Wnt/β-catenin regulation of the Sp1-related transcription factor *sp5l* promotes tail development in zebrafish

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Summary

Tail formation in vertebrates involves the specification of a population of multipotent precursors, the tailbud, which will give rise to all of the posterior structures of the embryo. Wnts are signaling proteins that are candidates for promoting tail outgrowth in zebrafish, although which Wnts are involved, what genes they regulate, and whether Wnts are required for initiation or maintenance steps in tail formation has not been resolved. We show here that both *wnt3a* and *wnt8* are expressed in the zebrafish tailbud and that simultaneous inhibition of both *wnt3a* and *wnt8* using morpholino oligonucleotides can completely block tail formation. In embryos injected with *wnt3a* and *wnt8* morpholinos, expression of genes in undifferentiated presomitic mesoderm is initiated, but not maintained. To identify genes that might function downstream of Wnts in tail formation, a DNA microarray screen was conducted, revealing that *sp5l*, a member of the Sp1 family of zinc-finger transcription factors, is activated by Wnt signaling. Moreover, we show that *sp5l* expression in the developing tail is dependent on both *wnt3a* and *wnt8* function. Supporting a role for *sp5l* in tail formation, we find that inhibition of *sp5l* strongly enhances the effects of *wnt3a* inhibition, and overexpression of *sp5l* RNA is able to completely restore normal tail development in *wnt3a* morphants. These data place *sp5l* downstream of *wnt3a* and *wnt8* in a Wnt/β-catenin signaling pathway that controls tail development in zebrafish.

Key words: Sp1, Tail development, Wnt, Zebrafish

Introduction

Structures of the posterior vertebrate body are derived from the tailbud, an undifferentiated group of cells at the caudal end of the embryo. The tailbud has qualities of a stem cell population, in that differentiated cell types that contribute to posterior outgrowth are continuously generated, while a population of multipotent precursors is maintained to contribute to further caudal development (Cambray and Wilson, 2002; Charrier et al., 1999; Gont et al., 1993). Within the tailbud, precise coordination of cell fate specification, proliferation and morphogenetic movements leads to caudal outgrowth of the embryo. A multitude of signaling pathways have been implicated in the regulation of these processes, among them the BMP, Nodal, FGF and Wnt pathways (Agathon et al., 2003; Griffin and Kimelman, 2003; Mathieu et al., 2004; Vasiliauskas and Stern, 2001).

Much of our current understanding of the role for Wnt/β-catenin signaling in posterior mesoderm formation in vertebrates comes from analysis of mouse mutants. For example, mice homozygous for a null allele of *Wnt3a* fail to form somites and notochord caudal to the forelimb bud, instead making ectopic neural tissue (Greco et al., 1996; Takada et al., 1994; Yoshikawa et al., 1997). These embryos form only the anterior-most seven to nine somites and completely lack a tailbud (Takada et al., 1994). Mice doubly homozygous for mutations in two downstream effectors of the Wnt pathway, *Lef1* and *Tcf1*, or homozygous for a null allele of the Wnt co-receptor, LRP6, show a similar phenotype (Galceran et al., 1999; Pinson et al., 2000).

Studies in zebrafish have also implicated Wnt/β-catenin signaling at an early step in posterior mesoderm formation. Transplantation studies show that the ventral marginal region of the zebrafish gastrula functions as a tail organizer, able to induce the formation of an ectopic tail when transplanted to the animal pole (Agathon et al., 2003). In the same study, overexpression experiments indicated that a combination of high levels of Wnt/β-catenin, Nodal and BMP signaling can induce the formation of ectopic tail organizers (Agathon et al., 2003).

wnt8 is highly expressed at the ventral margin and has been shown to be required for specification of ventrolateral mesoderm, which contributes to formation of the tailbud at the end of gastrulation (Erter et al., 2001; Lekven et al., 2001). Strong loss of *wnt8* function, either by mutation or by antisense morpholinos (MOs), results in tail formation defects (Agathon et al., 2003; Lekven et al., 2001). These defects are presaged during gastrulation by a dramatic loss of expression of ventrolateral mesoderm markers such as the T-box transcription factor *tbx6*, which is strongly expressed in the ventral margin and subsequently in the tailbud (Griffin et al., 1998; Hug et al., 1997), suggesting that *wnt8* acts at a very early step of tail formation.

While *Wnt3a* in the mouse and *wnt8* in the zebrafish clearly act at early steps in mesoderm induction and/or patterning that

subsequently affect tail development, whether Wnts also act later during tail development is less clear. In zebrafish, the continued expression in the tailbud throughout somitogenesis of both *wnt8* and another Wnt gene, *wnt3a*, as well as of a transgenic β-catenin-responsive reporter, TOPdGFP, suggests a role for continuous Wnt activity throughout tail formation (Buckles et al., 2004; Kelly et al., 1995; Lekven et al., 2001).

Relatively little is known about the identity of downstream effectors of Wnt/β-catenin signaling in tail development in any species. One direct transcriptional target of *Wnt3a* in the mouse is the *T(Brachyury)* gene, a T-box transcription factor that is also required for posterior development (Galceran et al., 2001; Yamaguchi et al., 1999). In zebrafish, the homolog of *T*, *no tail*, is required for tail formation (Halpern et al., 1993), but has not been shown to be a Wnt target. Conversely, while *tbx6* is directly regulated by Wnt signaling in zebrafish (Szeto and Kimelman, 2004), it has not been demonstrated to have a functional role in tail development.

We demonstrate that Wnt/β-catenin signaling, activated by *wnt3a* and *wnt8*, is required not only during gastrulation for specification of the tail organizer, but also during early somitogenesis for the maintenance of expression of presomitic mesoderm markers within the tailbud. We then show that the Sp1-related zinc finger transcription factor *sp5-like* (*sp5l*), is expressed in response to *wnt3a* and *wnt8* in the tailbud and acts downstream of these Wnts to regulate tail formation. Thus, Wnt signaling is required at multiple stages of tail development, acting at least in part by activating expression of *sp5l*.

Materials and methods

Zebrafish strains and maintenance

Zebrafish were raised and maintained under standard conditions. Our wild-type line is derived from AB. In addition, we used the previously published transgenic TOPdGFP line (Dorsky et al., 2002), and the hsΔTCF GFP line (Lewis et al., 2004).

In situ hybridizations and antibody staining

In situ hybridizations using digoxigenin-labeled mRNA probes were performed using standard methods (Oxtoby and Jowett, 1993). For assessing the mitotic index in the tailbud, we first processed the embryos for *tbx6* expression using a Fast Red color reaction, which produces a fluorescent product. Subsequently, embryos were incubated overnight in a 1:500 dilution of polyclonal rabbit anti-phosphohistone H3 antibody (Upstate, Charlottesville, VA, USA) in PBT+10% calf serum, washed 5× in PBT, and incubated overnight in a 1:1000 dilution of AlexaFluor 488 anti-rabbit secondary antibody (Molecular Probes, Eugene, OR USA). Mitotic cells within the *tbx6* domain were counted and divided by the total number of *tbx6*-expressing cells.

Morpholino and RNA injections

wnt3a, *wnt8* ORF1, *wnt8* ORF2 and *sp5l/spr2* morpholinos (Gene Tools, Philomath, OR, USA) have been previously described (Buckles et al., 2004; Lekven et al., 2001; Zhao et al., 2003). For sense RNA injections, mRNA was synthesized using the mMessage mMachine kit (Ambion). Both mRNA and morpholinos were diluted in Danieau's buffer prior to injection. *wnt3a* MO was injected at a concentration of 2 mg/ml, except as noted in some co-injection experiments with *sp5l* MO, where the concentration was 1 mg/ml. *wnt8* ORF1 and ORF2 MOs were injected at a concentration of 0.5 mg/ml each, and *sp5l* MO was injected at a concentration of 2.5 mg/ml. In all experiments, a volume of 3-5 nl was injected into the yolk of one-cell stage embryos.

Results

wnt3a and *wnt8* are required for posterior mesoderm formation

As part of an earlier analysis of the role of zebrafish *wnt3a* in neural patterning, we noted a mild shortening of the tail when *wnt3a* function was reduced using either of two antisense morpholino oligonucleotides (Buckles et al., 2004), suggesting that *wnt3a* might function in tail development. To determine if inhibition of *wnt3a* reduced β-catenin-mediated transcriptional activity in the developing tail, we studied the effects of knocking down *wnt3a* on expression of a transgenic β-catenin-responsive reporter, TOPdGFP (Dorsky et al., 2002). For these and all subsequent experiments, we used a splice-blocking morpholino previously shown to completely block splicing of the first intron of *wnt3a* (Buckles et al., 2004). TOPdGFP is normally robustly expressed in the tailbud at the end of gastrulation (bud stage) and during somitogenesis (Dorsky et al., 2002). Expression is only modestly reduced when *wnt3a* is knocked down (Fig. 1D,E), and *wnt3a* morphants are essentially indistinguishable from wild-type at 24 hours post-fertilization (hpf) (Fig. 1F), although they are slightly shorter by 48 hpf (data not shown).

This result suggested that additional Wnt(s) expressed in the tailbud could be acting redundantly with *wnt3a* in tail

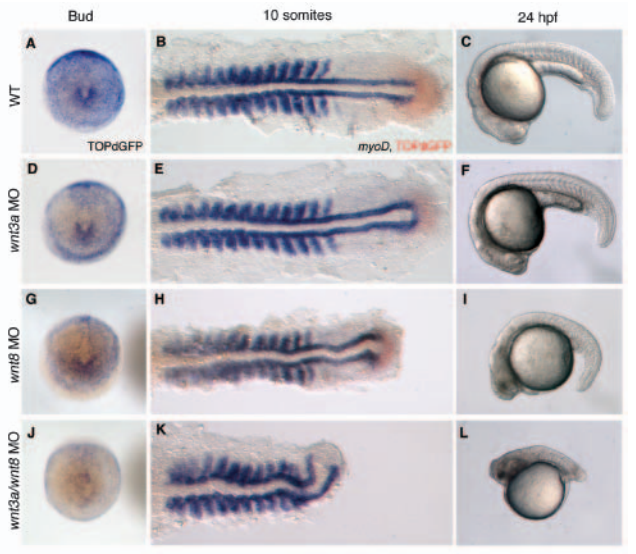

Fig. 1. *wnt3a* and *wnt8* coordinately regulate tail development. (A-C) Wild-type development. (D-L) Embryos from a transgenic TOPdGFP line were injected with *wnt3a* MOs (D,E,F), *wnt8* MOs (G,H,I) or both (J,K,L). Expression of the β-catenin-responsive reporter was examined at bud stage by in situ hybridization with a probe for GFP (A,D,G,J). Embryos are shown in a dorsal view of the tailbud, with anterior to the top. At the 10-somite stage (B,E,H,K), TOPdGFP expression was again assessed by in situ hybridization, in this case with a Fast Red color reaction. *myoD* expression, to visualize the defect in tail development, is in blue. Embryos are flat mounted and shown in a dorsal view with anterior to the left. At 24 hpf (C,F,I,L), living embryos of each type are shown in a lateral view, anterior to the left.

formation. We therefore tested whether the mild phenotype observed in *wnt3a* morphants was due to functional redundancy with *wnt8*, which is also expressed in the developing tail (Kelly et al., 1995; Lekven et al., 2001). To specifically address a potential later role for *wnt8* in tail development, we injected low doses of *wnt8* morpholinos that caused little or no effect on mesoderm patterning during gastrulation (see Fig. S1 in supplementary material, and C.J.T., unpublished results). In these experiments, we co-injected morpholinos against both open reading frames of the bicistronic *wnt8* locus, referred to hereafter for simplicity as *wnt8*. These morpholinos have previously been shown to specifically inhibit *wnt8* function (Lekven et al., 2001). Our partial knockdown of *wnt8* moderately reduced TOPdGFP expression in the tailbud (Fig. 1G,H), although tail formation was not greatly affected (Fig. 1I). In contrast, knocking down both *wnt3a* and *wnt8* results in a severe reduction in TOPdGFP expression at bud stage, and its complete absence in the tailbud by mid-somitogenesis (Fig. 1J,K). *wnt3a/wnt8* double morphants are dramatically shorter, with most embryos lacking almost all tail structures (Fig. 1L, 68% of embryos make only 1-3 tail somites, and 18% make none, n=276). We conclude that both *wnt3a* and *wnt8* are required for tail formation in zebrafish.

During gastrulation, *wnt8* is required for dorsoventral mesoderm patterning and for promoting posterior neural fates in the neurectoderm (Erter et al., 2001; Lekven et al., 2001). Since *wnt3a*, like *wnt8*, is expressed in the margin during gastrulation (data not shown), we tested whether *wnt3a* also plays a role in patterning of the mesoderm and neurectoderm by co-injecting *wnt3a* and *wnt8* MOs and examining the expression of marker genes. We found that while *wnt3a* is not required by itself for either of these processes, *wnt3a* knockdown modestly enhances the effects of partial loss of *wnt8* function in both dorsoventral patterning of the mesoderm and anterior-posterior patterning of the neurectoderm (see Fig. S1 in supplementary material). Thus, while *wnt8* is principally responsible for these two early patterning events, *wnt3a* also plays a more minor, redundant role. Consistent with this possibility, when higher doses of both *wnt3a* and *wnt8* morpholinos were injected, nearly all embryos were strongly dorsalized (data not shown). Since we wanted to specifically address a later role for *wnt3a* and *wnt8* in tail development, all further experiments were done using a hypomorphic dose of *wnt8* MOs to minimize early mesoderm patterning defects.

Wnt signaling is required during somitogenesis for maintenance of presomitic mesoderm

To gain further insight into the mechanism by which *wnt3a* and *wnt8* promote tail development, we examined the expression of several markers expressed in the tailbud at multiple time points from bud stage through early somitogenesis (Fig. 2). In both *wnt3a* (Fig. 2B,F) and *wnt8* (Fig. 2C,G) morphants, expression of *ntl* and *fgf8* is unaffected relative to wild-type (Fig. 2A,E) at bud stage. *tbx6* is expressed normally in *wnt3a* morphants (Fig. 2J, compare with wild-type in I), and is slightly reduced in *wnt8* knockdown embryos (Fig. 2K). Despite the much more severe tail phenotype in *wnt3a/wnt8* morphants, *ntl*, *fgf8* and *tbx6* are all expressed at bud stage (Fig. 2D,H,L), with *tbx6* slightly reduced, as seen in *wnt8* morphants. The relative lack of effect of the *wnt3a* and *wnt8*

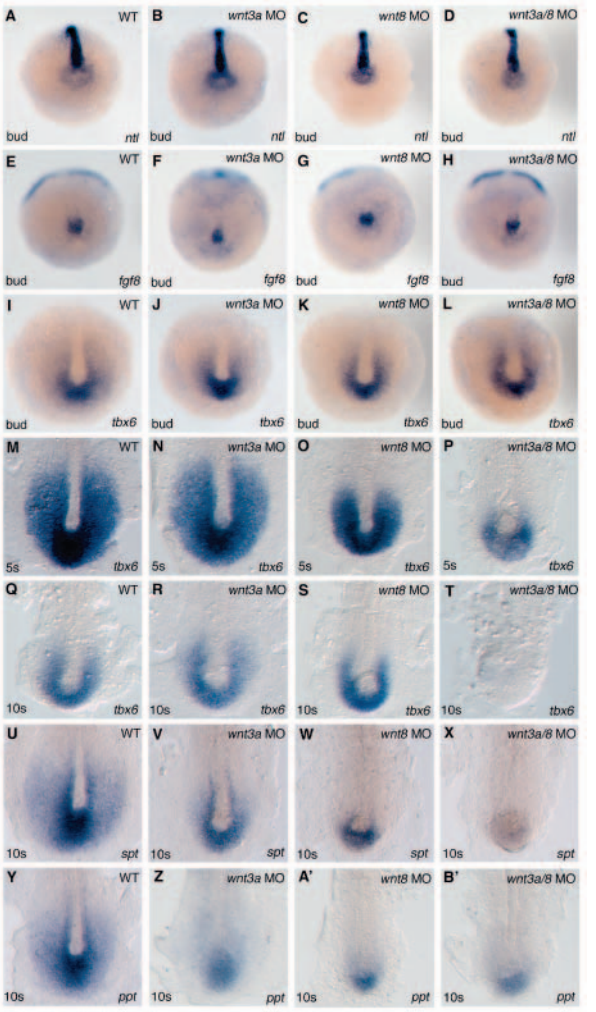

Fig. 2. *wnt3a* and *wnt8* are required for the maintenance, not initiation, of tailbud marker expression. (A-L) Embryos injected with the indicated morpholinos were fixed at tailbud stage and stained for *ntl* (A-D), *fgf8* (E-H) and *tbx6* (I-L). Embryos are shown in dorsal views, anterior to the top. (M-B′) Injected embryos were allowed to develop until the 5-somite stage (M-P) or 10-somite stage (Q-B′) and stained for *tbx6* (M-T), *spt* (U-X) or *ppt* (Y-B′). All embryos are flat mounted and shown in dorsal views with anterior to the top. By the 10-somite stage in the tailbud of *wnt3a/wnt8* MO embryos, *tbx6* (T) and *spt* (X) are not expressed, while *ppt* (B′) is still expressed at reduced levels compared with *wnt3a* MO (Z) or *wnt8* MO (A′).

MOs on the expression of these genes was somewhat surprising, since expression of the TOPdGFP reporter is dramatically reduced at this stage in *wnt3a/wnt8* morphants (Fig. 1J), suggesting that even low levels of Wnt signaling are sufficient for initial specification of the tailbud. We conclude that the block in tail development apparent by mid-somitogenesis in *wnt3a/wnt8* morphants is not due to a failure to specify early tailbud fates.

Since initial cell fates were properly specified, we then asked whether Wnt signals were required for maintenance of cell fates. As somitogenesis proceeds, *ntl* and *fgf8*, as well as *bmp4*, continue to be expressed in the tailbud of *wnt3a*, *wnt8* and *wnt3a/wnt8* morphants, although *ntl* is moderately reduced by the 10-somite stage in *wnt3a/wnt8* embryos (data not shown).

In contrast, markers of presomitic mesoderm such as *tbx6*, *spadetail* (*spt*) (Griffin et al., 1998) and *pipetail/wnt5* (*ppt*) (Krauss et al., 1992; Rauch et al., 1997) fade during early somitogenesis and are lost or drastically reduced by the 10-somite stage. In the case of *tbx6*, expression in *wnt3a/wnt8* morphants, is dramatically reduced at 5 somites (Fig. 2P; 34/41 embryos have significantly reduced *tbx6* expression relative to that seen in *wnt8* morphants, Fig. 2O), and *tbx6* expression is completely absent at 10 somites (Fig. 2T; 38/42 embryos lack expression). *spt* and *ppt/wnt5* are similarly affected in *wnt3a/wnt8* morphant embryos. At the 10-somite stage, expression of *spt* is reduced in both *wnt3a* MO and *wnt8* MO embryos compared with wild type (Fig. 2U-W), but is absent in *wnt3a/wnt8* morphants (Fig. 2X; 26/34 embryos lack *spt* expression). *ppt/wnt5* is expressed at reduced levels in *wnt3a* (Fig. 2Z) and *wnt8* (Fig. 2A′) morphants, and is even more strongly reduced in the double morphants (Fig. 2B′; 20/27 embryos). As *ppt/wnt5* is itself required for tail extension movements (Hammerschmidt et al., 1996; Rauch et al., 1997), it is possible that the reduction in *ppt/wnt5* expression could contribute to the tail truncation we observe in *wnt3a/wnt8* morphants. However, while we do observe some compression of somites along the anterior-posterior axis (see Fig. 1K), we do not observe the lateral expansion of posterior somites typical of *ppt/wnt5* mutants, nor do we observe any accumulation of cells in the tailbud as somitogenesis proceeds, but rather a pronounced reduction in size (see Fig. 1L). Thus, the effect of the reduction in *ppt/wnt5* expression is likely to be minor. Taken together, these results suggest that *wnt3a/wnt8* signaling is required for maintenance of presomitic mesoderm fates within the tailbud during early somitogenesis.

In the chick and mouse, Fgf signaling has been proposed to play a similar anti-differentiation role, such that higher levels of Fgf in the caudal tailbud inhibit the differentiation of presomitic mesoderm, and as cells move more anteriorly within the tailbud, they escape the influence of Fgf, differentiate, and form somites (Dubrulle et al., 2001; Dubrulle and Pourquie, 2004; Mathis et al., 2001). Since these data support a role for Fgf signaling in maintaining posterior cells in an undifferentiated state, we asked whether the failure to maintain presomitic fates in *wnt3a/wnt8* morphant embryos was due to a defect in Fgf signaling. Although we had already observed normal expression of *fgf8* in the tailbud (see above), at least three additional Fgf ligands, *fgf17b*, *fgf3* and *fgf24*, are also known to be expressed in this region (Cao et al., 2004; Draper et al., 2003; Phillips et al., 2001).

To test whether Fgf signaling is compromised in the tailbud of *wnt3a/wnt8* embryos, we examined expression of *sprouty4* (Fig. 3), a Fgf-induced inhibitor of the Fgf receptor that is expressed in the tailbud in response to Fgf activity (Furthauer et al., 2001). At both 5- and 10-somite stages, we observe robust expression of *sprouty4* in the tailbuds of *wnt3a/wnt8* morphants (Fig. 3D,H). In particular, at the 10-somite stage, when markers of presomitic mesoderm are completely absent or severely reduced (above, Fig. 2), *sprouty4* expression, and thus Fgf signaling, is still observed. These data indicate that a loss of Fgf signaling is not responsible for the failure to maintain presomitic mesoderm in *wnt3a/wnt8* morphants, and suggests a direct role for Wnt/β-catenin signaling in maintaining expression of presomitic mesoderm markers in the tailbud.

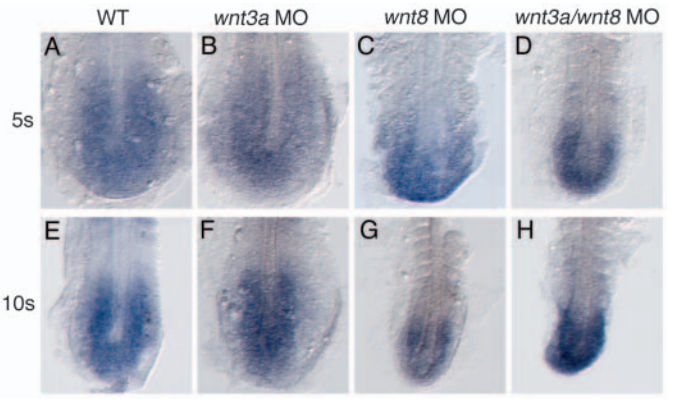

Fig. 3. Fgf signaling is not affected in the tailbud of *wnt3a/wnt8* morphants. The expression of *sprouty4* was examined at the 5-somite (A-D) and 10-somite (D-H) stages in wild-type (A,E), *wnt3a* MO (B,F), *wnt8* MO (C,G), and *wnt3a/wnt8* morphants (D,H). All embryos are shown in dorsal views of the tailbud, with anterior to the top.

We next tested whether a decrease in cell proliferation within the tailbud could account for the loss of presomitic mesodermal fates by staining embryos with an antibody to phosphohistone H3, a marker of mitotic cells, at the 4-somite stage. We observed no apparent reduction in cell proliferation in the tailbuds of *wnt3a*, *wnt8*, or *wnt3a/wnt8* morphant embryos (see Fig. S2 in supplementary material). We also measured cell death within the tailbud at several stages during somitogenesis using the TUNEL assay, and observed no obvious differences in levels of cell death between wild-type embryos and any of the morpholino-injected embryos (data not shown). In sum, our data indicate that Wnt signaling is required for maintenance of the expression of presomitic mesoderm markers, but not for regulation of cell proliferation or cell death in the tailbud.

wnt3a and *wnt8* are required for notochord and somite formation during tail development

The mouse *Wnt3a* knockout results in a severe early phenotype, with a loss of notochord and somites caudal to the forelimb bud, such that most mutant embryos make only 7-8 somites (Takada et al., 1994). Cells involuting through the primitive streak that would normally contribute to somitic tissue instead adopt a neural fate and form an ectopic neural tube (Yoshikawa et al., 1997). These results in the mouse raise the question of whether Wnt loss of function in zebrafish might result in similar fate transformations. Close examination of *wnt3a* morphants at 28 hpf shows an absence of notochord near the end of the tail (Fig. 4B, arrow; 91% of embryos have premature truncation of the notochord, *n*=253, compared with wild-type; Fig. 4A), indicating that *wnt3a* is required for late notochord development. In contrast, we observe no notochord defects in the tail of *wnt8* morphants (Fig. 4C). While most *wnt3a/wnt8* morphants completely lack or make only a rudimentary tail, the occasional weakly affected embryos have an even more penetrant and severe loss of notochord in the tail than that seen in *wnt3a* morphants (Fig. 4D, arrow; 100% of embryos have a notochord phenotype, *n*=78). We conclude that *wnt3a* and *wnt8* both function to specify notochord during tail outgrowth, with *wnt3a* playing the central role.

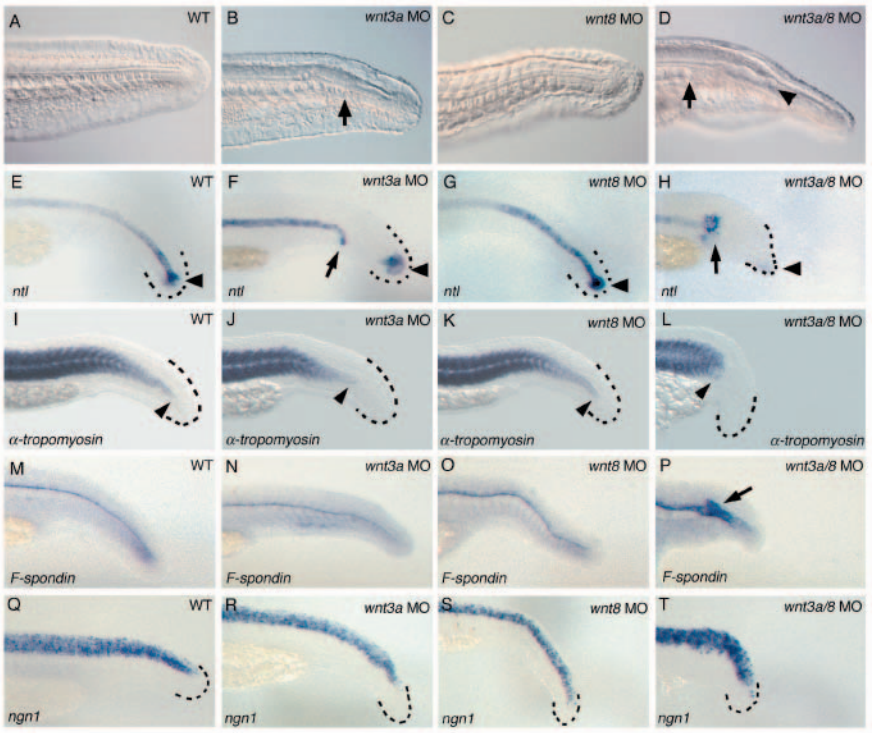

Fig. 4. *wnt3a* and *wnt3a/wnt8* morphants lack mesodermal fates at the expense of floor plate in the caudal tail. All embryos were examined at approximately 27-28 hpf. More mildly affected *wnt3a/wnt8* morphants that made some tail structures were selected for these analyses. (A-D) Tails of living 28 hpf embryos, anterior to the left. Arrows in B and D indicate premature termination of the notochord in *wnt3a* and *wnt3a/wnt8* morphants, respectively, and the arrowhead in D marks enlarged neural tube lumen. (E-P) Embryos were fixed at 26 hpf and stained with the indicated marker. The caudal tip of the tail is outlined in black dashes for clarity where necessary. (E-H) Embryos stained with *ntl* to visualize the notochord, arrowheads indicate *ntl* expression in the tailbud; arrows in F and H indicate the tip of the truncated notochord. (I-L) *α-tropomyosin* staining to visualize the somites; arrowheads mark the posterior extent of somite formation. (M-P) Embryos stained for *F-spondin* to visualize the floor plate; arrow in P indicates the expanded floor plate in *wnt3a/wnt8* embryos. (Q-T) Pan-neural *ngn1* expression in the neural tube is unaffected by *wnt3a* and *wnt8* inhibition.

The *ntl* gene is required for notochord formation in the tail (Amacher et al., 2002; Halpern et al., 1997; Halpern et al., 1993; Schulte-Merker et al., 1994), and its murine homolog, *T*, has been shown to be a direct transcriptional target of *Wnt3a* (Yamaguchi et al., 1999). We therefore tested whether *wnt3a* and/or *wnt8* regulate notochord formation in the tail by regulating *ntl* expression in the tailbud. While *wnt3a* morphants have a gap of *ntl* expression where the notochord has failed to form (Fig. 4F, arrow), expression within the tailbud is not affected at 28 hpf (Fig. 4F, arrowhead). *wnt8* morphants have normal notochord development (Fig. 4G, arrow) and also show normal levels of *ntl* expression in the tailbud (Fig. 4G, arrowhead). In contrast, *wnt3a/wnt8* morphants make no notochord tissue within their truncated tails (Fig. 4H, arrow) and also lack *ntl* expression in the tailbud (Fig. 4H, arrowhead; compare with wild type in Fig. 3E). The truncated notochords of *wnt3a/wnt8* embryos also display a characteristic sharp bend or kink at the terminus, leading to a broadened patch of *ntl* expression (Fig. 4H, arrow). This probably does not represent an anteriorward displacement of the tailbud domain of *ntl* expression, as we observe a similar pattern of notochord expression of *collagen2α*, which is not expressed in the tailbud (data not shown). Thus, while *ntl* expression is dependent on *wnt3a* and *wnt8*, loss of *ntl* expression does not account for the absence of notochord, since *wnt3a* morphants lack the caudal notochord, but still express *ntl* in the tailbud.

Since mouse *Wnt3a* is required for production of somites as well as notochord (Takada et al., 1994), we next investigated whether somites were properly formed in the tail of *wnt3a, wnt8* or *wnt3a/wnt8* morphants. Since most *wnt3a/wnt8* morphants make essentially no tail, we examined more weakly affected embryos that made a small tail. To assess the production of somites, we stained 26 hpf embryos for *α-tropomyosin* (Fig. 4I-L). While *α-tropomyosin* staining extends nearly to the tip of the tail in both wild-type (Fig. 4I) and *wnt8* morphants (Fig. 4K), we observe a slight reduction of expression in *wnt3a* morphants (Fig. 4J; 17/24 embryos), indicating a deficit in formation of somitic mesoderm. In *wnt3a/wnt8* MO embryos (Fig. 4L; 29/29 embryos), *α-tropomyosin* staining is completely absent from the caudal tail; absence of somites and premature termination of the notochord occur at a similar rostral/caudal level (compare Fig. 4H,L). Thus, *wnt3a* and *wnt8* are also required for production of somitic mesoderm in the tail.

In addition to the truncation of the notochord and loss of somites found in these embryos, we also observed an apparent expansion of neural tube tissue posterior to the end of the notochord in these more mildly affected *wnt3a/wnt8* embryos (4D, arrowhead indicates enlarged lumen of neural tube). To confirm this with molecular markers, we stained embryos at 26 hpf for *F-spondin*, which is expressed in the floor plate of the neural tube (Fig. 4M-P) (Higashijima et al., 1997). We observe a significant expansion of *F-spondin* expression in the posterior of *wnt3a/wnt8* morphants (Fig. 4P). Expression of *collagen 2α* in the floor plate is also expanded, confirming the expansion of floor plate tissue (data not shown). In contrast, the pan-neural marker *ngn1* (Korzh et al., 1998) is not expanded in *wnt3a/wnt8* morphants (Fig. 4T), suggesting that the loss of Wnt signaling results in an expansion only of the floor plate, and not of other fates within the neural tube.

Lastly, we examined the expression of markers of other tail tissues, including ventral fin epidermis (*msxb*) (Akimenko et al., 1995), blood (*gata1*) (Stainier et al., 1995), and vasculature (*fli1*) (Thompson et al., 1998), as well as the tailbud marker (*eve1*) (Joly et al., 1993). These data are presented in Fig. S3 in supplementary material. Briefly, while *eve1* expression in the tailbud is absent in *wnt3a/wnt8* morphants, blood and

vasculature is specified normally, suggesting that *wnt3a* and *wnt8* are not required for more lateral posterior mesodermal fates. Also, expression of *msxb* in the ventral tailfin was absent, possibly reflecting a mild dorsalization of *wnt3a/wnt8* embryos. Taken together, our data show that *wnt3a* and *wnt8* are required for the formation of notochord and somitic mesoderm in the tail, as well as for inhibiting production of floor plate cells.

sp5l is a downstream target of Wnt signaling

To identify genes that function downstream of Wnt signaling during early development, we performed a microarray screen for Wnt-responsive genes. Briefly, RNA from early gastrula stage embryos that had been injected with either *wnt8* RNA or GFP RNA was used to probe a microarray chip containing 8,000 zebrafish cDNAs from a mixed stage cDNA library. One gene, the RNA levels of which were significantly upregulated by overexpression of *wnt8,* was *sp5-like* (*sp5l*), a member of the Sp1 family of zinc-finger transcription factors. *sp5l* has previously been described as *spr2*, and has been implicated in mesoderm induction, acting downstream of FGF signaling (Zhao et al., 2003).

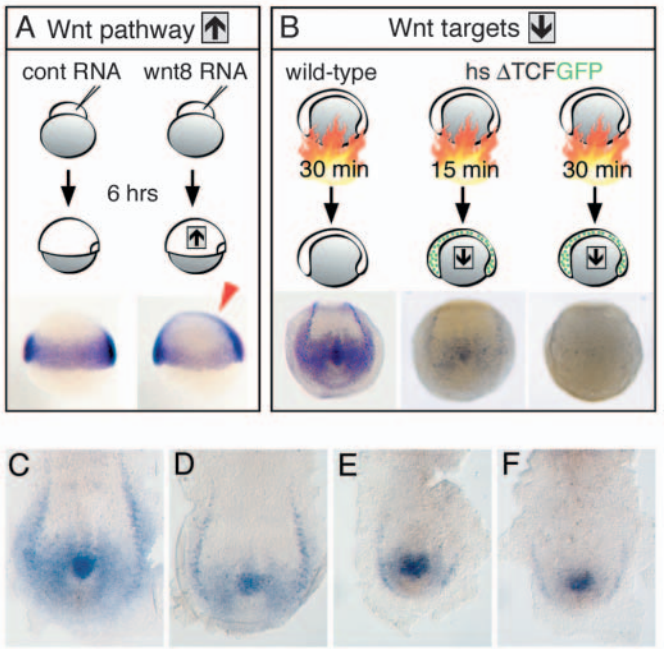

Fig. 5. *sp5l* transcription is induced by ectopic Wnt and repressed by interference with Wnt signaling. (A) Ectopic expression of *sp5l* at the animal pole of embryos injected with 10 pg *wnt8* RNA, 6 hours after injection at shield stage (right panel, dorsal view), compared with wild-type expression in embryos injected with equimolar amounts of GFP RNA (7 pg, left panel). (B) Overexpression of dominant-negative TCF rapidly abolishes *sp5l* expression. Fish heterozygous for heat-shock-inducible dominant-negative TCF-GFP [Tg (hsp70:ΔTCF-GFP)^{w26}] were outcrossed to wild type, the resulting embryos heat-shocked for 15 minutes or 30 minutes starting at the 1-somite stage and the whole clutch fixed immediately. In situ hybridization for GFP (light brown) in addition to *sp5l* (blue) was performed to identify transgenic embryos. *sp5l* expression is severely reduced in 100% (n=21) of transgenics 15 minutes after induction of the transgene (middle panel), and completely abolished in 100% (n=24) of transgenic embryos after 30 minutes (right panel). (C-F) Expression of *sp5l* in WT, *wnt3a*, *wnt8* and *wnt3a/wnt8* morphants at the 3-somite stage.

We used several independent assays to show that *sp5l* is regulated by Wnt signaling (Fig. 5). First, we injected *wnt8* RNA and examined *sp5l* expression at early gastrula stage. In embryos overexpressing Wnt8, we observed ectopic *sp5l* expression at the animal pole (arrowhead in Fig. 5A, right panel), confirming that activation of the Wnt pathway can activate *sp5l* expression. Conversely, we used a dominant-negative, heat shock-inducible, ΔTCF GFP transgenic line to block the activation of Wnt/β-catenin target genes (Lewis et al., 2004) to test whether *sp5l* was down-regulated. We found that expression of ΔTCF GFP can repress *sp5l* expression very rapidly: 15 minutes after induction of ΔTCF GFP expression at tailbud stage, *sp5l* RNA levels were already substantially reduced (Fig. 5B, middle panel) and hardly detectable after 30 minutes (Fig. 5B, right panel). This rapid downregulation suggests that ΔTCF GFP directly represses *sp5l* transcription and that *sp5l* may be a direct target of Wnt/β-catenin signaling. In support of this, the *sp5l* promoter contains six consensus Tcf/Lef binding sites within the 519 bp 5′ of exon 1, which bind Lef1 protein in vitro and are required for Wnt responsiveness in reporter assays conducted in zebrafish embryos (Weidinger et al., 2005). Thus, *sp5l* is a direct Wnt/β-catenin target gene.

As the above experiments did not reveal which endogenous Wnt ligand(s) regulate *sp5l*, we examined *sp5l* expression in *wnt3a*, *wnt8* and *wnt3a/wnt8* morphant backgrounds. During early gastrulation, *wnt8*, and not *wnt3a*, is required for full *sp5l* expression (Weidinger et al., 2005) (C.J.T., unpublished). At the 3-somite stage, when *sp5l* is robustly expressed in the tailbud of control embryos (Fig. 5C), its expression is significantly reduced in *wnt3a* morphants (Fig. 5D), and even more substantially reduced in *wnt8* (Fig. 5E) and *wnt3a/wnt8* (Fig. 5F) morphant embryos. Inhibition of *wnt3a* and *wnt8* does not completely eliminate *sp5l* expression, in contrast to what we observe following induction of ΔTCF GFP. This may be due to residual Wnt activity in our double morphant embryos. We conclude that by early somitogenesis, *sp5l* expression in the tailbud is largely dependent on Wnt signaling, principally *wnt8*.

s*p5l* inhibition enhances loss of *wnt3a* function

Since *sp5l* expression is regulated by *wnt3a* and *wnt8* in the tailbud, we examined whether knockdown of *sp5l* could enhance the defects seen in *wnt3a* or *wnt8* morphants. We used a translation blocking morpholino previously shown to specifically inhibit *sp5l* function (Zhao et al., 2003). We observed no enhancement of the *wnt8* morphant phenotype when *sp5l* MOs were co-injected (data not shown), perhaps because *sp5l* expression is already so dramatically reduced in *wnt8* morphants. In contrast, co-injection of *sp5l* MO strongly enhances the phenotype of *wnt3a* MO embryos. While *sp5l* morphants have no apparent tail defects at 48 hpf (Fig. 6C), and *wnt3a* MO embryos are only slightly shorter than wild-type embryos (Fig. 6E, compare with wild type in Fig. 6A), *wnt3a/sp5l* morphants are dramatically shorter (Fig. 6G; 93% of embryos, n=104). Similarly, at the 10-somite stage, *wnt3a/sp5l* morphants (Fig. 6H) have greatly reduced expression of *tbx6* relative to *sp5l* MO (Fig. 6D) or *wnt3a* MO (Fig. 6F) alone (32/34 embryos with

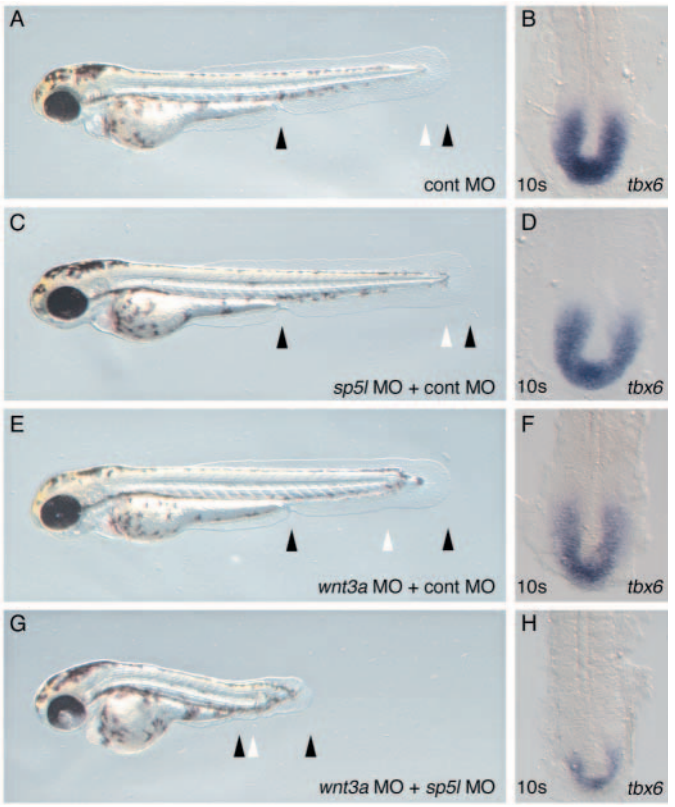

Fig. 6. *sp5l* MO enhances *wnt3a* loss of function. Embryos were injected with 3.5 mg/ml control morpholino (A,B), 2.5 mg/ml *sp5l* MO + 1 mg/ml control MO (C,D), 1 mg/ml *wnt3a* MO +2.5 mg/ml control MO (E,F), or 1 mg/ml *wnt3a* MO + 2.5 mg/ml *sp5l* MO (G, H). (A,C,E,G) Live embryos are shown at 48 hpf, anterior to the left. (B,D,F,H) Embryos stained for *tbx6* expression at the 10-somite stage and shown in a dorsal view of the tailbud region, anterior to the top.

significantly reduced staining relative to either single MO injection). Also, *sp5l* MO enhances the penetrance of the notochord truncation phenotype observed in *wnt3a* morphants [67% of embryos injected with 1 mg/ml *wnt3a* MO plus control MO have truncated notochords (n=73) vs 100% when co-injected with *sp5l* MO (n=75)]. Lastly, like *wnt3a/wnt8* morphants, *wnt3a/sp5l* embryos lack somites posterior to the truncated notochord (data not shown). These data indicate that *sp5l* functions redundantly with *wnt3a* in tail development, both in regulation of presomitic mesoderm markers such as *tbx6* and in promoting mesodermal fates in the caudal tail.

Since *sp5l* expression in the tailbud is regulated by Wnt/β-catenin signaling, and *sp5l* functionally interacts with *wnt3a* in tail formation, we next asked whether *sp5l* could be placed functionally downstream of *wnt3a*. We assessed this by co-injecting *sp5l* RNA with the *wnt3a* MO, and scoring for rescue of the notochord truncation phenotype at 48 hpf (Fig. 7). Co-injection of 200 pg *sp5l* RNA (Fig. 7B), but not control RNA (Renilla luciferase; Fig. 7A) resulted in significant rescue (see graph in Fig. 7C). Taken together, our observations indicate that *sp5l* is a functional target of Wnt signaling that plays key roles in development of the posterior body in zebrafish.

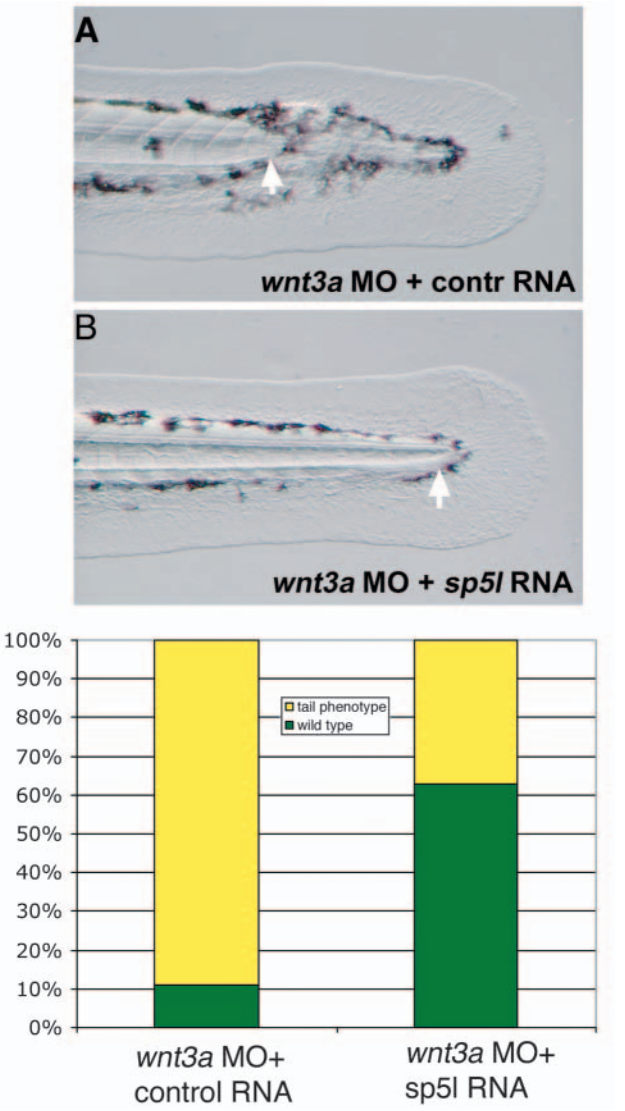

Fig. 7 *sp5l* RNA overexpression rescues *wnt3a* morphants. Embryos are shown at 48 hpf, with arrows marking the posterior end of the notochord. (A) *wnt3a* MO embryos co-injected with 200 pg of a control RNA (renilla luciferase) typically have a truncated notochord (89%, n=104). (B) Most embryos co-injected with *wnt3a* MO and 200 pg *sp5l* MO have normal tail development (33% have truncated notochord; n=107). (C) Graph showing penetrance of rescue.

Discussion

wnt3a and *wnt8* are required to maintain expression of presomitic mesoderm markers

Cell labeling experiments within the caudal presomitic mesoderm of zebrafish indicate that individual cells and their descendants reside in this region for variable amounts of time before exiting and beginning to differentiate (Muller et al., 1996). Thus, even as cells exit the tailbud and form somites, a population of undifferentiated precursors that will give rise to more caudal structures is maintained. Our data show that tailbud fates are initially specified correctly in *wnt3a/wnt8* morphants, followed by a rapid decline in the expression of presomitic mesoderm markers. As we observe no significant

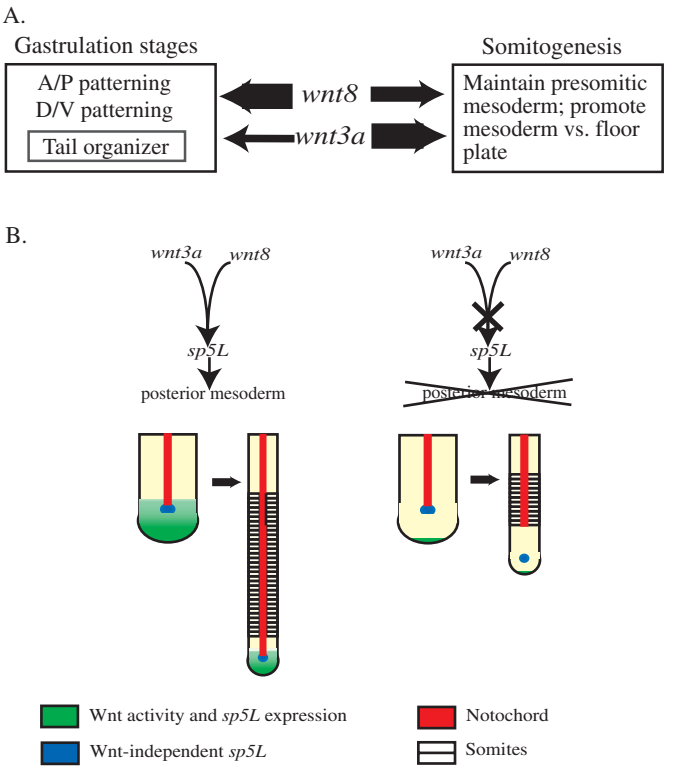

Fig. 8. Model for functions of *wnt3a*, *wnt8* and *sp5l* in tail development. (A) *wnt3a* and *wnt8* both regulate anterior-posterior (A/P) patterning and dorsal-ventral (D/V) patterning during gastrulation. The thicker arrow from *wnt8* represents the larger contribution to early patterning events by *wnt8* relative to *wnt3a*. Specification of the tail organizer is separately listed for clarity, but is probably tightly linked to D/V patterning. During somitogenesis, both genes are required for maintenance of presomitic mesoderm, but later roles for Wnt signaling in promoting mesoderm formation versus floor plate are more *wnt3a* dependent (represented by the thicker arrow). (B) *wnt3a* and *wnt8* both regulate *sp5l* expression in the tailbud. Overlap of *sp5l* expression with Wnt activity (visualized by TOPGFP reporter expression) in the tailbud is represented in green. When *wnt3a* and *wnt8* function is blocked, Wnt activity and *sp5l* expression in the tailbud is dramatically reduced, resulting in a failure in tail formation.

changes in cell death or gross levels of cell proliferation in the tailbud of *wnt3a/wnt8* morphants, what then is the cause of the loss of presomitic mesoderm and failure in tail development? One possible explanation to account for our observations is that Wnts could be acting as anti-differentiation factors in the presomitic mesoderm, where Wnt activity is localized, as determined by expression of the TOPdGFP reporter (Dorsky et al., 2002) (C.T., unpublished). In the absence of Wnt activity, presomitic mesoderm cells might prematurely differentiate and form somites, leaving no undifferentiated cells behind to maintain the tailbud. In this way, the cells initially allocated to the tailbud are quickly exhausted and tail development is dramatically truncated. Intriguingly, in *spadetail* mutants, presomitic cells fail to differentiate into somites and instead accumulate in the tailbud, and it has been proposed that this phenotype may be due to a failure to turn off genes such as *wnt8* that are normally highly expressed only in the caudal tailbud (Griffin and Kimelman, 2002). Although an exciting possibility, it will be necessary to carefully map the fates of presomitic precursors in *wnt3a/wnt8* embryos to directly test this model.

Alternatively, as *Wnt3a* has recently been shown to regulate oscillation of the segmentation clock (via regulation of *axin2*) in mouse presomitic mesoderm (Aulehla et al., 2003), it is also possible that a defect in segmentation underlies the failure of tail formation observed in *wnt3a/wnt8* morphants. We do not favor this hypothesis for two main reasons. First, the data from the mouse experiments indicates that repression of Wnt signaling in the presomitic mesoderm leads to larger somites being formed, while in our *wnt3a/wnt8* embryos, the posterior somites appear somewhat smaller than normal (see Fig. 4L). Secondly, in zebrafish segmentation mutants, as well as in the mouse *Wnt3a* mutant, *vestigial tail*, defects in segmentation are associated with a failure of somite boundary formation, leading to diffuse, unsegmented expression of somitic markers such as *myoD* (Aulehla et al., 2003; Holley et al., 2000). We do not observe this phenotype in *wnt3a/wnt8* morphants (see Fig. 1K,L; Fig. 4L), suggesting that segmentation is grossly normal. A detailed analysis of the expression of oscillating clock genes, such as *her1*, will be necessary to definitively address this issue, although it is noteworthy that *axin2* expression is not reported to cycle in zebrafish as it does in the mouse (Aerne and Ish-Horowicz, 2004), raising the possibility that regulation of segmentation in these two vertebrates may not be strictly orthologous.

Wnt signaling is required for production of both somitic mesoderm and notochord in the caudal tail

Zebrafish *wnt3a* is required for formation of caudal notochord, which is missing in nearly all *wnt3a* MO embryos. Since *Wnt3a* directly activates the transcription of *T* in the mouse (Yamaguchi et al., 1999), and the zebrafish *T* homolog, *ntl*, is required for notochord formation (Halpern et al., 1993; Schulte-Merker et al., 1994), it is tempting to speculate that the notochord defect in *wnt3a* morphants is due to a loss of expression of *ntl*. Although *ntl* continues to be expressed in the tailbud of *wnt3a* morphants (Fig. 3), *ntl* expression at the caudal end of the notochord – the chordoneural hinge – is lost, beginning at approximately the 20-somite stage (C.J.T., unpublished results). Fate mapping studies have shown that cells in this region can adopt a notochord or floor plate fate, and *ntl* has been shown to be important in promoting notochord fates; *ntl* mutants lack notochord and produce ectopic floor plate cells (Halpern et al., 1997). Although we observe a clear loss of notochord in the caudal tail of *wnt3a* morphants, we do not observe any increase in floor plate, suggesting that notochord progenitors may not be adopting floor plate identity. In the mouse *Wnt3a* mutant, the ectopic neural tissue observed in the absence of notochord and somites is derived from prospective somitic mesodermal cells (Takada et al., 1994; Yoshikawa et al., 1997). Our observation that ectopic floor plate is not produced when only notochord is missing (in the *wnt3a* morphant), but only when somitic mesoderm is also lacking (the *wnt3a/wnt8* double morphant), suggests that the same fate transformation could be occurring in the zebrafish.

Careful fate mapping within the tailbud to determine the origin of the ectopic floor plate will be required to directly address this possibility.

sp5l is a downstream target of Wnt signaling

Sp1-related transcription factors are characterized by having multiple zinc-finger DNA binding domains related to the *kruppel* gene from *Drosophila* (Pieler and Bellefroid, 1994). Sp1 proteins bind to GC-rich promoter regions, and while some members of this protein family are expressed ubiquitously and are thought to be required generally as enhancers of transcription, others, including members of the Sp5 subfamily, are expressed in restricted domains and are thought to participate in specific processes during development (Bell et al., 2003; Briggs et al., 1986; Harrison et al., 2000; Tallafuss et al., 2001; Treichel et al., 2001; Treichel et al., 2003).

Our data strongly suggest that *sp5l* functions downstream of Wnts in zebrafish tail development. What role might *sp5l* play in this process? One function could be to bind the promoters of downstream Wnt targets and enhance their activation. For example, in the mouse *T* promoter, both Tcf/Lef sites and regions containing Sp1 binding sites are important for normal expression of *T* in the primitive streak and tailbud (Clements et al., 1996; Yamaguchi et al., 1999), and mutations in the Sp1 family member *Sp5* enhance the tail truncation phenotype of heterozygous *T*/+ mice (Harrison et al., 2000), suggesting that *Sp5* could be functioning with *Wnt3a* to activate *T* transcription. Also, in vitro analysis indicates that LEF-1 can activate transcription from the HIV-1 promoter only when an Sp1-containing fraction, or purified Sp1 protein, is added to the transcription reaction (Sheridan et al., 1995).

The synergistic loss of *tbx6* expression observed when both *sp5l* and *wnt3a* are inhibited suggests that full activation of the *tbx6* promoter may also require the binding of both β-catenin/Tcf complexes to Tcf/Lef sites (Szeto and Kimelman, 2004) and Sp5l to putative Sp1 binding sites found in the *tbx6* promoter (C.J.T., unpublished). Additional deletion analysis of the *tbx6* promoter will be required to more directly address a requirement for *sp5l* in its activation.

Since the GC-rich promoter elements bound by Sp1 proteins are found upstream of many genes, it is conceivable that activation of multiple Wnt targets could be potentiated by *sp5l*. Since *sp5l* morphants, like the mouse *Sp5* knockout, have no discernable tail phenotype, this function may not be essential, or may be redundantly encoded. The latter is a distinct possibility, as in both mouse and zebrafish, another Sp5 homolog is largely co-expressed in the same tissues (Harrison et al., 2000; Tallafuss et al., 2001). Interestingly, the other identified zebrafish Sp5 homolog, called *buttonhead/Sp-related 1(bts1)*, is also a target of Wnt signaling (Harrison et al., 2000; Tallafuss et al., 2001; Weidinger et al., 2005). Also, a recent report suggesting that a mouse buttonhead homolog, *mBtd*, also called SP8, may play a role in maintaining expression of Wnt targets in the limb bud (Bell et al., 2003; Treichel et al., 2003) is an additional link between Sp1 family members and Wnt signaling during vertebrate development.

We suggest that in addition to a previously characterized role during gastrulation for specification of future tail fates, Wnt/β-catenin signaling is also required during somitogenesis for maintenance of presomitic mesoderm in the tailbud, and also to promote mesodermal fates and inhibit floor plate formation in subsequent tail outgrowth (Fig. 8). The early functions in dorsal-ventral patterning and specification of the tail organizer are principally carried out by *wnt8*. During early somitogenesis, both *wnt3a* and *wnt8* function to maintain presomitic mesoderm fates in the tailbud, while the later mesoderm/floor plate distinction is more sensitive to loss of *wnt3a* function. Thus, Wnt/β-catenin signaling, functioning in part through *sp5l*, is required throughout tail development to properly specify, pattern and maintain a precursor population in the tailbud. Further examination of the defects in mesoderm formation in Wnt-inhibited embryos and identification of additional Wnt/β-catenin targets will be important for a more complete understanding of tail development.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/8/1763/DC1

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