Corrigendum

Combinatorial gene regulation by Bmp and Wnt in zebrafish posterior mesoderm formation

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The labelling in Fig. 6 of this article is incorrect.
The label for A-F should read *mut1-2.0-gfp/Xex-bfp* and the label for G-L should read *mut1,2-2.0-gfp/Xex-bfp*
The authors apologise to the readers for this mistake.

Combinatorial gene regulation by Bmp and Wnt in zebrafish posterior mesoderm formation

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Summary

Combinatorial signaling is an important mechanism that allows the embryo to utilize overlapping signaling pathways to specify different territories. In zebrafish, the Wnt and Bmp pathways interact to regulate the formation of the posterior body. In order to understand how this works mechanistically, we have identified *tbx6* as a posterior mesodermal gene activated by both of these signaling pathways. We isolated a genomic fragment from the *tbx6* gene that recapitulates the endogenous *tbx6* expression, and used this to ask how the Bmp and Wnt signaling pathways combine to regulate gene expression. We find that the *tbx6* promoter utilizes distinct domains to integrate the signaling inputs from each pathway, including multiple Tcf/LEF sites and a novel Bmp-response element. Surprisingly, we found that overexpression of either signaling pathway can activate the *tbx6* promoter and the endogenous gene, whereas inputs from both pathways are required for the normal pattern of expression. These results demonstrate that both Bmp and Wnt are present at submaximal levels, which allows the pathways to function combinatorially. We present a model in which overlapping Wnt and Bmp signals in the ventrolateral region activate the expression of *tbx6* and other posterior mesodermal genes, leading to the formation of posterior structures.

Supplemental data available online

Key words: Bmp, Wnt, Posterior mesoderm, T-box genes, Zebrafish

Introduction

The posterior body of the zebrafish embryo is formed by a network of intercellular signaling molecules that pattern the formation of the ventrolateral mesoderm, which ultimately gives rise to the posterior (trunk and tail) mesoderm. These signaling factors include Bone morphogenetic proteins (Bmps) (reviewed by Hammerschmidt, 2002), Wnt8 (Kelly et al., 1995; Lekven et al., 2001), Fibroblast growth factors (Fgfs) (Amaya et al., 1993; Griffin et al., 1995; Rodaway et al., 1999; Draper et al., 2003; Griffin and Kimelman, 2003), and the Nodal-related proteins (Schier and Shen, 2000; Schier and Talbot, 2001). Whereas the Nodals appear to be critical for mesoderm induction and the Fgfs are important for maintaining mesodermal gene expression (reviewed by Kimelman and Schier, 2002), the exact roles of Bmp and Wnt signaling in forming the ventral/posterior mesoderm have been uncertain.

Three *bmps* are expressed in the mesoderm of the gastrula-stage embryo and their expression continues at the most posterior end of the embryo throughout somitogenesis (Martinez-Barbera et al., 1997; Nikaido et al., 1997). As in *Xenopus*, a large body of evidence demonstrates that the Bmps are key regulators of the posterior mesoderm patterning in zebrafish (reviewed by Hammerschmidt, 2002). For example, *bmp2b/swirl* is expressed in a gradient along the dorsoventral axis (Barth et al., 1999; Wagner and Mullins, 2002) and zebrafish mutants lacking *bmp2b/swirl* do not form tails (Mullins et al., 1996; Kishimoto et al., 1997). Like the *bmps*, zebrafish *wnt8* is also expressed in the ventrolateral mesoderm during gastrulation and at the posterior end of the embryo during somitogenesis (Kelly et al., 1995). In mutants lacking *wnt8*, the formation of the ventrolateral and posterior mesoderm is severely disrupted, demonstrating a requirement for Wnt signaling in the development of these mesodermal territories (Lekven et al., 2001).

Two studies have suggested that the Wnt and Bmp pathways cooperate to form the ventral and posterior mesoderm in *Xenopus* (Hoppler and Moon, 1998; Marom et al., 1999). The importance of such a cooperative interaction between these pathways has been further highlighted in a recent zebrafish study, demonstrating that combined overexpression of Wnt8 and Bmp is capable of inducing ectopic tail formation (Agathon et al., 2003). How these pathways integrate to regulate mesodermal gene expression is unknown.

In order to understand mechanistically how these two pathways interact, we sought a posterior mesodermal gene whose expression required input from both the Bmp and Wnt pathways. The T-box transcription factor *tbx6* (Hug et al., 1997) was found to meet this criterion, and we therefore identified a minimal region of the *tbx6* gene that reproduces the endogenous gene expression pattern. Analysis of the *tbx6* promoter shows that Bmp and Wnt8 regulate *tbx6* expression through different domains within the promoter, demonstrating that these pathways intersect by regulating transcription of specific target genes. Elimination of regions that respond to Bmp or Wnt signaling revealed that the promoter is able to respond to overexpression of a single pathway, whereas normally the promoter integrates responses to both pathways to provide normal *tbx6* expression. Analysis of the expression

of the endogenous gene under conditions in which either Bmp or Wnt signaling is blocked together with analysis of the *tbx6* promoter has allowed us to put forth a model in which submaximal levels of Bmp and Wnt signals cooperate to regulate the formation of the posterior mesoderm.

Materials and methods

Embryos and morpholino oligonucleotides injections

Zebrafish embryos were obtained by natural spawning of adult AB strain zebrafish. Embryos were raised and maintained at 28.5°C in system water and staged as described (Westerfield, 1995). *Wnt8*-specific morpholino antisense oligonucleotides were a generous gift from Randy Moon. At the one-cell stage, each embryo was injected with approximately 1 nl volume of morpholino oligonucleotides (5 ng) using a Picospritzer II (Parker Hannifin). Embryos were collected at the appropriate stages and fixed in 4% paraformaldehyde, pH 7.0, in phosphate-buffered saline (PBS), overnight at 4°C. Fixed embryos were dechorionated, washed three times with PBS and stored in methanol at –20°C.

Plasmids and constructs

Various *gfp* reporter constructs were made by cloning the *tbx6* promoter fragments into the *Bam*HI restriction site of the *gfp* reporter plasmid vector. The dual fluorescence system was created by inserting the *pXex-bfp* DNA (gift of Stephen C. Ekker) into the existing *gfp* reporter constructs. The *luciferase* reporter constructs were made by cloning the *tbx6* promoter fragments into the multiple cloning sites of the PGL3 promoter vector (Promega).

Site-directed mutagenesis

The two proximal Tcf site mutations in the *tbx6* promoter were generated using the QuikChange procedure (Stratagene). *Mut1-2.0* was constructed by using primers: 5′-gtgcacatacacacctctggccccccc-tcgaggtgatggaagaaaggtagaagcctag-3′ and 5′-ctaggcttctaccttcttccatca-cctcgaggggggccagaggtgtgtatgtgcac-3′. *Mut2-2.0* was constructed by using primers: 5′-gtgggctggctggagacaaaagacaggttaacgaggagactga-tgttttgacagaag-3′ and 5′-cttctgtcaaaacatcagtctcctcgttaacctgtcttttgtct-ccagccagcccac-3′. *Mut1,2-2.0* construct was generated by combining the two mutated Tcf sites.

DNA and RNA injections

DNA was isolated with a Qiagen midi kit and resuspended in RNase-free water. DNA at a concentration of 0.1 mg/ml was used for injection. RNAs were synthesized from *Sal*I linearized *SP64T-Xbmp4* (gift of Jim Smith), Asp718 linearized *CS2-zwnt8* (gift of Randy Moon) and *Not*I linearized *CS2-TVGR* (gift of Paul Wilson) templates using the mMessage Machine Kit (Ambion) and dissolved in RNase-free sterile water. RNA (at concentrations indicated in the text) was injected in the presence or absence of 0.1 μg of reporter DNA into one-cell zebrafish embryos. The expression of the reporter gene was analyzed at the appropriate stages using bright field or fluorescence microscopy.

In situ hybridization

Whole-mount in situ hybridization was performed using digoxigenin-labeled antisense RNA probes and visualized using anti-digoxigenin Fab fragments conjugated with alkaline phosphatase (Roche Molecular Biochemicals) as described (Griffin et al., 1998). Riboprobes were made from DNA templates, which were linearized and transcribed with either SP6 or T7 RNA polymerases. Embryos were processed and hybridized as described (Griffin et al., 1998), except that 5 μg/ml of Proteinase K in PBS/0.1% Tween-20 was used for 5 to 10 minutes depending on the age of the collected embryos.

Xenopus and zebrafish transgenesis

PAC DNA was isolated with the Qiagen midi kit. To generate *Xenopus* transgenic embryos, we used 200 pg of PAC DNA for each injection as described (Amaya and Kroll, 1999) with the exception that no restriction enzyme was used. To generate stable transgenic zebrafish lines, reporter plasmid DNA was digested with *Bss*HII to completion, and then separated on a 1% agarose preparative gel overnight. The gel slice containing the DNA insert was isolated and DNA was electroeluted using a Schleicher and Schuell ELUTRAP. DNA was extracted once with equal volume of phenol and once with an equal volume of chloroform. After extraction, DNA was ethanol precipitated and then resuspended in distilled water. For injection, 80 mg/ml of purified DNA insert was used to inject one-cell stage zebrafish embryos. Injected embryos were examined at the 15- to 18-somite stage for the presence of GFP fluorescence. Embryos showing specific GFP fluorescence in the tail region were collected and raised to sexual maturity as founders. Different combinations of founder crosses were set up for the identification of F1 embryos showing non-mosaic GFP fluorescence in the tail region at the 15- to 18-somite stage.

Luciferase assays

Injected embryos were collected at the shield stage and then separated into three pools of 10 embryos each for assay in triplicate. Experiments were repeated at least three times. Excess zebrafish embryo medium was removed, embryos were homogenized in 100 μl of 1×Cell Culture Lysis Reagent (Promega), and cleared by 10 minutes' microcentrifugation at room temperature. Fifty microliters of the resulting supernatant was used for luciferase activity assays that were performed according to the Promega protocol with a Berthold luminometer.

Results

Regulation of *tbx6* expression by Wnt and Bmp signals

In our initial attempts to find a gene regulated by both the Wnt and Bmp pathways, we identified the T-box transcription factor *tbx6* as a candidate. *Tbx6*, along with two other T-box genes, *spadetail* (*spt*) and *no tail* (*ntl*), is expressed in the posterior mesoderm from the gastrula stage through the end of somitogenesis (Schulte-Merker et al., 1994; Hug et al., 1997; Griffin et al., 1998). *Wnt8* was previously shown to be required for the expression of *tbx6* in the ventrolateral mesoderm (Lekven et al., 2001), which we confirmed using a pair of *wnt8*-specific morpholino oligonucleotides (MOs) (Fig. 1A,B) (n=55). Whereas the lateral expression was completely absent, some residual *tbx6* expression was observed in the ventral region (Fig. 1B). In contrast, *ntl* expression was unaffected by the inhibition of Wnt8 function (Fig. 1E,F) and *spt* expression was only partially affected (Fig. 1C,D), indicating that *tbx6* is the most dependent of the three mesodermal genes on Wnt function at the gastrula stage.

To determine if *tbx6* is regulated by Bmp signaling, we examined *tbx6* expression in *swirl* mutants, which have a defective *bmp2b* gene (Kishimoto et al., 1997; Nikaido et al., 1997). In approximately one quarter of the embryos collected from the crosses of *swirl* heterozygotes, we observed a strong reduction in the expression of *tbx6* (Fig. 1G,H) (n=75), whereas the expression of *spt* and *ntl* was normal in all the embryos examined (Fig. 1I-L). Interestingly, unlike the result observed with the *wnt8MOs* (Fig. 1B), *swirl* embryos showed residual *tbx6* expression in both the ventral and lateral regions (Fig. 1H). Similar results were obtained with embryos injected with RNA encoding the Bmp inhibitor Noggin (Fig. 2A,B)

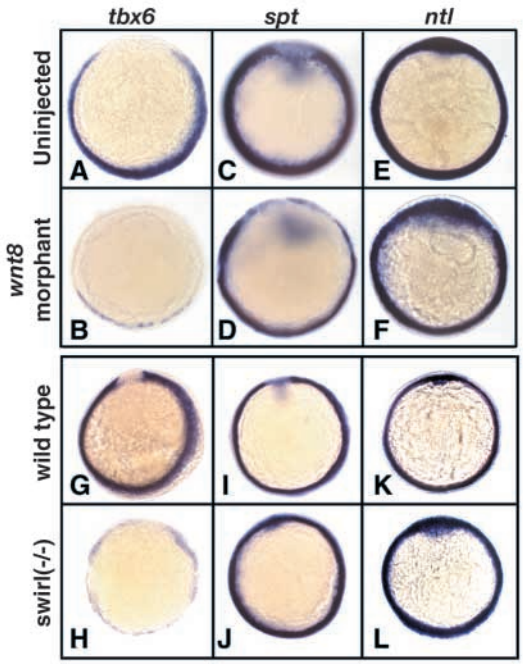

Fig. 1. Wnt8 and Bmp2b are required for *tbx6* expression. Animal pole views of shield stage embryos with dorsal to the top. The in situ probe is shown at the top of the Figure (*ntl, no tail; spt, spadetail*) and the genotype of the embryo is shown on the left side. *Wnt8* morphants are wild-type embryos injected with a pair of *wnt8*-specific morpholino oligonucleotides. Note that residual *tbx6* expression is only observed ventrally in the *wnt8* morphant embryos whereas residual *tbx6* expression is present laterally and ventrally in *swirl*–/– embryos.

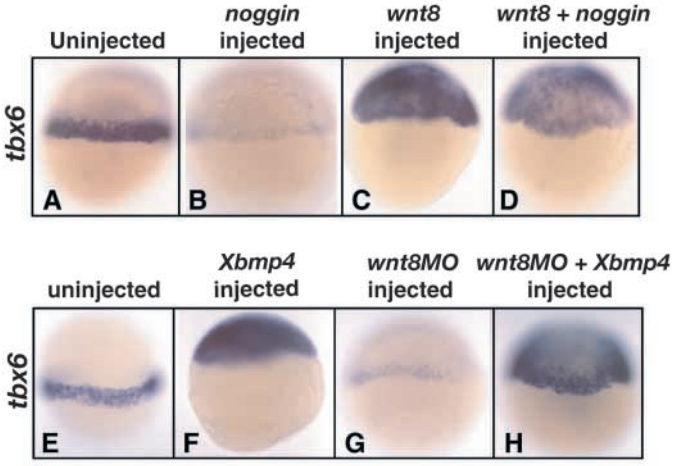

Fig. 2. Regulation of *tbx6* by Wnt and Bmp. (A-H) Lateral views with the animal pole to the top. (A,B) Expression of endogenous *tbx6* in an uninjected embryo (A) and in an embryo injected with 50 pg of *noggin* RNA (B). The injected embryo shows dramatic reduction of *tbx6* expression. (C) An embryo injected with 75 pg of *wnt8* RNA shows ectopic expression of *tbx6*. (D) An embryo coinjected with 75 pg of *wnt8* RNA and 50 pg of *noggin* RNA showing ectopic expression of *tbx6* in the animal pole. (E-H) Expression of endogenous *tbx6* in an uninjected embryo (E) and in an embryo injected with 150 pg of *Xbmp4* RNA (F). The injected embryo shows ectopic expression of *tbx6*. (G) An embryo injected with *wnt8MOs* shows decreased expression of *tbx6*. (H) A zebrafish embryo coinjected with *wnt8MOs* and 150 pg of *Xbmp4* RNA showing strong marginal and ectopic dorsal domains of *tbx6* expression.

(85%, *n*=65). These results demonstrate that normal *tbx6* expression requires input from both the Wnt and Bmp pathways, which is consistent with the observation that both Bmp and Wnt signals are required for the formation of the posterior body in zebrafish (Lekven et al., 2001; Agathon et al., 2003).

To further examine the regulation of *tbx6* by Bmp and Wnt signals, we overexpressed these factors in the early zebrafish embryo. We observed that overexpression of either signaling molecule caused ectopic *tbx6* expression throughout the embryo (Fig. 2C,F) (80%, *n*=43). These results suggest that *tbx6* can be separately activated by each of these signaling pathways alone, although it was possible that each of the overexpressed signals also activated the other signaling pathway. To eliminate the effect of the other pathway, we coinjected *wnt8* with the Bmp inhibitor *noggin*, and coinjected *Xenopus bmp4* (*Xbmp4*) RNA (*Xbmp4* causes the same effects as the zebrafish Bmps) (Kishimoto et al., 1997) with the *wnt8MOs*. Whereas 50 pg of *noggin* alone caused a strong reduction in *tbx6* expression, as was observed in *swr* mutant embryos (Fig. 2B, compare with Fig. 1H), *noggin* only caused a partial reduction in the ectopic *tbx6* induced by overexpression of *wnt8* (Fig. 2D) (70%, *n*=35). This result suggests that only a part of the *wnt8*-mediated activation of *tbx6* depends on the ability of Wnt8 to upregulate endogenous *bmp2b* expression (Agathon et al., 2003). Conversely, whereas the *wnt8MOs* strongly downregulated *tbx6* expression (Fig. 2G), the *wnt8MOs* did not inhibit marginal *tbx6* expression in *Xbmp4*-injected embryos, although it reduced the animal pole expression of *tbx6* (Fig. 2H) (87%, *n*=45). Because *bmp* overexpression does not alter zygotic *wnt8* expression (Agathon et al., 2003), our results suggest that maternal *wnt8* (Kelly et al., 1995) is required for the high-level ectopic animal pole expression of *tbx6* in *Xbmp4*-injected embryos, but it is not required for XBmp4 to activate *tbx6* expression at the margin. In summary, our results show that both Wnt and Bmp can induce *tbx6* expression when the other pathway is blocked, although the two factors alone can activate *tbx6* in specific patterns.

Identification and characterization of the *tbx6* promoter

In order to understand how these two pathways regulate *tbx6* expression, we screened a zebrafish PAC genomic library and obtained three *tbx6* positive clones. Using the frog transgenesis approach (Amaya and Kroll, 1999), we tested one of these *tbx6* positive PAC clones (*PAC-tbx6*) for its ability to target expression of zebrafish *tbx6* to the posterior mesoderm of developing *Xenopus* embryos. The advantage to using *Xenopus* is that we could examine the expression of the zebrafish *tbx6* gene using in situ hybridization because the fish and frog *tbx6* genes are sufficiently divergent (Uchiyama et al., 2001), without the need to make a reporter gene fusion in the PAC DNA. Approximately 20% of the transgenic embryos (7/36) showed zebrafish *tbx6* transcripts in the posterior paraxial mesoderm (see Fig. S1A,B at http://dev.biologists.org/supplemental), whereas the remainder of the embryos failed to express zebrafish *tbx6* from the injected PAC (see Fig. S1C

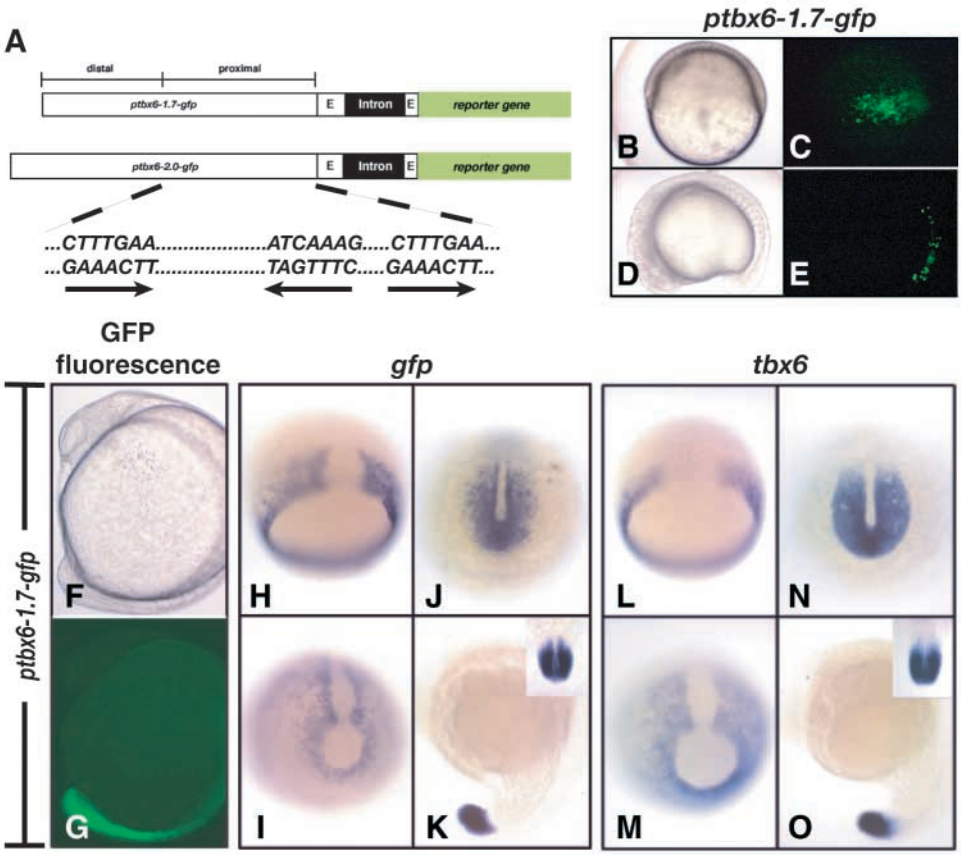

Fig. 3. *tbx6-gfp* reporter constructs are correctly expressed in zebrafish embryos. (A) Schematic diagrams of *gfp* reporter constructs, *ptbx6-1.7-gfp* and *ptbx6-2.0-gfp*, which contain 1.7 and 2.0 kb of upstream sequence, respectively. The 1.7 kb region is subdivided into a proximal 900 bp domain and a distal 800 bp domain. The sequences of three putative Tcf binding sites (Tcf-1, Tcf-2 and Tcf-3) are indicated with the arrows showing the orientation of the sites. (B-E) Lateral views with animal pole to the top of shield stage (B,C) and 15-somite (D,E) embryos injected at the one-cell stage with *ptbx6-1.7-gfp*. Bright field (B,D) and GFP fluorescence (C,E) pictures showing specific GFP fluorescence at the margin at shield stage (C) and in the posterior mesoderm at the 15-somite stage (G). Note that because these embryos are not transgenic, the GFP is expressed mosaically in the embryos from the injected DNA. (F-O) Transgenic embryos containing *ptbx6-1.7-gfp*. (F,G) Lateral views with anterior to the top. Bright field (F) and fluorescence (G) pictures of a *ptbx6-1.7-gfp* transgenic embryo at the 15- to 18-somite stage. (H-O) In situ staining for *gfp* (H-K) and *tbx6* (L-O). Embryos shown are at 70% epiboly (H,L; dorsal view with animal pole to the top), 95% epiboly (I,M; posterior view with dorsal to the top), 5-somite stage (J,N; posterior view with dorsal to the top) and 18-somite stage (K,O; lateral view with anterior to the top). The small inserts in K and O show a close-up posterior view of expression in the tip of the tail. Note the *tbx6* in situ probe was from the 3′ end of the gene and therefore does not bind to the transcripts from the transgene. E, exon sequence.

at http://dev.biologists.org/supplemental). This expression pattern matches the endogenous *Xenopus tbx6* (Uchiyama et al., 2001) as well as that of the *ntl* ortholog *Xbra* (Smith et al., 1991) (see Fig. S1D at http://dev.biologists.org/supplemental). These results demonstrate that *PAC-tbx6* has all of the regulatory elements necessary for expression within the posterior mesoderm, and that these regulatory sites are conserved between fish and frogs.

Having determined that *PAC-tbx6* targets expression to the posterior mesoderm in developing *Xenopus* embryos, we subcloned smaller fragments and characterized them in zebrafish. We initially focused on two constructs fused in frame to GFP, *ptbx6-1.7-gfp* and *ptbx6-2.0-gfp*, which contain 1.7 and 2.0 kb of DNA upstream from the start of translation, respectively, and the first intron (Fig. 3A). These constructs were injected into one-cell stage zebrafish embryos and then analyzed for GFP fluorescence at different stages of development. At 60-75% epiboly, we observed GFP fluorescence at the margin where endogenous *tbx6* is normally expressed (Fig. 3B,C, Table 1). At the 15- to 18-somite stage, GFP fluorescence was detected in the tail mesoderm, again matching the endogenous pattern of expression (Fig. 3D,E, Table 1). Although both reporter constructs were expressed in the same pattern, we noticed that *ptbx6-2.0-gfp* produced consistently higher fluorescence levels than *ptbx6-1.7-gfp*, suggesting the presence of a general enhancer between –1.7 and –2.0 kb. The fluorescence results were confirmed by in situ hybridization with a *gfp* probe on zebrafish embryos injected with either of the two reporter constructs (see Fig. S2 at http://dev.biologists.org/supplemental).

Because expression from injected DNA is mosaic (non-uniform), we wanted to confirm that the *tbx6* promoter constructs faithfully matched the endogenous gene expression by producing transgenic embryos. We therefore created stable transgenic zebrafish lines [Tg(*tbx6:gfp*)] expressing *ptbx6-1.7-gfp*. As shown in Fig. 3, the expression of transcripts from *ptbx6-1.7-gfp* was the same as the endogenous *tbx6* gene expression pattern (Fig. 3H-O). The GFP fluorescence matched the transcript pattern, although it extended more anteriorly (Fig. 3F,G). This was expected because the posterior mesodermal cells originate at the most posterior end and are incorporated into somites as the posterior end extends (Kanki and Ho, 1997). Because GFP is a stable protein, it perdures for a while in more anterior regions as the posterior end extends caudally, producing a posterior to anterior gradient of fluorescence (Fig. 3G). We also tested *ptbx6-1.7-gfp* in transgenic *Xenopus* embryos and found that it produced the same pattern of expression as *PAC-tbx6* (data not shown). These data demonstrate that *ptbx6-1.7-gfp* and *ptbx6-2.0-gfp* have all of the necessary regulatory elements to recapitulate

Table 1. Comparison of the targeting ability and promoter activity of different reporter constructs

Reporter constructs (GFP and Luciferase)	GFP expression		Relative promoter	Responsiveness	
	Margin	Tail	Activity	Bmp*	Wnt
ptbx6-2.0	91% (*n*=90)	91% (*n*=90)	100%	Yes	Yes†
ptbx6-1.7	88% (*n*=100)	88% (*n*=100)	34%	Yes	Yes‡
mut1-2.0	90% (*n*=50)	90% (*n*=50)	n/d	Yes	Yes†
mut2-2.0	85% (*n*=50)	85% (*n*=50)	n/d	Yes	Yes†
mut1,2-2.0	16% (*n*=50)§	22% (*n*=70)§	11%	Yes	Yes‡
ptbx6-0.9	0% (*n*=60)	0% (*n*=60)	6%	No	Yes‡

n/d, not determined.
*Tested with overexpression of *XBmp4*.
†Tested with overexpression of *TVGR* only.
‡Tested with overexpression of *wnt8* and *TVGR*.
§Only a few cells showed marginal GFP fluorescence.

expression of the *tbx6* gene in both transient expression assays and in transgenic embryos.

Regulation of the *tbx6* promoter by Wnt and Bmp

During the development of the posterior mesoderm, the endogenous *tbx6* promoter must be able to integrate inputs from both the Wnt and Bmp pathways, as shown above. We therefore tested the ability of *ptbx6-1.7-gfp* to respond to these signaling pathways by injecting the promoter with *wnt8* or *Xbmp4* and compared the response of the endogenous gene with the same levels of exogenous Wnt and Bmp signaling inputs. Similar to the response of the endogenous *tbx6* gene in the presence of Wnt8 overexpression, we observed ectopic GFP fluorescence in embryos coinjected with *wnt8* RNA and *ptbx6-1.7-gfp* (Fig. 4A-D), showing that the *tbx6* promoter is capable of responding to Wnt8. Ectopic expression of *Xbmp4* RNA also induced widespread expression of the *ptbx6-1.7-gfp* as in the case of the endogenous *tbx6* gene (Fig. 4E-H). These results show that the promoter is activated by either Wnt or Bmp signals, similar to that of the endogenous *tbx6* gene.

Construction of a dual fluorescence system for promoter analysis in zebrafish

We wanted to analyze the *tbx6* promoter in detail by deleting and mutating specific regions to determine which parts of the promoter are necessary for normal expression. Because of the variable and mosaic patterns of expression when DNA is injected into zebrafish embryos, it is difficult to accurately quantitate results because some embryos express the injected DNA in only a few cells and/or in regions outside the area of interest (in our case the ventral and lateral mesoderm). In order to improve this situation, we added an additional feature to our reporter constructs. We placed a *blue fluorescence protein* (*bfp*) reporter gene under the control of a ubiquitous *Xenopus EF1α* (*pXex*) promoter, which has been characterized and shown to produce ubiquitous BFP fluorescence in developing zebrafish embryos (Finley et al., 2001). Our dual fluorescence (blue and green) system allows simultaneous detection of the regions of the embryo that inherited the injected DNA by examining the location of BFP fluorescence, in addition to revealing the specific expression of GFP under the control of the *tbx*6 promoter. For example, a construct containing *ptbx6-2.0-gfp* and *pXex-bfp* (Fig. 5A) showed scattered BFP fluorescence throughout the embryo at 70% epiboly with specific GFP expression at the margin (Fig. 5C,D). At later stages, the BFP was ubiquitous but the GFP was restricted to the most posterior end of the embryo (Fig. 5F,G). We used this system in our subsequent studies such that we only scored the presence or absence of GFP fluorescence in embryos in which there was substantial BFP fluorescence in the ventral-lateral mesoderm during the gastrula stages or in the posterior mesoderm during somitogenesis. We found that this was a much more reliable method for analyzing gene expression from exogenous promoters in transient zebrafish assays.

Mutational analysis of the *tbx6* promoter

To understand how the *tbx6* promoter is regulated by Wnt and Bmp signals, we undertook a systematic analysis of the *tbx6* promoter. The canonical Wnt signaling pathway typically works through regulation of the Tcf transcription factor (reviewed by van Noort and Clevers, 2002). Consistent with this, ectopic expression of a constitutively activated Tcf (TVGR) (Darken and Wilson, 2001) caused widespread activation of *ptbx6-1.7-gfp* (Fig. 4I,J), as did ectopic *wnt8* expression (Fig. 4C,D). Examination of the *tbx6* promoter constructs revealed three consensus Tcf binding sites within the 900 bp proximal region of the promoter (Fig. 3A). The presence of multiple Tcf sites in the *tbx6* promoter suggested that the expression of *tbx6* is directly regulated by Wnt8 signaling during posterior mesoderm formation.

Using our dual fluorescence system, we asked whether the activation of *ptbx6-2.0-gfp* in the posterior mesoderm required these Tcf DNA binding sites. To do this, we mutated the two proximal Tcf sites (Tcf-1 and Tcf-2) (Fig. 3A), generating *mut1-2.0-gfp/Xex-bfp* containing a mutation in the first Tcf site, *mut2-2.0-gfp/Xex-bfp* containing a mutation in the second Tcf site, and *mut1,2-2.0-gfp/Xex-bfp* containing both mutations. We found that either single mutation alone did not dramatically affect the expression of the *tbx6* promoter in the developing posterior mesoderm as indicated by the presence of strong GFP fluorescence (Fig. 6A-F, Table 1). In contrast, zebrafish embryos injected with the double Tcf site mutation construct showed almost no detectable GFP fluorescence in the majority of the embryos and the remainder had only occasional BFP-positive mesodermal cell expressing GFP (Fig. 6G-L, Table 1). The double Tcf site mutant promoter was still functional, however, because overexpression of the constitutively active Tcf was able to activate transcription from this promoter, probably through the remaining Tcf site (Table 1). In addition, this promoter was activated when coinjected with *Xbmp4*,

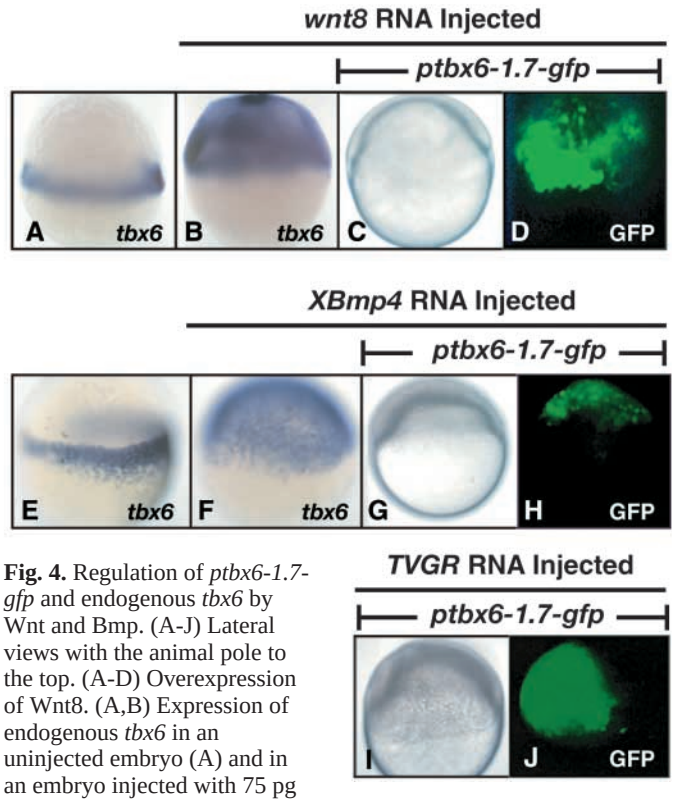

Fig. 4. Regulation of *ptbx6-1.7-gfp* and endogenous *tbx6* by Wnt and Bmp. (A-J) Lateral views with the animal pole to the top. (A-D) Overexpression of Wnt8. (A,B) Expression of endogenous *tbx6* in an uninjected embryo (A) and in an embryo injected with 75 pg of *wnt8* RNA (B). The injected embryo shows ectopic expression of *tbx6*. (C,D) Bright field (C) and fluorescence (D) pictures of a zebrafish embryo coinjected with *ptbx6-1.7-gfp* and 75 pg of *wnt8* RNA showing ectopic GFP fluorescence in the animal pole. (E-H) Expression of endogenous *tbx6* in an uninjected embryo (E) and in an embryo injected with 150 pg of *Xbmp4* RNA (F). The injected embryo shows ectopic expression of *tbx6*. (G,H) Bright field (G) and fluorescence (H) pictures of a zebrafish embryo coinjected with *ptbx6-1.7-gfp* and 150 pg of *Xbmp4* RNA at the shield stage showing ectopic GFP fluorescence in the animal pole. (I,J) Bright field (I) and fluorescence (J) pictures of a zebrafish embryo coinjected with *ptbx6-1.7-gfp* and 25 pg of *TVGR* RNA showing ectopic GFP fluorescence in the animal pole. TVGR is a constitutively active form of the Tcf transcription factor (Darken and Wilson, 2001). The in situ hybridizations are at shield stage whereas the bright field and fluorescence images are at 50% epiboly.

as was the wild-type promoter (Table 1). These results demonstrate that either of the two most proximal Tcf sites are required for normal *tbx6* expression, showing that Wnt signaling directly activates the *tbx6* gene.

Using a quantitative assay, we further confirmed the contribution of these Tcf binding sites to the overall promoter activity of *ptbx6-2.0*. We replaced the *gfp* gene with the *luciferase* gene to quantify the promoter activity of our reporter constructs. These *luc* reporter genes, *ptbx6-2.0-luc*, *ptbx6-1.7-luc* and *mut1,2-2.0-luc*, were injected into one-cell stage embryos and the luciferase activity of each reporter construct was determined in injected embryos at the 60% epiboly stage. Consistent with our early finding that *ptbx6-2.0-gfp* showed stronger GFP fluorescence than *ptbx6-1.7-gfp*, we found that the promoter activity of *ptbx6-1.7-luc* is 34% of *ptbx6-2.0-luc*. More importantly, we determined that the promoter activity of *mut1,2-2.0-luc* is 11% of *ptbx6-2.0-luc* (Table 1). Taken together, these data show that the normal expression of *tbx6* in the developing mesoderm is regulated directly by Wnt activation through the Tcf sites in the proximal region of the promoter.

A 900 bp *tbx6* promoter fragment is insufficient for normal expression

Because all three Tcf DNA binding sites are located in the 900 bp proximal promoter region, we wanted to know if this region of the promoter is sufficient to correctly target expression to the developing posterior mesoderm. To answer that, we constructed the reporter gene, *ptbx6-0.9-gfp/pXex-bfp*, by placing the sequence of the 900 bp proximal region of the *tbx6* promoter in front of the *gfp* gene, together with *pXex-bfp*. We observed no detectable GFP fluorescence in any of the embryos injected with *ptbx6-0.9-gfp/pXex-bfp*, even though these embryos had robust BFP expression (Table 1). In support of this, when the *ptbx6-0.9* was fused to *luciferase*, we found that it was only 6% as active as *ptbx6-2.0* (Table 1).

To determine if the Tcf sites were still functional in this construct, we examined the responsiveness of *ptbx6-0.9-gfp* to the constitutively active Tcf and to ectopic Wnt8. In both cases we observed ectopic GFP fluorescence in the embryos, showing that *ptbx6-0.9* is functional and capable of responding to Tcf activation (Table 1). Thus the 900 bp proximal region of the promoter is capable of responding to Tcf activation, but it is not sufficient to drive the expression of *gfp* in the developing posterior mesoderm, possibly because it lacks the ability to respond to Bmp signaling.

Identification of a Bmp responsive domain within *tbx6* promoter

To determine whether *ptbx6-0.9-gfp* can respond to Bmp, we coinjected either *ptbx6-1.7-gfp* or *ptbx6-0.9-gfp* with *Xbmp4* RNA into one-cell stage embryos and examined GFP fluorescence. At 50% epiboly, we observed no GFP fluorescence in embryos coinjected with *ptbx6-0.9-gfp* and *Xbmp4* RNA (Table 1). In contrast, we observed ectopic GFP fluorescence in embryos coinjected with *ptbx6-1.7-gfp* and *Xbmp4 RNA* (Fig. 4G,H, Table 1). These results show that *ptbx6-0.9-gfp* has a Wnt response element but lacks a promoter element necessary for the Bmp response.

Two regions were deleted in *ptbx6-1.7* to produce *ptbx6-0.9*, the distal 800 bp region (*dr800*) and the first intron. To ask whether either of these regions can mediate Bmp responsiveness, we placed them in front of the *SV40* minimal promoter driving the *luciferase* gene, generating *dr800-SV40-luc* and *intron-SV40-luc*. These reporter constructs were coinjected with and without *Xbmp4* RNA into one-cell stage zebrafish embryos and luciferase activity was assayed at the shield stage. Addition of *dr800* to *SV40-luc* enhanced the promoter activity approximately 3-fold, probably because of the endogenous Bmp present in the embryo (Fig. 7A). Coinjection of *Xbmp4* RNA and *dr800-SV40-luc* resulted in a further 3-fold increase in activity. In contrast, with *intron-SV40-luc* the promoter activity was the same with or without the addition of *Xbmp4* RNA (Fig. 7A). These data demonstrate that the Bmp response element is located in the 800 bp distal region of the *tbx6* promoter.

To better define the Bmp-responsive element of the *tbx6* promoter, we generated a series of deletion constructs of the

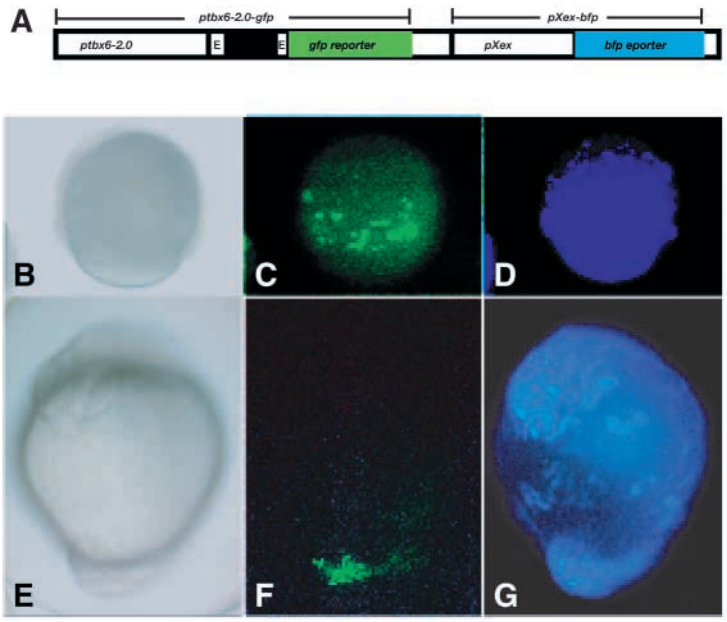

Fig. 5. A dual fluorescence reporter for examining promoter expression in zebrafish embryos. (A) Schematic diagram of the dual fluorescence reporter construct, *ptbx6-2.0-gfp/pXex-bfp*. The black box shows the *tbx6* first intron. (B-G) Pictures of embryos injected with the *ptbx6-2.0-gfp/pXex-bfp* promoter in bright field (B,E), showing GFP fluorescence (C,F) and BFP fluorescence (D,G). (B-D) Lateral views with animal pole to the top at 70% epiboly. (E-G) Ventral views with anterior to the top at the 16-somite stage. E, exon sequence.

dr800 region. We initially found that the 3′-half of the dr800 region retains the ability to respond to Bmp activation, whereas the 5′-half did not (Fig. 7B). Further analysis of this 3′-half of the dr800 region revealed that a 65 bp domain is capable of conferring Bmp responsiveness, and that further reduction of this 65 bp region into smaller domains eliminated the Bmp response (Fig. 7C). These data demonstrate that the core Bmp response element must be located within the 65 bp distal region of the *tbx6* promoter and suggest that multiple sites within this region are necessary to obtain the Bmp response. Collectively, our data show that the *tbx6* promoter has two distinct regulatory regions that confer the ability to respond to Wnt and Bmp signals. Moreover, we find that both regulatory elements must function cooperatively to confer full promoter activity in the developing posterior mesoderm.

Discussion

We show here that *tbx6* is regulated by combinatorial signaling from the Wnt and Bmp pathways. This fits well with a recent study demonstrating that the combination of Wnt and Bmp induces tail formation in zebrafish (Agathon et al., 2003), and suggests that *tbx6* is one of a series of posterior mesodermal genes regulated by these two pathways. Indeed, *eve1*, which has a similar expression pattern to *tbx6* (Joly et al., 1993), was also found to be jointly regulated by these two pathways (Agathon et al., 2003), although the mechanism of its regulation is at present unknown. Although the role of *eve1* in posterior mesoderm formation is unclear, a recent study indicates that *tbx6* functions as part of a network of T-box transcription factors to regulate domains within the posterior mesoderm (Goering et al., 2003).

Although the normal expression of *tbx6* is dependent on Wnt and Bmp signaling, we find that inhibition of either one of these signaling pathways does not lead to a complete loss of *tbx6* expression in the ventrolateral mesoderm. Interestingly, *wnt8* morphants show a graded reduction of *tbx6* expression with the strongest effect in the lateral domains of the ventrolateral mesoderm. In embryos injected with *wnt8MOs*, *bmp* expression becomes ventrally restricted even at the shield stage (see Fig. S3A-D at http://dev.biologists.org/ supplemental), and *tbx6* expression is restricted to this region. In contrast, loss of Bmp function causes a strong uniform reduction in *tbx6* expression, but does not significantly alter the expression of *wnt8* (see Fig. S3E-H at http:// dev.biologists.org/supplemental). These findings show that the Wnt and Bmp signaling pathways have

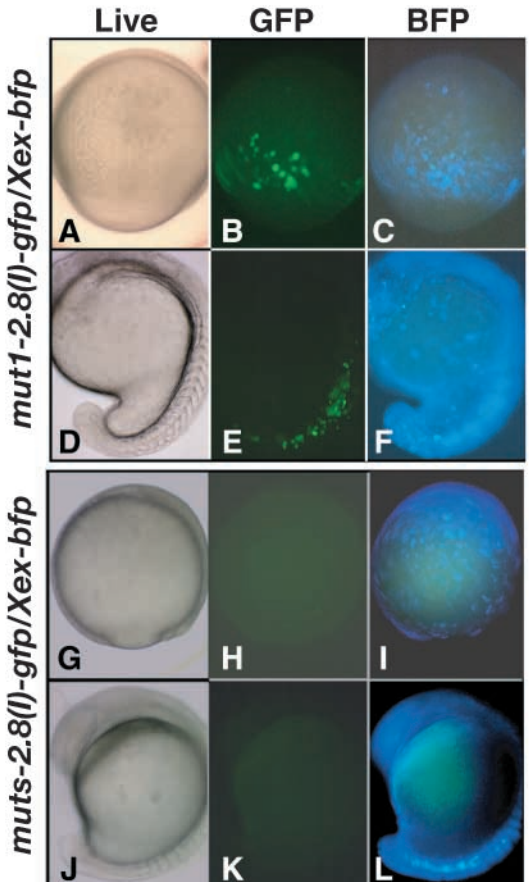

Fig. 6. Tcf sites are required for normal *tbx6* expression. Embryos were injected with the *tbx6* promoter containing a mutation in Tcf site 1 (*mut1-2.0-gfp/Xex-bfp*; A-F) or a promoter with mutations in Tcf sites 1 and 2 (*mut1,2-2.0-gfp/Xex-bfp*; G-L). Embryos were viewed in bright field, for GFP fluorescence or for BFP fluorescence as indicated on the right side of the panels. Embryos were at 70-90% epiboly (A-C,G-I) or at the 5-somite stage (D-F,J-L).

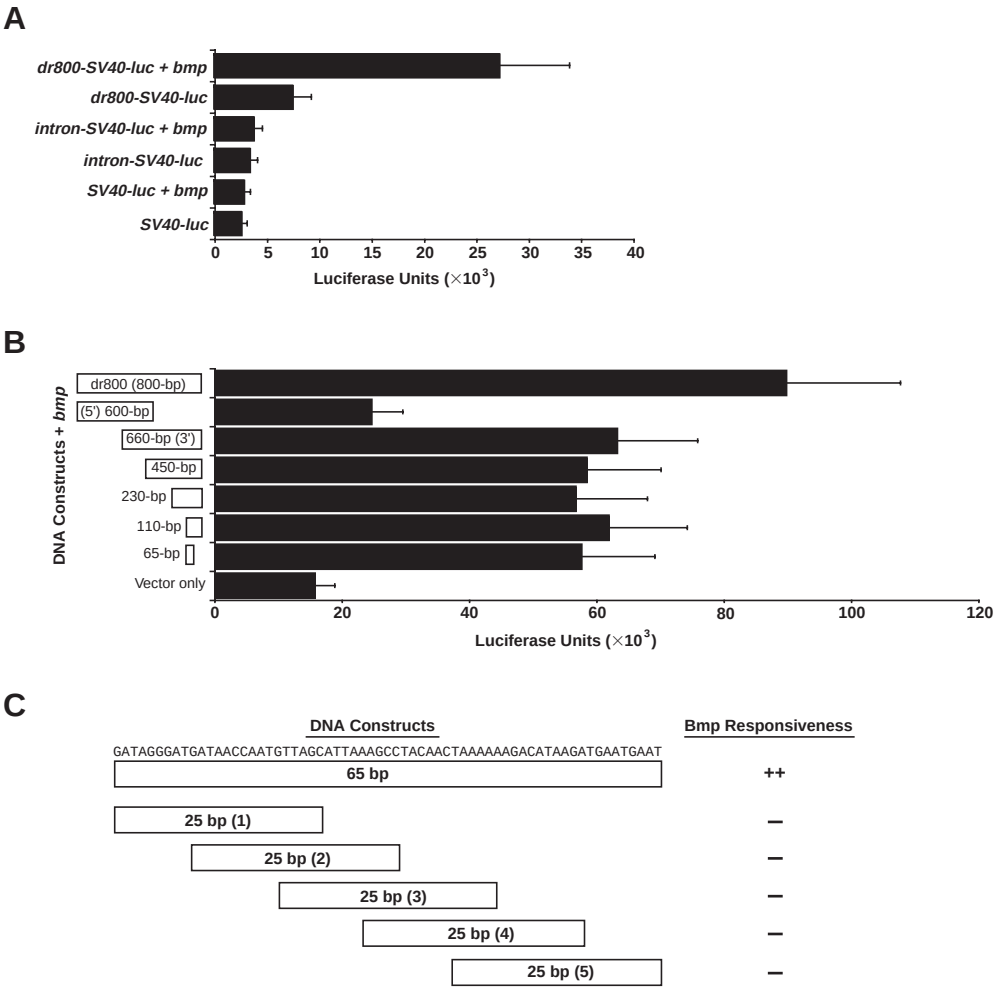

Fig. 7. Regulation of the *tbx6* promoter. (A) Luciferase activities of the indicated constructs measured at 50% epiboly, with and without the coinjection of *Xbmp4* (*bmp*). *dr800* is the distal 800 bp and *intron* is the first intron of the *tbx6* promoter. (B) Luciferase activities at 50% epiboly of the indicated constructs coinjected with *Xbmp4*, showing a 65 bp region of the *tbx6* promoter is fully capable of responding to Bmp activation. (C) Sequence and schematic diagram of the 65 bp region. Division of this region into domains of 25 bp [(1) to (5)] causes a complete loss of Bmp responsiveness.

distinct impacts on the overall expression of *tbx6* in the posterior mesoderm.

Although the contribution of Bmp and Wnt8 signals has a qualitative and quantitative difference in their ability to activate *tbx6* expression, their cooperation is essential to give the normal expression pattern during posterior mesoderm formation. This raises the question, why is combinatorial signaling used to regulate *tbx6* and other mesodermal genes? The expression patterns of the *bmp* genes and *wnt8* are very dynamic during early embryogenesis, yet *tbx6* expression is maintained in the ventrolateral mesoderm throughout somitogenesis. Combinatorial signaling not only restricts the domain in which *tbx6* is expressed to regions where both signals are present, but it permits *tbx6* to be expressed in regions where Bmp signaling is very low (the lateral regions) and where Wnt8 signaling is very low (the ventral regions from 90% epiboly onwards). Thus as the levels of the signals vary, *tbx6* expression is maintained.

Interestingly, a family of three *Drosophila* genes, *Dorsocross1-3*, encode T-box genes most closely related to *tbx6* (Reim et al., 2003). Although the expression patterns of these genes is complex, the expression of all three genes in the dorsal ectoderm and mesoderm requires both Wnt and Bmp signals (Reim et al., 2003). Whether this represents conservation of regulation or convergent evolution awaits further analysis, but it is an intriguing parallel.

Analysis of the *tbx6* promoter during posterior mesoderm development

Two models can be used to account for the required input of both Wnt and Bmp signals for the normal *tbx6* expression in the posterior mesoderm. One is that these pathways may signal independently of each other, but function together in a cooperative fashion to activate the expression of *tbx6*. Alternatively, these pathways may function in a linear pathway such that one signal is upstream of the other as was suggested from *Xenopus* studies (Hoppler and Moon, 1998; Marom et al., 1999) and a recent zebrafish study (Agathon et al., 2003).

To delineate the interplay between Wnt8 and Bmp signaling pathways in regulating the expression of *tbx6* during posterior mesoderm development, we isolated and characterized the promoter of *tbx6*. We found that a genomic fragment including sequences from 1.7 kb upstream of the start of translation through the second exon recapitulated the normal *tbx6* expression pattern in both zebrafish and *Xenopus* embryos, demonstrating that the regulatory elements are conserved between these species. The normal domain of *tbx6* expression was observed when the intron was removed from the promoter; however, we also observed some ectopic expression outside of the mesodermal domain, indicating that the intron contains a repressor of non-mesodermal expression (D.P.S. and D.K., unpublished).

Analysis of the *tbx6* promoter revealed independent

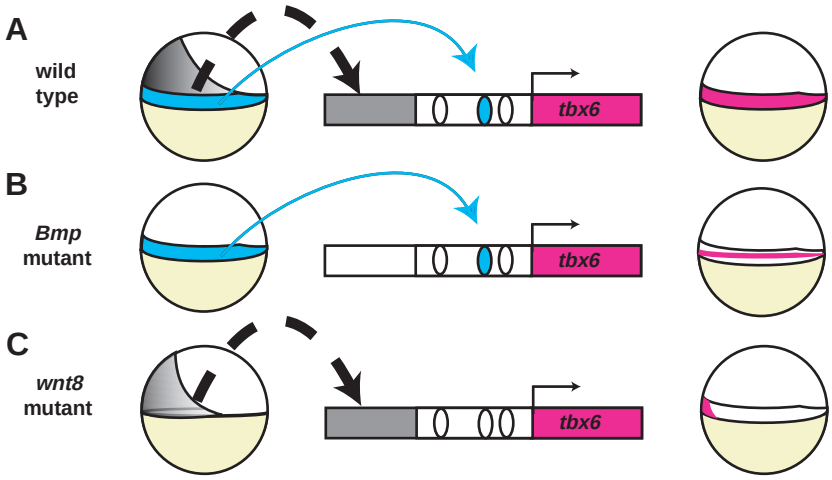

Fig. 8. Model for the combinatorial regulation of *tbx6* by Bmp and Wnt. (A) In the wild-type embryo, *tbx6* promoter utilizes two distinct domains to integrate the signaling inputs of both Bmp and Wnt (gray box, Bmp response domain; white box with three oval circles, Wnt response domain; oval circles, Tcf binding sites; black gradient, Bmp activity gradient; blue region, *wnt8* expression domain; blue filled oval circle, occupied Tcf site; pink color, *tbx6* gene and transcripts). *wnt8* expressed at the margin works combinatorially with *bmps* expressed ventrally to activate the *tbx6* promoter throughout the ventrolateral region. (B) In a *bmp* mutant or *noggin*-injected embryo, endogenous Wnt8 only weakly activates expression throughout the margin. (C) In a *wnt8* mutant or in an embryo injected with *wnt8MOs*, endogenous Bmp signaling activates *tbx6* only in the most ventral region where Bmp activity is the highest. When Wnt8 signaling is inhibited, Bmp signaling is confined to a more ventral domain than in wild-type embryos.

elements that respond cooperatively to Wnt and Bmp signals. In the proximal 900 bp, we discovered three Tcf sites and demonstrated that mutation of both of the two most proximal sites prevents normal *tbx6* expression. Because Tcf is activated by Wnt signaling, these results demonstrate that *tbx6* is a direct target of Wnt signaling. We located the Bmp response element to a distinct region of the *tbx6* promoter, located between 1.7 kb and 0.9 kb (dr800) from the start of translation. In the presence of high levels of Bmp signal, this region alone can activate transcription when attached to a SV40 minimal promoter, but the activity is much weaker than that of the full 1.7 kb promoter. Within this dr800 region, we identified a 65 bp domain, which is capable of responding to Bmp activation. Smaller fragments did not respond to Bmp signaling, suggesting that the Bmp response element is composed of more than one transcription factor binding site. How this element is activated by Bmp signaling is not yet known. A transcription factor required for the function of Bmp-type Smads called OAZ was shown to be required for the activation of a key Bmp target gene in *Xenopus* (Hata et al., 2000), but no OAZ site was found in the *tbx6* promoter. Moreover, during somitogenesis OAZ is expressed anteriorly (Hata et al., 2000), whereas *tbx6* is expressed at the most posterior end of the embryo (Hug et al., 1997), suggesting that a novel transcription factor regulates the Bmp response of *tbx6*.

Regulation of *tbx6* by combinatorial Wnt and Bmp signaling

Because the normal regulation of *tbx6* requires input from both the Wnt and Bmp pathways, we were surprised to find that ectopic expression of either signal at the level used to produce tails (Agathon et al., 2003) resulted in strong activation of the endogenous *tbx6* gene and of the *tbx6* promoter. Using inhibitors of Bmp and Wnt8 signaling, we found that overexpression of each signaling pathway alone is able to activate *tbx6* expression when the other pathway is blocked, demonstrating that Bmp and Wnt8 have the potential to activate *tbx6* without the contribution of the other signaling pathway. Because the endogenous Bmp and Wnt8 can not activate *tbx6* when the other pathway is inhibited, our results suggest that Bmp and Wnt signals are present in the embryo at relatively low levels such that they can combinatorially activate *tbx6* only when both are present. This mechanism ensures that *tbx6* is only expressed in the regions where these two factors overlap. How this works in regulating the promoter remains to be determined. At endogenous levels of signaling, it could be that the Wnt and Bmp response sites are only active transiently when a single signal is present, and only when both signals are present does a stable transcription complex form. At higher levels of signaling through one factor, the sites are occupied continuously and this promotes transcription of the *tbx6* gene.

From all of this data we have put together a model to explain how *tbx6* is regulated in the normal embryo and in mutants. In the normal embryo, *wnt8* is expressed at the margin in the gastrula-stage embryo and *bmps* are expressed in a ventral to dorsal gradient (Fig. 8A). Together they combine to activate *tbx6* at the margin, with both signals working at submaximal levels. In *bmp* mutants (and *noggin*-injected embryos), Wnt8 causes a low level of *tbx6* expression in the lateral and ventral regions (Fig. 8B). In *wnt8* mutants (or in embryos injected with *wnt8MOs*), Bmps activate *tbx6* in the ventral region where they are most abundant (Fig. 8C).

Tbx6: a platform for multiple levels of regulation

Our study has focused only on the regulation of *tbx6* by the Wnt and Bmp pathways. Earlier analyses have shown that *tbx6* is also strongly regulated by *spt* (Griffin et al., 1998) and weakly by *ntl* (Hug et al., 1997; Griffin et al., 1998). Although Agathon et al. (Agathon et al., 2003) demonstrated that tails can be induced by overexpression of Wnt and Bmp at levels similar to those used here, they also found that co-expression of a member of the Nodal pathway lowered the amount of Wnt and Bmp necessary to form tails. Because Nodal signaling regulates *spt* (Griffin and Kimelman, 2003), one explanation for the synergism between the Nodals and the Wnt and Bmp pathways is that Spadetail contributes directly or indirectly to *tbx6* expression. Thus *tbx6* will continue to serve as a useful paradigm for understanding how multiple regulatory inputs lead to the formation of the posterior mesoderm.

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