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, 1995; 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).
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 VTA YQN using Tfl polymerase (Epicon Technologies). Amplification products were analyzed by PAGE and ampiclons 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* originated from a stock with a complex genetic background (Kimmel et al., 1989). The *spt* mutation removes nucleotides +660-745 and introduces 8 extra bases (AAATTAAC) containing an in-frame stop (underlined). The mutation was identified in ENU-mutagenized stock (Haven et al., 1996) and has an 8-base insertion (TAAGGACCA) at position +589 that contains an in-frame stop (underlined). *spt* 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* is a gamma-ray-induced allele that eliminates polymorphic DNA markers located near the *spt* gene (S. L. A., unpublished data).

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 observed from intercrosses of *spt*/*spt*; *ntl*/*ntl* heterozygous adults; *ntl* mutant embryos from intercrosses of *ntl*/*ntl*; *ntl*/*ntl* double-mutant embryos from intercrosses of *spt*/*spt*; *ntl*/*ntl*; *ntl*/*ntl* 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 NotI and full-length *spt* cDNA was linearized with EcoRI. *ntl* probe was synthesized as described (Schulte-Merker et al., 1992).

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 supernantant (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 Brachury/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
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<sup>b104</sup>, is a small deletion that we used as a polymorphic marker to test linkage to the spt<sup>-</sup> phenotype. We found no recombinants among 473 haploid progeny of spt<sup>b104/+</sup> 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<sup>b104</sup> 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

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**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.*
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. 2](image-url) 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 stained with an antibody to mero-myosin (anterior, uppermost) show bilateral rows of expression either side of the notochord (Fig. 3E; Kimmel et al., 1990). 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 stained with an antibody to mero-myosin (anterior, uppermost) show bilateral rows of expression either side of the notochord (Fig. 3E; Kimmel et al., 1990).

![Fig. 3](image-url) 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 mosaically 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 (arrow) but not in the segmental plate mesoderm (compare with K).
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 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 sptb104 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 epiblast but, in the hypoblast, spt and tbx6 are expressed in segmental plate, whereas ntl expression is restricted to the notochord (Fig. 4D-F).

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<sup>−</sup> embryos differs dramatically from that in wild-type embryos (compare Fig. 3G and H). In mid-gastrula spt<sup>−</sup> 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<sup>−</sup> 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., 1995; 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<sup>−</sup> embryos. During gastrulation and up until the 4-somite stage, spt expression in ntl<sup>−</sup> 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 uninucleated 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<sup>−</sup> 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<sup>−</sup> 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<sup>−</sup> mutant segmental plate gradually diminishes and is undetectable at the 18-somite stage, when somites cease to form in ntl<sup>−</sup> 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<sup>−</sup> and spt<sup>−</sup>;ntl<sup>−</sup> 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<sup>−</sup> zebrafish, except that it occurs in a more posterior region (Chapman et al., 1996; Chapman and Papaioannou, 1998). Tbx6<sup>−/−</sup> 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<sup>−</sup> 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<sup>−</sup> embryos, tbx6 expression is initially distributed normally, although it is very weak, but is not upregulated in dorsolateral mesoderm (Fig. 6E,F). Expression of tbx6 in the hypoblast and segmental plate of spt<sup>−</sup> 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<sup>−</sup> embryos (Fig. 6H). Thus in spt<sup>−</sup> 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<sup>−</sup> embryos might be related to the activity of tbx6, we analysed tbx6 expression in spt<sup>−</sup>;ntl<sup>−</sup> double mutant embryos, which lack all mesoderm in the trunk (S. L. A. and C. B. K., unpublished data). The spt<sup>−</sup>;ntl<sup>−</sup> phenotype is far more severe than if the single mutant phenotypes were merely additive, raising the possibility that spt andntl 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<sup>−</sup> 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<sup>−</sup> embryos, tbx6 is expressed at high levels in the tail bud epiblast, but only weakly in the segmental plate (Fig. 7C,D).
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 Ruivinsky 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 (Kombi, 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 represor 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 eomesoderm, 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 \textit{spt\textsuperscript{-}} 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 \textit{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 \textit{Tbx6} functions similarly to \textit{spt}, except that it is required in a more posterior region of the embryo (Chapman and Papaioannou, 1998). \textit{Tbx6\textsuperscript{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, \textit{Tbx6\textsuperscript{tm1pa}} embryos have an accumulation of cells at the tip of the tail, suggestive of a cell movement defect similar to \textit{spt} mutant zebrafish. Similarly, \textit{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 \textit{Brachyury/ntl} function is required for cell movement towards the point of mesoderm involution, whereas one function of \textit{spt} and \textit{tbx6} is for movement of involuted cells away from these locations. Consistent with this idea, \textit{Brachyury} mutant cells accumulate in the murine primitive streak and \textit{ntl} mutant zebrafish develop a thickened tail bud epiblast (Halpern et al., 1993; Wilson et al., 1995), whereas \textit{spt} mutant zebrafish and \textit{Tbx6\textsuperscript{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 \textit{spt}, \textit{ntl} and \textit{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**

\textit{spt\textsuperscript{-};ntl\textsuperscript{-}} 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 \textit{tbx6} is synergistically regulated by \textit{spt} and \textit{ntl} provides a clear molecular basis for the interaction between these two genes. In \textit{ntl\textsuperscript{-}} embryos, \textit{tbx6} expression differs little from wild-type embryos; in \textit{spt\textsuperscript{-}} embryos, \textit{tbx6} expression is markedly reduced but, in \textit{spt\textsuperscript{-};ntl\textsuperscript{-}} embryos, it is totally absent. Although phylogenetic analysis (Ruvinsky et al., 1998) indicates that zebrafish \textit{tbx6} and murine \textit{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 \textit{tbx6} may have a \textit{spt}-like function in tail mesoderm, as \textit{Tbx6} does in the mouse (Chapman and Papaioannou, 1998). We suggest that the function of zebrafish \textit{tbx6} accounts for both the patchy trunk non-notochordal mesoderm as well as the recovery of tail mesoderm formation found in \textit{spt\textsuperscript{-}} 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 \textit{spt} and \textit{ntl}, but the regulatory relationship among \textit{spt}, \textit{ntl} and an important downstream target, \textit{tbx6}, differ in the two regions.

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

During early segmentation, the regulatory hierarchy switches (Fig. 8, right side). \textit{spt} and \textit{tbx6} expression becomes fully dependent upon \textit{ntl} function around the 4- to 8-somite stage, and \textit{tbx6} may take over the role fulfilled by \textit{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.,
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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