Wnt5a and Wnt11 regulate mammalian anterior-posterior axis elongation

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ABSTRACT
Mesoderm formation and subsequent anterior-posterior (A-P) axis elongation are fundamental aspects of gastrulation, which is initiated by formation of the primitive streak (PS). Convergent extension (CE) movements and epithelial-mesenchymal transition (EMT) are important for A-P axis elongation in vertebrate embryos. The evolutionarily conserved planar cell polarity (PCP) pathway regulates CE, and Wnts regulate many aspects of gastrulation including CE and EMT. However, the Wnt ligands that regulate A-P axis elongation in mammalian development remain unknown. Wnt11 and Wnt5a regulate axis elongation in lower vertebrates, but only Wnt5a, not Wnt11, regulates mammalian PCP signaling and A-P axis elongation in development. Here, by generating Wnt5a; Wnt11 compound mutants, we show that Wnt11 and Wnt5a play redundant roles during mouse A-P axis elongation. Both genes regulate trunk notochord extension through PCP-controlled CE of notochord cells, establishing a role for Wnt11 in mammalian PCP. We show that Wnt5a and Wnt11 are required for proper patterning of the neural tube and somites by regulating notochord formation, and provide evidence that both genes are required for the generation and migration of axial and paraxial mesodermal precursor cells by regulating EMT. Axial and paraxial mesodermal precursors ectopically accumulate in the PS at late gastrula stages in Wnt5a−/−; Wnt11−/− embryos and these cells ectopically express epithelial cell adhesion molecules. Our data suggest that Wnt5a and Wnt11 regulate EMT by inducing p38 (Mapk14) phosphorylation. Our findings provide new insights into the role of Wnt5a and Wnt11 in mouse early development and also in cancer metastasis, during which EMT plays a crucial role.

KEY WORDS: Convergent extension, EMT, Gastrulation, Notochord, Planar cell polarity, Wnt

INTRODUCTION
In vertebrate embryos, the basic body plan is established during gastrulation through the formation of three germ layers and a major rearrangement of cells. Gastrulation is initiated with primitive streak (PS) formation at the posterior of the embryo (Tam and Behringer, 1997). The PS elongates anteriorly during gastrulation. Within the PS, cells of the primitive ectoderm delaminate and undergo an epithelial-mesenchymal transition (EMT) (Arnold and Robertson, 2009) to give rise to mesodermal and endodermal structures (Beddington and Robertson, 1999; Tam and Loebel, 2007). Defective EMT results in severe phenotypes, as cells fail to migrate away from the PS and instead accumulate (Sun et al., 1999; Ciruna and Rossant, 2001). Besides its essential role in gastrulation, EMT is also crucial for other developmental processes, wound healing, fibrosis and cancer metastasis (Thiery et al., 2009). However, the molecular mechanisms that regulate EMT remain to be fully elucidated.

Anterior-posterior (A-P) body axis elongation requires continuous generation and proper organization of axial and paraxial mesoderm. Fate-mapping studies have revealed that cells emerging from the anterior PS give rise to the axial mesoderm that forms the notochord (Lawson et al., 1991). The notochord serves as an important signaling center that patterns the overlying neuroectoderm and adjacent somites through secretion of signaling molecules such as sonic hedgehog (Shh) (Echelard et al., 1993; Chiang et al., 1996; reviewed by Cleaver and Krieg, 2001). Anterior to the PS, an indentation called the posterior notochord (PNC, also referred to as the node or ventral node) forms at mid-gastrula stages (Blum et al., 2007). The PNC provides instructive signals during mouse left-right determination and is continuous with the notochord (Sulik et al., 1994; Nonaka et al., 2002). Cells of the trunk and tail notochord are derived from the PNC (Sulik et al., 1994; Yamanaka et al., 2007; Ukita et al., 2009). However, since PNC cells are ciliated and proliferate at a very low rate, a region consisting of the anterior-most streak and the posterior-most part of the node/PNC, called the node/streak border (NSB), is considered the origin of notochord precursors (Sulik et al., 1994; Bellomo et al., 1996; Cambray and Wilson, 2002, 2007; Ukita et al., 2009). PNC cells express Noto and the epithelial marker E-cadherin (Abdelkhalak et al., 2004; Plouhinec et al., 2004; Yamanaka et al., 2007). Genetic fate-mapping studies revealed that the Noto-expressing cells in the PNC contribute to notochord precursor cells (NPCs) that form the trunk and tail notochord as well as some of the paraxial mesoderm (Yamanaka et al., 2007; Ukita et al., 2009). In addition, paraxial mesodermal cells are derived from more posterior regions of the PS and migrate anteriorly to give rise to the somites (Tam and Beddington, 1987; Tam et al., 2000).

The trunk notochord is derived from the PNC and forms by convergent extension (CE) (Sausedo and Schoenwolf, 1994; Ybot-Gonzalez et al., 2007), whereas the tail notochord (posterior notochord extension) is generated by peripheral PNC cells actively migrating posteriorly (Sulik et al., 1994; Yamanaka et al., 2007). Although continuous production of notochord cells by the NPCs is crucial to establish the correct body plan (Ukita et al., 2009), the molecular and cellular regulation of NPC generation remains unknown. The Wnt signaling pathways regulate many fundamentally important developmental processes, including gastrulation. Although Wnts can signal through the canonical pathway mediated by β-catenin, the β-catenin-independent Wnt/planar cell polarity (PCP) pathway has recently emerged as a regulator of many key processes (Song et al., 2010; Gao et al., 2011; Gao et al., 2012).

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The PCP pathway was initially identified in Drosophila melanogaster, where it controls the establishment of a polarity within an epithelial sheet perpendicular to apical-basal polarity. A group of core PCP components has been identified in Drosophila, and these are functionally conserved in vertebrates (Tree et al., 2002; Seifert and Mlodzik, 2007; McNeill, 2010). In vertebrates, PCP regulates CE of axial and paraxial mesodermal cells during axis elongation, and defects in PCP signaling result in a shortened and widened A-P axis (Heisenberg et al., 2000). However, mouse embryos exhibited CE defects in the developing notochord (supplementary material Fig. S1E-H) (Kispert et al., 1996). CE movements are characterized by polarized cell behavior as cells converge along one axis, intercalate and finally extend along the axis perpendicular to the initial cell movements (Keller, 2002). In mammals, PCP signaling is essential for many morphological processes, including closure of the neural tube, inner ear hair cell polarity, left-right asymmetry and elongation of the A-P axis (Kim et al., 2001; Curtis et al., 2003; Montcouquiol et al., 2003; Wang et al., 2006; Ybot-Gonzalez et al., 2007; Borovina et al., 2010; Hashimoto et al., 2010; Song et al., 2010; Gao et al., 2011). Mutations in human core PCP components cause congenital neural tube defects (NTDs) such as spina bifida (Kibar et al., 2007; Lei et al., 2010). However, despite the importance of PCP signaling, the mechanism underlying Wnt regulation of PCP signaling is poorly understood.

Wnt5a and Wnt11 have been shown to regulate vertebrate PCP signaling. Loss of Wnt5a results in a severe shortening of the A-P axis and limb truncations (Yamaguchi et al., 1999a). Wnt5a regulates PCP establishment by inducing phosphorylation of Vangl2, a core PCP protein (Gao et al., 2011). In Xenopus embryos, expression of a dominant-negative Wnt5a results in NTDs and CE defects (Tada and Smith, 2000) and the zebrafish wnt11 mutant silberblick exhibits CE defects in the developing notochord that result in a shortened A-P axis (Heisenberg et al., 2000). However, mouse Wnt11−/− embryos do not show PCP defects, in contrast to the silberblick mutant (Majumdar et al., 2003), raising the question of whether Wnt11 regulates PCP signaling in mammals.

Here we show that, upon loss of both Wnt5a and Wnt11, the phenotype of Wnt5a−/− embryos is exacerbated as the A-P axis is further shortened, indicating functional redundancy of these two signaling molecules during axis formation in the murine embryo. We further show that Wnt5a and Wnt11 regulate CE, EMT and cell migration, disruption of which results in defects in notochord formation and in patterning of the neural tube and somites.

RESULTS

Wnt5a and Wnt11 are required for PCP during CE of notochord cells

wnt11 is required to regulate axis elongation through PCP in zebrafish (Heisenberg et al., 2000). The lack of similar defects in mouse wnt11 mutants (Majumdar et al., 2003) suggests that Wnt11 might play redundant roles with Wnt5a during mouse gastrulation. We first examined the expression of Wnt5a and Wnt11 in early mouse embryos and confirmed that Wnt5a is expressed in a caudal-to-rostral gradient in the PS (supplementary material Fig. S1A-D) (Yamaguchi et al., 1999a), whereas Wnt11 expression is more restricted (supplementary material Fig. S1E-H) (Kispert et al., 1996). Wnt11 was expressed in the PNC and in the forming heart, as previously reported (Kispert et al., 1996). To investigate a possible redundancy between Wnt5a and Wnt11 during early embryonic development, we generated Wnt5a; Wnt11 double-mutant mouse embryos. Wnt5a−/−; Wnt11−/− embryos were found at the expected Mendelian ratio between E8.5 and E10.5 (Fig. 1A-L), and died between E10.5 and E11.5. The phenotype of Wnt5a−/−; Wnt11−/− embryos became apparent at ~E8.5 (Fig. 1A-D) and was much more severe than that of the Wnt5a single mutant, as the A-P axis was further shortened (Fig. 1G,H,K,L). Therefore, Wnt11 plays redundant roles with Wnt5a in regulating early mouse embryonic development. There was no difference between Wnt5a−/−; Wnt11−/− and Wnt5a−/− embryos in terms of morphology and marker gene expression (data not shown).

A-P axis elongation is driven by PCP-mediated CE movements within the notochord and paraxial mesoderm. Defects in PCP signaling result in a shortened and widened A-P axis (Ybot-Gonzalez et al., 2007; Song et al., 2010). To test whether similar defects were caused by loss of Wnt5a and Wnt11, we examined the length/width ratio at E8.5. Wnt5a−/−; Wnt11−/− embryos displayed a decrease in the length/width ratio, which was enhanced in Wnt5a−/−; Wnt11−/− mutants (supplementary material Fig. S2A-D). Next, we investigated the expression of Shh, which marks the notochord and floor plate at E9.5 (Echelard et al., 1993), to assess notochord formation (Fig. 1M-P). Surprisingly, the notochord was not only reduced in length but also in width in Wnt5a−/−; Wnt11−/− embryos (Fig. 1M-P). To further understand the observed notochord malformation, we investigated expression of the transcription factor brachyury (T), a marker of the nascent mesoderm and the notochord (Wilson et al., 1995). At E8.0, we found a sparse and irregular T expression pattern, and fewer cells expressed T in Wnt5a−/−; Wnt11−/− than in control embryos (Fig. 2A,B).

To test whether the reduction in notochord length in Wnt5a−/−; Wnt11−/− embryos was due to defective CE, we performed in vitro Dil labeling experiments to trace cells of the PNC. After 12 h of in vitro culture, most labeled PNC cells had migrated anteriorly along the parallel axis. Additionally, the notochord cells were elongated, with irregular morphology (Fig. 2C,C′). The notochord cells exhibited asymmetric localization of the core PCP protein Vangl1, and the cells were less intercalated to form two adjacent rows of notochord cells. This is typical of highly conserved CE movements. The PCP pathway has been shown to be instructive for the correct alignment of notochord cells during CE and is regulated by Wnt5a (Qian et al., 2007; Ybot-Gonzalez et al., 2007; Gao et al., 2011). Therefore, we tested whether notochord cells exhibit asymmetric localization of the core PCP protein Vangl1, as the asymmetric localization of core PCP proteins is a hallmark of PCP signaling (Klein and Mlodzik, 2005; Song et al., 2010). In control embryos, in many notochord cells, as marked by T expression, Vangl1 was localized asymmetrically with an increase in cell membranes perpendicular to the A-P axis as compared with the parallel axis. Additionally, the notochord cells were elongated mediolaterally (Fig. 2E,E′). By contrast, in Wnt5a−/−; Wnt11−/− embryos, Vangl1 protein levels were much reduced. Very few cells showed asymmetrically localized Vangl1 and the cells were less elongated, with irregular morphology (Fig. 2F,F′). These results

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indicate that PCP signaling, and hence CE regulated by PCP, is reduced in the absence of Wnt5a and Wnt11.

The early heart tube of the Wnt5a−/−; Wnt11−/− embryo failed to undergo normal rightward looping and remained as a linear tube at E9.5 (Fig. 3A). This malformation may lead to embryonic lethality and confirmed the findings of a recent study (Cohen et al., 2012). Nkx2.5, a homeobox transcriptional regulator that is essential for heart looping (Lyons et al., 1995), was expressed throughout the myocardial layer of the heart tube in control embryos at E9.5 (Fig. 3A). Normal Nkx2.5 expression was maintained in the heart tube of Wnt5a−/−; Wnt11−/− embryos at E9.5 (Fig. 3C), raising the question of whether laterality determination is affected by loss of Wnt5a and Wnt11. We have previously shown that PCP breaks bilateral symmetry by positioning cilia to the posterior side of the PNC cells (Nonaka et al., 2005; Song et al., 2010), and in Xenopus embryos knocking down Wnt11b results in left-right defects.
Somitogenesis depends on proper Wnt5a and Wnt11 signaling

Apart from a shortened A-P axis, we also observed smaller and irregular somites in Wnt5a−/−; Wnt11−/− embryos, indicating defects in somite formation. In addition, the A-P axis reduction in Wnt5a−/−; Wnt11−/− embryos was most severe in posterior regions, where cells of the PS and later the tailbud give rise to the paraxial mesoderm, the precursor of the somites (Tam and Beddington, 1987; Tam et al., 2000). To understand the defects in somitogenesis in the absence of Wnt5a and Wnt11, we first investigated the expression of Wnt3a and Fgf8, which are essential for the formation of paraxial mesoderm (Takada et al., 1994; Crossley and Martin, 1995). The expression levels of Wnt3a and Fgf8 were similar in all investigated specimens (Fig. 3D-I). Furthermore, Wnt/β-catenin signaling indicated by TOPGAL (DasGupta and Fuchs, 1999) and Fgf signaling indicated by Spry4 expression (Minovoda et al., 1999) were normal in Wnt5a−/−; Wnt11−/− embryos (supplementary material Fig. S6A-F). However, the expression domains of Wnt3a and Fgf8 were significantly shortened along the A-P axis in Wnt5a−/−; Wnt11−/− as compared with control and Wnt5a−/−; Wnt111+− embryos (Fig. 3D-I).

This shortening might result from a reduction in paraxial mesoderm formation and/or defects in mesodermal cell migration. Mesodermal movements are controlled by T, which is expressed in the PS (Wilson et al., 1995). In the absence of Wnt5a and Wnt11, we observed a reduction of T expression as compared with the Wnt5−/−1−/− mutant, indicating defects in mesoderm formation and migration (Fig. 3J-L). To investigate paraxial mesoderm formation we compared the expression of Tbx6 at E8.5 and E9.5 (Fig. 3M-R). Wnt5a−/−; Wnt11−/− embryos were significantly shorter along the A-P axis in Wnt5a−/−; Wnt11−/− as compared with control and Wnt5a−/−; Wnt111+− embryos (Fig. 3D-I).

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Our analysis of notochord formation and Shh expression in the Wnt5a−/−; Wnt11−/− embryos suggests that the reduction in notochord size resulted in a smaller floor plate, as Shh expressed in the notochord induces floor plate formation (Echelard et al., 1993; Placzek et al., 1993). Since Shh secreted from the notochord and the floor plate is required for repression of dorsal and induction of ventral cell fates in the neural tube (NT) (Echelard et al., 1993; Chiang et al., 1996), we tested whether the reduction of Shh expression in Wnt5a−/−; Wnt11−/− embryos resulted in a dorsalized NT. Indeed, expression of Pax6, which is a dorsal NT marker repressed by Shh (Ericson et al., 1997), was expanded ventrally in the Wnt5a−/−; Wnt11−/− embryos (supplementary material Fig. S5A-C). Conversely, expression of the ventral markers Nkx2.2, Olig2 and Nkx6.1, which depend on Shh induction (Chiang et al., 1996), was reduced in the Wnt5a−/−; Wnt11−/− mutants (supplementary material Fig. S5D-L). To exclude the possibility that the reduction in ventral cells was the result of a reduced number of cells in the NT, we quantified the ratio of labeled to unlabeled NT cells and were able to confirm a dorsalized NT in the Wnt5a−/−; Wnt11−/− embryos (supplementary material Fig. S5M).
Wnt5a and Wnt11 promote cell proliferation and survival independently of Wnt3a and Fgf signaling in the paraxial mesoderm.

In Wnt5a−/−; Wnt11−/− embryos the somites were not only smaller but also irregular, suggesting that, apart from the defects in cell proliferation and survival, somite patterning was also disrupted. To test this, we examined the expression of Uncx4.1 (also known as Uncx), which marks the posterior compartment of mature somites (Mansouri et al., 1997). Uncx4.1 was only expressed in the caudal part of somites in control embryos (Fig. 4A). However, Uncx4.1 expression became continuous and sometimes asymmetric on the left and right side in the newly formed somites of Wnt5a−/−; Wnt11−/− embryos (n=3/6; Fig. 4C). These results suggest that the periodic and synchronized segmentation of the paraxial mesoderm and the A-P polarity of the somite were disrupted in the absence of Wnt5a and Wnt11.

As Notch signaling plays fundamental roles in the formation and patterning of somites (Conlon et al., 1995; Kageyama et al., 2007), we examined Notch signaling in Wnt5a−/−; Wnt11−/− embryos. Mesp2 acts downstream of Notch signaling to specify the rostral compartments of somites and its expression is restricted to the future rostral somitic half (Saga et al., 1997). Mesp2 expression was greatly reduced in Wnt5a−/−; Wnt11−/− but not in Wnt5a−/−; Wnt11−/− embryos (Fig. 4D-F). In addition, expression of Hes7 and Lfng, which are Notch signaling effectors (Bessho et al., 2001; Dale et al., 2003), was also significantly diminished in Wnt5a−/−; Wnt11−/− embryos (Fig. 4G-L), indicating that Notch signaling activity was reduced in the absence of Wnt5a and Wnt11. The expression of Notch1 and Notch2 was greatly reduced in the paraxial mesoderm of Wnt5a−/−; Wnt11−/− embryos (Fig. 4M-R). However, the expression levels of the Notch ligandsDll1 and DII3 were normal in the paraxial mesoderm of Wnt5a−/−; Wnt11−/− embryos (Bettenhausen et al., 1995; Dunwoodie et al., 1997) (Fig. 4S-X). These results suggest that the reduced Notch signaling activity is likely to be due to diminished expression of Notch1 and Notch2 in the Wnt5a−/−; Wnt11−/− embryos.

Consistent with the asymmetric expression pattern of Uncx4.1 (Fig. 4C), we observed lateral asymmetric expression patterns of Mesp2 and Lfng in Wnt5a−/−; Wnt11−/− embryos (Fig. 4F,L), indicating defects in somite synchronization. As retinoic acid (RA) signaling is required for the bilateral symmetry of somite formation and establishment of the determination front (Vernot et al., 2005; Vernot and Pourquie, 2005; Sirbu and Duester, 2006), we examined the expression of Raldh2 (Aldh1a2), the RA biosynthetic enzyme, and of Cyp26b1, the RA degrading enzyme (Zhao et al., 1996; White et al., 2000). Raldh2 was expressed in the segmented region of the control embryos and its expression was reduced in the somites of Wnt5a−/−; Wnt11−/− embryos at E9.5 (supplementary material Fig. S8A-C), whereas Cyp26b1 expression in the tail bud remained normal (supplementary material Fig. S8D-F). Taken together, these results indicate that significantly reduced Notch and RA signaling inhibit proper somite formation in Wnt5a−/−; Wnt11−/− embryos.

**Wnt5a and Wnt11 regulate EMT during late gastrula stages**

The thinner notochord and smaller somites in Wnt5a−/−; Wnt11−/− embryos prompted us to further investigate the molecular and cellular mechanisms underlying the defects in notochord and somite formation, focusing on posterior mesoderm formation and migration during gastrulation. Interestingly, we noticed an ectopic accumulation of cells in the Wnt5a−/−; Wnt11−/− embryos at the posterior of the PNC at E8.5 (Fig. 5A-B′), suggesting that Wnt5a and Wnt11 are additionally required for the specification and/or migration of mesodermal cells. We speculated that this ectopic cell
mass might be a result of accumulated precursors of midline and paraxial mesodermal cells failing to migrate to their proper destination. Therefore, impaired notochord and somite formation might be caused by a lack of normal motile precursor cells. To test this hypothesis, we characterized the accumulated cells in detail.

Cell labeling and genetic lineage-tracing experiments have revealed that NPCs are derived from relatively quiescent PNC cells (Sulik et al., 1994; Yamanaka et al., 2007; Ukita et al., 2009). In addition, the source of the axial and paraxial mesoderm precursors is believed to reside at the NSB (Sulik et al., 1994; Bellomo et al., 2010; Ukita et al., 2009). In our hypothesis, we characterized the accumulated cells in detail.

Tbx6 and Wnt5a expression was only observed in the dorsal part of the ectopic cell accumulation (arrow in F). The ventral border of the ectopic cell accumulation is indicated by a dotted line (F). Boxes in A–D indicate the areas shown at higher magnification in A′–D′, respectively.

As mesoderm generation from the ectodermal epiblast requires EMT (Shook and Keller, 2003) and cell accumulations in the PS have been reported in mouse mutants with defective EMT during gastrulation (Arnold et al., 2008), we hypothesized that incomplete EMT might be a cellular mechanism underlying the reduced mesoderm formation in Wnt5a−/−; Wnt11−/− embryos. To test this, we first examined the expression of Snail (also known as Snai1), a transcription factor that regulates the induction of EMT (Barrallo-Gimeno and Nieto, 2005). However, Snail expression was similar in Wnt5a−/−; Wnt11−/− and control embryos (supplementary material Fig. S9E,F), suggesting that initiation of EMT occurred normally. Next, we tested whether EMT takes place in late gastrulating embryos by examining the integrity of the basement membrane and the expression of epithelial and mesenchymal cell markers (Hay and Zuk, 1995; Smyth et al., 1999; Mendez et al., 2010). In the region posterior to the PNC in wild-type embryos at E7.75-E8.25, the basement membrane had broken down, as indicated by fragmented laminin staining (supplementary material Fig. S10A-F). Intact basement membrane was observed anterior to the PNC (supplementary material Fig. S9C,D). Therefore, we concluded that the loss of Wnt5a and Wnt11 led to reduced mesoderm formation.
active EMT occurs posterior to the PNC in wild-type embryos as a continuous process from mid to late gastrula stages, and thus NSB and anterior PS cells both undergo EMT in late gastrulation. However, in Wnt5a−/−; Wnt11−/− embryos, cells within the ectopic accumulation expressed E-cadherin ectopically (Fig. 6C-D″), while vimentin expression was reduced (Fig. 6E,F). These cells also expressed T at similar levels to that in notochord cells, indicating that these cells were early progenitors of the notochord (Fig. 6D,D″, arrows). These findings indicate that EMT in this region generates notochord progenitor cells and that, in the absence of Wnt5a and Wnt11, even though EMT was induced and the cells were on their way to becoming mesoderm, they failed to fully acquire mesenchymal character. Increased cell-cell adhesion due to persistent E-cadherin expression in these cells prevents them from migration. In support of this, we found that ZO-2 (Tjp2) expression, which marks tight junctions in epithelial and endothelial cells, was increased in the ectopically accumulated cells, although normal expression was maintained in the endodermal layer in the absence of Wnt5a and Wnt11 (Fig. 6G,H).

Proper cell migration not only requires downregulation of E-cadherin but also a functional extracellular matrix (ECM). After cell fate determination and germ layer formation, different germ layers are separated by ECM containing fibronectin (FN; also known as Fn1), which plays important roles in cell adhesion and migration (Pankov and Yamada, 2002). Deposition of FN has been linked to CE movements and PCP (Goto et al., 2005). In zebrafish, Wnt11 together induce more robust p38 phosphorylation. To further investigate this interaction, we tested whether JNK signaling, which has been identified as a target of Wnt5a and Wnt11 signaling, is involved in the activation of p38 MAPK (Pandur et al., 2007). In order to establish whether Wnt11 also activates p38 and if Wnt5a and Wnt11 act synergistically in this process, we treated primary mouse embryonic fibroblast (MEF) cells with recombinant Wnt5a and/or Wnt11 protein (Fig. 7C,D). We found that Wnt5a can induce p38 phosphorylation and that Wnt5a and Wnt11 together induce more robust p38 phosphorylation.
employed the RhoA inhibitor Rhosin (Shang et al., 2012). Inhibition of RhoA by Rhosin prevented Wnt5a/Wnt11-mediated p38 phosphorylation in MEF cells (Fig. 7H,I). We therefore concluded that Wnt5a and Wnt11 act synergistically to enhance p38 phosphorylation and that RhoA signaling can mediate this activity.

Finally, we examined whether Wnt5a and Wnt11 regulate EMT through PCP signaling, as both EMT and PCP pathways regulate cytoskeletal reorganization and play important roles in cell migration. We analyzed Vangl1−/−; Vangl2−/− embryos and were unable to detect a similar accumulation of cells posterior to the notochord as we observed in Wnt5a−/−; Wnt11−/− embryos (supplementary material Fig. S11A,B). However, there was a slight increase in E-cadherin in this region, although the increase in p38 phosphorylation was less profound than that in the Wnt5a−/−; Wnt11−/− embryos (supplementary material Fig. S11A,B). However, there was a slight increase in E-cadherin in this region, although the increase in p38 phosphorylation was less profound than that in the Wnt5a−/−; Wnt11−/− embryos. Inhibition of RhoA by Rhosin prevented Wnt5a/Wnt11-mediated changes in phosphorylation levels induced by the addition of Wnt5a and Wnt11 (D,E). (F,G) Activation of RhoA is induced by Wnt11 (F). aRhoA, activated RhoA; p38 phosphorylation and c-Jun phosphorylation are induced by recombinant Wnt5a and Wnt11 protein. Quantifications of three experiments reveal significant differences in RhoA activation levels.

**DISCUSSION**

Here we have found that Wnt5a and Wnt11 play redundant roles in regulating multiple developmental processes in the late gastrulating embryo. Although Wnt11−/− embryos do not have obvious developmental defects, the phenotypes of the early Wnt5a−/−− embryos were significantly enhanced by loss of Wnt11. Wnt11 is specifically expressed in the PNC (Kispert et al., 1996) and this expression accounts for its redundant role with Wnt5a in early embryonic development, as shown here. This explains why the defects observed are restricted to the notochord and the paraxial mesoderm that ingress in this region. Wnt5a and Wnt3a are both expressed strongly in the PS (Takada et al., 1994; Yamaguchi et al., 1999a). In Wnt5a−/−− embryos, the canonical Wnt signaling activity is largely normal, as judged by TOPGAL activity (DasGupta and Fuchs, 1999), confirming that both Wnt5a and Wnt11 act through β-catenin-independent pathways in early mouse embryos.

During the formation of the trunk notochord at E8.5, cell proliferation and cell death appeared normal in Wnt5a−/−−; Wnt11−/−− embryos (supplementary material Fig. S7). However, at later stages we observed an increase in cell death and reduced proliferation. This reduction in cell survival might be secondary to the heart defects observed and the reduction in Shh expression due to earlier defects in notochord formation. In the early
embryos, the PCP pathway controls CE that is required for notochord formation (Ybôt-Gonzalez et al., 2007). Both \( \text{wnt}5a \) and \( \text{wnt}11 \) regulate CE in zebrafish (Rauch et al., 1997; Heisenberg et al., 2000), but in mammalian embryos the role of \( \text{Wnt}11 \) in CE had not been demonstrated. We found in this study that the \( \text{Wnt}11 \) function can be mostly fulfilled by \( \text{Wnt}5a \) and that \( \text{Wnt}5a \) and \( \text{Wnt}11 \) both regulate CE of the trunk notochord cells and paraxial mesodermal cells through PCP (Fig. 2). However, in \( \text{Wnt}5a^{-/-}\); \( \text{Wnt}11^{-/-} \) embryos, asymmetrical localization of Vangl1 was still observed in some notochord cells and NT closure was normal, suggesting that other Wnt ligands present in early embryos (Yamaguchi, 2008) can also regulate PCP. Consistent with this, we did not detect any defects in neurulation, which requires PCP function. We also observed normal asymmetrical localization of Vangl1 in the ciliated PNC cells and low penetrance of left-right asymmetry defects in the \( \text{Wnt}5a^{-/-}\); \( \text{Wnt}11^{-/-} \) embryos.

CE defects in PCP mutants result in shortened and broadened notochord and floor plate (Greene et al., 1998; Ybôt-Gonzalez et al., 2007). However, in \( \text{Wnt}5a^{-/-}\); \( \text{Wnt}11^{-/-} \) embryos, the notochord is shortened, but thinner, resulting in reduced Shh expression and hence dorsalization of the NT and somites. We found that this peculiar phenotype is caused by reduced migration and generation of NPCs from the PNC. The source of trunk and tail notochord progenitors is believed to reside in the border region between the posterior PNC and the anterior PS (Cambray and Wilson, 2007). Since the PNC (node)/streak border region is believed to contain a population of stem cells, it is also possible that both trunk and tail notochord formation are dependent on proper migration of the descendants of these stem cells, which is regulated by \( \text{Wnt}5a \) and \( \text{Wnt}11 \) (Cambray and Wilson, 2007). Thus, the thinner notochord is likely to be caused by defects in two processes regulated by \( \text{Wnt}5a \) and \( \text{Wnt}11 \). First, as PCP is required to provide directionality to migrating cells, disruption of PCP due to loss of \( \text{Wnt}5a \) and \( \text{Wnt}11 \) leads to reduced directed anterior and posterior migration of NPCs, such that there are fewer cells to form trunk and tail notochord. Second, as the PNC is organized as an epithelium and the migrating cells display mesenchymal properties, this process involves EMT. Indeed, in the PNC (node)/streak border region, we have observed breakdown of the basement membrane and a reduction of epithelial markers (supplementary material Fig. S10). An increase in cell adhesion molecules in the NPCs of \( \text{Wnt}5a^{-/-}\); \( \text{Wnt}11^{-/-} \) embryos indicates that some of the precursors of the notochord are unable to fully adopt mesenchymal cell properties, resulting in fewer cells available for trunk and tail notochord formation. A similar EMT process has recently been shown to control the generation of limb mesenchyme cells during limb bud formation (Gros and Tabin, 2014).

Wnt/β-catenin signaling is a potent inducer of EMT in early gastrulation and it suppresses E-cadherin expression. Interestingly, blocking Wnt/β-catenin signaling by removing β-catenin in \( \text{Noto} \)-expressing cells led to persistent strong E-cadherin expression in NPCs and reduction of notochord elongation (Ukita et al., 2009), suggesting that Wnt/β-catenin signaling also regulates EMT in the generation of NPCs at late gastrulation stages. Consistent with this, it has been reported that \( \text{Wnt}5a \) controls paraxial mesoderm formation and Tbx6 expression (Nowotschin et al., 2012). The data in that report reveal that cells modulate the expression of \( \text{Wnt}5a \) as they traverse the PS: as they enter they upregulate \( \text{Wnt}3a \), and then subsequently downregulate \( \text{Wnt}5a \) as they exit. The study suggests that reduced tail bud formation in \( \text{Wnt}5a^{-/-} \) mutants might be caused by the first steps of late paraxial mesoderm formation, such as ingresson/EMT (Nowotschin et al., 2012). Compared with Wnt/β-catenin signaling, \( \text{Wnt}5a \) and \( \text{Wnt}11 \) are weak inducers of EMT. EMT occurred at early gastrulation stages in \( \text{Wnt}5a^{-/-}\); \( \text{Wnt}11^{-/-} \) embryos and the phenotypes were only manifested at later stages, possibly due to progressive accumulation of a weak EMT defect.

In addition to reduced migration of NPCs, we propose that reduced A-P axis elongation is also caused by reduced paraxial mesoderm formation in the absence of \( \text{Wnt}5a \) and \( \text{Wnt}11 \) (Fig. 5). In the PS of the late gastrula embryo there is continuous EMT (Arnold and Robertson, 2009). Cells ingress and then delaminate from the epiblast ectoderm to form a loose mesenchyme that will later form the paraxial mesoderm. In this process, the ingressing cells turn off the \( \text{Sox}2 \) enhancer N1, whereas \( \text{Tbx}6 \) expression is upregulated (Takemoto et al., 2011). Once formed, the mesoderm is then separated from the neural ectoderm by a layer of FN-containing basement membrane. In \( \text{Wnt}5a^{-/-}\); \( \text{Wnt}11^{-/-} \) embryos, reduced T expression and expanded \( \text{Sox}2 \) expression suggest that reduced paraxial mesoderm formation might also be caused by incomplete EMT. FN deposition requires dynamic cell rearrangements and the increase in cell adhesion that we observed in this region might thus be responsible for the lack of FN (Dzamba et al., 2009). The defective EMT therefore led secondarily to incomplete formation of the FN-containing basement membrane, reduced axial mesoderm migration causing a thinner notochord, and reduced paraxial mesoderm migration causing the formation of smaller and irregular somites. Therefore, in this study we have identified that \( \text{Wnt}5a \) and \( \text{Wnt}11 \) are required to control EMT in the late gastrula embryo.

Our results suggest that \( \text{Wnt}5a \) and \( \text{Wnt}11 \) regulate EMT through RhoA-mediated activation of p38. However, we cannot exclude the possibility that other pathways downstream of \( \text{Wnt}5a \) and \( \text{Wnt}11 \) might also be involved in regulating EMT, as \( \text{Wnt}5a \) and \( \text{Wnt}11 \) can stimulate multiple non-canonical Wnt pathways (reviewed by Yang, 2012). We show that JNK signaling is activated by the addition of \( \text{Wnt}5a \) and \( \text{Wnt}11 \), but it is not clear whether JNK activation is required for p38 activation. As JNK and p38 signaling pathways often act in parallel (Widenmaier et al., 2009; Das et al., 2011; Chen et al., 2013; Tanaka et al., 2013) and JNK signaling has been shown to be involved in the induction of EMT in various tissues (Pallet et al., 2012; Chen et al., 2013), both p38 and JNK might regulate EMT during embryonic A-P axis elongation.

As Wnt/β-catenin signaling is required for T expression and T regulates \( \text{Tbx}6 \) expression (Yamaguchi et al., 1999b), the reduced T expression dorsally may cause ventrally extended \( \text{Sox}2 \) expression, which is normally suppressed by \( \text{Tbx}6 \). One scenario is that \( \text{Wnt}5a \) and \( \text{Wnt}11 \) can signal weakly through the canonical Wnt pathway. However, the strong \( \text{Wnt}5a \) expression and normal TOPGAL activity in \( \text{Wnt}5a^{-/-}\); \( \text{Wnt}11^{-/-} \) embryos (Fig. 3F; supplementary material Fig. S6F) suggest that \( \text{Wnt}5a \) and \( \text{Wnt}11 \) might modulate T and \( \text{Tbx}6 \) expression independently of the canonical Wnt pathway. Further experiments are required to clarify this point.

**MATERIALS AND METHODS**

**Mouse lines and genotyping**

\( \text{Wnt}5a \), \( \text{Wnt}11 \), \( \text{Vangl1} \) and \( \text{Vangl2} \) mutant mouse strains and associated genotyping methods have been described previously (Yamaguchi et al., 1999a; Majumdar et al., 2003; Song et al., 2010). Animal care and experimental animal procedures were performed in accordance with the NHGRI institutional standards and were approved by the Institutional Animal Care and Use Committee, NHGRI, and by the NIH Institutional Review Board.
\textbf{In situ hybridization and immunofluorescence staining}

Whole-mount \textit{in situ} hybridization was performed according to standard protocols (Topol et al., 2003).

RNA probes have been described previously: Shh (Echelard et al., 1993), T (Wilson et al., 1995), Nx2.5 (Lyons et al., 1995), Wnt3a (Takada et al., 1994), Wnt5a (Yamaguchi et al., 1999a), Wnt11 (Kispert et al., 1996), Fgf8 (Crossey and Martin, 1995), Tbx6 (Chapman and Papaioannou, 1998), Uncx4.1 (Mansouri et al., 1997), Mesp2 (Saga et al., 1997), Hes7 (Bessho et al., 2001), Lfng (Dale et al., 2003), Noto (Abdelkhalak et al., 2004), Notch1 (Conlon et al., 1995), Notch2 (Bessho et al., 2001), Dll1 (Bettenhausen et al., 1995) and Dll3 (Dunwoodie et al., 1997). For immunofluorescence staining, embryos were fixed in 4\% paraformaldehyde for 30 min at 4°C and processed for cryosectioning and immunostaining according to standard protocols (Song et al., 2010). Unless indicated otherwise, each result presented was observed in at least two individual specimens with a penetrance of 100\%.

Primary antibodies used: brachyury (1:150; R&D Systems, AF2085), E-cadherin (1:200; R&D Systems, AF748), fibronectin (1:500; gift from K. Yamada, Bethesda, MD, USA), Sox17 (1:50; R&D Systems, AF1924), Sox2 (1:500; Abcam, ab97959), Vangl1 [1:50 (Song et al., 2010)], ZO-2 (1:100; Cell Signaling, 2847), β-catenin (1:500; BD Biosciences, 610153), phospho-p38 (1:200; Cell Signaling, #9211), Pax6 (1:50), Pax7 (1:50), Nkx2.2 (1:100), Islet1 (1:50), Lim1 (1:50), Nkx6.1 (1:50) (Developmental Studies Hybridoma Bank), phospho-histone H3 (1:100; Cell Signaling, 9071) and Olig2 (1:100; Abcam, ab33427). Secondary antibodies were obtained from Life Technologies and used at 1:400.

Apoptosis was analyzed using the ApopTag Apoptosis Kit (S7111, Millipore). Images were acquired using a Zeiss LSM 510 NLO Meta system (Carl Zeiss). Images were obtained from Life Technologies and used at 1:400.

\textbf{In vitro labeling}

Embryos were dissected in DMEM containing 10\% FBS and 25\% HEPES pH 7.4. Dil (Sigma) was injected at 0.2 mg/ml in 3 M sucrose using a glass micropipette into cells of the PNC. Embryos were subsequently cultured for 12 h at 37°C, 5\% CO2 in a roller culture (Rotator Genie, Scientific Industries) in 25\% rat serum and 75\% culture medium (DMEM 500 nM-15 µM SP600125 (Sigma). RhoA activity was determined using a RhoA Activity Kit (New East Biosciences, 80601). RhoA activity was inhibited by incubating the cells for 2 h in 1 µM Rhosin (EMD Millipore). JNK activity was blocked using 500 nM-15 µM SP600125 (Sigma).

\textbf{Research Article}


References


Das, J., Ghosh, J., Mann, P. and Sill, P. C. (2011). Taurine suppresses doxorubicin-triggered oxidative stress and cardiac apoptosis in rat via up-


**Fig. S1. Expression of Wnt5a and Wnt11.** Whole mount *in situ* hybridization showing expression of Wnt5a (A-D) and Wnt11 (E-F) at indicated stages. Wnt5a is expressed at the posterior part of the embryo and forms a gradient towards the anterior (A-D). Expression of Wnt11 is observed in the posterior region, allantois and the PNC (E-H). LS, Late Streak; LB, Late Bud; EHF, Early Headfold; LHF, Late Headfold.

**Fig. S2. Reduced length/width ratio in the Wnt5a−/−; Wnt11−/− embryos.** Length (A, B, C) and width (A’, B’, C’) measurements shown by red line in embryos of indicated genotypes at the 6-Somite stage. (D) Reduced length/width ratio in the Wnt5a−/−; Wnt11−/− embryos (n=4; student’s t test p=0.04).

**Fig. S3. DiI labeling in of notochord cells.** (A-D) sections of embryos labeled with DiI and cultured for 12h combined with immunofluorescent staining of T positive cells. (A, B) Sagittal sections of embryos shown in Fig. 2C, D with DiI and T co-staining (arrows). Transversal sections of DiI labeled embryos show co-localization of T and DiI (arrows).

**Fig. S4. Normal left-right asymmetry in most of the Wnt5a−/−; Wnt11−/− embryos.** (A-C) Whole-mount *in situ* hybridization showing left sided expression of Nodal in ventral views of E8.5 control and Wnt5a−/−; Wnt11−/− embryos. Inversed expression of Nodal in one Wnt5a−/−; Wnt11−/− embryo (C, n=1/4). (D, E) Immunofluorescent staining of Vangl1 in the PNC reveals
normal asymmetric localization of Vangl1 (arrows in D, E) in control and Wnt5a\textsuperscript{−/−}; Wnt11\textsuperscript{−/−} embryos at E8.0. a, anterior; p, posterior.

Fig. S5. Dorsalized neural tube in the Wnt5a\textsuperscript{−/−}; Wnt11\textsuperscript{−/−} embryos. (A-L) Immunofluorescent staining of dorsal (Pax6; A-C) and ventral markers (Nkx2.2, Olig2, Nkx6.1; D-L) of the neural tube at E9.5 showing increased expression of the dorsal marker at the expense of ventral markers in Wnt5a\textsuperscript{−/−}; Wnt11\textsuperscript{−/−} embryos (C, F, I, L). (M) Quantifications of ratio between labeled and unlabeled cells in the NT show reduction of ventral fated cells in Wnt5a\textsuperscript{−/−}; Wnt11\textsuperscript{−/−} embryos.

Fig. S6. Normal Fgf and canonical Wnt signaling in the Wnt5a\textsuperscript{−/−}; Wnt11\textsuperscript{−/−} embryos. Whole-mount in situ hybridization of Spr4 (A-C) and X-Gal staining of β-galactosidase activity in TOPGAL mice (D-F) in lateral views of the tail bud at E9.5. Normal expression of Spr4 in the Wnt5a\textsuperscript{−/−}; Wnt11\textsuperscript{−/−} embryo (C). Normal activity level of β-galactosidase in the Wnt5a\textsuperscript{−/−}; Wnt11\textsuperscript{−/−} embryo (F).

Fig. S7. Increased apoptosis and reduced proliferation in the posterior Wnt5a\textsuperscript{−/−}; Wnt11\textsuperscript{−/−} embryos. (A-R) Immunofluorescent staining of Phospho-Histone H3 (PPH3, cell proliferation, D-F, J-L, P-R) and TUNEL staining (apoptosis A-C, G-I, M-O) at E8.5 (A-F), E9.5 (G-L) and E10.5 (M-R) showing increased cell death and reduced proliferation in Wnt5a\textsuperscript{−/−}; Wnt11\textsuperscript{−/−} embryos (C, F, I, L, O, R). Transversal sections of posterior embryos in (A-F), sagittal sections of tail bud in (G-R).
Fig. S8. Reduced retinoic acid signaling in the Wnt5a<sup>–/–</sup>; Wnt11<sup>–/–</sup> embryos. Whole mount in situ hybridization of Raldh2 (A-C), Cyp26 and Uncx4.1 (D-F) in dorsal views of the tail bud at E9.5 showing reduced expression of Raldh2 in the Wnt5a<sup>–/–</sup>; Wnt11<sup>–/–</sup> embryos (C) and normal expression of Cyp26 in the Wnt5a<sup>–/–</sup>; Wnt11<sup>–/–</sup> embryos (F). Arrows in (D-F) point at Cyp26 expression.

Fig. S9. Normal expression of Sox17 and Snail in the Wnt5a<sup>–/–</sup>; Wnt11<sup>–/–</sup> embryo. (A-D) Immunofluorescent staining of Sox17 and Phospho-Histone H3 (PPH3) in sagittal sections of posterior E8.5 embryos (A, D) shows normal endodermal fate determination and proliferation in the Wnt5a<sup>–/–</sup>; Wnt11<sup>–/–</sup> embryo (B, D). (E-F’) Whole mount in situ hybridization of Snail at E8.5 in lateral (E, F) and ventral (E’, F’) views showing normal expression in the Wnt5a<sup>–/–</sup>; Wnt11<sup>–/–</sup> embryo (F, F’).

Fig. S10. EMT occurring posterior to the PNC in WT embryos. (A-F’) Immunofluorescent staining of Laminin, Vimentin and E-cadherin in WT embryos of different stages (E7.5 – E8.25) indicates a lack of basal membrane posterior to the PNC (marked by asterisks). Note the presence of a basal membrane in the PNC and anteriorly (arrowheads), which is absent posterior to the PNC (arrows). Scale bars in (A-F’) represent 50 μm. Boxes in (A-F) indicate areas shown at higher magnification in (A’-F’), respectively.LS, Late Streak; EB, Early Bud; EHF, Early Headfold; 1S, 1-Somite; 2S, 2-Somite; 3S, 3-Somite.

Fig. S11. Lack of ectopic accumulations in posterior in Vangl1<sup>–/–</sup>; Vangl2<sup>–/–</sup> embryos. (A, B) Immunofluorescent staining of sagittal sections of posterior parts of E8.5 embryos. No ectopic
cell accumulations in $\text{Vangl1}^+/^-; \text{Vangl2}^+/^-$ embryos but slight increase of E-cadherin in the posterior region (arrow in B and B’). Boxed regions in (A, B) are shown with higher magnification in (A’, B’) respectively. (C, D) Induction of p38 phosphorylation in $\text{Vangl1}^+/^-; \text{Vangl2}^+/^-$ MEF cells. Quantification of three experiments shown in (D).
Fig. S1

A: Wnt5a, LB
B: Wnt5a, LHF
C: Wnt5a, 3 Somites
D: Wnt5a, 4 Somites
E: Wnt11, LS
F: Wnt11, EHF
G: Wnt11, 3 Somites
H: Wnt11, 7 Somites
Fig. S2

**Wnt5a**\(^{+/−}\); **Wnt11**\(^{+/−}\)

**Wnt5a**\(^{−/−}\); **Wnt11**\(^{+/−}\)

**Wnt5a**\(^{−/−}\); **Wnt11**\(^{−/−}\)

**D** length/width ratio

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* p=0.04
Fig. S3

Wnt5a^{+/-}; Wnt11^{+/-}  Wnt5a^{-/-}; Wnt11^{-/-}

A

B

C

D

T Dil

T Dil

T Dil

T Dil
Fig. S4

A  B  C  D  E

Wnt5a+/−; Wnt11+/− Nodal  Wnt5a−/−; Wnt11−/−  Wnt5a+/−; Wnt11−/− Nodal  Wnt5a+/−; Wnt11+/−  Wnt5a−/−; Wnt11−/− Vangl1  Wnt5a−/−; Wnt11+/− Vangl1
Fig. S5

Wnt5a<sup>+/−;</sup> Wnt11<sup>+/−</sup>  
A. Pax6  
B. Pax6  
C. Pax6  

Wnt5a<sup>−/−;</sup> Wnt11<sup>+/−</sup>  
D. Nkx2.2  
E. Nkx2.2  
F. Nkx2.2  

Wnt5a<sup>−/−;</sup> Wnt11<sup>−/−</sup>  
G. Olig2  
H. Olig2  
I. Olig2  

Nkx6.1  
J.  
K.  
L.  

M labeled/unlabeled cells in neural tube

*p=0.001
*p=0.0002
*p=0.03

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Fig. S6

\[ \text{Wnt5a}^{+/+}; \text{Wnt11}^{+/+} \quad \text{Wnt5a}^{+/-}; \text{Wnt11}^{+/+} \quad \text{Wnt5a}^{+/-}; \text{Wnt11}^{-/-} \]

(A) Spry4 (B) Spry4 (C) Spry4

(D) TOPGAL (E) TOPGAL (F) TOPGAL
Fig. S7

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Fig. S9  

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Fig. S11

A  Vangl1+/−; Vangl2+/−
E-cadherin Fibronectin
A’  Vangl1+/−; Vangl2+/−
E-cadherin Fibronectin

B  Vangl1+/−; Vangl2−/−
E-cadherin Fibronectin
B’  Vangl1+/−; Vangl2−/−
E-cadherin Fibronectin

C  p38 phosphorylation
Wnt5a  -  -  +  -  +
Wnt11  -  -  -  +  +
P-p38
p38
5 min

D  p38 phosphorylation levels
* p=0.03

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