subsequent differentiation of the cranial neural crest, is also rescued. To investigate this, we examined whether Wnt11b is sufficient to restore the developmental fate of the neural crest in Pcdh7-disrupted embryos, specifically to form the cranial cartilage. Embryos were co-injected with Pcdh7MO and Wnt11b mRNA, together with GFP mRNA as a tracer, then fixed at stage 46 and stained with Alcian Blue to visualize cranial cartilage. Consistent with our results demonstrating neural crest apoptosis, cranial cartilage structures are absent or reduced in Pcdh7MO- or Pcdh7ΔE -injected embryos, as compared to CMO-injected embryos. Cartilage defects from either Pcdh7MO- or Pcdh7ΔE -injected embryos were mostly mild to moderate, with a reduction or loss of the branchial basket the predominant phenotype, at the posterior end of cranial cartilage (Fig. 8D). Confirming the associated roles of Wnt11b and Pcdh7 in cranial cartilage development, co-injecting Wnt11b with the Pcdh7 morpholino partially rescues these defects (Fig. 8E and Supplementary Table III). Similarly, co-injecting Bcl2l1 with Pcdh7MO also rescues cranial cartilage defects, indicating that preventing apoptosis and rescuing Snail2 expression, via either Wnt11b or the anti-apoptotic factor Bcl2l1, results in cranial neural crest cells that migrate and develop normally. Collectively, these results strongly suggest that one important role of Pcdh7 at the neural crest border is to maintain sensorial layer tissue integrity required for proper Wnt11b expression, which then acts to maintain Snail2 expression and neural crest survival.

3. Discussion

3.1. Pcdh7 is required for proper formation of the sensorial layer

In Xenopus, the sensorial layer of the ectoderm forms early during embryonic development by the radial intercalation of deeper cells during gastrulation (Keller, 1991; Keller, 1980). These cells express Pcdh7 and disrupting Pcdh7 inhibits radial intercalation, disrupting the formation and differentiation of the inner layer (Bradley et al., 1998; Heggem and Bradley, 2003). In frogs, the neural crest cells also arise from the inner sensorial layer of the ectoderm, just lateral to the neural ectoderm. We show here that the lateral domain of the presumptive neural crest expresses Pcdh7 and that loss of Pcdh7 disrupts the lateral border sensorial layer cells and results in neural crest apoptosis. We also see reduced, albeit not statistically significant, neural crest proliferation, which may reflect the loss of neural crest cells by apoptosis. While we cannot exclude the possibility that disrupting Pcdh7 in the neural plate or epidermal ectoderm (further lateral to the neural crest border) also contributes to the loss of neural crest cells, we feel that this

is unlikely: disrupting Pcdh7 in the neural or epidermal ectoderm leads to altered cell sorting, but apoptosis is rarely observed (Heggem and Bradley, 2003; Rashid et al., 2006). Apoptosis of the neural crest is only observed when Pcdh7MO or Pcdh7ΔE is targeted to the lateral neural crest border. We conclude that Pcdh7 is necessary for maintaining tissue integrity at the lateral border and the secretion of neural crest survival signals, such as Wnt11b.

3.2. Cadherin family members and neural crest development

The mechanism by which Pcdh7 functions to maintain the lateral border is likely as a cell-cell adhesion molecule. As shown here, disrupting Pcdh7 at the lateral neural crest border leads to a loss of cell-cell adhesion between the sensorial layer cells, as evidenced by the loss of β-catenin staining at the cell membrane and the general disorganized appearance of the sensorial layer. The mechanism by which Wnt11b expression is then lost in these cells is not clear, but may involve altered cell sorting and a fate change in the sensorial layer. This is supported by the loss of p63 expression in the sensorial layer and the increase in the outer pigmented epithelial layer and increased epidermal keratin staining observed (Fig. 5). Whether Pcdh7 acts homotypic or heterotypic, interacting with other cadherins, to maintain the sensorial layer is not well understood. Previous results demonstrate that Pcdh7-expressing ectodermal cells sort together in a cohesive cluster, suggestive of a homophilic adhesion molecule (Bradley et al., 1998). However, Pcdh7 has also been shown to interact with connexin 43 and promote gap junction formation between carcinoma cells and astrocytes (Chen et al., 2016). Altogether, these results indicate that Pcdh7 plays a role in cell-cell adhesion and/or cell communication within the sensorial layer, without which the cells do not properly differentiate and lose expression of Wnt11b.

Several other cadherin family members are involved in neural crest development, particularly during EMT and migration. In avians these include N-cadherin, Cadherin-6B, Cadherin-7 and Protocadherin-1, while in frogs these include Cadherin-11, Protocadherin 8 (paraxial protocadherin) and Protocadherin-8-like (PCNS). Disrupting any of these cadherins in embryos results in defects in neural crest emigration, migration and/or ultimately cell fate, in concert with their roles in promoting cell adhesion (Bononi et al., 2008; Borchers et al., 2001; Coles et al., 2007; Nakagawa and Takeichi, 1995; Nakagawa and Takeichi, 1998; Rangarajan et al., 2006; Schneider et al., 2014; Shoval et al., 2007). However, as shown here for Pcdh7, proper cadherin function may promote neural crest survival and migration beyond strictly regulating adherens junctions. For example, in Xenopus, Cadherin-11 promotes adhesion to fibronectin and affects cranial neural crest motility by its involvement with small Rho-GTPases (Abbruzzese et al., 2016; Becker et al., 2013; Kashef et al., 2009; Langhe et al., 2016).

3.3. Wnt11b acts as a survival factor for the neural crest

Our results indicate that at least one function of an intact sensorial layer at the neural crest border is as a source of the secreted factor Wnt11b. A role for Wnt proteins in neural crest differentiation is well established. There is strong evidence that canonical Wnt signaling is required for NC induction (Deardorff et al., 2001; LaBonne and Bronner-Fraser, 1998; Steventon et al., 2009; Vallin et al., 2001). In contrast, neural crest specification and migration requires noncanonical Wnt signaling, via Wnt11 and Wnt11b (De Calisto et al., 2005; Matthews et al., 2008; Mayor and Theveneau, 2014; Ossipova and Sokol, 2011). In Xenopus, Wnt11 is expressed in the neural plate, medial to the Snail2 expressing neural crest cells, while Wnt11b is expressed lateral to the neural crest, overlapping with Pcdh7 expression. While both Wnt11 and Wnt11b are expressed adjacent to the neural crest, neither appears to overlap the neural crest domain prior to neural crest migration. Disrupting either Wnt11 or Wnt11b in Xenopus does not alter neural crest induction or Snail2 expression, but does result in a failure of cranial neural crest to migrate, with the cells accumulating next to the hindbrain (De Calisto et al., 2005; Matthews et al., 2008). This differs somewhat from our results, as when Pcdh7 is inhibited and Wnt11b expression is downregulated, neural crest cells undergo apoptosis. One possibility that could account for this disparity is that inhibiting Pcdh7 may affect the expression of additional factors secreted by the sensorial layer. Other secreted factors reported to be in the ectoderm and implicated in neural crest differentiation include Wnt7b, Wnt10a and BMP4 (Chang and Hemmati-Brivanlou, 1998; Garriock et al., 2007; Steventon et al., 2009). Thus, in this scenario, disrupting the lateral neural crest border via Pcdh7 knockdown results in a loss of Wnt11b plus some additional factor(s), which then results in loss of a survival and migration signal. However, that this loss is at least partially rescued by ectopic expression of Wnt11b strongly implies that Wnt11b is involved in more than just neural crest migration. A role for Wnt11b as a survival factor has not previously been described, although the related mammalian Wnt11 has been shown to promote survival and migration of several cell types (Ouko et al., 2004; Railo et al., 2008; Uysal-Onganer et al., 2010).

Our results suggest that the mechanism by which Wnt11b acts as a survival factor in Xenopus is by maintaining Snail2 expression in the neural crest. In support of this, neural crest apoptosis in Pcdh7 knockdown embryos is rescued by ectopic expression of either Snail2 or Wnt11b, with ectopic Wnt11b rescuing Snail2 expression (Figs. 7, 8). Ectopic expression of Wnt11b was previously reported to have no effect on neural crest induction and Snail2 expression in early neurulas (De Calisto et al., 2005). In our hands, we observed an increase in Snail2 expression in a proportion of embryos injected with Wnt11b (Supplementary Table II and data not shown). Though the experiments are different, our results may further point to a role for Wnt11b in regulating Snail2 expression after induction, during neural crest specification. In addition, we cannot rule out a role for Wnt11b in increasing neural crest proliferation as well. Both Snail2 and Twist are anti-apoptotic and can positively regulate expression of the anti-apoptotic protein Bcl2l1 as well as negatively regulate pro-apoptotic caspase-9 expression, likely acting via the NFkB signaling cascade (Inukai et al., 1999; Maestro et al., 1999; Tribulo et al., 2004; Zhang et al., 2006; Zhang and Klymkowsky, 2009). Morpholinos that inhibit Snail2 or Twist in early embryos result in cranial neural crest apoptosis and cartilage defects that are similar to those we observe upon inhibition of Pcdh7 (Tribulo et al., 2004; Zhang et al., 2006; Zhang and Klymkowsky, 2009). In the case of a Snail2 morpholino, this loss in neural crest is partially rescued by ectopic expression of either BMP4 or Wnt8, with the two together acting synergistically, supporting the likelihood that several factors are involved in neural crest survival (Shi et al., 2011). Wnt8, along with Wnt3a, FGF3 and FGF8 are primarily expressed in the underlying mesoderm (Betancur et al., 2010; Sauka-Spengler and Bronner-Fraser, 2008; Stuhlmiller and Garcia-Castro, 2012). However, since Pcdh7 is not expressed in mesoderm and disrupting Pcdh7 does not affect the morphological integrity of the mesoderm, it is unlikely that these factors are directly involved in the apoptosis observed in Pcdh7 morphants (Heggem and Bradley, 2003; Rashid et al., 2006). Thus, by disrupting Pcdh7, as reported here, we can directly discern that the sensorial layer, lateral to the neural crest domain, plays an important role in neural crest survival and differentiation. In our model, Pcdh7 is required for the integrity of this layer, which secretes Wnt11b, and possibly additional factors, required at or before neural crest specification and prior to migration.

In the neural crest, Wnt11b is thought to function via the Frizzled 7 receptor (Fzd7). Fzd7 is expressed in a subset of premigratory NC cells adjacent to the Wnt11b expression domain (De Calisto et al., 2005) and interestingly, is a known survival factor for colon and breast cancer cells (Simmons Jr. et al., 2014; Ueno et al., 2009). As Pcdh7 is expressed in the lateral neural crest cells, where it likely overlaps the Fzd7

domain, we cannot rule out that Pcdh7 is also required for proper reception of the Wnt11b signal by the lateral neural crest domain.

3.4. Pcdh7 and cranial neural crest cells

In Xenopus, the pharyngeal cranial neural crest forms from the border region between the hindbrain and the non-neural ectoderm and begins migration shortly before neural tube closure, migrating ventrally in three streams towards the pharyngeal pouches to give rise to the cranial cartilages. Neural crest cells from the most anterior pharyngeal stream migrate around the developing eye into the mandibular arch to form Meckel's cartilage. Neural crest in the second stream migrate to the hyoid arch to form the ceratohyal cartilage, while cells in the posterior stream migrate to the anterior and posterior branchial arches and differentiate into branchial cartilage (Sadaghiani and Thiebaud, 1987). Disrupting Pcdh7 in the cranial neural crest results in cranial cartilage defects, primarily in the branchial basket cranial cartilage, as opposed to Meckel's and hyal cartilages. This may indicate that the most posterior branchial neural crest stream is more sensitive to Pcdh7 knockdown, which is substantiated by the in situ hybridization results that show highest Pcdh7 expression at the posterior end of the cranial neural crest domain (Fig. 1). While we cannot rule out preferential targeting of the Pcdh7MO or Pcdh7ΔE construct to the branchial arch neural crest stream, we generally observed morpholino or Pcdh7ΔE/GFP positive cells well anterior to the pharyngeal arch neural crest region, which makes this possibility less likely. Importantly, only neural crest-derived cranial cartilages are altered in Pcdh7 knockdown embryos; the basihyobranchial structure, which forms from mesoderm and protrudes central to the ceratohyal cartilage (Olsson and Hanken, 1996), was unaffected (Fig. 8F).

It has been proposed that neural crest cells are especially sensitive to perturbation, causing them to undergo apoptosis, as compared to neighboring tissue. This sensitivity is likely due to the multiple genes required for precisely coordinated secreted signals and tissue interactions, at varying stages, from induction to EMT, migration, and ultimately to final differentiation (Betancur et al., 2010; Driever et al., 1996; Neuhauss et al., 1996; Schille and Schambony, 2017). This is supported by the relatively high prevalence of neural crest defects in humans (1 per 300 to 2500 births) caused by genetic defects or prenatal exposures, many of which result in apoptosis of the neural crest (Chappell Jr. et al., 2009; McCarthy and Eberhart, 2014; WHO, 2004; Stanier and Moore, 2004). In the zebrafish embryo, neural crest cells were reported to be especially sensitive to morpholino knockdown, as compared to genetic knockouts of the same gene, and similar phenomena have been demonstrated for siRNAs (Boer et al., 2016; Robu et al., 2007). This may be due to non-specific effects of the morpholino, mosaic distribution of the morpholino, or compensatory mechanisms that are specifically upregulated in a genetic knockout, but not in a knockdown (Rossi et al., 2015). Due to this potential increased sensitivity, we verified that the apoptosis we observed in Pcdh7MO knockdown embryos is specific to inhibition of Pcdh7 by rescuing the defects with full-length Pcdh7 mRNA. Furthermore, we also utilized Pcdh7ΔE, a dominant negative version of Pcdh7 that lacks the extracellular domain and competes with the intracellular domain of endogenous Pcdh7. Both Pcdh7MO and Pcdh7ΔE cause similar neural tube closure and sensorial layer defects (Bradley et al., 1998; Heggem and Bradley, 2003; Rashid et al., 2006). In this study, loss of neural crest specification markers, apoptosis and downstream craniofacial cartilage defects were all recapitulated with the Pcdh7ΔE dominant negative, strongly implying that the neural crest defects are specific to loss of Pcdh7 function.

In sum, our results add Pcdh7 to the cadherin family members involved in neural crest development in Xenopus, albeit in a unique role. While Pcdh7 is expressed in a subset of neural crest cells, its main function in relation to neural crest is to maintain tissue integrity of the sensorial layer. Not only do neural crest cells derive from the sensorial layer in Xenopus, but this layer, lateral to the neural crest domain, is also responsible for critical secreted signals for neural crest development. We show here that knockdown of Pcdh7 results in a loss of Wnt11b from the sensorial layer and subsequent defects in cranial cartilage, demonstrating that Pcdh7 is a critical factor for neural crest survival and differentiation.

4. Materials and methods

4.1. In situ hybridization

In situ hybridizations were performed according to standard protocols (Broadbent and Read, 1999; Harland, 1991). Digoxygenin- and FITC-labeled riboprobes were generated from cDNAs encoding Pcdh7 (Bradley et al., 1998), Pax3 (Espeseth et al., 1995), Snail2 and Zic1 (Mayor et al., 1995; Milet et al., 2013) (kind gifts of C. Merzdorf), c-myc (Bellmeyer et al., 2003) (kind gift of C. LaBonne), Twist and Wnt11b (EXRC) and epidermal keratin (Kintner and Melton, 1987). Sox2, Sox15, Six1 and Sox9 cDNAs were generated by PCR from a stage 17 Xenopus cDNA library (Kintner and Melton, 1987) and cloned into pBSSKII. Detection reagents for single in situs utilized BCIP/NBT (Millipore), while double in situs were detected with BCIP and magenta phosphate or BM-purple (Sigma-Aldrich).

4.2. Microinjections

Synthesis and injection of mRNA encoding Pcdh7, Pcdh7ΔE, LacZ and green fluorescent protein (eGFP) and the antisense Pcdh7 morpholino and control morpholino were as previously described (Heggem and Bradley, 2003; Rashid et al., 2006). To minimize off-target effects, 1 ng morpholino was injected per embryo. Constructs encoding full-length Wnt11b or Bcl2l1 were obtained from EXRC and Thermo Scientific, respectively, and 1 ng RNA was injected per embryo for rescue experiments. Embryos were injected at the 4-cell stage and fixed in MEMFA (100 mM MOPS, 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde pH 7.4) at the indicated stages and stained for β-galactosidase activity as previously described (Bradley et al., 1998).

4.3. Immunostaining

Morpholino- or Pcdh7ΔE -injected embryos were fixed, embedded in OCT (Tissue-Tek), then cryosectioned. For activated Caspase-3 (Cell Signaling Technology) embryos were fixed in Dent's (80% MeOH, 20% DMSO), and stained at 1:200 overnight at 4 °C, followed by an Alexafluor goat-anti mouse 568-conjugated secondary antibody (Molecular Probes). Sections were analyzed on a Zeiss Axioscope and imaged using ProgRes Capture Pro software. The average number of caspase positive cells per section was determined for 6–12 embryos per injection. Cells within the cranial neural crest domain (defined by the region bounded by, but not including, the neural tube, epithelial/ectodermal boundary, and endoderm) were counted and compared between injected and uninjected sides of embryos, and with control morpholino injected embryos. The most anterior third of each embryo was sectioned and counted, correlating to the approximate boundary of cranial neural crest. For sensorial layer staining, embryos were co-injected with Pcdh7MO or Pcdh7ΔE, together with LacZ or GFP mRNA, fixed in MEMFA, cryosectioned, treated in acetone and incubated in anti-p63 (Oncogene Research Products) or anti-β-catenin (Sigma-Aldrich) at 1:100 overnight at 4 °C followed by an Alexafluor goat-anti mouse 568-conjugated secondary antibody. The *t*-test for independent groups was used to determine statistical difference between conditions (vassarstats.net).

4.4. Cartilage staining

To examine cranial cartilage, embryos were injected at the 4–8 cell stage with the above constructs together with RNA encoding eGFP.

Embryos were sorted at stage 28 based on right/left cranial GFP expression, then fixed in MEMFA at stage 46 and dehydrated in ethanol. Cartilage staining was performed as described (Bellmeyer et al., 2003). Briefly, embryos were stained for 3–5 days with Alcian Blue (0.04% Alcian Blue in 30% acetic acid, 70% ethanol), then rehydrated by washing in a graded series of ethanol:potassium hydroxide solutions. Finally, embryos were passed through a graded series of glycerol in 2% KOH solutions. Ectoderm tissue was removed by dissection to reveal the underlying cranial cartilage. Defects were judged as mild if the injected side cartilage was reduced in size; moderate if morphological abnormalities were observed; or severe if cartilage on the injected side was absent.

Acknowledgments

We thank C. West, B. Murphy and M. Hedinger for technical assistance. This study was supported by grants from NSF (IOS 0923651). Undergraduate research funding for P.P. and E.R. provided by grants from NIH (NIGMS P20GM103474) and The Howard Hughes Medical Institute (HHMI 52006931).

Conflicts of interest: none

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mod.2018.01.002.

References

Abbruzzese, G., Becker, S.F., Kashef, J., Alfandari, D., 2016. ADAM13 cleavage of cadherin-11 promotes CNC migration independently of the homophilic binding site. Dev. Biol. 415, 383–390.

Batle, E., Sancho, E., Franci, C., Dominguez, D., Monfar, M., Baulida, J., Garcia de Herreros, A., 2000. The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. Nat. Cell Biol. 2.

Becker, S.F., Mayor, R., Kashef, J., 2013. Cadherin-11 mediates contact inhibition of locomotion during Xenopus neural crest cell migration. PLoS One 8, e85717.

Bellmeyer, A., Krase, J., Lindgren, J., LaBonne, C., 2003. The protooncogene c-Myc is an essential regulator of neural crest formation in Xenopus. Dev. Cell 4, 827–839.

Betancur, P., Bronner-Fraser, M., Sauka-Spengler, T., 2010. Assembling neural crest regulatory circuits into a gene regulatory network. Annu. Rev. Cell Dev. Biol. 26, 581–603.

Boer, E.F., Jette, C.A., Stewart, R.A., 2016. Neural crest migration and survival are susceptible to morpholino-induced artifacts. PLoS One 11, e0167278.

Bolos, V., Peinado, H., Perez-Moreno, M.A., Fraga, M.F., Esteller, M., Cano, A., 2003. The transcription factor slug represses E-cadherin expression and induces epithelial to mesenchymal transitions: comparison with snail and E47 repressors. J. Cell Sci. 116, 499–511.

Bononi, J., Cole, A., Tewson, P., Schumacher, A., Bradley, R., 2008. Chicken protocadherin-1 functions to localize neural crest cells to the dorsal root ganglia during PNS formation. Mech. Dev. 125, 1033–1047.

Borchers, A., David, R., Wedlich, D., 2001. Xenopus cadherin-11 restrains cranial neural crest migration and influences neural crest specification. Development 128, 3049–3060.

Bradley, R.S., Espeseth, A., Kintner, C., 1998. NF-protocadherin, a novel member of the cadherin superfamily, is required for Xenopus ectodermal differentiation. Curr. Biol. 8, 325–334.

Broadbent, J., Read, E.M., 1999. Wholemount in situ hybridization of Xenopus and zebrafish embryos. Methods Mol. Biol. 127, 57–67.

Chang, C., Hemmati-Brivanlou, A., 1998. Neural crest induction by Xwnt7b in Xenopus. Dev. Biol. 194, 129–134.

Chappell Jr., J.H., Wang, X.D., Loeken, M.R., 2009. Diabetes and apoptosis: neural crest cells and neural tube. Apoptosis 14, 1472–1483.

Chen, Q., Boire, A., Jin, X., Valiente, M., Er, E.E., Lopez-Soto, A., Jacob, L., Patwa, R., Shah, H., Xu, K., Cross, J.R., Massague, J., 2016. Carcinoma-astrocyte gap junctions promote brain metastasis by cGAMP transfer. Nature 533 (7604), 493–498.

Coles, E.G., Taneyhill, L.A., Bronner-Fraser, M., 2007. A critical role for Cadherin6B in regulating avian neural crest emigration. Dev. Biol. 312, 533–544.

De Calisto, J., Araya, C., Marchant, L., Riaz, C.F., Mayor, R., 2005. Essential role of non-canonical Wnt signalling in neural crest migration. Development 132, 2587–2597.

Deardorff, M.A., Tan, C., Saint-Jeannet, J.P., Klein, P.S., 2001. A role for frizzled 3 in neural crest development. Development 128, 3655–3663.

Driever, W., Solnica-Krezel, L., Schier, A.F., Neuhauss, S.C., Malicki, J., Stemple, D.L., Stainier, D.Y., Zwartkruis, F., Abdelilah, S., Rangini, Z., Belak, J., Boggs, C., 1996. A genetic screen for mutations affecting embryogenesis in zebrafish. Development 123, 37–46.

Espeseth, A., Johnson, E., Kintner, C., 1995. Xenopus F-cadherin, a novel member of the cadherin family of cell adhesion molecules, is expressed at boundaries in the neural tube. Mol. Cell. Neurosci. 6, 199–211.

Garriock, R.J., Warkman, A.S., Meadows, S.M., D'Agostino, S., Krieg, P.A., 2007. Census of vertebrate Wnt genes: isolation and developmental expression of Xenopus Wnt2, Wnt3, Wnt9a, Wnt9b, Wnt10a, and Wnt16. Dev. Dyn. 236, 1249–1258.

Harland, R.M., 1991. In situ hybridization: an improved whole-mount method for Xenopus embryos. Methods Cell Biol. 36, 685–695.

Heggem, M.A., Bradley, R.S., 2003. The cytoplasmic domain of Xenopus NF-protocadherin interacts with TAF1/set. Dev. Cell 4, 419–429.

Inukai, T., Inoue, A., Kurosawa, H., Goi, K., Shinjyo, T., Ozawa, K., Mao, M., Inaba, T., Look, A.T., 1999. SLUG, a ces-1-related zinc finger transcription factor gene with antiapoptotic activity, is a downstream target of the E2A-HLF oncoprotein. Mol. Cell 4, 343–352.

Kashef, J., Kohler, A., Kuriyama, S., Alfandari, D., Mayor, R., Wedlich, D., 2009. Cadherin-11 regulates protrusive activity in Xenopus cranial neural crest cells upstream of Trio and the small GTPases. Genes Dev. 23, 1393–1398.

Keller, R.E., 1980. The cellular basis of epiboly: an SEM study of deep-cell rearrangement during gastrulation in *Xenopus laevis*. J. Embryol. Exp. Morphol. 60, 201–234.

Keller, R., 1991. Early embryonic development of *Xenopus laevis*. Methods Cell Biol. 36, 61–113.

Kintner, C.R., Melton, D.A., 1987. Expression of Xenopus N-CAM RNA in ectoderm is an early response to neural induction. Development 99, 311–325.

Klymkowsky, M.W., Rossi, C.C., Artinger, K.B., 2010. Mechanisms driving neural crest induction and migration in the zebrafish and *Xenopus laevis*. Cell Adhes. Migr. 4, 595–608.

Koster, M.I., Kim, S., Mills, A.A., DeMayo, F.J., Roop, D.R., 2004. p63 is the molecular switch for initiation of an epithelial stratification program. Genes Dev. 18, 126–131.

LaBonne, C., Bronner-Fraser, M., 1998. Neural crest induction in Xenopus: evidence for a two-signal model. Development 125, 2403–2414.

Langhe, R.P., Gudzenko, T., Bachmann, M., Becker, S.F., Gonnermann, C., Winter, C., Abbruzzese, G., Alfandari, D., Kratzer, M.C., Franz, C.M., Kashef, J., 2016. Cadherin-11 localizes to focal adhesions and promotes cell-substrate adhesion. Nat. Commun. 7, 10909.

Le Douarin, N.M., Kalcheim, C., 1999. The Neural Crest. second edition. Cambridge University Press.

Lu, P., Barad, M., Vize, P.D., 2001. Xenopus p63 expression in early ectoderm and neurectoderm. Mech. Dev. 102, 275–278.

Maestro, R., Dei Tos, A.P., Hamamori, Y., Krasnokutsky, S., Sartorelli, V., Kedes, L., Doglioni, C., Beach, D.H., Hannon, G.J., 1999. Twist is a potential oncogene that inhibits apoptosis. Genes Dev. 13 (17), 2207.

Matthews, H.K., Broders-Bondon, F., Thiery, J.P., Mayor, R., 2008. Wnt11r is required for cranial neural crest migration. Dev. Dyn. 237, 3404–3409.

Mayor, R., Theveneau, E., 2013. The neural crest. Development 140, 2247–2251.

Mayor, R., Theveneau, E., 2014. The role of the non-canonical Wnt-planar cell polarity pathway in neural crest migration. Biochem. J. 457, 19–26.

Mayor, R., Morgan, R., Sargent, M.G., 1995. Induction of the prospective neural crest of Xenopus. Development 121, 767–777.

McCarthy, N., Eberhart, J.K., 2014. Gene–ethanol interactions underlying fetal alcohol spectrum disorders. Cell. Mol. Life Sci. 71, 2699–2706.

Meulemans, D., Bronner-Fraser, M., 2004. Gene-regulatory interactions in neural crest evolution and development. Dev. Cell 7, 291–299.

Milet, C., Monsoro-Burq, A.H., 2012. Neural crest induction at the neural plate border in vertebrates. Dev. Biol. 366, 22–33.

Milet, C., Maczkowiak, F., Roche, D.D., Monsoro-Burq, A.H., 2013. Pax3 and Zic1 drive induction and differentiation of multipotent, migratory, and functional neural crest in Xenopus embryos. Proc. Natl. Acad. Sci. U. S. A. 110, 5528–5533.

Mizuseki, K., Kishi, M., Matsui, M., Nakanishi, S., Sasai, Y., 1998a. Xenopus Zic-related-1 and Sox-2, two factors induced by chordin, have distinct activities in the initiation of neural induction. Development 125 (4), 579–587.

Mizuseki, K., Kishi, M., Shiota, K., Nakanishi, S., Sasai, Y., 1998b. SoxD: an essential mediator of induction of anterior neural tissues in Xenopus embryos. Neuron 21 (1), 77–85.

Nakagawa, S., Takeichi, M., 1995. Neural crest cell-cell adhesion controlled by sequential and subpopulation-specific expression of novel cadherins. Development 121, 1321–1332.

Nakagawa, S., Takeichi, M., 1998. Neural crest emigration from the neural tube depends on regulated cadherin expression. Development 125, 2963–2971.

Neuhauss, S.C., Solnica-Krezel, L., Schier, A.F., Zwartkruis, F., Stemple, D.L., Malicki, J., Abdelilah, S., Stainier, D.Y., Driever, W., 1996. Mutations affecting craniofacial development in zebrafish. Development 123, 357–367.

Olsson, L., Hanken, J., 1996. Cranial neural-crest migration and chondrogenic fate in the oriental fire-bellied toad *Bombina orientalis*: defining the ancestral pattern of head development in anuran amphibians. J. Morphol. 229, 105–120.

Ossipova, O., Sokol, S.Y., 2011. Neural crest specification by noncanonical Wnt signaling and PAR-1. Development 138, 5441–5450.

Ouko, L., Ziegler, T.R., Gu, L.H., Eisenberg, L.M., Yang, V.W., 2004. Wnt11 signaling promotes proliferation, transformation, and migration of IEC6 intestinal epithelial cells. J. Biol. Chem. 279, 26707–26715.

Pandur, P.D., Moody, S.A., 2000. Xenopus Six1 gene is expressed in neurogenic cranial placodes and maintained in the differentiating lateral lines. Mech. Dev. 96 (2), 253–257.

Pegoraro, C., Monsoro-Burq, A.H., 2013. Signaling and transcriptional regulation in neural crest specification and migration: lessons from Xenopus embryos. Wiley Interdiscip. Rev. Dev. Biol. 2, 247–259.

Piper, M., Dwivedy, A., Leung, L., Bradley, R.S., Holt, C.E., 2008. NF-Protocadherin and TAF1 regulate retinal axon initiation and elongation in vivo. J. Neurosci. 28, 100–105.

Plouhinec, J.L., Roche, D.D., Pegoraro, C., Figueiredo, A.L., Maczkowiak, F., Brunet, L.J., Milet, C., Vert, J.P., Pollet, N., Harland, R.M., Monsoro-Burq, A.H., 2014. Pax3 and Zic1 trigger

the early neural crest gene regulatory network by the direct activation of multiple key neural crest specifiers. Dev. Biol. 386, 461–472.
Railo, A., Nagy, I.I., Kilpelainen, P., Vainio, S., 2008. Wnt-11 signaling leads to down-regulation of the Wnt/beta-catenin, JNK/AP-1 and NF-kappaB pathways and promotes viability in the CHO-K1 cells. Exp. Cell Res. 314, 2389–2399.
Rangarajan, J., Luo, T., Sargent, T.D., 2006. PCNS: a novel protocadherin required for cranial neural crest migration and somite morphogenesis in Xenopus. Dev. Biol. 295, 206–218.
Rashid, D., Newell, K., Shama, L., Bradley, R., 2006. A requirement for NF-protocadherin and TAF1/set in cell adhesion and neural tube formation. Dev. Biol. 291, 170–181.
Robu, M.E., Larson, J.D., Nasevicius, A., Beiraghi, S., Brenner, C., Farber, S.A., Ekker, S.C., 2007. p53 activation by knockdown technologies. PLoS Genet. 3, e78.
Rogers, C.D., Jayasena, C.S., Nie, S., Bronner, M.E., 2012. Neural crest specification: tissues, signals, and transcription factors. Wiley Interdiscip. Rev. Dev. Biol. 1, 52–68.
Rossi, A., Kontarakis, Z., Gerri, C., Nolte, H., Holper, S., Kruger, M., Stainier, D.Y., 2015. Genetic compensation induced by deleterious mutations but not gene knockdowns. Nature 524, 230–233.
Sadaghiani, B., Thiebaud, C.H., 1987. Neural crest development in the *Xenopus laevis* embryo, studied by interspecific transplantation and scanning electron microscopy. Dev. Biol. 124, 91–110.
Sauka-Spengler, T., Bronner-Fraser, M., 2008. A gene regulatory network orchestrates neural crest formation. Nat. Rev. Mol. Cell Biol. 9, 557–568.
Schille, C., Schambony, A., 2017. Signaling pathways and tissue interactions in neural plate border formation. Neurogenesis (Austin). 4, e1292783.
Schneider, M., Huang, C., Becker, S.F., Gradl, D., Wedlich, D., 2014. Protocadherin PAPC is expressed in the CNC and can compensate for the loss of PCNS. Genesis 52, 120–126.
Shi, J., Severson, C., Yang, J., Wedlich, D., Klymkowsky, M.W., 2011. Snail2 controls mesodermal BMP/Wnt induction of neural crest. Development 138, 3135–3145.
Shoval, I., Ludwig, A., Kalcheim, C., 2007. Antagonistic roles of full-length N-cadherin and its soluble BMP cleavage product in neural crest delamination. Development 134, 491–501.
Simmons Jr., G.E., Pandey, S., Nedeljkovic-Kurepa, A., Saxena, M., Wang, A., Pruitt, K., 2014. Frizzled 7 expression is positively regulated by SIRT1 and beta-catenin in breast cancer cells. PLoS One 9, e98861.
Spokony, R.F., Aoki, Y., Saint-Germain, N., Magner-Fink, E., Saint-Jeannet, J.P., 2002. The transcription factor Sox9 is required for cranial neural crest development in Xenopus. Development 129, 421–432.
Stanier, P., Moore, G.E., 2004. Genetics of cleft lip and palate: syndromic genes contribute to the incidence of non-syndromic clefts. Hum. Mol. Genet. 13, R73–R81.
Steventon, B., Araya, C., Linker, C., Kuriyama, S., Mayor, R., 2009. Differential requirements of BMP and Wnt signalling during gastrulation and neurulation define two steps in neural crest induction. Development 136, 771–779.
Stuhlmiller, T.J., Garcia-Castro, M.I., 2012. Current perspectives of the signaling pathways directing neural crest induction. Cell. Mol. Life Sci. 69, 3715–3737.
Taneyhill, L.A., Schiffmacher, A.T., 2017. Should I stay or should I go? Cadherin function and regulation in the neural crest. Genesis 55.
Taneyhill, L.A., Coles, E.G., Bronner-Fraser, M., 2007. Snail2 directly represses cadherin6B during epithelial-to-mesenchymal transitions of the neural crest. Development 134, 1481–1490.
Theveneau, E., Mayor, R., 2012. Neural crest delamination and migration: from epithelium-to-mesenchyme transition to collective cell migration. Dev. Biol. 366, 34–54.
Tien, C.L., Jones, A., Wang, H., Gerigk, M., Nozell, S., Chang, C., 2015. Snail2/slug cooperates with Polycomb repressive complex 2 (PRC2) to regulate neural crest development. Development 142, 722–731.
Tribulo, C., Aybar, M.J., Sanchez, S.S., Mayor, R., 2004. A balance between the anti-apoptotic activity of slug and the apoptotic activity of msx1 is required for the proper development of the neural crest. Dev. Biol. 275, 325–342.
Ueno, K., Hazama, S., Mitomori, S., Nishioka, M., Suehiro, Y., Hirata, H., Oka, M., Imai, K., Dahiya, R., Hinoda, Y., 2009. Down-regulation of frizzled-7 expression decreases survival, invasion and metastatic capabilities of colon cancer cells. Br. J. Cancer 101, 1374–1381.
Uysal-Onganer, P., Kawano, Y., Caro, M., Walker, M.M., Diez, S., Darrington, R.S., Waxman, J., Kypta, R.M., 2010. Wnt-11 promotes neuroendocrine-like differentiation, survival and migration of prostate cancer cells. Mol. Cancer 9, 55.
Vallin, J., Thuret, R., Giacomello, E., Faraldo, M.M., Thiery, J.P., Broders, F., 2001. Cloning and characterization of three Xenopus slug promoters reveal direct regulation by Lef/beta-catenin signaling. J. Biol. Chem. 276, 30350–30358.
World Health Organization, 2004. Global strategies to reduce the health care burden of craniofacial anomalies: report of WHO meetings on international collaborative research on craniofacial anomalies. Cleft Palate Craniofac. J. 238–243.
Zhang, C., Klymkowsky, M.W., 2009. Unexpected functional redundancy between twist and slug (Snail2) and their feedback regulation of NF-kappaB via nodal and cerberus. Dev. Biol. 331, 340–349.
Zhang, C., Carl, T.F., Trudeau, E.D., Simmet, T., Klymkowsky, M.W., 2006. An NF-kappaB and slug regulatory loop active in early vertebrate mesoderm. PLoS One 1, e106.