

No tail co-operates with non-canonical Wnt signaling to regulate posterior body morphogenesis in zebrafish

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Accepted 6 October 2003

Development 131, 203–216
Published by The Company of Biologists 2004
doi:10.1242/dev.00915

Summary

The vertebrate posterior body is formed by a combination of the gastrulation movements that shape the head and anterior trunk and posterior specific cell behaviors. Here, we investigated whether genes that regulate cell movements during gastrulation [*no tail (ntl)/brachyury*, *knypek (kny)* and *pipetail (ppt)/wnt5*] interact to regulate posterior body morphogenesis. Both *kny;ntl* and *ppt;ntl* double mutant embryos exhibit synergistic trunk and tail shortening by early segmentation. Gene expression analysis in the compound mutants indicates that anteroposterior germ-layer patterning is largely normal and that the tail elongation defects are not due to failure to specify or maintain posterior tissues. Moreover, *ntl* interacts with *ppt* and *kny* to synergistically regulate the posterior expression of the gene encoding bone morphogenetic protein 4 (*bmp4*) but not of other known T-box genes, fibroblast growth factor genes or *caudal* genes. Examination of mitotic and apoptotic cells indicates that impaired tail elongation is not simply due to decreased cell proliferation or increased cell

death. Cell tracing in *ppt;ntl* and *kny;ntl* mutants demonstrates that the ventral derived posterior tailbud progenitors move into the tailbud. However, gastrulation-like convergence and extension movements and cell movements within the posterior tailbud are impaired. Furthermore, subduction movements of cells into the mesendoderm are reduced in *kny;ntl* and *ppt;ntl* mutants. We propose that Ntl and the non-canonical Wnt pathway components Ppt and Kny function in parallel, partially redundant pathways to regulate posterior body development. Our work initiates the genetic dissection of posterior body morphogenesis and links genes to specific tail-forming movements. Moreover, we provide genetic evidence for the notion that tail development entails a continuation of mechanisms regulating gastrulation together with mechanisms unique to the posterior body.

Key words: Convergence, Extension, Gastrulation, *knypek*, *silberblick (wnt11)*, *pipetail (wnt5)*, subduction

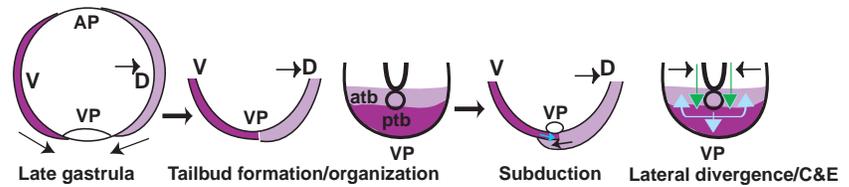
Introduction

The rostral vertebrate body, including the head and anterior trunk, is generated by a set of gastrulation movements largely conserved among vertebrates (Schoenwolf and Smith, 2000). The germ layers form through the internalization of prospective mesendodermal cells beneath the ectoderm, and epiboly enlarges their surface. Convergence and extension movements narrow the germ layers from belly to back and lengthen them rostrocaudally. Formation of the posterior body uses the mechanisms that shape the anterior body during gastrulation as well as posterior-specific behaviors (Griffith et al., 1992; Kanki and Ho, 1997; Schoenwolf, 1984). In zebrafish, chick and mouse embryos, a cell aggregate (the tailbud, composed of dorsal and ventral derived cells) contributes to the tail and posterior trunk tissues (Hamburger and Hamilton, 1992; Hogan et al., 1994; Kanki and Ho, 1997; Tam, 1984; Tam, 1986). The zebrafish tailbud forms at late gastrulation; cells originating from dorsal and ventral gastrula regions arrive at the vegetal pole and close over the ventral yolk plug (Fig. 1) (Kimmel et al., 1995; Westerfield, 1995). Within the tailbud, the cells are spatially restricted according to their gastrula origin, such that the ventrally originating cells

constitute the posterior half, whereas the dorsal derived cells occupy the anterior tailbud (Fig. 1) (Kanki and Ho, 1997). Cell fates and gene expression patterns are restricted within the tailbud of chick, frog, and zebrafish embryos, indicating that tail formation is a conserved process that is not simply mediated by the addition of cells to the posterior end (Beck and Slack, 1998; Catala et al., 1995; Gaertner, 1949; Gont et al., 1993; Hammerschmidt and Nüsslein-Volhard, 1993; Joly et al., 1992; Joly et al., 1993b; Kanki and Ho, 1997; Schoenwolf, 1977; Schulte-Merker et al., 1992; Talbot et al., 1995; Tucker, 1995). However, elevated cell proliferation in dorsal medial regions might contribute to tail extension in zebrafish (Kanki and Ho, 1997).

Tracing cell movements within the zebrafish tailbud revealed a mechanism for posterior body elongation that combines gastrulation (convergence and extension) and novel tailbud-specific movements (subduction and laterad divergence) (Fig. 1) (Kanki and Ho, 1997). Within the extending tail, dorsal convergence of paraxial cells and anteroposterior (AP) tissue extension as observed during gastrulation continues until the tail everts (Kanki and Ho, 1997). Concurrently, posterior tailbud cells move anteriorly and laterally (laterad divergence)

Fig. 1. Gastrulation movements position posterior body progenitors in the tailbud region. As gastrulation ends ventral (dark purple) and dorsal (light purple) derived marginal cell layers close over the yolk to form the tailbud. The ventral derived cells contribute to the posterior tailbud and are spatially separated from the dorsal derived anterior bud cells with Kupffer's vesicle as a morphological boundary (open circle). As tail-specific movements begin, the posterior bud cells subduct beneath the anterior bud cells. Gastrulation-like convergence and axial extension contribute to tailbud elongation; anterior tailbud cells (light green arrows) advance posteriorly and posterior tailbud cells (light blue arrows) move within the posterior flow and then laterally, avoiding the midline and anterior bud cells.



to avoid the midline (Kanki and Ho, 1997). Posterior tailbud cells undergo subduction, movement underneath the anterior tailbud cells, at the boundary between the ventral-derived posterior and dorsal-derived anterior tailbud. This type of movement is not observed in the gastrula, where the ventral and dorsal blastopore lips do not meet (Kanki and Ho, 1997).

Several genes required for tail formation have been identified and recent studies suggest that a combination of high levels of Bmp, canonical Wnt and Nodal signaling activity in the ventral region of the zebrafish gastrula specify a tail-organizer region that can induce ectopic tails upon transplantation (Agathon et al., 2003). Little, however, is known about how the diverse cell movements underlying posterior body morphogenesis are regulated. Fibroblast growth factor (Fgf) signaling is important for normal gastrulation and trunk and tail formation, upstream of Tbx genes (which encode T-box transcription factors) (Amaya et al., 1991; Amaya et al., 1993; Griffin et al., 1995; Griffin et al., 1998; Isaacs et al., 1994; Isaacs et al., 1992; Schulte-Merker and Smith, 1995). Heterozygous *brachyury* mutant mice and mutants in its zebrafish homologue, *no tail (ntl)*, exhibit tail truncations (Chesley, 1935; Halpern et al., 1993). Although homozygous mutant mice have more severe caudal mesoderm and gastrulation defects than zebrafish *ntl* mutants, this is probably due to compensation by partially redundant Tbx genes that act downstream of Fgf signaling during gastrulation and trunk development in zebrafish (Chesley, 1935; Griffin et al., 1995; Griffin et al., 1998; Halpern et al., 1993; Wilson et al., 1995). Fgf and Tbx are proposed to regulate cell adhesion during gastrulation, but it is not clear which/whether tail-specific cell movements require their function (Griffin et al., 1995; Ho and Kane, 1990; Isaacs et al., 1992; Wilson et al., 1995; Yamamoto et al., 1998).

Zebrafish Nodal-related signaling is essential for endoderm and dorsolateral mesoderm induction. Yet elimination of Nodal signaling in *cyclops;squint* compound mutants and in maternal-zygotic mutants for the Nodal cofactor *one-eyed pinhead (MZOep)*, permits relatively normal posterior mesoderm formation and has relatively little effect on tail extension (Feldman et al., 1998; Griffin and Kimelman, 2002; Schier and Shen, 2000). The role of Nodal signaling in posterior body development has been revealed by genetic interactions; *oep* cooperates with the Tbx genes *ntl* and *spadetail (spt)* to regulate posterior tissue specification (Griffin and Kimelman, 2002; Schier et al., 1997). Although Spt has also been implicated in modulating cell adhesion and motility as a positive regulator of *paraxial protocadherin* expression (Yamamoto et al., 1998), its interaction with *Zoep* affects presomitic mesoderm progenitor differentiation rather than

motility (Griffin and Kimelman, 2002). These studies suggest that several pathways coordinate tissue specification and morphogenesis within the posterior body.

Mutational analyses in zebrafish identified many genes regulating cell movements and behaviors required for normal convergence and extension during gastrulation (Hammerschmidt et al., 1996; Solnica-Krezel et al., 1996), including those that encode components of a non-canonical Wnt signaling pathway (Heisenberg et al., 2000; Kilian et al., 2003; Topczewski et al., 2001). Altered activity of non-canonical Wnt ligand genes *silberblick (slb)/wnt11* and *pipetail (ppt)/wnt5*, results in morphogenesis defects without affecting cell fates (Heisenberg et al., 2000; Makita et al., 1998; Rauch et al., 1997). Similarly, inactivation of the putative Wnt signaling modulator glypican Knypek (Kny) impairs mediolateral (ML) cell elongation underlying convergence and extension movements (Topczewski et al., 2001). Zebrafish *ppt* and *kny* but not *slb* mutant embryos exhibit shorter tails, suggesting a role for these genes in tail morphogenesis (Hammerschmidt et al., 1996; Heisenberg et al., 2000; Rauch et al., 1997; Solnica-Krezel et al., 1996; Topczewski et al., 2001).

To investigate whether non-canonical Wnt signaling regulates cell movements within the developing tail and to identify genes that it might cooperate with during this process, we generated double mutants for loss of *ntl* and non-canonical Wnt signaling components, *slb*, *ppt* and *kny*. Here, we show that *ppt;ntl* and *kny;ntl* compound mutants exhibit synergistic posterior body shortening. These defects are not due to impaired posterior mesoderm specification and patterning, nor decreased proliferation or excess apoptosis. Cell tracing during tail development reveals that both the gastrulation-like movements and tailbud unique movements are impaired in double mutants. Specifically, *ntl* interacts genetically with both *ppt* and *kny* to regulate convergence and extension movements within the posterior tailbud, and to promote normal subduction movements. We demonstrate that these genes co-operate to regulate specific cell movements during posterior body morphogenesis through a mechanism parallel to Fgf signaling, *cad1* or known Tbx genes.

Materials and methods

Zebrafish maintenance, embryo generation, staging and genotyping

The *ppt^{ta98+/-}*, *kny^{m119+/-}* and *ntl^{b195+/-}* and double mutant zebrafish strains were maintained as described previously (Solnica-Krezel et al., 1994). Embryos were obtained from natural spawnings and morphologically staged as described previously (Kimmel et al., 1995).

Embryos were genotyped using linked z markers for *ntl*^{b195}, by PCR amplification followed by restriction digest for *pp*^{ta98} (primers: forward, GATTACTGCCTGCGCAATGAAAC; reverse, GGTCTTG-AACTGGTCGTACACGCGG; followed by *Sac*II digestion, giving 29 bp and 100 bp products for the mutant allele), *slb*^{z216} (primers: forward, GTGAGGCAGCGTTTGTGGTT; reverse, CGTAGTAGCG-AAGGTTATCTCCACATT; followed by *Mse*I digestion, giving 27 bp and 141 bp products for the mutant allele) and *kny*^{m119} (primers: forward, TCCATGTTAGGTCTCGCTGA; reverse, CCTCAGGGCT-GTAGGGTCTA; followed by *Eco*RV digestion, giving a 310 bp fragment for the wild type and 230 and 80 bp fragments for the mutant allele).

In situ hybridization

Whole mount in situ hybridization was performed according to Thisse and Thisse (Thisse and Thisse, 1998). Embryos were photographed with a Zeiss Axiophot using either Axiocam digital camera or a 35 mm camera.

Cell labeling/movement analysis

Photoactivation of caged fluorescein in cells at the ventral blastoderm margin was performed as described in Myers et al. (Myers et al., 2002a), except that embryos were fixed at bud and six-somite stages. For tailbud uncaging, embryos were loaded with caged dye as described by Sepich et al. (Sepich et al., 2000) and then posterior tailbud cells were uncaged at the bud to two-somite stages and fixed at 20 somites. For cell transplantations, donor embryos were labeled with Rhodamine dextran (Molecular Probes) and transplanted to unlabeled hosts at blastula stages as described by Westerfield (Westerfield, 1996).

Cell proliferation

Embryos were fixed and stained as described previously (Topczewski et al., 2001) except that, in situ hybridization with digoxigenin-labeled

paraxial protocadherin (*papc*), detection with fast red preceded immunohistochemistry. The primary antibody was a monoclonal mouse anti-phosphohistone (Sigma) and the secondary antibody was a CY2 anti-mouse IgG (Jackson Immuno). Images were acquired using the Zeiss LSM 510 laser scanning inverted microscope.

Results

ntl interacts with *kny* and *ppt* during tail and trunk formation

In mouse and frog, interference with *Brachyury* function impairs gastrulation and results in posterior truncations (Beddington et al., 1992; Chesley, 1935; Conlon and Smith, 1999; Wilson et al., 1995). By contrast, zebrafish null *ntl* mutants lack notochord and posterior tail tissues but exhibit a milder gastrulation defect characterized by impaired convergence but normal extension (Amacher et al., 2002; Glickman et al., 2003; Griffin et al., 1995; Griffin et al., 1998; Halpern et al., 1993). Therefore, other genes might compensate for loss of *ntl* in zebrafish gastrula. Although zebrafish embryos harboring mutations in non-canonical Wnt signaling components also exhibit shortened AP axes by early segmentation stages, they form the normal number of somites (Rauch et al., 1997; Solnica-Krezel et al., 1996). To investigate whether *ntl* interacts with non-canonical Wnt signaling we constructed *ppt;ntl*, *kny;ntl* and *slb;ntl* double mutants (Fig. 2). At 26 hours post-fertilization (hpf), compared to the individual mutants (Fig. 2A-E), the *slb;ntl* (Fig. 2F) mutants exhibited only the combined deficiencies expected for loss of *ntl* (Fig. 2E) and *slb* (Fig. 2B) function: they lacked differentiated notochord and displayed tail truncations and variable

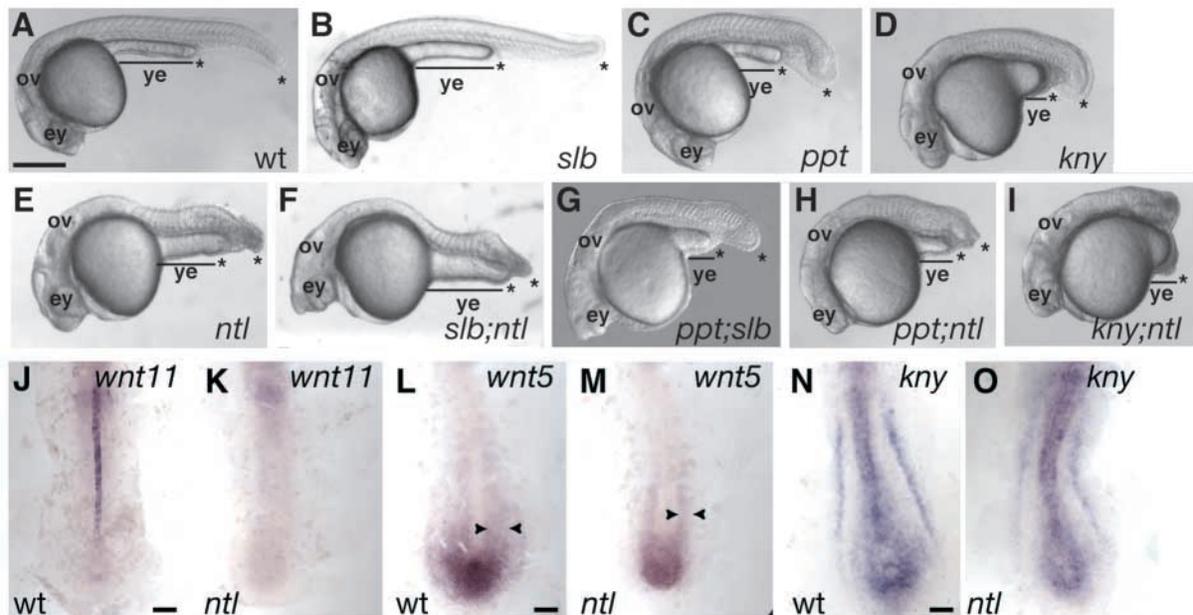


Fig. 2. The *ntl* gene interacts with *kny* and *ppt*, but not *slb*. At 26 hpf, the tail extends beyond the yolk extension (ye) in wild-type embryos (A). Embryos with the *slb* mutation (B) have normal tails, whereas *ppt* (C), *kny* (D) and *ntl* (E) embryos have shorter tails (*). Embryos with the *slb;ntl* double mutation (F) have cyclopic eyes (ey) and tail defects like individual mutants, and *slb;ppt* (G) double mutants are shorter than individual mutants. The tail does not extend beyond the yolk tube in *ppt;ntl* (H) and *kny;ntl* (I) double-mutant embryos. At the ten-somite stage, *slb/wnt11* is expressed in the notochord of wild-type (J) embryos but is absent in *ntl* mutants (K). Posterior *ppt/wnt5* expression is mediolaterally broader in wild-type (L) but persists in *ntl* (M) embryos, and *kny* expression is comparable between the wild type (N) and *ntl* mutants (O). (A-I) Lateral views. (J-O) Dorsal posterior views. Scale bar=50 μ m (A-I) and 100 μ m (J-O).

synophthalmia and cyclopia (Halpern et al., 1993; Heisenberg and Nusslein-Volhard, 1997). By contrast, *ppt;ntl* (Fig. 2H) and *kny;ntl* (Fig. 2I) double mutants showed a synergistic phenotype, characterized by a dramatically shortened axis and yolk extension, such that only head and anterior trunk are clearly distinguishable. The additive *slb;ntl* phenotype was not surprising, because posterior *wnt11* expression has previously been shown to require *ntl* (Fig. 2J,K) (Makita et al., 1998). By contrast, compared to the wild type (Fig. 2L), tailbud and lateral aspects of *wnt5* expression were only slightly reduced in *ntl* mutants (Fig. 2M), indicating that posterior *wnt5* expression does not absolutely require *ntl*. Similarly, compared with the wild type (Fig. 2N), *kny* tailbud expression was mildly reduced in *ntl* (Fig. 2O) mutants, indicating that it does not entirely depend on *ntl* function. The persistence of *ppt* and *kny* expression in *ntl* mutants is consistent with these genes functioning in parallel pathways during posterior morphogenesis.

Given its abundant expression in posterior tissues, *ppt(wnt5)* could compensate for *slb* function in posterior body extension. Indeed, recent studies of *ppt(wnt5);slb(wnt11)* double mutants

revealed that the two genes display some functional redundancy in the anterior mesendoderm movements (Kilian et al., 2003). Morphological analysis of *ppt(wnt5);slb(wnt11)* double mutants at 1 day post-fertilization (dpf) revealed more pronounced shortening of the posterior body in the double mutants (Fig. 2G), compared with single mutants (Fig. 2B,C) (Westfall et al., 2003). Therefore, whereas *slb(wnt11)* largely compensates for *ppt(wnt5)* function in more anterior parts of the gastrula (Kilian et al., 2003), the reverse is true in the posterior region, where *ppt(wnt5)* activity suffices for normal posterior body extension in the absence of *slb(wnt11)*.

By the six-somite stage, wild-type (Fig. 3A), *ppt* (Fig. 3B), *kny* (Fig. 3C) and *ntl* (Fig. 3D) embryos were morphologically distinct from the shorter *ppt;ntl* (Fig. 3E) and *kny;ntl* (Fig. 3F) double mutants. At this stage, the differences between *ppt;ntl* (Fig. 3E) embryos were greater than those between *kny;ntl* (Fig. 3F) and the individual mutants. At the 16-somite stage, wild-type (Fig. 3G), *ppt* (Fig. 3H) and *kny* (Fig. 3I) somites were separated by the midline chordamesoderm tissue. Although anterior somites were normal in *ntl* (Fig. 3J), *ppt;ntl* (Fig. 3K) and *kny;ntl* (Fig. 3L), the caudal somites were medially fused as previously reported for *ntl* (Halpern et al., 1993). In the wild type (Fig. 3G) and *ppt* (Fig. 3H) and *ntl* (Fig. 3J) individual mutants, caudal tissues extended beyond the posterior limit of the yolk tube extension, whereas posterior tissue ended abruptly in *ppt;ntl* embryos (Fig. 3K). Although the yolk tube had not extended in both *kny* (Fig. 3I) and *kny;ntl* (Fig. 3L) mutant embryos, the double mutants exhibited a shorter axis.

Double mutant phenotypes are not due to loss or failure to specify posterior tissues

To determine whether *ppt;ntl* and *kny;ntl* mutants were shorter than single mutants owing to failure to specify or maintain posterior cell fates, we examined tissue specific marker expression. At the six-somite stage, *gata2* (*gta2*) is expressed in bilateral stripes marking blood precursors (Dorfman et al., 1992). In wild-type (Fig. 4A), *ppt* (Fig. 4C) and *kny* (Fig. 4D) embryos, the stripes were separated in the posterior tailbud region. By contrast, the *gata2* stripes were less well separated in *ntl* (Fig. 4B) and were fused caudally to form a horseshoe-shaped domain in *ppt;ntl* (Fig. 4E) and *kny;ntl* (Fig. 4F) embryos. The expression of *pax2.1* in the optic stalks, otic vesicles and midbrain-hindbrain boundary (MHB) (Krauss et al., 1991) was normal in all individual and double mutant embryos. However, pronephric duct expression extended more caudomedially in *ntl*, *ppt;ntl* and *kny;ntl* double mutants, and, like *gta1* and *gta2* expression, it surrounded the tailbud (data not shown). Double mutant embryos lacked *myoD* expression in posterior adaxial cells and displayed broader somites (Fig. 4A-F) (Hammerschmidt et al., 1996; Solnica-Krezel et al., 1996; Topczewski et al., 2001; Weinberg et al., 1996).

Functional redundancy between *ntl* and *spt* Tbx genes during trunk and tail mesoderm formation has already been demonstrated (Amacher et al., 2002; Griffin et al., 1998). Therefore, we examined *spt*

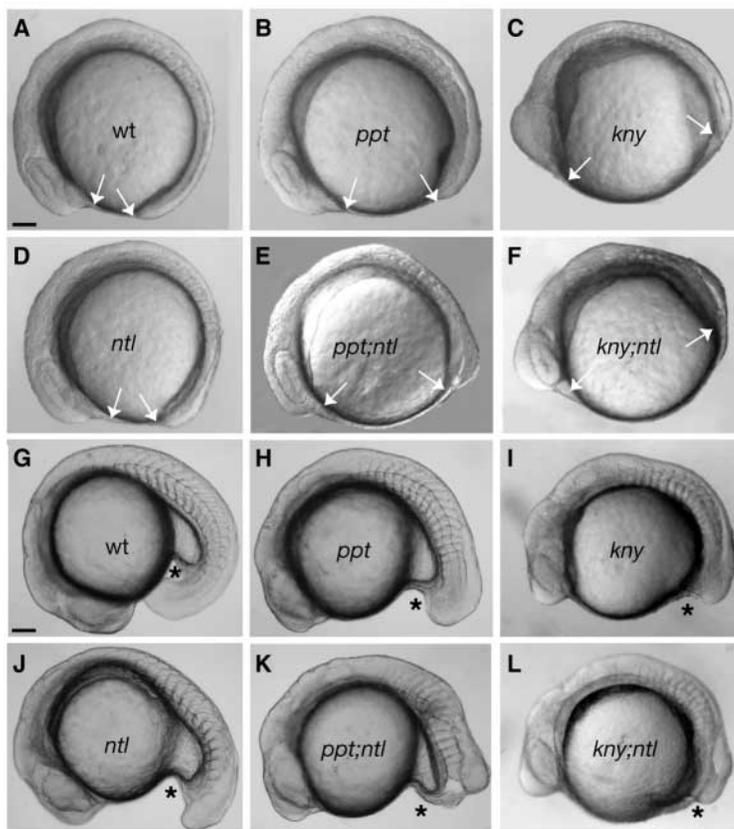


Fig. 3. The *ppt;ntl* and *kny;ntl* double mutants exhibit synergistic tail elongation defects. At the eight-somite stage, the AP axis of the wild-type embryo (A) is elongated such that the head and tail almost touch. This distance is greater (arrows) in *ppt* (B), and *kny* (C) mutants exhibiting shortened AP axes. In *ntl* (D) mutants, the AP axis is only slightly shorter at this stage, whereas *ppt;ntl* (E) and *kny;ntl* (F) double mutants are shorter than the individual mutants. At 18 somites, the tail extends beyond the yolk tube (*) in wild-type (G) embryos and is shorter in *ppt* (H), *kny* (I) and *ntl* (J) mutants. At this stage, the tail has not extended in *ppt;ntl* (K) and *kny;ntl* (L) double mutants. (A-L) Lateral views. Scale bars=100 μ m.

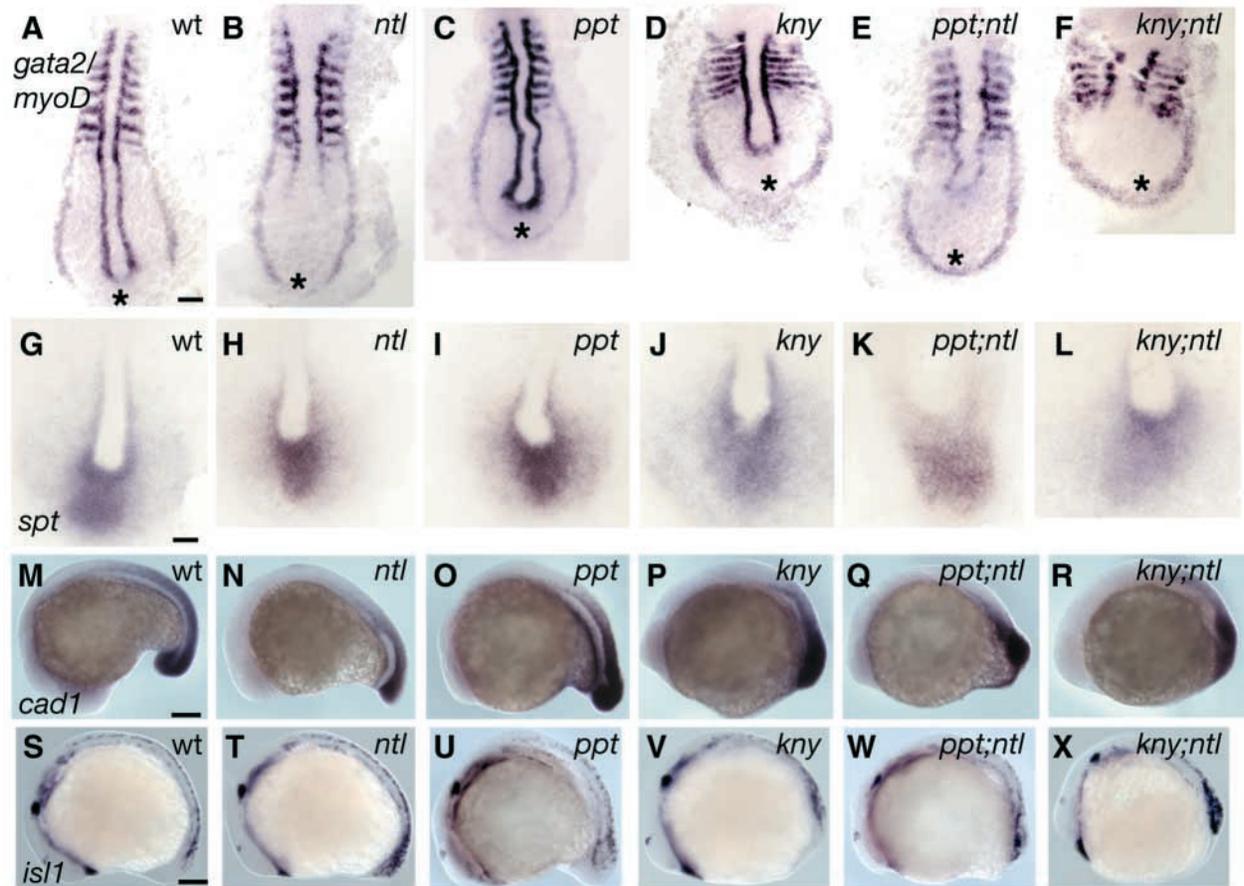


Fig. 4. Posterior tissues are specified in *ppt;ntl* and *kny;ntl* mutants. At nine somites, *myoD* is expressed in the somites and adaxial cells, and *gta2* is expressed in two bilateral stripes of prospective blood in wild-type (A) embryos. In *ntl* mutants (B), adaxial cell expression of *myoD* is lost and the stripes of *gta2* are less separated posteriorly. In *ppt* mutants (C), adaxial cell expression of *myoD* is kinked and *gta2* is similar to the wild type. In *kny* mutants (D), somites are mediolaterally broader and the AP lengths of adaxial cells and the *gta2* domain are shorter. The *ppt;ntl* (E) and *kny;ntl* (F) mutants exhibit *myoD* expression patterns as expected for the combined individual mutants, and *gta2* stripes are fused posteriorly. (G-L) Expression of *spt* in the paraxial mesoderm of wild-type (G), *ntl* (H), *ppt* (I), *kny* (J), *ppt;ntl* (K) and *kny;ntl* (L) embryos. (M-R) Expression of *Caudal* in the tailbud of wild-type (M), *ntl* (N), *ppt* (O), *kny* (P), *ppt;ntl* (Q) and *kny;ntl* (R) embryos. (S-X) Expression of *isl1* in developing neurons at the 16 somite stage. Ectopic *isl1* expression is observed in *ntl* (T), *ppt;ntl* (W) and *kny;ntl* (X) mutants. (A-L) Dorsal posterior flat mounts. (M-X) Lateral views. Scale bars=100 μ m (A-F,M-X), 50 μ m (G-L).

expression in double mutant clutches (Fig. 4G-L). Compared with the wild-type (Fig. 4G), *ppt* (Fig. 4I) and *kny* (Fig. 4J) embryos exhibited broader *spt* expression domains, consistent with their respective convergence and extension defects. Expression of *spt* was weaker in the double mutants (Fig. 4K,L) than in *ntl* (Fig. 4H) single mutants, suggesting that reduced *spt* might contribute to their posterior shortening. However, because *ppt;ntl* and *kny;ntl* mutants do not phenocopy *spt;ntl* double mutants and *spt* expression is not abolished in *ppt;ntl* and *kny;ntl*, the phenotypic differences between these double mutant classes probably reflect distinct underlying mechanisms.

Mutation of the zebrafish *caudal* (*cad1*) homeobox gene results in a shortened trunk and loss of the tail (Golling et al., 2002), prompting us to investigate whether Tbx and Wnt proteins interact to regulate *cad1* expression. Zebrafish *cad1* was expressed in the neuroectoderm and mesoderm of the caudal mass in wild-type (Fig. 4M), *ppt* (Fig. 4O) and *kny* (Fig. 4P) embryos during segmentation stages (Joly et al., 1992).

Expression of *cad1* was reduced in the mesoderm of *ntl* (Fig. 4N) embryos and was similarly reduced in the caudal mass of *ppt;ntl* (Fig. 4Q) and *kny;ntl* (Fig. 4R) mutants. Therefore, Ntl and non-canonical Wnt components interact to mediate posterior development via a mechanism that does not involve regulation of *cad1* expression.

Murine T and zebrafish *ntl* mutants exhibit excess neuronal markers in the posterior body (Nguyen et al., 2000). We detected an increase relative to the wild type of *isl1* neural marker expression in *ppt;ntl* and *kny;ntl* mutants that was comparable to that observed in single *ntl* mutants (Fig. 4S-X). We concluded that *ntl* and non-canonical Wnt components do not co-operate to limit neural cell fates in the posterior body. Together, these expression data indicate that gross AP patterning of the embryo is intact in the double mutants. Furthermore, the persistence of posterior mesodermal and neuroectodermal markers indicates that defective specification or maintenance of posterior tissues does not underlie the compound mutant phenotypes.

Tailbud expression of *bmp4* but not *Fgf* genes requires both *Ntl* and non-canonical Wnt signaling

Fgf signaling is important for mesoderm induction and patterning, gastrulation movements, and maintenance of *Tbx* gene expression through a mutually dependent feedback mechanism (Gerhart, 1989; Griffin et al., 1995; Isaacs et al., 1992; Kimelman and Kirschner, 1987; Slack et al., 1987; Smith et al., 1991). Furthermore, dominant negative *Fgf* receptor overexpression results in more severe defects than observed for *ntl* mutants, including loss of trunk and tail in *Xenopus* (Amaya et al., 1991) and zebrafish (Griffin et al., 1995). However, inactivation of the zebrafish *acerebellar (ace)/fgf8* gene does not produce posterior body deficiencies, possibly owing to overlapping and redundant *Fgf* genes (Reifers et al., 1998). Thus, the *ppt;ntl* and *kny;ntl* posterior defects might be a consequence of further impairment of *Fgf* activity owing to synergistic regulation by *Ntl* and non-canonical Wnts. To test this possibility, we analysed the expression of *fgf8*, *fgf3* and the *Fgf*-induced feedback antagonist *sef* (Furthauer et al., 2002; Kiefer et al., 1996; Tsang et al., 2002) during gastrulation and somitogenesis (Fig. 5A-L, and data not shown). At the 18-somite stage, *fgf8* was expressed in the forebrain, MHB, somites and tailbud (Reifers et al., 1998) of wild-type (Fig. 5A), *ppt* (Fig. 5B) and *kny* (Fig. 5C) embryos. Despite normal *fgf8* expression in all rostral tissues and the trunk somites, its tailbud expression was reduced to a similar degree in *ntl* (Fig. 5D), *ppt;ntl* (Fig. 5E) and *kny;ntl* (Fig. 5F) mutants. As for *fgf8*, expression of *fgf3* was similarly reduced in *ntl* and double mutants (data not shown). At the 16-somite stage, *sef* transcripts were detected in the forebrain, MHB, somites and ectoderm and mesoderm of the caudal mass of wild-type (Fig. 5G), *ppt* (Fig. 5H) and *kny* (Fig. 5I) embryos. In *ntl* (Fig. 5J), *ppt;ntl* (Fig. 5K) and *kny;ntl* (Fig. 5L) mutants *sef* expression was normal in rostral tissues and somites, but was equally reduced in the caudal mass, particularly in the ectoderm. Together, these data suggest that *ntl* and non-canonical Wnt components do not interact to regulate *Fgf* activity, so reduced *Fgf* signaling is an unlikely cause for the posterior defects of double-mutant embryos.

Bmp4 signaling was proposed to act upstream of Notch to promote outgrowth and patterning of the developing tailbud in *Xenopus* embryos (Beck et al., 2001). In zebrafish and mice, null mutations in *Bmp* pathway components result in ventral tail tissue deficiencies and patterning defects (Connors et al., 1999; Kishimoto et al., 1997; Winnier et al., 1995; Yang et al., 1999) and are associated with decreased tail progenitor migration into the tailbud in the zebrafish (Myers et al., 2002a). Hence, we examined *bmp4* expression at gastrula and tailbud stages. During early gastrulation, individual and double mutants could not be distinguished from wild-type gastrulae (data not shown). By contrast, at the five-somite stage, *bmp4* was expressed in the anterior prechordal plate of wild-type, single- and double-mutant embryos (Fig. 5M-R), whereas tailbud expression was severely reduced in *ntl* (Fig. 5P) embryos and was absent in *ppt;ntl* (Fig. 5Q) and *kny;ntl* (Fig. 5R) mutants. These data indicate that *ntl* and non-canonical Wnt components act together to promote *bmp4* expression in the developing tailbud, which might contribute to the synergistic double mutant phenotype.

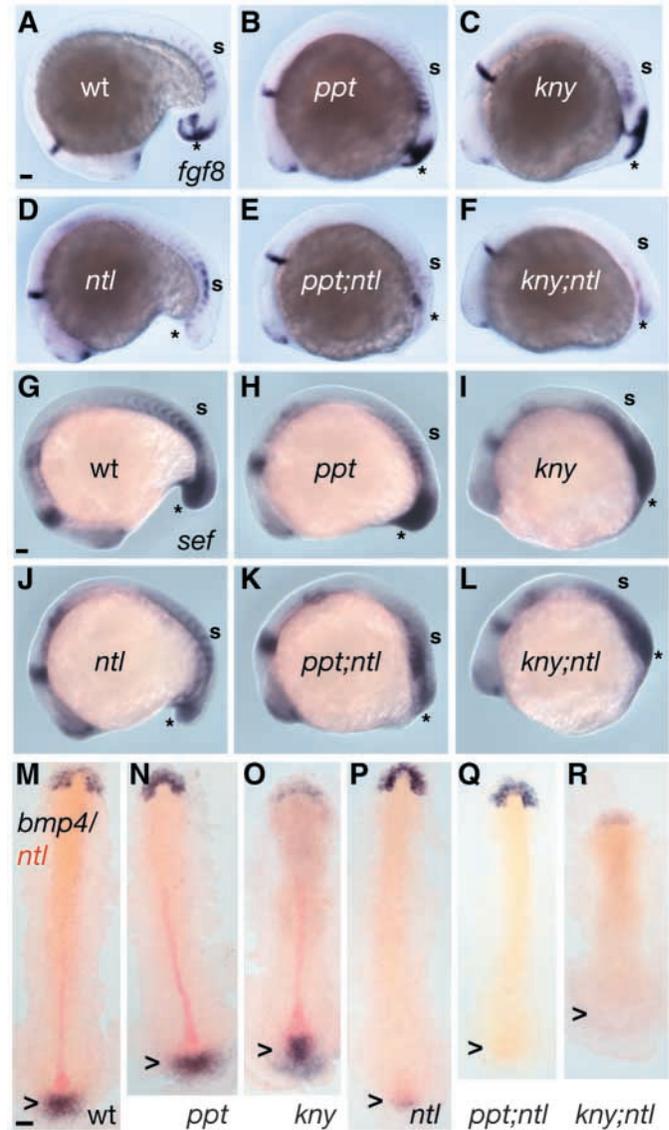


Fig. 5. *Ntl* and non-canonical Wnts synergistically regulate the expression of *bmp4*, but not *Fgf* genes. At 16 somites, *fgf8* is expressed in the brain and somites, and posteriorly in ectoderm and mesoderm in the wild type (A) and in the *ppt* (B) and *kny* (C) mutants. Despite normal anterior *fgf8* expression in *ntl* mutants (D), tail expression is extremely reduced but is not further reduced in *ppt;ntl* (E) or *kny;ntl* (F) double mutants. At 16 somites, *sef* expression in the brain, somites and tail is comparable in wild-type (G), *ppt* (H) and *kny* (I) embryos, but is reduced in *ntl* (J) mutants. Expression of *sef* is not further reduced in *ppt;ntl* (K) and *kny;ntl* (L) mutants. At five somites, *bmp4* is expressed in the prechordal plate and tailbud of wild-type (M), *ppt* (N) and *kny* (O) embryos. In *ntl* mutants (P), tailbud expression of *bmp4* is severely reduced and is absent in *ppt;ntl* (Q) and *kny;ntl* (R) double mutants. (A-L) Lateral views. (M-R) Dorsal flat mounts; arrowheads indicate tailbud. Scale bars=100 μ m.

Cell proliferation and death cannot account for the tail extension defects in *kny;ntl* and *ppt;ntl* double mutants

During vertebrate development, *Bmp* signaling regulates both cell proliferation and cell death (Ashique et al., 2002; Hogan,

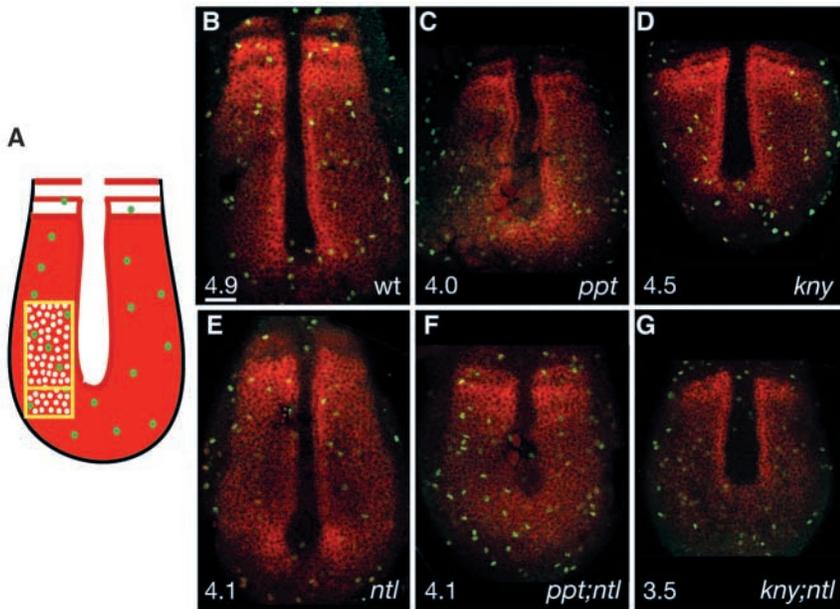


Fig. 6. Abnormal cell proliferation cannot account for the tail elongation defects in double mutants. Schematic representation of the dorsal medial paraxial region, where the ratio of phosphorylated-histone-positive cells (green) to *papc*-expressing cells (red) was determined (A). Confocal images of dorsal posterior section of the wild type (B), *ppt* (C), *kny* (D) and *ntl* (E) mutants, and *ppt;ntl* (F) and *kny;ntl* (G) double mutants at five somites, phosphorylated-histone-positive cells (green) and *papc*-expressing cells (red). (B-G) Scale bar=50 μm .

1996a; Hogan, 1996b). To investigate whether loss of *bmp4* expression in the developing tail was associated with decreased cell proliferation or enhanced cell death in compound mutant embryos, we analysed cell proliferation using an anti-phosphohistone antibody (pH3) that recognizes M-phase cells (Ajiro et al., 1996; Chadee et al., 1995; Mahadevan et al., 1991). Given the altered double mutant morphology, we used *paraxial protocadherin* (*papc*) expression in presomitic mesoderm (PSM) (Yamamoto et al., 1998) as a landmark to ensure that equivalent cell populations were evaluated. We determined the incidence of pH3-positive cells within the paraxial mesoderm adjacent to the notochord, previously shown to exhibit the highest mitotic indices during zebrafish tailbud development (Fig. 6A) (Kanki and Ho, 1997). At the five-somite stage, the *papc* expression domain included the forming somites, adaxial cells and undifferentiated paraxial mesoderm in wild-type (Fig. 6B), *ppt* (Fig. 6C) and *kny* (Fig. 6D) embryos, but was broader mediolaterally and shortened anteroposteriorly in *ppt* and *kny*, as expected for their convergence and extension defects (Topczewski et al., 2001; Rauch et al., 1997). In *ntl* mutants, *papc* expression was weaker in paraxial mesoderm and absent in adaxial cells (Fig. 6E) (Odenthal et al., 1996). In *ppt;ntl* (Fig. 6F) and *kny;ntl* (Fig. 6G) embryos, *papc* expression was altered as predicted for the combined individual mutant phenotypes. The mitotic indices (MIs) for wild-type (MI=4.9; $n=13$ embryos; 5568 cells), *ppt* (MI=4.0; $n=6$ embryos; 2875 cells; $p > 0.3$), *ppt;ntl* (MI=4.1; $n=4$ embryos; 1706 cells; $P > 0.1$), *ntl* (MI=4.1; $n=5$ embryos; 2366 cells; $P > 0.1$), *kny* (MI=4.5; $n=7$ embryos; 2737 cells; $P > 0.5$) and *kny;ntl* (MI=3.5; $n=5$ embryos; 2262 cells; $P > 0.1$) embryos were not significantly different. Furthermore; the MI

for double mutants did not statistically differ from the individual mutants (*ppt* vs *ppt;ntl*, $P > 0.8$; *kny* vs *kny;ntl*, $P > 0.2$; *ntl* vs *ppt;ntl*, $P > 0.9$; *ntl* vs *kny;ntl*, $P > 0.3$); therefore, reduced cell proliferation in the paraxial mesoderm cannot account for the severe tail extension defects in compound mutants.

Next, we tested whether increased cell death might underlie the double-mutant phenotypes. TUNEL staining, performed at the 5-, 13-, 16- and 22-somite stages, revealed apoptotic cells along the entire rostro-caudal axis with more cell death in the head than the tail of all embryos, consistent with previous reports (Cole and Ross, 2001) (data not shown). Compared with the wild type and individual mutants, *ppt;ntl* and *kny;ntl* embryos did not exhibit increased cell death in the tail region during early tail formation (data not shown). By contrast, increased cell death was observed in *ntl* and double mutants at the 22-somite stage (data not shown), when apoptosis of the tail was morphologically apparent in *ntl* and double mutants. However, this was well after the double-mutant tail extension defects were morphologically manifested (Fig. 3). Therefore, increased cell death probably does not underlie the early tail extension defects, although it contributes to later truncation of the tail.

Impaired movements of posterior tailbud cells underlie defective tail extension

Tailbud formation is initiated when blastoderm margin cells arrive at the ventral yolk plug forming the 'core' of cells that will mostly contribute to the posterior body (Kimmel et al., 1995; Westerfield, 1995). Fate mapping studies in the zebrafish have shown that posterior tailbud cells are ventral derived (Kanki and Ho, 1997; Kimmel and Law, 1985; Myers et al., 2002a; Warga and Nüsslein-Volhard, 1998). One possible explanation for the posterior defects in the double mutants is failure of progenitor cells to reach the posterior bud. To investigate whether double mutants enter tail extension stage with a deficit in contributing cells, we labeled cells in the ventral blastoderm margin during early gastrulation and monitored their position at bud stage and before the tailbud extension stage (Fig. 7A) (Myers et al., 2002a). Because gene expression analysis revealed *gta1*-positive cells in posterior medial regions of the compound mutants' tailbuds (Fig. 4E,F), we also asked whether these ectopic cells arose because of impaired cell movements or altered fate. In the wild type and in all mutants, the labeled cell groups moved from the ventral blastoderm margin into the posterior tailbud region (Fig. 7B,C). Subsequently, during early segmentation, they became displaced laterally and extended anteroposteriorly (Fig. 7D; $n=49$). By the six-somite stage, the labeled cell groups formed an elongated array positioned laterally, overlapping with *gta1* expression. In *ppt* (Fig. 7E) ($n=4$) and *kny* (Fig. 7F) ($n=16$), the labeled cells overlapped with lateral *gta1* expression, but their (and *gta1* cells') convergence and extension were reduced compared with the wild type (Fig. 7D) and the *ntl* mutant (Fig.

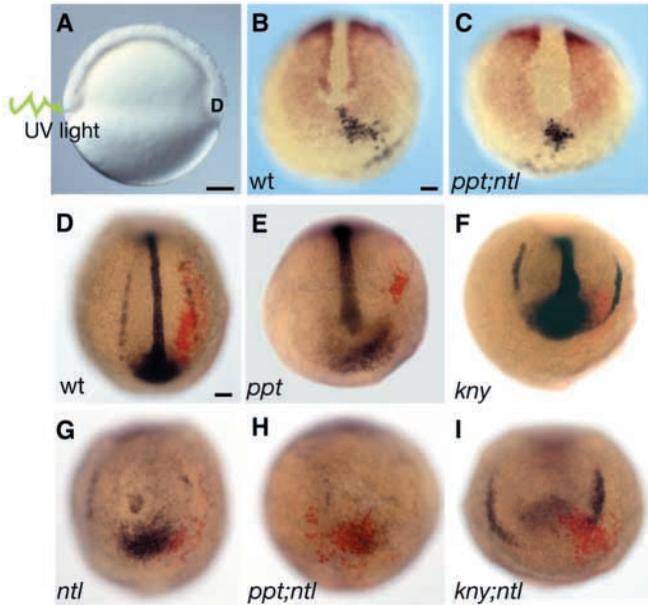


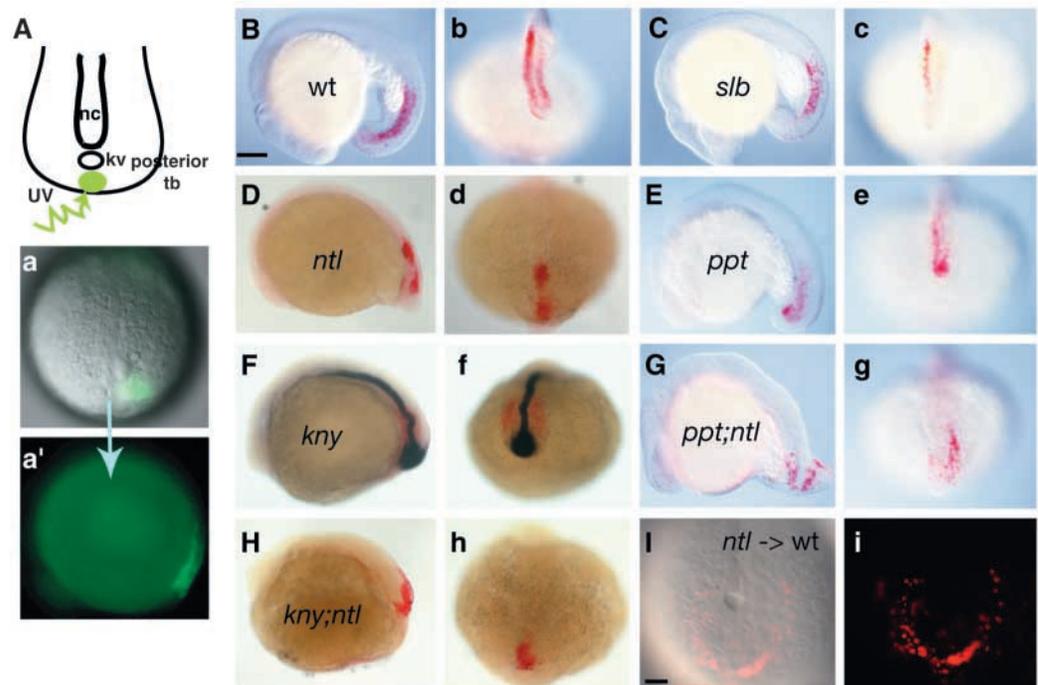
Fig. 7. Cell movements are impaired in double mutants before the elongation stage of tail morphogenesis. Prospective posterior tailbud cells were labeled 180° from the shield by uncaging with ultraviolet light (A). Labeled cells (black) move to the tailbud region marked by *papc* expression (red) in wild-type (B) and in single and double mutant embryos, including *ppt;ntl* (B), at bud stage. At five somites, the position of the labeled cells (red) relative to the ventral derived *gta1*-expressing cells (blue) was determined. In wild-type embryos (D), labeled cells form elongated arrays that overlap with *gta1*-expressing cells. In *ppt* (E), *kny* (F) and *ntl* (G) embryos, the labeled cells form shorter arrays that overlap with *gta1* expression. By contrast, labeled cells in *ppt;ntl* (H) and *kny;ntl* (I) mutants do not form elongated arrays and remain in the posterior bud. (A) Lateral view. (B-I) Dorsal posterior views. Scale bars=100 μm.

7G) ($n=9$). Notably, in *ppt;ntl* (Fig. 7H) ($n=6$) and *kny;ntl* (Fig. 7I) ($n=3$), the labeled cells remained in the posterior bud, overlapping the ectopic *gta1* expression domain. These data indicate that the movements that bring the posterior body precursors to the tailbud region are normal in the double mutants. However, the movements that shift cells from the posterior tailbud into paraxial positions and extend the posterior body are impaired in double mutants.

As tail extension continues, convergence and extension movements similar to those observed in the gastrula contribute

to posterior body elongation along with movements that are unique to the developing tail (Kanki and Ho, 1997). Specifically, posterior tailbud cells move beneath the advancing anterior tailbud cells (subduction) and anteriorly but laterally around them (extension and laterad divergence, respectively) (Kanki and Ho, 1997). To examine these movements directly in *ppt;ntl* and *kny;ntl* mutants, we labeled uniformly sized small cell groups within the posterior tailbud before the tail extension phase as described previously (Topczewski et al., 2001) and assessed their position and extension at the 18-somite stage (Fig. 8A). In wild-type embryos (Fig. 8a,a'; $n=32$) the labeled cells were positioned beneath the ectoderm (Fig. 8B) and lateral to the midline (Fig. 8b) as expected for cells that have undergone subduction and anterior laterad movements. Similar position of labeled cells was observed in *slb* (Fig. 8C, c; $n=6$) mutants, and measurements revealed that AP extension of the labeled cell array was not significantly different from the wild type (98%

Fig. 8. *Ntl* synergistically interacts with *Ppt* and *Kny* to regulate cell movements in the developing posterior body. At one somite, cells within the posterior tailbud were labeled by uncaging with ultraviolet light and, at 18 somites, the position of the labeled cells was determined (A). The labeled cells (red) are within the mesendoderm and lateral to the notochord (*ntl* in blue) in the wild-type embryos (B,b) and *slb* mutants (C,c). In *ntl* mutants (D,d), labeled cells are also within the mesendoderm (D) but are positioned medially (d). In *ppt* mutants (E,e), the cells occupy the mesendoderm (E) and are lateral to the notochord (e). In *kny* mutants, cells are within the mesendoderm (F) and lateral to the notochord (f). In *ppt;ntl* (G,g) and *kny;ntl* (H,h) mutants, labeled cells are



present in both the mesendoderm and ectoderm and fail to move from the posterior bud laterally or to extend (g,h). (B-H) Lateral views. The *ntl* mutant cells transplanted into wild-type embryos undergo subduction and lateral divergence (I,i). (b-h,I,i) Dorsal posterior views. Scale bar=100 μm.

of wild-type extension; $P > 0.42$). In *ppt* (Fig. 8E,e; $n=5$) and *kny* single mutants (Fig. 8F,f; $n=9$) the labeled cells were found lateral to the midline in the deep mesendoderm, but the array extension was reduced compared to wild type (85% of wild-type extension for *ppt*; $P < 0.05$; 82% of wild-type extension for *kny*). Whereas, in *ntl* embryos (Fig. 8D,d; $n=11$), labeled cells were positioned within the mesendoderm indicative of normal subduction, their position along the midline revealed that subsequent anterior laterad movements did not occur. Measurements indicated that extension of the labeled cell array was not reduced compared to the wild type (97% of wild-type length; $n=6$, $P > 0.38$). These cell movement analyses, together with morphological observations (Fig. 2), support the notion that the functions of *ntl* and *slb* alone are not required for posterior body extension, but that *ppt* and *kny* are both essential for these movements. Furthermore, these studies reveal the requirement for *ntl* function in laterad divergence movements. In *ppt;ntl* (Fig. 8G,g) and *kny;ntl* (Fig. 8H,h), although some labeled cells were found within mesendoderm, others remained within the epiblast, indicating that subduction movements were impaired. Additionally, labeled cell arrays did not extend much (47% of wild-type length for *ppt;ntl*, $n=5$; 33% of wild-type length for *kny;ntl*, $n=5$), nor did they move laterally. Therefore, Ntl and non-canonical Wnt components synergistically regulate posterior tailbud extension cell movements. In addition, they co-operate to mediate normal subduction movements.

The failure of posterior tailbud cells in *ntl* mutants to undergo laterad divergence movements could simply reflect the absence of the midline tissues that normally extend from the anterior tailbud (Kanki and Ho, 1997). Alternatively, *ntl* mutant cells could be unable to respond to divergence signals or to execute such a movement. To distinguish between these possibilities, we carried out mosaic analyses. When *ntl* mutant cells were transplanted into the ventral region of wild-type gastrulae, they underwent normal subduction and, notably, also normal divergence (Fig. 8I,i; $n=4$). These results demonstrate that the divergence movement defect in *ntl* mutants is non-cell-autonomous and probably occurs because of the failure of the anterior tailbud tissue to extend posteriorly and form the posterior midline.

Discussion

Combined activities of non-canonical Wnt and Ntl regulate tail formation

The transcription factor No tail and the non-canonical Wnt signaling pathway components Slb (Wnt11), Ppt (Wnt5) and glypican Kny are all essential for convergence and extension movements during gastrulation (Heisenberg et al., 2000; Kilian et al., 2003; Topczewski et al., 2001). Here, we have analysed genetic interactions between these genes to ask whether they functionally interact during posterior body morphogenesis. The *slb;ntl* compound mutants display only additive defects, without further caudal deficiencies compared with *ntl* mutants. Accordingly, late expression of *wnt11* in the posterior body (Fig. 2), but not anterior nor early gastrula expression, depends on *ntl* function (Makita et al., 1998). The absence of tail defects in *slb* single mutants suggests that additional partially overlapping signals must compensate for loss of posterior Wnt11 during tail formation, a notion supported by the

stronger phenotype observed for *slb;ppt* double mutants (Fig. 1) (Kilian et al., 2003; Westfall et al., 2003). Similar to *ntl* mutants, *ppt;wnt5* and *kny* single mutants exhibit shortened tails without loss of tail tissues (Hammerschmidt et al., 1996; Solnica-Krezel et al., 1996). In contrast to *slb;ntl* mutants, both *ppt;ntl* and *kny;ntl* double mutants display early tail elongation defects beyond those expected for the combined individual mutant phenotypes. Furthermore, *ppt* and *kny* expression persist in *ntl* mutants. Together, these data indicate that *kny* and *ppt* act in a parallel pathway that cooperates with *ntl* during posterior body development.

ppt;ntl and *kny;ntl* do not co-operate to specify posterior tissues

Genetic lesions and dominant negative interference have revealed roles for several genes in trunk and tail formation including *ntl*, other Tbx genes, *papc*, Fgf genes and *caudal* (Amaya et al., 1991; Chesley, 1935; Golling et al., 2002; Griffin et al., 1995; Halpern et al., 1993; Ho and Kane, 1990; Yamamoto et al., 1998). Because mutations in the murine *brachyury* gene or interference with Fgf signaling in frog and fish result in more severe posterior defects than those observed in zebrafish *ntl* mutants, redundant mechanisms have been proposed to operate downstream of Fgf during zebrafish trunk and tail development (Griffin et al., 1995). Therefore, it was possible that simultaneous inactivation of *ntl* and *ppt* or *kny* genes leads to synergistic loss of Fgf activity and the severe posterior defects in double mutants. However, we found no further reduction in expression of *fgf3*, *fgf8* or the Fgf feedback antagonist *sef* compared with individual mutants. Based on these data, we propose that impaired Fgf activity does not account for the synergistic double mutant defects.

Previous studies in zebrafish have demonstrated overlapping function of Tbx genes during tail and trunk morphogenesis, with *spt;ntl* double mutants displaying more severe posterior defects than either individual mutant (Amacher et al., 2002; Griffin et al., 1998). Significant *spt* expression persists in *ppt;ntl* and *kny;ntl* double mutants, indicating that it is not synergistically regulated by Ntl and non-canonical Wnt components. In support of this, the *ppt;ntl* and *kny;ntl* compound mutants do not phenocopy the *spt;ntl* defects. Although *spt;ntl* double mutants exhibit severe trunk and tail mesoderm deficits, *ppt;ntl* and *kny;ntl* double mutants only have mesoderm deficiencies comparable to those observed in *ntl* single mutants (Amacher et al., 2002). Furthermore, expression of *myoD* in posterior somites is absent from *spt;ntl* and from double mutants for the Nodal cofactor *oep* and either *ntl* or *spt* (Amacher et al., 2002; Griffin et al., 1995; Griffin et al., 1998; Griffin and Kimelman, 2002; Schier et al., 1997) but not in *ppt;ntl* and *kny;ntl* double mutants.

The *cad1* homeobox gene regulates posterior body specification in *Drosophila* and vertebrates (Golling et al., 2002; Joly et al., 1992; Macdonald et al., 1986; Macdonald and Struhl, 1986; Mlodzik et al., 1990; Subramanian et al., 1995). Mutational inactivation of and interference with Cad1 leads to posterior truncations in the zebrafish and *Drosophila*, and to anterior homeotic transformations in the mouse (Golling et al., 2002; Joly et al., 1992; Macdonald and Struhl, 1986; Subramanian et al., 1995). However, persistent expression of *cad1* in the posterior tissues of double-mutant embryos indicates that their tail elongation defects must occur through

a *cad1*-independent mechanism. Therefore, in contrast to Tbox and Nodal interactions and the *cad1* gene, which promote posterior tissue specification and/or differentiation (Amacher et al., 2002; Griffin et al., 1998; Griffin and Kimelman, 2002; Schier et al., 1997), the *ppt;ntl* and *kny;ntl* mutant phenotypes probably arise by a distinct mechanism that involves regulation of morphogenetic processes.

How might *bmp4* contribute to tail elongation?

Bmps regulate cell fate, proliferation, survival and cell movements throughout development (reviewed in Ashique et al., 2002; Hammerschmidt and Mullins, 2002; Hogan, 1996a; Hogan, 1996b; Myers et al., 2002b). Recent studies in *Xenopus* revealed Notch-dependent and -independent functions for Bmp during tail outgrowth and patterning (Beck et al., 2001). Here, we show that *bmp4* expression is lost at earlier developmental stages in *ppt;ntl* and *kny;ntl* double mutant embryos than in *ntl* individual mutants raising the possibility that the severe posterior defects are due to the earlier loss of *bmp4* activity. According to the *Xenopus* model, Bmp4 signaling upstream of Notch promotes tailbud outgrowth. However, we did not observe enhanced reduction of *notch6*, *notch1* or *deltaC* expression in double mutants compared with *ntl* single mutants (data not shown), although we cannot exclude the possibility that other *notch* and *delta* genes are involved. Further supporting the notion that Bmp4-dependent regulation of Notch signaling is probably not responsible for the synergistic tail phenotypes, single and double zebrafish mutants in Notch/Delta and their target genes exhibit segmentation and/or neurogenic phenotypes without loss of tail (Appel et al., 1999; Henry et al., 2002; Itoh et al., 2003; Oates and Ho, 2002; van Eeden et al., 1998). In *Xenopus*, Bmp signaling mediates tail somite formation by a mechanism that does not use Notch (Beck et al., 2001). Consistent with a role for Bmp signaling in tail mesoderm specification, *ntl* and compound mutants fail to form tail somites. However, this process does not appear to be synergistically affected in the compound mutants. In zebrafish, Bmp signaling was proposed to mediate posterior somite formation by ensuring proper tail progenitor movements during gastrulation (Myers et al., 2002a). Zebrafish mesodermal cells residing in the ventral no-convergence no-extension zone (NCEZ), where Bmp activity levels are the highest, do not undergo convergence and extension movements during gastrulation. Instead, they move vegetally to occupy the posterior tailbud (Myers et al., 2002a). In dorsalized zebrafish mutants with diminished Bmp activity and reduced NCEZ, the ventral cells fail to reach the tailbud, leading to tail truncations (Connors et al., 1999; Kishimoto et al., 1997; Miller-Bertoglio et al., 1997; Myers et al., 2002a). However, during early and late gastrulation, dorsal and ventral marker expression revealed overtly normal dorsoventral patterning in individual *kny*, *ppt* and *ntl* mutants (Rauch et al., 1997; Solnica-Krezel et al., 1996; Topczewski et al., 2001) and in *kny;ntl* and *ppt;ntl* compound mutants. Therefore, altered dorsoventral patterning in the gastrula does not underlie the tail elongation defects (data not shown). Accordingly, cell tracing experiments revealed normal movement of ventral mesodermal cells into the posterior tailbud in single and double mutants. Therefore, the posterior defects in double mutants are not due to a failure of contributing progenitors to move into the tailbud.

After the zebrafish tailbud forms, posterior Bmp signaling is

promoted by the Chordin antagonist Tolloid/Minifin (Mfn) (Connors et al., 1999). Mutations that disrupt the *mfn* gene lead to mild dorsalization, loss of ventral tail tissues and reduction of somitic mesoderm. Despite these patterning defects and reduction of *bmp4* and *eve1* expression, extension of the tail is largely normal in *mfn* mutants (Connors et al., 1999). Furthermore, a dramatic downregulation of *eve1* expression in *ntl* has been reported previously (Joly et al., 1993a) and we observed a comparable reduction of *eve1* expression in *kny;ntl* and *ppt;ntl* double mutants (data not shown). Given normal extension of posterior body in *ntl* (Figs 2, 8) and *mfn* mutants, downregulation of *eve1* in posterior tissues of *kny;ntl* and *ppt;ntl* double mutants during segmentation is unlikely to underlie their morphogenetic defects. However, as neither *mfn* nor *ntl* embryos exhibit complete loss of tailbud *bmp4* expression as observed in *kny;ntl* and *ppt;ntl* compound mutants, the specific contribution of Bmp signaling to tail elongation will require further investigation.

Aberrant cell proliferation and death do not account for early posterior body shortening

Given that Bmp regulates cell proliferation and survival (Ashique et al., 2002), loss of *bmp4* expression in *ppt;ntl* and *kny;ntl* mutants could lead to elevated cell death or reduced cell proliferation during tail elongation. In either case, double-mutant embryos would have fewer cells contributing to the posterior body. Our findings do not support this hypothesis. Rather, we found comparable levels of cell proliferation and death between compound and individual mutants before and during tail extension stages. Increased cell death occurred well after the double-mutant phenotype was manifest morphologically. At this time, cell death is visible in *ntl* and in compound mutants, and probably leads to the subsequent truncation and/or loss of posterior tissues. In support of this, *Xenopus* embryos with perturbed *brachyury* function and *brachyury* mouse mutants only exhibit apoptosis in posterior tissues after gastrulation defects are apparent (Chesley, 1935; Conlon and Smith, 1999; Yanagisawa et al., 1981). This increase in programmed cell death was proposed to be an indirect consequence of altered adhesion as reported for epithelial cells that occupy an inappropriate location (Conlon and Smith, 1999; Khwaja et al., 1997). Our cell labeling analysis supports this notion because ventral-derived posterior tailbud cells occupy the midline region, from which they are normally excluded (Kanki and Ho, 1997).

Convergence and extension genes interact with *ntl* to regulate tail-forming movements

Zebrafish tailbud morphogenesis entails regionalized cell movements (Kanki and Ho, 1997). Throughout the anterior and posterior tailbud, cells continue to undergo convergence and extension movements as observed during gastrulation until the tail eversion stage (Fig. 9) (Kanki and Ho, 1997). We link *ntl* with *ppt* and *kny* genes to the gastrulation-like convergence and extension movements within the tailbud. Between tailbud formation and the onset of tail extension, convergence and extension movements within the tailbud are normal in *ntl* mutants and impaired in single *kny* and *ppt* mutants, whereas double mutants have most severe tail convergence defects (Fig. 9). Tail convergence defects were apparent by broader gene expression domains before the onset of the tailbud extension

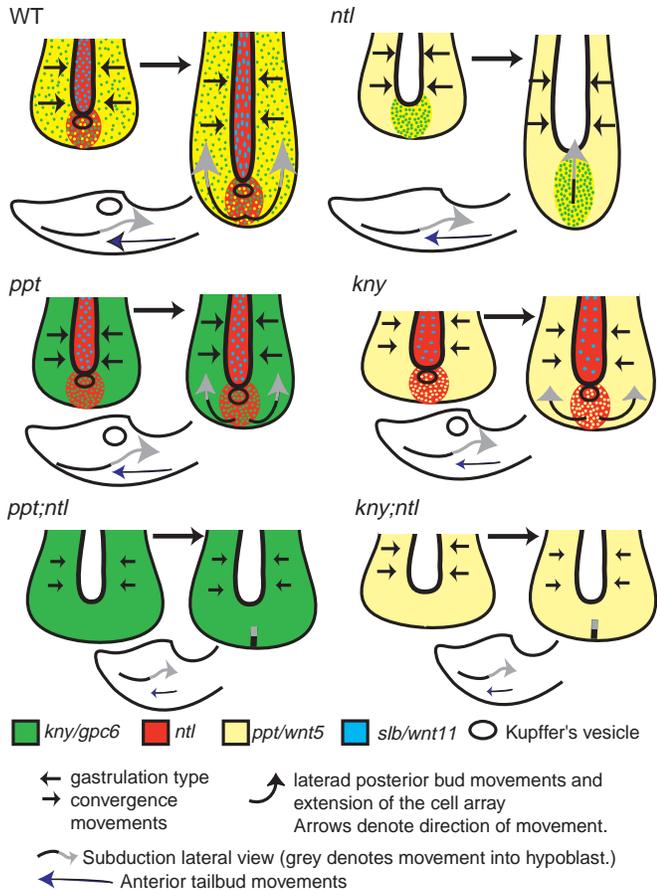


Fig. 9. Synergistic interactions between Ntl and Ppt and Kny regulate specific cell movements underlying tail morphogenesis. During posterior body morphogenesis, convergence and extension movements as observed in the gastrula contribute to tail elongation until the tail everts. At the same time, laterad divergence movements of the posterior tailbud cells occur to avoid the midline, and they enter the mesendoderm by subducting beneath the advancing anterior tailbud cells at Kupffer's vesicle in the wild type. In *ntl* mutants, convergence movements are relatively normal despite a lack of *wnt11* in the notochord. During tail elongation, convergence and extension movements continue relatively normally. At this time, posterior tailbud cells fail to undergo laterad divergence and instead extend anteriorly. Movement of posterior tailbud cells into the mesendoderm occurs normally despite the lack of Kupffer's vesicle, suggesting that the boundary between dorsal- and ventral-derived cells is maintained (if this distinction is required for normal subduction). In *ppt* mutants, gastrula-like convergence movements are impaired although initial positioning of cells within the posterior tailbud is normal. These cells undergo laterad divergence from the midline but it is reduced compared with the wild type, as is extension; movement into the mesendoderm is normal. In *kny* mutants, convergence and extension movements are impaired although ventral-derived posterior body precursor cells undergo normal epiboly movements (Topczewski et al., 2001) and contribute to the tailbud. In addition, tail-specific laterad divergence occurs and subduction movements position cells within the mesendoderm. In *ppt;ntl* and *kny;ntl* embryos, gastrulation-like convergence and extension movements are impaired, although posterior tailbud cells arrive at the bud on time. Like *ppt;ntl* double mutants, *kny;ntl* double mutant cells fail to undergo laterad divergence and do not move from the posterior bud. In addition, subduction movements into the mesendoderm are impaired but not completely blocked suggesting an additional role for Kny, Ppt and Ntl function.

stages. Therefore, *ntl* co-operates with *ppt/wnt5* and *kny/gpc4/6*, possibly by regulating *wnt11*, to mediate the gastrulation-like convergence and extension movements throughout the tailbud.

Anterior mesendodermal tailbud cells ingress and move posteriorly within the dorsomedial posterior flow until exiting the flow and occupying more lateral regions. By contrast, posterior tailbud cells enter the mesendoderm beneath the dorsal derived anterior cells through a tailbud-specific subduction movement (Figs 1, 9) (Kanki and Ho, 1997). The posterior tailbud cell groups also diverge from the midline via laterad movement and extend anteroposteriorly (Fig. 9) (Kanki and Ho, 1997). In *ntl* single mutants, cells enter the mesendoderm but do not exhibit laterad movements even though the array extends normally (Fig. 9). Similarly, normal extension of dorsal mesoderm despite impaired convergence was observed in *ntl* gastrulae, but was attributed to epiboly movements (Glickman et al., 2003). Recent studies in *Xenopus* show that *Xbra/ntl* function promotes convergence and extension while inhibiting cell migration; therefore, it is possible that increased migration in the dorsal mesoderm of zebrafish *ntl* mutants contributes to its extension (Kwan and Kirschner, 2003). The cell behaviors driving extension of posterior tissues remain to be characterized.

In mosaic mouse embryos, *brachyury* mutant cells accumulate along the length of the primitive streak but are largely absent from the lateral paraxial mesoderm, a defect that has been associated with altered adhesion (Wilson and

Beddington, 1997; Wilson et al., 1995). In the mouse, restoring Ntl activity in the streak is sufficient for cells to move into paraxial regions. Likewise, we found that *ntl* mutant cells in a wild-type host can undergo normal laterad divergence movements. Thus, the requirement for Ntl activity to promote lateral movement into paraxial mesoderm might be conserved between mouse and zebrafish (Wilson and Beddington, 1997). We hypothesize that the posterior tailbud cells upon subduction encounter the midline tissues extending from the anterior tailbud (Kanki and Ho, 1997), which serve as a barrier for their migration, resulting in the laterad divergence of their movement. It will be important to test whether a simple mechanical barrier or/and repulsive signals are involved. During tailbud extension stages, *ntl* and non-canonical Wnt signaling interact to regulate convergence and extension and tailbud unique movements. Cell labeling revealed a synergistic function between *ntl* and *ppt*, and between *ntl* and *kny* in facilitating posterior tailbud cell movements. In *ppt;ntl* and *kny;ntl* compound mutants, posterior tailbud cells fail to move from the posterior bud, fail to extend and exhibit impaired subduction movements (Fig. 9). We suggest that the tail extension defects in double-mutant embryos are due to the combined loss of *ntl* and parallel, partially overlapping non-canonical Wnt signaling inputs, which synergistically regulate posterior-specific cell movements but not posterior specification during tail morphogenesis. Moreover, genes required for cell movements in the gastrula interact to regulate cell movements within the developing posterior body,

supporting the notion that tail formation is in part a continuation of mechanisms mediating gastrulation.

We thank L.S.-K. lab members for discussions and critical comments. We thank J. Clanton and W. Rogers for excellent fish care, C. Kimmel, C.-P. Heisenberg and M. Hammerschmidt for mutant lines, M. Westerfield, H. Takeda, P. Ingham, M. Ekker, Y. Grinblat, B. Thisse, C. Thisse, E. Weinberg and T. Jowett for probes, and R. Harland, S. Sokol and M. Tada for constructs. The Zeiss confocal microscope is supported by NIH grant 1S10RR015682. Work in E.M.G. laboratory is supported by grants from the government of Spain (FIS01/796 and MCYTBM2000-0540). Work in L.S.-K. lab is supported by NIH grant GM62283.

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