BMP and non-canonical Wnt signaling are required for inhibition of secondary tail formation in zebrafish

Yi Yang and Chris Thorpe

SUMMARY

The role of bone morphogenetic protein (BMP) signaling in specifying cell fate in the zebrafish tailbud has been well established. In addition to a loss of ventral tissues, such as ventral tailfin and cloaca, some embryos with compromised BMP signaling produce an additional phenotype: a ventrally located secondary tail containing both somitic muscle and notochord. This phenotype has been proposed to reflect a fate-patterning defect due to a change in a hypothesized BMP activity gradient. Here, we show that a defect in morphogenetic movements, not fate patterning, underlies the formation of secondary tails in BMP-inhibited embryos. Our data indicate that BMP signaling is activated in the ventroposterior tailbud to promote cell migration during tailbud protrusion, and that defective migration of these cells in BMP mutants ultimately leads to bifurcation of the caudal notochord. Additionally, we show that non-canonical Wnt signaling is also required for proper tail morphogenesis, possibly by maintaining cohesion of notochord progenitors by regulation of cadherin localization. We propose a model in which BMP and the non-canonical Wnt pathway regulate tail morphogenesis by controlling cell migration and cell adhesion within the tailbud.

KEY WORDS: Zebrafish, BMP, Wnt, Tailbud, Notochord, Morphogenesis

INTRODUCTION

The vertebrate tailbud is a mass of undifferentiated cells that gives rise to posterior tissues of the body during tail development. In zebrafish, the tailbud is derived from cells originating at both the dorsal and ventral margins, which come together at the vegetal pole of the embryo upon completion of epiboly (Kimmel et al., 1995). Ventrally derived cells constitute the posterior portion of the tailbud and are fated to develop into somitic muscle in the tail, whereas the dorsally derived cells constitute the anterior tailbud and form primarily axial tissues of the tail, such as notochord and floor plate (Kanki and Ho, 1997; Myers et al., 2002; Agathon et al., 2003).

Extensive genetic analyses have demonstrated roles for several conserved signaling pathways in the specification, patterning and morphogenesis of the tailbud and its derivatives, including the bone morphogenetic protein (BMP), fibroblast growth factor (FGF), Nodal, and both the Wnt/β-catenin and non-canonical Wnt pathways (Schier and Talbot, 2005). For example, the BMP pathway is required (along with Wnt/β-catenin and Nodal signaling) during gastrulation for the formation of a tail organizer at the ventral margin (Agathon et al., 2003). In embryos completely lacking BMP activity during gastrulation, such as *swirl* (*swr*; *bmp2b* – Zebrafish Information Network) mutants, tail development is essentially nonexistent (Mullins et al., 1996; Kishimoto et al., 1997). During gastrulation, BMP is thought to function as a morphogen, patterning cell fates along the dorsal-ventral (DV) axis, with highest BMP activity inducing the ventral-most fates, including tail mesoderm, intermediate levels specifying lateral fates such as trunk mesoderm, and the absence of BMP activity allowing for the development of dorsal fates such as the notochord (Little and Mullins, 2006).

Division of Biology, Kansas State University, Manhattan, KS 66506, USA.

*Author for correspondence (thorpe@ksu.edu)

Independent of its role in fate patterning, BMP signaling also regulates morphogenetic movements during gastrulation, promoting the convergence of lateral mesodermal cells towards the dorsal midline (Myers et al., 2002; von der Hardt et al., 2007). The BMP activity gradient is proposed to negatively regulate cell adhesion, establishing distinct patterns of migratory behavior among mesodermal cells along the DV axis (von der Hardt et al., 2007). Presumably, simultaneous control of cell fate patterning and cell movements by BMP ensures tight coupling of these two processes.

During somitogenesis, BMP acts to pattern cell fates derived from the tailbud. Post-gastrula inhibition of BMP signaling results in embryos showing reduced ventral tailfin and cloaca tissue (Pyati et al., 2005; Pyati et al., 2006; Tucker et al., 2008; Yu et al., 2008). Such embryos show a similar phenotype to that observed in embryos carrying mutations in several conserved components of the BMP pathway: *bmp4*, *mini fin* (*mfn*; also known as *tolloid-like 1*, *tll1* – Zebrafish Information Network), *lost-a-fin* (*laf*; also known as *alk8* and *acvr1l*), as well as hypomorphic *smad5* alleles (Connors et al., 1999; Mintzer et al., 2001; Kramer et al., 2002; Stickney et al., 2007). It has been suggested that a gradient of BMP signaling specifies distinct cell types in posterior tissues, with the highest levels of BMP activity being required for production of ventral tailfin and cloaca and a lower amount being sufficient for presomitic mesoderm and blood (Stickney et al., 2007).

Cell tracing experiments have shown that the anterior and posterior portions of the tailbud undergo distinct morphogenetic movements during tail outgrowth (Kanki and Ho, 1997). Cells in the anterior (dorsally derived) tailbud continue the convergence and extension (CE) movements observed during gastrulation, with presomitic and somitic mesoderm converging to the midline, driving the posteriorward extension of the embryo. By contrast, posterior (ventrally derived) tailbud cells move laterally away from the midline and subduct beneath the posteriorly migrating anterior tailbud cells.

Some genes that regulate cell movements during gastrulation have also been shown to contribute to proper tail morphogenesis. For example, the non-canonical Wnt pathway, which promotes

dorsal convergence independently of BMP during gastrulation (Sepich et al., 2005), is also required for proper tailbud morphogenesis (Marlow et al., 2004). Whether the BMP pathway regulates morphogenesis as well as fate patterning in the tailbud is not known.

A subset of BMP-deficient embryos with defective fate patterning in the tail also produce a curious additional phenotype: a ventrally located secondary tail containing both somitic muscle and notochord (Connors et al., 1999; Pyati et al., 2005; Stickney et al., 2007; Yu et al., 2008). It has been proposed that these secondary tails form as a result of a mis-specification of cell fates due to a change in the postulated gradient of BMP activity in the tail (Stickney et al., 2007). According to this hypothesis, a mild reduction in BMP activity could lead to a shallower slope of the gradient such that it would be insufficient to specify ventral tailfin, but would expose more cells to the intermediate level of activity sufficient for formation of tail mesoderm, leading to an expansion of this tissue.

However, this model does not explain why the excess tail mesoderm would form such a morphologically distinct structure or why the secondary tails observed in BMP-compromised embryos include notochord, which is not hypothesized to be responsive to the BMP gradient. Also, although *bmp4* mutant embryos show the loss of ventral tailfin and expansion of blood progenitors and somitic muscle predicted by the gradient model, these embryos do not form secondary tails (Stickney et al., 2007). Furthermore, when expression of a dominant-negative BMP receptor (dnBMPR) is induced at the bud stage, the population of blood progenitors is not expanded, even though ~15% of these embryos form secondary tails (Pyati et al., 2005). Taken together, these observations are inconsistent with the hypothesis that secondary tails form as a consequence of defects in fate patterning.

We show here that a defect in morphogenesis, not fate patterning, underlies formation of secondary tails in BMP mutants, and demonstrate a role for BMP in regulating the migration of presomitic mesodermal cells in the tailbud. We further show that non-canonical Wnt signaling functions in a parallel pathway to prevent secondary tail formation, perhaps by regulating cohesion of caudal notochord cells.

MATERIALS AND METHODS
Zebrafish strains
Mutant lines used were *mfn*[tc263a] (Connors et al., 1999), *kny*[fr6] (Topczewski et al., 2001), *smad5*[m169] (Kramer et al., 2002), *smad5*[dty40] (Kramer et al., 2002) and *snh*[ty68a] (Schmid et al., 2000). *mfn/+;Tg(flh:EGFP)* and *kny/+;Tg(flh:EGFP)* strains were created by crossing *mfn* and *kny* heterozygotes to the *Tg(flh:EGFP)* transgenic line (Gamse et al., 2003).

In situ hybridization and antibody staining
Whole-mount in situ hybridization was carried out using standard methods (Oxtoby and Jowett, 1993). The following probes were used: *col2a* (Yan et al., 1995), *myoD* (Weinberg et al., 1996), *neurog1* (Blader et al., 1997), *ntl* (Schulte-Merker et al., 1992), *tbx6* (Hug et al., 1997), *gata1* (Detrich et al., 1995), *fli1* (Brown et al., 2000) and *flh* (Talbot et al., 1995). Fluorescent in situ hybridization (FISH) for *flh* expression was performed using a Fast Red color reaction (Hauptmann and Gerster, 1994), followed by incubation with P-Smad1/5/8 antibody (1:100; Cell Signaling Technology). β-catenin antibody (BD Transduction Laboratories) was applied at 1:400; pan-cadherin antibody (Sigma) was applied at 1:100; Tbx6 antibody (ZIRC) was applied at 1:1000. Appropriate Alexa Fluor (Molecular Probes) secondary antibodies were used.

Morpholino and RNA injection
Morpholino antisense oligonucleotides (*dvl1* MO: 5′- ATATGATTTTA-GTCTCCGCCATGAG-3′) were purchased from Gene Tools. *mfn, dvl2, dvl3* and *cdh2* morpholinos have been described previously (Lele et al.,

2002; Angers et al., 2006; Jasuja et al., 2006). The standard control morpholino by Gene Tools was used in some experiments. Morpholinos were diluted in Danieau's buffer before injection. *mfn* MO was injected at a concentration of 1 mg/ml; concentrations of 0.5 mg/ml and 1.5 mg/ml were injected as low and high dose. *dvl1, dvl2 and dvl3* MOs were injected at a suboptimal concentration of 1 mg/ml each; a concentration of 1.5 mg/ml was injected as high dose; to fully inhibit *dvl2* and *dvl3*, the concentration was 3 mg/ml. *cdh2* MO was injected at a suboptimal concentration of 0.1 mg/ml and a high concentration of 0.25 mg/ml. Synthetic mRNA of membrane-bound RFP (mRFP) was made from pCS2+ constructs with mMessage mMachine kit (Ambion) and injected at a concentration of 25 mg/ml. In all experiments, a volume of 3-5 nl was injected into the yolk of one-cell-stage embryos.

Confocal microscopy
Tg(flh:EGFP), mfn;Tg(flh:EGFP) and *kny;Tg(flh:EGFP)* embryos were injected with mRFP mRNA to provide contrast. To analyze tail morphogenesis, embryos were mounted at the ten-somite stage in 0.7% low-melt agarose in Ringer's solution. Agarose surrounding the posterior body was removed to ensure the free extension of the tailbud. Images were acquired every 1.5 minutes using a Zeiss LSM 5 Pascal confocal microscope and a 20× lens. Videos were re-aligned using ImageJ.

BMP Inhibition
We treated embryos with dorsomorphin (AMPK Inhibitor, Compound C, Calbiochem) as described with slight modifications (Yu et al., 2008). Dorsomorphin stock solution in DMSO was dissolved in fish water at 60 μM. Embryos were dechorionated and reared in the solution for the duration of the experiment. Control embryos were dechorionated and raised in water containing an equivalent amount of DMSO.

RESULTS
Mesoderm tissues produced in the secondary tail of *mini fin*
To develop a clearer understanding of the origin of secondary tails in BMP-compromised embryos, we analyzed this process in the *mfn* mutant, the only mutant reported to consistently produce secondary tails (Connors et al., 1999). To determine which tissues are located in the secondary tail, we examined the expression of the notochord marker *col2a* (*col2a1a* – Zebrafish Information Network), muscle marker *myoD* (*myod1* – Zebrafish Information Network), neural marker *neurog1* and the notochord/tailbud marker *ntl* (*ntla* – Zebrafish Information Network) by in situ hybridization. In *mfn* embryos at 26 hours post-fertilization (hpf), ectopic *col2a* (Fig. 1A,B) and *myoD* (Fig. 1C,D) expression was observed in 53% and 30% of the embryos, respectively (Table 1). Consistent with previous studies, no embryo showed ectopic *neurog1* expression (Fig. 1E,F), indicating that secondary tails do not contain neural tissue (Pyati et al., 2005). Ectopic *ntl* expression (Fig. 1G,H) was detected in 64% of the embryos. In all embryos with a secondary tail, we also observed ectopic expression of two posterior tailbud markers, *tbx6* and *eve1* (Fig. 1I,J, and data not shown).

Secondary tail formation is not accompanied by changed mesoderm fates
The BMP morphogen model in tail development suggests that secondary tail formation only occurs at a modestly reduced level of BMP signaling in the tailbud, whereas stronger loss of BMP activity results in reduced ventral mesoderm, with no secondary tails formed (Stickney et al., 2007). BMP mutants are variably dorsalized, with the degree of dorsalization typically scored using the dorso-anterior index (DAI), which ranges from very weak (C1) to completely dorsalized (C5). Secondary tail formation is proposed to occur only in embryos with a phenotype intermediate
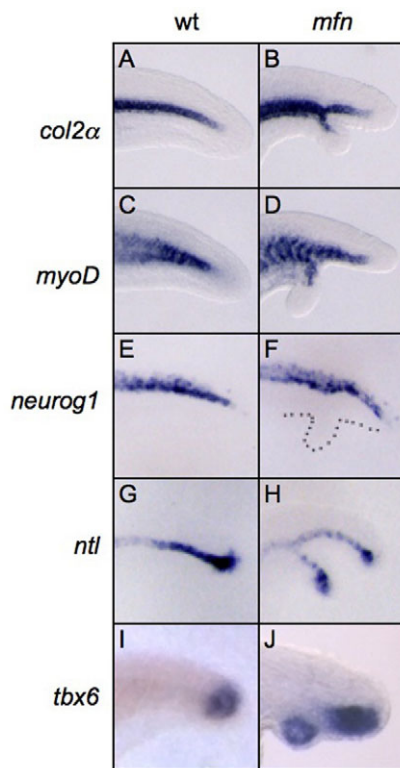
Fig. 1. Mesoderm tissues are mis-specified in the secondary tails of *mfn*. (**A-J**) Lateral view of expression of *col2a* (col2α; A,B), *myoD* (C,D), *neurog1* (E,F), *ntl* (G,H) and *tbx6* (I,J) in the posterior tail of wild-type and *mfn* zebrafish embryos. At 26 hpf, *col2a* (B), *myoD* (D) and *ntl* (H) are ectopically expressed in the secondary tail, but *neurog1* is not (F). At 24 hpf, *tbx6* (J) is also ectopically expressed in the secondary tail. Embryos in all images are mounted with anterior to the left. Dotted line in F indicates secondary tail.

between C1 and C2, which is included in the range of phenotypes typically seen in *mfn* embryos (Stickney et al., 2007). To test this model, we examined *ntl* expression in other mutants that reduce BMP signaling: *piggytail* (*smad5* – Zebrafish Information Network) and *snailhouse* (*bmp7a* – Zebrafish Information Network). These mutants display a range of dorsalized phenotypes from weak (C1) to severe (C4) (Mullins et al., 1996). In the weak *piggytail* (*smad5^{m169}*) allele, we observed that 49% of the C1 homozygotes had a secondary tail (see Table S1 in the supplementary material). In clutches of embryos from an incross of *piggytail* (*smad5^{dty40}*) carriers, we observed a range of phenotypes spanning the C1, C2 and C3 classes. The overall penetrance of secondary tail formation in all the mutant embryos was 36% (see Table S1 in the supplementary material). Notably, we observed secondary tail formation in each phenotypic class (Fig. 2A-D). Furthermore, in *snailhouse* (*bmp7^{ty68}*) embryos, which are severely dorsalized (C4), 38% formed secondary tails (Fig. 2E

and see Table S1 in the supplementary material). Taken together, we observed secondary tails in multiple BMP mutant lines independent of the severity of dorsalization. These results suggest that secondary tail formation does not require BMP signaling to be reduced to a specific level, and it is unlikely to result from expanded mesoderm, as secondary tails can form in embryos with limited tail mesoderm.

BMP signaling is required during early somitogenesis to inhibit secondary tail formation

To determine the temporal requirements for BMP in this process, we used the BMP antagonist dorsomorphin (DM) (Yu et al., 2008). We treated embryos at progressively later stages, beginning at the 1-somite stage, then scored for the presence of a secondary tail at 24 hpf. Nearly all embryos treated with DM beginning at 6 somites lacked ventral tailfin at 24 hpf, with ~30% also forming a secondary tail (Fig. 2F). Embryos treated with DM after 7 somites showed normal or slightly reduced ventral tailfin with only a few embryos forming a secondary tail (Fig. 2F). In rare cases, such embryos formed a secondary tail even in the presence of a fully formed ventral tail fin (Fig. 2G). This time frame is very similar to that observed in dnBMPR transgenic embryos (Pyati et al., 2005). Our data indicate that the crucial periods for specifying ventral tailfin and for prevention of secondary tail formation overlap significantly. However, the two processes appear to be separable, indicating that secondary tails might be formed in the absence of overt fate patterning defects.

Although *bmp4* mutants do not form secondary tails, it has been suggested that the expansion of blood and muscle fates observed in *bmp4* embryos could contribute to their formation in other BMP-compromised embryos (Stickney et al., 2007). To investigate whether expansion of blood and somitic mesoderm is associated with secondary tail formation, we examined *gata1* (*gata1a* – Zebrafish Information Network), *fli1* (*fli1a* – Zebrafish Information Network) and *myoD* expression in DM-treated embryos. For each marker, we treated 60 embryos with DM beginning at the 5-somite stage. We fixed half of these at the 13-somite stage and stained for *gata1*, *fli1* or *myoD*. To get an estimate of the penetrance of secondary tails in each group of embryos, we stained the other 30 embryos for *ntl* at 24 hpf. *gata1* expression was indistinguishable from wild type in 27/30 embryos (Fig. 2H,I), although three showed weak expansion of *gata1* expression at the posterior end (data not shown). Our *ntl* staining indicated that 11/30 embryos formed a secondary tail in this experiment. Similarly, we observed no expansion of *fli1* or *myoD* in DM-treated embryos (Fig. 2J-M), despite approximately a third (11/30) of the sibling embryos going on to form a secondary tail. As a further test of whether somitic muscle is expanded in embryos producing secondary tails, we stained *mfn* embryos at 22 hpf with *myoD*. Whereas *myoD* expression has been reported to be expanded across the midline of the caudal tail in *bmp4* embryos (Stickney et al., 2007), we observed no such expansion in *mfn* embryos (Fig. 2N,O). Taken together, our data suggest that the role of BMP in preventing secondary tail formation might be independent of its role in cell fate patterning.

Non-canonical Wnt signaling functions with BMP in inhibiting secondary tail formation

Non-canonical Wnt signaling plays an essential role in governing morphogenesis of the posterior body (Veeman et al., 2003; Seifert and Mlodzik, 2007). *ppt* (*wnt5* – Zebrafish Information Network) and *kny* (*glypican4*, *gpc4* – Zebrafish Information Network) mutants are defective in components of the non-canonical Wnt

Table 1. Ectopic tail tissues produced in *mini fin* mutants

	col2a	*myoD*	*neurog1*	*ntl*
mfn embryos scored (*n*)	77	83	69	70
ectopic tissue (%)	53.2	30.1	0	64
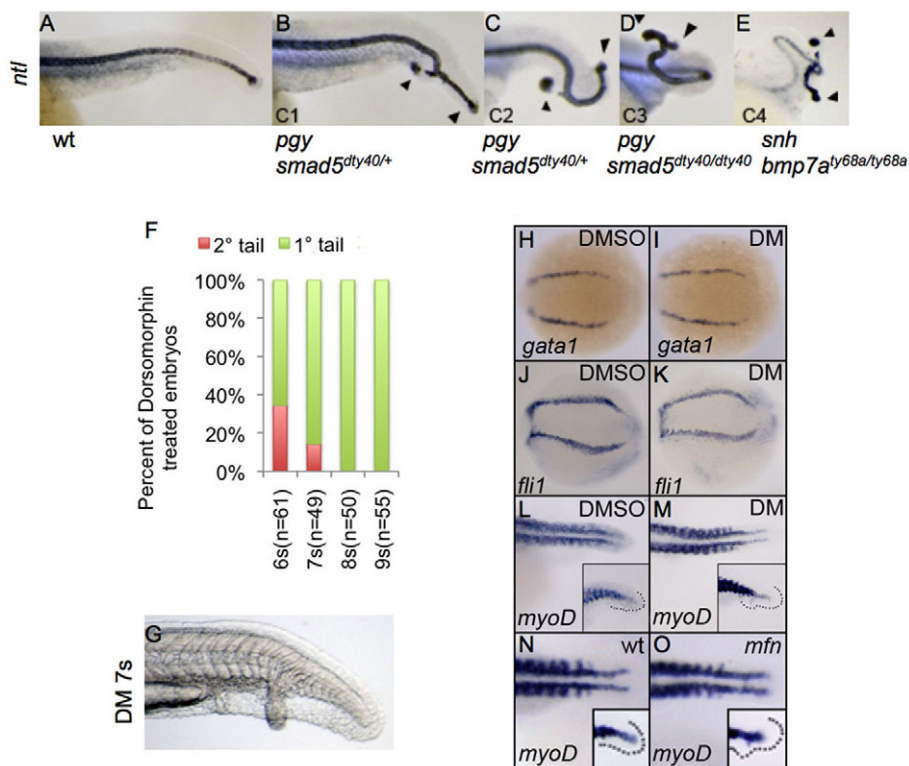
Fig. 2. Secondary tail formation is independent of the role of BMP signaling in fate patterning. (**A-E**) Lateral view of zebrafish tails in wild-type and dorsalized embryos of indicated genotypes at 24 hpf, after whole-mount in situ for *ntl*. Primary and secondary tails are marked with arrowheads. C1-C4 refer to the degree of dorsalization using the dorso-anterior index. (**F**) Distribution of secondary tail phenotype in embryos following dorsomorphin (DM) treatment at the indicated time points. Secondary tails were scored by the presence of ectopic *ntl* expression. (**G**) Lateral view of the tail of a live embryo at 24 hpf, after DM treatment at 7 somites (7s). Note the secondary tail with fully developed ventral fin. (**H-M**) Dorsal view of embryos expressing *gata1* (H,I), *fli1* (J,K) at the 13-somite stage and *myoD* (L,M) at 24 hpf in the tail, after treatment with DMSO (H,J,L) and DM (I,K,M) at the 5-somite stage. (**N,O**) Dorsal view of *myoD* expression in the tail of wild-type (wt; J) and *mfn* (K) embryos at 22 hpf. Inset shows the lateral view. Expression of *gata1* (I), *fli1* (K) and *myoD* (M,O) is unaffected.

pathway and have short tails due to impaired cell movements (Marlow et al., 2004). Strikingly, *kny* mutants have also been reported to exhibit ectopic *eve1* and *shh* (*shha* – Zebrafish Information Network) expression domains in the tail (Solnica-Krezel et al., 1996; Marlow et al., 1998), resembling the expression pattern of these markers in secondary tails of dnBMPR embryos (Pyati et al., 2005). Upon examination of later stage (72 hpf) *kny* embryos, we could observe clear bifurcations of the posterior notochord (Fig. 3C), suggesting that a secondary tail might form in these embryos. We confirmed this by observing ectopic *ntl*, *col2a* and *myoD* expression at 24 hpf (Fig. 3D-I; Table 2).

We next tested whether this phenotype is unique to *kny* mutants or is common to other embryos in which non-canonical Wnt signaling is impaired. First, we knocked down three *dishevelled* homologs, *dvl1*, *dvl2* and *dvl3*, by morpholino (MO). Knockdown of each dvl gene individually did not result in a secondary tail phenotype (data not shown). However, we found that inhibition of *dvl2* and *dvl3* simultaneously did give rise to a secondary tail in a subset of embryos (Fig. 3A,B) in addition to the previously reported mild CE defect (Angers et al., 2006).

In contrast to our observations in *kny* or *dvl2/dvl3* MO embryos, we never observed secondary tail formation in embryos in which *strabismus* (*stbm; vangl2* – Zebrafish Information Network), *prickle 1a* (*pk1a*), or *prickle 2* (*pk2*) had been inhibited, separately or in combination (see Table S2 in the supplementary material). Lastly, we tested several known non-canonical Wnt ligands (*wnt5*, *wnt11* and *wnt11r*), knocking them down using morpholinos, singly and in combination, and observed no evidence for a role in secondary tail formation for any of these genes (data not shown).

To investigate whether non-canonical Wnt signaling interacts with BMP signaling in inhibiting secondary tail formation, we tested for synergy between *mfn* and *dvl* by injecting suboptimal amounts of morpholinos for each gene and scoring embryos for secondary tail formation (Fig. 3J). Although partial knockdown of

mfn showed a very low penetrance of secondary tail formation, we observed moderate enhancement with partial knockdown of *mfn* and *dvl3* simultaneously, and greater enhancement with partial knockdown of *mfn*, *dvl2* and *dvl3*. Injection of suboptimal amounts of *dvl2/dvl3* MO into *mfn* embryos also caused a robust enhancement (Fig. 3J). Lastly, we scored the penetrance of secondary tails in *mfn;kny* double mutant embryos. In *mfn;kny*, strong enhancement of secondary tail formation was observed (88% of double mutant embryos formed a secondary tail, Fig. 3K), approximately what one would expect for a simple additive effect on penetrance of this phenotype. Together, these genetic interactions are consistent with a model in which non-canonical Wnt signaling and BMP signaling function in parallel to inhibit secondary tail formation.

BMP and non-canonical Wnt signaling are required to prevent bifurcation of the caudal notochord during tail outgrowth

During our earlier observations in BMP-deficient embryos, we did not observe a clean bifurcation of the notochord when stained with *ntl*, but, instead, the normally compact caudal end of the notochord appeared stretched and elongated (data not shown), suggesting the possibility that a defect in notochord morphogenesis could underlie the defect. As the caudal end of the notochord is a source of notochord progenitors, mislocalization of cells from this region could account for the ectopic notochord cells we observe in secondary tails. For simplicity, we will refer to these progenitor cells as caudal notochord (CN) cells.

Table 2. Ectopic tail tissues produced in *knypek* mutants

	col2a	*myoD*
kny embryos scored (*n*)	112	107
2° tail (%)	56.3	51.4
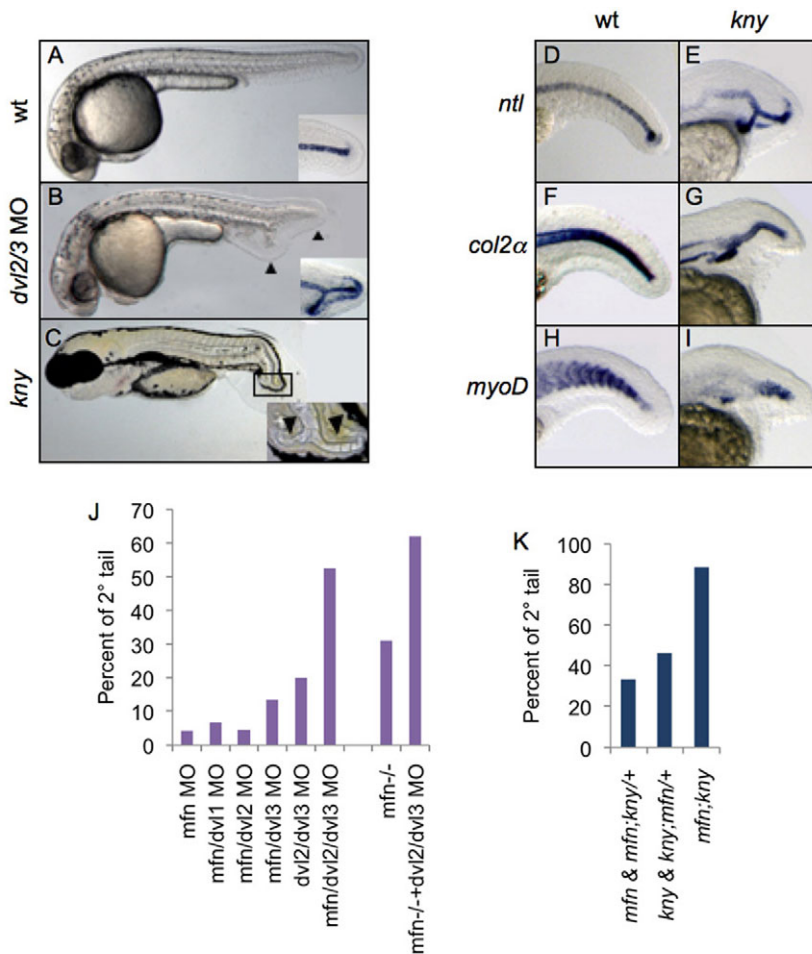
Fig. 3. Non-canonical Wnt signaling functions with BMP to inhibit secondary tail formation. (**A-C**) Lateral view of wild-type (A, 24 hpf), *dvl2/3* morphant (B, 24 hpf) and *kny* mutant (C, 72 hpf) zebrafish. Insets of A and B show *ntl* expression in the posterior tail. Inset of C shows a close-up of posterior tail. Primary and secondary tails are marked with arrowheads. (**D-I**) Lateral view of expression of *ntl* (D,E), col2a (*col2α*; F,G) and *myoD* (H,I) in the tail of wild-type and *kny* embryos fixed at 24 hpf. *ntl* (E), *col2a* (G), and *myoD* (I) exhibit ectopic expression within the tails of *kny*. (**J**) Percentages of secondary tail formation in embryos injected with indicated combination of morpholinos (MO; 1 mg/ml each, except for 'dvl2/dvl3 MO' condition, where a concentration of 3 mg/ml for each MO was injected). For each column over 100 embryos from three separate experiments were scored. (**K**) Percentages of secondary tail formation in embryos from incross of *mfn/+;kny/+* double mutant carriers. Embryos are categorized by phenotypes. Secondary tails in J and K were scored by *ntl* expression.

We first tested whether the secondary tails contained ectopic CN cells by performing in situ hybridization for *flh*, which is expressed in the CN during tail development. In both *mfn* and *kny* embryos at 24 hpf, *flh* was ectopically expressed in the region of the secondary tail (Fig. 4A-C). We confirmed this result by observing ectopic expression of a second CN marker, *fgf4* (see Fig. S1 in the supplementary material).

In order to understand the emergence of the ectopic CN cells, we performed a time-course experiment by fixing *mfn*, *snh* and *kny* embryos hourly during tail outgrowth and using *flh* expression to determine the onset of the secondary CN domain (see Fig. S2 in the supplementary material). For each mutant, we initially observed a single domain of *flh* expression in all embryos, but between 18-24 hpf, we observed increasing numbers of embryos with either elongated CN domains or two distinct CN domains (see Fig. S2A-C in the supplementary material). Although these data cannot rule out the possibility that new *flh* expression is contributing to the ectopic expression that we observe, we favor the interpretation that the CN domain is specified normally in both *mfn* and *kny* embryos, but becomes split during tail extension. This suggests that a defect in morphogenetic movements could underlie the secondary tail phenotype, which we confirmed by time-lapse analysis (see below).

N-cadherin is required to maintain tailbud integrity

Proper regulation of intercellular adhesion is important for proper cell movements (Halbleib and Nelson, 2006). Both the BMP pathway and non-canonical Wnt signaling have been demonstrated to regulate cell movements during gastrulation, in part by regulation of the localization or activity of cadherins (Ulrich et al., 2005; von der Hardt et al., 2007). As a recent study has shown that a semi-dominant allele of N-cadherin (*cdh2*) perturbs cell cohesion in the tailbud (Harrington et al., 2007), we asked whether disruption of cell adhesion in the tailbud affects CN morphogenesis. We performed genetic interaction assays between *dvl2/3* and *cdh2*. Co-injection of suboptimal amounts of *dvl2/3* MO and *cdh2* MO, which did not cause any tail defects when injected separately, induced notochord bifurcations (Fig. 5A,C-E). At higher doses of *dvl2/3* MO and *cdh2* MO, we observed dramatically scattered notochord cells rather than simple bifurcation (Fig. 5F). Interestingly, in *kny* embryos, we occasionally observed embryos with triple-branched notochords (see Fig. S3 in the supplementary material), which resemble these severe *dvl/cdh2* MO morphants (Fig. 5F). Similarly, *cdh2* MO significantly enhanced the secondary tail penetrance in *mfn* morphants (Fig. 5B). Together, these results indicate that proper regulation of cell adhesion is essential for maintaining the coherence of the CN during tail development, suggesting the possibility that BMP and/or the non-canonical Wnt pathway could act to prevent tail bifurcation by regulating intercellular adhesion in the tailbud.

Non-canonical Wnt signaling is required for proper localization of cadherin

During gastrulation, *wnt11* promotes coherence of the anteriorly migrating prechordal plate cells by regulating the localization of E-cadherin (Ulrich et al., 2005). To determine whether cadherin localization is regulated by non-canonical Wnt signaling in the

flh
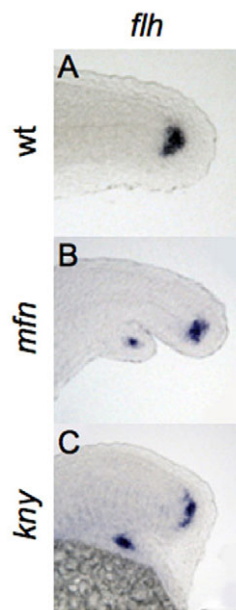
Fig. 4. Bifurcation of the caudal end of notochord in BMP and non-canonical Wnt mutants. (**A-C**) Lateral view of expression of *flh*, marking the caudal end of notochord in wild-type, *mfn* and *kny* zebrafish embryos fixed at 24 hpf. *flh* is expressed in the secondary tail of *mfn* (B) and *kny* (C) mutants.

tailbud, we stained embryos with a pan-cadherin antibody. In wild-type embryos, cadherin was localized to the plasma membrane of CN cells (Fig. 5H), whereas in the CN of *kny* embryos, cadherin was localized more intracellularly (Fig. 5I). In most *kny* embryos, we also observed a similar mislocalization in more anterior notochord cells also (data not shown). We never observed this aberrant cadherin localization in posterior tailbud cells or presomitic mesoderm cells of *kny* embryos. We also analyzed the localization of β-catenin in *kny* embryos, and observed no defects in its normal membrane localization (see Fig. S4 in the supplementary material). Lastly, both cadherin and β-catenin were localized normally in *mfn* embryos (Fig. 5J and see Fig. S4 in the supplementary material). These results indicate that non-canonical Wnt signaling, but not BMP, is required for proper localization of cadherin both within notochord progenitors and the differentiated notochord.

CN bifurcation occurs during tailbud protrusion

Our temporal studies with dorsomorphin indicate that the 6- to 7-somite stage is a crucial period for BMP signaling, as inhibition of BMP beginning at this stage, but not later, is capable of inducing formation of secondary tails. Known BMP ligands in the tailbud are expressed at this stage in the posterior tailbud, suggesting that the action of BMP could be indirect (Dick et al., 2000; Thisse et al., 2004). To determine more precisely where in the tailbud BMP is active, we performed immunostaining with anti-phosphorylated Smad1/5/8 (P-Smad5) antibody in embryos at the 6-somite stage, and combined this assay with fluorescent in situ hybridization (FISH) using *flh* probe to visualize the CN. Consistent with a recent study (Esterberg et al., 2008), we observed activation of BMP signaling in the posterior mesoderm of the tailbud, separate from the CN, persisting at least until the 14-somite stage (Fig. 6A,B).

By contrast, we observed no activation of BMP signaling in the tailbud of *mfn* embryos (Fig. 6C), although these embryos showed normal phosphorylation of Smad1/5/8 in the notochord.

To understand how BMP signaling regulates CN morphogenesis by its activation in the posterior tailbud, we performed confocal time-lapse imaging of the developing tail in *Tg(flh:EGFP)* and *mfn;Tg(flh:EGFP)* embryos. During tailbud extension in wild-type embryos, the CN moved along the surface of the yolk cell in close association with Kupffer's vesicle (KV) (Fig. 6D and see Movie 1 in the supplementary material). As the tailbud entered the protrusion phase at the 12-somite stage, the most ventral posterior tailbud cells, coming from a region of high BMP activity (see Fig. 6B), moved anteriorly and began to undercut KV (Fig. 6D′). Progressively, more cells from the ventral side moved in between KV and the yolk, and this was accompanied by notochord extension and yolk constriction (Fig. 6D″). As a result, the CN, together with KV, was lifted away from the surface of the yolk and the tail began to extend off the yolk ball (Fig. 6D‴).

In *mfn* embryos, after a comparatively normal extension stage (Fig. 6E and see Movie 2 in the supplementary material), the most ventral cells failed to undercut KV. Instead, they moved towards the yolk, often forming an indentation (Fig. 6E′). This had the effect of drawing KV and the CN ventrally towards, or even into, the yolk cell. As tail extension continued, the lengthening notochord eventually caused the CN to adopt an elongated shape (Fig. 6E″). At the end of tailbud protrusion, the stretching of the CN became more severe (Fig. 6E‴). Depending on the embryo, these ectopic CN domains could either be completely 'left behind' during tail elongation, or remain connected to the main axis by branching of the notochord.

We also performed time-lapse imaging in *kny;Tg(flh:EGFP)* embryos (see Movie 3 in the supplementary material). We did not notice any change of CN morphology until at about the 14 somite stage, when the tailbud began to protrude off the yolk (Fig. 6F′). As the most ventral cells in *kny* undercut KV, we observed cells gradually detaching away from the CN (Fig. 6F″). Over time, the ectopic CN cells formed an elongated cluster (Fig. 6F‴). Our observations suggest that cell movements occur roughly normally in *kny* embryos, albeit at a reduced rate, and that the CN is unable to maintain coherence during tail extension and protrusion.

BMP regulates migration of posterior mesoderm cells

To determine the identity of the cells that undercut KV and the CN, we performed immunostaining with a Tbx6 antibody to label presomitic mesoderm cells in *Tg(flh:EGFP)* and *mfn;Tg(flh:EGFP)* embryos at several stages of tail development. In wild type, we observed a band of Tbx6-positive cells located beneath the CN at the 16-somite stage (Fig. 7A), suggesting that those cells migrating beneath the CN are posterior mesodermal cells. Notably, these cells show high levels of P-Smad5 staining (see Fig. S5 in the supplementary material). In *mfn* embryos, a cluster of Tbx6-positive cells is retained in the tailbud (Fig. 7B). Consistent with our time-lapse video of *mfn* embryos, at least some of these posterior mesoderm cells eventually moved anteriorly and gradually surrounded the ectopic CN domain (Fig. 7D). Taken together, our data suggest that splitting of the CN upon loss of BMP signaling is likely to be a secondary consequence of the aberrant migration of ventral-posterior presomitic mesoderm cells. The defective movement of these Tbx6-positive cells at least partially accounts for our observation that secondary tails always contain cells expressing markers of presomitic mesoderm (*eve1* or *tbx6*).
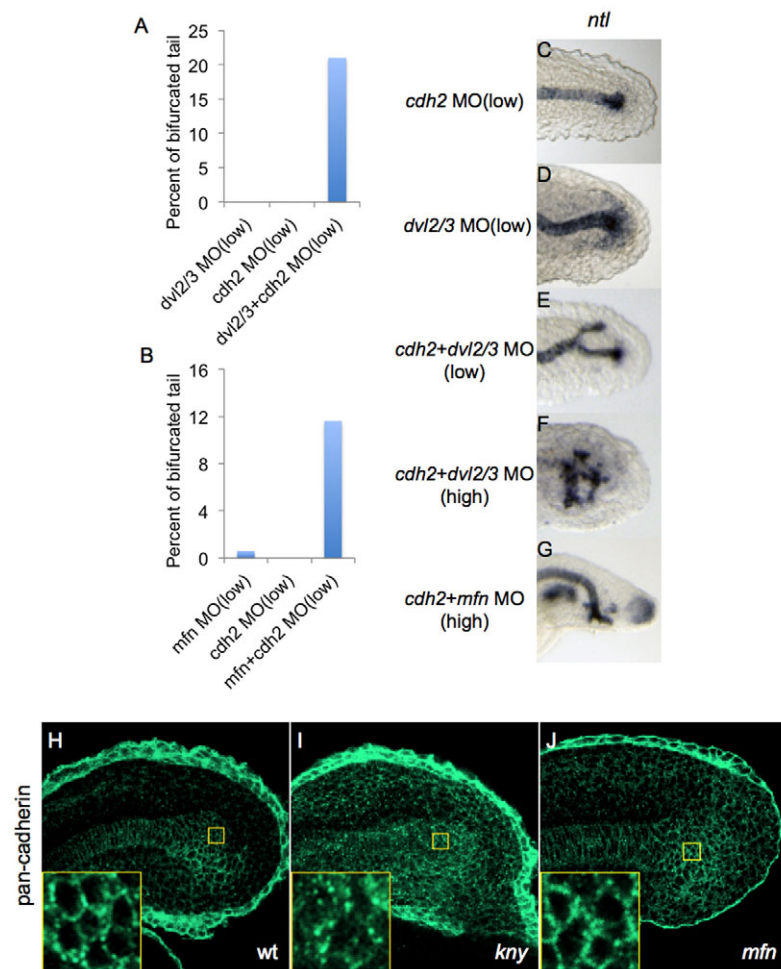
Fig. 5. N-cadherin is required to maintain tailbud integrity. (**A**,**B**) Percentages of bifurcated tail in zebrafish embryos injected with the indicated combination of morpholinos (MOs). For low concentrations, *dvl2/3* (1 mg/ml each), *mfn* (0.5 mg/ml) and *chd2* (0.1 mg/ml) MOs were injected. For each column over 100 embryos from three separate experiments were scored. (**C-G**) Lateral view of tails of embryos at 24 hpf injected with the indicated combination of morpholinos, after in situ hybridization for *ntl*. *ntl* expression in *cdh2* and *dvl2/3* MO morphants (C,D) is similar to wild type. Note that co-injection of a high concentration of *chd2* and *dvl2/3* MOs further enhanced the *ntl* expression from a single fork shaped pattern (E) to a multi-fork pattern (F), but co-injection of high concentration of *chd2* and *mfn* MOs (G) did not result in a similar phenotype. This difference is consistent with the following antibody staining results (see below). For high concentrations, *dvl2/3* (1.5 mg/ml each), *mfn* (1.5 mg/ml) and *chd2* (0.25 mg/ml) MOs were injected. (**H-J**) Representative confocal microscopy images of tailbud in wild-type, *kny* and *mfn* embryos stained with pan-cadherin antibody at 21 somites; inset, close-up of the caudal end of notochord. Membrane staining pattern in the caudal end of notochord is shown in the wild type (H) and the *mfn* (J) mutant, whereas diffused staining pattern is shown in the *kny* mutant (I).

We performed a similar time-course experiment in *kny;Tg(flh:EGFP)* embryos. Migration of the Tbx6-positive cells is delayed in *kny* embryos; these cells are retained posteriorly at 18 hpf (Fig. 7F). Only later, at the 21-somite stage, do we observe some Tbx6-positive cells located ventrally beneath the CN (Fig. 7H). At 23 hpf, we observed Tbx6-positive cells colocalized with ectopic CN cells (Fig. 7J), suggesting that these Tbx6 cells were pushed along as the ectopic CN cells drifted out from the main axis. Consistent with our time-lapse video (Fig. 6F″), the undercutting movement of the posterior mesoderm cells still occurs in *kny* embryos, although with delayed timing.

DISCUSSION
BMP signaling inhibits secondary tail formation
A recently proposed model suggests that a gradient of BMP activity in the tailbud patterns the fates of tail mesoderm, and that within a narrow range of BMP inhibition, ectopic presomitic mesoderm is produced that leads to formation of a secondary tail (Stickney et al., 2007). Two lines of evidence indicate that the gradient model does not adequately explain the formation of secondary tails. First, our observation that secondary tails are produced in several BMP pathway mutants of widely varying strengths indicates that secondary tails are not a consequence of a precise adjustment of a BMP activity gradient within a narrow range. Second, secondary tails are formed in the absence of the fate patterning defects predicted by the BMP gradient model. Thus, our findings do not support the BMP morphogen model as an explanation of secondary

tail formation. Rather, we propose that BMP signaling independently regulates morphogenesis and cell fate patterning in the tailbud during early somitogenesis. Our results are consistent with observations of dnBMPR embryos by Pyati and Kimelman, which led them to suggest that mesodermal progenitors were being left behind during tail extension, subsequently forming a secondary tail (Pyati et al., 2005).

During gastrulation, BMP is thought to create a gradient of intercellular adhesion along the DV axis, from low levels on the ventral side, where BMP activity is highest, to high levels dorsally, where BMP activity is low. Migrating mesodermal cells are therefore moving away from regions of high BMP signaling/low adhesion towards regions of low BMP activity/higher adhesion (Myers et al., 2002; von der Hardt et al., 2007). Although we observe a similar directionality (movement of mesodermal cells away from a region of high BMP activity), we do not know if an analogous gradient of adhesion is established in the tailbud by BMP. A key phenotypic distinction between the role of BMP in regulation of cell migration during gastrulation and the defects we observe here is that during gastrulation, dorsalward movement of lateral mesoderm is completely blocked in BMP-compromised embryos, whereas in the tailbud, posterior mesodermal cells still move anteriorly in *mfn* mutants, but move aberrantly into the yolk cell instead of undercutting KV. Precisely why these cells take this path remains unclear, although one possible explanation is that KV becomes more tightly associated with the yolk cell in BMP-compromised embryos, thereby blocking the movement of the
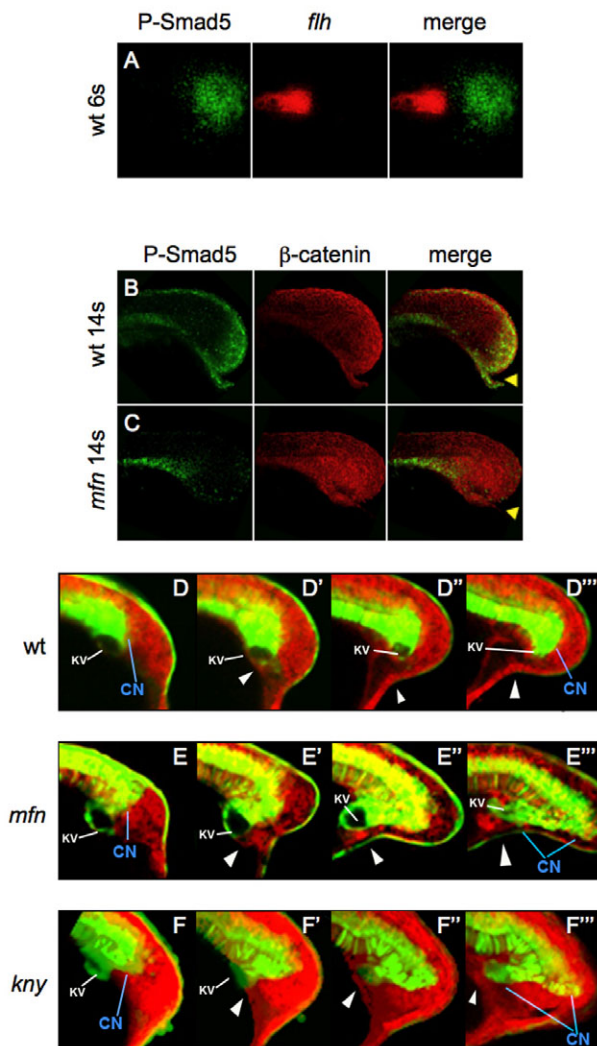
Fig. 6. Defects of the caudal end of notochord in *mfn* and *kny* initiate during tailbud protrusion. (**A**) Dorsal view of flat-mounted tailbud of a 6-somite wild-type zebrafish embryo, after fluorescence in situ hybridization (FISH) for *flh* (red) and antibody staining for P-Samd5 (green); no colocalization (merge) is seen. (**B,C**) Lateral view of tails of 14-somite wild-type and *mfn* embryos exposed to P-Smad5 (green) and β-catenin (red) antibodies. P-Smad5 staining is absent in the posterior tailbud of *mfn* (C, merge). Arrowheads indicate the ventroposterior cells in the tailbud that are normally positive for P-Smad5 (B, merge). (**D-F′′′**) Confocal time-lapse recording of tailbuds in embryos expressing *Tg(flh:EGFP)* transgene (green) and mRFP (red) from the 10-somite stage. Shown are wild type (D-D′′′) and *mfn* (E-E′′′) and *kny* (F-F′′′) mutants. Note that transgenic EGFP is retained in the caudal notochord, floorplate and periphery of Kupffer's vesicle (KV; marked by the white line). The caudal end of notochord (CN) is indicated by blue line. Arrowheads mark the ventroposterior cells in the tailbud. Embryos were all mounted laterally.

presomitic mesoderm cells. However, in embryos where the dorsal organizer region was surgically extirpated near the end of gastrulation, and also in embryos where formation of KV was blocked by blastula-stage injection into the yolk cell of *ntl* and *spt* MOs, we still observed ectopic *tbx6*-postive cells associated with the yolk extension (Y.Y., unpublished results), indicating that these cells migrate aberrantly even in the absence of KV or the CN.

During gastrulation, BMP is proposed to regulate cell adhesion in a cadherin-dependent manner (von der Hardt et al., 2007). We observed a strong enhancement of the secondary tail phenotype when *cdh2* (encoding N-cadherin) was knocked down in conjunction with partial knockdown of BMP. Although this result indicates that regulation of cell adhesion is important for proper morphogenesis of the tailbud, whether BMP functions in this process via regulation of cadherin function is unclear. For example, *cdh2* is expressed in the notochord as well as the tailbud (Lele et al., 2002; Harrington et al., 2007), raising the possibility that the synergy we observe is due to separate effects of BMP on the posterior tailbud and N-cadherin on the notochord. Alternatively, *cdh2* and BMP might both regulate migration of posterior mesoderm without necessarily functioning in the same pathway. More work, including a detailed examination of morphogenetic movements occurring in the tailbuds of *mfn;cdh2* MO embryos, will be required to address these possibilities more definitively.

## Somitic muscle formation in secondary tails

Our data indicate that although nearly all secondary tails contain ectopic presomitic mesoderm cells, only a subset of them actually form differentiated somitic muscle. It could be that in their ectopic location, presomitic mesoderm progenitors might not be fully competent to produce somitic muscle or, if they do, they might tend to be incorporated into somites produced by the primary tailbud. When we do observe ectopic *myoD* cells in the secondary tail, they are invariably part of a group of cells branching off from the main axis (see Fig. 1D).

Interestingly, in other vertebrates, the caudal end of the notochord has been shown to comprise a population of cells, termed the chordoneural hinge (CNH), which has been shown to act as a pool of progenitor cells that can give rise to multiple tissues, including notochord, floor plate and somitic muscle (Charrier et al., 1999; Davis and Kirschner, 2000; Cambray and Wilson, 2002; Cambray and Wilson, 2007). In *Xenopus*, when the CNH is grafted to host embryos, it is able to produce an ectopic tail (Gont et al., 1993). It is tempting to speculate that the caudal notochord in zebrafish could function similarly, and thus serve as a source for both the ectopic notochord and somitic muscle cells that we observe in secondary tails. However, in zebrafish, there is as yet no evidence that this region possesses the same mix of progenitor cells observed in other vertebrates, so at this time we favor the hypothesis that the ectopic notochord and muscle cells in the secondary tail arise from two distinct populations of cells: the CN and the aberrantly migrating Tbx6-positive cells, respectively.

Non-canonical Wnt signaling and Cadherin localization

To date, although we have observed secondary tails in *kny* and *dvl2/dvl3* MO embryos, the ligand for this putative non-canonical Wnt pathway remains unidentified. Our observation that other genes that regulate CE movements during gastrulation, such as *stbm*, *pk1* and *pk2*, are not required to prevent secondary tail formation suggests that secondary tails are not formed merely as a by-product of earlier defects in morphogenesis. However, we do observe that inhibition of *stbm* or *pk1* can partially suppress the penetrance of secondary tail formation in *kny* and *dvl2/3* MO embryos (data not shown), suggesting that *stbm* and *pk1* might act antagonistically to *kny* and *dvl* in this context. Although such antagonism has been shown between *stbm* (*Van Gogh* – FlyBase), *pk* and *frizzled* signaling in
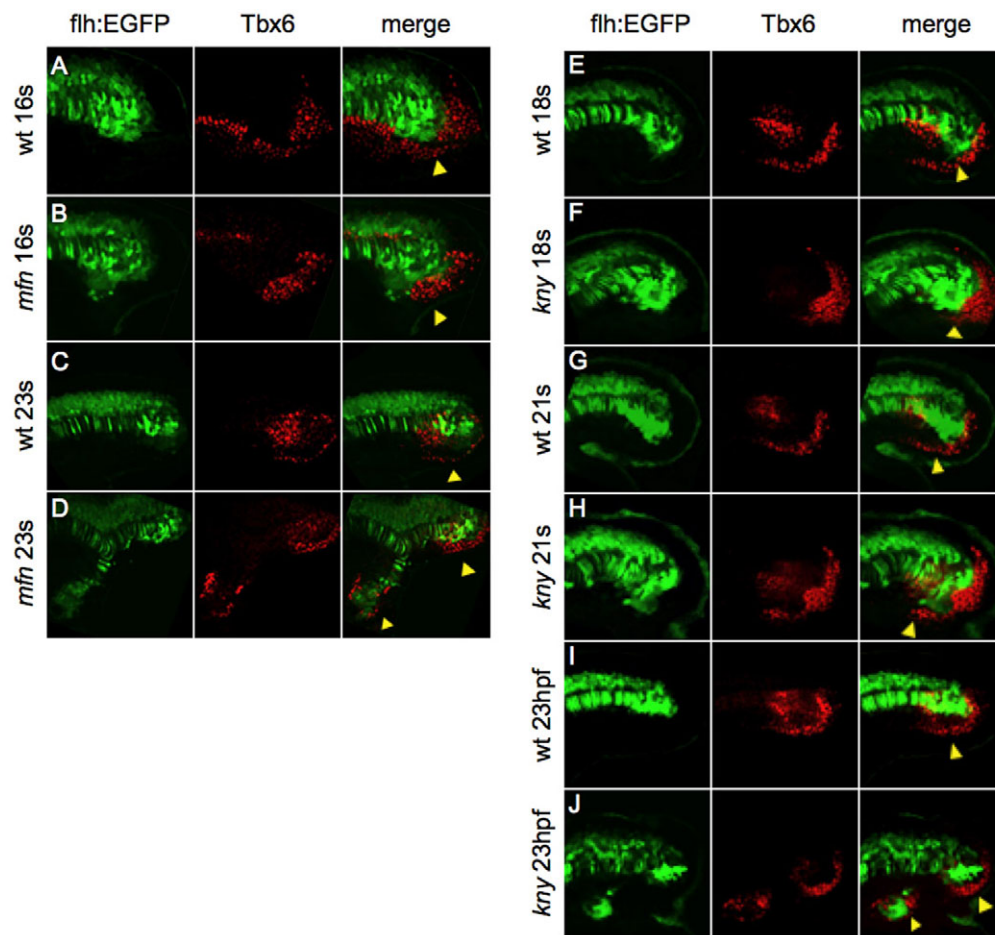
Fig. 7. BMP regulates posterior mesoderm cells of the tailbud to inhibit secondary tail formation. (**A-J**) All panels show the midline section of tails of *Tg(flh:EGFP)*-expressing zebrafish embryos (green) at the indicated time points after anti-Tbx6 (red) immunostaining. Tbx6-positive mesoderm cells in *mfn* embryos (B) shows detention in the posterior tailbud, when compared with wild-type embryos (A) at the same stage. A more wild-type-like movement of posterior mesoderm cells in *kny* embryos is shown in F and H. Note that eventually ectopic Tbx6-positive mesoderm cells are shown in the secondary tail domain of *mfn* (D) and *kny* (J) embryos. Arrowheads mark the ventroposterior mesoderm cells in the taillbud. All tails are flat mounted laterally.

Drosophila, much work remains to be done in order to clarify the roles of these genes and to identify additional genes that participate in this pathway.

During gastrulation, Wnt11 has been proposed to promote the cycling of E-cadherin between the plasma membrane and endosomes in migrating prechordal plate cells (Ulrich et al., 2005). In the absence of Wnt11, E-cadherin is predominantly localized at the plasma membrane, whereas in Wnt11-overexpressing cells, E-cadherin is observed most often in endosomes. Curiously, in the CN, we observe the opposite effect: cadherin becomes more intracellularly localized in the absence of Wnt signaling, rather than in its presence. The significance of this difference is unclear. One possibility is that prechordal plate cells, which actively migrate across a substrate of other cells and extracellular matrix, need to constantly remodel adhesion complexes in order to both move over their substrate and remain associated with other prechordal plate cells. It is not clear whether CN cells behave in the same way; posteriorward extension of the CN appears to be more passive, driven predominantly by CE movements occurring more anteriorly. Perhaps in this situation, more static localization of cadherin to the plasma membrane is sufficient to maintain coherence of the CN.

A model for the roles of BMP and non-canonical Wnt signaling in tail morphogenesis

Our data suggest that BMP and non-canonical Wnt signaling function independently of each other to ensure proper morphogenesis of the tail (Fig. 8). BMP signaling, acting on ventroposterior mesodermal precursors, promotes the anteriorward

migration of these cells, undercutting KV and disrupting the association of the CN with the yolk prior to tail protrusion. When BMP signaling is blocked, aberrant migration of the mesodermal progenitors into the yolk can capture the CN, leading to its bifurcation as tail development proceeds.
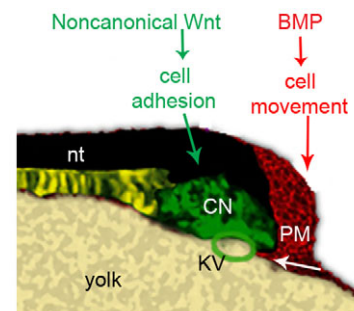
Fig. 8. A model for the roles of BMP and non-canonical Wnt signaling in regulating tail development. Depiction of the tailbud of a 10-somite wild-type zebrafish embryo. During tailbud protrusion, BMP signaling in the posterior tailbud plays a crucial role in guiding the ventroposterior mesoderm cells to move beneath the Kupffer's vesicle properly. Non-canonical Wnt signaling promotes intercellular adhesion of cells in the caudal end of notochord, allowing them to maintain cohesion during tail elongation. CNH, the caudal end of notochord; nt: notochord; PM, posterior mesoderm; KV, Kupffer's vesicle.

Non-canonical Wnt signaling promotes the localization of cadherin to the plasma membrane in CN cells, possibly increasing intercellular adhesion. As the CN moves posteriorly during tail extension, it encounters a continuous stream of anteriorly migrating mesodermal progenitors from the posterior tailbud. We hypothesize that the CN might experience some shear stress as the result of moving through this field of cells, and that in the absence of non-canonical Wnt signaling, it is unable to maintain sufficient cohesion, leading to a sloughing off of CN cells. When both pathways are blocked, nearly all embryos form a secondary tail, possibly because a less cohesive CN will nearly always split when 'caught' in the yolk. Thus, we propose that these two pathways, which function independently to regulate convergence-extension during gastrulation, work in parallel later in development to govern morphogenesis in distinct regions of the tailbud.

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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