The role of Ppt/Wnt5 in regulating cell shape and movement during zebrafish gastrulation

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Abstract

Wnt genes play important roles in regulating patterning and morphogenesis during vertebrate gastrulation. In zebrafish, \textit{slb/wnt11} is required for convergence and extension movements, but not cell fate specification during gastrulation. To determine if other Wnt genes functionally interact with \textit{slb/wnt11}, we analysed the role of \textit{ppt/wnt5} during zebrafish gastrulation. \textit{ppt/wnt5} is maternally provided and zygotically expressed at all stages during gastrulation. The analysis of \textit{ppt} mutant embryos reveals that Ppt/Wnt5 regulates cell elongation and convergent extension movements in posterior regions of the gastrula, while its function in more anterior regions is largely redundant to that of Slb/Wnt11. \textit{Frizzled-2} functions downstream of \textit{ppt/wnt5}, indicating that it might act as a receptor for Ppt/Wnt5 in this process. The characterisation of the role of Ppt/Wnt5 provides insight into the functional diversity of Wnt genes in regulating vertebrate gastrulation movements.

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1. Introduction

During vertebrate gastrulation, large cellular rearrangements lead to the formation of the three germ layers, ectoderm, mesoderm and endoderm. One of the major cell movements during gastrulation is convergent extension (CE). In CE, both mesendodermal and ectodermal cells move to the dorsal side of the embryo (convergence), which is accompanied by an elongation of the forming embryo along its anterior–posterior axis (extension). The cellular mechanisms that underlie CE movements have been well studied in the amphibian \textit{Xenopus} (Keller et al., 2000). In \textit{Xenopus}, cells converging towards the dorsal side of the gastrula undergo medio-lateral cell intercalations that drive the elongation of the forming embryonic axis along its anterior–posterior extent (Keller and Tibbetts, 1989). Cells undergoing medio-lateral cell intercalations are elongated along the medio-lateral axis and exhibit polarised protrusive activity (Concha and Adams, 1998; Keller et al., 1992; Shih and Keller, 1992).

Wnt genes are thought to be key players in regulating gastrulation movements (reviewed in Tada et al., 2002; Wallingford et al., 2002). Although in \textit{Xenopus}, mis-expression of wild type and dominant negative versions of several Wnt genes, including \textit{wnt4}, \textit{wnt5} and \textit{wnt11}, have been shown to interfere with CE movements, their precise endogenous requirements have not yet been analysed (Du et al., 1995; Tada and Smith, 2000; Wallingford et al., 2001). In zebrafish \textit{slb/wnt11} mutant embryos, CE movements of anterior and posterior mesendodermal and neuroectodermal cells are reduced, leading to a transiently shortened body axis at the end of gastrulation (Heisenberg et al., 1996; Heisenberg and Nusslein-Volhard, 1997; Heisenberg et al., 2000). In contrast, zebrafish \textit{ppt/wnt5} mutants exhibit defects in tail elongation, while no obvious CE phenotype has yet been described (Hammerschmidt et al., 1996; Rauch et al., 1997).

Several studies have addressed the signaling pathways that mediate the morphogenetic function of Wnt genes during gastrulation. Interestingly, genes involved in the establishment of epithelial planar cell polarity (PCP) in \textit{Drosophila} appear to play an important role in mediating and/or modulating Wnt function during gastrulation (reviewed in Tada et al., 2002; Wallingford et al., 2002).
Shared components between these signaling pathways include homologues of frizzled (fz), dishevelled (dsh), strabismus/van gogh (stbm/vang), the small GTPases rhoA and cdc42, and the rhoA effector rho kinase 2 (rok2) (Tada and Smith, 2000; Wallingford et al., 2000; Heisenberg et al., 2000; Djiane et al., 2000; Habas et al., 2001; Park and Moon, 2001; Goto and Keller, 2002; Darken et al., 2002; Jessen et al., 2002; Marlow et al., 2002). In addition to the PCP pathway, Wnt genes have also been reported to regulate gastrulation movements via a dsh-independent pathway as demonstrated by the ability of Wnt5a to induce the release of intracellular Ca²⁺ and activate protein kinase C (PKC) (Sheldahl et al., 1999; Kuhl et al., 2000).

In this study, we analyse the function of Ppt/Wnt5 during zebrafish gastrulation. We show that ppt/wnt5 is required for regulating CE movements in posterior mesendodermal and ectodermal regions while its function in the anterior mesendoderm is largely redundant to that of Slb/Wnt11. We further demonstrate that Ppt/Wnt5 influences cell elongation and CE movements in posterior parts of the gastrula. Finally, epistasis experiments suggest that Fz2 might function as a receptor for Ppt/Wnt5 during gastrulation.

2. Results

2.1. Ppt/wnt5 is maternally provided and zygotically expressed during gastrulation

To determine the temporal and spatial distribution of ppt/wnt5 mRNA, we analysed the expression pattern of ppt/wnt5 at various stages during gastrulation. ppt/wnt5 is maternally provided (Fig. 1A), and zygotic expression of ppt/wnt5 can be detected from the shield stage onwards, first at the germ ring within epi- and hypoblast cells and then in posterior paraxial mesendodermal cells (Fig. 1B and data not shown). At the end of gastrulation, ppt/wnt5 is expressed within the posterior paraxial and axial mesendoderm adjacent to the slb/wnt11 expression domain in the anterior paraxial mesendoderm and paraxial protocadherin (papc) (Fig. 1C–F and data not shown).

2.2. Ppt/Wnt5 function is required for regulating CE movements in the posterior mesendoderm

To analyse the function of Ppt/Wnt5 in regulating CE movements, we measured the anterior–posterior extension of axial mesendodermal tissues such as the notochord (marked by the expression of notail) and prechordal plate (marked by the expression of hatching gland gene 1) in ppt mutant embryos during the course of gastrulation (Fig. 2A). We chose to measure the notochord and prechordal plate because they are easily recognizable and constitute a reliable readout for the degree of CE movements in both axial and paraxial tissues (Fig. 2a–d and Heisenberg et al., 2000). In wild type embryos, prechordal plate cells first migrate towards the animal pole and subsequently accumulate within the polster at the end of gastrulation. This is reflected by an initial increase in the anterior–posterior extension of the prechordal plate followed by a pronounced reduction towards the end of gastrulation (Fig. 2B). In contrast, CE movements within posterior mesendodermal regions of wild type embryos lead to a continuous increase in the anterior–posterior extension of the notochord throughout the course of gastrulation (Fig. 2C). In ppt mutants, the anterior–posterior extension of the notochord is reduced from mid-gastrulation stages onwards (Fig. 2C) while the extension of the prechordal plate is indistinguishable from wild type embryos (Fig. 2B). In contrast, slb mutant embryos not only show a reduction in the anterior–posterior extension of the notochord from early gastrulation stages onwards (Fig. 2C), but also an increase in the length of the prechordal plate towards the end of gastrulation, indicating that prechordal plate morphogenesis is also defective (Fig. 2B). Taken together, these results suggest...
that Ppt/Wnt5 is predominantly required for CE movements in posterior mesendodermal tissues such as the notochord, whereas Slb/Wnt11 is needed for morphogenesis of both anterior (prechordal plate) and posterior (notochord) mesendoderm.

To test more directly if the defect in extension of the notochord in ppt mutant embryos is primarily due to reduced CE movements in posterior mesendodermal regions, we monitored the distribution of cells within this region during the course of gastrulation. Biotin-labeled cells were transplanted into a region of the germ ring at shield stage that will give rise to posterior paraxial mesendoderm. During the course of gastrulation, these cells are redistributed along the anterior–posterior axis of the forming embryo (Fig. 3A and Warga and Kimmel, 1990; Heisenberg et al., 2000). In ppt embryos (n = 20), the anterior-posterior distribution of cells within the posterior paraxial mesendoderm was reduced, as compared to wild type controls (n = 20), indicating that Ppt/Wnt5 is required for convergent extension movements within this region (Fig. 3E).

Elongation of ectodermal and mesendodermal cells along the medio-lateral axis is thought to be a prerequisite for CE movements during gastrulation (Keller et al., 1992; Shih and Keller, 1992; Concha and Adams, 1998; Jessen et al., 2002). To test if medio-lateral cell elongation is dependent on Ppt/Wnt5 activity, we determined the morphology of cells in the posterior paraxial ectoderm of wild type and ppt mutant embryos at the end of gastrulation (Fig. 3B). We decided to focus on the ectodermal germ layer as their cell morphologies are easier to measure and compare than in the underlying mesendoderm at late gastrulation stages. Posterior ectodermal cells do not express high levels of ppt/wnt5 at bud stage (Fig. 1F), however, they are directly adjacent to Ppt/Wnt5 producing
cells in the underlying mesendoderm and express ppt/wnt5 themselves at earlier gastrulation stages (Fig. 1B, F and data not shown). As an expression of cell elongation we measured the degree of cell roundness in both wild type and ppt mutant embryos (for details see Fig. 3B, C, F, G). We found that in wild type embryos, ectodermal cells exhibit a significantly lower degree of roundness (Fig. 3C, F) than cells in similar regions of ppt mutant embryos ($n > 100$; $P < 0.05$; Fig. 3F, G). This indicates that Ppt/Wnt5 is required for the medio-lateral elongation of ectodermal cells during gastrulation.

To obtain a more detailed image of the morphological differences of single ectodermal and mesendodermal cells in posterior regions of the gastrula between wild type and ppt embryos, we labeled those cells with a mixture of cytosolic and membrane-bound green fluorescent protein (GFP) and recorded three dimensional images, using a two-photon confocal microscope. To ensure that we were analyzing the strongest possible ppt mutant phenotype, we used wild type embryos, which were injected with 4 ng of wnt5 morpholino antisense oligonucleotides (MOs). Injection of similar amounts of wnt5 MOs has previously been shown to lead to a morphant phenotype similar but also slightly stronger than the ppt mutant phenotype, indicating that the ppt allele used (ta98) represents a hypomorphic allele (Lele et al., 2001).

Wild type ectodermal cells are elongated along their medio-lateral axis and extend short processes preferentially directed both in medial and lateral directions (Fig. 2I). In contrast, ectodermal cells from wild type embryos, which were injected with wnt5 MOs, are less elongated along their...
2.4. Mis-expression of ppt/wnt5 can rescue the slb mutant phenotype

Similarly, mesendodermal cells from wnt5 MO-injected embryos are less elongated and show cellular extensions that appear less well aligned along the medio-lateral axis as compared to equivalent wild type cells (Fig. 2K, L). These observations suggest that ppt/wnt5 is needed for the medio-lateral elongation and orientation of cellular processes in ectodermal and mesendodermal cells at late gastrulation stages.

We also transplanted wild type cells into ppt embryos (number of analysed clones n = 20) and ppt cells into wild type embryos (n = 20) at shield stage to determine the cell-autonomy of Ppt/Wnt5 function for the medio-lateral elongation of ectodermal cells at the end of gastrulation. ppt cells in wild type embryos looked indistinguishable from surrounding wild type cells, while wild type cells in ppt embryos had a similar morphology to the surrounding ppt cells (Fig. 3D, H). This indicates that Ppt/Wnt5 function is required cell-non-autonomously for the medio-lateral elongation of ectodermal cells during gastrulation.

2.3. Ppt/Wnt5 function is redundant to Slb/Wnt11 within the anterior mesendoderm

To reveal if there is any redundant function between Ppt/Wnt5 and Slb/Wnt11 we generated slb;ppt double mutant embryos (Fig. 4A–D). In slb;ppt double mutants, the notochord is strongly shortened and broadened as expected for an additive phenotype between slb and ppt (Fig. 4I–L). However, the prechordal plate of slb;ppt double mutants was more strongly affected than expected from a simple addition of the single phenotypes (Fig. 4E–H) indicating that Ppt/Wnt5 and Slb/Wnt11 display some functional redundancy in the anterior mesendoderm.

To address the function of maternal ppt/wnt5, we also generated embryos mutant for maternal and zygotic ppt/wnt5 (mzppt). mzppt mutant embryos looked indistinguishable from zygotic ppt mutants, indicating that maternal ppt/wnt5 is not required for regulating CE movements (data not shown and Rauch et al., 1997). We also analysed if the slb;ppt double mutant phenotype is enhanced in the absence of maternal ppt/wnt5 by generating mzppt;slb double mutant embryos. mzppt;slb double mutant embryos displayed a phenotype indistinguishable from zygotic slb;ppt double mutants (data not shown), suggesting that even in the absence of zygotic slb/wnt11 and ppt/wnt5 there is no requirement of maternal ppt/wnt5 to regulate CE movements.

2.5. Mox2 is required downstream of ppt/wnt5 for CE of posterior mesendodermal tissues

To further compare the functions of slb/wnt11 and ppt/wnt5 in regulating CE movements, we tested if we can rescue the slb mutant phenotype by mis-expression of ppt/wnt5 RNA. Injection of 10 pg ppt/wnt5 RNA in slb mutants is sufficient to rescue the slb phenotype in the anterior axial mesendoderm (position and shape of prechordal plate), indicating that ppt/wnt5 can partially replace slb/wnt11 during gastrulation (Table 1). However, a comparison between ppt/wnt5 and slb/wnt11 for their efficiency to rescue the slb mutant phenotype showed that consistently higher amounts of ppt/wnt5 RNA compared to slb/wnt11 were needed to fully rescue the slb prechordal plate phenotype (Table 1). This suggests that, while ppt/wnt5 can functionally replace slb/wnt11 in the anterior mesendoderm, it is less efficient in doing so.

Surprisingly, we were not able to rescue the ppt mutant phenotype by mis-expressing ppt/wnt5 or slb/wnt11 RNA. Instead, ppt embryos injected with various amounts of slb/wnt11 or ppt/wnt5 RNA exhibited variable malformations such as a shortened/ truncated notochord, a misshaped and displaced prechordal plate and, at higher amounts (>100 pg/embryo), a split body axis and severe dorsi-salisation from early gastrula stages onwards (data not shown). The reason for this might be that the posterior mesendoderm is generally more sensitive to the dose of Ppt/Wnt5 or Slb/Wnt11 and that ubiquitous mis-expression interferes with normal CE movements in this region.

<table>
<thead>
<tr>
<th>RNA</th>
<th>pg/embryo</th>
<th>ppl (%)</th>
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<tr>
<td>wnt11</td>
<td>10</td>
<td>52</td>
<td>107</td>
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<tr>
<td>wnt5</td>
<td>10^d</td>
<td>15</td>
<td>87</td>
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<tr>
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<td>5</td>
<td>5</td>
<td>167</td>
</tr>
<tr>
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<td>1</td>
<td>17</td>
<td>90</td>
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<tr>
<td>wnt5</td>
<td>1</td>
<td>4</td>
<td>96</td>
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a Amount of RNA injected per embryo.

b Percentage of rescued prechordal plate (ppl) phenotypes; the position and shape of the prechordal plate were analyzed at bud stage.

c n, total number of injected embryos.

d Higher amounts (>10 pg/embryo) of wnt5 led to variable malformations of the injected embryos and were therefore not included in this Table.
these observations, we also injected fz2 MOs (8 ng/embryo) into embryos \( n > 100 \), which had also been injected with MOs against \( \text{wnt}5 \) (4 ng/embryo; Lele et al., 2001). \( \text{wnt}5 \) MO injected embryos showed a reduction in the length of the notochord slightly stronger than seen in \( \text{ppt} \) mutant embryos (Fig. 5E and Lele et al., 2001). No further enhancement of the \( \text{wnt}5 \) morphant notochord phenotype was observed after \( \text{fz}2 \) MO injection, confirming that \( \text{fz}2 \) functions downstream of \( \text{ppt} \) and \( \text{wnt}5 \) (Fig. 5E, F). In contrast, \( \text{slb} \) embryos injected with an equal amount of \( \text{fz}2 \) MO (\( n > 100 \)) showed a strongly enhanced CE phenotype (Fig. 5G, H) reminiscent of the \( \text{slb};\text{ppt} \) double mutant phenotype (Fig. 4H, L) suggesting that \( \text{fz}2 \) acts in a parallel pathway to \( \text{slb}/\text{wnt}11 \).

3. Discussion

We show that \( \text{ppt}/\text{wnt}5 \) is required for cell elongation and CE movements in posterior mesendodermal and ectodermal regions during late gastrulation. We further present evidence that \( \text{ppt}/\text{wnt}5 \) also displays a function in regulating convergence and extension in more anterior parts of the gastrula. This function is overlapping and largely redundant to that of \( \text{slb}/\text{wnt}11 \) in the same region. \( \text{Fz}2 \) functions downstream of \( \text{ppt}/\text{wnt}5 \) suggesting that it might act as a receptor of Ppt/Wnt5 during gastrulation.

3.1. The function of Ppt/Wnt5 during gastrulation

The observation from this study that Ppt/Wnt5 regulates elongation and medio-lateral intercalations of cells within posterior regions of the gastrula provides insight into the role of Ppt/Wnt5 during gastrulation. In \textit{Xenopus}, mis-expression of \( \text{wnt}5a \) has been shown to interfere with normal CE movements during gastrulation and the elongation of activin-treated animal caps and dorsal marginal zone explants (Du et al., 1995; Wallingford et al., 2001). Although this is indicative for a function of Wnt5a in regulating gastrulation movements, neither the endogenous requirement of Wnt5a nor the mechanism by which Wnt5a functions during gastrulation has yet been analysed. This study, therefore, provides genetic and cellular evidence that Ppt/Wnt5 is needed for normal cell movements during vertebrate gastrulation.

Interestingly in \textit{Xenopus}, Wnt5a, in combination with rFz5 or xFz7, is also capable of interfering with dorso-ventral patterning of the gastrula through activation of the canonical Wnt signalling pathway (He et al., 1997; Sumanas et al., 2000; Umbhauer et al., 2000). In contrast, we have not been able to observe any endogenous requirement of Ppt/Wnt5 for dorso-ventral patterning during gastrulation (data not shown), suggesting that maternal and zygotic Ppt/Wnt5 are not required for this process in zebrafish. However, it is also possible that the \( \text{ppt} \) allele we have analysed in this

Fig. 4. Comparison of the shape of axial tissues between wild type (A,E,I), \( \text{ppt} \) (B,F,J), \( \text{slb} \) (C,G,K) and \( \text{slb};\text{ppt} \) (D,H,L) embryos at bud stage. (A–D) Live images showing wild type (A), \( \text{ppt} \) (B), \( \text{slb} \) (C) and \( \text{slb};\text{ppt} \) double mutant embryos (D) at bud stage; lateral views; anterior to the left. (E–H) Position and shape of the prechordal plate, marked by the expression of \textit{hatching gland gene-1} (\textit{hgg}), in wild type (E), \( \text{ppt} \) (F), \( \text{slb} \) (G) and \( \text{slb};\text{ppt} \) double mutant embryos (H) relative to the anterior edge of the neural plate, outlined by the expression of \textit{dlx}3; animal views; anterior to the left. (I–L) Length of the notochord, marked by the expression of \textit{notail} (\textit{ntl}), in wild type (I), \( \text{ppt} \) (J), \( \text{slb} \) (K) and \( \text{slb};\text{ppt} \) double mutant embryos (L); dorsal views, anterior to the left.
study (ta98, a point mutation leading to the exchange of a conserved Cys at position 323 into Arg; Rauch et al., 1997) exhibits only an incomplete loss-of-function phenotype and that the morphogenesis function is more sensitive to the dose of Wnt5 than the function in dorso-ventral patterning. The observation that injection of wnt5 MO at high concentrations can lead to a morphant phenotype, which is slightly stronger than the ppt mutant phenotype (Lele et al., 2001) argues in favour of this hypothesis. However, no defect in dorso-ventral patterning has been detected in wnt5 MO-injected embryos (Lele et al., 2001) suggesting that maternal and zygotic wnt5 mRNA are not involved in dorso-ventral patterning of the gastrula.

3.2. Distinct local requirements of Ppt/Wnt5 and Slb/Wnt11 during gastrulation

In ppt mutant embryos, CE movements in the posterior mesendoderm are reduced (this study and Hammerschmidt et al., 1996), whereas in slb mutants, convergence and extension movements in both anterior and posterior mesendodermal tissues are affected (Heisenberg et al.,

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**Fig. 5. frizzled-2 (fz2) requirements in wild type and ppt mutant embryos.** (A,B) Wild type embryos (A) and wild type embryos injected with 8ng fz2 morpholino antisense oligonucleotides (MO) (B) at bud stage. (C,D) ppt embryos (C) and ppt injected with 8 ng of fz2MO (D) at bud stage. The embryo shown in (E) belongs to the ~50% of injected embryos that showed a slightly enhanced notochord phenotype while the remaining ~50% appeared indistinguishable from un.injected ppt mutants. (E,F) Wild type embryos injected with 8 ng wnt5MO (E) and wild type embryos injected with both 8 ng wnt5MO and 8 ng fz2MO (F) at bud stage. (G,H) slb embryos (G) and slb embryos injected with 8 ng of fz2MO (H) at bud stage. All embryos were stained for ntl (notochord), hgg1 (prechordal plate) and dlx3 (anterior edge of neuroectoderm). In each panel, the left part shows an animal view (prechordial plate and anterior edge of neural plate) with anterior to the left, and the right part is a dorsal view (notochord) with anterior to the top. Black bars outline the length of the notochord.
These observations indicate that Ppt/Wnt5 and Slb/Wnt11 possess distinct and non-redundant functions in regulating zebrafish gastrulation movements.

There are different possible cellular and molecular mechanisms that could underlie the functional diversification of ppt/wnt5 and slb/wnt11 during gastrulation. Variations in the temporal and spatial expression of ppt/wnt5 and slb/wnt11 might account for their observed distinct activities. While both of them are expressed in ingressing mesendodermal cells at the germ ring throughout gastrulation, slb/wnt11 is specifically expressed in anterior and ppt/wnt5 in posterior mesendodermal tissues at the end of gastrulation (this study and Heisenberg et al., 2000). It is conceivable that both of them are predominantly required at the place of their own expression in the anterior (slb/wnt11) and posterior (ppt/wnt5) mesendoderm at the end of gastrulation, while the function of Slb/Wnt11 in the posterior and the redundant requirement of Ppt/Wnt5 in the anterior mesendoderm are due to their earlier expression in the germ ring.

In addition, spatial and temporal differences in the competence of cells to respond to Ppt/Wnt5 and Slb/Wnt11, e.g. through the differential expression of the appropriate receptor(s), might also account for the distinct activities of ppt/wnt5 and slb/wnt11. frizzled-7 (fz7) is expressed in an overlapping domain with slb/wnt11 during zebrafish gastrulation (El-Messaoudi and Renucci, 2001) and, by binding to Wnt11, can function as a downstream mediator of the morphogenetic activity of Wnt11 both in zebrafish and in Xenopus (Djiane et al., 2000; Sumanas et al., 2002). In contrast, fz2 is expressed in the posterior paraxial mesendoderm overlapping with the ppt/wnt5 expression domain and functions downstream of ppt/wnt5 within the posterior mesendoderm (this study and Sumanas et al., 2001). The spatial and temporal restriction of Wnt5 and Wnt11 functions might therefore be at least partially due to the differential competence of tissues to respond to these Wnt ligands through Fz2 and Fz7, respectively.

3.3. Distinct cell behaviours in relation to the local requirements of Ppt/Wnt5 and Slb/Wnt11

It is likely that the different local requirements of ppt/wnt5 and slb/wnt11 along the anterior–posterior axis of the gastrula reflect some functional and/or structural subdivision of the developing mesendoderm. Indeed, ongoing in vivo analyses of cell behaviour in zebrafish show that anterior mesendodermal cells seem not to undergo the classical CE movements described for more posterior tissues but instead exhibit a highly co-ordinated type of ‘migration over a substrate’ cell movement (Miguel Concha, M.T. and C.P.H., unpublished data). It is, therefore, conceivable that Slb/Wnt11 regulates the migration of anterior mesendodermal cells while both Ppt/Wnt5 and Slb/Wnt11 are required for CE movements in more posterior mesendodermal tissues. The molecular mechanisms, which underlie the differences in cell behaviour along the anterior–posterior axis are still unclear, although there are several genes implicated in the regulation of gastrulation movements such as snail-2 and paraxial protocadherin that are exclusively expressed in the zebrafish anterior and posterior mesendoderm, respectively (Thisse et al., 1995; Yamamoto et al., 1998).

4. Conclusions

The observation that ppt/wnt5 and slb/wnt11 are required at distinct levels along the anterior–posterior axis of the gastrula to regulate CE movements provides insight into the functional diversification of Wnt genes in this process. Future studies will have to address the differences in the molecular and cellular mechanisms underlying convergence and extension movements between anterior and posterior mesendodermal regions during gastrulation.

5. Experimental procedures

5.1. Zebrafish maintenance and genetics

Zebrafish were maintained as previously described (Mullins et al., 1994). Alleles used were pptta98 (kindly provided by Henry Roehl, MPI Tübingen) and slb2226. For cell transplantation and quantification studies ppt and slb mutant embryos were obtained by crossing homozygous ppt or slb carriers. slb;ppt double heterozygous fish were produced by crossing double heterozygous carriers for ppt and slb. The resulting egg-lays contained slb, ppt and slb;ppt mutant phenotypes in the expected ratio although some of the embryos scored as ‘wild type’ in a few crosses showed a very mild shortening of the embryonic axis indicative of some dominant phenotype(s) of slb and/or ppt. Maternal-zygotic (mz) slb;ppt double mutant embryos were obtained by crossing carriers, which were homozygous for ppt and heterozygous for slb.

5.2. In situ hybridisation

Antisense RNA probes were produced with a digoxigenin RNA labeling kit (Roche, Switzerland) according to the manufacturer’s instructions using plasmids containing cDNA of wnt5, wnt11, ntl, hgg1, and dlx3. Whole mount in situ hybridisations were performed essentially as previously described (Heisenberg et al., 1996).

5.3. RNA and morpholino antisense oligonucleotide (MO) injection

Sense RNA of wnt11 and wnt5 was produced using a mMessage mMachine kit (Ambion, USA). MO against fz2 and wnt5 have been described previously (Sumanas et al.,
and were obtained from Gene Tools (USA). All injections were performed on one-cell stage embryos.

5.4. Cell transplantation

For transplantations, donor embryos were injected with a mixture of rhodamine and biotin–dextran. Cells were transplanted into host embryos as described previously (Ho and Kane, 1990). Transplanted cells were detected with a Vectastain kit (Vector Laboratories, USA) to reveal biotin.

5.5. Confocal analysis

Bud to 1 somite stage embryos were manually dechorionated and mounted in 1% agarose in E3 fish medium. Life images were obtained at 18°C with a 60 x water immersion objective, NA 1.2, on a Nikon TE 300 Microscope at a BioRad Radiance 2000 Multiphoton Confocal Microscope setup; as a software, LaserSharp 2000 version 1.4 on a Windows NT based PC was used. Confocal z-stacks were acquired by scanning an area of 104.2 x 104.2 μm² (0.2 μm/pixel) with 500 lines per second and 0.5 μm steps over a total vertical distance of 60 μm. Three image stacks were taken continuously with no time gap in between. A mode-locked infrared laser line between 890 and 910 nm were used as a pump laser, a 532 nm laser source with 5 W output. Three image stacks were rendered in three dimensions using Volocity 2.0 (Improvisation, USA).

The obtained image stacks were subsequently processed using Image J version 1.28 (http://rsb.info.nih.gov/ij/) and rendered in three dimensions using Velocity 2.0 (Improvisation, UK).

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