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Molecular identification of *spadetail:* regulation of zebrafish trunk and tail mesoderm formation by T-box genes

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SUMMARY

Inhibition of fibroblast growth factor (FGF) signaling prevents trunk and tail formation in *Xenopus* and zebrafish embryos. While the T-box transcription factor Brachyury (called No Tail in zebrafish) is a key mediator of FGF signaling in the notochord and tail, the pathways activated by FGF in non-notochordal trunk mesoderm have been uncertain. Previous studies have shown that the *spadetail* gene is required for non-notochordal trunk mesoderm formation; *spadetail* mutant embryos have major trunk mesoderm deficiencies, but relatively normal tail and notochord development. We demonstrate here that *spadetail* encodes a T-box transcription factor with homologues in *Xenopus* and chick. Spadetail is likely to be a key mediator of FGF signaling in trunk non-notochordal mesoderm, since *spadetail* expression is regulated by FGF signaling. Trunk and tail development are therefore dependent upon the complementary actions of two T-box genes, *spadetail* and *no tail*. We show that the regulatory hierarchy among *spadetail*, *no tail* and a third T-box gene, *tbx6*, are substantially different during trunk and tail mesoderm formation, and propose a genetic model that accounts for the regional phenotypes of *spadetail* and *no tail* mutants.

Key words: *spadetail*, *brachyury*, *no tail*, *tbx6*, Posterior development, FGF, Zebrafish, T-box

INTRODUCTION

Mesoderm in the trunk and tail of vertebrate embryos differs fundamentally from mesoderm in the head. A dramatic example of this, first demonstrated by Amaya et al. (1991), is the critical requirement during gastrulation for fibroblast growth factor (FGF) signaling in trunk and tail mesoderm. Inhibition of FGF signaling in both *Xenopus* and zebrafish embryos using a dominant negative FGF receptor (dnFGFR) results in embryos with normal head development but lacking in trunk and tail mesoderm (Amaya et al., 1991; Griffin et al., 1995). Genes that function downstream of FGF signaling must therefore serve important roles during trunk and tail mesoderm formation.

A key target of FGF signaling in mesoderm is the *Brachyury* gene, called *no tail* (*ntl*) in zebrafish. *Brachyury* homologues encode T-box-containing transcription factors (Kispert et al., 1995a; Conlon et al., 1996), which are highly conserved and are found throughout the metazoan lineage (Herrmann et al., 1990; Smith et al., 1991; Kispert et al., 1994, 1995b; Schulte-Merker et al., 1994). In vertebrate embryos, *Brachyury* is expressed transiently by all mesodermal progenitors and expression persists in notochord cells (Wilkinson et al., 1990; Smith et al., 1991; Schulte-Merker et al., 1992; Northrop and Kimelman, 1994). FGF signaling is required for the expression of *Xenopus Brachyury* (Amaya et al., 1993; Isaacs et al., 1994; Schulte-Merker and Smith, 1995) and *no tail* in zebrafish (Griffin et al., 1995), and *Brachyury* mutant mice and *ntl* mutant zebrafish both lack notochord and have severe defects in posterior development (Chesley, 1935; Halpern et al., 1993).

However, it is clear from studies in zebrafish that loss of *ntl* function alone does not account for all of the defects caused by inhibition of FGF signaling. *ntl* mutant embryos lack only the notochord and tail (Halpern et al., 1993), whereas FGF signaling is required throughout trunk and tail mesoderm (Griffin et al., 1995). Thus another gene or set of genes, collectively referred to as *'no trunk'*, must operate in trunk mesoderm to mediate the effects of FGF (Griffin et al., 1995). Additional evidence supports this view that the mechanism of trunk mesoderm formation is genetically separable from the mechanism underlying tail mesoderm formation. Mutant analysis in zebrafish has shown that the *spadetail* (*spt*) gene controls mesodermal cell fate and morphogenesis in the trunk (Kimmel et al., 1989; Ho and Kane, 1990; Weinberg et al., 1996; Amacher and Kimmel, 1998; Yamamoto et al., 1998). *spt* mutant embryos are severely deficient in trunk non-notochordal mesoderm, but have relatively normal tail development (Kimmel et al., 1989).

We wished to identify the hypothetical gene *'no trunk'*, to further understand the pathways underlying trunk mesoderm

formation. We reasoned that, since *ntl* is expressed by trunk paraxial mesoderm but is not required in those cells, 'No Trunk' might be functionally redundant with Ntl and thus be a member of the T-box gene family (Bollag et al., 1994; Papaioannou and Silver, 1998). We therefore cloned novel zebrafish T-box genes and show here that one of these is the *spt* gene. *spt* is highly homologous to recently identified genes from *Xenopus* and chick (Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996; Horb and Thomsen, 1997; Knezevic et al., 1997) and its expression is regulated by FGF. Based on both the regulation of *spt* and the *spt* mutant phenotype, we argue that Spt mediates the effects of FGF signaling in trunk non-notochordal mesoderm, thereby accounting for the activity previously attributed to the hypothetical *'no trunk'* gene. Both *spt* and *ntl* are expressed in trunk and tail mesodermal progenitors, yet *spt* predominantly regulates trunk mesoderm formation and *ntl* predominantly regulates tail formation. To gain insight into why these genes are essential in only a subset of cells that express them, we examined the regulatory hierarchy between *spt*, *ntl* and a third T-box gene, *tbx6* (Hug et al., 1997). We show that a major change in the regulatory hierarchy among these T-box genes occurs as tail mesoderm begins to form. This regulatory hierarchy supports a genetic model that explains the regional defects in ntl^{-} and spt^{-} mutants.

MATERIALS AND METHODS

RT-PCR and cloning

RNA was isolated from early gastrula stage embryos (shield stage, Kimmel et al., 1995) using the hot phenol method (Schulte-Merker et al., 1992). First-strand cDNA was prepared from 5 μg total RNA using oligo(dT) primer and Superscript II reverse transcriptase (Gibco BRL) according to manufacturers' instructions; reactions were performed at 50˚C for 2 hours. 1/10 of the reaction was amplified for 30 cycles by PCR with a pair of degenerate primers encoding the amino acid sequences Y(I/M)HPDS and VTAYQN using *Tfl* polymerase (Epicenter Technologies). Amplification products were analyzed by PAGE and amplicons in the expected size range (220-250 bp) eluted from gel slices, subcloned and sequenced. Amplicons with homology to other T-box genes were identified using NCBI-BLAST. Several novel sequences were obtained, these were used as probes to screen a gastrula stage cDNA library (4-8 hours postfertilization (h.p.f.); gift of T. Lepage). A 2.6 kb cDNA of the *spt* candidate gene was sequenced fully on both strands using automated sequencing and found to contain a single complete open-reading frame of 1410 nucleotides (GenBank accession number AF077225).

Molecular analysis of *spt* mutant alleles

mRNA was isolated from mid-somite stage embryos showing the spt^{-} phenotype (18 h.p.f.) using Tri-Reagent (Molecular Research Center, Inc.), and first-strand cDNA was synthesized as described. The coding region of the *spt* candidate gene was amplified by PCR with *Vent* DNA polymerase (New England Biolabs), subcloned and sequenced fully on both strands using automated sequencing. spt^{b104} originated from a stock with a complex genetic background (Kimmel et al., 1989). The spt^{b104} mutation removes nucleotides +660-745 and introduces 8 extra bases (AAATTAAA) containing an in-frame stop (underlined). spt^{b433} was isolated from ENU-mutagenized stock (Henion et al., 1996) and has an 8-base insertion (TAAGACCA) at position +589 that contains an in-frame stop (underlined). spt^{m423} was isolated from ENU-mutagenized stock (Solnica-Krezel et al., 1996) and contains a T→A point mutation at position +234 that creates a TAA stop codon. spt^{b333} is a gamma-ray-induced allele that eliminates polymorphic DNA markers located near the *spt* gene (S. L. A., unpublished data).

To test for linkage between the *spt* candidate gene and *spt*, genomic DNA was prepared (Johnson et al., 1994) from wild-type and mutant haploid embryos obtained from $spt^{b104}/+$ females, followed by PCR to genotype individual haploid embryos using the following primer pair: CCTTTACTGCCGTCACTGCCTACC and GCCTTCACCTCCAGCTCTTTACG. These primers flank the intragenic deletion found in spt^{b104} and amplify a 1.6 kb product in *spt* mutant haploids and an approximately 2.5 kb product in wild-type haploids. This primer pair is not recommended for genotyping of diploid embryos.

Embryo handling, mutant strains and riboprobe synthesis

Adult zebrafish and embryos were reared at 28.5˚C as described (Westerfield, 1995). For in situ hybridisation experiments, *spt* mutant embryos were obtained from intercrosses of $spt^{b104}/+$ heterozygous adults; *ntl* mutant embryos from intercrosses of $ntl^{b195}/+$ heterozygous adults and $spt^{-};ntl^{-}$ double-mutant embryos from intercrosses of $spt^{b104}/+$; $ntl^{b195}/+$ doubly heterozygous adults. Riboprobes were synthesized from linearized plasmids containing full-length cDNAs using T7 RNA polymerase (Boehringer-Mannheim) and standard conditions. Full-length *tbx6* (Hug et al., 1997) was linearized with *Not*I and full-length *spt* cDNA was linearized with *Eco*RI. *ntl* probe was synthesized as described (Schulte-Merker et al., 1992).

mRNA encoding a dominant-negative FGF receptor, XFD (Amaya et al., 1991) was synthesized using the mMessage mMachine kit (Ambion Inc.) and injected into newly fertilized eggs from AB strain adult zebrafish as described (Griffin et al., 1995).

In situ hybridisation, immunocytochemistry and photography

Whole-mount in situ hybridization was performed using digoxigenin-labeled antisense RNA and visualized using anti-digoxigenin Fab fragments conjugated with alkaline phosphatase (Boehringer Mannheim) as previously described (Griffin et al., 1995; Melby et al., 1997). Whole-mount antibody staining was performed as described (Griffin et al., 1995). Mero-myosin was visualized using MF20 supernatant (Developmental Studies Hybridoma Bank) at 1:10 dilution, followed by HRP-conjugated goat anti-mouse Ig (Zymed) at 1:200. HRP was developed for 30 minutes at room temperature as described (Westerfield, 1995). Embryos stained by in situ hybridisation or immunocytochemistry were cleared in methyl salicylate (Sigma) or 1:2 benzyl alcohol: benzyl benzoate, mounted in Permount (Fisher) on bridged slides and photographed. For sectioning, stained embryos were mounted in Epon and sectioned as described (Melby et al., 1997). Live embryos were anesthetized with tricaine (Sigma), oriented in 0.6-1.2% agar and photographed.

RESULTS

Molecular identification of *spadetail*

FGF signaling is required for mesoderm formation in the trunk and tail. One mediator of FGF signaling is Brachyury/No Tail, a T-box transcription factor required for notochord and tail formation. We sought to identify the mediator of FGF signaling in trunk non-notochordal mesoderm, previously referred to as *'no trunk'* (Griffin et al., 1995), and reasoned that *'no trunk'* might also be a member of the T-box gene family. In order to identify potential candidates for *'no trunk'*, we performed RT-PCR using degenerate primers to highly conserved regions within the T-box, a 180 amino acid region responsible for DNA binding and dimerization (Bollag et al., 1994; Müller and Herrmann, 1997; Papaioannou and Silver, 1998). Several

previously unidentified zebrafish T-box genes were isolated, one of which is the likely orthologue of a gene identified by several groups in *Xenopus* (*Xombi*, Lustig et al., 1996; *Antipodean*, Stennard et al., 1996; *VegT*, Zhang and King, 1996; *BraT*, Horb and Thomsen, 1997) and also in chick (*ChTbx6L*, Knezevic et al., 1997). The proteins are highly conserved within the T-box, but are also homologous outside of this domain (Fig. 1).

We anticipated that a mediator of FGF signaling in trunk mesoderm might also be the *spt* gene, as *spt* mutant embryos are severely deficient in trunk non-notochordal mesoderm (Fig. 2A,D; Kimmel et al., 1989). Muscle-specific staining is markedly reduced and disorganized in *spt*$^{-}$ embryos (Fig. 2B,E), but tail muscle development is remarkably normal (Kimmel et al., 1989). Furthermore, the expression pattern during gastrulation of the T-box gene that we had cloned (see below) is very similar to that of *paraxial protocadherin* (*papc*), a downstream genetic target of *spt* (Yamamoto et al., 1998). We therefore tested whether this T-box gene was *spt*, and found that we could not detect the cloned sequence in *spt*$^{-}$ embryos carrying a deficiency allele, *b333*, either by in situ hybridization (Fig. 2C,F) or by gene-specific PCR (Fig. 2G).

Sequencing data and linkage analysis demonstrated that the T-box gene that we identified was indeed *spt*. We sequenced the coding region of the candidate gene from *spt*$^{-}$ embryos carrying three putative point mutant alleles of *spt* and found mutations that introduce a premature stop codon in all cases (see Methods for details of molecular lesions). The mutations all remove the COOH-terminal putative regulatory region (Kispert et al., 1995a; Conlon et al., 1996), and two of the mutations also remove some or most of the T-box, which is the DNA-binding and dimerisation domain (Fig. 2H; Müller and Herrmann, 1997). Cosegregation analysis demonstrated that the candidate gene was tightly linked to *spt*. The molecular lesion identified in one of the alleles, *spt*^{b104}, is a small deletion that we used as a polymorphic marker to test linkage to the *spt*$^{-}$ phenotype. We found no recombinants among 473 haploid progeny of *spt*^{b104}/$_{+}$ females genotyped for this polymorphism (0±0.42 cM; data not shown). We conclude that we have identified the *spt* gene and that *spt* encodes a T-box-containing transcription factor. The three *spt* alleles that we have characterized are all likely to be null alleles; homozygous *spt* embryos carrying either *b104*, *b433* and *m423* all have very similar phenotypes at day 1 of development, and the phenotype during gastrulation and early segmentation of *trans*-heterozygous mutant embryos carrying *spt*^{b104} over the deficiency is not more severe than the phenotype of homozygous *b104* embryos (S. L. A., unpublished data).

spt is expressed at sites of mesoderm formation and involution

spt is extensively expressed in regions of the gastrula embryo and tail bud where mesoderm forms. In zebrafish embryos, mesoderm forms at the blastoderm margin and eventual mesodermal fates correlate with the dorsoventral position of the progenitors in the early gastrula (Kimmel et al., 1990). During gastrulation, mesodermal progenitors involute or ingress at the margin (Warga and Kimmel, 1990; Shih and Fraser, 1995), forming two layers of cells: an outer layer of non-involuted cells, the epiblast, and an inner layer of involuted mesodermal and endodermal cells, the hypoblast. At the margin, these two layers are called the germ ring (Kimmel et al., 1995). *spt* expression is first detected in the late blastula and is initially ubiquitous (Fig. 3A). This contrasts with the likely *Xenopus* orthologue, which is present in the oocyte as a maternal RNA localized to the vegetal pole, and is never

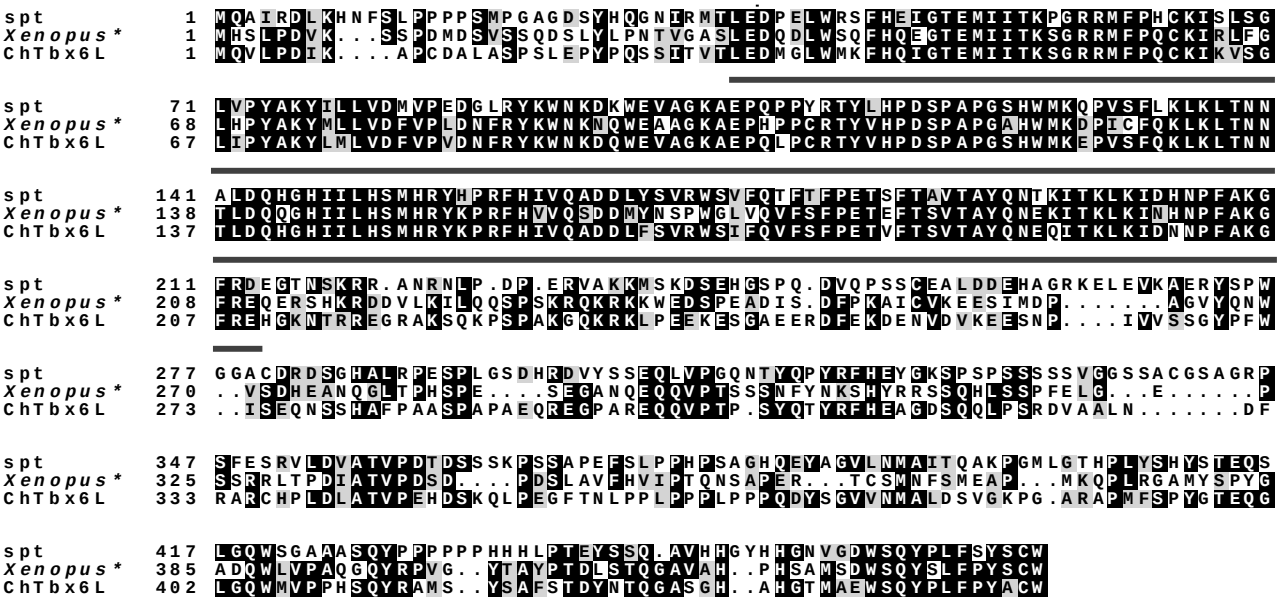

Fig. 1. Comparison of the amino acid sequences of Spt with likely orthologues from *Xenopus* and chicken (ChTbx6L, Knezevic et al., 1997). The murine *spt* orthologue has not yet been identified. *The *Xenopus* orthologue was independently identified by four groups (*Xombi*, Lustig et al., 1996; *Antipodean*, Stennard et al., 1996; *VegT*, Zhang and King, 1996; *BraT*, Horb and Thomsen, 1997). Since each group reported slightly different sequences protein sequences, only one (Antipodean) is shown here. Darkly shaded residues are conserved, light-shaded residues are similar, dots indicate deletions. The T-box is underlined.

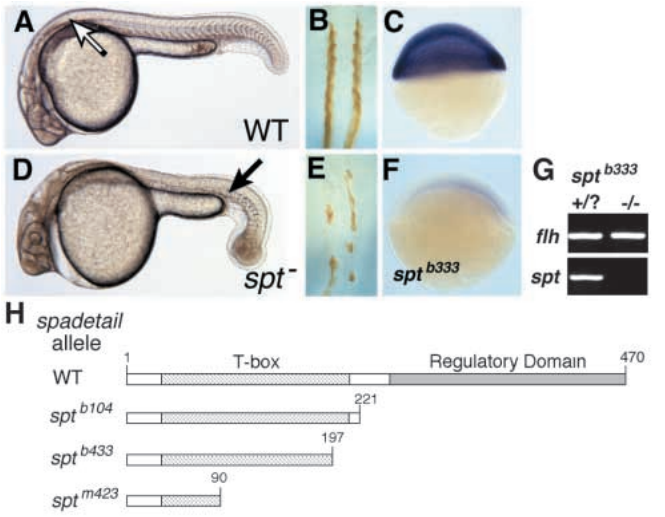

Fig. 2. Appearance of (A) wild-type (WT) and (D) *spt*⁻ live embryos at 24 hours postfertilization (h.p.f.; anterior, left). In both panels, arrow marks the first visible somite. Chevron-shaped somites are visible in the trunk and tail of WT embryos, but are absent from the trunk of *spt*⁻ embryos. In *spt*⁻ embryos, presumptive trunk mesodermal progenitors migrate abnormally and form a mass of cells at the tail tip (Ho and Kane, 1990). (B,E) Dorsal views of 24 h.p.f. embryos stained with an antibody to mero-myosin (anterior, uppermost). Bilateral rows of expression are visible either side of the notochord in the WT embryo (B) but staining in the *spt*⁻ embryo (E) is significantly reduced and is patchy. (C,F) Embryos at 50% epiboly obtained from intercrosses of *spt*^{b333}/+ adults hybridized with *spt* probe (blue stain). 75% of the embryos (C) express *spt*, whereas 25% of the embryos from such a cross, which presumably represent the mutant embryos (F), had no detectable *spt* expression, even after a prolonged colour reaction. (G) Bulk-segregant analysis of *spt*^{b333} genomic DNA by PCR. As a control, *floating head* (*flh*) was amplified from pooled DNA isolated from phenotypically WT and *spt*⁻ embryos, whereas the *spt* candidate gene could not be amplified from the mutant DNA. (H) Schematic maps of WT and truncated Spt proteins; the putative regulatory domain is inferred from studies of the Brachyury protein (Kispert et al., 1995a). Numbers above the coding regions refer to amino acid position. See Methods for details of the molecular lesions.

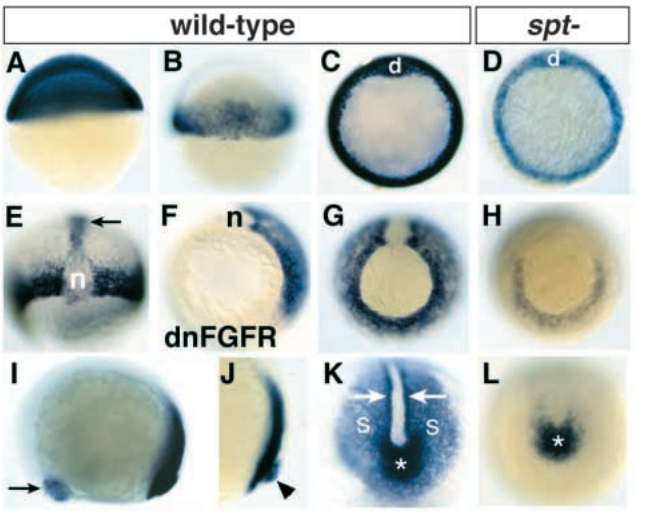

Fig. 3. Expression of *spt* in WT and *spt*⁻ embryos and regulation by FGF signaling. (A) *spt* expression, first detected between sphere and dome stages, is ubiquitous in the late blastula embryo (40% epiboly, 5 h.p.f., lateral view). (B) By the early gastrula stage (shield, 6 h.p.f., dorsal view), *spt* expression is specific to the mesoderm and, in an animal pole view (C), is found throughout all regions of the germ ring (d, dorsal). (E) *spt* expression refines during gastrulation and by 60% epiboly (7 h.p.f., dorsal view), *spt* is not expressed in the notochord progenitors (n), whereas lateral and ventral germ ring and head mesoderm (arrow) express *spt*. (F,G) Vegetal views of *spt* expression in late-gastrula embryos (80% epiboly, 8.3 h.p.f., dorsal uppermost). (F) Expression of a dominant-negative FGF receptor (dnFGFR), which is mosaicly distributed (Griffin et al., 1995), causes loss of *spt* expression in large areas of the germ ring; compare with WT in G. (I) At the 4-somite stage (11.5 h.p.f., anterior to left) expression is localized to the hatching gland progenitors (arrow), the tail bud and the segmental plate mesoderm. (J,K) In the tail bud, *spt* is expressed in epiblast cells at the site of involution (arrowhead in J, * in K), and in the segmental plate (S); *spt* is also expressed in adaxial cells (arrows; Devoto et al., 1996). (J, 8 somites, 13 h.p.f., lateral view, anterior to left; K, 4 somites, 11.5 h.p.f., posterior view, dorsal uppermost). (D,H,L) *spt* expression in *spt*⁻ embryos. (D) In early gastrula *spt*⁻ embryos (shield, 6 h.p.f., animal pole view), *spt* expression is weak but has normal spatial distribution (d, dorsal); compare with C. (H) During gastrulation (80% epiboly, 8.3 h.p.f., vegetal view), *spt* expression is not maintained in hypoblast or dorsolateral epiblast, but is weakly expressed on the ventral side; compare with G. (L) In early-somite stage *spt*⁻ embryos (4 somite, 11.5 h.p.f., posterior view, dorsal uppermost), *spt* is expressed in the tail bud (*) but not in the segmental plate mesoderm (compare with K).

expressed in the presumptive ectoderm (Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996; Horb and Thomsen, 1997). In the late blastula and early gastrula embryo, *spt* expression becomes rapidly restricted to marginal cells (Fig. 3B,C). After involution of the head mesoderm, *spt* is not expressed in dorsal marginal cells, which are fated to become notochord (Fig. 3E; Kimmel et al., 1990; Shih and Fraser, 1995; Melby et al., 1996).

To determine which germ layers express *spt*, we characterized *spt* expression at single cell resolution in sectioned mid-gastrula-staged embryos that were hybridized for *spt* mRNA in whole mount. We observed that *spt* is expressed in both epiblast and hypoblast cells of the lateral and ventral germ ring. In the epiblast, *spt* is expressed in marginal cells close to the point of involution but, in the hypoblast, *spt* is extensively expressed in cells located up to several cell diameters away from the margin (Fig. 4A). At this and later stages, *spt* expression is almost identical to that of a related T-box gene, *tbx6* (Fig. 4B, Hug et al., 1997), and both *spt* and *tbx6* are co-expressed with *ntl* in non-notochord mesoderm closest to the margin (Fig. 4C).

In the tail bud, *spt* is expressed in the region of the epiblast where mesodermal involution has been shown to occur (Kanki and Ho, 1997), and broadly throughout the hypoblast, with the exception of the notochord (Fig. 3I-K). *spt* is also prominently expressed in adaxial cells flanking the notochord (Fig. 3K). *spt* expression in the segmental plate is downregulated before cells coalesce into somites, and *spt* expression in the tail bud and paraxial mesoderm persists until the end of somitogenesis (data not shown). Even though *spt* is expressed throughout all stages of trunk and tail development, mutant analysis indicates that it is only required in the trunk since tail somites form normally

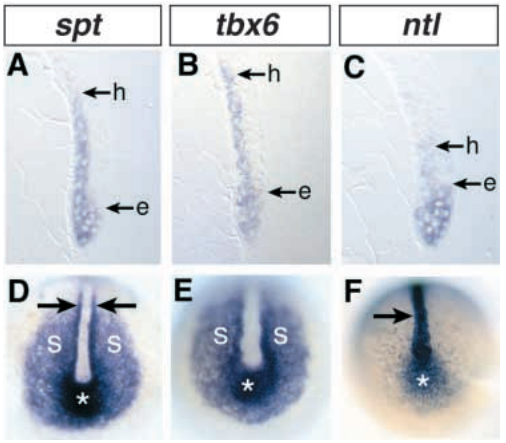

Fig. 4. Overlapping and complementary expression of T-box genes in the early mesoderm. (A-C) Sections through lateral germ ring at mid-gastrulation (8.5 h.p.f.); arrows mark limits of expression in epiblast (e) and hypoblast (h). (A) *spt* and (B) *tbx6* are expressed in epiblast cells closest to the margin, and broadly in the hypoblast. (C) *ntl* expression is expressed only near the margin in both the epiblast and hypoblast. (D-F) Posterior views of 4-6-somite embryos (12 h.p.f., dorsal uppermost). (D) *spt* and (E) *tbx6* are co-expressed in the region of the tail bud epiblast (*) where involution occurs (Kanki and Ho, 1997) and in the underlying hypoblast and segmental plate (S), but only *spt* is prominently expressed in adaxial cells (arrows in D). (F) *ntl* is expressed more broadly in the tail bud epiblast (*); *ntl* expression in the hypoblast is restricted to the notochord (arrow).

in *spt*⁻ embryos (Fig. 2D, Kimmel et al., 1989). The sequence and expression of this gene was recently reported by Ruvinsky et al. (1998), who refer to it as *tbx16*. In addition to the mesodermal expression, *spt/tbx16* is also expressed in a small number of spinal cord cells (data not shown, see Ruvinsky et al., 1998), as described for the likely *Xenopus* orthologue (Lustig et al., 1996; Zhang and King, 1996).

Expression of *spt* in the tail bud is extremely similar to *tbx6* expression, except that *tbx6* is not prominently expressed in adaxial cells (Fig. 4D,E; Hug et al., 1997). *spt*, *tbx6* and *ntl* are all expressed in uninvoluted mesodermal cells in the tail bud

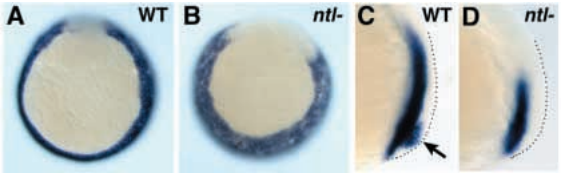

Fig. 5. Dynamic regulation of *spt* expression in *ntl*⁻ embryos. (A,B) Mid gastrula (7.5 h.p.f., vegetal view). *spt* is expressed at normal levels in the *ntl* mutant embryo. Although the spatial distribution of *spt* expression is slightly different in *ntl*⁻ embryos, the broader region of non-expressing cells represent dorsal mesoderm as they express the axial mesoderm marker *floating head* (data not shown). (C,D) 8-somite stage (13 h.p.f., lateral view, anterior is left). *spt* expression in the tail bud epiblast (arrow) is readily detected in WT embryos (C), but this expression domain is not seen in *ntl*⁻ embryos at a similar stage (D). The domain of *spt* expression in involuted mesoderm is only partially reduced in the mutant embryo. The increased thickness of the epiblast in *ntl*⁻ embryos is already apparent at this stage (dots mark outer surface of epiblast).

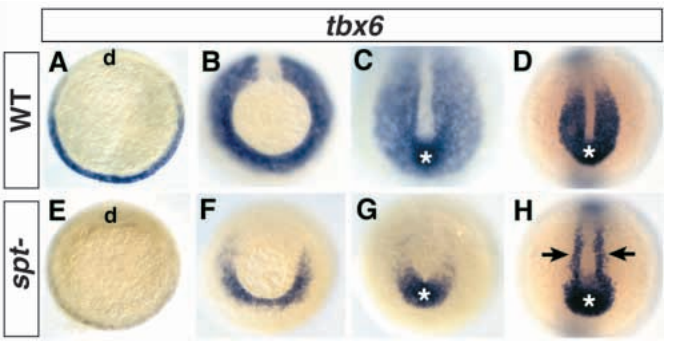

Fig. 6. *spt* function regulates *tbx6* expression in trunk but not tail mesoderm. (A) In WT embryos at the early gastrula stage (shield, 6 h.p.f., animal pole view; d, dorsal) *tbx6* expression is restricted to ventral mesoderm. During gastrulation (B; 80% epiboly, 8.3 h.p.f., vegetal view) *tbx6* expression is up-regulated in the dorsolateral mesoderm, and during segmentation (C,D) *tbx6* is extensively expressed in tail bud epiblast (*) and segmental plate (C, 2-4 somites, 11 h.p.f.; D, 7-8 somites, 13 h.p.f.; posterior view, dorsal uppermost). In *spt*⁻ embryos, *tbx6* expression is initially weak (E), remains restricted to the ventral side during gastrulation (F) and is barely detectable in the segmental plate at 2-4 somites (G). However, at 7-8 somites (H) *tbx6* has a relatively normal distribution compared to WT, and is easily detected in segmental plate mesoderm (arrows) in addition to the tail bud (*).

epiblast but, in the hypoblast, *spt* and *tbx6* are expressed in segmental plate, whereas *ntl* expression is restricted to the notochord (Fig. 4D-F).

spt expression is abnormal in *spt*⁻ embryos

The expression of *spt* can be monitored in *spt*^{b104} embryos since the mutant mRNA is expressed, but it does not produce functional protein. Although the distribution of *spt* transcripts in *spt*⁻ embryos is normal in blastula and early gastrula embryos, the level of expression is much reduced (compare

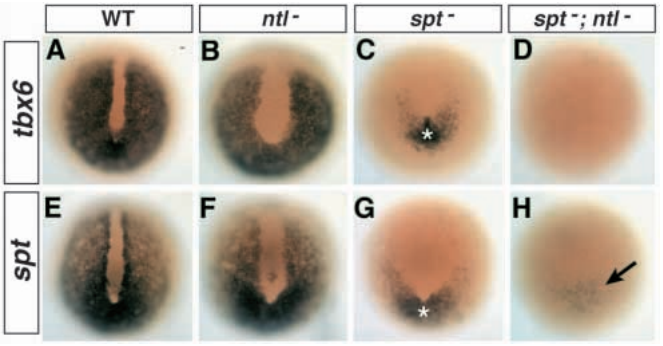

Fig. 7. Synergistic regulation of *spt* and *tbx6* by *spt* and *ntl*. Expression of *tbx6* (A-D; bud, 10 h.p.f.) and *spt* (E-H; 95% epiboly, 9.5 h.p.f.). In *ntl*⁻ embryos expression of *tbx6* (B) and *spt* (F) are similar to WT (A,E) except for broadening of the non-expressing notochord domain (Melby et al., 1997). In addition, *spt* is no longer prominently expressed by adaxial cells. In *spt*⁻ embryos, expression of both *tbx6* (C) and *spt* (G) are very weak or absent from hypoblast cells but remain detectable in the tail bud epiblast (*). In *spt*⁻;*ntl*⁻ embryos, expression of *tbx6* (D) is entirely undetectable, and *spt* (H) is only barely detected (arrow).

Fig. 3C and D), suggesting either that *spt* may autoregulate, or that the mutant mRNA may have a shorter half-life than the wild-type mRNA. As gastrulation proceeds, however, the distribution of *spt* transcripts in *spt*⁻ embryos differs dramatically from that in wild-type embryos (compare Fig. 3G and H). In mid-gastrula *spt*⁻ embryos, *spt* expression is very weak or absent throughout the hypoblast and is absent entirely from the dorsolateral germ ring; *spt* is still expressed ventrally, however (Fig. 3H). *spt* expression is detectable in the tail bud epiblast of *spt*⁻ embryos during segmentation, but expression in the segmental plate is at best very weak, even during tail somitogenesis (Fig. 3L, and data not shown).

spt is regulated by FGF signaling

We tested whether *spt* expression was dependent upon FGF signaling using expression of a dominant-negative FGF receptor (dnFGFR). We find that *spt* is indeed regulated by FGF, like its *Xenopus* and chick homologues (Lustig et al., 1996; Horb and Thomsen, 1997; Knezevic et al., 1997). While expression of *spt* in the early gastrula is not significantly affected by dnFGFR expression (data not shown), *spt* expression is dependent upon FGF signaling from the mid-gastrula stage onward (Fig. 3F,G). This demonstrates that FGF signaling does not activate *spt* expression but is required to maintain it, and is in contrast to *ntl* expression, which requires FGF signaling even in the early gastrula (Griffin et al., 1995). Therefore, the dramatic defects in posterior development caused by inhibition of FGF signaling (Amaya et al., 1991; Griffin et al., 1995) are largely attributable to the functions of these two T-box transcription factors.

spt becomes dependent upon *ntl* during tail mesoderm development

One possible explanation for the regulation of *spt* expression by FGF signaling is that *spt* is downstream of *ntl*. To address this possibility, we analysed *spt* expression in *ntl*⁻ embryos. During gastrulation and up until the 4-somite stage, *spt* expression in *ntl*⁻ embryos is similar to the wild-type expression pattern, except for widening of the non-expressing dorsal mesoderm (Fig. 5A,B). In the tail bud, however, expression of *spt* in the uninvoluted mesoderm of the epiblast becomes completely dependent upon *ntl*. At the 8-somite stage (approximately when progenitors of tail mesoderm begin to involute; J. Kanki, personal communication), *spt* is no longer expressed in the tail bud epiblast of *ntl*⁻ embryos but is still abundantly expressed in segmental plate (Fig. 5C,D). The loss of *spt* expression in epiblast cells coincides with the onset of abnormal thickening in the *ntl*⁻ tail bud epiblast (Fig. 5D; Halpern et al., 1993; Melby et al., 1997). At later stages, the size of the *spt*-expressing region in the *ntl* mutant segmental plate gradually diminishes and is undetectable at the 18-somite stage, when somites cease to form in *ntl*⁻ embryos (data not shown; Halpern et al., 1993). Thus during gastrulation and early segmentation, when trunk mesoderm is forming, *spt* expression is independent of *ntl* function, indicating that FGF signaling must regulate these two genes independently of one another. However, as tail mesoderm begins to involute, *spt* expression becomes genetically downstream of *ntl*.

tbx6 expression in *spt*⁻ and *spt*⁻;*ntl*⁻ embryos correlates with the severity of mesodermal defects

We wished to understand the regional specificity of the *spt* mutant phenotype, which shows that *spt* is only required in trunk mesoderm, even though *spt* is also expressed by tail mesoderm progenitors. We reasoned that the activity of a third T-box gene, *tbx6* (Hug et al., 1997) might be involved. *tbx6* is expressed in a very similar manner to *spt* (Fig. 4D, E) and the two transcription factors are closely related in residues important for DNA binding (data not shown; Müller and Herrmann, 1997). Although the function of zebrafish *tbx6* is still unclear, a null mutation in a related murine gene, also called *Tbx6*, causes a dramatic mesodermal deficiency that has many similarities to that seen in *spt*⁻ zebrafish, except that it occurs in a more posterior region (Chapman et al., 1996; Chapman and Papaioannou, 1998). *Tbx6*^{tm1pa} mouse embryos have mostly normal rostral somites but lack somites caudal to the forelimb bud, and also have an abnormal accumulation of mesenchymal cells in the tail bud. Curiously, neural tissue forms in place of the missing somites. If zebrafish *tbx6* functions similarly to murine *Tbx6*, then zebrafish *tbx6* may have a similar role in tail mesoderm as *spt* has in trunk mesoderm. But if *tbx6* and *spt* both have similar functions and are similarly expressed, why then do *spt*⁻ mutants have any trunk phenotype at all?

We examined the possibility that *spt* might regulate *tbx6* in trunk but not tail mesoderm. In wild-type gastrulae, *tbx6* expression is initially ventral (Fig. 6A) but is dramatically upregulated in dorsolateral mesoderm between early and mid-gastrulation and from then on resembles *spt* expression (Figs 4, 6B-D; Hug et al., 1997). In *spt*⁻ embryos, *tbx6* expression is initially distributed normally, although is very weak, but is not upregulated in dorsolateral mesoderm (Fig. 6E,F). Expression of *tbx6* in the hypoblast and segmental plate of *spt*⁻ embryos up to the 4-somite stage is weak and is spatially more restricted (Fig. 6F,G). However at the 7- to 8-somite stage, which is approximately when tail mesodermal progenitors begin to involute (J. Kanki, personal communication), *tbx6* expression becomes detectable in the segmental plate in *spt*⁻ embryos (Fig. 6H). Thus in *spt*⁻ embryos, *tbx6* cannot fully substitute for *spt* function in trunk mesoderm since *spt* is a major regulator of *tbx6* expression. However, in tail mesoderm, *tbx6* expression is independent of *spt* function and may account for the recovery of non-notochordal mesoderm formation in this region in *spt* mutant embryos.

Neither trunk non-notochord mesoderm (Fig. 2E; Kimmel et al., 1989) nor *tbx6* expression (Fig. 3E-G) are completely dependent upon *spt* function, however. To test the possibility that the patchy trunk non-notochordal mesoderm found in *spt*⁻ embryos might be related to the activity of *tbx6*, we analysed *tbx6* expression in *spt*⁻;*ntl*⁻ double mutant embryos, which lack all mesoderm in the trunk (S. L. A. and C. B. K., unpublished data). The *spt*⁻;*ntl*⁻ phenotype is far more severe than if the single mutant phenotypes were merely additive, raising the possibility that *spt* and *ntl* act synergistically. We hypothesised that *spt* and *ntl* might both regulate *tbx6* since all three T-box genes are co-expressed in mesodermal progenitors (Fig. 4).

In *ntl*⁻ embryos at the end of gastrulation, *tbx6* expression is similar to wild type, except for broadening of the non-expressing notochord domain and a reduction in epiblast expression, as described previously (Fig. 7A,B; Hug et al., 1997). In *spt*⁻ embryos, *tbx6* is expressed at high levels in the tail bud epiblast, but only weakly in the segmental plate (Fig.

7C). In *spt⁻;ntl⁻* double mutant embryos, however, *tbx6* expression is entirely undetectable (Fig. 7D). Similar observations were made of *spt* expression in *spt⁻;ntl⁻* embryos, except that *spt* was still weakly expressed in the *spt⁻;ntl⁻* tail bud (Fig. 7F-H). These data demonstrate that *ntl* is required for *tbx6* and *spt* expression at these early stages, but that its role is minor in comparison with the requirement for *spt* function. The spatial and temporal regulation of *tbx6* expression in *spt⁻* and *spt⁻;ntl⁻* embryos is consistent with the possibility that trunk mesoderm is partially dependent upon *tbx6* function and that *tbx6* functionally replaces *spt* in the tail mesoderm.

DISCUSSION

spadetail encodes a T-box transcription factor

In this paper, we describe our successful attempt to identify FGF-dependent regulators of mesoderm formation in the zebrafish trunk. We have molecularly identified the *spt* gene, which was previously only known by its mutant phenotype (Kimmel et al., 1989). Coincidentally, this T-box gene was also recently identified by Ruvinsky et al. (1998) as *tbx16*, and using phylogenetic analysis they show that *spt/tbx16* is the likely orthologue of *Xombi/Antipodean/BraT/VegT* and *ChTbx6L*. The molecular identification of *spt* provides important insights into the control of trunk mesoderm formation and the genetically separable mechanisms underlying trunk and tail development. In accordance with our proposition that *spt* is the putative *'no trunk'* gene, *spt* expression is FGF dependent and in *spt* mutants most, but not all, trunk mesoderm fails to develop whereas tail mesoderm is fairly normal. Like Ntl, which is required for notochord and tail development, Spt is a T-box transcription factor. The dependence of trunk and tail mesoderm upon FGF (Amaya et al., 1991; Griffin et al., 1995) is therefore accounted for by the combined functions of only two targets of FGF signaling, both of which are T-box genes (see also Lustig et al., 1996). Trunk paraxial mesoderm depends mainly upon *spt* function, whereas tail and notochord depend upon *ntl* function.

spt orthologues in other vertebrates

Sequence and expression analysis indicates that likely orthologues of *spt* have been identified in *Xenopus* (*Xombi*, Lustig et al., 1996; *Antipodean*, Stennard et al., 1996; *VegT*, Zhang and King, 1996; *BraT*, Horb and Thomsen, 1997) and chick (*ChTbx6L*, Knezevic et al., 1997). Although a murine *spt* orthologue has not yet been reported, the murine *FGFR1* mutant phenotype (Deng et al., 1994; Yamaguchi et al., 1994) has many similarities to the zebrafish *spt⁻* phenotype, suggesting that murine *spt* may be functionally downstream of this FGF receptor. Since fundamental mechanisms are frequently conserved among vertebrates, the loss-of-function phenotype of zebrafish *spt* is likely to be a good indication of the role of *spt* orthologues in other vertebrates.

One attempt to define the role of the *Xenopus spt* orthologue involved injection of a chimeric protein containing the repressor domain of *Drosophila engrailed* fused to the T-box domain of *BraT* (Horb and Thomsen, 1997). Ectopic expression of this mRNA during embryogenesis substantially blocked mesoderm induction and gastrulation, a phenotype far more severe than the zebrafish *spt⁻* phenotype. Although it is possible that *spt* might have a more prominent role in *Xenopus* mesoderm formation than in zebrafish, an alternative possibility is that the chimeric repressor protein also interferes with the functions of other T-box proteins. In zebrafish, *spt* appears to function in concert with other members of the T-box family and at least one of these, *tbx6*, is highly related to *spt* in sequence, expression and possibly also in function (Hug et al., 1997; Chapman and Papaioannou, 1998).

The *Xenopus spt* orthologue might also have novel functions during *Xenopus* development compared with *spt* in zebrafish. First, Lustig et al. (1996) have shown that the *Xenopus spt* orthologue can induce the formation of ectopic bottle cells, a specialised cell type normally found at the blastopore lip. Although zebrafish *spt* is also active in this assay (K. J. P. G., unpublished observations), zebrafish embryos do not have an obvious counterpart to bottle cells. Second, maternal transcripts of the *Xenopus spt* orthologue are vegetally localized and are proposed to act as a maternal determinant of mesoderm (Stennard et al., 1996; Zhang and King, 1996) and/or endoderm (Lustig et al., 1996; Horb and Thomsen, 1997). In addition, the zygotic expression is specific to the mesoderm and endoderm. In zebrafish, however, *spt* is not maternally expressed and the zygotic expression of *spt* is not initially mesoderm-specific (Fig. 3A). Perhaps the mechanisms that define the early mesodermal territory in *Xenopus* and zebrafish embryos differ fundamentally or else employ different genes. Consistent with this, a zebrafish gene highly homologous to *eomesodermin*, a *Xenopus* T-box gene expressed very early in mesoderm development (Ryan et al., 1996), is not detectably expressed by zebrafish mesoderm (K. J. P. G. and D. K., unpublished data).

T-box genes: an important link between morphogenesis and cell fate

One important role of *spt* is to regulate convergence movements of paraxial mesoderm during gastrulation (Ho and Kane, 1990). Yamamoto et al. (this issue) have shown that a cell adhesion molecule, *paraxial protocadherin* (*papc*), lies downstream of *spt* function and may mediate the effects of *spt* on convergence movements. Moreover, in the tail of *spt⁻* embryos, where convergence appears normal, *papc* expression does not depend upon *spt* (Yamamoto et al., 1998). The identification of Spt as transcription factor and the close parallels between expression of *spt* and *papc*, especially during gastrulation, suggests that *spt* directly activates *papc* expression in trunk mesoderm. Furthermore, because *tbx6* expression is largely independent of *spt* in the tail (this study), we propose that, in the tail, *tbx6* takes over the upstream regulatory role that *spt* plays in the trunk and accounts for the return of *papc* expression in the tail of *spt* mutants (see below).

spt also has a major role in regulating mesodermal cell fate. In the absence of function of the notochord-specific gene *floating head* (*flh*), presumptive notochord cells express *spt* ectopically and develop into muscle rather than notochord (Yamamoto et al., 1998; Halpern et al., 1995; Melby et al., 1996) and studies of *spt⁻;flh⁻* double mutant embryos show that *spt* is required for this *trans*-fating event (Amacher and Kimmel, 1998). Conversely, a muscle-to-notochord *trans*-fating event may also occur in *spt⁻* mutants. In addition to the severe depletion of trunk muscle in *spt⁻* embryos (Kimmel et al., 1989), mesoderm from the lateral and ventral germ ring can

adopt the notochord fate and there may also be an increase in the number of cells in the notochord anlage during gastrulation (Warga, 1996). It is unclear, however, whether these fate changes and the alterations in mesodermal gene expression patterns in *spt*⁻ embryos (Figs 3H, 6F; Hammerschmidt and Nüsslein-Volhard, 1993; Thisse et al., 1993; Weinberg et al., 1996) are secondary to cell movement defects, or whether *spt* directly activates mesodermal genes (Ho and Kane, 1990).

At least two other mesodermally expressed T-box genes also regulate cell fate and morphogenesis. Murine *Tbx6* functions similarly to *spt*, except that it is required in a more posterior region of the embryo (Chapman and Papaioannou, 1998). *Tbx6*^{tm1pa} mutant mice do not form somites caudal to the forelimb bud (approximately somite 9-10) and instead ectopic neural tissue forms in these locations. In addition, *Tbx6*^{tm1pa} embryos have an accumulation of cells at the tip of the tail, suggestive of a cell movement defect similar to *spt* mutant zebrafish. Similarly, *Brachyury* is essential for specifying the notochord fate in mouse and zebrafish and also regulates cell movements (Chesley, 1935; Halpern et al., 1993; Schulte-Merker et al., 1994; Wilson et al., 1995; Melby et al., 1997). One intriguing possibility is that *Brachyury/ntl* function is required for cell movement towards the point of mesoderm involution, whereas one function of *spt* and *tbx6* is for movement of involuted cells away from these locations. Consistent with this idea, *Brachyury* mutant cells accumulate in the murine primitive streak and *ntl* mutant zebrafish develop a thickened tail bud epiblast (Halpern et al., 1993; Wilson et al., 1995), whereas *spt* mutant zebrafish and *Tbx6*^{tm1pa} mutant mice develop accumulations of mesenchymal cells in the tail bud (Kimmel et al., 1989; Ho and Kane, 1990; Chapman and Papaioannou, 1998).

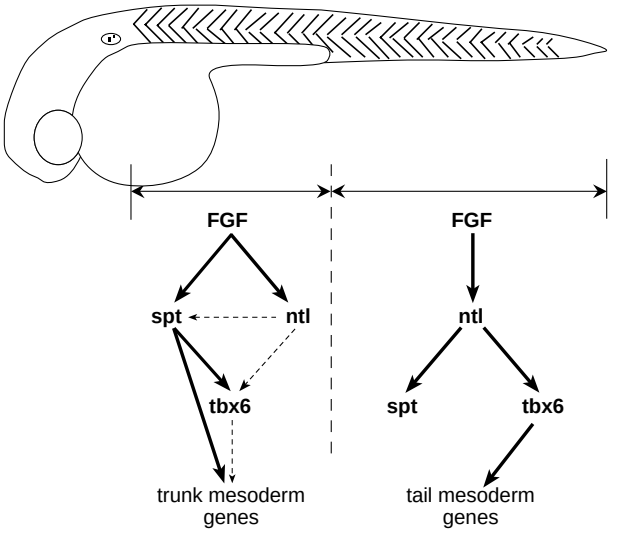

Fig. 8. Dynamic interactions between *spt*, *ntl* and *tbx6* control trunk and tail non-notochordal mesoderm development. This simplified genetic model summarizes the regulatory relationships among these genes in trunk (left-side) and tail (right-side) mesodermal progenitors, based upon our data and Chapman and Papaioannou (1998). Bold arrows indicate strong influences, dashed arrows indicate weak influences. The relationships may not be direct. See text for full explanation. The control of notochord development is not included and is described elsewhere (Halpern et al., 1997; Amacher and Kimmel, 1998).

Dynamic interactions among T-box genes regulate trunk and tail formation

spt⁻;*ntl*⁻ double mutant embryos have a much more severe phenotype in trunk mesoderm than if the single mutant phenotypes were merely additive (S. L. A. and C. B. K., unpublished data), raising the possibility that these genes act synergistically. Our observation that *tbx6* is synergistically regulated by *spt* and *ntl* provides a clear molecular basis for the interaction between these two genes. In *ntl*⁻ embryos, *tbx6* expression differs little from wild-type embryos; in *spt*⁻ embryos, *tbx6* expression is markedly reduced but, in *spt*⁻;*ntl*⁻ embryos, it is totally absent. Although phylogenetic analysis (Ruvinsky et al., 1998) indicates that zebrafish *tbx6* and murine *Tbx6*, are probably not orthologous, their expression patterns suggest that these two genes may be at least functionally homologous (Hug et al., 1997; Chapman and Papaioannou, 1998). Thus zebrafish *tbx6* may have a *spt*-like function in tail mesoderm, as *Tbx6* does in the mouse (Chapman and Papaioannou, 1998). We suggest that the function of zebrafish *tbx6* accounts for both the patchy trunk non-notochordal mesoderm as well as the recovery of tail mesoderm formation found in *spt*⁻ embryos.

We propose that the functional and regulatory interplay of three T-box genes underlies mesoderm development during trunk and tail formation, and have formulated a genetic model illustrating the pathways downstream of FGF signaling in zebrafish trunk and tail mesoderm. For the sake of clarity, we consider only the formation of non-notochordal mesoderm, as the regulation of notochord development is discussed elsewhere (Halpern et al., 1997; Amacher and Kimmel, 1998). In both trunk and tail mesoderm, FGF is required to regulate expression of *spt* and *ntl*, but the regulatory relationship among *spt*, *ntl* and an important downstream target, *tbx6*, differ in the two regions.

In trunk mesoderm (Fig. 8, left side), *spt* and *ntl* expression are mostly independent, and *spt* is the principle effector of mesodermal gene expression in the trunk. Since *spt*⁻ embryos produce some trunk mesoderm, we suggest that another gene must also activate trunk mesoderm genes, albeit less effectively. *tbx6* is a good candidate for this additional gene, as it is similar to *spt* in the residues important for DNA binding, is co-expressed with *spt*, and may have a similar function (data not shown; Hug et al., 1997; Müller and Herrmann, 1997; Chapman and Papaioannou, 1998). Since *tbx6* expression in the trunk partially depends upon *spt* function, the residual expression of *tbx6* in *spt*- embryos only partially compensates for the lack of *spt* function, resulting in patchy trunk muscle formation. Analysis of *spt*⁻;*ntl*⁻ embryos uncovers a role for *ntl* function in regulating *spt* and *tbx6* expression, although this role is minor relative to the role of *spt*. The regulation of *tbx6* expression by *ntl* is likely to account for both the patchy trunk mesoderm in *spt*⁻ embryos, as well as the total absence of trunk muscle seen in *spt*⁻;*ntl*⁻ embryos (S. L. A. and C. B. K., unpublished data).

During early segmentation, the regulatory hierarchy switches (Fig. 8, right side). *spt* and *tbx6* expression becomes fully dependent upon *ntl* function around the 4- to 8-somite stage, and *tbx6* may take over the role fulfilled by *spt* in the trunk. The phenotypes of several mouse mutants indicate that a similar switch may occur but at a more rostral axial level, adjacent to the forelimb bud (Chesley, 1935; Takada et al.,

1994; Goh et al., 1997; Chapman and Papaioannou, 1998). Murine *Brachyury* and *Tbx6* are both expressed by all paraxial progenitors but loss-of-function mutations only result in mesodermal defects posterior to the forelimb bud. Although it is not yet known whether somites rostral to the forelimb depend upon the function of a murine *spt* homologue, formation of paraxial mesoderm in this rostral domain does require FGFR1 function (Deng et al., 1994; Yamaguchi et al., 1994) and the regulation of myotome formation in these rostral somites is substantially different than in somites caudal to the forelimb (Soriano, 1997).

A major question is what controls the change in the regulatory hierarchy from trunk to tail mesoderm? Potentially, all of the genetic interactions between these three T-box genes remain the same, but the availability of accessory signals and/or transcription factors could change the relative importance of each of the interactions that we have defined. With the identification of these crucial genes involved in establishing mesodermal cell fate and the future isolation of new mutants that perturb this process, the molecular nature of these interactions will be revealed.

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