Available online at www.sciencedirect.com

SCIENCE DIRECT®

Developmental Biology 295 (2006) 206–218

DEVELOPMENTAL BIOLOGY

www.elsevier.com/locate/ydbio

PCNS: A novel protocadherin required for cranial neural crest migration and somite morphogenesis in *Xenopus*

Janaki Rangarajan, Ting Luo, Thomas D. Sargent *

Laboratory of Molecular Genetics, NICHD, NIH, Bethesda, MD 20892, USA

Received 21 November 2005; revised for publication 16 March 2006; accepted 20 March 2006
Available online 3 May 2006

Abstract

Protocadherins (Pcdhs), a major subfamily of cadherins, play an important role in specific intercellular interactions in development. These molecules are characterized by their unique extracellular domain (EC) with more than 5 cadherin-like repeats, a transmembrane domain (TM) and a variable cytoplasmic domain. *PCNS* (*P*roto*c*adherin in *N*eural crest and *S*omites), a novel Pcdh in *Xenopus*, is initially expressed in the mesoderm during gastrulation, followed by expression in the cranial neural crest (CNC) and somites. PCNS has 65% amino acid identity to *Xenopus* paraxial protocadherin (PAPC) and 42–49% amino acid identity to Pcdh 8 in human, mouse, and zebrafish genomes. Overexpression of PCNS resulted in gastrulation failure but conferred little if any specific adhesion on ectodermal cells. Loss of function accomplished independently with two non-overlapping antisense morpholino oligonucleotides resulted in failure of CNC migration, leading to severe defects in the craniofacial skeleton. Somites and axial muscles also failed to undergo normal morphogenesis in these embryos. Thus, *PCNS* has essential functions in these two important developmental processes in *Xenopus*.

Keywords: Somitogenesis; Cadherin; Cell adhesion; Epitheliomesenchymal transition

Introduction

Every embryo undergoes three stages of development: gastrulation, neurulation and organogenesis, as part of its transformation into a multi-cellular organism. During these stages, cells undergo different morphogenetic processes like proliferation, adhesion, migration, differentiation, and apoptosis. Two important developmental processes that take place in the vertebrate embryo are somitogenesis or the segmentation of the paraxial mesoderm into somites and the migration of the neural crest (NC) cells. Somitogenesis, which eventually gives rise to the vertebrae, ribs, dermis and their associated muscles, begins at the anterior end of the presomitic mesoderm (PSM), and passes down the axis of the embryo in a wave-like manner to generate somites at regular intervals. In *Xenopus*, the PSM is initially patterned by selector genes that specify segmental identity, leading to the formation of segmental boundaries. After the formation of the boundaries through the segregation of the myotomal cells from the PSM, the somite cells rotate 90° to conclude the formation of one somite segment (Hamilton, 1969; Youn and Malacinski, 1981). Theoretical models including the "clock and wavefront model", with the involvement of different genes, have been proposed for the segmentation of somites (Pourquie, 2001a,b, 2003a,b). Recent evidence suggests that the oscillating expression of genes like those involved in the Notch signaling pathway, the hairy-like WRPW-bHLH proteins, bHLH transcription factors of the Mesp family, retinoic acid and PAPC, match the periodicity of the somite formation and, thus, may be involved in the segmentation of the PSM (Jen et al., 1997; Kim et al., 2000; Moreno and Kintner, 2004; Rida et al., 2004; Sparrow et al., 1998). It is thought that these genes are either regulated by or components of the somite differentiation clock (Andrade et al., 2005; Pourquie, 2003a). Furthermore, manipulating the expression of these genes results in disruption of segmental identity (Kim et al., 2000). A recent study indicating that synchronized gene oscillations in the PSM cells require cell–cell contact suggests that adhesion molecules might be an integral part of signaling pathways controlling somitogenesis (Maroto et al., 2005).

The segregation of the NC cells from the dorsal neural tube and their migration throughout the embryo to give rise to different lineages is another dramatic morphogenetic event in

* Corresponding author.

0012-1606/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2006.03.025

embryogenesis (Erickson and Perris, 1993). The NC cell population that originates at the border between the neural plate and the non-neural ectoderm gives rise to a wide variety of cell lineages like the craniofacial skeleton, connective tissue, neurons, glia of the peripheral nervous system, melanocytes, and the neuroendocrine cells of the adrenal medulla (Basch et al., 2004; LaBonne and Bronner-Fraser, 1999). The cranial neural crest (CNC) plays an especially important role in determining the shape of the vertebrate head, as the source of most of the cranial bone and cartilages, as well as some of the musculature (Le Douarin and Kalcheim, 1999; Noden, 1991). In *Xenopus*, CNC cells migrate from the hindbrain in three streams and populate the pharyngeal arches. CNC cells from the anterior stream migrate around the eye vesicle, populate the mandibular arch and contribute to the formation of the Meckel's cartilage; those from the second stream populate the hyoid arch and give rise to the ceratohyal cartilage and those from the posterior stream give rise to the anterior and the posterior branchial arches (Sadaghiani and Thiebaud, 1987). The first step towards migration, the epitheliomesenchymal transition (EMT), is a complex process involving changes in basal lamina, cell adhesion, extracellular matrix, and the cytoskeleton (Erickson and Perris, 1993; Halloran and Berndt, 2003; Hay, 1995; Savagner, 2001). All the steps involved in the EMT require a combination of interaction between CNC cells and interactions between CNC cells and the surrounding environment or the extracellular matrix (ECM). One group of important molecules involved in the process of regulation of cell contacts and cytoskeletal dynamics underlying cell motility are the cadherins (Halloran and Berndt, 2003; Pla et al., 2001).

Cadherins are a superfamily of transmembrane glycoproteins which mediate homophilic calcium-dependent cell adhesion (Sano et al., 1993). Members of the cadherin superfamily are characterized by their unique extracellular (EC) domains, which are primarily composed of multiple repeats of a cadherin motif. Classical cadherins are likely to have evolved from ancestral proteins resembling protocadherins (Pcdhs), which constitute the biggest subfamily within the cadherin superfamily (Kim et al., 1998). Pcdh molecules contain more than five cadherin repeats in their EC domain with sequences more similar to each other than to the classical cadherins and with divergent and distinct cytoplasmic domains that lack β-catenin binding sites (Angst et al., 2001; Suzuki, 1996). Pcdh α, β, and γ gene clusters comprising many Pcdh genes have been found in different species including human, mouse, rat, chicken, and zebrafish (Noonan et al., 2004; Sugino et al., 2000, 2004; Tada et al., 2004; Wu and Maniatis, 1999; Wu et al., 2001; Yanase et al., 2004). At least 2 clusters of Pcdhγ genes have been found in the genome of *Xenopus tropicalis* (Ishii et al., 2004). Pcdh genes presumably appeared during evolution of the vertebrate central nervous system, since they have not been found in invertebrates such as *Drosophila* or *C. elegans* (Tada et al., 2004). Various studies on the role of Pcdhs in different species have shown their importance in development. (Aoki et al., 2003a; Bradley et al., 1998; Hirano et al., 1999; Kim et al., 1998; Kohmura et al., 1998; Kuroda et al., 2002; Obata et al., 1995; Yamagata et al., 1999). Especially in *Xenopus* and zebrafish, the functions of three Pcdh molecules, NF-Pcdh (NFPC), PAPC and Axial Pcdh (AXPC) have been studied during embryogenesis. NFPC was found to mediate differential adhesiveness required for proper ectodermal differentiation in *Xenopus* (Bradley et al., 1998). AXPC expression, limited to the notochord at neurula stages, has been shown to mediate cell sorting activity associated with axis formation (Kuroda et al., 2002), while PAPC has been implicated in partitioning gastrulating mesoderm into paraxial and axial domains (Kim et al., 1998) and in the maintenance of segmental gene expression and somite formation (Kim et al., 2000). Furthermore, the interaction between PAPC and the Frizzled receptor contributed to the separation of ectodermal and mesodermal cells during gastrulation by the activation of Rho and protein kinase Cα (Medina et al., 2004). PAPC was also found to functionally interact with the Wnt/planar cell polarity (PCP) pathway in the control of convergent extension movements in *Xenopus* by modulating the activity of Rho GTPase and jun N-terminal-kinase (JNK; Unterseher et al., 2004). In zebrafish, PAPC is a downstream target of Spadetail, a transcription factor necessary for morphogenetic movements during gastrulation (Yamamoto et al., 1998).

In this report, we have analyzed a novel Pcdh molecule, PCNS (*P*roto*c*adherin in *N*eural Crest and *S*omites), which was identified from a pool of genes that were found to be activated in *Xenopus* in response to overexpression of transcription factor AP2α (TFAP2α; Luo et al., 2005). TFAP2α has been found to play a significant role in NC induction and/or epidermal gene regulation in *Xenopus* (Luo et al., 2002, 2003), zebrafish (Barrallo-Gimeno et al., 2004; Knight et al., 2003) and mouse (Schorle et al., 1996; Zhang et al., 1996). *PCNS* is intensely expressed in the CNC and somites during the development of the frog embryo. Failure of CNC migration along with severe defects in craniofacial skeleton posterior to the mandible resulted from loss of function using antisense morpholino oligonucleotides (MOs) targeted to the untranslated region or to the translational initiation site of PCNS. Formation of the somites and axial muscles was also severely disrupted. From this study, it is evident that PCNS possesses a dual function in the migration of the CNC and somitogenesis in *Xenopus*.

Materials and methods

Embryo manipulation and in situ hybridization

Embryos were obtained from adult *X. laevis* females by hormone-induced egg-laying, artificially fertilized using standard methods (Sive et al., 2000) and staged (Nieuwkoop and Faber, 1994). Following microinjection, embryos were cultured in 3% Ficoll–400/1× MMR (Sive et al., 2000) for 1–2 h, then transferred to 0.3× MMR until they reached the desired stage. Whole-mount in situ hybridization was performed according to Harland (1991) with some modifications (Luo et al., 2001). Antisense probes labeled with digoxigenin were synthesized using a labeling kit from Roche Molecular Biochemicals with cDNA templates encoding *PCNS*, *Slug* (Mayor et al., 1995), *Sox 9* (Spokony et al., 2002), *Sox 10* (Aoki et al., 2003b; Honore et al., 2003), *Krox-20* (Bradley et al., 1993), *X-Delta-2* (Jen et al., 1997), *MyoD* (Hopwood et al., 1989), and *Thylacine 1* (Sparrow et al., 1998). Embryos were bleached after in situ hybridization by treatment with 10% hydrogen peroxide in 70% methanol.

DNA constructs and RNA synthesis for injection

The cDNA clone including the full open reading frame of PCNS (Genbank accession number: BG020773, pCMV-SPORT6 vector) was obtained from Open Biosystems Inc. (www.openbiosystems.com). The plasmid insert from the full-length PAPC clone (FL-PAPC, pBS-SK; a gift of Eddy De Robertis) was subcloned into the *Stu*-*Xho*I sites of pCTS, a derivative of pCS2+ (Feledy et al., 1999; Turner and Weintraub, 1994). For enhanced GFP (eGFP), the open-reading frame from pEGFP (Clontech) was subcloned into pCTS. The PCNS-eGFP construct was generated by amplifying the *PCNS* mRNA from −79 to +297 (Forward primer: TCG GGC GGA TCC CCC ACG CGT CCG CCC ACG; Reverse primer: CTG CAG ATC GAT CAA AGC CAG GTT ACA ATG AAG) and ligating this *Bam*H1-*Cla*1 fragment to the 5′ end of the eGFP sequence, maintaining the reading frame. Synthetic capped mRNA for injection for all plasmids was obtained by linearizing with *Not*I and transcribing with SP6 polymerase (Ambion Message Machine kit). RNAs were checked for size by denaturing agarose gel electrophoresis and tested for translational efficiency using a rabbit reticulocyte kit from Promega.

Dissociation and reaggregation assay

Embryos were injected with either 15 ng tetramethylrhodamine-labeled dextran, MW 10,000 (RDx; Molecular Probes Inc.), 400 pg eGFP mRNA alone or 400 pg eGFP mRNA and *PCNS* mRNA (3.4 ng) or FL-PAPC mRNA (3.4 ng) (Kim et al., 1998), apportioned into all four animal blastomeres at the eight-cell stage to ensure even distribution. Animal caps were excised at stage 9 in 0.3× MMR and dissociated by transferring to 1% agarose-coated dishes with CMFM medium (Sargent et al., 1986). After 20 min, the outer cell layer, which did not dissociate, was carefully separated from the inner cell layer and discarded. Dissociated inner layer cells from three RDx-injected caps were mixed with cells from five eGFP, eGFP + PCNS or eGFP + PAPC caps and reaggregated overnight at 22°C by transferring them to 1% agarose dishes containing 0.7× MMR (Detrick et al., 1990; Kim et al., 1998).

Northern blot hybridization

Whole embryo RNAs were isolated according to standard procedures (Sargent et al., 1986) and analyzed using glyoxal RNA gels (Ambion Northernmax Glyoxal gel system). The gel was stained with ethidium bromide to monitor equal loading of the RNA samples. Probes for expression of *PCNS* were labeled with ^{32}P-dCTP by primer extension (Amersham Pharmacia). Northern blots were washed using 0.2× SSPE at 62–63°C.

Antisense morpholino oligonucleotides

Two antisense morpholino oligonucleotides (MOs), M1 and M2, were synthesized to block translation of PCNS (GeneTools). The sequence for M1 was GTGGTGTAGTTCTTAGAAGTAACAT (homologous to mRNA positions +1 through +25, the complement of the ATG methionine codon = +1, underlined). The sequence of M2 was AAGTTGCACTAACAAAGTTGCTGTG (homologous to mRNA positions −22 through −46). The sequence of the control MO (Co), which was derived from M1, mismatched at five positions (lower case), was GTtGTtTAGTTtTTAGAAtTAAtAT. When the MOs (25 ng) were injected into one blastomere at the two-cell stage, 2 ng of a fluorescently labeled standard control MO (GeneTools) was co-injected. Embryos were then sorted into right- or left-side injected samples under a fluorescence dissecting microscope.

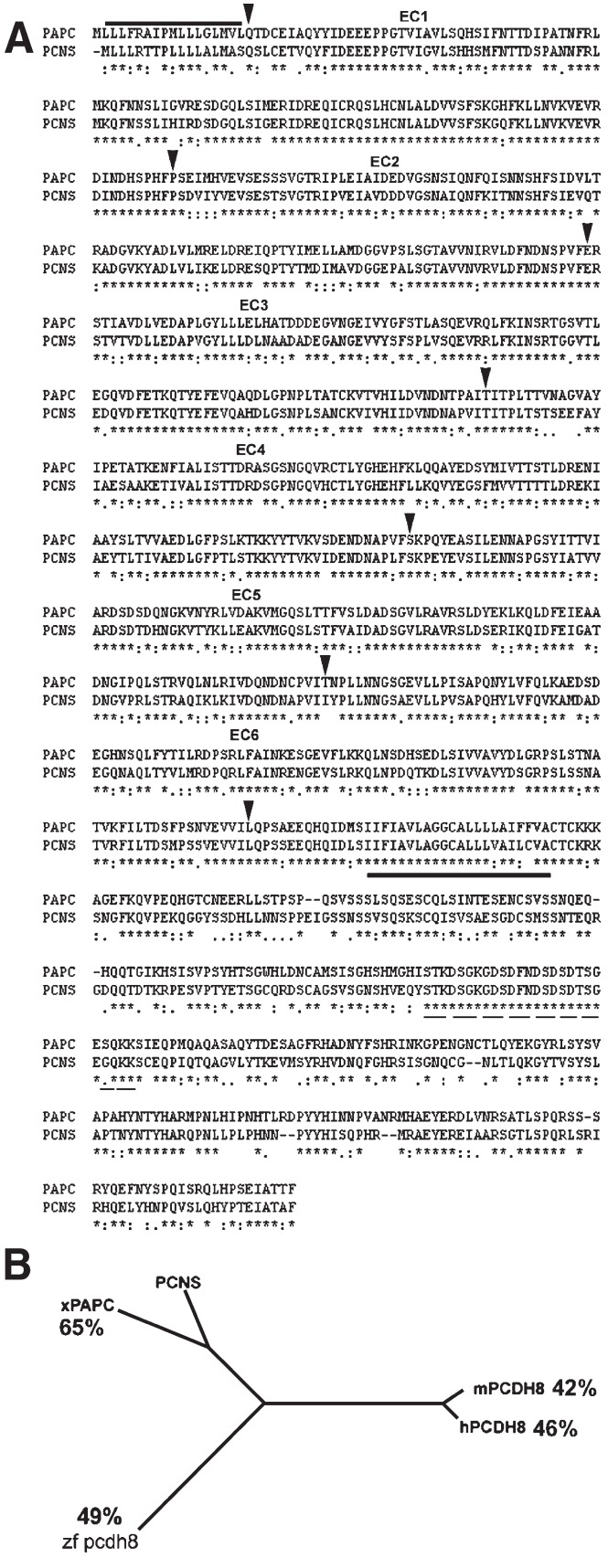

Fig. 1. *Xenopus* PCNS is a protocadherin. (A) Amino acid sequences of *Xenopus laevis* PAPC and PCNS. The solid black overline indicates the signal peptide and the solid black underline the transmembrane domains. The 25-residue conserved domain of PAPC required for mesoderm elongation is indicated with the dashed underline. Extracellular cadherin repeat domains are indicated with arrowheads. (B) Dendrogram showing the homology of PCNS to Pcdhs in other species. The percentages refer to amino acid identity compared to PCNS.

Alcian blue cartilage staining

Stage 44 embryos were fixed in 3.7% formaldehyde, rinsed in 1× phosphate-buffered saline (PBS), 0.1% Tween-20, dehydrated using a 30%, 50%, 70%, 100% ethanol series, then stained overnight in 0.05% Alcian blue in 80% ethanol/20% acetic acid (acid alcohol). After destaining for 48 h with several changes of acid alcohol, the embryos were dissected. Then the specimens were gradually rehydrated and transferred into 1% KOH/3% H_2O_2 for an hour. The tissue was cleared in 0.05% trypsin in saturated sodium tetraborate for another hour and washed with 1× PBS (Pasqualetti et al., 2000).

Cranial neural crest explants

Vitelline membranes from stage 17 embryos were removed in 0.3× MMR and CNC explants were dissected (Borchers et al., 2000) in 2% agarose-coated dishes containing 1× MMR and 50 μg/ml gentamycin sulphate according to procedure previously described (Alfandari et al., 2003). The explants were transferred to agarose-coated dishes containing sterile Steinberg's balanced salt solution (BSS) (Jones and Elsdale, 1963) before culturing overnight at 23°C on fibronectin-coated tissue culture slides in 500 μl BSS (89%), Leibowitz medium (10%) supplemented with 1 mM L-glutamine, 100 μg/ml gentamycin and fetal calf serum (1%) (Yaniv et al., 2003).

Immunohistochemistry and histology

To visualize somite morphology, embryos were stained with a muscle specific monoclonal antibody, 12/101 (Kintner and Brockes, 1984). At stage 24, embryos were fixed in MEMFA and incubated with 1:10 dilution of 12/101 culture supernatant in 20% goat serum and 0.1% Triton X-100 in PBS for 48 h at 4°C. After four 2-h washes, the embryos were incubated in HRP-conjugated goat anti-mouse secondary antibody (1:500) for 48 h at 4°C. Then the embryos were washed extensively, stained using diaminobenzidene, bleached, and photographed (Kim et al., 2000; Sive et al., 2000; Tucker and Slack, 1995). To visualize the cellular morphology of the somites, embryos were embedded using the JB-4 embedding kit (Polysciences, Inc.) and sliced into 10-μm parasagittal sections. The sections were mounted using a mounting medium with DAPI (Vector Laboratories) for visualization of nuclei.

Results

Identification and expression of PCNS

The original cDNA clone for PCNS (Accession number XL097h19; National Institute of Basic Biology, Okazaki, Japan http://xenopus.nibb.ac.jp) was identified in a microarray-based screen for genes under the control of the transcription factor TFAP2α (Luo et al., 2005). This clone lacked amino terminal protein coding sequence, and a plasmid with 100% homology to the original but including the full *PCNS* open reading frame was identified by screening public databases, obtained and sequenced on both strands. The *PCNS* coding sequence

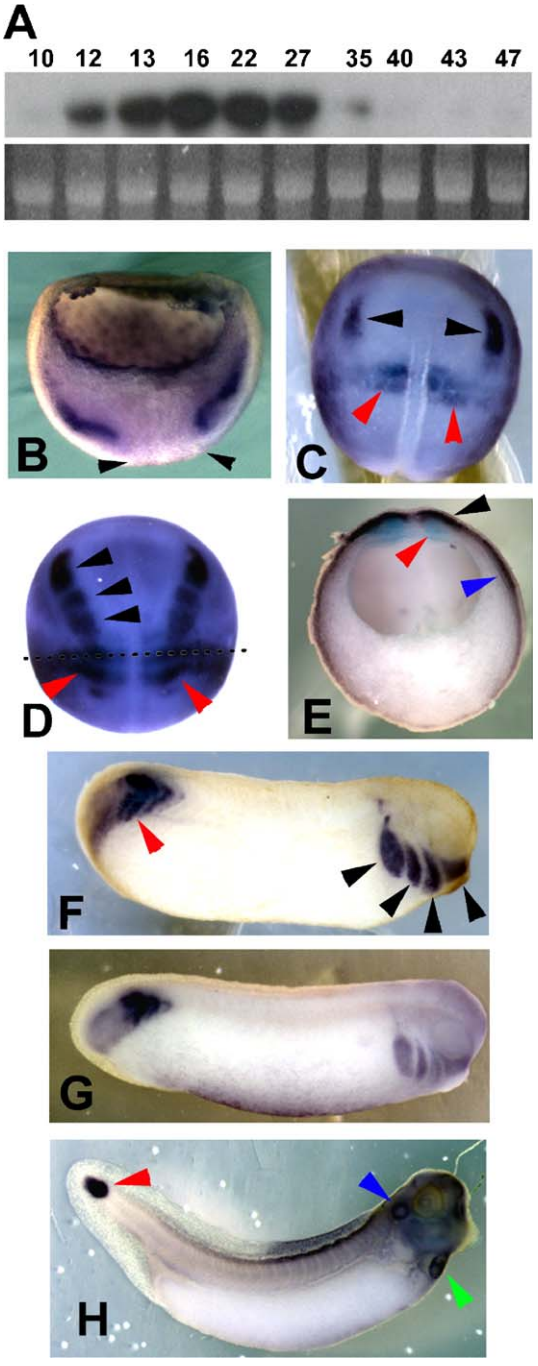

Fig. 2. *PCNS* expression in *Xenopus* embryos. (A) Developmental northern showing the temporal expression of *PCNS*. *Xenopus* embryo stages (Nieuwkoop and Faber, 1994) are indicated above the lanes. Strong expression is observed from late gastrula (stage 12) until late tailbud (stage 27) after which *PCNS* mRNA declines to background level. The gel was stained with ethidium bromide to assure equal loading of lanes. (B) Sagittal section of a mid-gastrula embryo (stage 11), showing expression in the deep mesodermal cells. Animal pole at the top, dorsal on the right. The edges of the blastopore are indicated with arrowheads. (C) Expression in the CNC (black arrowheads) and somites (red arrowheads) in an early neurula stage embryo (stage 14). (D) Individual domains of CNC expression corresponding to prospective pharyngeal arches (black arrowheads) and a more posterior set of somites (red arrowheads) in late neurula stage embryo (stage 17). (E) Transverse section of embryo in panel D (plane indicated by dashed line) showing expression in the somite (red arrowhead), trunk NC (black arrowhead) and lateral plate mesoderm (blue arrowhead). Note that the color differences are an artifact of BM Purple staining. (F) Tailbud embryo (stage 25) expression in pharyngeal arches (black arrowheads) and posterior band of somites (red arrowhead). Anterior is to the right. (G) Stage 31 embryo; pharyngeal arch expression has begun to fade as somite bands proceed further to the posterior. (H) Early tadpole (stage 34) somite expression exclusively at the most posterior tip of the tail (red arrowhead). *PCNS* RNA now detected in otic vesicle (blue arrowhead) and heart anlage (green arrowhead).

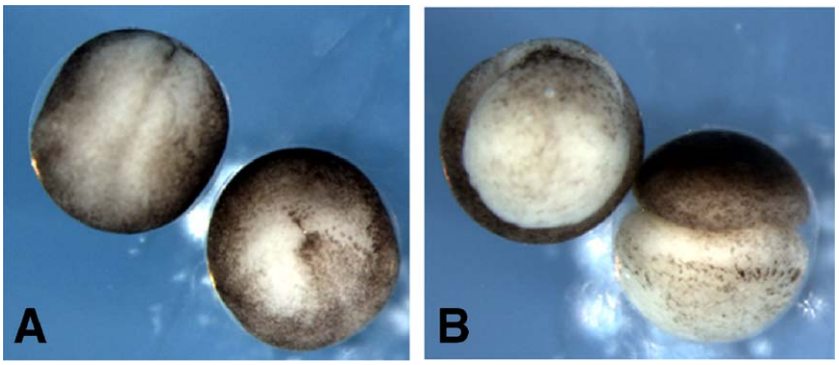

Fig. 3. *PCNS* overexpression phenotype—early neurula (stage 14). Injection of 1 ng RNA encoding PCNS resulted in gastrulation failure (Panel B; 96%, $n = 28$). Uninjected embryos were normal (panel A; 100%, $n = 28$).

(Genbank accession # DQ178630) was 2934 bp predicting a 978-amino-acid protein with overall structural similarity to Pcdh family members. PCNS has an amino terminal signal peptide, an extracellular domain containing six cadherin repeats of about 110 amino acids each, a transmembrane domain and a unique cytoplasmic domain (Fig. 1A). Pairwise comparisons using BLAST (http://www.ncbi.nlm.nih.gov) show that PCNS has 65% amino acid identity to *Xenopus laevis* PAPC (Kim et al., 1998), and homology to Pcdh8 in human (NP_116567; 46% identity), mouse (NP_067518; 42% identity) and zebrafish (NP_571284; 49% identity; Fig. 1B).

PCNS mRNA which was initially detected by Northern blot analysis at late gastrula (stage 12) increases through later developmental stages until stage 27 after which the transcripts declined to undetectable levels (Fig. 2A). *PCNS* mRNA could be detected by whole-mount in situ hybridization at stage 11 in the involuting mesoderm (Fig. 2B). By neurula stages, *PCNS* transcripts were localized to the CNC and the somites and also to lateral plate mesoderm, which also appeared to be segmented (Figs. 2C–E). At tailbud stages (stage 25, 31), CNC expression was restricted to the mandibular, hyoid and the branchial arches and the posterior somites. The expression in the mandibular arch was limited to the sector ventral to the optic vesicle (Figs. 2F, G). By early tadpole (stage 34), the branchial arch expression had disappeared while the somite signal was restricted to the extreme tail tip. At this stage expression also becomes apparent in otic vesicle and heart anlage (Fig. 2H).

PCNS gain of function analysis

Ectopic overexpression of PCNS in whole embryos was accomplished by injection of 1 ng of *PCNS* mRNA into the animal region of a one-cell stage embryo. Cleavage through blastula stages occurred normally, but 96% ($n = 28$) of injected embryos failed to complete gastrulation, resulting in a large mass of mesoendodermal cells remaining on the exterior of the embryo at mid-neurula (Fig. 3B). The extracellular domain of PCNS is similar to other protocadherins like PAPC and AXPC that function as cell adhesion molecules (Kim et al., 1998; Kuroda et al., 2002), suggesting the possibility that such gastrulation failure might be due to abnormally high levels of cell adhesion. This was investigated for PCNS using an ectodermal cell dissociation and reaggregation assay. All four animal blastomeres of an eight-cell stage embryo were injected with RDx or mixtures of RNAs encoding eGFP, PCNS, or PAPC, and animal caps isolated, dissociated, and reaggregated as described in Materials and methods. As shown in Fig. 4, cells expressing PAPC sorted out to a significant degree from non-expressing cells, as has been previously reported (Kim et al., 1998) (panel B), but in contrast, cells expressing PCNS remained intermingled with non-expressing cells (panel C), similar to the control with eGFP alone (panel A). Both PCNS and PAPC RNAs were translated with approximately equal efficiency in vitro (data not shown), so these results suggest that PCNS may not function as effectively as PAPC in conferring specific cell–cell adhesion, and that the disruption of gastrulation by PCNS overexpression may be

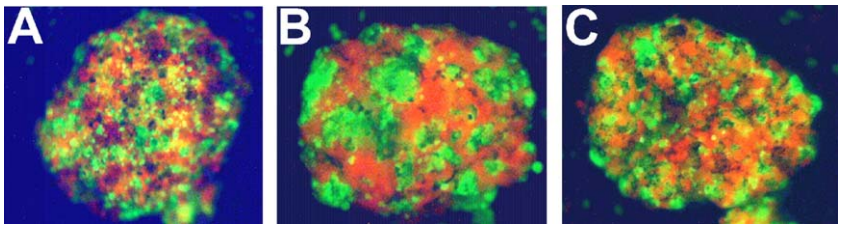

Fig. 4. Dissociation and reaggregation assay. Dissociated inner ectodermal cells from embryos injected with 15 ng of RDx were mixed with inner ectodermal cells from embryos injected with RNAs encoding eGFP (panel A) or eGFP and PAPC (panel B) or eGFP and PCNS (panel C). Reaggregated cells were cultured until sibling embryos reached stage 28. PCNS expression yielded essentially the same degree of segregation as eGFP alone, while PAPC-expressing cells (green) largely separated from non-expressing cells (red). Each sample replicated in quadruplicate with similar results.

due to some other mechanism, for example interference with cell–cell communication.

PCNS is essential for migration of CNC cells in Xenopus

To evaluate the effects of *PCNS* loss of function, antisense MOs were used to inhibit translation of *PCNS* mRNA. As a control to rule out nonspecific effects, two independent, non-overlapping MOs (M1 and M2) were designed, and effects that were observed with both of these MOs were considered to be valid. As an additional control, a third MO with a 5-bp mismatch to M1 was also constructed (Co). To test the effectiveness of these MOs in blocking the translation of *PCNS* mRNA, the 5′ untranslated portion of *PCNS* mRNA along with the amino terminal 297 nucleotides was fused in frame to eGFP (PCNS-eGFP). MOs were injected individually into fertilized eggs (50 ng), followed by PCNS-eGFP RNA, or eGFP RNA as a negative control (200 pg each). Translation was evaluated at midblastula by viewing in a fluorescence microscope. None of the MOs had any noticeable effect on eGFP, whereas both M1

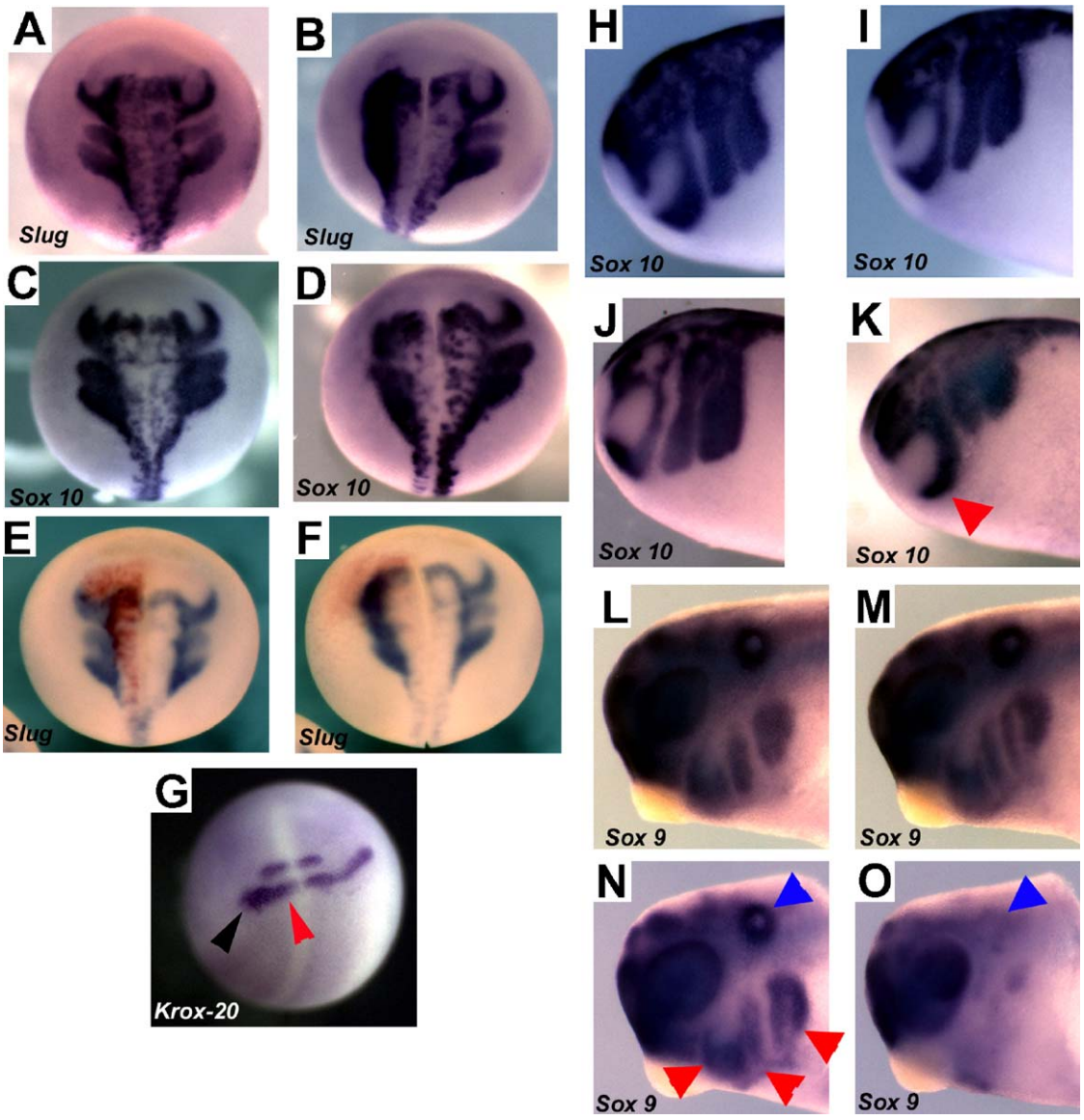

Fig. 5. *PCNS* loss of function. Whole-mount in situ hybridizations with NC markers to stage 19 (A–G), stage 22 (H–K) or stage 31 (L–O) embryos injected into one cell at the two-cell stage with 25 ng of M1 (B, D, G, K, O) or Co (A, C, I, M), or into one cell at the eight-cell stage with 6 ng of M1 (F) or Co (E), along with lacZ RNA as a lineage tracer. In situ probes are indicated on the individual panels at lower left. In each case, M1 resulted in severe inhibition of CNC cell migration into pharyngeal arches, and Co had no effect. In panels E and F, the lineage tracer is visible as red staining on the left side of the embryos. M1 injection inhibited migration of NC from rhombomere 5 (G; black arrowhead) without affecting the neural tube expression (red arrowhead). Panels A–G are dorsal views with the injected side on the left. In some cases, images were flipped horizontally to maintain this orientation. In panels H–O, the uninjected sides are shown on the left (H, J, L, N) and injected sides on the right (I, K, M, O). Images have been flipped horizontally to facilitate comparison of injected and uninjected sides. M1 strongly inhibited migration into posterior pharyngeal arches at stage 22 but had less effect on the mandibular arch migration (K; red arrowhead). Injection of Co had no effect (I). By stage 31 (L–O), *Sox 9* expression in pharyngeal arches (N; red arrowheads) was essentially undetectable on the M1-injected side (O). Expression of *Sox 9* in the otic vesicle was also strongly reduced (O; blue arrowhead). Injection of Co had no effect (M). Result statistics are summarized in Table 1.

Table 1
Summary of MO experiments

Stage	Injection	Co	M1	M2	Probe
		Normal	Migration inhibited	Migration inhibited	
19	1/2 cell	90%, $n = 52$	100%, $n = 19$	84%, $n = 37$	*Slug*
19	1/8 cell	94%, $n = 18$	86%, $n = 28$	ND	*Slug*
19	1/2 cell	90%, $n = 29$	100%, $n = 26$	87%, $n = 60$	*Sox 10*
19	1/2 cell	ND	89%, $n = 37$	71%, $n = 17$	*Krox-20*
22	1/2 cell	90%, $n = 31$	100%, $n = 47$	65%, $n = 53$	*Sox 10*
31	1/2 cell	100%, $n = 7$	73%, $n = 15$	ND	*Sox 9*

Embryos injected with Co, M1 or M2 at two-cell or eight-cell stage as indicated, and scored for normal or inhibited migration of CNC into pharyngeal arches at the indicated stages, based on the pattern of expression of NC probes (right column). ND, not done.

and M2 strongly inhibited fluorescence in embryos injected with PCNS-eGFP. Co had no effect (Supplementary Fig. 1).

To disrupt expression of *PCNS*, these MOs were injected (25 ng) into one blastomere of a two-cell stage embryo, with the uninjected side serving as a further control. The embryos were then cultured until stage 19 and examined by in situ hybridization with NC markers *Slug* (Mayor et al., 1995), *Sox 10* (Aoki et al., 2003b; Honore et al., 2003), and the NC/hindbrain marker *Krox20* (Bradley et al., 1993). As shown in Fig. 5, all NC marker genes were induced to comparable levels on the injected and uninjected sides, although in some cases, MO injection resulted in a slight delay (Supplementary Fig. 2). Both M1 and M2 led to an essentially identical loss of CNC migration, although M2 was somewhat less effective. Co had no effect. (Figs. 5A–D, Supplementary Fig. 3; results summarized in Table 1). Since *PCNS* is expressed in early mesoderm (Fig. 2B), one possible mechanism for the disruption of CNC migration might be a secondary effect of altered mesoderm function. To rule this out, targeted injections were carried out at the eight-cell stage with M1, into dorsal/animal blastomeres that contribute little to mesodermal tissue. As shown in Figs. 5E–F, this resulted in a phenotype that was similar to that obtained with the earlier two-cell stage injections. Furthermore, hybridization of two-cell stage MO-injected embryos with *Krox-20* showed that the hindbrain expression of this marker was not affected, indicating that the MO-induced defect was specific for the NC and not due to a general inhibition of neuroectodermal differentiation (Fig. 5G).

Similar results were obtained at later stages (stage 22, Figs. 5H–K and stage 31, Figs. 5L–O), using as markers *Sox 10* and *Sox 9* (Spokony et al., 2002). Although there was some variability in the degree of inhibition of migration into the mandibular arch, more posterior arch migration was almost entirely inhibited on the injected side (Figs. 5K, O). There was not much effect on migration into the more proximal and dorsal sector of mandibular arch, consistent with the absence of expression of *PCNS* in this region. Co had no effect at this stage (Figs. 5I, M). As at the earlier stage, equivalent results were obtained with both M1 and M2 (Supplementary Figs. 3–5), as summarized in Table 1. M1-injected embryos cultured to tadpole stage (stage 44), exhibited abnormal head morphology and severe curving towards the injected side (Fig. 6D). Alcian blue staining of the cranial cartilage revealed partial loss of the mandibular and a complete loss of the hyoid and branchial arch derivatives on the injected side (Fig. 6C). As with earlier stages, Co had no effect (Figs. 6A, B).

Migratory behavior is an intrinsic property of NC cells, which will actively migrate from explanted neural fold tissue on a suitable substrate, such as a fibronectin-coated slide (Alfandari et al., 2003). To test the effect of loss of *PCNS* on this characteristic, 25 ng of M1 or Co was injected into one cell of two-cell stage embryos. These were cultured to stage 17 and

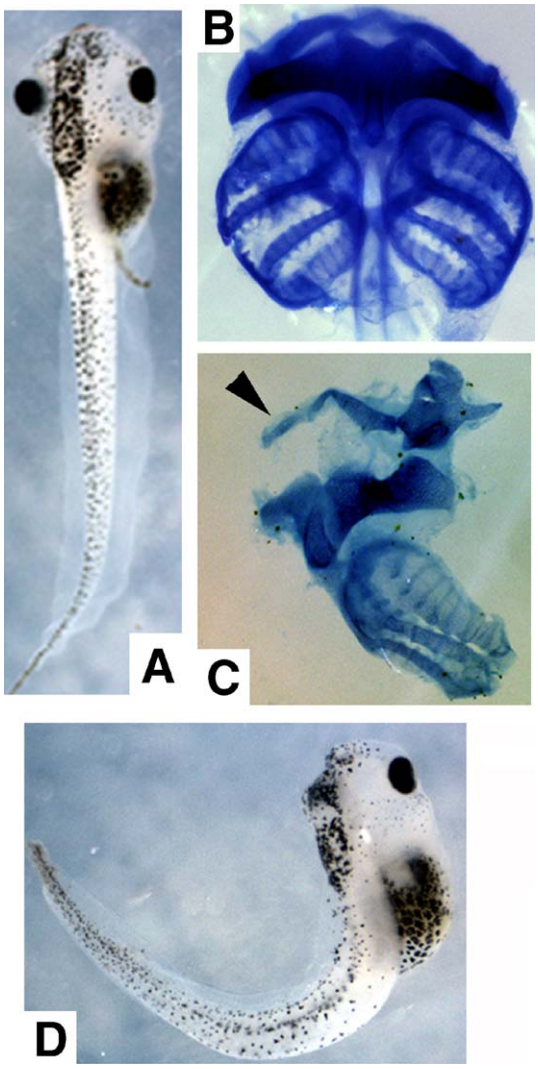

Fig. 6. Craniofacial phenotype in tadpoles (stage 44). Embryos injected with 25 ng Co (A, B) or M1 (C, D) into one blastomere at the two-cell stage. The injected side is on the left in each panel. (A) Embryos injected with Co were normal overall (100%, $n = 17$) and had normal cranial cartilage as visualized with Alcian blue staining (panel B). (D) M1 resulted in cranial hypoplasia on the injected side in addition to a curved tail (87%, $n = 15$) and virtually complete loss of cranial cartilage, especially more posterior elements and gills. Some Meckel's cartilage was retained (arrowhead, panel C).

explants of tissue from the CNC region of the injected side were dissected and cultured on fibronectin until sibling embryos reached stage 30 (Fig. 7). CNC cells from control morpholino (Co) injected embryos spread and migrated (Fig. 7A). However, explants from M1-injected embryos largely disaggregated. These cells had a round morphology and failed to bind or spread on the substrate (Fig. 7B). To show that explants were correctly isolated from CNC-containing tissue, embryos were fixed following microsurgery and hybridized in situ to a *Slug* probe, showing the expected gap in *Slug* expression (Supplementary Figs. 6A–C). The explants continued to express CNC markers, as shown by in situ hybridization with *Slug* and *Sox 10* probes (Supplementary Figs. 6D–I). Thus, the CNC defect associated with loss of PCNS occurs after the induction of NC gene expression but during the EMT, since the cells fail to adopt the flattened mesenchymal morphology, do not adhere to fibronectin in vitro, and do not migrate normally, either in the embryo or in vitro.

PCNS in the segmentation of the somites

The other major site of *PCNS* expression is in somites. To investigate *PCNS* function in this tissue, embryos injected as above with 25 ng of Co or M1 into one cell of a two-cell stage embryo were probed with somite-specific markers *MyoD* (Hopwood et al., 1989), *X-Delta-2* (Jen et al., 1997), and *Thylacine-1* (Sparrow et al., 1998). In all cases, marker gene expression levels were approximately normal, but loss of *PCNS* resulted in a disorganization of somite morphology. Some segmentation was present, but definition between segments was poor (Fig. 8). Injection of M2 yielded a similar phenotype (data not shown), while Co had no effect. The disorganized morphology of somites was shown more clearly by staining sections of stage 24 M1-injected embryos with 12/101 monoclonal antibody, which recognizes an epitope present on the surface of differentiated muscle cells (Kintner and Brockes, 1984), or with DAPI to visualize nuclei of muscle cells (Fig. 9). About 75% of M1-injected embryos had somites that were poorly defined and also lacked the normal chevron shape found at this stage (Fig. 9B). The nuclear staining also showed abnormal alignment, suggesting that somite rotation had been disrupted (Fig. 9D). From these results, it is clear that *PCNS* is an essential component of somite morphogenesis in the frog embryo.

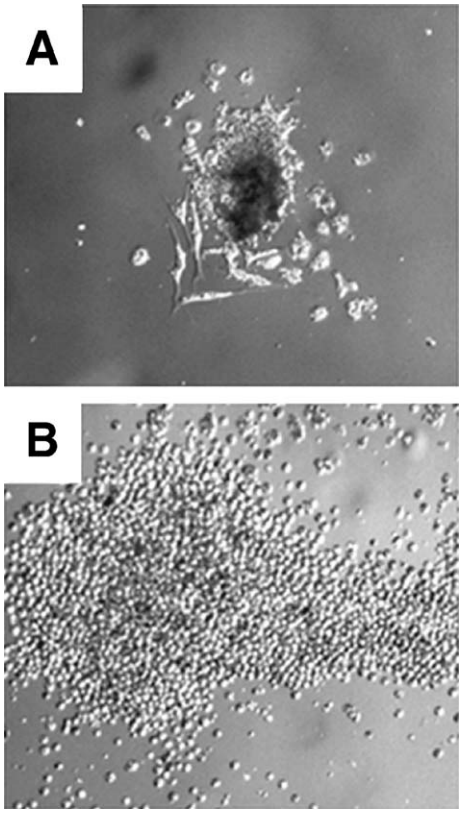

Fig. 7. In vitro migration assay. Migration of cells from explants of embryos injected with 25 ng of Co or M1 on one side at the two-cell stage. Explants were dissected at stage 17 and cultured on glass slides coated with fibronectin until sibling embryos reached stage 30. Spreading and migration were observed from control morpholino injected samples (A), but explants from M1-injected embryos (B) disaggregated (100%, $n = 8$ for each). Also, M1-injected cells remained rounded and did not adhere well to the fibronectin substrate.

Discussion

PCNS and other protocadherins

The Pcdh most closely related to PCNS is Pcdh8 in human, mouse, and zebrafish genomes. *Xenopus* PAPC shares approximately the same degree of homology to these Pcdh8 proteins. For example, BLAST comparison of *Xenopus* PCNS and PAPC to zebrafish Pcdh8 yields identities of 49% and 48%, respectively. There is a highly conserved ~25-amino-acid motif present in the cytoplasmic domains of Pcdh8, PCNS and PAPC, which is essential for the PAPC-mediated mesodermal elongation activity in *Xenopus* (Kim et al., 1998; Makarenkova et al., 2005; Yamamoto et al., 2000) (Fig. 1A). Mouse Pcdh8/PAPC is not expressed in the CNC. Targeted inactivation of this gene has little if any phenotype in mouse (Yamamoto et al., 2000). However, experiments with a dominant-negative PAPC in organ culture support a role for this protein in somite segment border organization (Rhee et al., 2003). It is nevertheless unclear which is the actual homolog of *PCNS* in other vertebrates. Given the large size (~70 or more genes) in the Pcdh gene family and potential for redundancy, drift, recombination, etc., such an unambiguous homology assignment might not be possible. In *Xenopus*, there is clearly some phylogenetic and functional relationship between PAPC and PCNS. The sequence identity is 65% overall (Fig. 1A), so these two genes are more similar to each other than to their closest relatives in other vertebrates. Both are expressed in gastrula stage mesoderm, although PAPC is more localized to the dorsal region. *PCNS* expression in somites is confined to a few segments at the leading edge of the segmentation wave and is thus more transient than PAPC, which is transcribed in posterior presomitic axial mesoderm, and is downregulated in the anterior trailing edge of somite development at approximately the same anteroposterior level as *PCNS* (Kim et al., 1998). The initial studies on PAPC relied on a dominant negative approach to loss

of function. More recent work using MOs (Medina et al., 2004) suggest a more subtle phenotype. While there are other interpretations, one possibility is that the PAPC dominant negative protein has some cross-interference with PCNS, and to some extent, the resultant phenotype is due to disturbing both PAPC and PCNS, or even PCNS alone. This might also be the basis for the disruption of gastrulation by overexpression of PCNS. Taken together, these observations show that PCNS is a novel protocadherin related to and possibly having functional overlap with PAPC/Pcdh8.

Cell adhesion properties of PCNS

By virtue of the extracellular cadherin repeat (EC) domains, Pcdh proteins, including PAPC, can act as homophilic adhesion factors, although somewhat less efficiently than cadherins (Bradley et al., 1998; Kim et al., 1998; Kuroda et al., 2002). With 83% similarity between the EC domains of PAPC and PCNS (Fig. 1A), PCNS might be expected to have this characteristic and lead to segregation of *PCNS*-expressing ectodermal cells in *Xenopus* reaggregation assays. However, this is not what we observed; while PAPC expression resulted in segregation, PCNS did not (Fig. 4). One problem with such a comparison is that the protein expression is via injected synthetic mRNAs, which although translated with similar efficiency in vitro, this may not be the case in vivo. Furthermore, it is possible that different optima may exist for PAPC and PCNS expression in this assay. Nevertheless, to a first approximation, it is likely that PCNS is not particularly potent as an adhesion molecule, even by the standard for Pcdhs. This is interesting in the context of the overexpression phenotype, which severely disrupted gastrulation. If this effect were not due to artificially enhanced cell–cell adhesion, what might be the molecular mechanism? One possibility is that PCNS overexpression interferes with one or more signaling pathways. Studies in zebrafish and *Xenopus* have revealed the importance of non-canonical Wnt signaling mechanisms in vertebrate gastrulation (Darken et al., 2002; Keller, 2002; Myers et al., 2002; Ueno and Greene, 2003; Veeman et al., 2003), and PAPC has been found to interact with the Wnt/PCP pathway to regulate gastrulation in *Xenopus* (Unterseher et al., 2004). Interestingly, knockdown of the PCP/Wnt-Ca^{++} pathway has resulted in inhibition of migration of CNC (De Calisto et al., 2005). Furthermore, both PAPC and AXPC derivatives lacking cytoplasmic domains exhibit much stronger adhesive properties, suggesting a negative regulatory mechanism mediated by these domains (Kim et al., 1998; Kuroda et al., 2002). The cytoplasmic domain of PCNS is particularly divergent, so if it has a signal transduction function, this probably differs significantly from other Pcdh proteins. This will be an important issue for future studies on PCNS.

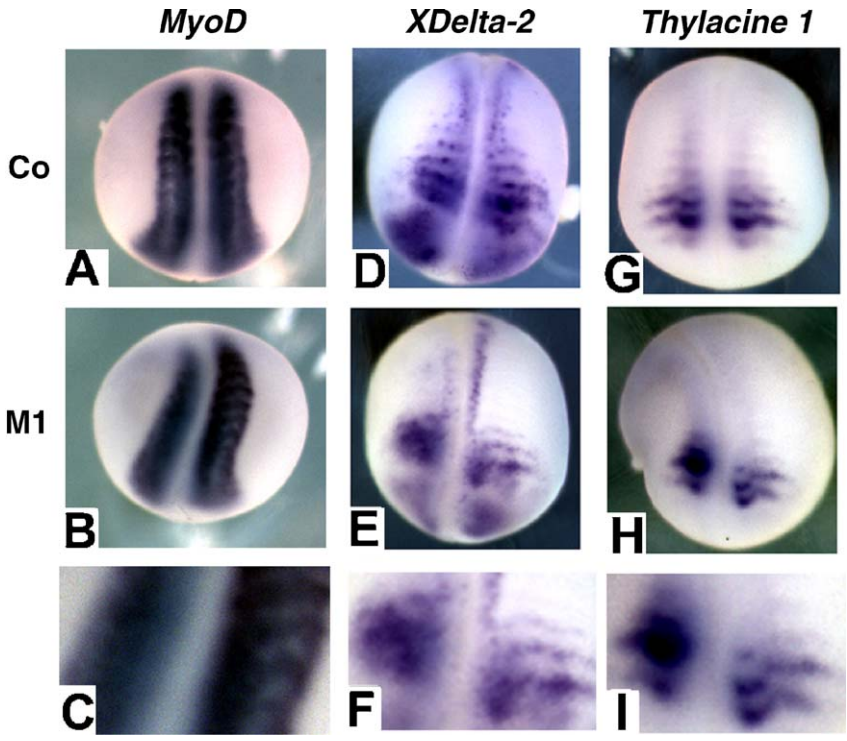

Fig. 8. Impaired somitogenesis in M1-injected embryos. (A) Whole-mount in situ hybridizations with *MyoD* (A–C), *X-Delta-2* (D–F) or *Thylacine 1* (G–I) to stage 20 (A–C) or stage 21 (D–I) embryos injected with 25 ng of Co or M1 into one cell at the two-cell stage. Injection of M1 inhibited somite segmentation for *MyoD* (panel B; 100%, $n = 15$) *X-Delta-2* (panel E; 54%, $n = 28$) and *Thylacine 1* (panel H; 53%, $n = 15$). Magnified images of somites in M1 embryos are shown in separate panels for *MyoD* (C), *X-Delta-2* (F) and *Thylacine 1* (I). Injection of Co had no effect (100% for *MyoD*, $n = 29$; 100%, $n = 25$ for *X-Delta*-2; 100%, $n = 15$ for *Thylacine 1*; panels A, D and G, respectively). All panels show dorsal view of embryos with anterior to the top and the injected side to the left.

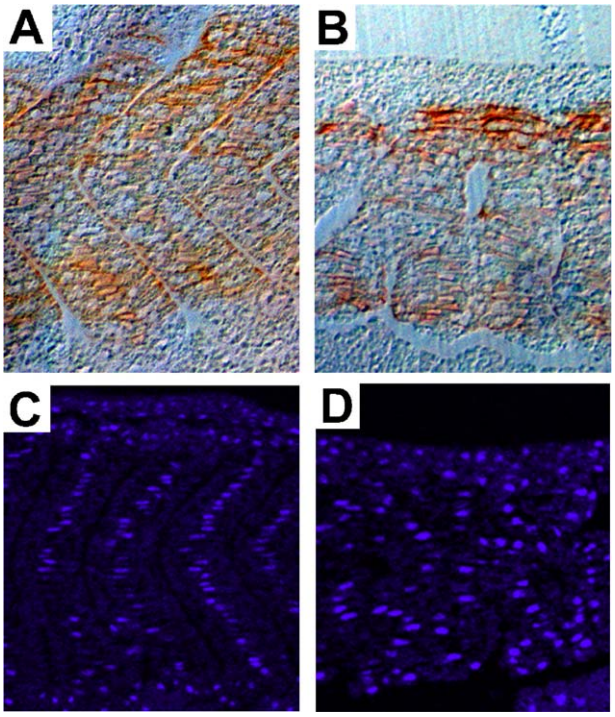

Fig. 9. Parasagittal sections of a stage 24 embryo injected in one cell at two-cell stage with 25 ng M1. Immunostaining with 12/101, a muscle-specific antibody to section of (A) uninjected and (B) M1-injected side shows disturbed somite morphology. DAPI staining of (C) uninjected and (D) injected side sections shows that cell orientation is also disrupted.

PCNS in epitheliomesenchymal transition and migration of CNC

CNC cell migration is a complex process commencing with EMT followed by movement through the extracellular matrix (ECM). EMT is itself a complex process involving rearrangement of the actin and microtubule cytoskeletons and delamination. Typically a non-polarized epithelial cell is transformed into a more flattened, polarized migratory mesenchymal cell (Raftopoulou and Hall, 2004). This involves many molecules with adhesive and/or signaling functions such as the ephrins and their receptors (Robinson et al., 1997; Smith et al., 1997), type II classical cadherins such as cadherin-11, cadherin-6, cadherin-7 (Hadeball et al., 1998; Hoffman and Balling, 1995; Inoue et al., 1997; Nakagawa and Takeichi, 1995; Nakagawa et al., 1998; Tanihara et al., 1994; Vallin et al., 1998) and other Pcdhs including PAPC and AXPC (Kim et al., 1998; Kuroda et al., 2002).

Two independent, non-overlapping PCNS MOs yielded an essentially identical effect on CNC, i.e., a strong inhibition of migration from the dorsal neural tube into the branchial arches, while a mismatched control MO had no phenotype. These data strongly support an essential role for PCNS in this process. The inhibition was particularly severe in the CNC corresponding to the more posterior branchial arches; little migration could be observed in the intact embryo by in situ hybridization (Fig. 5) and even less in the in vitro migration assay (Fig. 7). In the latter assay, the CNC explants from PCNS MO injected embryos largely disaggregated into rounded cells that did not adhere well to the fibronectin substrate. This suggests that the blockage resulting from *PCNS* loss of function takes place following CNC induction, since marker gene activation was not prevented, but during EMT, since the explants disaggregated, the cells failed to adopt a mesenchymal morphology, did not spread, develop filopodia, or attach to the substrate. Further analysis of CNC cytoskeletal and adhesive properties, and signal transduction pathways, and more detailed studies of cell morphology should help identify the molecular events that are disrupted by loss of PCNS.

The apparent differential effect of *PCNS* loss of function on posterior versus anterior CNC could point to some difference in the function of this protein at different axial levels, although expression is evidently uniform. It is also possible, however, that the MO treatments have reduced but not completely eliminated PCNS protein synthesis, and that more anterior CNC cells require a lower threshold to initiate and complete migration.

PCNS in somitogenesis

One of the most important morphogenetic processes involves the segmentation of the PSM into metameric units called somites along the anteroposterior (A–P) axis of the vertebrate embryo. In *Xenopus*, a somite forms when a group of myotomal cells segregate, rotate 90 degrees, and orient parallel to the A–P axis (Jen et al., 1997). A wave-like process which starts with the prepatterning of the cells of the PSM by selector genes leads to the translation of the segmental prepattern into the morphogenetic changes that accompany formation of the somite boundaries and finally to the patterning and the differentiation of the somites. A number of studies have shown the role of various molecules in this process, including components of the Notch signaling pathway, the Hairy-like proteins and bHLH transcription factors of the Mesp family (Jen et al., 1997; Joseph and Cassetta, 1999; Sparrow et al., 1998). Eph/ephrin signaling was also found to be important in the prepatterning of PSM and formation of the somites (Durbin et al., 1998).

As was the case with the CNC, *PCNS* knockdown did not interfere with the induction of somite marker gene expression, but rather disrupted morphogenesis. Similar results have been obtained from loss of function of *X-Delta-2* (Jen et al., 1997), which is expressed within the PSM in a set of stripes corresponding to the prospective somite boundaries. Since expression of this regulatory gene was not inhibited by *PCNS* MO injection although the pattern was disturbed, *PCNS* is likely to function either downstream from *X-Delta-2*, or alternatively in an independent pathway in somite morphogenesis. It will be interesting to determine the extent to which PCNS interacts with signaling pathways and to learn if there is any overlap in this regard with other Pcdh proteins, particularly with *PAPC* which shares some expression domain as well as sequence similarity with *PCNS*.

Acknowledgments

We thank Drs. Eddy De Robertis for the gift of plasmids and Yanhua Xu for helpful discussions. This research was supported

by the Intramural Research Program of the National Institute of Child Health and Human Development, NIH.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.03.025.

References

Alfandari, D., Cousin, H., Gaultier, A., Hoffstrom, B.G., DeSimone, D.W., 2003. Integrin alpha5beta1 supports the migration of *Xenopus* cranial neural crest on fibronectin. Dev. Biol. 260, 449–464.

Andrade, R.P., Pascoal, S., Palmeirim, I., 2005. Thinking clockwise. Brain Res. Brain Res. Rev. 49, 114–119.

Angst, B.D., Marcozzi, C., Magee, A.I., 2001. The cadherin superfamily. J. Cell Sci. 114, 625–626.

Aoki, E., Kimura, R., Suzuki, S.T., Hirano, S., 2003a. Distribution of OL-protocadherin protein in correlation with specific neural compartments and local circuits in the postnatal mouse brain. Neuroscience 117, 593–614.

Aoki, Y., Saint-Germain, N., Gyda, M., Magner-Fink, E., Lee, Y.H., Credidio, C., Saint-Jeannet, J.P., 2003b. Sox10 regulates the development of neural crest-derived melanocytes in *Xenopus*. Dev. Biol. 259, 19–33.

Barrallo-Gimeno, A., Holzschuh, J., Driever, W., Knapik, E.W., 2004. Neural crest survival and differentiation in zebrafish depends on mont blanc/tfap2a gene function. Development 131, 1463–1477.

Basch, M.L., Garcia-Castro, M.I., Bronner-Fraser, M., 2004. Molecular mechanisms of neural crest induction. Birth Defects Res. Part C Embryo Today 72, 109–123.

Borchers, A., Epperlein, H.H., Wedlich, D., 2000. An assay system to study migratory behavior of cranial neural crest cells in *Xenopus*. Dev. Genes Evol. 210, 217–222.

Bradley, L.C., Snape, A., Bhatt, S., Wilkinson, D.G., 1993. The structure and expression of the *Xenopus* Krox-20 gene: conserved and divergent patterns of expression in rhombomeres and neural crest. Mech. Dev. 40, 73–84.

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.

Darken, R.S., Scola, A.M., Rakeman, A.S., Das, G., Mlodzik, M., Wilson, P.A., 2002. The planar polarity gene strabismus regulates convergent extension movements in *Xenopus*. EMBO J. 21, 976–985.

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.

Detrick, R.J., Dickey, D., Kintner, C.R., 1990. The effects of N-cadherin misexpression on morphogenesis in *Xenopus* embryos. Neuron 4, 493–506.

Durbin, L., Brennan, C., Shiomi, K., Cooke, J., Barrios, A., Shanmugalingam, S., Guthrie, B., Lindberg, R., Holder, N., 1998. Eph signaling is required for segmentation and differentiation of the somites. Genes Dev. 12, 3096–3109.

Erickson, C.A., Perris, R., 1993. The role of cell–cell and cell–matrix interactions in the morphogenesis of the neural crest. Dev. Biol. 159, 60–74.

Feledy, J.A., Beanan, M.J., Sandoval, J.J., Goodrich, J.S., Lim, J.H., Matsuo-Takasaki, M., Sato, S.M., Sargent, T.D., 1999. Inhibitory patterning of the anterior neural plate in *Xenopus* by homeodomain factors Dlx3 and Msx1. Dev. Biol. 212, 455–464.

Hadeball, B., Borchers, A., Wedlich, D., 1998. *Xenopus* cadherin-11 (Xcadherin-11) expression requires the Wg/Wnt signal. Mech. Dev. 72, 101–113.

Halloran, M.C., Berndt, J.D., 2003. Current progress in neural crest cell motility and migration and future prospects for the zebrafish model system. Dev. Dyn. 228, 497–513.

Hamilton, L., 1969. The formation of somites in *Xenopus*. J. Embryol. Exp. Morphol. 22, 253–264.

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

Hay, E.D., 1995. An overview of epithelio-mesenchymal transformation. Acta Anat (Basel) 154, 8–20.

Hirano, S., Ono, T., Yan, Q., Wang, X., Sonta, S., Suzuki, S.T., 1999. Protocadherin 2C: a new member of the protocadherin 2 subfamily expressed in a redundant manner with OL-protocadherin in the developing brain. Biochem. Biophys. Res. Commun. 260, 641–645.

Hoffman, I., Balling, R., 1995. Chromosomal localization of the murine cadherin-11. Mamm. Genome 6, 304.

Honore, S.M., Aybar, M.J., Mayor, R., 2003. Sox10 is required for the early development of the prospective neural crest in *Xenopus* embryos. Dev. Biol. 260, 79–96.

Hopwood, N.D., Pluck, A., Gurdon, J.B., 1989. MyoD expression in the forming somites is an early response to mesoderm induction in *Xenopus* embryos. EMBO J. 8, 3409–3417.

Inoue, T., Chisaka, O., Matsunami, H., Takeichi, M., 1997. Cadherin-6 expression transiently delineates specific rhombomeres, other neural tube subdivisions, and neural crest subpopulations in mouse embryos. Dev. Biol. 183, 183–194.

Ishii, Y., Asakawa, S., Taguchi, Y., Ishibashi, S., Yagi, T., Shimizu, N., 2004. Construction of BAC library for the amphibian *Xenopus tropicalis*. Genes Genet. Syst. 79, 49–51.

Jen, W.C., Wettstein, D., Turner, D., Chitnis, A., Kintner, C., 1997. The Notch ligand, X-Delta-2, mediates segmentation of the paraxial mesoderm in *Xenopus* embryos. Development 124, 1169–1178.

Jones, K.W., Elsdale, T.R., 1963. The culture of small aggregates of amphibian embryonic cells in vitro. J. Embryol. Exp. Morphol. 11, 135–154.

Joseph, E.M., Cassetta, L.A., 1999. Mespo: a novel basic helix–loop–helix gene expressed in the presomitic mesoderm and posterior tailbud of *Xenopus* embryos. Mech. Dev. 82, 191–194.

Keller, R., 2002. Shaping the vertebrate body plan by polarized embryonic cell movements. Science 298, 1950–1954.

Kim, S.H., Yamamoto, A., Bouwmeester, T., Agius, E., Robertis, E.M., 1998. The role of paraxial protocadherin in selective adhesion and cell movements of the mesoderm during *Xenopus* gastrulation. Development 125, 4681–4690.

Kim, S.H., Jen, W.C., De Robertis, E.M., Kintner, C., 2000. The protocadherin PAPC establishes segmental boundaries during somitogenesis in *Xenopus* embryos. Curr. Biol. 10, 821–830.

Kintner, C.R., Brockes, J.P., 1984. Monoclonal antibodies identify blastemal cells derived from dedifferentiating limb regeneration. Nature 308, 67–69.

Knight, R.D., Nair, S., Nelson, S.S., Afshar, A., Javidan, Y., Geisler, R., Rauch, G.J., Schilling, T.F., 2003. Lockjaw encodes a zebrafish tfap2a required for early neural crest development. Development 130, 5755–5768.

Kohmura, N., Senzaki, K., Hamada, S., Kai, N., Yasuda, R., Watanabe, M., Ishii, H., Yasuda, M., Mishina, M., Yagi, T., 1998. Diversity revealed by a novel family of cadherins expressed in neurons at a synaptic complex. Neuron 20, 1137–1151.

Kuroda, H., Inui, M., Sugimoto, K., Hayata, T., Asashima, M., 2002. Axial protocadherin is a mediator of prenotochord cell sorting in *Xenopus*. Dev. Biol. 244, 267–277.

LaBonne, C., Bronner-Fraser, M., 1999. Molecular mechanisms of neural crest formation. Annu. Rev. Cell Dev. Biol. 15, 81–112.

Le Douarin, N., Kalcheim, C., 1999. The Neural Crest. Cambridge Univ. Press, Cambridge, NY.

Luo, T., Matsuo-Takasaki, M., Lim, J.H., Sargent, T.D., 2001. Differential regulation of Dlx gene expression by a BMP morphogenetic gradient. Int. J. Dev. Biol. 45, 681–684.

Luo, T., Lee, Y.H., Saint-Jeannet, J.P., Sargent, T.D., 2003. Induction of neural crest in *Xenopus* by transcription factor AP2alpha. Proc. Natl. Acad. Sci. U. S. A. 100, 532–537.

Luo, T., Matsuo-Takasaki, M., Thomas, M.L., Weeks, D.L., Sargent, T.D., 2002. Transcription factor AP-2 is an essential and direct regulator of epidermal development in *Xenopus*. Dev. Biol. 245, 136–144.

Luo, T., Zhang, Y., Khadka, D., Rangarajan, J., Cho, K.W., Sargent, T.D., 2005. Regulatory targets for transcription factor AP2 in *Xenopus* embryos. Dev. Growth Differ. 47, 403–413.

Makarenkova, H., Sugiura, H., Yamagata, K., Owens, G., 2005.

Alternatively spliced variants of protocadherin 8 exhibit distinct patterns of expression during mouse development. Biochim. Biophys. Acta 1681, 150–156.

Maroto, M., Dale, J.K., Dequeant, M.L., Petit, A.C., Pourquie, O., 2005. Synchronised cycling gene oscillations in presomitic mesoderm cells require cell–cell contact. Int. J. Dev. Biol. 49, 309–315.

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

Medina, A., Swain, R.K., Kuerner, K.M., Steinbeisser, H., 2004. *Xenopus* paraxial protocadherin has signaling functions and is involved in tissue separation. EMBO J. 23, 3249–3258.

Moreno, T.A., Kintner, C., 2004. Regulation of segmental patterning by retinoic acid signaling during *Xenopus* somitogenesis. Dev. Cell 6, 205–218.

Myers, D.C., Sepich, D.S., Solnica-Krezel, L., 2002. Convergence and extension in vertebrate gastrulae: cell movements according to or in search of identity? Trends Genet. 18, 447–455.

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., Matsunami, H., Takeichi, M., Matsunami, H., 1998. Selective aggregation assays for embryonic brain cells and cell lines. Curr. Top. Dev. Biol. 36, 197–210.

Nieuwkoop, P.D., Faber, J., 1994. Normal Table of *Xenopus laevis* (Daudin): A Systematical and Chronological Survey of the Development from the Fertilized Egg Till the End of Metamorphosis. Garland Pub, New York.

Noden, D.M., 1991. Cell movements and control of patterned tissue assembly during craniofacial development. J. Craniofac. Genet. Dev. Biol. 11, 192–213.

Noonan, J.P., Grimwood, J., Schmutz, J., Dickson, M., Myers, R.M., 2004. Gene conversion and the evolution of protocadherin gene cluster diversity. Genome Res. 14, 354–366.

Obata, S., Sago, H., Mori, N., Rochelle, J.M., Seldin, M.F., Davidson, M., St John, T., Taketani, S., Suzuki, S.T., 1995. Protocadherin Pcdh2 shows properties similar to, but distinct from, those of classical cadherins. J. Cell Sci. 108 (Pt. 12), 3765–3773.

Pasqualetti, M., Ori, M., Nardi, I., Rijli, F.M., 2000. Ectopic Hoxa2 induction after neural crest migration results in homeosis of jaw elements in *Xenopus*. Development 127, 5367–5378.

Pla, P., Moore, R., Morali, O.G., Grille, S., Martinozzi, S., Delmas, V., Larue, L., 2001. Cadherins in neural crest cell development and transformation. J. Cell. Physiol. 189, 121–132.

Pourquie, O., 2001a. The vertebrate segmentation clock. J. Anat. 199, 169–175.

Pourquie, O., 2001b. Vertebrate somitogenesis. Annu. Rev. Cell Dev. Biol. 17, 311–350.

Pourquie, O., 2003a. The segmentation clock: converting embryonic time into spatial pattern. Science 301, 328–330.

Pourquie, O., 2003b. Vertebrate somitogenesis: a novel paradigm for animal segmentation? Int. J. Dev. Biol. 47, 597–603.

Raftopoulou, M., Hall, A., 2004. Cell migration: Rho GTPases lead the way. Dev. Biol. 265, 23–32.

Rhee, J., Takahashi, Y., Saga, Y., Wilson-Rawls, J., Rawls, A., 2003. The protocadherin papc is involved in the organization of the epithelium along the segmental border during mouse somitogenesis. Dev. Biol. 254, 248–261.

Rida, P.C., Le Minh, N., Jiang, Y.J., 2004. A Notch feeling of somite segmentation and beyond. Dev. Biol. 265, 2–22.

Robinson, V., Smith, A., Flenniken, A.M., Wilkinson, D.G., 1997. Roles of Eph receptors and ephrins in neural crest pathfinding. Cell Tissue Res. 290, 265–274.

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.

Sano, K., Tanihara, H., Heimark, R.L., Obata, S., Davidson, M., St John, T., Taketani, S., Suzuki, S., 1993. Protocadherins: a large family of cadherin-related molecules in central nervous system. EMBO J. 12, 2249–2256.

Sargent, T.D., Jamrich, M., Dawid, I.B., 1986. Cell interactions and the control of gene activity during early development of *Xenopus laevis*. Dev. Biol. 114, 238–246.

Savagner, P., 2001. Leaving the neighborhood: molecular mechanisms involved during epithelial–mesenchymal transition. BioEssays 23, 912–923.

Schorle, H., Meier, P., Buchert, M., Jaenisch, R., Mitchell, P.J., 1996. Transcription factor AP-2 essential for cranial closure and craniofacial development. Nature 381, 235–238.

Sive, H.L., Grainger, R.M., Harland, R.M., 2000. Early Development of *Xenopus laevis*: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Smith, A., Robinson, V., Patel, K., Wilkinson, D.G., 1997. The EphA4 and EphB1 receptor tyrosine kinases and ephrin-B2 ligand regulate targeted migration of branchial neural crest cells. Curr. Biol. 7, 561–570.

Sparrow, D.B., Jen, W.C., Kotecha, S., Towers, N., Kintner, C., Mohun, T.J., 1998. Thylacine 1 is expressed segmentally within the paraxial mesoderm of the *Xenopus* embryo and interacts with the Notch pathway. Development 125, 2041–2051.

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.

Sugino, H., Hamada, S., Yasuda, R., Tuji, A., Matsuda, Y., Fujita, M., Yagi, T., 2000. Genomic organization of the family of CNR cadherin genes in mice and humans. Genomics 63, 75–87.

Sugino, H., Yanase, H., Hamada, S., Kurokawa, K., Asakawa, S., Shimizu, N., Yagi, T., 2004. Distinct genomic sequence of the CNR/Pcdhalpha genes in chicken. Biochem. Biophys. Res. Commun. 316, 437–445.

Suzuki, S.T., 1996. Protocadherins and diversity of the cadherin superfamily. J. Cell Sci. 109 (Pt. 11), 2609–2611.

Tada, M.N., Senzaki, K., Tai, Y., Morishita, H., Tanaka, Y.Z., Murata, Y., Ishii, Y., Asakawa, S., Shimizu, N., Sugino, H., Yagi, T., 2004. Genomic organization and transcripts of the zebrafish protocadherin genes. Gene 340, 197–211.

Tanihara, H., Sano, K., Heimark, R.L., St John, T., Suzuki, S., 1994. Cloning of five human cadherins clarifies characteristic features of cadherin extracellular domain and provides further evidence for two structurally different types of cadherin. Cell Adhes. Commun. 2, 15–26.

Tucker, A.S., Slack, J.M., 1995. Tail bud determination in the vertebrate embryo. Curr. Biol. 5, 807–813.

Turner, D.L., Weintraub, H., 1994. Expression of achaete-scute homolog 3 in *Xenopus* embryos converts ectodermal cells to a neural fate. Genes Dev. 8, 1434–1447.

Ueno, N., Greene, N.D., 2003. Planar cell polarity genes and neural tube closure. Birth Defects Res. C Embryo Today 69, 318–324.

Unterseher, F., Hefele, J.A., Giehl, K., De Robertis, E.M., Wedlich, D., Schambony, A., 2004. Paraxial protocadherin coordinates cell polarity during convergent extension via Rho A and JNK. EMBO J. 23, 3259–3269.

Vallin, J., Girault, J.M., Thiery, J.P., Broders, F., 1998. *Xenopus* cadherin-11 is expressed in different populations of migrating neural crest cells. Mech. Dev. 75, 171–174.

Veeman, M.T., Axelrod, J.D., Moon, R.T., 2003. A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling. Dev. Cell 5, 367–377.

Wu, Q., Maniatis, T., 1999. A striking organization of a large family of human neural cadherin-like cell adhesion genes. Cell 97, 779–790.

Wu, Q., Zhang, T., Cheng, J.F., Kim, Y., Grimwood, J., Schmutz, J., Dickson, M., Noonan, J.P., Zhang, M.Q., Myers, R.M., Maniatis, T., 2001. Comparative DNA sequence analysis of mouse and human protocadherin gene clusters. Genome Res. 11, 389–404.

Yamagata, K., Andreasson, K.I., Sugiura, H., Maru, E., Dominique, M., Irie, Y., Miki, N., Hayashi, Y., Yoshioka, M., Kaneko, K., Kato, H., Worley, P.F., 1999. Arcadlin is a neural activity-regulated cadherin involved in long term potentiation. J. Biol. Chem. 274, 19473–19479.

Yamamoto, A., Amacher, S.L., Kim, S.H., Geissert, D., Kimmel, C.B., De Robertis, E.M., 1998. Zebrafish paraxial protocadherin is a downstream target of spadetail involved in morphogenesis of gastrula mesoderm. Development 125, 3389–3397.

Yamamoto, A., Kemp, C., Bachiller, D., Geissert, D., De Robertis, E.M., 2000.

Mouse paraxial protocadherin is expressed in trunk mesoderm and is not essential for mouse development. Genesis 27, 49–57.

Yanase, H., Sugino, H., Yagi, T., 2004. Genomic sequence and organization of the family of CNR/Pcdhalpha genes in rat. Genomics 83, 717–726.

Yaniv, K., Fainsod, A., Kalcheim, C., Yisraeli, J.K., 2003. The RNA-binding protein Vg1 RBP is required for cell migration during early neural development. Development 130, 5649–5661.

Youn, B.W., Malacinski, G.M., 1981. Somitogenesis in the amphibian *Xenopus laevis*: scanning electron microscopic analysis of intrasomitic cellular arrangements during somite rotation. J. Embryol. Exp. Morphol. 64, 23–43.

Zhang, J., Hagopian-Donaldson, S., Serbedzija, G., Elsemore, J., Plehn-Dujowich, D., McMahon, A.P., Flavell, R.A., Williams, T., 1996. Neural tube, skeletal and body wall defects in mice lacking transcription factor AP-2. Nature 381, 238–241.