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The zebrafish T-box genes *no tail* and *spadetail* are required for development of trunk and tail mesoderm and medial floor plate

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SUMMARY

T-box genes encode transcriptional regulators that control many aspects of embryonic development. Here, we demonstrate that the mesodermally expressed zebrafish *spadetail* (*spt*)/*VegT* and *no tail* (*ntl*)/*Brachyury* T-box genes are semi-redundantly and cell-autonomously required for formation of all trunk and tail mesoderm. Despite the lack of posterior mesoderm in *spt*$^{-}$;*ntl*$^{-}$ embryos, dorsal-ventral neural tube patterning is relatively normal, with the notable exception that posterior medial floor plate is completely absent. This contrasts sharply with observations in single mutants, as mutations singly in *ntl* or *spt* enhance posterior medial floor plate development. We find that *ntl* function is required to repress medial floor plate and promote notochord fate in cells of the wild-type notochord domain and that *spt* and *ntl* together are required non cell-autonomously for medial floor plate formation, suggesting that an inducing signal present in wild-type mesoderm is lacking in *spt*$^{-}$;*ntl*$^{-}$ embryos.

Key words: *VegT*, *Brachyury*, Genetic mosaic, Fate map, Genetic redundancy, Genetic synergy, Zebrafish

INTRODUCTION

T-box transcription factors are a large family of transcriptional regulators involved in many aspects of embryonic development (Smith, 1999). The founding family member, *Brachyury* (or *T*), was originally identified by mutation in the mouse (Dobrovolskaïa-Zavadskaïa, 1927). The *Brachyury* gene is expressed in the notochord and tail bud (Herrmann et al., 1990). Similar to their mouse *Brachyury* mutant counterparts, zebrafish *no tail* (*ntl)* mutant embryos lack a notochord and a tail (Halpern et al., 1993; Schulte-Merker et al., 1994b). Inhibition of *Brachyury* function in *Xenopus laevis* also prevents notochord and tail development (Conlon et al., 1996). Conversely, *Brachyury* expression in *X. laevis* animal caps induces mesodermal gene expression in a dose-dependent fashion (Cunliffe and Smith, 1992; O'Reilly et al., 1995).

Zebrafish cell lineage analyses indicate that some *ntl*$^{-}$ cells located in a region of the dorsal organizer from which notochord cells would originate in wild-type embryos become mesenchymal cells that lie beneath the spinal cord (Halpern et al., 1993; Melby et al., 1996). Additionally, *ntl*$^{-}$ embryos have a wider medial floor plate (MFP), a ventral row of midline spinal cord cells that is usually only one cell wide (Strähle et al., 1996; Odenthal et al., 1996; Halpern et al., 1997). This observation prompted the idea that some *ntl*$^{-}$ dorsal organizer cells in the wild-type 'notochord domain' may adopt a floor plate fate (Halpern et al., 1997). Consistent with both ideas, mouse and zebrafish chimera analysis has shown that *Brachyury/ntl* mutant cells form disorganized mesodermal patches near wild-type notochord and show a propensity to form floor plate (Halpern et al., 1993; Wilson et al., 1995).

At least three additional T-box genes, *Eomesodermin, Tbx6* and *VegT*, are involved in mesodermal fate specification. *Eomesodermin (Eomes)* was first identified in *X. laevis* and is implicated in mesoderm development (Ryan et al., 1996). Mouse *Eomes* mutant embryos arrest soon after implantation, and tetraploid chimera analyses demonstrates that *Eomes* is required in the embryo for mesoderm formation (Russ et al., 2000). *Eomes* mutant cells can occasionally adopt mesodermal fates in chimeras, suggesting that *Eomes* may function to recruit cells into the primitive streak (Russ et al., 2000). In the mouse embryo, *Tbx6* is expressed in the primitive streak, the paraxial mesoderm, and the tail bud (Chapman et al., 1996). In *Tbx6* mutant embryos, posterior paraxial mesoderm develops as neural tissue, suggesting that *Tbx6* is required in paraxial mesoderm to block neural development (Chapman and Papaioannou, 1998). The zebrafish *tbx6* gene, although not considered the true ortholog of mouse *Tbx6* (see Ruvinsky et al., 1998) is expressed very similarly to the mouse gene (Hug et al., 1997). A third gene, *X. laevis VegT*, was isolated independently by several groups (Zhang and King, 1996; Lustig et al., 1996; Stennard et al., 1996; Horb and Thomsen, 1997). Maternal *VegT* transcripts are localized vegetally in *X. laevis* oocytes; *VegT* is zygotically expressed in the presumptive mesoderm of the marginal zone and is restricted to the lateral and ventral mesoderm by late gastrulation

(reviewed by Smith, 1999). Depletion experiments show that VegT function is required for endoderm and mesoderm development by regulating TGFβ family signaling molecules, with evidence for a cell-autonomous, dose-sensitive VegT requirement as well (Zhang et al., 1998a; Clements et al., 1999; Kofron et al., 1999; Kavka and Green, 2000; Xanthos et al., 2001).

The zebrafish *spadetail (spt)* gene, originally identified by mutation, is likely a *VegT* ortholog (Griffin et al., 1998). Although zygotic *spt* is expressed similarly to *X. laevis VegT*, the two genes probably have functional differences, since *spt/VegT* transcripts are maternally provided in frogs but not fish. Later, the gene is expressed in lateral mesoderm in both fish and frogs and in prechordal plate mesoderm in fish, but not frogs (Griffin et al., 1998; Ruvinsky et al., 1998). The *X. laevis VegT* expression pattern reflects expression of two differentially spliced forms: one is provided maternally, whereas the other is expressed zygotically in a pattern similar to zebrafish *spt* (Stennard et al., 1999). Homozygous *spt* mutant embryos lack trunk somites and later are deficient in trunk muscle; additional analyses demonstrate that *spt* is required in trunk somitic precursors for convergence movements and for muscle cell fate decisions (Kimmel et al., 1989; Ho and Kane, 1990; Amacher and Kimmel, 1998; Yamamoto et al., 1998). Other mesodermal derivatives, such as blood, pronephros, and pectoral fin, are variably deficient in *spt*⁻ embryos (Kimmel et al., 1989; Solnica-Krezel et al., 1996; Thompson et al., 1998).

The phenotypes of *ntl*⁻ and *spt*⁻ embryos are less severe than mutations in or depletion of their mouse and frog counterparts. This could be due to partial functional redundancy among zebrafish T-box genes. For example, *tbx-c* shows expression overlap with *ntl* (Dheen et al., 1999) and *tbx6* is expressed very similarly to *spt* (Hug et al., 1997; Griffin et al., 1998; Ruvinsky et al., 1998).

Previous work suggested that *spt* and *ntl* genes function synergistically, as expression of zebrafish *tbx6* is completely abolished in *spt*⁻*;ntl*⁻ embryos, a more severe effect than if the mutations were merely additive (Griffin et al., 1998). Here, we confirm that *spt* and *ntl* function together in mesoderm patterning. Using double mutant and genetic mosaic analyses, we demonstrate that *spt* and *ntl* are cell-autonomously required for development of all trunk and tail mesoderm. Although most dorsal-ventral patterning in the *spt*⁻*;ntl*⁻ neural tube is relatively normal, the posterior medial floor plate (MFP) is completely absent. The lack of posterior MFP is the most striking synergistic interaction we observe, especially since mutations in *ntl* or *spt* appear to enhance MFP development and can suppress MFP defects in some mutant backgrounds. Currently, there is some controversy about the origin and timing of floor plate development (see Le Douarin and Halpern, 2000; Placzek et al., 2000). The fate mapping data we present support the idea that floor plate originates from a midline precursor population and that *ntl* function is required during early gastrulation in cells that normally make notochord to repress floor plate and promote notochord fate. However, the genetic mosaic analysis we present suggests that the lack of posterior MFP in *spt*⁻*;ntl*⁻ embryos results from loss of an inducing signal from mesoderm, suggesting that a requirement for non cell-autonomous factors as well. We discuss the possible mesodermal signals and cell interactions that may be involved and the role that T-box genes play in development of posterior mesoderm and medial floor plate.

MATERIALS AND METHODS

Mutant alleles, stocks and fish husbandry

Fish were reared at 28.5°C and cared for as described elsewhere (Westerfield, 1995). Homozygous mutant embryos were obtained from natural matings of heterozygous carriers. Embryos were collected and sorted (Westerfield, 1995) during early cleavage stages and maintained in embryo medium at 28.5°C until the desired developmental stage. Embryos were staged according to Kimmel et al. (Kimmel et al., 1995).

The mutant alleles used were *spt*^{b104}, *cyclops*^{b16} (*cyc*^{b16}) and *ntl*^{b195}. The molecular lesions in each of these alleles are described as definite or likely null alleles: *spt*^{b104} (Griffin et al., 1998) (this paper); *cyc*^{b16} (Rebagliati et al., 1998; Sampath et al., 1998); *ntl*^{b195} (Schulte-Merker et al., 1994b). *spt/tbx16* maps to LG 8 (S. L. A., unpublished data), *cyc/znr1/ndr2* maps to LG 12 (Talbot et al., 1998; Sampath et al., 1998; Rebagliati et al., 1998), and *ntl* maps to LG 19 (Postlethwait et al., 1994).

Carriers doubly heterozygous for *spt*^{b104} and *cyc*^{b16} mutations and for *spt*^{b104} and *ntl*^{b195} mutations were obtained by crossing fish heterozygous for one mutation to fish heterozygous for the other and raising their progeny. Doubly heterozygous fish were intercrossed to produce homozygous double mutant embryos. At 24 hours postfertilization (hpf), the progeny of two doubly heterozygous carriers can be sorted into four phenotypic classes in the 9:3:3:1 Mendelian ratio expected for two independently assorting mutations. Progeny from *spt;cyc* doubly heterozygous mutant carriers were obtained in a ratio of 8.84 : 3.04 : 3.13 : 0.98 (WT : *cyc*⁻ : *spt*⁻ : *spt*⁻*;cyc*⁻, n=1534, χ^2=0.91, P>0.80). Progeny from *spt;ntl* doubly heterozygous mutant carriers were obtained in a ratio of 8.99 : 3.03 : 2.97 : 1.01 (WT : *ntl*⁻ : *spt*⁻ : *spt*⁻*;ntl*⁻, n=1972, χ^2=0.10, P>0.95). Approximately two-thirds of the *spt*⁻ embryos from the latter intercross had a more severe phenotype (reduced posterior notochord and floor plate, severe muscle reduction, more necrotic cell accumulation in tail) that we suspected was due to heterozygosity for the *ntl* mutation. To test this idea, we crossed fish that were heterozygous for the *spt* mutation (but homozygous for the wild-type *ntl* allele) to fish that were doubly heterozygous for both the *spt* and *ntl* mutations. Approximately one-quarter of the progeny had the *spt*⁻ phenotype, with half of those having more severe phenotypic disturbances (WT : *spt*⁻ : *spt*⁻ *severe*=2.94 : 0.52 : 0.54; n=884; χ^2=1.3, P>0.70). In these experiments, it did not matter whether the male or female was the doubly heterozygous carrier. The severe phenotype will be described in detail elsewhere (L. Goering and D. J. Grunwald, personal communication).

Antibody generation and immunohistochemistry

Polyclonal anti-Spt antibodies were generated by immunizing mice with a purified 6-histidine-tagged fusion protein containing Spt amino acids 215-382, produced using the pQE expression system (QIAGEN). Whole-mount antibody staining was performed on paraformaldehyde-fixed embryos following in situ hybridization or incubation at 65°C overnight in buffer containing 50% formamide, 2× SSC, 0.1% Tween 20, pH 6.0. Embryos were incubated for 4 hours in antibody staining buffer (PBS containing 1% DMSO, 0.1% Triton X-100, 2% normal goat serum, 2 mg/ml BSA), followed by overnight incubation in a 1:1000 dilution of mouse anti-Spt polyclonal antiserum in antibody staining buffer. Following incubation with horseradish peroxidase (HRP)-conjugated secondary antibody and extensive washes in wash buffer (PBS containing 1% DMSO, 0.1% Triton X-100), protein localization was visualized using the Vectastain ABC HRP kit as recommended (Vector Laboratories).

For co-localization of Spt and Ntl proteins, fixed embryos were embedded in agarose and cryostat sectioned as previously described (Westerfield, 1995). Sectioned embryos were stained with a 1:600 dilution of mouse anti-Spt polyclonal antiserum and a 1:5,000 dilution of rabbit anti-Ntl polyclonal antiserum (Schulte-Merker et al., 1992). Following washes, sections were incubated simultaneously with a 1:200 dilution each of goat anti-rabbit Alexa Fluor 594 and goat anti-mouse Alexa Fluor 488 (Molecular Probes). Sectioned embryos were examined using a Zeiss Axiophot microscope or a Zeiss LSM laser scanning confocal microscope.

In situ hybridization and histological sectioning

Embryos were processed for whole-mount in situ hybridization as described by Thisse et al. (Thisse et al., 1993), with the modifications described by Melby et al. (Melby et al., 1997). Digoxigenin-labeled RNA probes were synthesized from the following plasmid templates: *no tail (ntl)* (Schulte-Merker et al., 1992), *spadetail (spt)* (Griffin et al., 1998), *myoD* (Weinberg et al., 1996), *pax2* (Krauss et al., 1991), *sonic hedgehog (shh)* (Krauss et al., 1993), *islet1* (Appel et al., 1995), *her1* (Müller et al., 1996), *tropomyosin* (Thisse et al., 1993), and *krox20* (Oxtoby and Jowett, 1993). Following probe detection, embryos were dehydrated, cleared and mounted either between coverslips or on bridged slides (Melby et al., 1997). For preparation of sections after whole-mount in situ hybridization, embryos were dehydrated and embedded in Epon and sectioned as described (Westerfield, 1995). For the sections shown in Fig. 2E-H, embryos were first fixed in Bouin's solution and processed as above. Embryos were photographed on a Zeiss Universal microscope using a 35 mm camera or on a Zeiss Axioplan II using a Zeiss Axiocam digital camera.

Cell transplantation

Transplantations were performed between blastula stages (4 hpf) and the onset of gastrulation (5.2 hpf) as described previously (Amacher and Kimmel, 1998), except that all transplants were done isochronically. Donor embryos from an intercross of *spt;ntl* doubly heterozygous carriers were uniformly labeled as described previously (Halpern et al., 1993) by injecting lineage tracer dye (a mixture of 4% tetramethyl rhodamine-dextran and 4% lysine-fixable biotinylated dextran in 0.2 M KCl) at the 1- to 4-cell stage. Wild-type control donor embryos were similarly labeled using a 3% solution of lysine-fixable dextran-conjugated fluorescein in 0.2 M KCl. Cells were removed from a rhodamine-labeled donor and from a wild-type fluorescein-labeled donor and transplanted together into the blastoderm margin of an unlabeled host embryo. In some experiments, only rhodamine-labeled donor cells were transplanted. Because transplantations were performed before mutant phenotypes are distinguishable, donor embryos were kept alive to score phenotype later. During the pharyngula stage (post-24 hpf), transplanted cells were visualized in host embryos using a low light silicon-intensified camera (Videoscope). In some experiments, host embryos were fixed in 4% paraformaldehyde overnight, processed to detect *shh* transcripts by in situ hybridization and to detect biotinylated dextran-labeled cells using a Vectastain kit (Vector Laboratories).

Caged fluorescein fate-mapping

Embryos for fate mapping experiments were generated by injecting 1% lysine-fixable caged fluorescein (Molecular Probes) in 0.2 M KCl at the 1-4 cell stage. Caged fluorescein was injected into embryos from a cross of ntl^{b195} heterozygotes or into embryos co-injected with antisense *ntl* morpholino-modified oligomer (*ntl*-MO, kind gift from Steve Ekker). *ntl*-MO was injected at a concentration of 0.5 mg/ml in 0.1 M KCl containing 0.25% Phenol Red, basically as described by Nasevicius and Ekker (Nasevicius and Ekker, 2000), except that 1% caged fluorescein was injected simultaneously.

Fluorescein was uncaged with pulses of a 375 nm nitrogen laser (Micropoint Laser System, Photonic Instruments) as described previously (Gritsman et al., 2000). The beam was focused through a 50× Leitz water immersion objective on a Zeiss standard microscope. Early gastrula stage embryos (shield stage to 60% epiboly) were positioned shield-up in 0.2% agarose in embryo medium supplemented with 10 mM Hepes, and 'dorsal-up' positioning was verified by the presence of forerunner cells just beyond the shield on the yolk syncytial layer (YSL) surface (Melby et al., 1996). To activate fluorescein in a patch of about 20 notochord-domain cells, the laser beam was successively focused on 4 adjacent cells, at and just one cell behind the blastoderm margin, and about 3 cells deep (the shield is about 6 cell-diameters deep at this stage). Firing the laser at each position yields a string of several brightly fluorescing cells along the path of the beam (including a single surface EVL cell) and a few dimly fluorescing neighboring cells.

Uncaged fluorescein label was detected either by fluorescence using a Zeiss Axiophot microscope or by a more sensitive procedure using anti-fluorescein Fab antibody conjugated to alkaline phosphatase (Boehringer Mannheim). Addition of anti-fluorescein antibody and subsequent detection of alkaline phosphatase activity was done essentially as described previously (Cornell and Eisen, 2000; Gritsman et al., 2000), except that digestion by Proteinase K (Boehringer Mannheim; 10 μg/ml, 4 minutes) was performed before BSA blocking.

RESULTS

The zebrafish T-box genes *no tail* and *spadetail* are co-expressed at the blastoderm margin

Spt protein is localized to cell nuclei (Fig. 1). The Spt protein expression pattern correlates well with the *spt* mRNA expression pattern (Griffin et al., 1998; Ruvinsky et al., 1998). During early segmentation, Spt is expressed strongly in adaxial and tail bud cells and more weakly in presomitic and lateral mesodermal cells (Fig. 1A) and prechordal plate cells (data not shown) of wild-type embryos. Co-labeling for Spt protein and *ntl* RNA confirm expression overlap in lateral presumptive pronephros (Fig. 1B). Co-labeling for Spt protein and *myoD* RNA confirms that Spt is expressed in adaxial cells and that expression is confined to presomitic (not somitic) mesoderm, with Spt disappearing before somites form (Fig. 1C).

To position cells co-expressing Spt and Ntl more precisely, we double-labeled sections of gastrula and segmentation-staged embryos with Spt and Ntl antibodies. During mid-gastrulation, Spt and Ntl are co-expressed in a few cells of the lateral germ ring at the margin of the blastoderm (Fig. 1D). At later stages, sagittal sections through the embryonic dorsal midline shows that Spt and Ntl are co-expressed in tail bud cells (Fig. 1E). Similar sections of doubly stained spt^{-} embryos shows that the number of Ntl-expressing tail bud cells is expanded (Fig. 1F), but Spt-expressing cells are lacking (Fig. 1G), substantiating our previous prediction that the spt^{b104} allele is a functional null (Griffin et al., 1998).

spt and *ntl* are required together for trunk and tail mesoderm development

To examine the functional consequence of removing both *ntl* and *spt* T-box genes, we constructed the *spt;ntl* double mutant (Fig. 2). Anterior morphology appears normal in $spt^{-};ntl^{-}$ embryos; however, there is an extreme deficit of mesodermal cells in the trunk and tail (Fig. 2A-D). Some deficiencies were expected based upon single mutant phenotypes. For example, ntl^{-} embryos lack a tail and a differentiated notochord (Halpern

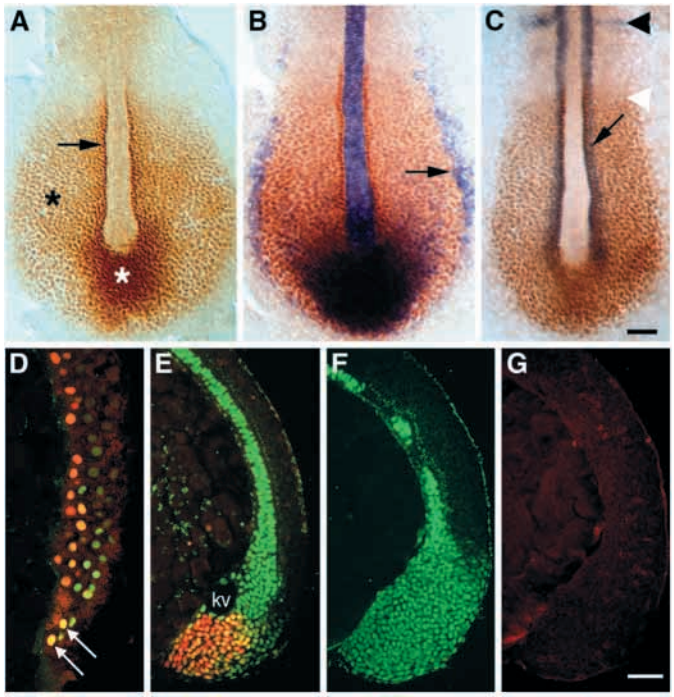

Fig. 1. Two zebrafish T-box genes, *ntl* and *spt*, are co-expressed in gastrula cells at the blastoderm margin. Spt protein expression in wild-type (A-E) and *spt*⁻ (F,G) embryos at midgastrula stage (70% epiboly) (D) and at the 6-somite stage (12 hpf) (A-C,E-F). (A) Spt is expressed strongly in adaxial (arrow) and tail bud (white asterisk) cells and more weakly in presomitic and lateral mesodermal cells (asterisk). (B) Double-labeling for Spt protein (brown) and *ntl* transcripts (blue); arrow indicates lateral presumptive pronephros. (C) Double-labeling for Spt protein (brown) and *myoD* transcripts (blue). Arrow indicates adaxial cells, black arrowhead indicates the posterior limit of *myoD* expression, and white arrowhead indicates the anterior extent of Spt protein expression. *myoD* is expressed in adaxial cells and in posterior cells of each formed somite (Weinberg et al., 1996). (D) Section through the lateral region of a midgastrula embryo (70% epiboly) double-labeled for Ntl (green) and Spt (orange) protein; white arrows indicate cells expressing both Ntl and Spt. (E) Sagittal section through the dorsal midline, double-labeled for Ntl (green) and Spt (orange); kv, Kuppfer's vesicle. (F,G) Similar sections of *spt*⁻ embryos double-labeled for Ntl (F) and Spt (G). Scale bars: 50 μm in C for A-C and in G for D-G.

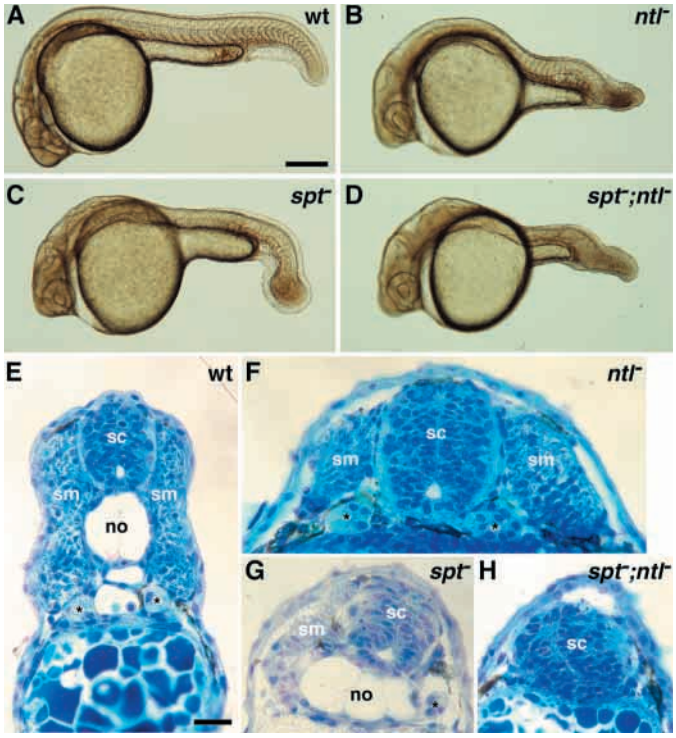

Fig. 2. *spt* and *ntl* are required together for trunk and tail mesoderm development. (A-D) Live wild-type and mutant embryos at 24 hpf. Embryos doubly homozygous for *spt* and *ntl* mutations (D) have more severe posterior mesodermal deficiencies than *ntl*⁻ (B) and *spt*⁻ (C) embryos. Anterior development appears unperturbed. (E-H) Tranverse sections through trunk regions of wild-type (E) and mutant embryos (F-H) at 30 hpf show that (F) *ntl*⁻ embryos lack a differentiated notochord, (G) *spt*⁻ embryos have reduced muscle tissue, and (H) *spt*⁻;*ntl*⁻ embryos lack all recognizable mesoderm, having only a spinal cord covered by skin. sc, spinal cord; no, notochord; sm, somitic muscle; *, pronephric tubule. Scale bars, 250 μm (A), 25 μm (E).

et al., 1993), whereas *spt*⁻ embryos are deficient in ventrolateral mesoderm (Kimmel et al., 1989) (compare Fig. 2A with 2B,C). Muscle development is affected in both mutants to varying extents (Kimmel et al., 1989; Halpern et al., 1993). About two-thirds of the *spt*⁻ embryos display reductions in notochord, floor plate and tail muscle due to heterozygosity at the *ntl* locus (data not shown), which we confirmed by observing the same severe phenotype in crosses of *spt;ntl* heterozygous fish to *spt* heterozygous fish (see Materials and Methods). Interestingly, *spt*⁻;*ntl*⁻ embryos lack the characteristic *spt*⁻ 'spade' tail (Fig. 2D). The *spt*⁻ spade cells are trunk somitic precursors that fail to migrate properly during gastrulation and express *ntl* (Ho and Kane, 1990) (Fig. 1F). Thus, the abnormal accumulation of cells in the *spt*⁻ tail bud requires *ntl* function. Histological sections of wild-type and mutant embryos at 30 hpf reveal that the trunk region of *spt*⁻;*ntl*⁻ embryos consists of a spinal cord covered by epidermis (Fig. 2E-H). Trunk mesodermal cell types, such as muscle and pronephros, that are present to various extents in both single mutant embryos (Fig. 2F,G), are absent in *spt*⁻;*ntl*⁻ embryos (Fig. 2H).

Posterior mesodermal development, but not mesodermal induction, is blocked in *spt*⁻;*ntl*⁻ embryos

Panmesodermal expression

Widespread expression of *ntl/Brachyury* is an immediate early response to mesoderm-inducing signals (Smith et al., 1991). Embryos carrying the *ntl*^{b195} mutation do not produce functional protein and antibodies fail to detect Ntl protein in *ntl*⁻ and *spt*⁻;*ntl*⁻ embryos (see Fig. 3J,L). However, *ntl*^{b195} mutants produce *ntl* mRNA. During midgastrula stages, we find that *ntl* is expressed in epiblast cells at the blastoderm margin in wild-type, *spt*⁻, *ntl*⁻ and *spt*⁻;*ntl*⁻ embryos (Fig. 3A-D), suggesting that *spt*⁻;*ntl*⁻ cells respond normally to early mesoderm-inducing signals.

Somitic precursor cells

Presomitic cells express *her1*, a homolog of the *Drosophila*

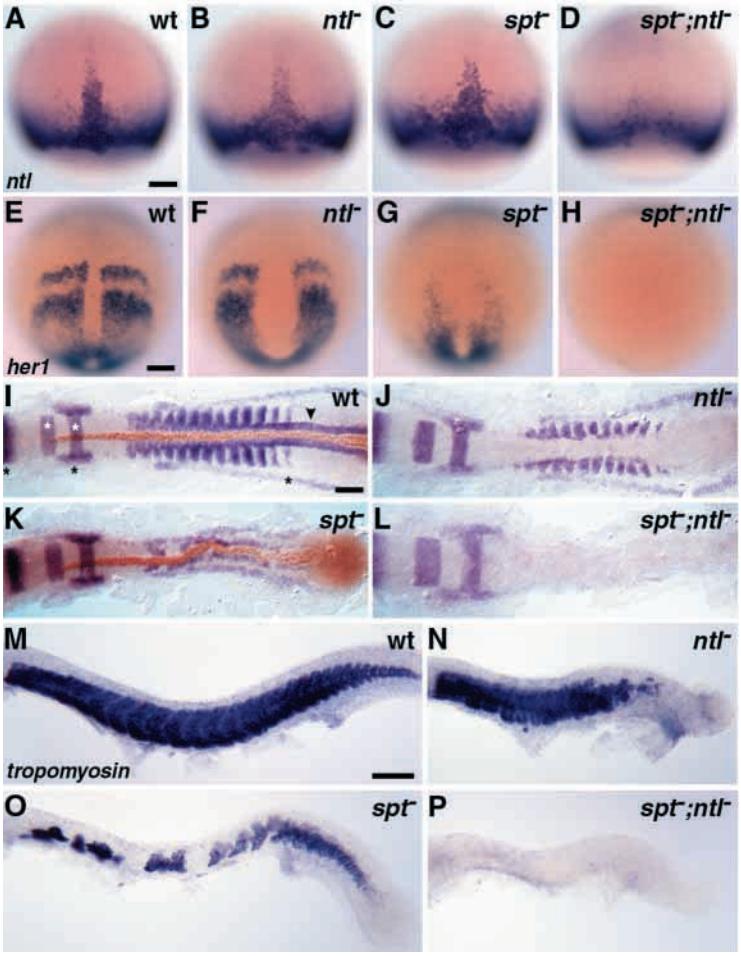

Fig. 3. Expression analyses reveal that mesoderm development, but not induction, is disupted in *spt;ntl* double mutant embryos. In situ hybridization and immunohistochemistry in wild-type (A,E,I,M), *ntl*$^{-}$ (B,F,J,N), *spt*$^{-}$ (C,G,K,O) and *spt*$^{-}$*;ntl*$^{-}$ (D,H,L,P) embryos reveal synergistic and partially redundant interaction of *spt* and *ntl* during mesoderm formation. (A-D) *ntl* expression at mid-gastrulation (75% epiboly, 8 hpf) in wild-type and mutant embryos. (E-H) *her1* expression during late gastrulation (95% epiboly, 9.5 hpf) in wild-type and mutant presomitic mesoderm. In I-L, *pax2.1*, *krox20*, and *myoD* transcripts are visualized in blue and Ntl protein is visualized in brown. In 10-somite stage (14 hpf) wild-type embryos (I), *pax2.1* is expressed in the mid-hindbrain boundary, the otic placode and the developing pronephros (black asterisks), *krox20* in hindbrain rhombomeres 3 and 5 (white asterisks), *myoD* in adaxial cells (arrowhead) and a subset of cells within each formed somite and the two most anterior forming somites, and Ntl in developing notochord cells. As discussed in the text, mesodermal gene expression is variably disrupted in *ntl*$^{-}$ and *spt*$^{-}$ embryos (J,K) and is abolished in *spt*$^{-}$*;ntl*$^{-}$ embryos (L). (M-P) *tropomyosin* expression at 24 hpf. Embryos with intact yolks (A-H) are dorsal views, with anterior to the top. Dorsal (I-L) and lateral (M-P) views of deyolked embryos are shown with anterior to the left. Scale bars: 100 μm in A for A-D; E for E-H; I for I-L and M for M-P .

melanogaster pair-rule gene *hairy* and one of the earliest segmentally expressed zebrafish genes (Müller et al., 1996). Near the end of gastrulation, *her1* is expressed in tail bud cells and in stripes in the presomitic mesoderm of wild-type and *ntl*$^{-}$ embryos (Fig. 3E,F), and is expressed in tail bud cells and weakly in scattered presomitic cells in *spt*$^{-}$ embryos (Fig. 3G). In contrast, *her1* is not expressed in *spt*$^{-}$*;ntl*$^{-}$ embryos (Fig. 3H), suggesting that presomitic mesoderm development is abolished in the absence of *ntl* and *spt* function.

Myogenic cells and differentiated muscle

During normal development, muscle precursors express *myoD* several hours before completion of the gastrula period (Weinberg et al., 1996). In contrast, *myoD* expression is not initiated in either *spt* or *ntl* single mutant embryos until the end of the gastrula period, although *myoD* expression partially recovers in both single mutants during segmentation stages (Weinberg et al., 1996). In *ntl*$^{-}$ embryos, *myoD* is expressed in the posterior border of somites as they form, but only very weakly in adaxial cells of the presomitic mesoderm, whereas its expression is variable in *spt*$^{-}$ embryos in cells that flank the developing midline (Fig. 3J,K) (Weinberg et al., 1996). In contrast, in *spt*$^{-}$*;ntl*$^{-}$ embryos, *myoD*-expressing cells are never detected posterior to the head at any stage (Fig. 3L). Although differentiated posterior muscle (visualized by *tropomyosin* expression) is not observed in *spt*$^{-}$*;ntl*$^{-}$ embryos (compare Fig. 3M-O with P), head musculature and a small heart are present (data not shown).

Pronephric precursors and embryonic pronephric tubules

During segmentation stages, *pax2.1* is expressed at the midbrain-hindbrain boundary, in developing otic placodes, and in presumptive pronephros (Krauss et al., 1991). Cells expressing *pax2.1* are found in all these domains in wild-type, *spt*$^{-}$, and *ntl*$^{-}$ embryos (Fig. 3I-K), but are missing at the edge of the lateral plate mesoderm (pronephros) in *spt*$^{-}$*;ntl*$^{-}$ embryos (Fig. 3L). The absence of early *pax2.1* staining in the presumptive pronephros correlates well with the absence of pronephric tubules in older *spt*$^{-}$*;ntl*$^{-}$ embryos (Fig. 2H).

Taken together, the expression analyses suggest that *spt* and *ntl* together are not required for initial mesoderm induction, but are required and are partially redundant for further development of mesodermal cell types. Because mesodermally derived signals have been implicated in neural patterning, we next investigated the anterior-posterior and dorsal-ventral patterning of the *spt*$^{-}$*;ntl*$^{-}$ neural tube.

Neither *spt* nor *ntl* function is required for most dorsal-ventral spinal cord patterning

General anterior-posterior patterning of the *spt*$^{-}$*;ntl*$^{-}$ neural tube, as revealed by markers of midbrain, hindbrain and primary

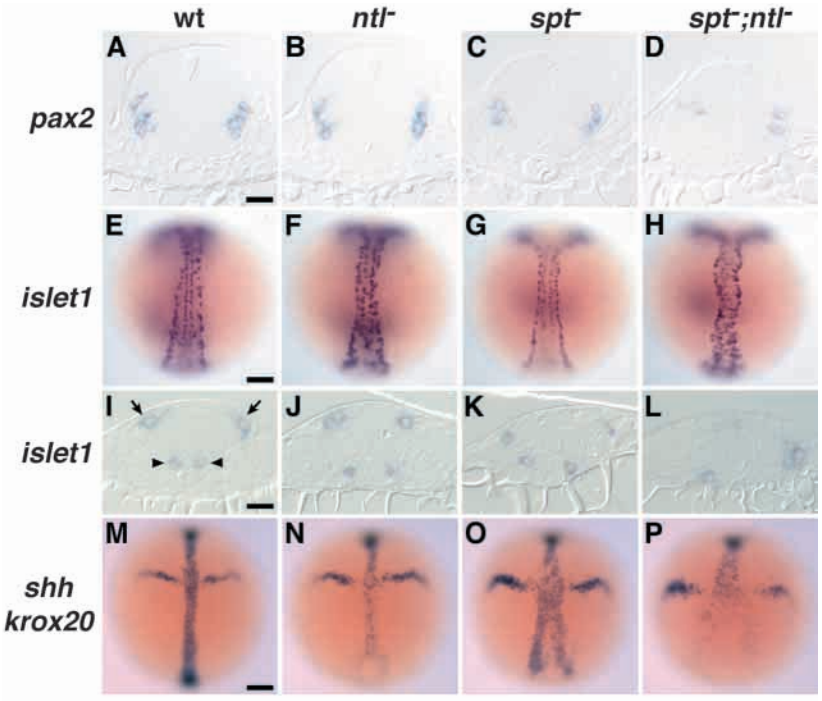

Fig. 4. Neither *spt* nor *ntl* function is required for most dorsal-ventral spinal cord patterning. (A,E,I,M) Wild-type, (B,F,J,N) *ntl*$^{-}$, (C,G,K,O) *spt*$^{-}$, and (D,H,L,P) *spt*$^{-}$;*ntl*$^{-}$ embryos. (A-D) *pax2.1* interneuron expression at 24 hpf, (E-L) *islet1* expression at the 8-10 somite stage (13-14 hpf); arrowheads indicate primary motoneurons and arrows indicate Rohon-Beard neurons. (M-P) *sonic hedgehog (shh)* and *krox20* expression at the late gastrula stage (~9.5 hpf). Transverse sections (A-D; I-L) are approximately from midtrunk levels. Anterior is to the top. Scale bars: 25 μm (A-D and I-L), 100 μm (E-H and M-P).

motoneurons (*pax2.1* and *krox20* in Fig. 3; *islet1* in Fig. 4), appears normal. To characterize dorsal-ventral neural tube patterning, we examined several markers that are differentially expressed along the dorsal-ventral axis (Fig. 4). The genes *msxb* and *pax3* (Ekker et al., 1997; Seo et al., 1998) are expressed in the normal dorsal territory in *spt*$^{-}$;*ntl*$^{-}$ spinal cord at 24 hpf (data not shown). In the intermediate neural tube of *spt*$^{-}$;*ntl*$^{-}$ embryos, *pax2.1*-expressing interneurons (Krauss et al., 1991) are present, but reduced in number, and histological sections reveal that they are sometimes medially displaced (Fig. 4A-D). Early expression of *islet1* is in dorsally located Rohon-Beard sensory neurons and in ventrally located primary motoneurons (Inoue et al., 1994; Tokumoto et al., 1995; Appel et al., 1995). At 13-14 hpf (8- to 10-somite stage), both types of *islet1*-expressing cells appear present in single and double mutant embryos based upon their position (Fig. 4E-H; sections in Fig. 4I-L). However, fewer ventral *islet1*-expressing cells are observed in *spt*$^{-}$;*ntl*$^{-}$ embryos, and histological sections reveal that they sometimes are located in midline positions (Fig. 4L).

As in other vertebrates, Hedgehog signaling is required for induction of zebrafish primary motoneurons (Jessell, 2000; Lewis and Eisen, 2001; Varga et al., 2001; Chen et al., 2001). We examined expression of a zebrafish *hedgehog* gene, *sonic hedgehog (shh)*, at the end of gastrulation (Fig. 4M-P). At this time, midline cells of wild-type and *spt*$^{-}$ embryos strongly express *shh* (Fig. 4M,O), although *shh* expression in *spt* mutants is broader and flares posteriorly. At this stage, midline *ntl*$^{-}$ cells express *shh*, albeit only weakly posteriorly (Fig. 4N) (Krauss et al., 1993). In *spt*$^{-}$;*ntl*$^{-}$ embryos, *shh* is barely detectable posterior to the hindbrain, although a few *shh*-positive cells are seen, and these cells are often distant from the dorsal midline (Fig. 4P). In agreement with other observations (Lewis and Eisen, 2001; Varga et al., 2001; Chen et al., 2001), we hypothesize that transient Hedgehog expression is sufficient to promote primary motoneuron development in *spt*$^{-}$;*ntl*$^{-}$ embryos.

spt and *ntl* together are required for trunk and tail medial floor plate formation

The most ventral neural tube tissue is the floor plate, and in zebrafish, the floor plate is composed of a single midline row of medial floor plate (MFP) cells flanked by lateral floor plate (LFP) cells (Odenthal and Nusslein-Volhard, 1998). A wide medial floor plate (MFP) forms in *ntl*$^{-}$ embryos (Fig. 5B) (Odenthal et al., 1996; Strähle et al., 1996; Halpern et al., 1997). Similarly, the *spt*$^{-}$ posterior MFP is often more than one-cell wide, and frequently, cells expressing MFP markers are observed ventral to the notochord (Fig. 5C; Fig. 6C) (Amacher and Kimmel, 1998). In contrast to both single mutant phenotypes, the *spt*$^{-}$;*ntl*$^{-}$ MFP is severely truncated and extends only slightly posterior of the hindbrain (Fig. 5D). The single and double mutant floor plate phenotypes observed using *shh* as a marker are also observed with other MFP markers, including *tiggy-winkle hedgehog* (Ekker et al., 1995), *α-collagen2* (Yan et al., 1995), and *axial/HNF3β* (Strähle et al., 1993) at the 20-somite stage (data not shown). Thus, although posterior MFP develops in excess in *spt*$^{-}$ and *ntl*$^{-}$ embryos, posterior MFP formation is abolished in *spt*$^{-}$;*ntl*$^{-}$ embryos.

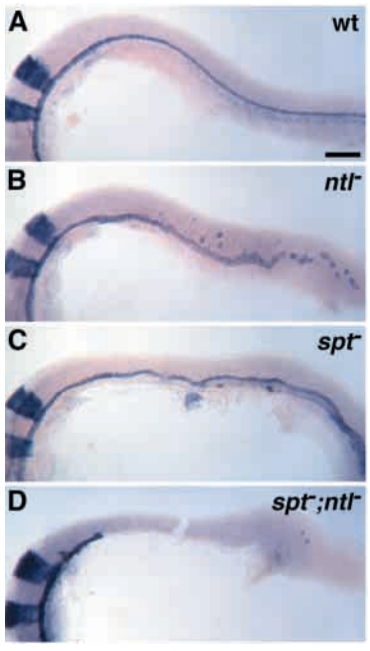

Fig. 5. *spt* and *ntl* together are required for spinal cord medial floor plate formation. Expression of *sonic hedgehog (shh)* in ventral brain, medial floor plate (MFP), and posterior notochord and of *krox20* in wild-type (A) and mutant (B-D) embryos at 24 hpf. Posterior MFP irregularities, such as broadening, are noted in *ntl*$^{-}$ (B) and *spt*$^{-}$ (C) embryos, but MFP is conspiciously missing in *spt*$^{-}$;*ntl*$^{-}$ embryos (D), beginning approximately at the end of the hindbrain. Lateral views, with anterior to the left. Scale bar: 100 μm.

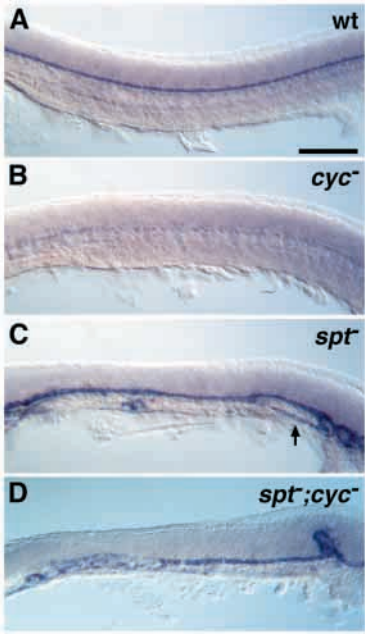

Fig. 6. The *nodal*-related gene *cyclops* (*cyc*) is not required for medial floor plate development in the absence of *spt* function. *sonic hedgehog (shh)* expression in trunk medial floor plate (MFP) of wild-type (A) and mutant (B-D) embryos at 24 hpf. As noted already, *spt*– MFP is often broader and some MFP cells are located ventral to the notochord (arrow) (C). In contrast, MFP is completely absent in the trunk of *cyc*– embryos (B), but is significantly restored in *spt*–;*cyc*– embryos (D). Ventral brain *shh* expression in *spt*–;*cyc*– embryos is comparable to that in *cyc*– embryos (data not shown); thus, suppression of the *cyc*– MFP defect by the *spt* mutation is restricted to the trunk and tail. Lateral views, with anterior to the left. Scale bar: 100 μm.

The *nodal*-related gene *cyclops* is not required for floor plate development in the absence of *spt* gene function

Previous work has shown that the *nodal*-related gene *cyclops (cyc)* is required for MFP formation (Hatta et al., 1991; Rebagliati et al., 1998; Sampath et al., 1998), but that mutations in *ntl* partially suppress the *cyc* MFP defect (thus, posterior MFP forms in *cyc*–;*ntl*– embryos) (Halpern et al., 1997). We therefore asked if a mutation in *spt* can similarly suppress the *cyc*– MFP defect and we found that it can (Fig. 6). MFP (detected by *shh* expression) develops in wild-type and *spt*– embryos (Fig. 6A,C), but does not form in *cyc*– embryos, except for a few scattered cells in the tail (Fig. 6B). In contrast, many trunk and tail MFP cells are present in *spt*–;*cyc*– embryos (Fig. 6D). Thus, the *nodal*-related gene *cyc* is not required for posterior MFP development in the absence of either *spt* or *ntl* function.

spt and *ntl* together are required cell-autonomously in mesodermal cells, but neither gene is required in medial floor plate cells for their fate

To determine whether *spt* and *ntl* are required cell-autonomously in mesoderm and/or floor plate, we created genetic mosaic embryos. We transplanted blastula cells from a rhodamine-labeled donor embryo derived from an intercross of two heterozygous *spt;ntl* carriers, together with blastula cells from a fluorescein-labeled wild-type donor, into the presumptive mesoderm region of a wild-type host embryo (Fig. 7; see Materials and Methods). Because transplantations were performed before mutant phenotypes are distinguishable, the donor genotype was retrospectively determined by examining each donor embryo at later stages when the morphological phenotype is obvious (see Fig. 2). When wild-type donor cells adopted mesoderm or floor plate fates, we assessed whether mutant donor cells co-transplanted into the same fate map position could also adopt those fates. In control transplants (*n*=72), where both fluorescein- and rhodamine-labeled donor cells were wild type, we observed near-perfect overlap in cell fates adopted by cells from both donors. In over 97% of the cases in which rhodamine-labeled wild-type donor cells formed mesoderm or floor plate, the fluorescein-labeled cells also contributed cells to the same tissue. When *spt* or *ntl* mutant cells were transplanted into wild-type hosts, we observed that they could adopt floor plate and some mesodermal fates (Fig. 7C-E, data not shown). *ntl*– cells fail to form notochord in wild-type host embryos (Fig. 7D) (Halpern et al., 1993), whereas *spt*– cells fail to form trunk muscle and instead contribute to various tail derivatives (Fig. 7E) (Ho and Kane, 1990). In striking contrast to control and single mutant results, we never observed *spt*–;*ntl*– cells adopting mesodermal fates in a wild-type environment (*n*=12). The co-transplanted wild-type cells adopted either mesodermal fates only (4/12) or mixed mesodermal and neural fates (7/12). In one case, the co-transplanted wild-type cells contributed only to neural tissue. In every case, the co-transplanted *spt*–;*ntl*– cells never adopted mesodermal fates, but instead adopted ectodermal fates (Fig. 7F).

In some transplants, we observed *spt*–;*ntl*– cells contributing to the wild-type host floor plate (Fig. 7G). As posterior MFP fails to form in *spt*–;*ntl*– embryos, this suggests that *spt* and *ntl* are required non cell-autonomously for posterior MFP fate. To distinguish MFP unambiguously from neighboring cells, we transplanted fewer rhodamine-labeled mutant cells into wild-type hosts, and omitted co-transplanting fluorescein-labeled wild-type cells. As in the preceding experiments, *spt*–;*ntl*– cells contributed only to ectodermal fates (*n*=13). Almost all of the transplants contained neurons (12/13), and of those containing neurons, many (8/12) contained MFP cells. When host embryos were fixed and processed to detect expression of *shh*, a MFP marker, we observed donor-derived *spt*–;*ntl*– cells within the *shh*-expressing wild-type donor floor plate (Fig. 7H). Our genetic mosaic experiments demonstrate that *spt* and *ntl* are required cell-autonomously for posterior mesodermal development. In contrast, neither gene is required in posterior MFP cells for their fate, suggesting that *spt* and *ntl* are required in non-MFP cells to promote MFP fate.

Origin of the floor plate in *ntl* single mutants

We and others have suggested that *ntl* gene function is required to promote notochord and repress floor plate development in midline cells (see Halpern et al., 1997). If loss of *ntl* function leads to excess floor plate at the expense of notochord, one would predict that the ectopic *ntl*– floor plate cells would arise from a domain of the gastrula fate map normally corresponding to notochord. To determine the origin of floor plate cells in *ntl*– embryos, we injected embryos with caged fluorescein-dextran during early cleavage stages and then uncaged the fluorophore in a small population of approximately 20 dorsal organizer cells during early gastrulation (Fig. 8A; see Materials and Methods). Uncaged fluorescein label was detected by fluorescence microscopy and/or using anti-fluorescein antibody. Because the fluorophore was uncaged in approximately 3 tiers of cells, we expected to find labeled cells in derivatives of the enveloping layer (EVL) and of the deep layer (DEL). EVL cells do not involute or undergo convergence

movements, but instead stay on the surface and differentiate as periderm (Kimmel et al., 1990). In contrast, deep layer (DEL) cells located at the blastoderm margin adopt mesodermal and endodermal fates, depending upon developmental stage (Kimmel et al., 1990; Melby et al., 1996). In control wild-type embryos (n=6), the fluorophore was detected in large populations of labeled notochord cells and a dorsal patch of labeled cells in the enveloping layer (EVL). In three of the six wild-type embryos, labeled DEL derivatives were restricted to the notochord (Fig. 8B,D). In two embryos (those uncaged at the earliest stage), labeled DEL derivatives also included hatching gland (like notochord, hatching gland derives from the shield margin) (Melby et al., 1996). In one embryo, labeled DEL derivatives included a small number of labeled floor plate cells along with a larger population of notochord cells. A strikingly different distribution of labeled progeny was observed when fluorescein was uncaged in the same domain in embryos depleted of *ntl* function (by injecting *ntl*-MO; see Materials and Methods). *ntl*-depleted embryos (n=8), all contained large numbers of labeled floor plate cells and a dorsal patch of labeled EVL (Fig. 8C,E). In seven of the eight *ntl*-depleted embryos, DEL labeling was restricted to the floor plate; in one, a few mesenchymal cells underlying the floor plate were also labeled.

In a separate experiment, cells at the same position were uncaged in gastrula stage embryos from a cross of two *ntl* heterozygotes. At the time of uncaging, the genotype is unknown; however, we assessed phenotype and distribution of labeled cells later during the pharyngula stage (after 24 hpf). Phenotypically wild-type embryos (n=7) all had labeled cells in a dorsal patch of EVL, and DEL labeling was restricted to the notochord (n=6) or to the hatching gland (n=1). All homozygous *ntl*$^{-}$ embryos (n=6) had DEL labeling restricted to the floor plate (n=3), floor plate and dorsal mesenchyme under the floor plate (n=2), or to dorsal mesenchyme alone (n=1). Our results show that excess *ntl*$^{-}$ floor plate cells originate from a fate map position corresponding to the wild-type notochord domain.

DISCUSSION

spt and *ntl* act synergistically to promote trunk and tail mesoderm fates

The results suggest that *spt/VegT* and *ntl/Brachyury* can substitute for each other for a crucial early function, the specification of all posterior mesoderm. However, the same genes are required individually for what may be later functions, promoting development of distinct mesodermal types. Because both Spt and Ntl are both T-box transcription factors, they might be able to activate common transcriptional target genes, suggesting that they can functionally substitute for one another, at least partially, in regions of the embryo where they are co-expressed. In vitro binding site selection experiments show that Brachyury binds to a specific palindromic sequence (Kispert and Herrmann, 1993; Conlon et al., 2001), and crystallography structure analysis confirms that the Brachyury T-box domain can bind DNA as a dimer (Müller and Herrmann, 1997). Binding site selection experiments demonstrate that Brachyury, VegT and Eomesodermin all recognize pairs of the same core sequence, but that spacing and orientation of paired sites differs for each protein (Conlon et al., 2001). However, no promoter analyzed to date contains sites that are perfect matches to the in vitro selected sites (Tada and Smith, 2001). At least three types of *X. laevis* direct T-box target genes whose promoters have been analyzed, namely *Bix* genes, *fgf*s and *nodal*-related genes of the TGFβ family (see Tada and Smith, 2001), are expressed in the blastoderm margin and are potential candidates for mesoderm specification genes that might be activated by either *spt* or *ntl*. FGFs and the TGFβ family member Derriére are particularly intriguing candidates because of their proposed roles in posterior mesoderm development.

The lack of posterior mesoderm in *spt*$^{-}$*;ntl*$^{-}$ embryos is very similar to the phenotype of zebrafish and frog embryos in which FGF signaling has been disrupted (Amaya et al., 1991; Griffin et al., 1995; Griffin et al., 1998). To date, several zebrafish FGF genes (*fgf8, fgf3, gfgf, fgf4*) have been isolated that are expressed (at least transiently) in mesodermal precursors (Furthauer et al., 1997; Reifers et al., 1998; Furthauer et al., 2001; Phillips et al., 2001) (B. W. D. and C. B. K., unpublished data). Gene expression analyses in *ntl*, *spt* and *fgf8/ace* single mutants and compound heterozygotes indicate that zebrafish T-box genes and *fgf8* are involved in a regulatory loop (B. W. D. and C. B. K., unpublished data), similar to the auto-regulatory loop described for *X. laevis Brachyury* and *eFGF* (Issacs et al., 1994; Schulte-Merker and Smith, 1995; Casey et al., 1998). The *X. laevis* TGFβ family member Derriére is involved in mesoendoderm development and appears to function in posterior regions of the embryo (Sun et al., 1999). It has been proposed that Derriére, zygotic VegT and Brachyury operate in an FGF-dependent regulatory loop in the early gastrula to specify posterior mesoderm development (Sun et al., 1999). A zebrafish *derriére* homolog has not yet been described, but may prove to be an important *spt* and/or *ntl* target gene.

The floor plate 'paradox' – why is there excess floor plate in *spt* and *ntl* single mutant embryos, but lack of posterior medial floor plate in *spt;ntl* double mutant embryos?

We propose that *spt* and *ntl* function at two different times during posterior MFP development, just as we suggest they have early and late functions during posterior mesoderm development. An early requirement for *spt* or *ntl* promotes MFP fate, but at later times, *spt* and *ntl* each function to restrict MFP fate. Thus, *spt;ntl* double mutant embryos lack posterior MFP, because an early promoting influence (both *spt* and *ntl* function) is missing. In contrast, single mutant embryos have excess MFP, because the early promoting influence (*spt* or *ntl* function) is present, but a later repressive influence (*spt* or *ntl* function) that restricts MFP number, is lacking. First, we consider the role of *spt* and *ntl* in promoting MFP fate, and then in the sections that follow, we detail the possible roles of each T-box gene in restricting MFP fate.

How do *spt* and *ntl* function to promote MFP fate?

We show that *spt*$^{-}$*;ntl*$^{-}$ embryos lack posterior MFP (Fig. 5), but that *spt*$^{-}$*;ntl*$^{-}$ cells can form posterior MFP when placed into a wild-type environment (Fig. 7). The simplest explanation for the *spt*$^{-}$*;ntl*$^{-}$ MFP defect is that a mesodermal derivative important for posterior MFP induction is missing in *spt*$^{-}$*;ntl*$^{-}$ embryos. Alternatively, embryos lacking *spt* and *ntl* function may fail to generate a midline precursor population (a cell

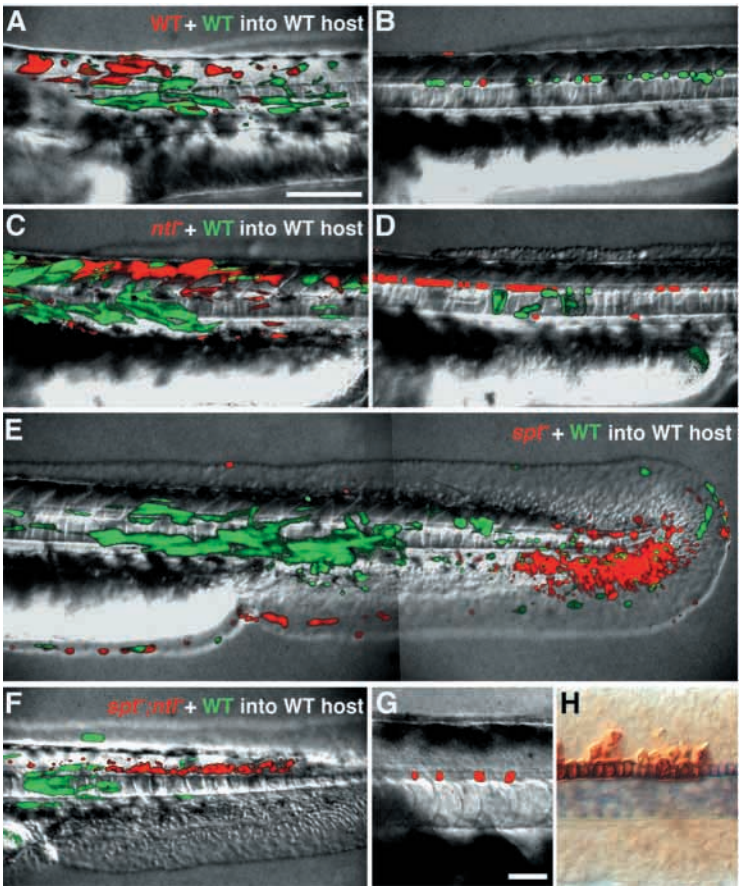

Fig. 7. *spt* and *ntl* together are required cell-autonomously in mesodermal cells, but neither gene is required in medial floor plate cells. Genetic mosaic embryos were generated by isochronic transplantation of blastula cells from a rhodamine-labeled donor embryo derived from an intercross of two heterozygous *spt;ntl* carriers, together with blastula cells from a fluorescein-labeled wild-type donor, into the presumptive mesoderm region of a wild-type host embryo (see Materials and Methods for details). Shown are live embryos at approximately 36 hpf with rhodamine- and fluorescein-labeled cells visualized in red and green, respectively (A-G), and an embryo fixed at 22 hpf and processed to detect *shh* expression (blue) and a co-injected biotinylated-dextran lineage tracer (brown) (H). In A-F, the fluorescein-labeled cells are wildtype, and rhodamine-labeled cells are either wildtype (A,B), *ntl*⁻ (C,D), *spt*⁻ (E), or *spt*⁻*;ntl*⁻ (F,G). Cells doubly homozygous for *spt* and *ntl* mutations never make mesoderm, but instead contribute to non-neural ectoderm, spinal cord, and MFP (F,G). Some genetic mosaics containing *spt*⁻*;ntl*⁻ cells were fixed at approximately 22 hpf and processed to detect *shh* transcripts (blue) and the co-injected biotinylated-dextran lineage tracer (brown) to show that donor-derived *spt*⁻*;ntl*⁻ floor plate cells express *shh*, a MFP marker (H). Scale bars: 100 μm (A-F), 25 μm (G,H).

population normally giving rise to both notochord and floor plate), yet individual *spt*⁻*;ntl*⁻ cells can be recruited into the midline precursor population and adopt a floor plate fate if nudged along by wild-type cells in a genetic mosaic. One way we are distinguishing between these two possibilities is by transplanting wild-type cells into *spt*⁻*;ntl*⁻ embryos to ask if wild-type cells or tissues can restore *spt*⁻*;ntl*⁻ MFP development. To date, we have observed large stretches of posterior *spt*⁻*;ntl*⁻ MFP in two hosts transplanted with wild-type cells (S. L. A., unpublished observations). In both cases, the wild-type cells were located in presumptive midline mesodermal derivatives at midbrain and hindbrain levels of the head, suggesting that signals from anterior mesoderm (the prechordal plate or nearby tissues) can induce posterior MFP. Thus, we favor the first possibility, that a mesodermally derived signal is required to induce MFP fate. (If the second possibility were true, we would predict that *spt*⁻*;ntl*⁻ host MFP cells would be co-mingled with wild-type donor MFP cells in the same region.)

Are there prechordal plate mesoderm defects in *spt;ntl* double mutant embryos? We note that anterior mesoderm (e.g., hatching gland and head muscle) is present in *spt*⁻*;ntl*⁻ embryos. However, *spt* is expressed in prechordal plate mesoderm (Griffin et al., 1998; Ruvinsky et al., 1998) and *ntl* may be transiently expressed there (judged by the early gastrula co-expression of *ntl* and *goosecoid*, a marker of anterior mesoderm) (Schulte-Merker et al., 1994a), suggesting that *spt* or *ntl* expression in anterior mesoderm may be required to generate a posterior MFP-inducing signal or cell interaction.

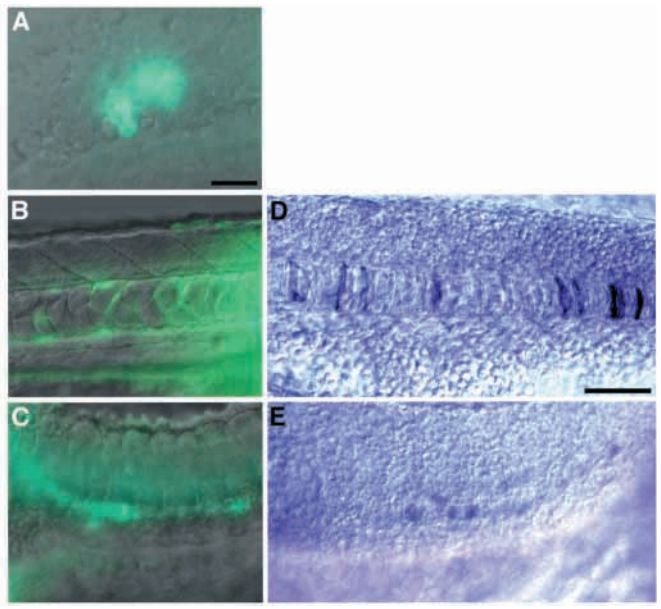

Fig. 8. Excess *ntl* mutant floor plate cells originate from a fate map position corresponding to the wild-type notochord domain. Caged fluorescein fate mapping (see Materials and Methods and text) was used to determine fates of dorsal marginal cells of *ntl*⁻ embryos. (A) The fluorophore was uncaged in the early gastrula in a small population of approximately 20 dorsal marginal cells of wild-type embryos and embryos depleted of *ntl* function (using *ntl*-MO). (B-E) Uncaged fluorescein label was detected at 24 hpf by fluorescence (B,C) and/or using anti-fluorescein antibody (D,E). Representative examples of deep layer cell (DEL) fates are shown. (B,D) In control wild-type embryos, the fluorophore was always detected in large populations of notochord cells, occasionally in hatching gland, and only rarely in floor plate. (C,E) In *ntl*-depleted embryos, the fluorophore was detected in large numbers of floor plate cells. See text for details. Scale bars: 50 μm, in A for A-C and in D for D,E.

Floor plate induction is differentially regulated along the anterior/posterior axis

In chick and mouse, Sonic hedgehog (Shh) is a potent floor plate-inducing molecule (Dodd et al., 1998; Placzek et al., 2000). In zebrafish, MFP induction requires Nodal signaling, but not Shh signaling, whereas LFP formation requires Shh signaling (Sampath et al., 1998; Rebagliati et al., 1998; Zhang et al., 1998b; Schauerte et al., 1998; Karlstrom et al., 1999; Pogoda et al., 2000; Sirotkin et al., 2000; Odenthal et al., 2000; Chen et al., 2001; Etheridge et al., 2001; Lewis and Eisen, 2001; Varga et al., 2001). One Nodal signal, Cyclops, is required in the prechordal plate mesoderm, but not in the notochord, for MFP induction along the entire axis (Sampath et al., 1998). Furthermore, the EGF-CFC Nodal cofactor One-eyed pinhead (Oep) (Gritsman et al., 1999) is required in floor plate cells for MFP fate, presumably for reception of Nodal signals (Strähle et al., 1997; Shinya et al., 1999). Because *spt*$^{-}$*;ntl*$^{-}$ embryos lack posterior MFP, one might suspect that Nodal signaling is disrupted. However, there are important differences between *spt*$^{-}$*;ntl*$^{-}$ embryos and embryos deficient in Nodal signaling. Nodal signaling mutants completely lack MFP and have severe anterior defects including cyclopia and prechordal plate mesoderm deficiencies (Sampath et al., 1998; Rebagliati et al., 1998; Zhang et al., 1998b; Pogoda et al., 2000; Sirotkin et al., 2000). In contrast, *spt*$^{-}$*;ntl*$^{-}$ embryos only lack posterior MFP (Fig. 5) and anterior development is morphologically normal, suggesting that Nodal signaling, at least anteriorly, is not disrupted (Fig. 2). Characterization of the *spt*$^{-}$*;ntl*$^{-}$ phenotype demonstrates that anterior versus posterior MFP formation is differentially regulated along the anterior-posterior axis. Whether novel, non-Nodal signals are involved in posterior MFP induction is an open question.

ntl functions to repress floor plate fate in midline precursor cells

A function for *ntl* in repressing floor plate fate was first proposed because posterior MFP forms in *ntl*$^{-}$*;cyc*$^{-}$ embryos, whereas *cyc* single mutant embryos lack MFP (Halpern et al., 1997). Although lack of *ntl* function bypasses the *cyc* requirement for posterior MFP development (Halpern et al., 1997), it only partially bypasses the requirement for the Nodal cofactor Oep (Schier et al., 1997; Strähle et al., 1997). Together, these data suggest that cells lacking *ntl* function may be diverted to a MFP fate, but that Nodal signals are likely required for the transfating event. Therefore, *ntl* appears to have a dual role during floor plate formation; in addition to promoting MFP fate (as revealed by analyses of the *spt*$^{-}$*;ntl*$^{-}$ mutant), *ntl* also functions to repress MFP development.

Recent studies in chick and zebrafish have suggested that a pool of precursor cells in the organizer region (the gastrula embryonic shield or the chordoneural hinge of later stage embryos) contains both notochord and floor plate precursors (see Le Douarin and Halpern, 2000). Segregation of notochord and floor plate fates occurs while cells are still in the organizer or chordoneural hinge, long before differentiation begins (see Le Douarin and Halpern, 2000). We provide fate mapping data to support the idea that notochord and floor plate precursors segregate early into distinct populations within the organizer (Fig. 8). A role for zebrafish *ntl* in notochord versus floor plate fate choice was first hypothesized by Halpern et al. (Halpern et al., 1997) (see also Le Douarin and Halpern, 2000). Here, we demonstrate that *ntl*$^{-}$ cells in the organizer domain that would form notochord in wild-type embryos, adopt a floor plate fate instead (Fig. 8), clearly establishing a role for *ntl* in repressing floor plate fate. Cell fate choice in this domain may also be mediated by Notch-Delta signaling, since overexpression of zebrafish *deltaA* (*dlA*) results in excess floor plate at the expense of notochord (Appel et al., 1999). Conversely, inhibition of Delta-Notch signaling leads to excess notochord at the expense of floor plate (Appel et al., 1999). These observations led to the proposal that Notch activity represses notochord fate, allowing cells to respond to factors that specify the alternate floor plate midline fate. Considering the opposing roles of *ntl* and *deltaA* in notochord and floor plate development, it will be interesting to examine the epistatic relationship of these two genes in midline cell fate selection.

What is the role of *spt* in midline cell fate choice?

We show that posterior MFP is slightly expanded and sometimes forms in ectopic postions in *spt*$^{-}$ embryos (Figs 5, 6) (Amacher and Kimmel, 1998). Additionally, we show that loss of *spt* function partially suppresses the MFP defect of *cyc* mutant embryos (Fig. 6) and the MFP and notochord defects of *flh* single mutant embryos (Amacher and Kimmel, 1998). Together, these results suggest a normal role for *spt* in repressing posterior midline fates (notochord and floor plate) in addition to its well-established positive role in trunk somitic precursor cell movements and cell fate. A repressive role is further strengthened by the observation that non-midline *spt*$^{-}$ cells can produce notochord in response to ectopic *fgf4* expression. When *fgf4* mRNA is injected into wild-type embryos, notochord expands laterally, but when *fgf4* mRNA is injected into *spt*$^{-}$ embryos, notochord gene expression encompasses the entire embryo (B. W. D. and C. B. K., unpublished results). Thus, *spt* function may be required to limit cell number in the midline precursor cell population (i.e., Spt-positive cells are not responsive to dorsalizing signals). Indeed, a significant amount of MFP forms in *spt*$^{-}$*;cyc*$^{-}$ embryos when compared to almost complete lack of MFP in *cyc*$^{-}$ embryos (Fig. 6). In *spt*$^{-}$*;cyc*$^{-}$ embryos, a probable source of early acting Nodal is Squint (Feldman et al., 1998), a molecule shown to act as a morphogen over considerable distance (Chen and Schier, 2001). Another possible explanation for expanded MFP in *spt*$^{-}$ embryos and MFP presence in *spt*$^{-}$*;cyc*$^{-}$ embryos is that slower convergence of *spt*$^{-}$ midline cells (Thisse et al., 1995; Warga and Nüsslein-Volhard, 1998) allows MFP-inducing signals to be sent over wider distances or for longer times. Whether the expanded MFP in *spt*$^{-}$ embryos is explained by increased midline precursor cell number, or broader or prolonged signaling, our genetic mosaic data (Fig. 7) suggest that the signal itself (not the ability to respond to signal) is diminished when both *spt* and *ntl* functions are lacking.

Future directions

Zebrafish embryos lacking function of two T-box genes, *spt* and *ntl*, lack all mesoderm and MFP in the trunk and tail. The downstream target genes that mediate *spt*- and *ntl*-dependent signaling function are unknown, but intriguing possibilities are FGFs and TGFβ family members (Nodals and Derrière). Additionally, our work suggests that some targets of *spt* and

ntl must function within mesodermal cells, since the genetic mosaic data demonstrate that *spt⁻;ntl⁻* cells cannot adopt mesodermal fates in the trunk and tail even when surrounded by wild-type cells. The identification of such target genes, as well as a posterior MFP-inducing signal, are important goals for the future.

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