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A role of *glypican4* and *wnt5b* in chondrocyte stacking underlying craniofacial cartilage morphogenesis

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ABSTRACT

The Wnt/Planar Cell Polarity (PCP) pathway controls cell morphology and behavior during animal development. Several zebrafish mutants were identified as having perturbed Wnt/PCP signaling. Many of these mutants have defects in craniofacial formation. To better understand the role that Wnt/PCP plays in craniofacial development we set out to identify which of the mutants, known to be associated with the Wnt/PCP pathway, perturb head cartilage formation by disrupting chondrocyte morphology. Here we demonstrate that while *vang-like 2* (*vangl2*), *wnt11* and *scribbled* (*scrib*) mutants have severe craniofacial morphogenesis defects they do not display the chondrocyte stacking and intercalation problems seen in *glypican 4* (*gpc4*) and *wnt5b* mutants. The function of Gpc4 or Wnt5b appears to be important for chondrocyte organization, as the neural crest in both mutants is specified, undergoes migration, and differentiates into the same number of cells to compose the craniofacial cartilage elements. We demonstrate that Gpc4 activity is required cell autonomously in the chondrocytes and that the phenotype of single heterozygous mutants is slightly enhanced in embryos double heterozygous for *wnt5b* and *gpc4*. This data suggests a novel mechanism for Wnt5b and Gpc4 regulation of chondrocyte behavior that is independent of the core Wnt/PCP molecules and differs from their collaborative action of controlling cell movements during gastrulation.

1. Introduction

The Wnt/Planar Cell Polarity (PCP) pathway, initially identified in *Drosophila* (Gubb and Garcia-Bellido, 1982), plays an important role in patterning the polarity of epithelial and mesenchymal cells in invertebrates and vertebrates. In zebrafish, this pathway is particularly linked to convergence and extension cell movements during gastrulation (Heisenberg et al., 2000; Jessen et al., 2002; Park and Moon, 2002; Rauch et al., 1997; Topczewski et al., 2001; Wada et al., 2005) and neuronal migration (Wada et al., 2005; Wada and Okamoto, 2009). The pathway also regulates aspects of zebrafish organogenesis (Dale et al., 2009) such as establishing hair cell polarity within the neuromasts of the lateral line, the sensory system, of the zebrafish (Lopez-Schier and Hudspeth, 2006; Lopez-Schier et al., 2004). In addition, two of the mutants that exhibit convergence and extension defects, *glypican 4* (*gpc4*; *knypek*) and *wnt5b* (*pipe tail*), also have shortened craniofacial cartilage elements (Hammerschmidt et al., 1996; LeClair et al., 2009; Piotrowski et al., 1996; Solnica-Krezel et al., 1996; Topczewski et al., 2001).

Craniofacial cartilage elements are derived from the cranial neural crest. In zebrafish, Wnt/PCP signaling plays a role in the migration of the cranial neural crest (De Calisto et al., 2005). As neural crest cells migrate they extend polarized protrusions allowing the cells to migrate in a directed motion. The polarization of these protrusions is controlled in part by Wnt/PCP elements Wnt11, Fzd7 and Disheveled (Carmona-Fontaine et al., 2008; Clay and Halloran, 2011; Matthews et al., 2008). The cranial neural crest cells migrate in four distinct streams, (1) premandibular, (2) mandibular (3) hyoid and (4) branchial. These streams then form the seven pharyngeal arches, with the third stream forming three of these arches (Knight and Schilling, 2006; Schilling et al., 1996). Following migration, the neural crest cells condense and begin to form the craniofacial cartilage elements. At the early larval stage, ~3.5 mm standard length (SL), the bones of the viscerocranium begin to form through either endochondrial ossification of the cartilage elements (e.g. hyosymplectic) or directly by membranous ossification around cartilage elements (e.g. Meckel's cartilage) or without a cartilage mold (e.g. branchiostegal ray) (Cubbage and Mabee, 1996; Parichy et al., 2009).

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The cartilage elements of *gpc4* and *wnt5b* mutants are shorter and built of rounded chondrocytes that do not stack properly (Hammerschmidt et al., 1996; Piotrowski et al., 1996; Solnica-Krezel et al., 1996; Topczewski et al., 2001). The morphology of the chondrocyte stacking defects in the *gpc4* and *wnt5b* mutant cartilages are similar to the defects found in the axial mesoderm of Wnt/PCP mutants during gastrulation (Glickman et al., 2003; Lin et al., 2005) suggesting a role of the pathway in cartilage morphogenesis. Furthermore, rescued adult *gpc4*^{tm818} mutants have smaller skulls and display a lack of elongated cartilage in the palaquadrate elements (LeClair et al., 2009). While *gpc4* and *wnt5b* mutants exhibit shortened cartilage elements, they are not the only core PCP pathway members reported to display craniofacial skeleton defects. The zebrafish *wnt11* mutant (*silberblick*) also have smaller craniofacial cartilage, particularly in the jaw (Heisenberg et al., 1996) and embryos mutant for *vang-like 2* (*vangl2*; *trilobite*) exhibit cyclopia and have a smaller head (Marlow et al., 1998).

Due to their role in convergence and extension and the similar body phenotypes found in *gpc4* and *wnt5b* mutants compared to other mutants disrupting core members of the PCP pathway, one might assume that all of the core genes of the Wnt/PCP pathway play a similar role in the morphogenesis of craniofacial cartilage. This hypothesis has yet to be tested. Here we explore the craniofacial cartilage phenotypes of members of the Wnt/PCP pathway within the zebrafish: *gpc4*, *wnt5b*, *wnt11*, *scrib* (*scribble1*; *llk*), and *vangl2*. We demonstrate that roles of Wnt11, Scrib or Vangl2 are in contrast with Gpc4 and Wnt5b which are required for the proper elongation of cartilage elements by regulating the process of chondrocyte elongation and stacking. This defect is not due to improper neural crest specification or migration, or a decrease in cell number. Furthermore, we show that Gpc4 acts cell autonomously in the control of cartilage morphogenesis and that *wnt5b* can enhance the cartilage phenotype of *gpc4* heterozygotes. Taken together these results suggest that Wnt5b and Gpc4 are specifically required for normal chondrocyte stacking.

2. Results

2.1. Genes involved in the Wnt/PCP signaling pathway are expressed in the pharyngeal arches

As craniofacial cartilage phenotypes are observed in mutants of the PCP pathway, we sought to verify that these genes were expressed in the craniofacial cartilage during chondrocyte condensation and stacking (55 h post fertilization (hpf) and 3 days post fertilization (dpf), respectively). Whole mount in situ hybridizations were performed on 55 hpf and 3 dpf embryos with probes for *vangl1*, *vangl2*, *gpc4*, *wnt5b*, and *wnt11* (Fig. 1). As expected, of the Wnt/PCP genes with known craniofacial defects, all were expressed in the pharyngeal arches (Fig. 1A–H′, K–L′) except *vangl1* (Jessen and Solnica-Krezel, 2004) (Fig. 1A–B′). Furthermore, *wnt5b* maintains its expression in the pharyngeal arches in *gpc4*^{fr6} mutants (Fig. 1I–J′). Given the craniofacial phenotypes observed in *gpc4* and *wnt5b* mutants, we wanted to specifically observe the expression pattern of these genes within the pharyngeal arches at 55 hpf when the cartilage is still condensing. Whole mount in situ hybridizations for *gpc4* and *wnt5b* were performed on 55 hpf wild type embryos and cyrosectioned (Fig. 1N, O). To provide a reference for the location of the neural crest at this time point Sox10:GFP embryos, which express GFP in the neural crest, were also sectioned (Fig. 1P). This demonstrates that while *gpc4* is expressed in the pharyngeal endoderm, neural crest and mesoderm at 55 hpf, *wnt5b* has a more restricted pattern and is only expressed in the neural crest and mesoderm. Whole mount in situ

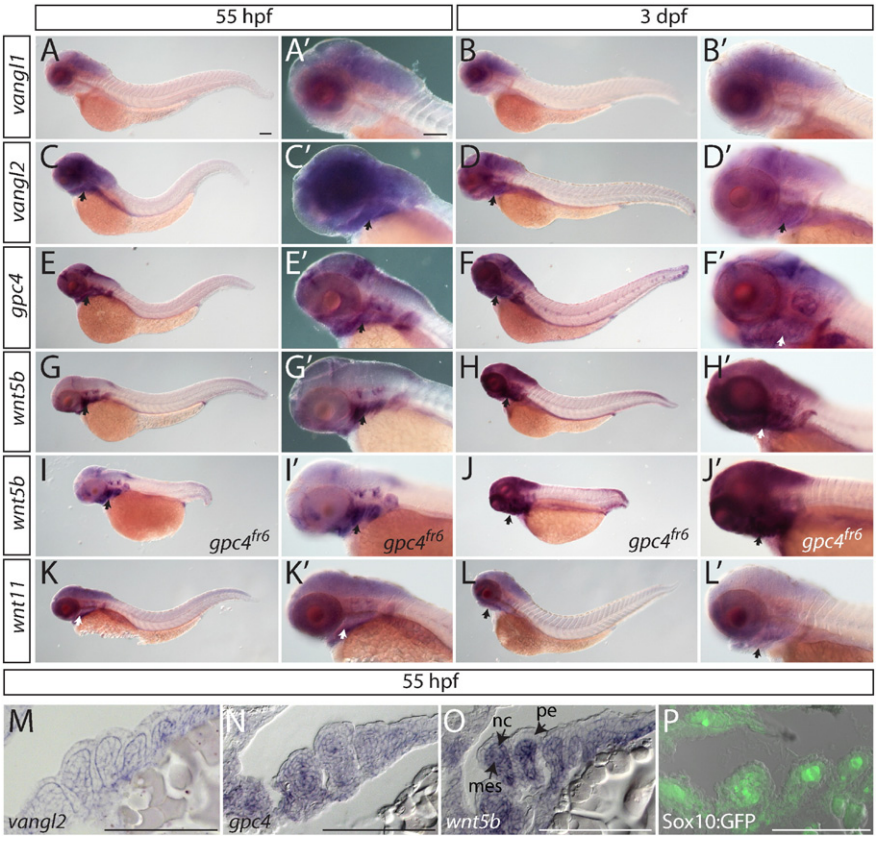

Fig. 1. Expression of Wnt/PCP molecules in the zebrafish head of 55 h hpf and 3 dpf embryos. (A–H′, K–L′) *In situ* hybridizations of whole mount wild type embryos. (I–J′) *wnt5b* expression in *gpc4*^{fr6} mutant embryos. (A–L′) Arrows indicate staining in the pharyngeal arches. (M–P) Sections of whole mount in situ hybridizations. (M) *vangl2* is expressed in the mes and nc. (N) *gpc4* is expressed in the pe, mes and the nc. (O) *wnt5b* is expressed in the mes and nc. (P) Sox10:GFP expression in the mes and in the nc. Abbreviations: mes = mesoderm, nc = neural crest, pe = pharyngeal endoderm. Scale bar = 100 μm.