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Zebrafish *tbx-c* functions during formation of midline structures

Thameem Dheen[1], Inna Sleptsova-Friedrich[1], Yanfei Xu[2], Matthew Clark[3], Hans Lehrach[3], Zhiyuan Gong[2] and Vladimir Korzh[1,*]

[1]Institute of Molecular Agrobiology and [2]Department of Biological Sciences, National University of Singapore, Singapore
[3]Max-Planck Institute of Molecular Genetics, Dahlem, Berlin
*Author for correspondence (e-mail: vlad@ima.org.sg)

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SUMMARY

Several genes containing the conserved T-box region in invertebrates and vertebrates have been reported recently. Here, we describe three novel members of the T-box gene family in zebrafish. One of these genes, *tbx-c*, is studied in detail. It is expressed in the axial mesoderm, notably, in the notochordal precursor cells immediately before formation of the notochord and in the chordoneural hinge of the tail bud, after the notochord is formed. In addition, its expression is detected in the ventral forebrain, sensory neurons, fin buds and excretory system. The expression pattern of *tbx-c* differs from that of the other two related genes, *tbx-a and tbx-b*.

The developmental role of *tbx-c* has been analysed by overexpression of the full-length *tbx-c* mRNA and a truncated form of *tbx-c* mRNA, which encodes the dominant-negative Tbx-c. Overexpression of *tbx-c* causes expansion of the midline mesoderm and formation of ectopic midline structures at the expense of lateral mesodermal cells. In dominant-negative experiments, the midline mesoderm is reduced with the expansion of lateral mesoderm to the midline. These results suggest that *tbx-c* plays a role in formation of the midline mesoderm, particularly, the notochord. Moreover, modulation of *tbx-c* activity alters the development of primary motor neurons.

Results of in vitro analysis in zebrafish animal caps suggest that *tbx-c* acts downstream of early mesodermal inducers (*activin* and *ntl*) and reveal an autoregulatory feedback loop between *ntl* and *tbx-c*. These data and analysis of midline ($ntl^{-/-}$ and $flh^{-/-}$) and lateral mesoderm ($spt^{-/-}$) mutants suggest that *tbx-c* may function during formation of the notochord.

Key words: T-box gene, Notochord, Somite, Chordoneural hinge, Motor neuron, Overexpression, Dominant-negative, myoD, shh, ntl, flh, islet1, tbx6, spt, Animal cap

INTRODUCTION

During gastrulation in vertebrate embryos, three definitive germ layers (ectoderm, mesoderm and endoderm) are formed by organized and coordinated cell movements. In zebrafish, further subdivision of mesoderm gives rise to axial, adaxial and paraxial mesoderm. The axial mesoderm contributes to the prechordal plate and notochord whereas the adaxial and paraxial cells give rise to different types of muscles (Devoto et al., 1996; Blagden et al., 1997; Currie and Ingham, 1998). In recent years, T-box genes, which encode transcriptional regulatory proteins containing the conserved DNA-binding domain (T-domain), have gained attention as they have been shown to play an essential role in the specification and differentiation of early mesoderm (Papaioannou and Silver, 1998). *Brachyury (T)*, a founder member of the vertebrate T-box gene family, regulates the specification and differentiation of posterior mesoderm during gastrulation (Herrmann, 1995). Furthermore, maternally expressed T-box gene, VegT alone controls the pattern of primary germ layer specification in *Xenopus* embryos (Zhang et al., 1998). Recently, murine *Tbx6* has been shown to be required for cells to choose between mesodermal and neuronal differentiation during gastrulation (Chapman and Papaioannou, 1998). All these data establish the proteins encoded by T-box genes as the molecular switches acting in various cell lineages during determination of cell fates.

Fate mapping has shown that the embryonic shield (the zebrafish organizer) contains future dorsal derivatives, including mesodermal and ectodermal lineages (Kimmel et al., 1990; Shih and Fraser, 1995). It has been reported that cell groups in the organizer region are under the control of zygotically expressed genes (Krauss et al., 1993; Strähle et al., 1993; Ekker et al., 1995; Weinberg et al., 1996). Expression patterns of these genes reflect the functional complexity of the organizer region. For example, *gsc* is specifically expressed in the primordium of the anterior axial mesoderm that contributes to the prechordal plate (Stachel et al., 1993; Thisse et al., 1994). Conversely, *ntl*, a Brachyury homologue and *flh*, a homeobox gene, are expressed in the prospective posterior axial mesoderm that forms the notochord (Schutle-Merker et al., 1994; Talbot et al., 1995). Analysis of the zebrafish mutants, $ntl^{-/-}$ and $flh^{-/-}$, has revealed that both of these genes play essential roles in

notochord development (Halpern et al., 1993, 1995; Talbot et al., 1995). Recent analysis of *flh;ntl* double mutant embryos suggests that Ntl may function in the cell fate choice between the notochord and the floor plate, whereas Flh may act in the choice between the notochord and the muscle (Halpern et al., 1997). Furthermore, the T-box genes, *ntl* and *spt*, interact antagonistically during specification of the notochord and paraxial mesoderm (Amacher and Kimmel, 1998; Griffin et al., 1998).

Here we present three novel zebrafish T-box-containing genes, *tbx-a*, *tbx-b* and *tbx-c*, which are related to the *Drosophila optomotor-blind* (*omb*) gene, a member of the T-box gene family (Pflugfelder et al., 1992). The three zebrafish T-box genes form, within the T-box gene family, a subfamily of *omb*-related genes. Expression patterns of the *omb*-related genes in mouse and chick have been reported (Chapman et al., 1996; Gibson-Brown et al., 1996), but the functions of these genes are largely unknown. More recently, chick *Tbx2* has been shown to act in the lateral mesoderm during early limb development (Gibson-Brown et al., 1998).

We describe the expression pattern of *tbx-c* in wild-type embryos as well as $ntl^{-/-}$, $flh^{-/-}$ and $spt^{-/-}$ embryos and the results of both gain-of-function and loss-of-function experiments, which produce nearly opposite effects. In addition, we studied regulation of *tbx-c* expression and its interaction with mesodermal determinants in an animal cap assay. These results reveal that *tbx-c* plays a role in the specification of late notochordal precursor cells and formation of the differentiated notochord.

MATERIALS AND METHODS

Animals

Wild-type zebrafish embryos were obtained from the fish facility of the Institute of Molecular Agrobiology (IMA), Singapore. Mutant embryos were obtained from fish lines established in the IMA. Heterozygous mutant fish, larvae and embryos were kindly provided by Drs C. Kimmel, B. Trevarrow, M. Halpern, D. Grunwald, U. Strähle and H.-G. Frohnhoefer. The developmental stages are presented as hours postfertilisation (hpf) (Westerfield, 1995; Kimmel et al., 1995).

Cloning and sequencing

A cDNA clone of 1.8 kb encoding a T-box-related gene that lacks the 5′-terminal of the coding region was initially identified by random sequencing from the cDNA library of mixed stages (Gong et al., 1997) and was named the zebrafish *tbx-c*. After sequencing, polymerase chain reaction (PCR) was performed using a specific reverse primer (5′-ACCATCCAGCGAGAGTTG-3′) and forward T3 primer in order to amplify the full-length coding sequence of *tbx-c* from the cDNA library. One of two cDNA fragments obtained was identified as the 5′-terminal of *tbx-c* and used to generate the full-length cDNA of *tbx-c* (GenBank accession number AF 136946). The second cDNA fragment, which contains 700 nucleotides at the 5′-terminal including a part of the T-box, is distinct from the *tbx-c* and has been named as *tbx-a*. In addition, screening of an embryonic 24 hpf zebrafish cDNA gridded library (Max-Planck-Institute of Molecular Genetics, Berlin, Germany) by high-stringency hybridization (using *tbx-c* as a probe) resulted in isolation of another cDNA clone containing a partial coding region of a third T-box gene. This clone, which lacks the 5′-terminal, was named *tbx-b*. Both strands of these clones were sequenced by the dideoxy chain termination method using the T7 Sequencing Kit (Pharmacia, Sweden). Sequences were analyzed using DNA Works, Clustal and MacVector softwares. Work is in progress to obtain the complete coding regions of *tbx-a* and *tbx-b*.

Whole-mount in situ hybridization and immunohistochemistry

Whole-mount in situ hybridization using RNA probes labelled with digoxigenin (Dig; Boehringer Mannheim, Germany) was carried out as previously reported by Oxtoby and Jowett (1993). The stained embryos were cryosectioned (10-15 μm), when necessary. After whole-mount in situ hybridization, some embryos were stained with an anti-Pan-islet antibody and anti-ntl antibody as described previously (Korzh et al., 1993; Schulte-Merker et al., 1992). Two-colour in situ hybridization was carried out according to protocol kindly provided by Dr Elke Ober, Tubingen, Germany.

Synthesis of capped mRNA and microinjection

cDNAs encoding either full-length (*tbx-c*+) or truncated (*dn-tbx-c*) *tbx-c* were subcloned into the pSP64T vector (a gift of Dr D. Melton). The *dn-tbx-c* construct was made by deleting the 3′-terminal region of *tbx-c* from the position of Pro306. The plasmids were linearized and the capped RNAs were synthesized using the mESSAGE mACHINE SP6 transcription kit (Ambion, USA). Activin mRNA was synthesized using linearised pSP64T-XActivin β plasmid (a gift of Dr D. Melton). Unless otherwise stated (see Figure Legends), 1 ng of *tbx-c*+ and *dn-tbx-c*, or 100 pg of β-gal synthetic RNAs (as controls) were injected into embryos at the 1- to 2-cell stage. After injection, embryos were allowed to develop at 28°C and then fixed in 4% paraformaldehyde (PFA) at the stages indicated.

Animal cap assay

Zebrafish animal caps were isolated as reported by Sagerström et al. (1996), with minor modifications. Briefly, embryos at the 1- to 2-cell stage were injected with *tbx-c*+ (1 ng), *ntl* (800 pg), *activin* (2 or 7 pg) or *lacZ* (100 pg) mRNA and left to develop until the late blastula stage (4 hpf). The caps were dissected and incubated in a L15 Leibovitz medium (Sigma, USA) supplemented with 10% horse serum and antibiotic (100 U Penicillin-100 μg Streptomycin/ml; GIBCO BRL, USA,) at 28°C and harvested at various stages. The caps were fixed in 4% PFA and analyzed by whole-mount in situ hybridization.

RESULTS

Sequence analysis

Three novel zebrafish T-box genes from the *omb*-related subfamily were isolated and sequenced as described in Materials and Methods. The conceptual protein sequence of zebrafish *tbx-c* consists of 672 amino acid (aa) residues (Fig. 1A). Alignment with sequences of mouse Tbx2, *Drosophila* Omb, zebrafish Ntl and *Xenopus* ET within the T-domain shows that the T-domain of zebrafish Tbx-c is more closely related to murine Tbx2 than to ET, Omb or Ntl (Fig. 1B,E). Furthermore, the BLAST homology analysis (Altschul et al., 1997) revealed that the C-terminal end, excluding the T-domain, of *tbx-c* has similarity only with mouse Tbx2. Overall, the open reading frame of this clone has 70.2% conservation in comparison to that of mouse Tbx2.

Tbx-b contains a partial T-domain (130 aa residues), which is almost 80% similar to murine and human Tbx3 (Fig. 1C). The third clone encoding the N-terminal end of Tbx-a, has 87% similarity with the zebrafish Tbx-c (Fig. 1D).

Expression pattern of *tbx-c*

The pattern of expression of *tbx-c* in the wild-type zebrafish

embryos has been studied by whole-mount in situ hybridization. Weak expression of *tbx-c* was first detected in the axial mesoderm at 80% epiboly (not shown). At the end of gastrulation, *tbx-c* expression was observed in the prospective ventral forebrain, the single eye field and the presumptive notochord (Fig. 2A,B). By 12 hpf, the eye field has separated at the midline into two *tbx-c*-positive domains of expression (not shown). At 20 hpf, *tbx-c* expression in the eye was found to be restricted to the dorsal retina (Fig. 2C,E). In addition, *tbx-c* expression was evident in the epiphysis, otic vesicles and sensory neurons of cranial ganglia including the trigeminal, and the anterior and the posterior lateral line ganglia (Fig. 2C,E,F). In the dorsal spinal cord, *tbx-c* expression mapped to the population of mechanosensory Rohon-Beard (RB) cells (Figs 2G, 3A). Posteriorly, the *tbx-c* transcripts were localized to the excretory system (Figs 2G, 3A). At 22 hpf, *tbx-c* expression was detected in buds of pectoral fins (Fig. 2D) and, at 30 hpf, in a subset of interneurons in the anterior spinal cord (not shown).

After 12 hpf, expression of *tbx-c* in the midline was found only at the posterior end of the notochord where it could be detected until the end of segmentation (Fig. 3A,D,F,G). The midline precursor cells in the tail bud form a bulb-like structure and segregate into specific cell lineages to form midline tissues, the notochord, the hypochord and the floor plate. Morphologically (Pasteels, 1943; D′Amico and Cooper, 1997) and genetically by the expression of *flh* and *ntl* (Gont et al., 1996; Catala et al., 1995, 1996), this structure has been defined as the chordoneural hinge (CNH). As differentiation of the notochord proceeds, the round-shaped early midline precursor cells in the posterior region of the CNH transform into the oval-shaped cells. These cells further undergo morphological changes and become spindle-shaped cells, characteristic of the notochord (Fig. 3H).

The expression pattern of *tbx-c* differs from that of other two T-box genes presented in this study. In the posterior body, *tbx-c* was expressed in the CNH, RB cells and the excretory system (Fig. 3A), whereas expression of *tbx-b* and *tbx-a* mapped to the differentiated notochord (Fig. 3B) and excretory system (Fig. 3C), respectively. This suggests that

Fig. 1A

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MAYHPFHAHR PTDFPMSAFL AAAQPSFFPA LTLPPGALTK PIPDHTLAGA AEAGLHPALS HHHQAAHLRS LKSLEPEEEV EDDPKVTLEA KDLWDQFHKL GTEMVITKSG RRMFPPFKVR 120
INGLDKKAKY ILLMDIVAAD DCRYKFHNSR WMVAGKADPE MPKRMYIHPD SPATGEQWMA KPVAFHKLKL TNNISDKHGF TILNSMHKYQ PRFHIVRAND ILKLPYSTFR TYVFPETDFI 240
KPVAFHKLKL TNNISDKHGF TILNSMHKYQ PRFHIVRAND ILKLPYSTFR TYVFPETDFI AVTAYQNDKI TQLKIDNNPF AKGFRDTGNG RREKRKQLTL PSLRMYEDQC KVDRDGADSD 300
AVTAYQNDKI TQLKIDNNPF AKGFRDTGNG RREKRKQLTL PSLRMYEDQC KVDRDGADSD ASSSEPTTGR DAGHSPGPVS SPLRFNRGSR DDKTCTDSEH EMDHQNDRCG GSSSPAPEPS 360
ASSSEPTTGR DAGHSPGPVS SPLRFNRGSR DDKTCTDSEH EMDHQNDRCG GSSSPAPEPS SPFRSRSEDW GREKPIAEKK DDYPDSRKTS DSIFSIRNLE KDKLESRSRK DTDSSKKDTE 420
SPFRSRSEDW GREKPIAEKK DDYPDSRKTS DSIFSIRNLE KDKLESRSRK DTDSSKKDTE NSGISGSKDS FSPLMVQTES PSHFGAGHLQ SLALSGLHSQ QFFNPLNTGQ PLLFHPGQFA 480
NSGISGSKDS FSPLMVQTES PSHFGAGHLQ SLALSGLHSQ QFFNPLNTGQ PLLFHPGQFA MAPGAFSAMG MGHLLASVSG AGGLENGSLS AQGTGSTPSP FPFHLSQHML ASQGIPMPTF 540
MAPGAFSAMG MGHLLASVSG AGGLENGSLS AQGTGSTPSP FPFHLSQHML ASQGIPMPTF GGLFPYPYTY MAAAAAAASA LPASSSTASS LSRNPFLSSS TRPRLRFNPY QLPVSIPQST 600
GGLFPYPYTY MAAAAAAASA LPASSSTASS LSRNPFLSSS TRPRLRFNPY QLPVSIPQST NLLTTGLPSG LNPSSESSKC GSREASPVPD HKSGASQRNG SPKTTMKESI NELSKYSETS 660
KRPREPAGDF VT                                                                                                                    672
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Fig. 1B

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ntl         36 .LS..DAE.. TK.KE.TN.. IV..T..... .VLRAS.T.. .PN.M.SV.L .F....NNRW KYVNGEW-VP G..PE.QS.S CV.......N F.AH..KA.. 134
omb        305 .....G.D.. EK........ .......Q.. .QM.F..... .A.......L .......-Y. .......... .......... .........T ......Q.V. 403
ET           1 ---------- ---------- -----..... ......C... .......... .......H.. .......... .......... .L........ ......S.I.  84
Tbx-c       85 .....A.D.. DQ........ .......... ......IN.. .......... .....-.D.. .......... .......... .......... ......A... 183
mTbx2       96 .....A.E.. DQ........ .......... .......... .......... .....-.D.. .......... .......... .......... ......A... 194
Consensus    1 KVTLE.K.LW ..FHKLGTEM VITKSGRRMF PPFKVRVSGL DKKAKYILLM DIVAADD.CR YKFHNSRWMV AGKADPEMPK RMYIHPDSPA TGEQWM.KPV 100

ntl        135 ..S.V..S.K LNGGGQI--- -M...L...E ..I...KVGG .Q.M----IS SQS....Q.. .......EE. .A...KH... ..A.L.AK             214
omb        404 .......... .......VST .......... ....L..... .......... ....K..E.. .......E.. .......... ...L....             495
ET          85 .......... .......GGQ .......... ..S....... ..NS...... S..S..K... ....------ ---------- --------             148
Tbx-c      184 A......... .........--- .......... .......... .......... .......... .......D.. .......... ........             268
mTbx2      195 A......... .........--- .......... .......... .......... .......... .......D.. .......... ........             279
Consensus  101 SFHKLKLTNN ISDKHGF--- TILNSMHKYQ PRFHIVRAND ILKLPYSTFR TYVFPETDFI AVTAYQN.KI TQLKIDNNPF AKGFRDTG             185
```

Fig. 1C

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Tbx-b        1 .......... .......... .......... ..R....... ......N... .......... .......... .......... .......... .......... 100
Tbx-c      137 .......... .......... .......... .......... ..A P.A... .......... .......... .......... .......... .......... 236
hTbx3      157 .......... .......... .......... .......... ......T... .......... .......... .......... .......... .....L.... 256
Consensus    1 AADDCRYFKH NSRWMVAGKA DPEMPKRMYI HPDSPATGEQ WMSKVV.FHK LKLTNNISDK HGFTILNSMH KYQPRFHIVR ANDILKLPYS TFRTYVFPET 100

Tbx-b      101 .......... .........H .......... .                    131
Tbx-c      237 .......... .......... .......... .                    267
hTbx3      257 E......... .......... .......... .                    287
Consensus  101 DFIAVTAYQN DKITQLKIDN NPFAKGFRDT G                    131
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Fig. 1D

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Tbx-a        1 .......... .......... .......... ......-.S. ..A..A.S.. ...-------- ---...-A.. .......... .F.G.....D .G........  88
Tbx-c        1 .......... .T........ .......... ........T. .T...T.... ...-------- -------.P.. S......... .......... ..........  89
mTbx2        1 ........P. .......... .......... .A......G. ....PG.... .A.AAAAAAA AEA...VS.. .P.PP..... ........D. ..........  100
Consensus    1 MAYHPFHAHR PADFPMSAFL AAAQPSFFPA LTLPPGAL.K PLPDH.LAGA AEA------- ---GLH-.AL GHHHQAAHLR SLKSLEPEEE VEDDPKVTLE  89

Tbx-a       89 .......... I......... .K........ .......... .......... .E........ ....                                        152
Tbx-c       90 ..D....... .......... .......... .I........ .......... .......... ....                                        153
mTbx2      101 .......... .......... .......... ..S....... .......... .......... ....                                        164
Consensus   90 AKELWDQFHK LGTEMVITKS GRRMFPPFKV RVNGLDKKAK YILLMDIVAA DDCRYKFHNS RWMV                                          153
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Fig 1E

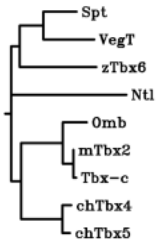

Fig. 1. Sequence analysis of *omb*-related zebrafish T-box proteins. (A) Deduced amino acid sequence of the zebrafish *tbx-c* gene. The T-domain is underlined. Tbx-c has been truncated at Pro306 (in bold) to generate dn-Tbx-c. (B) Comparison of the T-domains of zebrafish Tbx-c, mouse Tbx2, *Drosophila* Omb, *Xenopus* ET, zebrafish Ntl. (C) Comparison of partial amino acid sequences of T-domains of Tbx-b, human Tbx3 and Tbx-c. (D) Alignment of N-terminal regions of the zebrafish Tbx-a, Tbx-c and the murine Tbx2. (E) A phylogenetic tree of T-domains showing relationship of Tbx-c with other proteins of T-box family. The dots represent residues that are identical and the dashes represent missing aminoacid residues.

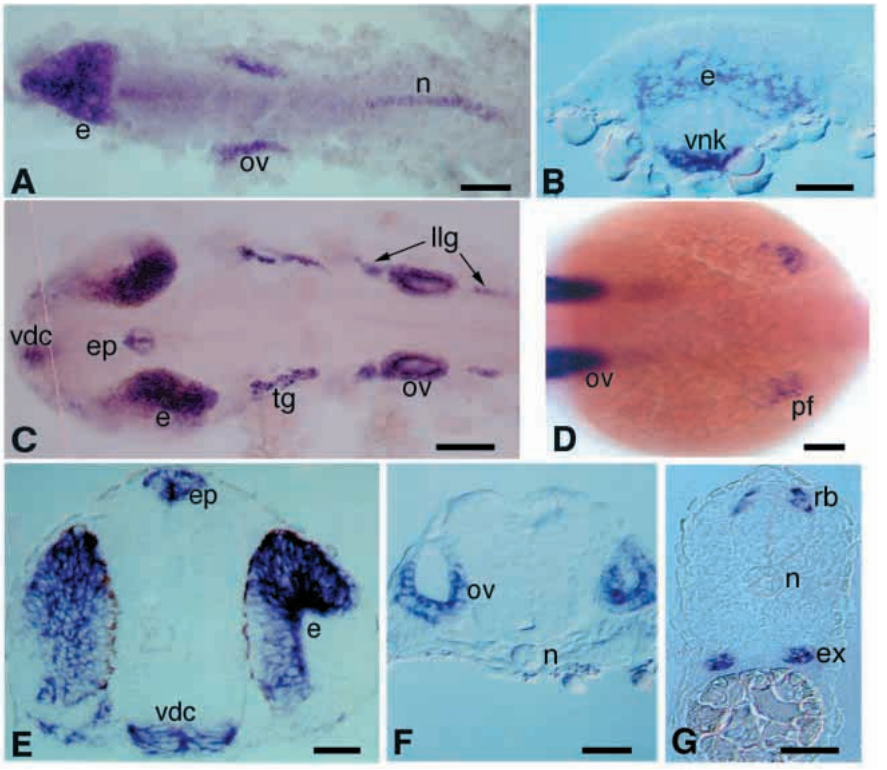

Fig. 2. Expression pattern of *tbx-c* in the zebrafish embryo. (A) Dorsal view of a flat-mounted zebrafish embryo at 10 hpf. Note the expression in the anterior ventral neural keel, single eye field, the otic vesicle and the early notochord. (B) Transverse section of the 10 hpf embryo showing *tbx-c* transcripts in the eye field as well as in the anterior ventral neural keel. (C) Dorsal view showing expression of *tbx-c* at 20 hpf. The expression is localized to the ventral forebrain, the dorsal eye, the epiphysis, the trigeminal as well as lateral line ganglia and the otic vesicle. (D) Dorsal view of whole-mounted embryo (22 hpf) showing expression in pectoral fin buds and the otic vesicle. (E) Transverse section at the eye level shows *tbx-c* transcripts in the epiphysis, the ventral diencephalon and retinal cells in the dorsal eye. (F) Transverse section shows expression of *tbx-c* in the otic vesicle. (G) Transverse section through the caudal spinal cord shows expression of *tbx-c* in mechanosensory Rohon-Beard cells and in the excretory system. e, eye; ep, epiphysis; ex, excretory system; llg, lateral line ganglia; n, notochord; ov, otic vesicle; rb, Rohon-Beard cells; tg, trigeminal ganglion; vdc, ventral diencephalon; vnk, ventral neural keel. Anterior is at the left, unless otherwise stated. Scale bars, 100 μm (A), 50 μm (B-G).

these three genes may have specific functions during formation of the posterior body.

The timing of expression of several developmental genes in the midline precursor cells correlates with important events of notochordal morphogenesis such as differentiation and formation of the notochord. These genes, including *shh*, *ntl* and *flh*, are expressed in discrete regions of the CNH. At 20 hpf, expression of Ntl was localized to early mesodermal cells, the CNH as well as the notochord (Fig. 3D,H; Schulte-Merker et al., 1992). *shh* expression was found in the CNH, the notochord and the floor plate (Fig. 3E). In contrast, *flh* expression was confined to the posterior region of the CNH containing round-shaped cells (Fig. 3F,H). Interestingly, *tbx-c* expression was localized to the oval-shaped cells in the anterior region of the

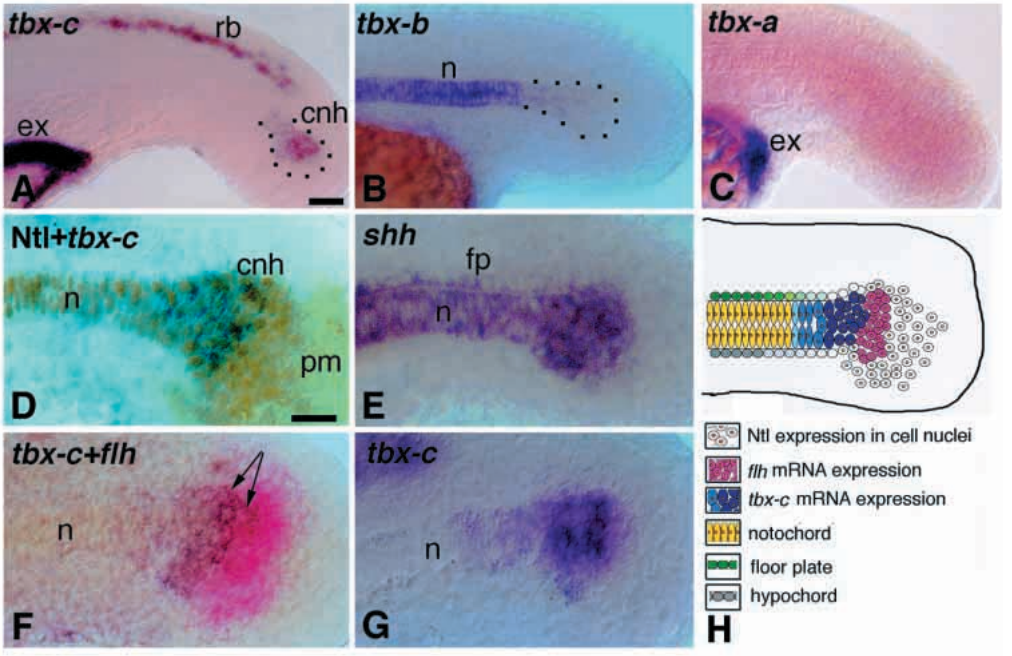

Fig. 3. Expression pattern of *tbx-c*, *tbx-b* and *tbx-a* in the tail and discrete cell groups in the chordoneural hinge (CNH) of zebrafish at 18-20 hpf. (A) *tbx-c* is expressed in the RB cells, the excretory system and the CNH (outlined). (B) *tbx-b* is expressed in the notochord. (C) *tbx-a* is expressed in the excretory system. (D) Double staining for Ntl protein (brown) and *tbx-c* mRNA (blue). Ntl is expressed in the posterior mesoderm, the CNH and the notochord. *tbx-c* transcripts map to the anterior CNH. (E) *shh* is expressed in the CNH, the notochord and the floor plate. (F) Two-colour in situ hybridization staining for *tbx-c* (blue) and *flh* (magenta). *tbx-c* is expressed in the anterior CNH, whereas *flh* is expressed in the posterior part of the CNH. Expression domains of *flh* and *tbx-c* overlap in the mid-CNH (arrows). (G) *tbx-c* is expressed in the anterior part of the CNH. (H) A model summarizing the distribution of gene products in the CNH and the notochord. cnh, chordoneural hinge; ex, excretory system; fp, floor plate; n, notochord; pm, posterior mesoderm. Scale bars, 25 μm.

CNH (Fig. 3D,F-H) and ceased once the oval-shaped cells were transformed into spindle-shaped cells of the notochord. Expression of *tbx-c* and *flh* was found in adjacent domains of the CNH and these domains partially overlap in the middle of the CNH (Fig. 3F).

tbx-c acts during specification of axial mesoderm

Tbx-c contains the highly conserved T-domain, which may be involved in DNA binding, similar to *Bra* and *omb* (Kispert and Herrmann, 1993; Pflugfelder et al., 1992) (Fig. 1A). It was shown that removal of the 3′-terminal region of the *Bra* gene, encoding the transactivation (TA) domain produces a dominant-negative mRNA with antimorphic effects (Stott et al., 1993; Rao, 1994). We used a similar strategy to synthesize *dn-tbx-c* mRNA encoding a truncated protein. We also synthesized a full-length *tbx-c* mRNA. These mRNAs were injected into zebrafish embryos, which were analyzed by whole-mount in situ hybridization using specific molecular markers for mesodermal (Table 1A,B) and neuroectodermal derivatives.

Mesoderm

First, the effect of *tbx-c* was analyzed using midline markers *shh* and *ntl* (Fig. 4A-H). *ntl* is expressed in the undifferentiated mesoderm and the notochord (Schulte-Merker et al., 1992) (Fig. 4A). *shh* is expressed in the notochord (Fig. 4D,G), the prechordal plate and the ventral neural tube (Krauss et al., 1993). As revealed by these markers, the midline mesoderm was expanded mediolaterally in the *tbx-c*+ embryos (Fig. 4B,E). In contrast, *dn-tbx-c* acts antimorphically, causing attenuation of the midline (Fig. 4C,F). Some embryos injected with *tbx-c*+ mRNA displayed axis bifurcation anteriorly (Fig. 4H). Multiple notochords were observed in about 20% (12 out of 61) of the embryos injected with a higher dose (3 ng) of *tbx-c*+ mRNA (Fig. 5B), indicating that the effect of *tbx-c* on axis formation is dose-dependent.

We next examined the effect of *tbx-c*+ on formation of other mesodermal derivatives. *tbx6* and *spt*, T-box containing genes, are co-expressed in the ventrolateral paraxial mesoderm contributing to somites (Hug et al., 1997; Griffin et al., 1998; Fig. 4I,L). Expression of these genes was reduced in *tbx-c*+ embryos (Fig. 4J,M) and expanded across the midline in *dn-tbx-c* embryos (Fig. 4K,N). *myoD* expression maps to adaxial and paraxial cells of somites (Weinberg et al., 1996; Fig. 4O,R). The *tbx-c*+ embryos displayed a broader midline surrounded by adaxial cells and partially or completely reduced somitic tissue (Fig. 4P,S). In *dn-tbx-c* embryos, somites were fused at the dorsal midline

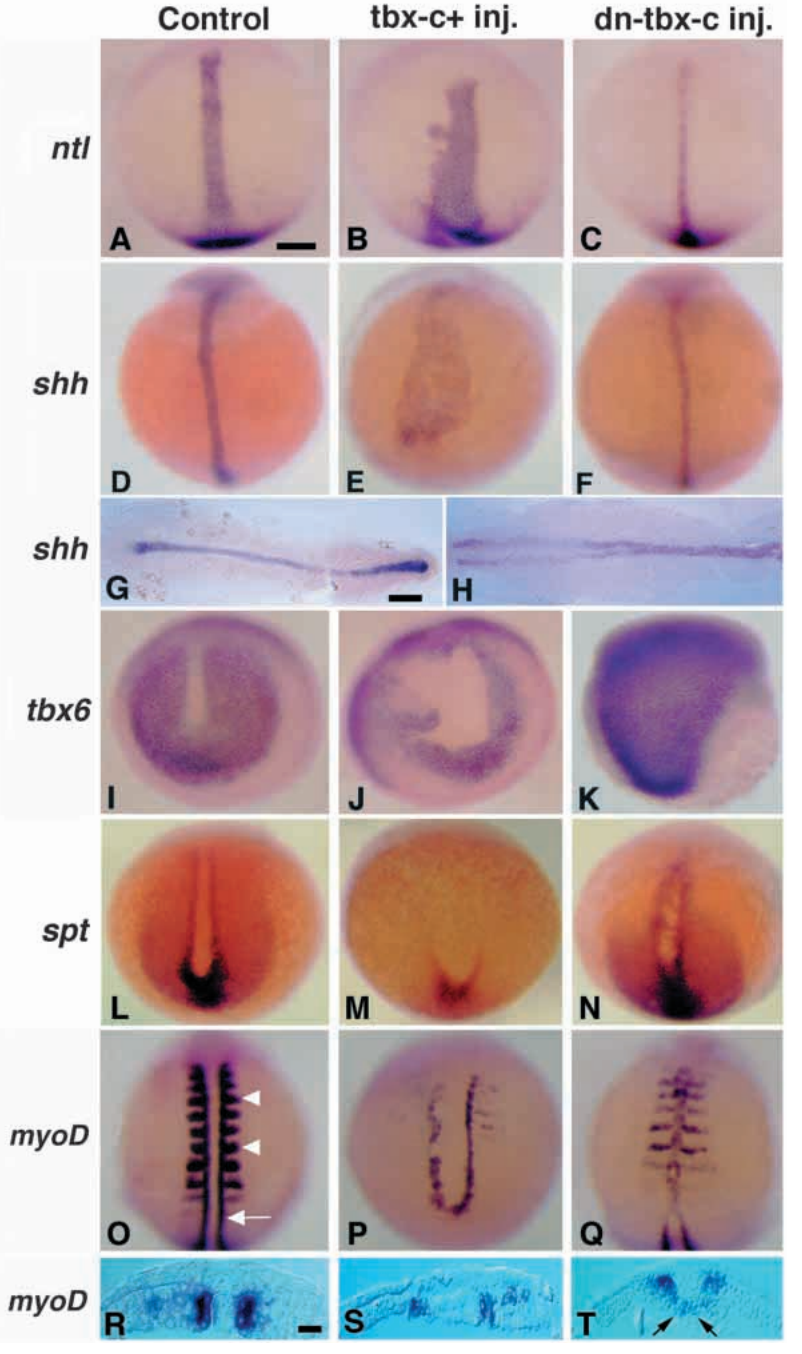

Fig. 4. Effects of ectopic expression of *tbx-c*+ and *dn-tbx-c* in zebrafish embryos at 10 hpf. For each marker, control embryos injected with *lacZ* mRNA, *tbx-c*+ embryos and *dn-tbx-c* embryos were included. (A-H) Expansion of the notochord in *tbx-c*+ embryos (B,E) and attenuation of the notochord in *dn-tbx-c* embryos (C,F) are shown by expression of *ntl* and *shh*. Dorsal view of a flat-mounted control (G) as well as *tbx-c*+ (H) embryos show that overexpression of *tbx-c*+ causes bifurcation of anterior midline (H). (I-K) *tbx6* expression in the lateral mesoderm (I) is reduced in *tbx-c*+ embryos (J) and expanded to the midline in *dn-tbx-c* embryos (K). (L-N) *spt* expression (L) like *tbx6*, is reduced in the lateral mesoderm of *tbx-c*+ embryos (M) and expanded in *dn-tbx-c* embryos (N). (O-T) *myoD* is expressed in adaxial (arrow) and paraxial (arrowheads) cells (O). Its expression reveals a reduction of somitic tissue in *tbx-c*+ embryos (P,S) and fusion of somites at the midline in *dn-tbx-c* embryos (Q,T). Transverse sections reveal that the midline is expanded about 2.5-fold in *tbx-c*+ embryos (P,S) in comparison to that of the control (O,R). In contrast, the midline mesoderm is reduced and *myoD*-positive muscle cells appear in the midline of *dn-tbx-c* embryos (arrows; T). Scale bars, 100 μm (A-Q), 25 μm (R-T).

Table 1. Microinjection of *tbx-c* mRNA in zebrafish embryos

A. Effect of ectopic expression of *tbx-c*+ on mesoderm derivatives			
Marker	Phenotype	%	no.of embryos*
ntl	Expanded notochord	48	(26/54)
shh	Expanded midline	31	(25/80)
tbx6	Wider midline, reduced lateral expression	50	(32/64)
spt	(ditto)	45	(27/60)
myoD	Reduced no. or complete absence of somitic cells	53	(41/78)
B. Effect of ectopic expression of *dn-tbx-c* on mesoderm derivatives			
ntl	Attenuated notochord	38	(18/47)
shh	Attenuated midline	31	(16/52)
tbx6	Expanded expression to the posterior midline	37	(22/60)
spt	(ditto)	50	(26/52)
myo D	Somites fused at the midline and/or laterally extended	39	(22/57)
C. Effect of ectopic expression of both *tbx-c*+ and *dn-tbx-c* mRNAs on mesodern derivatives			
myoD	Reduced no. or absence of somitic cells.	15.3	(8/52)
	Somites fused at the midline and/or laterally extended.	11.5	(6/52)
ntl	Expanded notochord	18.5	(10/54)
	Attenuated notochord	15	(8/54)

*****Number of affected embryos/number of analysed embryos.

and often expanded laterally (Fig. 4Q,T). Transverse sections reveal the presence of *myoD*-positive cells at the midline of *dn-tbx-c* embryos. These results suggest that *tbx-c* acts by promoting midline axial fates and suppressing lateral mesodermal fates.

Motor neurons (MNs)

It has been reported that local signals from the notochord induce differentiation of MNs (Placzek et al., 1990; Yamada et al., 1993; Ericson et al., 1992; Goulding et al., 1993). We have analyzed whether ectopic expression of *tbx-c*+ and *dn-tbx-c* influences specification of these neurons.

In wild-type embryos, MNs are arranged in a row on both sides of the midline and express *islet1* (Korzh et al., 1993; Inoue et al., 1994; Appel et al., 1995; Fig. 5A,D). Embryos injected with *tbx-c*+ displayed a substantial increase in the number of MNs (40%; 26/65). Several of these embryos developed 3-4 lines of MNs along the midline (not shown). The frequency of extranumerary MNs was increased when a higher dose of *tbx-c*+ mRNA (3 ng) was injected. Double staining for *ntl* and Islet1 confirmed the increase in the number of MNs with expanded or extranumerary notochords in *tbx-c*+ embryos (Fig. 5B,E). Conversely, number of MNs was significantly reduced (36%; 20/55; Fig. 5C,F) in *dn-tbx-c* embryos. Loss of Islet-1-positive MNs correlates with decrease or lack of *ntl* expression in the dorsal midline.

To examine whether *tbx-c*+ can rescue phenotypes caused by the injection of *dn-tbx-c* mRNA, we next injected *tbx-c*+ and *dn-tbx-c* mRNAs together (1 ng each) into embryos and analyzed these embryos by in situ hybridization with *myoD* and *ntl* probes. This experiment showed that *tbx-c*+ rescued phenotypes caused by *dn-tbx-c* in a majority of injected embryos (Table 1C).

In order to address possible regulatory interactions between *tbx-c* and other notochordal determinants, we analyzed expression of *tbx-c* in mutant embryos lacking Ntl and Flh. In $ntl^{-/-}$ mutants, the midline mesoderm was segregated from the paraxial mesoderm (Fig. 6B) as in wild-type embryos (Fig. 6A). Weak expression of *tbx-c* was found in the midline mesoderm of mutants (Fig. 6B). We injected *tbx-c*+ into a pool of unidentified embryos (88 embryos) derived from the heterozygous $ntl^{-/+}$ pair (ntl^{b160}) to study whether *tbx-c* can induce differentiation of notochord cells in the absence of *ntl*. At 11 hpf, embryos were stained for *ehh* mRNA, a marker for differentiated notochord (Currie and Ingham, 1996; V. K., unpublished data), followed by Ntl protein (Fig. 6C) to distinguish mutant embryos. Control $ntl^{-/-}$ embryos (ntl^{b160}) do not express *ehh* mRNA and Ntl protein (not shown). Similarly, injected $ntl^{-/-}$ embryos (18/88 embryos) did not express both *ehh* and Ntl (Fig. 6D). In contrast, wild-type siblings of $ntl^{-/-}$ embryos showed expression of *ehh* and Ntl in the expanded notochord caused by the injection of *tbx-c*+ (Fig. 6E). This result indicates that *tbx-c* can promote differentiation of notochord only in the presence of *ntl*.

In $flh^{-/-}$ mutants, there was no segregation of midline and paraxial mesoderm and *tbx-c* expression was completely absent in the midline (Fig. 6F). The injection of *tbx-c*+ did not induce notochord formation in $flh^{-/-}$ embryos as revealed by the expression of *ntl* (Fig. 6H). A few *ntl*-positive cells were found in the midline of both uninjected (Fig. 6G) and injected $flh^{-/-}$ embryos at 11 hpf. This result suggests that *flh* is required for the function of *tbx-c*. In both mutants, *tbx-c* expression appeared to be unaltered in anterior structures including the eye field and the ventral neural keel compared to the wild-type embryos.

As *spt* expression was strongly reduced in *tbx-c*+ embryos, we analyzed *tbx-c* expression in $spt^{-/-}$ embryos, which are deficient of trunk somites (Kimmel et al., 1989). *tbx-c* expression in $spt^{-/-}$ (Fig. 6J) embryos was similar to the wild-type expression pattern (Fig. 6I). However, the notochord in $spt^{-/-}$ embryos appeared to be widened as already reported (Amacher and Kimmel, 1998; Warga and Nusslein-Volhard, 1998).

Analysis of *tbx-c* regulation in animal caps

It has been shown that the expression of several genes encoding T-related proteins in *Xenopus* (VegT, Eomes and

Xbra) and in zebrafish (Ntl and Tbx6) is responsive to the dorsal mesodermal inducer, Activin. In *Xenopus* animal caps, Activin can respecify prospective ectodermal tissue to form different mesodermal cell types in a dose-dependent manner. Moreover, Activin can function in the embryo as a long-range morphogen (Gurdon et al., 1994, 1995; Jones et al., 1996; Reilly and Melton, 1996; Hug et al., 1997; for review see Smith, 1997).

In our experiments, expression of *ntl* and *tbx-c* was induced in animal caps dissected from the embryos injected with *activin* mRNA (Fig. 7B,D). There was no transcription of either *ntl* or *tbx-c* in the control caps (Fig. 7A,C). These data suggest that *tbx-c*, like *ntl*, can be a downstream target gene of general mesoderm inducers like Activin.

During normal development, *ntl* is expressed much earlier than *tbx-c* (Schulte-Merker et al., 1992). Correspondingly, *ntl* transcripts accumulated much faster than that of *tbx-c* in animal caps injected with *activin* mRNA. This suggests that activation of *tbx-c* expression can be mediated by *ntl* which is induced by *activin* mRNA. To test this possibility, we injected *ntl* RNA and assessed *tbx-c* expression in animal caps derived from injected embryos. *tbx-c* was strongly induced in these caps (Fig.7E). These results indicate that *ntl* acts upstream of *tbx-c* and can induce its transcription. In contrast, changes in *ntl* expression observed in whole embryos after injection of *tbx-c*+ and *dn-tbx-c* RNA suggest that *tbx-c* can regulate *ntl* expression. It seems that *tbx-c* function is necessary for the maintenance of *ntl* expression in the notochord via a positive feed-back regulatory loop. We further found that *tbx-c*+ can induce *ntl* expression in animal caps (Fig. 7F). However, *tbx-c* is not essential for the activation of *ntl* expression by the early mesoderm inducer, since *ntl* expression was induced in animal caps derived from embryos injected with *activin* and *dn-tbx-c* together (Fig. 7G). This result further suggests that effects of *dn-tbx-c* are attributed to the specific function of *tbx-c*.

DISCUSSION

Analysis of the structure of three T-box genes presented here reveals that they belong to the subfamily of zebrafish *omb*-related genes. The founder gene of this subfamily, *omb*, plays an essential role in development of the optic lobe and the distal wing of *Drosophila* (Pflugfelder et al., 1992; Grimm and Pflugfelder, 1996). A number of *omb*-related genes has been identified in many species, including *ET* in *Xenopus*, and *Tbx2* and *Tbx3* in chick, mice and human (Agulnik et al., 1995; Campbell et al., 1995; Law et al 1995; Li et al., 1997; Gibson-Brown et al., 1996; for review see Papaioannou and Silver, 1998). Among the T-box genes identified in vertebrates so far, only *Bra/ntl* is expressed in the notochord (Papaioannou and Silver, 1998). In contrast, *tbx-c* is expressed not only in the notochord but also in other cell lineages including the CNS, the limb and the excretory system. Amino acid sequence comparison suggests that *tbx-c* is closely related to *Tbx2* of higher vertebrates. However, the expression pattern of *tbx-c* differs from that of *Tbx2* identified in other species.

Tbx-c acts as a determinant of midline mesoderm

The unique expression pattern of *tbx-c* in the CNH suggests that *tbx-c* has a potential role in morphogenesis and differentiation of midline structures, especially the notochord. This was confirmed by functional experiments that include overexpression of full-length as well as dominant-negative Tbx-c. Changes in the midline of *tbx-c*+ and *dn-tbx-c* embryos indicate that Tbx-c is important for specification of midline precursor cells and formation of the notochord. The concurrent reduction of somites in *tbx-c*+ embryos and their partial fusion at the midline in *dn-tbx-c* embryos provides evidence that Tbx-c, similar to Flh, may promote axial fates by acting in the cell fate choice between axial and lateral mesoderm.

Moreover, stage-specific expression of *flh* in the early CNH and *tbx-c* in the late CNH suggests that these genes can be responsible for distinct phases of notochord differentiation. It is also possible that both Flh and Tbx-c may act synergistically in the regulation of differentiation of midline precursor cells initiated by early inducers of dorsal mesoderm such as Ntl, since the ectopic effects of Tbx-c and Flh homologue in *Xenopus* (Gont et al., 1996) on notochord development are similar. Probably, initiation of *tbx-c* expression is not dependent on *flh* as *tbx-c* is expressed in several cell lineages that do not express *flh*. In addition, the role of *tbx-c* differs from that of *flh* since *tbx-c*+ could not rescue the notochord in $flh^{-/-}$ embryos.

Analysis of *tbx-c* expression in $ntl^{-/-}$ embryos suggests that Ntl may not be involved in initiation of *tbx-c* expression. However, finding of the positive regulatory loop between *tbx-c* and *ntl* in the present study indicates that, during development of the notochord, Ntl may be essential for the maintenance of *tbx-c* expression and vice versa.

The gain-of-function phenotype observed in the present study is reminiscent to that of zebrafish $spt^{-/-}$ embryos that fail to form trunk somites but form a wider notochord (Kimmel et al., 1989; Thisse et al., 1995; Griffin et al., 1998). *spt* function appears to be essential for the development of the relative size of the axial and paraxial fields (Warga and Nusslein-Volhard, 1998). In the same fashion, it can be speculated that *tbx-c* may be responsible for establishing the relative size of the notochord, since the size of the notochord was altered in *tbx-c*+ and *dn-tbx-c* embryos. In addition, suppression of *spt* expression in *tbx-c*+ embryos suggests that Spt and Tbx-c act antagonistically during specification and segregation of axial and paraxial mesoderm lineages, like Flh and Spt (Amacher and Kimmel, 1998).

Tbx-c acts as a stage-specific regulator during notochord differentiation

The principal roles of two T-box genes, *ntl* and *tbx-c*, during mesoderm development may differ since their expression patterns are distinct. *ntl* is expressed in undifferentiated mesodermal cells and subsequently in the notochord. These data, together with mutant analysis suggest that the functional role of Ntl is limited to the notochord where it acts as a lineage-specific factor. In contrast, *tbx-c* is expressed in the CNH as well as in other cell lineages outside the mesoderm. Therefore, it is suggested that Tbx-c may function as a stage-specific differentiation factor during formation of the notochord. A number of developmentally important molecules including Flh and Islet1 function in a similar fashion in several cell lineages (Talbot et al., 1995; Pfaff et al., 1996; Masai et al., 1997).

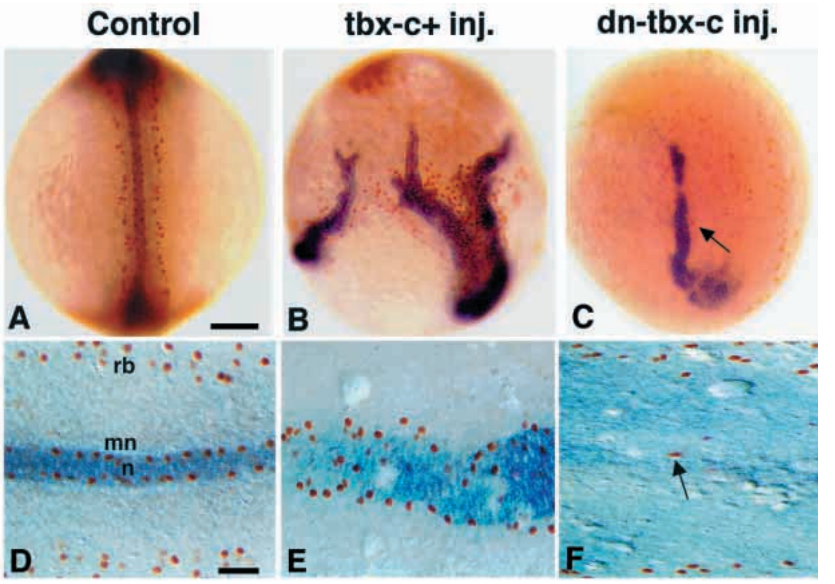

Fig. 5. Double localization of the *ntl* and the Islet-1 reveals concurrent changes in the notochord and MNs of *tbx-c+* and *dn-tbx-c* embryos at 11 hpf. The notochord is detected by *ntl* expression (blue) and neurons by the expression of Islet-1 (brown). (A,D) Control embryos featuring two bilateral lines of MNs along the midline and Rohon-Beard cells along the lateral neural plate. (B) Multiple notochords and extranumerary Islet-1-positive neurons in an embryo injected with a high dose of *tbx-c+* mRNA. (C) Reduced notochord and a decreased number of MNs (arrow) in the *dn-tbx-c* embryo. (E) Extranumerary MNs with an expanded notochord in the *tbx-c+* embryos. (F) The *dn-tbx-c* embryo showing a reduced number of MNs (arrow) and undetectable notochord. (A-C) Whole-mount embryos; (D-F) dorsal view of flat-mount embryos. mn, motor neurons; n, notochord; rb, Rohon-Beard cells. Scale bars, 100 μm (A-C); 25 μm (D-F).

Regulation of Tbx-c influences differentiation of primary motor neurons.

Sonic hedgehog (Shh), a secreted protein of the hedgehog family (Hh), is expressed in the midline precursor cells during gastulation and subsequently in the differentiated notochord and floor plate (Krauss et al., 1993). *shh* appears to induce differentiation of a subset of neurons in the ventral neural tube (Krauss et al., 1993; Roelink et al., 1994; Ekker et al., 1995; Hammerschmidt et al., 1996). Early specification of primary MNs takes place before formation of the differentiated notochord and has been suggested to depend on Hh signaling from midline precursor cells (Appel et al., 1995; Hammerschmidt et al., 1996; Beattie et al., 1997). These reports led us to speculate that the overproduction of MNs observed in *tbx-c+* embryos could be due to increased expression of Hh caused by expansion of the midline. In *dn-tbx-c* embryos, synthesis of Hh might be reduced as a result of attenuation of midline structures, thereby resulting in a reduced number of MNs. Therefore, it is suggested that the effect of Tbx-c on development of MNs can be mediated via Hh signaling. This is interesting in connection with recent observations that *Drosophila omb* and chick *Tbx2* are regulated by Hh (Kopp and Duncan, 1997; Gibson-Brown et al., 1996).

Alternatively, reduced expression of *tbx6* in *tbx-c+* embryos could also be the causative factor for the formation of extranumerary MNs since a mutation in the mouse *Tbx6* induces ectopic neural tissue including MNs, at the expense of somitic tissue (Chapman and Papaioannou, 1998). However, further analysis is required to determine the possible molecular mechanisms and involvment of Tbx-c in MNs development.

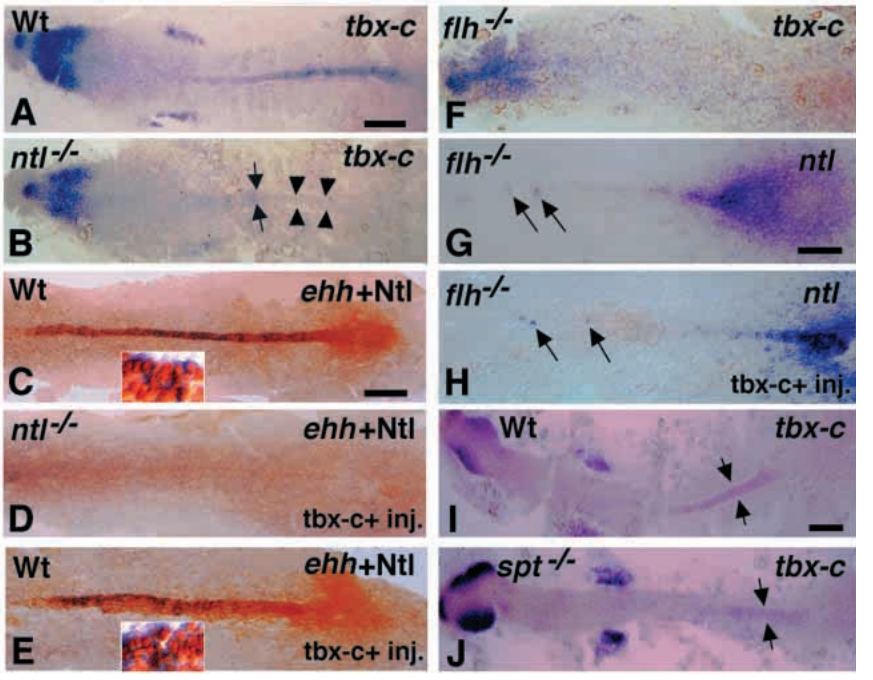

Fig. 6. Analysis of *tbx-c* expression and effect of ectopic expression of *tbx-c+* in mutants. **(**A,B) Expression of *tbx-c* in wild-type (A) and $ntl^{-/-}$ mutant (B) embryos. Note the segregation of axial mesoderm (arrowheads) and a low level expression of *tbx-c* in the midline at the trunk of the mutant (arrows). (C-E) Co-localization of *ehh* (blue) and Ntl (brown) in wild-type (C), *tbx-c+* injected $ntl^{-/-}$ (D) and normal sibling(E) embryos obtained by crossing heterozygous $ntl^{-/+}$ pair. Expression of both *ehh* and Ntl is expanded in the *tbx-c+* injected wild-type sibling embryo (E) and absent in the *tbx-c+* injected $ntl^{-/-}$ mutant embryo (D). (F) *tbx-c* expression in the $flh^{-/-}$ embryo. The notochord is absent and *tbx-c* is undetectable in the midline region of $flh^{-/-}$ embryo. (G,H) *ntl* expression in the $flh^{-/-}$ embryo (G) and the $flh^{-/-}$ embryo injected with *tbx-c+* (H). A few *ntl*-positive cells (arrows) can be seen in the midline of both group of embryos. (I,J) *tbx-c* expression in wild-type (I) and $spt^{-/-}$ embryos (J) at 14 hpf. Arrows indicate the *tbx-c* expression in the notochord. Insert (C,E): high magnification to show co-expression of *ehh* and Ntl in the notochord. Scale bars, 100 μm.

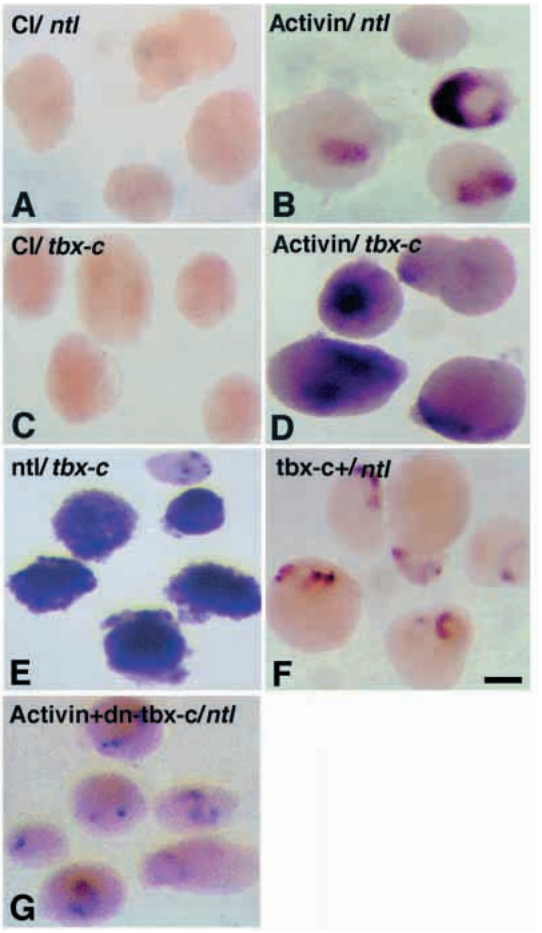

Fig. 7. (A-G) Analysis of expression of *ntl* and *tbx-c* in animal cap explants. Expression of *ntl* (A,C) and *tbx-c* was not detected in the control caps dissected from the uninjected embryos. Injection of *activin* mRNA induces expression of *ntl* (B) and *tbx-c* (D). Injection of *tbxc+* induces *ntl* expression (E) and injection of *ntl* induces *tbx-c* expression (F). *ntl* is induced in caps after coinjection of activin and *dn-tbx-c* mRNA (G). Scale bar, 100 μm.

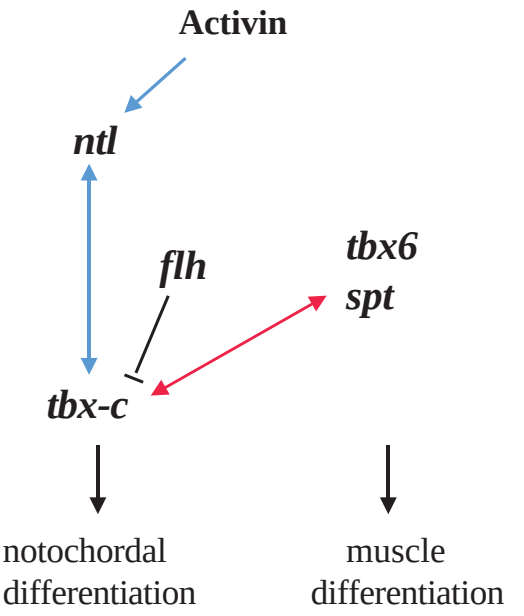

Fig. 8. A model illustrating possible genetic interactions during differentiation of cells in notochordal and muscle lineages. *ntl*, *flh* and *tbx-c* are consecutively expressed in the dorsal midline mesoderm and induce notochordal differentiation during embryogenesis. *ntl* acts synergistically with other factors to induce notochordal differentiation (see Discussion). *flh* and *tbx-c* antagonize the influence of *tbx6/spt* expressed in lateral mesoderm to promote notochordal fates in dorsal mesoderm. Blue arrow indicates positive regulation; red arrow indicates negative regulation.

A regulatory feedback loop involving Tbx-c is required for specification of midline structures

Our finding of an autoregulatory loop between *ntl* and *tbx-c* adds a new element to the understanding of mechanism of notochordal specification in zebrafish. The early mesodermal induction triggered by maternally expressed TGF-β and FGFs, activates early function of T-box genes such as *VegT* and *Bra* (Smith et al., 1991; Zhang et al., 1998). *ntl/Bra* interacts with eFGF in a positive regulatory feedback loop (Isaacs et al., 1994; Schulte-Merker and Smith, 1995) and acts synergistically with HNF-3β-related proteins (O'Reilly et al., 1995) to induce development of dorsal mesoderm. It seems that further development of the notochord requires stage-specific regulators including *flh* and *tbx-c*. Flh and Tbx-c act to equilibrate the antagonistic influence of determinants of the lateral mesoderm such as *tbx6/spt* in order to specify the identity of the notochordal cells (Hug et al., 1997; Chapman and Papaioannou, 1998; Amacher and Kimmel, 1998; Griffin et al., 1998). Similar antagonistic interactions could exist between specific regulators of the notochord and floor plate as well as the notochord and hypochord.

In this paper, we have described three novel genes that belong to the subfamily of *omb*-related zebrafish T-box genes. The expression pattern and functional analysis of *tbx-c* suggests that Tbx-c acts immediately before formation of the notochord and may be involved in the process of notochordal differentiation.

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Note added in proof

During preparation of this manuscript a partial sequence of the zebrafish *tbx-3* gene has been published (Yonei-Tamura et al., 1999). Within 188aa of the incomplete T-domain of *zf-tbx3* reported, we found only one aa mismatch comparing it to the corresponding region of the Tbx3-related *tbx-b* described here. This fact and similarity of expression of these two clones in the differentiated notochord suggests that the *zf-tbx3* and our *tbx-b* may represent independent isolates of the same gene.

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