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Identification of direct T-box target genes in the developing zebrafish mesoderm

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The zebrafish genes *spadetail* (*spt*) and *no tail* (*ntl*) encode T-box transcription factors that are important for early mesoderm development. Although much has been done to characterize these genes, the identity and location of target regulatory elements remain largely unknown. Here, we survey the genome for downstream target genes of the Spt and Ntl T-box transcription factors. We find evidence for extensive additive interactions towards gene activation and limited evidence for combinatorial and antagonistic interactions between the two factors. Using in vitro binding selection assays to define Spt- and Ntl-binding motifs, we searched for target regulatory sequence via a combination of binding motif searches and comparative genomics. We identified regulatory elements for *tbx6* and *deltaD*, and, using chromatin immunoprecipitation, in vitro DNA binding assays and transgenic methods, we provide evidence that both are directly regulated by T-box transcription factors. We also find that *deltaD* is directly activated by T-box factors in the tail bud, where it has been implicated in starting the segmentation clock, suggesting that *spt* and *ntl* act upstream of this process.

KEY WORDS: Enhancer prediction, Gene regulation, No tail/brachyury, Spadetail/tbx16

INTRODUCTION

The T-box genes encode a large family of transcription factors with about 20 members in most vertebrate species and are important for many developmental processes, including limb, heart and craniofacial development (Naiche et al., 2005). In addition, T-box genes play an integral role during vertebrate gastrulation, highlighted first in the mouse by the identification of the *T/Brachyury* gene (Dobrovolskaïa-Zavadskaïa, 1927), the first T-box gene to be molecularly identified (Herrmann et al., 1990). Since the cloning of *Brachyury*, orthologs have been found in *Xenopus* (Smith et al., 1991), zebrafish [*ntl* (Schulte-Merker et al., 1992) and *brachyury* (Martin and Kimelman, 2008)], *Ciona intestinalis* (Corbo et al., 1997), chick (Kispert et al., 1995) and many others (Naiche et al., 2005), including species as distant from vertebrates as hydra (Technau and Bode, 1999), suggesting that *Brachyury* is an ancient gene. In vertebrates, *Brachyury* homologs are expressed throughout the presumptive mesoderm during gastrulation and later in the tail bud and notochord (Kispert et al., 1995; Schulte-Merker et al., 1992; Smith et al., 1991; Wilkinson et al., 1990). Loss of *Brachyury* function causes a range of deficiencies in posterior mesoderm and notochord (Herrmann et al., 1990; Halpern et al., 1993; Schulte-Merker et al., 1994; Conlon et al., 1996; Martin and Kimelman, 2008).

Members of the Tbx6/16 subfamily of T-box factors also play important roles in mesoderm formation. In mouse, *Tbx6* is expressed in the presumptive mesoderm during gastrulation and in the tail bud and presomitic mesoderm during segmentation (Chapman et al., 1996). Strikingly, the paraxial mesoderm develops as neural tissue in *Tbx6* mutants (Chapman and Papaioannou, 1998). Zebrafish contain paralogs of mouse *Tbx6*, including *spadetail* (*spt*, *tbx16*), *tbx6* and *tbx24*, that are expressed in the presumptive non-axial mesoderm (Ruvinsky et al., 1998; Griffin et al., 1998; Hug et al., 1997; Nikaido et al., 2002). Zebrafish *spt* mutants have severe defects in trunk paraxial mesoderm formation as a result of incorrect cell migration and specification (Kimmel et al., 1989; Ho and Kane, 1990; Amacher and Kimmel, 1998).

Although *spt* and *ntl* mutant phenotypes have been well characterized and some Spt/Ntl targets have been identified (Yamamoto et al., 1998; Goering et al., 2003; Gourronc et al., 2007), identification of Spt- and Ntl-responsive regulatory elements is necessary to fully understand how these genes regulate mesoderm development. First, the identification of gene regulatory regions reveals which genes are direct targets of the factors and how they fit into the regulatory hierarchy of mesoderm patterning. Second, regulatory element identification may uncover features, such as binding site number and/or orientation of co-regulator binding sites, that are required for maximal response. The importance of T-box factor co-regulators is highlighted by the finding that Xbra directly interacts with the transcription factor Smad1, which is crucial for the activation of some targets in *Xenopus* (Messenger et al., 2005).

We have designed a moderate throughput method for identifying and characterizing Spt- and Ntl-responsive regulatory elements. First, we identified putative downstream targets by measuring genome-wide transcription in Spt- and Ntl-deficient embryos. We performed in vitro binding selection assays to generate binding site models to search non-coding sequence around putative targets for binding motif clusters, and prioritized these clusters using comparisons to other fish genomes. We demonstrate that Spt and Ntl bind two putative regulatory regions in vivo and in vitro, and have tested both regions for transcriptional activity using a transgenic reporter assay. Our results place Spt and Ntl directly upstream of *tbx6*, a T-box gene involved in posterior mesoderm specification (Hug et al., 1997).

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In addition, we show that T-box factors directly regulate *deltaD* (*dld*) in the posterior tail bud. As *dld* has been suggested to prime the segmentation clock (Mara et al., 2007), we suggest that Spt and Ntl play an important role in initiating cyclic gene expression in the presomitic mesoderm. Finally, the T-box sites that we defined within the *tbx6* regulatory region cluster with functional Tcf/Lef transcription factor binding sites (Szeto and Kimelman, 2004), suggesting that Spt and Ntl are required together with Wnt signaling to regulate some targets. Several other putative target genes we have identified are known targets of vertebrate Wnt signaling, including the mouse *dld* homolog *Delta-like 1* (Hofman et al., 2004), suggesting that an interaction between T-box genes and Wnt signaling in gastrula mesoderm is widespread.

MATERIALS AND METHODS

Zebrafish stocks and husbandry

Adult fish strains were kept at 28.5°C on a 14-hour light/10-hour dark cycle. Embryos were obtained from natural crosses or in vitro fertilizations and staged as previously described (Kimmel et al., 1995).

Morpholino injections

The morpholino (MO) combination needed to phenocopy *spt*$^{-}$;*ntl*$^{-}$ mutant embryos was determined by injecting translation-blocking MOs targeting either *spt* or *ntl* into the complementary single mutant. Injections were performed as described by Nasevicius and Ekker (Nasevicius and Ekker, 2000). A combination of four MOs reliably phenocopied *spt*$^{-}$;*ntl*$^{-}$ mutants (see Fig. S1 in the supplementary material).

mRNA isolation and microarray hybridization

RNA was collected at 75% epiboly from embryos injected with MOs against *spt* and/or *ntl* or control embryos injected with Danieau solution plus 0.25% Phenol Red. Samples were prepared and hybridized as previously described (Ouyang et al., 2008). Array probes consisted of the Compugen/Sigma-Genosys zebrafish oligo library (GEO platform accession number GPL7343). Three biological replicates were performed for each treatment and genes with significantly different expression levels in MO-injected embryos versus controls were identified using a Student-Newman-Keuls post hoc test.

Gene expression time course profiling

Gene expression profiles were downloaded from http://giscompute.gis.a-star.edu.sg/~govind/zebrafish/data_download.html (Mathavan et al., 2005) and visualized using MapleTree (http://rana.lbl.gov/EisenSoftware.htm). We normalized expression levels by setting the point of maximum expression for each gene to 1.

Recombinant Spt-GST and Ntl-GST protein preparation and in vitro DNA binding assays

spt- and *ntl*-GST fusion constructs were created in the vector pGEX-5X-1. Proteins were purified basically as described by Yagi et al. (Yagi et al., 2004). SELEX experiments were performed as described previously (Senger et al., 2004) and details can be provided on request. After 3-4 rounds of selection bound oligomers were subcloned and sequenced. Binding competition assays with genomic sequences (Fig. 5) were carried out as described previously (Chan et al., 2003).

Binding site score matrix construction and genome searches

Using Spt and Ntl SELEX data and previously identified binding sites for Tcf/Lef and Suppressor of Hairless (see Fig. S6 in the supplementary material), the program PATSER (Hertz and Stormo, 1999) was used to generate binding site score matrices. Sequences matching a score matrix with a *P*-value of ≤0.001 were counted as binding sites. We obtained gene models from the RefSeq database (Pruitt et al., 2005) and genomic positions from the UCSC genome browser (Kent et al., 2002; Karolchik et al., 2008) (Sanger Institute, 2004: ftp://ftp.sanger.ac.uk/pub/sequences/zebrafish). UCSC genome browser-annotated repetitive sequences (Smit et al., 2007; Jurka, 2000) were not included in genome searches.

Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitation on 75-85% epiboly zebrafish embryos was performed as previously described (Wardle et al., 2006; Morley et al., 2009) using anti-Ntl antibody (Schulte-Merker et al., 1992) or anti-Spt antibody (Amacher et al., 2002). The microarrays used contain probes ranging from 9 kb upstream and 3 kb downstream of transcription start sites. ChIP PCR was carried out using standard techniques (Morley et al., 2009).

Reporter plasmid construction

The I-*Sce*I-based plasmids mCherry::pXex:GFP and the Tol2-based mCherry::pXex:GFP construct were based on the previously described GFP::pXex:BFP construct (Szeto and Kimelman, 2004) (details can be provided on request).

Regulatory element fragments were amplified from genomic or plasmid DNA with high fidelity polymerase and inserted into reporter constructs using standard molecular biology techniques. The *dld* Tol2 construct was made using Gateway cloning procedures and plasmids from Tol2 Gateway kits (Villefranc et al., 2007; Kwan et al., 2007). Site-specific mutagenesis was carried out using PCR-based methods. Primer sequences can be provided on request.

Generation of transgenic zebrafish

Reporter constructs were injected as previously described for I-*Sce*I-based transgenesis (Thermes et al., 2002) and Tol2-based transgenesis (Kawakami, 2004; Kwan et al., 2007). Transiently transgenic embryos were grown to the appropriate stage and those with GFP expression at the blastoderm margin or in the tail bud and somites were classified as expressing mCherry in these regions robustly, faintly or not at all. To generate stable transgenic lines, germline transgenic founders were identified by crossing to wild-type fish. Embryos from these crosses were raised and their offspring analyzed.

In situ hybridization, photography and mounting

Whole-mount in situ hybridization was performed as described (Thisse et al., 1993) with modifications (Melby et al., 1997). Digoxigenin-labeled RNA probes were synthesized from templates as cited in Fig. 1. Embryos were cleared and mounted as described previously (Griffin et al., 1998).

RESULTS

Expression analysis identifies *spt* and *ntl* target genes

Mutational analyses have shown that whereas *spt* and *ntl* have distinct functions (Kimmel et al., 1989; Halpern et al., 1993) they also have partially redundant roles (Amacher et al., 2002). We measured genome-wide gene expression in *spt* and/or *ntl*-deficient embryos to identify their putative downstream targets, focusing on downregulated genes as Spt and Ntl homologs appear to act mainly as transcriptional activators (Horb and Thomsen, 1997; Conlon et al., 1996). We depleted embryos of Spt and Ntl activity using antisense morpholinos (MOs) targeting *spt* and *ntl* transcripts (see Fig. S1 in the supplementary material). To identify genes most likely to be direct targets, we measured transcript levels at mid-gastrulation [75% epiboly, 8 hours post-fertilization (hpf)], soon after *spt* and *ntl* expression normally begins and before mutant phenotypes become morphologically apparent. Genes with significantly different expression levels in depleted embryos (compared with control embryos) that were downregulated ≥2-fold were considered further. The list of 44 hits (representing 41 candidate target genes) is shown in Fig. 1A. Microarray results are available from the Gene Expression Omnibus (Accession Number GSE12857).

Downregulated genes include several known Spt and/or Ntl targets, such as *protocadherin 8* [a *spt* target (Yamamoto et al., 1998)], *no tail dependent gene 5* [a *ntl* target (Goering et al., 2003)], *mesogenin1* [a target of both *spt* and *ntl* (Goering et al., 2003)], and *tbx6* [which is down-regulated in *spt*$^{-}$ embryos and not expressed in *spt*$^{-}$;*ntl*$^{-}$ embryos (Griffin et al., 1998)] (Fig. 1A). Some

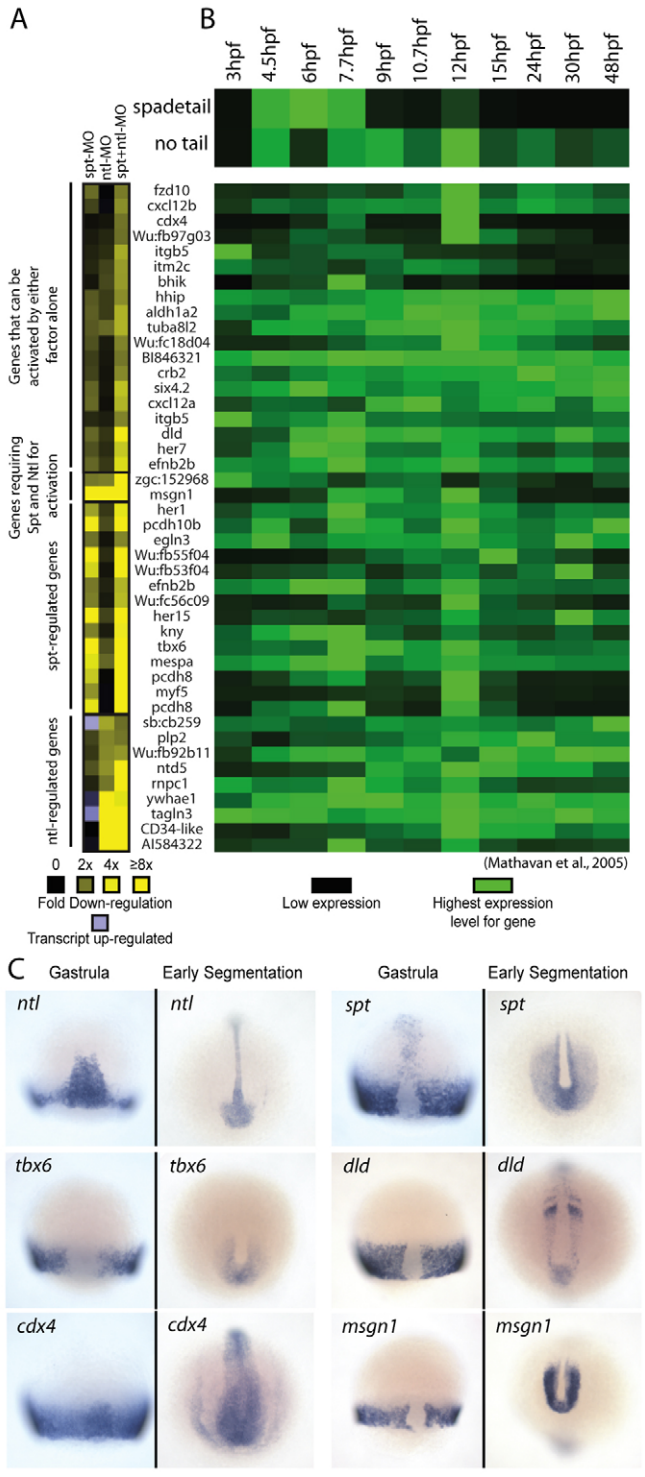


Fig. 1. Microarray analysis identifies candidate *spt* and *ntl* target genes. (**A**) Gene expression levels in MO-injected embryos relative to wild-type embryos at 75% epiboly. Downregulation is shown in shades of yellow (2 to >8-fold) and upregulation (2 to 3-fold) is shown in light purple. Only genes with a two-fold or greater decrease in expression level are shown. (**B**) *spadetail* (*spt*) and *no tail (ntl*) gene expression profiles between 3 and 48 hpf are shown (top) with the expression profiles of putative targets below. These gene expression profiles were retrieved from another dataset (Mathavan et al., 2005). The expression level for each gene was normalized to its highest expression level over the time course. (**C**) mRNA expression of *spt*, *ntl* and four targets at midgastrula (75% epiboly, 8 hpf) and early segmentation (11-13 hpf). These patterns have been previously reported: *ntl* (Schulte-Merker et al., 1992), *spt* (Griffin et al., 1998), *tbx6* (Hug et al., 1997), *dld* (Haddon et al., 1998; Hans and Campos-Ortega, 2002), *cdx4* (Joly et al., 1992) and *msgn1* (Yoo et al., 2003).

downregulated genes have known mutant phenotypes: for example, *cdx4* mutants have severe posterior mesoderm truncations (Davidson et al., 2003); mesogenin 1 (*Msgn1*) mutant mice fail to properly specify trunk and tail mesoderm (Yoon and Wold, 2000); *dld*, *her1* and *her7* mutants have mesodermal segmentation defects (Holley et al., 2000; Henry et al., 2002); and *knypek* mutants have convergence and extension defects (Topczewski et al., 2001). These phenotypes are reminiscent of *spt* and *ntl* mutant phenotypes, and thus consistent with the genes being targets of T-box factors.

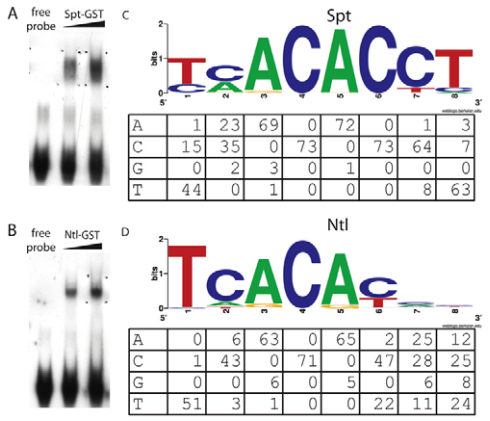
Of the >16,000 genes on the microarray: 14 are downregulated in the absence of *spt*, but not *ntl*, activity; nine are downregulated in the absence of *ntl*, but not *spt*, activity; 19 are downregulated when both factors are absent; and two are downregulated when either gene product is absent. The distinctions among categories are somewhat arbitrary because in some cases gene expression is reduced, but not below the level of our cut-off. For example, *tbx6* expression decreases 5-fold in *spt*-deficient embryos, 1.7-fold in *ntl*-deficient embryos, and 25-fold in *spt;ntl*-deficient embryos; it is labeled a *spt* target by our criteria, even though *ntl* also contributes to its activation.

spt and *ntl* target genes are spatially and temporally co-expressed with *spt* and *ntl*

We presumed that direct *spt* and *ntl* target genes would be expressed in spatial and temporal patterns overlapping with those of *spt* and *ntl*. To examine temporal overlap, we used an existing microarray dataset (Mathavan et al., 2005) that reports gene expression levels for >16,000 genes at 12 early developmental time points (0-48 hpf) using the same Compugen probe set as our experiments. We visualized the normalized expression profiles of *spt/ntl* target genes from our microarray, as well as the profiles for *spt* and *ntl*, using the program MapleTree (Fig. 1B). Although the target genes have a variety of expression profiles, they are generally expressed at similar times as *spt* and *ntl*. The spatial expression patterns of many target genes listed in Fig. 1A have been previously determined and, for most of them, expression partially overlaps with *spt* and/or *ntl* (Thisse et al., 2001) (see Fig. S2 in the supplementary material). Most are expressed in the non-axial gastrula margin and later in the tail bud, whereas surprisingly few are expressed in the axial midline. Typical target gene expression patterns, including those for the genes whose regulation we characterized further, are shown in Fig. 1C. The apparent lack of more targets (such as *ntd5*) expressed in the axial midline may be due to the function of a second *ntl*-like gene, *brachyury*, which can functionally substitute for *ntl* in anterior mesoderm (Martin and Kimelman, 2008).

Spt and Ntl bind similar sequences in vitro

Many regulatory elements have been characterized by identifying large genomic regions that drive appropriate reporter expression, followed by deletion and mutational analyses to determine crucial regulatory sequences. To identify Spt- and Ntl-responsive regulatory elements, we took a more streamlined and targeted



A	1	23	69	0	72	0	1	3
C	15	35	0	73	0	73	64	7
G	0	2	3	0	1	0	0	0
T	44	0	1	0	0	0	8	63

A	0	6	63	0	65	2	25	12
C	1	43	0	71	0	47	28	25
G	0	0	6	0	5	0	6	8
T	51	3	1	0	0	22	11	24

Fig. 2. Spt and Ntl bind similar sites in vitro. (**A**,**B**) Representative gel shifts using recombinant Spt-GST (A) and Ntl-GST (B). (**C**,**D**) WebLogo (Crooks et al., 2004) binding site models based on the sequences of >70 bound oligos from the in vitro selection assays are shown for Spt-GST (C) and Ntl-GST (D) with the count matrices for the sequenced binding sites below. Regions of binding sites contained in the non-random primer region of the oligonucleotide were not counted, therefore some columns have fewer total counts.

approach by searching for Spt- and Ntl-binding motif enrichment in genomic sequence around target genes. We first generated binding site models using in vitro binding selection assays (SELEX assays) (Fig. 2A,B). We performed gel mobility shift assays with random double-stranded oligonucleotides and GST-tagged Spt and Ntl, and amplified bound DNA using PCR (Gold et al., 1995). The binding selection/amplification cycle was performed four times and bound oligomers from round four were sequenced. Limited sequencing revealed that the Spt-bound oligomer pool from round four consisted almost entirely of near exact matches to previously identified consensus T-box binding sites (Kispert and Herrmann, 1993). As Spt-responsive regulatory elements probably contain both high- and low-affinity sites, we also sequenced oligomers from the third SELEX round and used these to generate binding site models. More than 70 bound oligonucleotides were sequenced for each protein and are represented by WebLogo diagrams (Crooks et al., 2004) (Fig. 2C,D). The Spt model has the consensus sequence TCACACCT and the Ntl model has the consensus sequence TCACACC(C/T), both of which are similar to sequences bound by T-box factors Brachyury, VegT, Eomesodermin and Tbx6 from other species (Kispert and Herrmann, 1993; Conlon et al., 2001; White and Chapman, 2005). This experiment also revealed that Spt, but rarely Ntl, can bind oligomers with a C at position 1; we confirmed this using binding assays with oligomers differing only at the first position (see Fig. S3 in the supplementary material).

Genome searches for *spt*/*ntl*-responsive regulatory elements

To identify putative T-box regulated regulatory elements, we searched genomic sequence within and around 23 putative Spt/Ntl target genes (those that could be assigned to an mRNA in the RefSeq database and aligned to the zebrafish genome) for conserved clusters of Spt- and Ntl-binding sites. We used the program PATSER (Hertz and Stormo, 1999) to construct scoring matrices based upon our SELEX data and background base frequencies derived from zebrafish intergenic sequence (36% G-C; 64% A-T). Using a 500 bp sliding window, we searched 5 kb upstream of potential target genes and within intronic sequence for motifs significantly matching the Spt- or Ntl-binding site models (P-value ≤0.001) and ranked each 500 bp region based upon the number of matches. Similar binding site cluster searches have proven effective in identifying *Drosophila* regulatory elements (Berman et al., 2002; Markstein et al., 2002). The regions with the greatest numbers of Spt and Ntl motifs in our dataset are shown in Table S1 in the supplementary material. In *Drosophila*, conservation of binding site clusters among species is a good indicator of regulatory activity, even when the primary sequence of the regulatory elements is divergent (Berman et al., 2004). To determine whether T-box site clusters were conserved, and thus more likely to identify Spt/Ntl-responsive regulatory elements, we aligned the genomic loci listed in Table S1 with the orthologous loci from *Fugu* (Aparicio et al., 2002), *Tetraodon* (Jaillon et al., 2004), *Gasterosteus* (Broad Institute, 2007: http://www.broad.mit.edu/models/stickleback/) and *Medaka* (Kasahara et al., 2007) obtained from the ENSEMBL database (Flicek et al., 2008), and assessed the number of Spt/Ntl motifs in the regions aligning with the 500 bp zebrafish T-box binding site clusters (an example from the *dld* locus is shown in Fig. S4 in the supplementary material).

We chose two putative regulatory regions for detailed analysis: the *dld* second intron and the upstream region of *tbx6*. The *dld* region (+911 bp to +1316) shows conservation among all four species for which sequence is available. In addition, previous work showed that a *dld* region between +1 and +2800 bp can drive gene expression in the gastrula margin and in the tail bud during segmentation, although regulatory motifs have not been identified (Hans and Campos-Ortega, 2002). Because we recognize that sequences from other fish species will not always be available for approaches such as ours, we also chose to examine a candidate region from the *tbx6* gene that contains six putative T-box binding sites within 500 bp of the transcription start site for which orthologous sequence was unavailable for comparison (Flicek et al., 2008). Additionally, this *tbx6* region is known to drive Wnt- and BMP-dependent gene expression in the forming mesoderm (Szeto and Kimelman, 2004). Thus, although several regions identified in our searches are promising candidates for regulation by T-box factors, we selected regions near *dld* and *tbx6* for further analysis for the above reasons and because of the predicted high affinity of the T-box binding motifs they contain.

Spt and Ntl bind predicted regulatory elements in vivo and in vitro

To determine whether Spt and Ntl bind *tbx6* and *dld* genomic regions containing conserved binding site clusters in the embryo, we performed chromatin immunoprecipitation (ChIP) at 75-85% epiboly using Spt and Ntl antibodies (Figs 3 and 4). Ntl-bound regions were detected using a promoter microarray containing probes corresponding to sequences up to 9 kb upstream and 3 kb downstream of transcription start sites (Fig. 3) and Spt-bound regions were detected using promoter-specific qPCR (Fig. 4). The conserved T-box binding site cluster upstream of *tbx6* is enriched 5-fold in the Ntl-bound sample (Fig. 3A,B). Although our promoter microarray does not include probes corresponding to the *dld* intron 2 T-box site cluster, a probe corresponding to the intron 2-exon 3 junction of *dld* is 2.3-fold enriched in Ntl-bound DNA (Fig. 3C,D). Using promoter-specific qPCR (Morley et al., 2009), we found that the *tbx6* upstream region and *dld* second intron T-box clusters are enriched 7.9±1.8 fold and

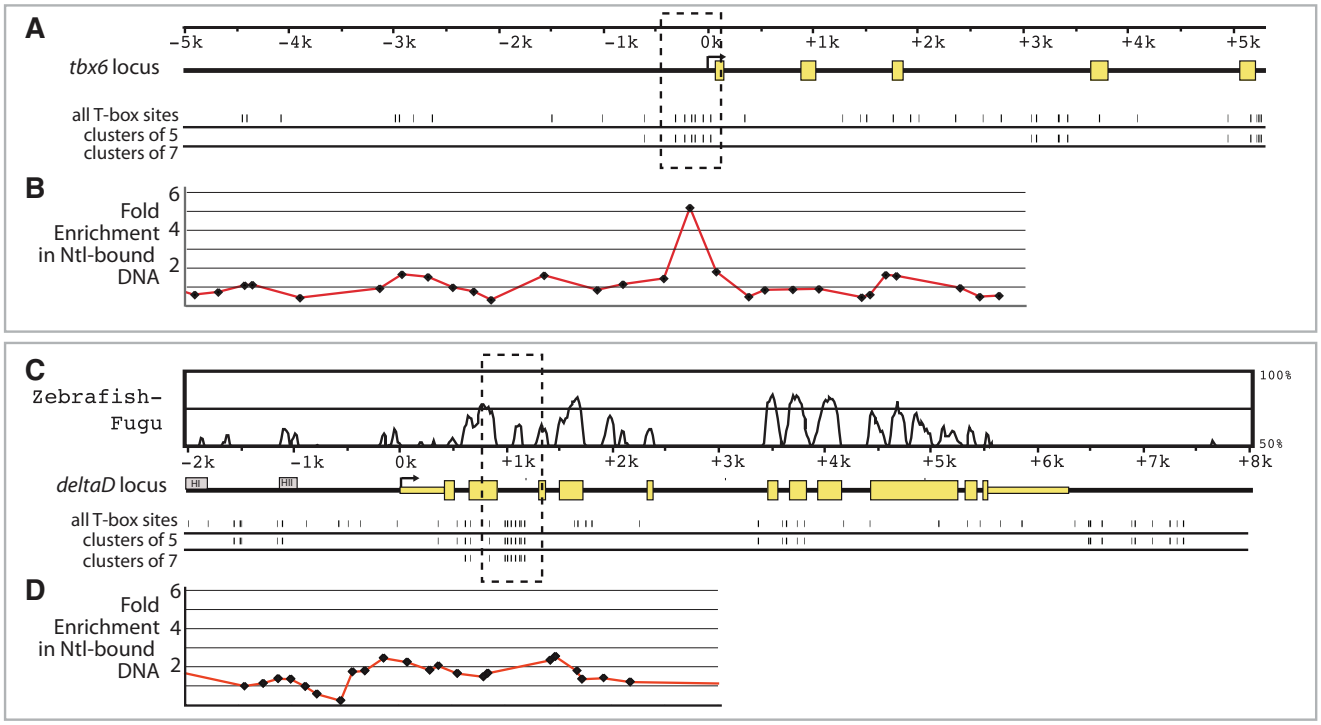


Fig. 3. Ntl binds clusters of T-box sites near *tbx6* and *deltaD* in vivo. (**A**,**C**) The genomic loci of *tbx6* (A) and *dld* (C) are schematized with sequence identity between the orthologous zebrafish and *Fugu* sequences plotted above the gene schematics using VISTA (Mayor et al., 2000; Frazer et al., 2004) (the *Fugu tbx6* ortholog has not yet been sequenced). Matches to the Spt and/or Ntl binding site models with a PATSER *P*-value ≤0.001 are depicted as hatch marks below the gene schematics, with all T-box sites shown first, followed by those that belong to a cluster of 5 and 7 sites within 500 bp of one another. Previously identified *dld* neural regulatory elements HI and HII (Hans and Campos-Ortega, 2002) are shown in grey in C. (**B**,**D**) Chromatin immunoprecipitation was carried out using an antibody against Ntl. Fold enrichment in the Ntl-immunoprecipitated samples is shown. Error bars are not assigned to the Ntl-ChIP data since it represents the median of ratios.

4.3±0.95 fold, respectively, in Spt-bound DNA relative to untreated DNA (Fig. 4A). Additional details for the ChIP experiments can be provided on request. The data indicate that Spt and Ntl bind both candidate regulatory regions in vivo.

To determine whether Spt and Ntl can bind the predicted regulatory regions in vitro, we tested whether a mixture of Spt-GST and Ntl-GST could bind the relevant *tbx6* (Fig. 5A,B) and *dld* (Fig. 5D,E) genomic fragments in gel mobility shift assays. A combination of equal volumes of bacterially produced Spt-GST and Ntl-GST bind the regions in vitro (compare lanes 1 and 2 in Fig. 5C,F). Because each radiolabeled genomic fragment contains multiple T-box binding sites, shifted fragments are probably bound by different numbers of transcription factor molecules and thus migrate more diffusely than when bound to a single T-box site (e.g. Fig. 2); however, most of the probe migrates as a higher mobility complex. To determine whether T-box sites are required for factor binding to the *tbx6* and *dld* regions, we performed an in vitro binding competition assay. Probe binding is eliminated with increasing concentrations of unlabeled wild-type competitor DNA (Fig. 5C,F, lanes 3-5). However, a competitor in which positions 4 and 5 in T-box motifs are changed from 'CA' to 'AT' (merely 8/256 bp changes for *tbx6* and 6/140 bp for *dld*) fails to compete efficiently (Fig. 5C,F, lanes 6-8), indicating that intact T-box sites are required for binding. Taken together, our in vivo and in vitro binding assays demonstrate that Spt and Ntl bind regions within the *tbx6* and *dld* genes that contain T-box binding sites and that these binding sites are required for this interaction. It will be interesting in the future to determine the specificity of Spt and Ntl for particular T-box binding motifs within each region.

Table 1. Embryo counts for transient transgenesis assays

	mCherry expression level (75% epiboly)				mCherry expression level (14 somites)			
Construct	Bright (%)	Faint (%)	None (%)	Number scored (*n*)	Bright (%)	Faint (%)	None (%)	Number scored (*n*)
tbx6:mCherry	93	5	2	110	96	4	0	110
tbx6:mCherry+spt and ntl MOs	0	6	94	47				
tbx6:mCherry mut a	0	87	13	24	89	11	0	38
tbx6:mCherry mut b	22	65	13	60	98	0	2	57
tbx6:mCherry mut c	0	87	13	63	98	0	2	65
tbx6:mCherry mut d	17	40	43	52	85	10	4	50
tbx6:mCherry mut a-d	0	0	100	79	1	15	84	105
–1.2 to +1.3 kb dld:mCherry	98	0	2	88	98	0	2	81
–1.2 to +1.3 kb dld:mCherry mut c-e	21	65	14	109	11	41	48	56

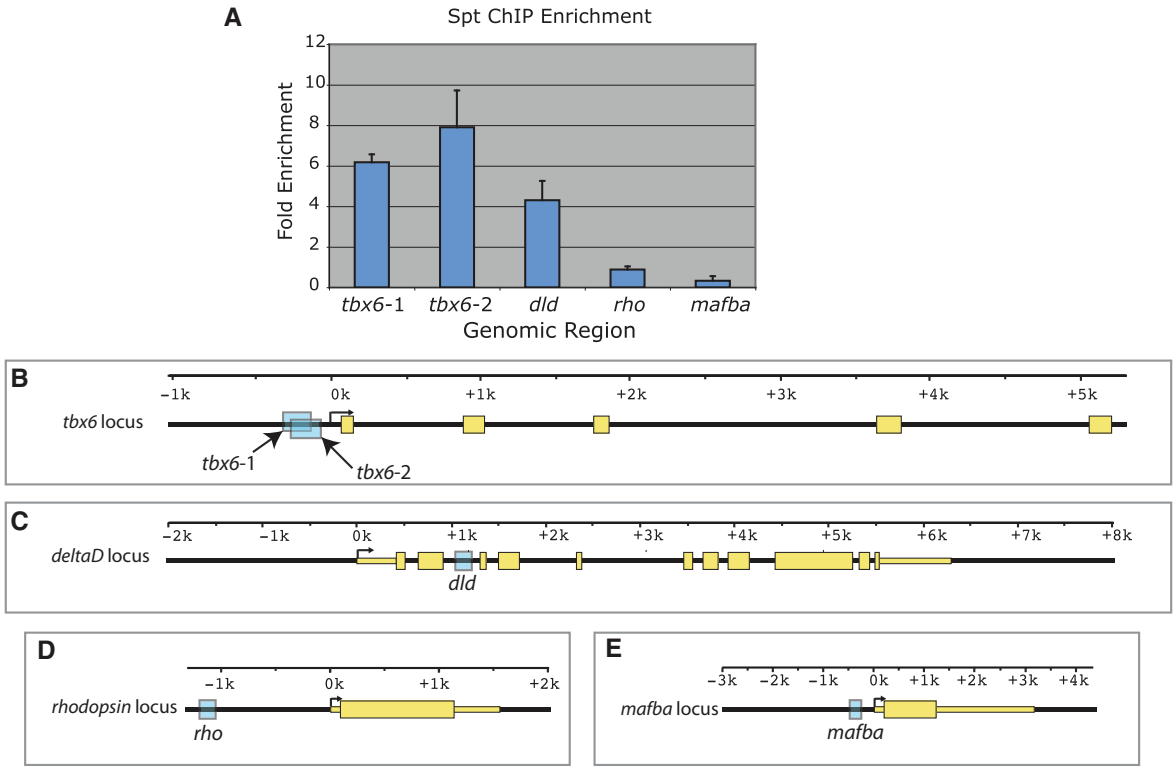


Fig. 4. Spt binds clusters of T-box sites near *tbx6* and *deltaD* in vivo. We used qPCR to measure the enrichment of the genomic sequences highlighted in blue in B-E in DNA precipitated using a Spt antibody. (**A**,**B**) Regions of the T-box motif cluster upstream of *tbx6* (B) are 7.9±1.8 and 6.2±0.39-fold enriched in Spt-bound DNA (A). A region of the intronic T-box motif cluster in the *dld* second intron (**C**) is enriched 4.3±0.95 fold in Spt-bound DNA (A). As a negative control, we determined the enrichment level of parts of the *rhodopsin* (*rho*) (**D**) and *v-maf musculoaponeurotic fibrossarcoma oncogne protein b* (*mafba*) (**E**) upstream regions in Spt-bound DNA using qPCR and saw no enrichment in Spt-bound DNA (A).

T-box binding sites are required for *tbx6* expression

tbx6 contains six Spt/Ntl binding motifs within 500 bp of the transcription start site (Fig. 3A; Fig. 6A), within a region previously shown to drive Wnt- and BMP-dependent gene expression during gastrulation and segmentation stages (Szeto and Kimelman, 2004). Two Tcf/Lef-binding sites were shown to be crucial for the Wnt responsiveness of this regulatory element (Szeto and Kimelman, 2004) and their positions are shown (Fig. 7B). To test the ability of the *tbx6* upstream region to drive gene expression in vivo, we built a dual fluorescence reporter construct based on one designed by Szeto and Kimelman (Szeto and Kimelman, 2004) that allows one to assess the activity and tissue specificity of a test regulatory region in transient transgenic assays compared with that of a ubiquitously expressed reporter on the same plasmid. The construct contains *tbx6* upstream sequence (–1.7 kb to the start codon) driving mCherry (Shaner et al., 2004) expression and a ubiquitous *pXex* promoter driving GFP expression to mark cells that received the plasmid (Fig. 6A). We used I-*Sce*I-mediated transgenesis (Thermes et al., 2002) to introduce the constructs into zebrafish embryos and analyzed GFP and mCherry expression in the injected embryos between mid-gastrulation and early segmentation stages. We observed that mCherry expression was restricted to the blastoderm margin during gastrulation (Fig. 6B; Fig. 7C, compare with Fig. 1C) and later to the tail bud and somites (Fig. 7D), as previously reported (Szeto and Kimelman, 2004). As previously reported (Szeto and Kimelman, 2004), we also observed that the fluorescent reporter was more stable than endogenous *tbx6* transcripts (Fig. 1C); thus, fluorescence perdures in presomitic cells as they incorporate into somites and become muscle cells. Reporter expression is detected in a majority of embryos at shield stage (6 hpf) (see Fig. S5A in the supplementary material), when endogenous *tbx6* is initially expressed (Hug et al., 1997), suggesting the regulatory element is involved in initiation of *tbx6* expression. Double in situ hybridization of reporter construct-injected embryos reveals that mCherry expression overlaps extensively with endogenous *tbx6* expression (see Fig. S5B,C in the supplementary material). Similar to the endogenous *tbx6* gene, mCherry expression is eliminated when *spt* and *ntl* MOs are injected with the reporter construct (Fig. 6B-G; see Fig. S5A broken versus unbroken line).

To assess whether T-box binding sites contribute to regulatory element activity, we mutated each of the four most proximal T-box sites independently by changing positions 4 and 5 of the binding site from ‘CA’ to ‘AT’ (Fig. 7E,H,K,N) and tested each modified regulatory element in transient expression assays. Each single T-box mutant construct drove weak mCherry expression at the gastrula margin relative to the wild-type construct (Fig. 7F,I,L,O); however, at segmentation stages, expression was indistinguishable from that driven by the wild-type construct (Fig. 7G,J,M,P). When all four T-box sites were mutated in the context of the full-length regulatory element (Fig. 7Q), reporter expression was completely lost (Fig. 7R,S), demonstrating that 2-4 T-box sites are crucial for *tbx6* expression in the forming mesoderm and consistent with our in vitro binding data showing that mutation of T-box sites a-d eliminates Spt and Ntl binding (Fig. 5C). Although both Spt and Ntl bind this region in vivo (Fig. 3B; Fig. 4A), Spt appears to play a greater role in activating *tbx6* than Ntl (Fig. 1A; Fig. 6D-G) (Griffin et al., 1998). Consistent with this, T-box sites a, b and c

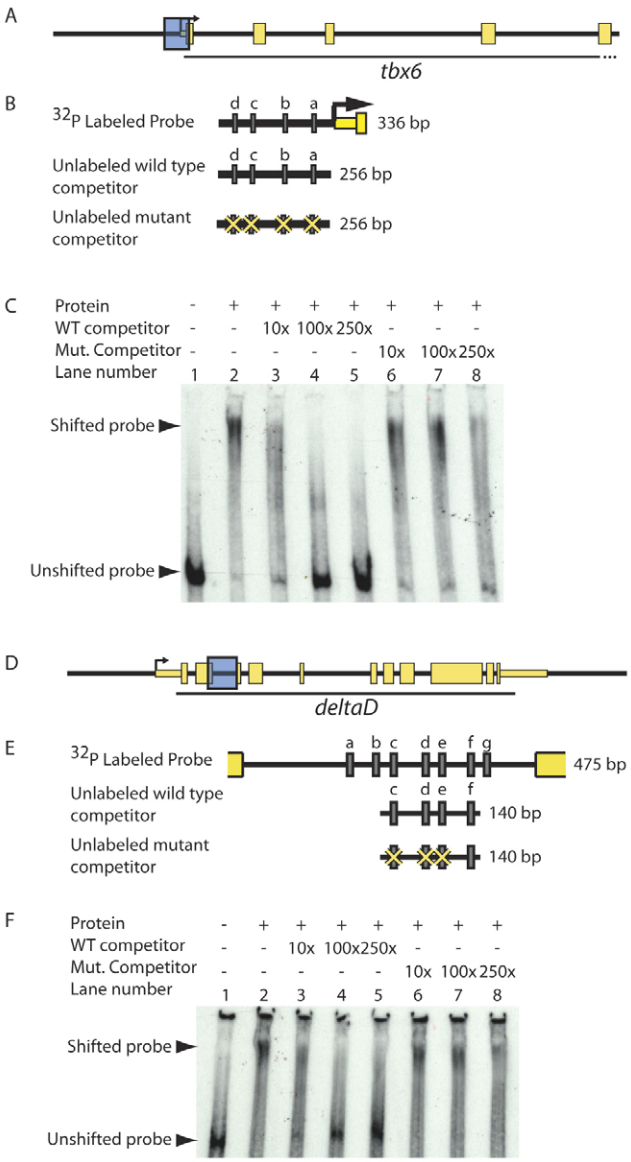


Fig. 5. T-box proteins bind putative regulatory sequences in vitro in a T-box binding site-dependent manner. (**A-F**) To test whether Spt and Ntl bind putative regulatory sequences in vitro, we performed gel shifts using equal volumes of recombinant Spt-GST and Ntl-GST, and radiolabeled DNA corresponding to putative *tbx6* and *dld* genomic regulatory sequences (shown as blue boxes in A and D and magnified in B and E to indicate Spt/Ntl binding motifs a-d and a-g, respectively, as grey rectangles). Spt and Ntl bind a 336 bp region containing four T-box sites just upstream of *tbx6* (C; lane 1 versus lane 2). Addition of 10-250× excess unlabeled competitor competes well for binding and virtually eliminates DNA-protein complex formation (C; lanes 3-5); however, excess unlabeled competitor containing mutated T-box sites competes poorly (C; lanes 6-8). Similarly, a mixture of equal volumes of Spt-GST and Ntl-GST binds a 475 bp fragment from the *dld* second intron containing seven putative T-box sites (F, lane 1 versus 2). Unlabeled competitor containing four of the T-box sites competes for binding (F; lanes 3-5), but fails to compete when three of the T-box sites are mutated (F, lanes 6-8).

have PATSER *P*-values <0.001 using the Spt matrix, but not the Ntl matrix, suggesting that Spt has a higher affinity for these sites. Site d has a similar predicted affinity for both factors.

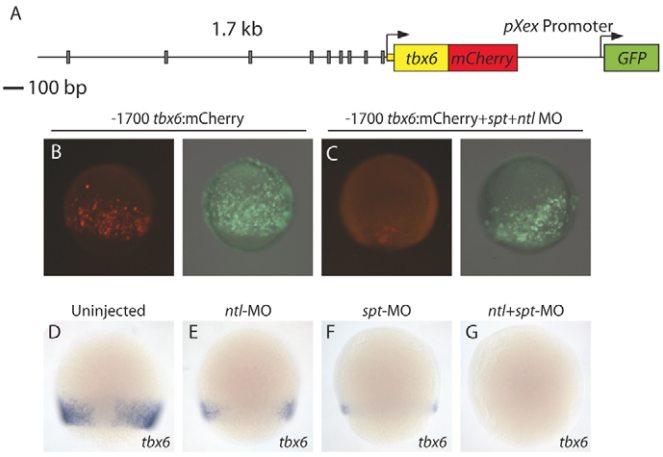


Fig. 6. The *tbx6* regulatory element drives mesodermal gene expression and requires Spt and Ntl for activity. The 1.7 kb region upstream of *tbx6* is shown with gray rectangles representing predicted Spt- and/or Ntl-binding site motifs (**A**). This region drives reporter expression at the margin at mid-gastrulation (**B**), but not if embryos are co-injected with *spt* and *ntl* MOs (**C**) (mCherry, red; GFP, green). (**D-G**) Endogenous *tbx6* expression is decreased in *spt*- (F) or *ntl*-depleted (E) embryos when compared with expression in wild-type embryos (D). *tbx6* expression is undetectable when both *ntl* and *spt* are depleted (G).

The early mesodermal *deltaD* regulatory element is directly regulated by T-box factors

We identified seven Spt/Ntl binding site motifs in the 405 bp *dld* second intron in a region of high sequence identity with *Fugu* (Fig. 3C). Importantly, other fish species also have multiple T-box sites in this region (Fig. 8A; see Table S1 in the supplementary material). The *dld* second intron from most fish species also contains putative binding sites for other relevant factors, including Tcf/Lef and Suppressor of Hairless (Fig. 8A). Previous *dld* regulatory analysis identified two upstream regulatory elements (labeled 'HI' and 'HII' in Fig. 3C) that drive neural expression in zebrafish embryos (Hans and Campos-Ortega, 2002). The same study found that the region between the *dld* transcription start site and +2.8 kb (including intron 2) contains regulatory information for driving mesodermal expression, but did not further define the sequences required. We tested activity of a *dld* reporter construct containing the 1.2 kb upstream sequence, the first two exons and introns and partial exon 3 sequence, fused in frame to mCherry (Fig. 8C) in transient transgenic embryos. This region drove mCherry expression in the blastoderm margin during gastrulation and in the tailbud during segmentation (Fig. 8E,F) and reporter expression overlaps well with endogenous *dld* expression (see Fig. S5D,E in the supplementary material).

To determine whether T-box sites in the second *dld* intron are required for activity, we mutated binding motifs in the context of the full-length regulatory element. The *dld* second intron from each fish species contains an assortment of Spt/Ntl binding motifs and two of these (sites c and d) are conserved in orientation and some flanking sequence (Fig. 8B). These two sites are followed closely by a consensus T-box binding site (site e) in zebrafish, so we mutated sites c, d and e. When these sites were mutated (Fig. 8G), mCherry expression substantially decreased (compare Fig. 8H,I with Fig. 8E,F). The remaining regulatory activity could be mediated by any of the four remaining T-box sites or other regulatory sequences contained in the construct.

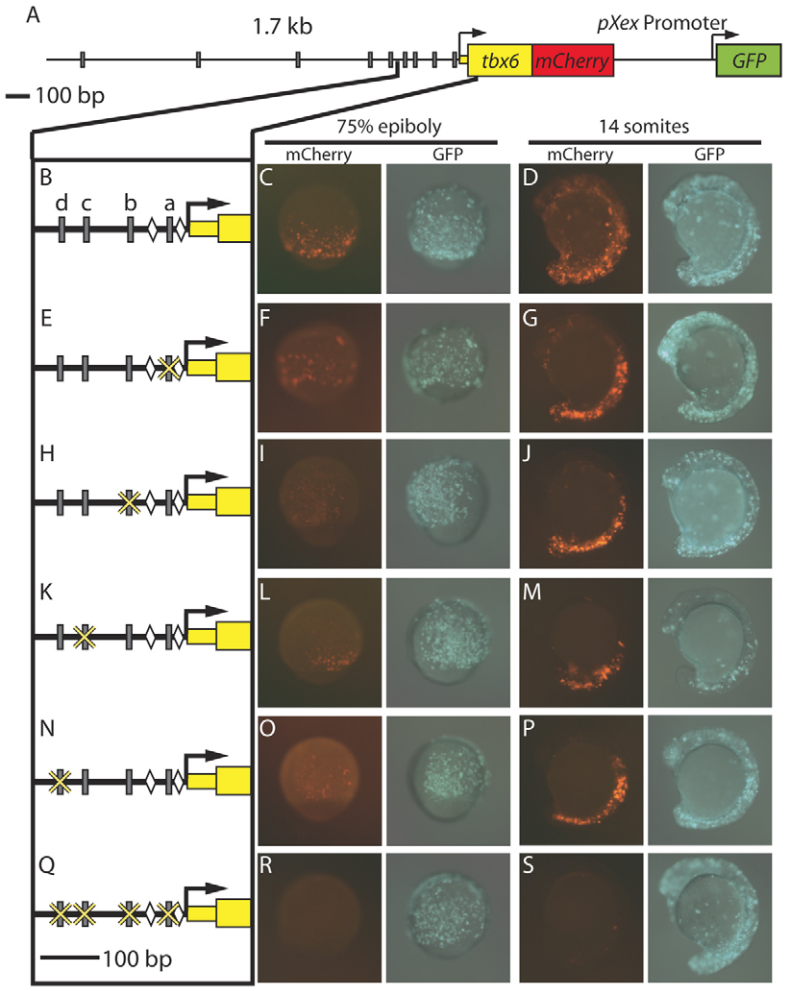


Fig. 7. T-box sites are required for activity of the *tbx6* mesodermal regulatory region. (**A**) The 1.7kb region upstream of *tbx6* is shown with gray rectangles representing predicted Spt- and/or Ntl-binding site motifs. (**B**) A detailed view of 300 bp upstream of the *tbx6* transcription start site [gray rectangles indicate putative T-box sites (a-d) and diamonds indicate Tcf/Lef-binding sites important for regulatory element function (Szeto and Kimelman, 2004)]. (**C**,**D**) mCherry expression driven by the full-length construct is robust at mid-gastrulation (8 hpf) (C) and mid-segmentation (14 somites) (D). (**E-P**) During gastrulation, mCherry expression driven by constructs in which one T-box site has been mutated (E,H,K,N) is reduced (F,I,L,O), but is normal during segmentation (G,J,M,P). (**Q-S**) When all four sites are mutated (Q) expression is abolished at both stages (R,S). The percentages of embryos with robust, faint or no mCherry expression are given in Table 1.

To further characterize the expression pattern driven by the full-length *dld* construct and facilitate functional analyses, we generated stable lines (Fig. 9). In both lines analyzed, we observed mCherry expression in a subset of locations where *dld* is normally expressed (Fig. 9B-I). Specifically, we observed mCherry transcripts in regions where *spt* and *ntl* are strongly co-expressed: at the blastoderm margin (Fig. 9E-G) and later in the posterior tail bud (Fig. 9C,D,H,I). mCherry was also faintly expressed in neural tissue (Fig. 9H), which was expected as the construct contains an regulatory element that drives expression in the hindbrain and other neural tissues (Hans and Campos-Ortega, 2002). The stable lines express mCherry at the margin by 30% epiboly (Fig. 9E), the earliest time at which *dld* is expressed (Hans and Campos-Ortega, 2002), suggesting the regulatory element initiates expression of *dld*.

To test whether *dld* regulatory element activity requires *spt* and *ntl* function, we analyzed reporter expression in transgenic embryos injected with *spt* and *ntl* MOs. Control and *ntl*-depleted embryos showed no difference in expression level (Fig. 9J,K). *spt*-depleted embryos exhibited decreased reporter expression (Fig. 9L) and embryos depleted of both Spt and Ntl fail to express mCherry (Fig. 9M). These results demonstrate that the *dld* regulatory element requires *spt* function for full activity, but that *ntl* also contributes to regulatory element activity. Endogenous *dld* responds similarly to treatment with these MOs (Fig. 1A; Fig. 9N-Q).

DISCUSSION

The use of T-box site clustering and conservation in regulatory element detection

Here, we show that T-box responsive regulatory elements can be found by searching for conserved clusters of T-box sites near target genes. Because we concentrated our search to introns and 5 kb upstream sequence and because we did not characterize regions with only a few T-box sites, we probably missed a subset of Spt/Ntl-responsive regulatory elements. Even with these possible limitations, we identified two mesodermal regulatory elements using our search criteria and provide evidence that both are directly regulated by T-box factors.

The use of comparative genomics to identify zebrafish regulatory sequence has been successfully implemented in the past (Dickmeis et al., 2004; Yang et al., 2007; Allende et al., 2006). One difficulty with this approach is that the positions of regulatory elements and of transcription factor binding sites within them often change over evolution, making them difficult to identify through alignments, particularly across large evolutionary distances (Sanges et al., 2006; Moses et al., 2006). Our method avoids some of these difficulties. First, we compared zebrafish sequence to that of closely related species, increasing the likelihood that the regulatory elements will be conserved. Second, we evaluated the conservation of a sequence based on the presence of transcription factor binding sites, allowing for some flexibility in the positions of the regulatory element regions and the binding sites they contain.

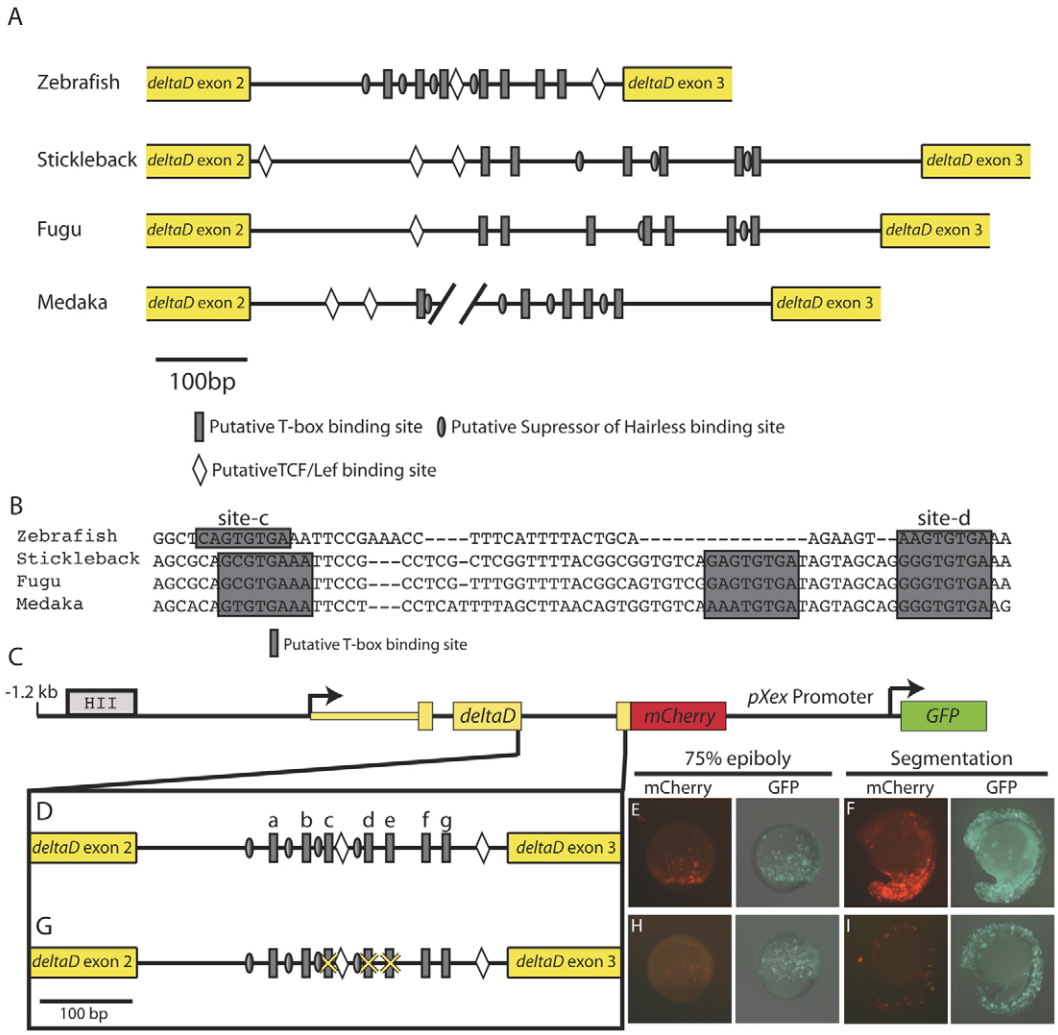


Fig. 8. The second intron of *deltaD* contains a conserved cluster of T-box sites required for T-box-dependent mesodermal expression. (**A**) The second intron from fish *dld* orthologs (there is an unsequenced gap in the Medaka intron). Grey rectangles represent T-box binding motifs, diamonds indicate putative Tcf/Lef sites and grey ovals represent putative Suppressor of Hairless-binding sites. (**B**) An alignment of the second intron of fish *dld* orthologs reveals two particularly well-conserved Spt/Ntl-binding motifs (sites c and d). (**C-F**) A construct containing 1.2 kb upstream and 1.3 kb downstream sequences relative to the *dld* transcription start site, including the known neural regulatory element (HII) (Hans and Campos-Ortega, 2002) and the T-box site cluster (C,D) drives robust mCherry expression in the non-axial margin during gastrulation (E) and in the tail bud and somites during segmentation (F). (**G-I**) When two well-conserved T-box sites (c,d) and a T-box consensus sequence (e) are mutated (G), mCherry expression is greatly reduced (H,I). The percentages of embryos with robust, faint or no mCherry expression is given in Table 1.

Regulatory interactions among T-box factors in mesoderm development

Detailed characterization of *spt*$^{-}$, *ntl*$^{-}$ and *spt*$^{-}$;*ntl*$^{-}$ embryos reveals that *spt* and *ntl* function in a partially redundant manner (Amacher et al., 2002). Other experiments suggest *spt*, *ntl* and *tbx6* interact in three ways: (1) two factors can activate target genes that neither factor can activate on its own (combinatorial interactions); (2) two factors can contribute to activation additively; and (3) one factor can prevent activation by another factor (competitive antagonism) (Goering et al., 2003). Our microarray-based approach indicates that additive and redundant interactions between Spt and Ntl are the most prevalent as many target genes have moderately decreased expression levels when one of the two T-box genes is depleted, but a greater decrease when both are depleted (Fig. 1A). Our data indicate combinatorial interactions are less common, as shown by the fact that only two targets had a greater than twofold decrease in expression level in both single knockdowns. The distinction between additive and combinatorial interactions is somewhat arbitrary as we define categories based on discrete cutoffs. Two genes were downregulated in *ntl*-depleted embryos, but upregulated in *spt*-depleted embryos. These targets may represent examples of competitive antagonism of Ntl function by Spt. One of the two genes, sb:cb259, encodes a transforming acidic coiled-coil-containing protein that is expressed strongly in the axial mesoderm and very weakly in the non-axial margin during gastrulation (Thisse et al., 2001), suggesting that Spt may repress expression in the non-axial margin. The other gene (*transgelin3*) is not spatially restricted during gastrula stages (Thisse et al., 2001) (see Fig. S2 in the supplementary material). Microarray experiments with other treatments (including *tbx6* and

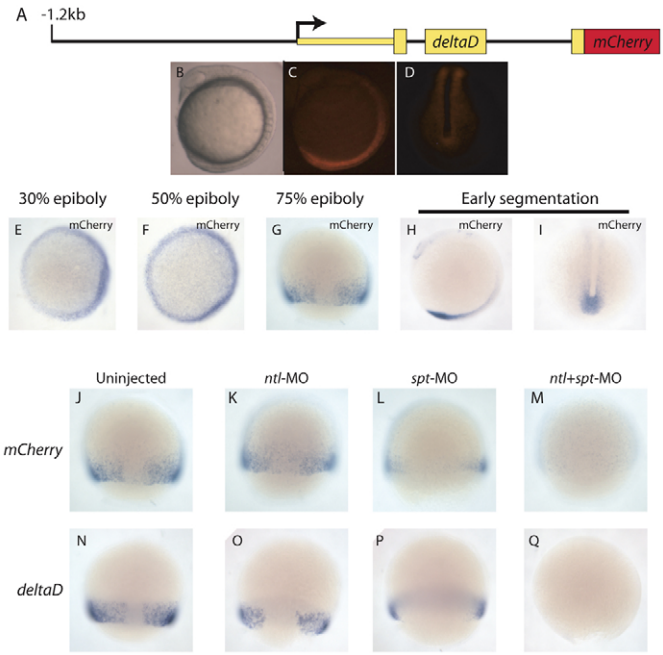


Fig. 9. The mesodermal *deltaD* regulatory element requires *spt* and *ntl* function. (**A-I**) Stable transgenics carrying the *dld* reporter construct (A) express mCherry at the blastoderm margin during mid-gastrulation (G), and in the tail bud and hindbrain (C,D,H,I). Transgene expression begins by 30% epiboly (E,F; animal pole views). (**J-M**) Stable *dld* transgenics were injected with MOs targeting *ntl*, *spt* or both transcripts, and analyzed by whole-mount in situ hybridization for mCherry. Most uninjected control (J) and Ntl-depleted embryos (K) expressed mCherry [76% (38/50) and 72% (26/36), respectively]. Only 67% (22/33) of Spt-depleted embryos expressed mCherry, and only 9% (3/33) did so at a level comparable with that of uninjected controls (L). No *spt*+*ntl* MO-injected embryos (0/54) expressed mCherry (M). (**N-Q**) In situ hybridization revealed that endogenous *dld* responds to Spt and Ntl depletion in a similar manner to the transgene.

bra depletion, and *spt*, *ntl*, *bra* and *tbx6* overexpression), as well as other functional experiments, will allow the interactions among T-box genes to be more fully characterized.

Interactions between T-box genes and Wnt signaling during posterior mesoderm development

Interference with Wnt signaling in the mesoderm increases the severity of *spt* and *ntl* phenotypes, suggesting that *spt*, *ntl* and Wnt signaling function in concert to generate trunk and tail mesoderm (Lekven et al., 2001). Consistent with this, we find several known Wnt target genes among the T-box targets identified in our microarray screen, including *tbx6* (Szeto and Kimelman, 2004), *cdx4* (Shimizu et al., 2005), *mesogenin1* (Wittler et al., 2007), *myf5* (Shi et al., 2002) and *aldh1a2* (Weidinger et al., 2005). During gastrulation, *wnt8a* and *ntl* positively regulate one another (Ueno et al., 2007; Goering et al., 2003; Martin and Kimelman, 2008), which may explain part of the overlap in Wnt/T-box target genes. However, we show that *tbx6* is directly regulated by T-box factors, which, taken in together with previous results demonstrating its direct regulation by Tcf/Lef factors (Szeto and Kimelman, 2004), indicates that, in some cases, the requirement for Wnt signaling and T-box genes is a result of direct activation by both players. The *dld* regulatory element we characterized also contains Tcf/Lef-binding motifs and further analysis will reveal if Wnt signaling contributes to activity. Several recent studies in mouse indicate that T-box and Tcf/Lef transcription factor binding are important for gene activation in the forming mesoderm, including expression of Delta homologs (Dunty et al., 2008; Wittler et al., 2007; Hofmann et al., 2004). This requirement could result from a direct physical interaction between Tcf/Lef and T-box factors, as *Xenopus* VegT (a close Spt homolog) can physically interact with Tcf3 in vitro and because protein domains of VegT and Tcf3 that are crucial for physical interaction include their well-conserved DNA-binding domains (Cao et al., 2007). Two T-box binding sites in the zebrafish *tbx6* regulatory element are close to Tcf/Lef binding sites (8 bp and 28 bp away), and the mouse *Dll1* regulatory element also contains T-box sites within 20 bp of Tcf/Lef binding sites (Hofman et al., 2004). This close clustering of binding sites could result in a physical interaction between the proteins.

Distinct T-box responsive regulatory elements drive *delta* expression in the tail bud of mouse and zebrafish

Previous studies have shown that *Dll1* expression in mouse is driven in the tail bud by a combination of Tbx6 and Tcf/Lef factors acting through an upstream element (Beckers et al., 2000; Hoffman et al., 2004; White and Chapman, 2005). By contrast, *dld* expression in the zebrafish tail bud is driven by an element downstream the transcription start site (Figs 8 and 9) (Hans and Campos-Ortega, 2002). We show the zebrafish *dld* second intron contains a tail bud regulatory element that is bound by Spt and Ntl, and requires the proteins for activation (Figs 3, 4, 8 and 9). Thus, *Dll1* and *dld* are both directly regulated by T-box factors, but through regulatory elements in different locations. Further experiments will determine whether Wnt signaling activates the *dld* tail bud regulatory element, as it does the *Dll1* regulatory element in mouse. It could be that these two elements are controlled by the same set of transcription factors and are functionally very similar, but located in different parts of the gene. This type of regulatory element shuffling may be common in vertebrate evolution (Sanges et al., 2006). Alternatively, it could be that the two regulatory elements are regulated by different sets of factors and the regulatory network has changed over evolution, but the *Delta* expression pattern in the tail bud has remained consistent.

dld is a component of the segmentation clock in the posterior tail bud, and has been described as a factor required to ‘prime’ cyclic gene expression in the presomitic mesoderm (Mara et al., 2007). Because T-box factors directly regulate *dld* expression in this posterior zone, *spt* and *ntl* act upstream of the priming process. Several other target genes identified in our microarray study are components of the segmentation clock, including the cyclic genes *her1*, *her7* and *her15.1* (Henry et al., 2002; Gajewski et al., 2003; Oates and Ho, 2002; Shankaran et al., 2007). Consistent with previous studies, recent work has shown that cyclic gene expression starts early in gastrulation (Riedel-Kruse et al., 2007). Our results implicate Spt and Ntl in initiating cyclic gene expression.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/5/749/DC1

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