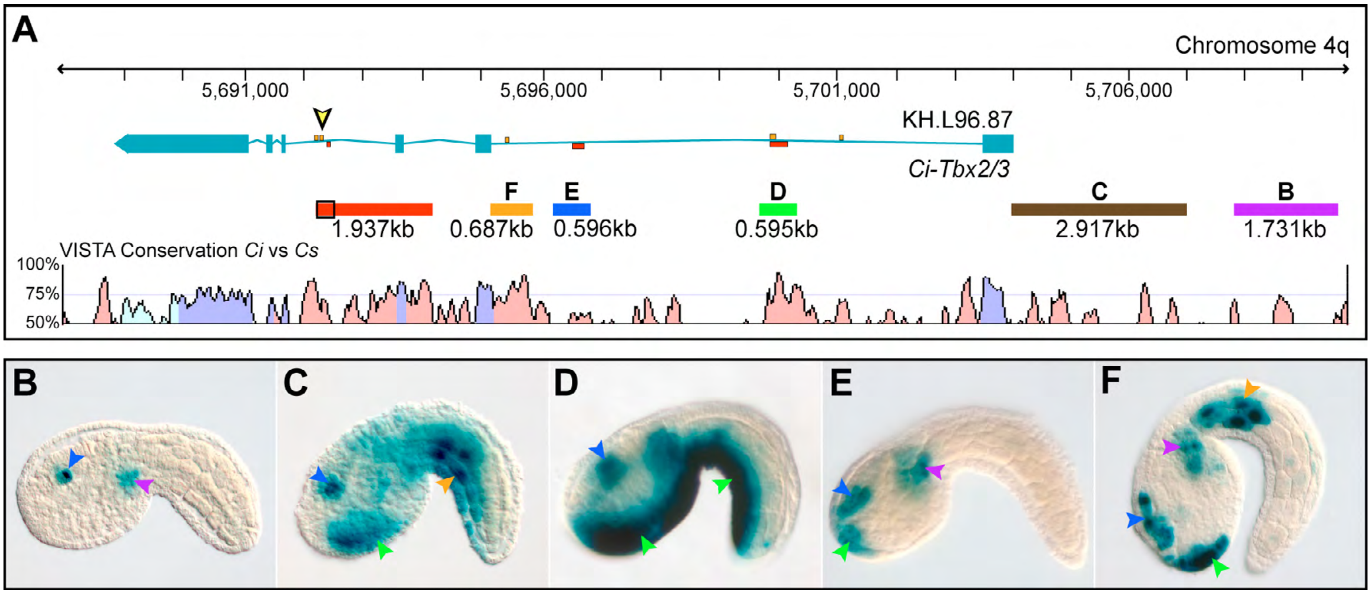


Fig. S1. Identification of CRMs with activity in *Ci-Tbx2/3* expression domains outside the notochord. (**A**) Top: map of the *Ci-Tbx2/3* locus. Teal rectangles and lines denote exons and introns, respectively. Areas with at least threefold enrichment for Ci-Bra and Ci-Tbx6b binding, ascertained from ChIP-chip data (Kubo et al., 2010), are shown as small red and orange boxes, respectively, within the *Ci-Tbx2/3* locus. The locations of the various CRMs tested are indicated by large boxes of different colors. The letters above each box indicate the panels showing representative transgenic embryos carrying that CRM (B-F). The 1937 kb notochord CRM (Fig. 2) is shown in red for reference; the minimal notochord enhancer is outlined in black and this area of the locus is further highlighted with a yellow arrowhead. Bottom: VISTA alignment illustrating the sequence conservation across the *Ci-Tbx2/3* locus between *Ciona intestinalis* (*Ci*) and *Ciona savignyi* (*Cs*), obtained using the following parameters: calculation window, 100 bp; minimum conservation width, 100 bp; conservation identity, 50%. Conserved coding regions are depicted as blue peaks, conserved portions of the 5′- or 3′-UTR are shown in aqua, whereas the conserved non-coding regions are illustrated as pink peaks. (**B-F**) X-Gal staining of *Ciona intestinalis* mid-tailbud stage embryos electroporated with the *Ci-Tbx2/3* CRMs indicated in A with staining in discrete regions of the sensory vesicle (B-F), ventral trunk epidermis (C,D) and palps (E,F). Expression domains are highlighted with arrowheads colored as follows: orange, muscle; blue, CNS; green, epidermis; purple, mesenchyme.

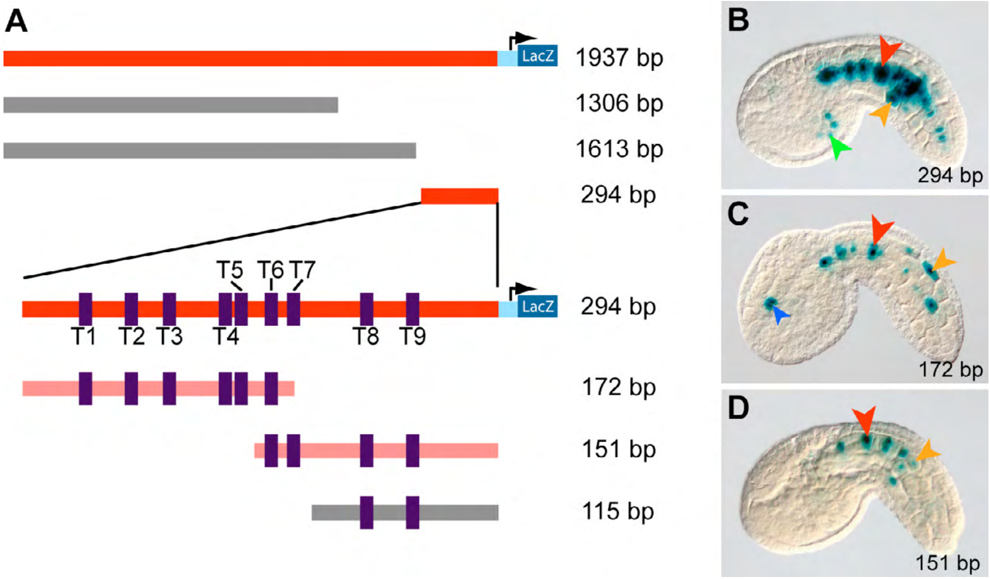


Fig. S2. Constructs used to define the *Ci-Tbx2/3* notochord CRM. (**A**) Schematic representation of the constructs tested to characterize the sequences controlling the activity of the *Ci-Tbx2/3* notochord CRM. Red and gray boxes represent fragments exhibiting or lacking notochord expression of the *lacZ* reporter, respectively. Light-red boxes depict constructs with weakened notochord staining. T-box binding sites are illustrated as purple rectangles. (**B-D**) Microphotographs of representative X-Gal stained embryos harboring select constructs from A.

A

```
Ci   GGGGAATGGGGATTACGGTAGTCGACGAAAAATAATTGTTTCGGTTTAGTGCTATGTATT
              ||||||          || |||||||||||||   ||     |  ||||||
Cs   CAAAGCAACGGATTAT---------CGGAAAATAATTGTTTTTCTT-----CGTTGTATT

Ci   ACTCGACCACCAAAGATGTGAAAGCCACGCAGTTGATCTAGCGTTAGATTTCGCCCCTTC
     ||     |||||   | |||| | || |||||||||| | |||| | | ||||| | |||
Cs   AC-----CACCACCAACGTGATACCCGCGCAGTTGATTTGGCGTCATAATTCGCGCGTTC
       T7           T6                  T5
Ci   GTGTTATTTGTTCTTGTCACATTGCTTATAGATTAACACTTACGTGTCAGAGAATAAAC-
     |||| |||||| ||  || |     |    || ||||||| | ||| ||  ||| |  |
Cs   GTGTCATTTGTCCTGATCGCGCAATTGGGCGAGTAACACTGAAGTGACAACGAAAAGCCA

Ci   ----CGTTTTATTTTATAACTAGTCCCACTATACGAATAAACACAAGTGTCATAAAGTAC
         | || || ||| |   |   |   || |||||| ||  || | | |  | ||
Cs   TCAGCTTTATAATTTCTCGTTTAGCGTTCTCTACGAA-AATTACCATTTTATTTAAACCG

Ci   AATACTGTGC-TGGGTGGCAAATGAACTTTGTAACGTATTTAACTACCCTGTCTGTCCAC
     |  |   ||| | |||  ||              | |  ||| | |  | || |||
Cs   ACCATAATGCATAGGTAACA--------------CTTTCTTAGCAAAACAGTGTGTT---
```

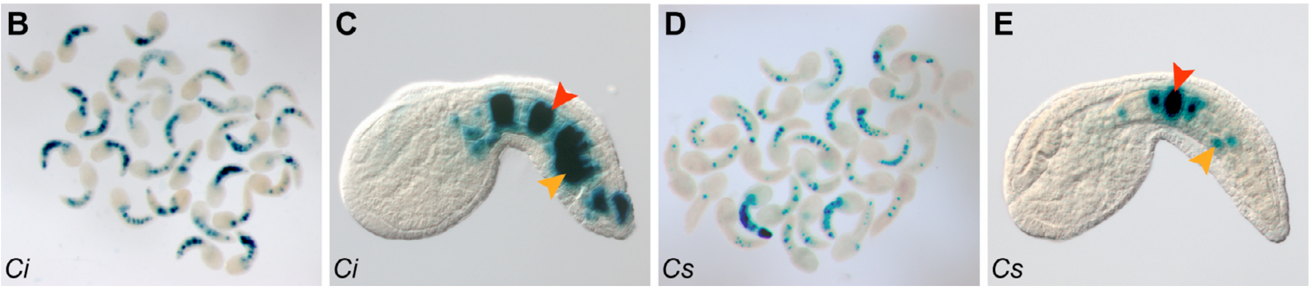


Fig. S3. Interspecific conservation of the *Ci-Tbx2/3* 294-bp notochord CRM. (**A**) Sequence alignment of the *Ciona intestinalis* (*Ci*) 294-bp *Ci-Tbx2/3* notochord CRM with the corresponding region of *Ciona savignyi* (*Cs*), as obtained from the whole-genome VISTA alignment between the two species (http://pipeline.lbl.gov/cgi-bin/gateway2). The T-box sites are highlighted in red in *Ci* and in pink in *Cs*. The T-box sites found to significantly contribute to notochord activity in *Ci* (T5, T6 and T7) are indicated in bold. (**B-E**) Group (B,D) and individual (C,E) microphotographs of *Ci* (B,C) and *Cs* (D,E) embryos electroporated with the 294 bp *Ci-Tbx2/3* notochord CRM. Red arrowheads indicate stained notochord cells, whereas orange arrowheads denote muscle activity.

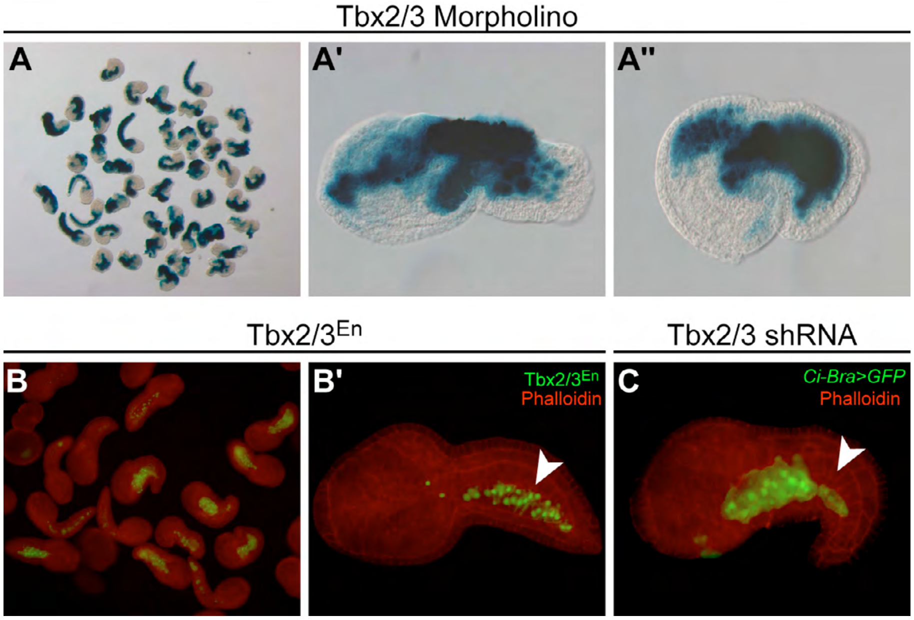


Fig. S4. Phenotypes induced through knockdown or expression of a repressor form of Ci-Tbx2/3. (**A-A″**) Group (A) and individual (A′,A″) microphotographs of embryos co-injected at the one-cell stage with an antisense morpholino oligonucleotide directed against *Ci-Tbx2/3* and the *Ci-Bra >lacZ* notochord reporter (blue staining). (**B**,**B′**) Group (B) and individual (B′) photos of embryos expressing *Ci-Bra >Tbx2/3*^{En}*::GFP*. (**C**) Embryo co-electroporated with the *Ci-Bra >GFP* notochord marker and an shRNA plasmid targeting Ci-Tbx2/3. Embryos in B-C have been counterstained with rhodamine-phalloidin. White arrowheads in B′ and C denote normal non-transgenic notochord cells.

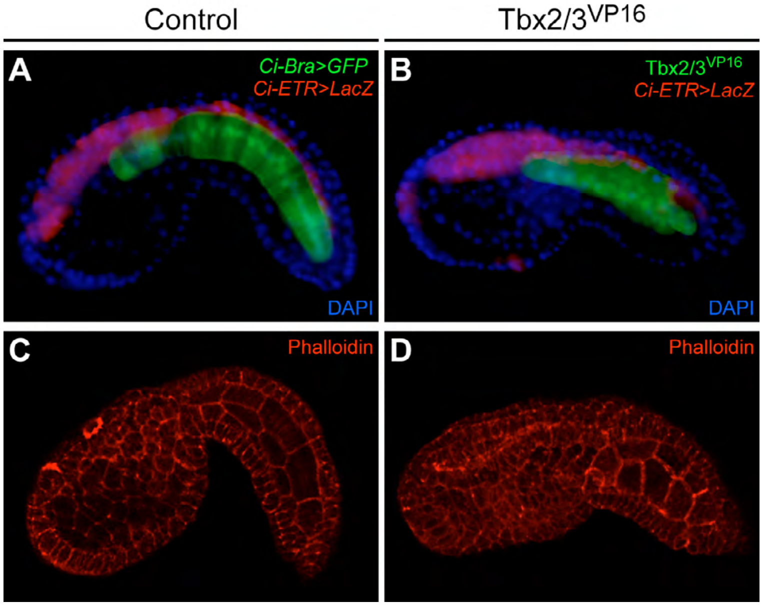


Fig. S5. Effects of expression of altered forms of Ci-Tbx2/3 in the notochord on surrounding tissues. (**A**,**B**) Immunofluorescence of embryos co-electroporated with the *Ci-ETR >lacZ* plasmid (red), which directs expression throughout the sensory vesicle and nerve cord, and either *Ci-Bra >GFP* (A, control) or the *Ci-Tbx2/3*^{VP16} construct (B), both green. Nuclei are counterstained in blue with DAPI. (**C**,**D**) Confocal stacks selected from the embryos shown in Fig. 4G,H, displaying a comparison between muscle cells from the control (C) and *Ci-Tbx2/3*^{VP16} transgenic embryos (D). Cell boundaries are highlighted by phalloidin staining (red).

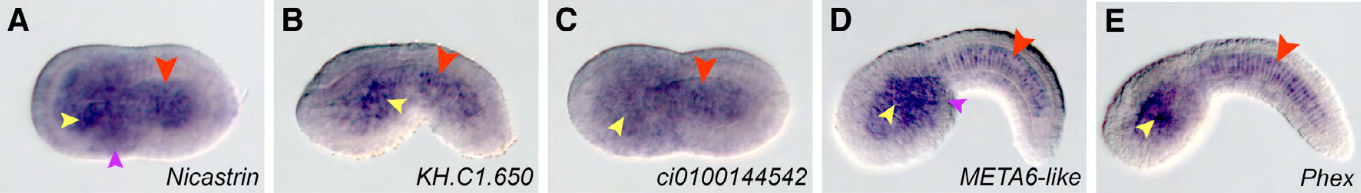
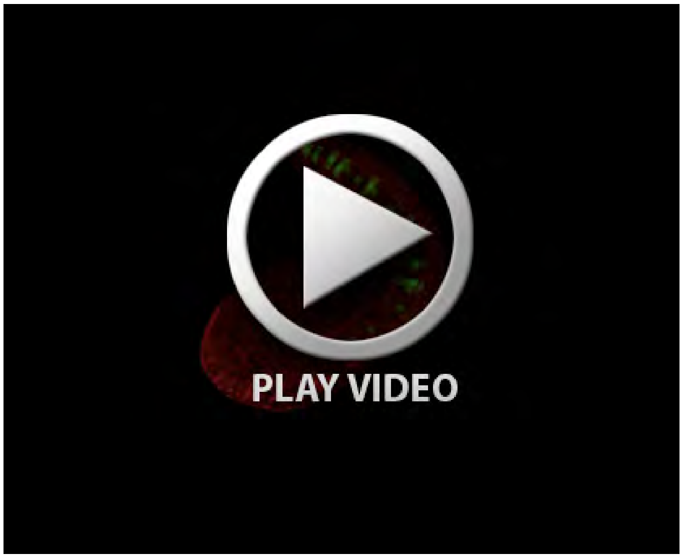
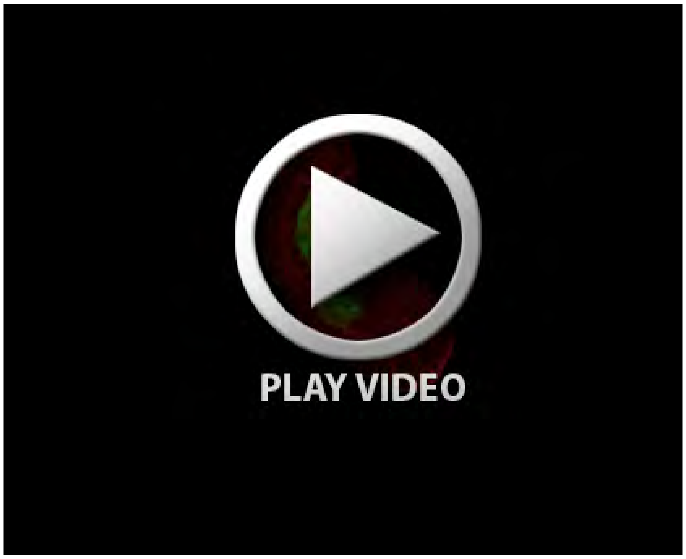


Fig. S6. Expression patterns of additional Ci-Tbx2/3 notochord targets identified via microarray screen. (A-E) Whole-mount *in situ* hybridization for the genes listed in the lower right corner of each panel. Territories stained are denoted by colored arrowheads as follows: red, notochord; purple, mesenchyme; yellow: endoderm. Larger arrowheads signal expression in domains matching that of *Ci-Tbx2/3* whereas smaller arrowheads denote expression in other areas.



Movie 1. Notochord morphology of a control embryo. Three-dimensional confocal volume rendering of a mid-tailbud stage embryo expressing *Ci-Bra>GFP* (green) to highlight the notochord. The embryo has been counterstained with phalloidin (red) to mark cell boundaries.



Movie 2. Notochord morphology in a Ci-Tbx2/3^{VP16} embryo. Three-dimensional confocal volume rendering of a mid-tailbud stage embryo expressing a *Ci-Bra>Ci-Tbx2/3*^{VP16}*::GFP* fusion gene (green) in the notochord. The embryo has been counterstained with phalloidin (red) to mark cell boundaries.

Table S1. Non-notochord gene targets from microarray screen of embryos expressing *Tbx2/3^{DBD}* or *Tbx2/3^{VP16}*

Gene	**KH gene model***	**JGI v1.0 gene model**	**Data set**	**Fold change[‡]**	**Pertinent expression domain(s)**
Hif1an	KH.C2.600	ci0100143509	DBD	4.91	Sensory vesicle
Rgs	KH.C9.235	ci0100139814	DBD	3.57	Head epidermis
Uncharacterized transcript	KH.C14.122	ci0100150875	VP16[§]	–3.01	Sensory vesicle
SLC20a	KH.S1022.1	ci0100142796	VP16	2.89	Sensory vesicle
SCO-spondin	KH.L144.2	ci0100140956	VP16[§]	–2.67	Nerve cord
ATP6V1E1/2	KH.C13.308	ci0100144075	DBD	2.65	Sensory vesicle
CathepsinH	KH.C3.765	ci0100135797	DBD	–2.42	Sensory vesicle, nerve cord
LRP-like	KH.C1.1037	ci0100131948	DBD	2.27	Sensory vesicle
Claudin gene family member 3	KH.C11.627	ci0100140153	VP16	2.22	Sensory vesicle
TRAF-3-like-1	KH.S2039.1	ci0100142385	VP16	2.11	Sensory vesicle
Amid	KH.C4.411	ci0100151268	VP16	2.07	Palps

*Satou et al., 2008.

[‡] Negative and positive scores denote a downregulation and upregulation, respectively, compared with *Bra>GFP* controls.

ZF, zinc-finger; v1.0, version 1.0; N/A, not applicable; DBD, DNA-binding domain (dominant negative).

[§] In these cases, Tbx2/3^{VP16} appears to be acting as a dominant negative (see text).

Table S2. Primers used for the characterization of *Ci-Tbx2/3* regulation and function

Primer/construct name	Primer sequence (5′-3′)*	Restriction site(s) used
I. CRM identification		
Tbx2/3 UR#1 (-3008 to -91) F	ATgcatgcTGCACATAAGCCAGTTTATCGCTG	*Sph*I
Tbx2/3 UR#1 (-3008 to -91) R	TGccatggATCTATGGAAAAGTTGAGTTCTAAGG	*Nco*I
Tbx2/3 UR #2 (-5566 to -2872) F	TtctagaTTCGAGTCCGTCGTATATCACAC	*Xba*I
Tbx2/3 UR #2 (-5566 to -2872) R	TggatccTGCGCTTTCCGCATACTCTAAAG	*Bam*HI
Tbx2/3 intron #1 cluster F	TtctagaGTTAGGGCTGCCATTTATCCGC	*Xba*I
Tbx2/3 intron #1 cluster R	ATccatggCAGCCCTAACTTTTTTGCCAAGC	*Nco*I
Tbx2/3 intron #3 cluster F (Notochord CRM 1-1937)	TtctagaCATGGTGCTTTGGACGCGTTACC	*Xba*I
Tbx2/3 intron #3 cluster R (Notochord CRM 1-1937)	ATccatggGGAATGGGGATTACGGTAGTCGAC	*Nco*I
II. CRM minimization		
1-1306	N/A	*Sty*I
1-1613	N/A	*Eco*NI/*Nco*I
1644-1937 (294bp) F	AtctagaGTGGACAGACAGGGTAGTTAAATAC	*Xba*I/*Nco*I‡
1644-1815 (172bp) R	TAccatggGTTATTTGTTCTTGTCACA	*Xba*I/*Nco*I‡
1787-1937 (151bp) F	AtctagaCTATAAGCAATGTGACAAG	*Xba*I/*Nco*I‡
1823-1937 (115bp) F	AtctagaGGCGAAATCTAACGCTAGATCAAC	*Xba*I/*Nco*I‡
III. Mutational analysis		
1644-1937 T5 F	CTCTGACACGTAAataTTAATCTATAAG	*Xba*I/*Nco*I
1644-1937 T5 R	CTTATAGATTAAtatTTACGTGTCAGAG	
1644-1937 T6 F	CTATAAGCAATataACAAGAAC	*Xba*I/*Nco*I
1644-1937 T6 R	GTTCTTGTtatATTGCTTATAG	
1644-1937 T7 F	CAAGAACAAATAAtatAAAGGGGCG	*Xba*I/*Nco*I
1644-1937 T7 F	CGCCCCTTTAtatTATTTGTTCTTG	
IV. shRNA construction		
Tbx2/3 shRNA #3 A1c	AGATGTGGATGGCGCTCCTAATTTTT	*Eco*RV/*Eco*RI
Tbx2/3 shRNA #3 A2c	aattAAAAATTAGGAGCGCCATCCACATCTGGAC	
Tbx2/3 shRNA #3 S1c	TTAGGAGTGCCGTCCATATCTGTGTGCTGTCC	
Tbx2/3 shRNA #3 S2c	AGCACACAGATATGGACGGCACTCCTAA	
V. Tbx2/3 full-length cDNA		
Tbx2/3 cDNA 5′ half F	TTCGGTTTATAGCATGTTAAAGTGTTGG	*Xho*I/*Pst*I
Tbx2/3 cDNA 5′ half R	GGGTAAGCCGCAGctgcagCACC	
Tbx2/3 cDNA 3′ half F	CAGGctcgagAAGCGAGCCACG	
Tbx2/3 cDNA 3′ half R	TTGAAGTTAAATTATTGAACAACTTTTCGC	

*Included restriction sites are in lowercase; mutated sites are lowercase and underlined.

‡Corresponding primer in the opposite direction is specific to the reporter plasmid.

Table S3. *Ci*-Tbx2/3 target genes and ESTs used for target gene expression analysis

Gene	KH gene model*	JGI v1.0 gene model	EST	Embryonic expression data source(s)	Fig. 5A category
Atlastin	KH.C12.101 KH.C12.437	ci0100131717	16a03	This study (Fig. 5B)	Notochord
SULT	KH.C7.375	ci0100133920	38i23	(Christiaen et al., 2008) (Fig. 5C)	Notochord
FN1-cont.	KH.C10.317	N/A	32i22	This study (Fig. 5D)	Notochord
Thsd1-cont.	KH.C2.1123	ci0100153405	10j14	This study (Fig. 5E)	Notochord
MLKL	KH.C4.411	ci0100152391	27f21	This study (Fig. 5F)	Notochord
Uncharacterized Transcript	KH.C8.749	N/A	27a16	This study (Fig. 5G)	Notochord
Uncharacterized Transcript	KH.C12.600	N/A	RT-PCR	This study (Fig. 5H)	Notochord
Nicastrin	KH.C1.1147	ci0100130440	22a09	(Satou et al., 2001) and this study (Fig. S6A)	Notochord
Uncharacterized Transcript	KH.C1.650	ci0100132205	12b13	This study (Fig. S6B)	Notochord
Uncharacterized Transcript	N/A	ci0100144542	38k04	This study (Fig. S6C)	Notochord
Ci-META6-like	KH.S655.4	ci0100144948	01j04	This study (Fig. S6D)	Notochord
Phex	KH.C12.669	ci0100141711	31a06	This study (Fig. S6E)	Notochord
Casp9-like	KH.C3.375	ci0100130387	83K02	(Satou et al., 2001)	Notochord
Fos-a	KH.C11.314	ci0100130316	63M13	(José-Edwards et al., 2011)	Notochord
Noto4	KH.L18.30	ci0100138226	33g19	(Hotta et al., 2000)	Notochord
ZF105	KH.C2.23	ci0100143717	10o09	(Miwata et al., 2006)	Notochord
GPV-like	KH.C7.584	ci0100154658	90M19	This study (Fig. 5L)	Multiple Tbx2/3
Chd8/9	KH.C12.278	N/A	25024	(Kusakabe et al., 2002) and this study (Fig. 5M)	Multiple Tbx2/3
Duox-c	KH.C2.477	ci0100134721	44c15	This study (Fig. 5N)	Multiple Tbx2/3
Slc23a	KH.C7.199	ci0100152342	59M01	(Kusakabe et al., 2002) and this study (Fig. 5O)	Multiple Tbx2/3
Hif1an	KH.C2.600	ci0100143509	13l12	This study (Table S1)	Non-Noto Tbx2/3
TRAF-3-like-1	KH.S2039.1	ci0100142385	06o12	This study (Fig. 5J)	Non-Noto Tbx2/3
Amid	KH.C4.411	ci0100151268	57I17	This study (Fig. 5K)	Non-Noto Tbx2/3
Rgs	KH.C9.235	ci0100139814	27c19	This study (Fig. 5I)	Non-Noto Tbx2/3
Slc20a	KH.S1022.1	ci0100142796	77A22	This study (Table S1)	Non-Noto Tbx2/3
ATP6V1E1/2	KH.C13.308	ci0100144075	17h02	This study (Table S1)	Non-Noto Tbx2/3
LRP-like	KH.C1.1037	ci0100131948	46i12	This study (Table S1)	Non-Noto Tbx2/3
Claudin gene family member 3	KH.C11.627	ci0100140153	33g09	This study (Table S1)	Non-Noto Tbx2/3
CathepsinH	KH.C3.765	ci0100135797	17b13 83K17	This study (Table S1)	Non-Noto Tbx2/3
SCO-spondin	KH.L144.12	ci0100140956	RT-PCR	This study (Table S1)	Non-Noto Tbx2/3
Uncharacterized transcript	KH.C14.122	ci0100150875	19l13	This study (Table S1)	Non-Noto Tbx2/3
Ethe1	KH.C10.362	N/A	22c23	This study	Mesenchyme
Polr1b	KH.C4.597	ci0100131534	88J16	This study	Mesenchyme
1810029B16Rik	KH.C2.576	ci0100140719	14k01	This study	Mesenchyme
Mcm7	KH.C1.124	ci0100147736	85K03	(Kusakabe et al., 2002) and this study	Mesenchyme
Astacin	KH.C9.212	ci0100137601	67N08 13h05	(Satou et al., 2001) (Kusakabe et al., 2002)	Mesenchyme
Eef2K	KH.L18.61	ci0100147355	80K10	This study	Mesenchyme

Sqstm1	KH.C9.881	ci0100152432	82K10	This study	Mesenchyme
Trappc6b	KH.C14.493	ci0100138847	20o12 73P21	This study	Mesenchyme
Mccc1	N/A	ci0100151489	69K20	This study	Mesenchyme
KIAA0664	KH.C14.234	ci0100151022	79N23 22o09	(Satou et al., 2001)	Mesenchyme
B4GalT2b	KH.L5.14	ci0100130848	30d21	This study	Mesenchyme
Uncharacterized Glycoprotein	KH.C1.1051	ci0100134090	37j01	This study	Mesenchyme
Uncharacterized Transcript	KH.C2.853	N/A	38o07	This study	Mesenchyme
Uncharacterized Transcript	KH.L84.3	N/A	26n02	This study	Mesenchyme
Uncharacterized Transcript	KH.C3.501	ci0100130481	28e07	(Kusakabe et al., 2002) and this study	Mesenchyme
Uncharacterized Transcript	KH.C1.935	ci0100131404	64P22	(Satou et al., 2001)	Mesenchyme
Uncharacterized Transcript	KH.L96.95	N/A	44e10	This study	Mesenchyme
Uncharacterized Transcript	KH.S1585.1	ci0100138863	43l07	This study	Mesenchyme
Apip	KH.C1.417	ci0100144911	92G24	This study	Other tissues
Duox-b	KH.C1.5	ci0100149417	73M12 15k18	This study	Other tissues
Mech3	KH.C4.351	N/A	26j17	(Tassy et al., 2010) and this study	Other tissues
Ptpn12/18 /22	KH.S424.10	ci0100146073	43e20	This study	Other tissues
Mos	KH.C2.835	ci0100132839	N/A	(Satou et al., 2001)	Other tissues
Aldh5	KH.C3.588	ci0100136576	71B04	This study	Other tissues
Ldh	KH.C14.591	ci0100149293	56M20	(Tassy et al., 2010)	Other tissues
Slc25a33/36	KH.C1.90	ci0100136825	19p09	(Kusakabe et al., 2002) and this study	Other tissues
GPCR-like	KH.C14.240	ci0100143862	40o18	This study	Other tissues
CD36	KH.L153.96	ci0100147772	12k09 61M01	This study	Other tissues
B4GalT3	KH.C10.488	ci0100146739	45p23	This study	Other tissues
Acot2	KH.C12.1	ci0100138543	24j15	This study	Other tissues
Uncharacterized Transcript	KH.C14.355	N/A	02o21	This study	Other tissues
Uncharacterized Transcript	KH.C7.89	N/A	93M11	This study	Other tissues
Uncharacterized Transcript	KH.L142.1	ci0100151283	02l10	This study	Other tissues
Uncharacterized Transcript	KH.C8.483	ci0100133176	63B02	This study	Other tissues
Uncharacterized Transcript	KH.C14.36	N/A	32c05	This study	Other tissues
Uncharacterized Transcript	KH.C12.235	ci0100141785	28j18	This study	Other tissues
Uncharacterized Transcript	KH.C1.451 (v1)	N/A	09h04	This study	Other tissues
Uncharacterized Transcript	N/A	N/A	74N01	This study	Other tissues

Uncharacterized Transcript	KH.L20.3	N/A	66A24	This study	Other tissues
Laminin β2-like	KH.C11.28	ci0100131107	04j21	N/A	No signal
Nahoda-like	KH.C4.350	ci0100131465	RT-PCR	N/A	No signal
Plcxd2/3	KH.L141.35	ci0100133233	RT-PCR	N/A	No signal
Uncharacterized Transcript	KH.C12.430	ci0100135710	01g18	N/A	No signal
Uncharacterized Transcript	N/A	ci0100138339	60O09	N/A	No signal
Uncharacterized Transcript	KH.S1489.1	ci0100142881	N/A	N/A	Not assayed
Uncharacterized Transcript	KH.S1745.2	N/A	N/A	N/A	Not assayed
Uncharacterized Transcript	KH.C11.582	N/A	N/A	N/A	Not assayed
Uncharacterized Transcript	KH.C1.3	ci0100149819	N/A	N/A	Not assayed
Uncharacterized Transcript	N/A	N/A (GRAIL2005.756.3.1)	N/A	N/A	Not assayed
Uncharacterized Transcript	N/A	N/A (GRAIL2005.189.1.1)	N/A	N/A	Not assayed

*Satou et al., 2008