**brachyury** null mutant-induced defects in juvenile ascidian endodermal organs

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We report the isolation of a recessive ENU-induced short-tailed mutant in the ascidian *Ciona intestinalis* that is the product of a premature stop in the *brachyury* gene. Notochord differentiation and morphogenesis are severely disrupted in the mutant line. At the larval stage, variable degrees of ectopic endoderm staining were observed in the homozygous mutants, indicating that loss of *brachyury* results in stochastic fate transformation. In post-metamorphosis mutants, a uniform defect in tail resorption was observed, together with variable defects in digestive tract development. Some cells misdirected from the notochord lineage were found to be incorporated into definitive endodermal structures, such as stomach and intestine.

**KEY WORDS: Notochord, Endoderm, Metamorphosis, Ascidian, Ciona intestinalis**

**INTRODUCTION**

The T-box transcription factor *brachyury* has an essential role in chordate mesoderm development (Herrmann and Kispert, 1994; Showell et al., 2004). The structure and function of *brachyury* has been extensively characterized in a number of chordates, including the ascidian (Satoh, 2003; Showell et al., 2004). In ascidians, *brachyury* is expressed in the notochord precursor and the notochord (Corbo et al., 1997; Imai et al., 2000; Yasuo and Satoh, 1993), and experimental manipulations have confirmed its role in notochord development (Di Gregorio et al., 2002; Satou et al., 2001; Yamada et al., 2003; Yasuo and Satoh, 1998). Both the molecular pathway inducing *brachyury* expression in the ascidian notochord precursor, and direct and indirect downstream targets of *brachyury* have been well characterized (Hotta et al., 2000; Hotta et al., 2008; Imai et al., 2006; Nishida, 2005; Takahashi et al., 1999; Yagi et al., 2004).

Functional study of *brachyury* in ascidians has relied largely on the misexpression of DNA constructs, the downregulation of *brachyury* expression by targeting upstream factors, and morpholino knockdown. Here, we report an N-ethyl-N-nitrosourea (ENU)-induced null mutant allele of *brachyury* in juvenile ascidian notochord precursor, and direct and indirect downstream targets of *brachyury* have been well characterized (Hotta et al., 2000; Hotta et al., 2008; Imai et al., 2006; Nishida, 2005; Takahashi et al., 1999; Yagi et al., 2004).

**MATERIALS AND METHODS**

**Animals**

Adult *C. intestinalis* were collected at the Santa Barbara Yacht Harbor. ENU treatment and culturing of animals was as described (Hendrickson et al., 2004; Moody et al., 1999).

**SNP linkage mapping**

Larvae from crossed heterozygous mutant 411 adults were segregated by phenotype and then pooled in groups of 50 to 200. Genomic DNA was isolated from pooled larvae as described (Hendrickson et al., 2004). Forty-six PCR primer sets that amplify loci on the various chromosome arms were designed based on the *C. intestinalis* genome sequence. The full sequences of the panel of primers are available on request.

**Sequence of mutant *brachyury* allele**

Genomic DNA for dideoxy sequencing of the *brachyury* gene from mutant 411 was amplified by specific primers that covered the entire ORF: 5'-ATGACGTCATCAGATAGTAAGTTAAGC-3' (*bra1F*) and 5'-TCACAAAGAAGTGCGGTAG-3' (*bra1R*), or *bra1F* and 5'-GGTTCGTAATTAGTGAGTATTTTG-3' (*bra4R*).

**Microarray analysis**

Microarray analysis was performed using the *C. intestinalis* Oligoarray ver.1 (Yamada et al., 2005). Two hundred nanograms of Trizol-isolated (Invitrogen) total RNA from each sample were used. Replicates included swapping of the fluorescent dyes between the samples.

**Immunohistochemical staining and whole-mount in situ hybridization**

Embryos were stained with BODIPY-FL phallacidin (Invitrogen), DAPI (Sigma) and rabbit anti-GFP (Invitrogen). Alexa 488 (Invitrogen) was used as a secondary antibody for the anti-GFP antibody. Immunostaining was as described previously (Veeman et al., 2008). Alkaline phosphatase (AP) activity was detected as described (Whittaker and Meedel, 1989). AP staining reactions were allowed to proceed for 40-60 minutes at room temperature.

For whole-mount in situ hybridization, antisense RNA probes were transcribed from cDNA clones in the *C. intestinalis* gene collection release 1 (Satou et al., 2002). In situ hybridization was as described (Satou et al., 1995).

**Single tadpole PCR**

Single larvae were genotyped for the *brachyury* locus after AP staining or in situ hybridization. Genomic DNA was isolated for PCR from single larvae as described previously (Veeman et al., 2008). Four μl samples of the digested larvae or negative controls (from 11 μl total) were PCR-amplified for 40 cycles using the *bra1F* and *bra4R* primers (see above).

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Reverse transcription-PCR (RT-PCR)

RNA was isolated with Trizol at the following stages: 110-cell, early tailbud, larva, and stage 3 juvenile (Chiba et al., 2004). The SMART RACE cDNA amplification kit (Clontech) was used for cDNA synthesis. The cDNA samples were amplified with primers br1F and br1R for brachyury, or with 5’-GGTTTCCCCATCCATCGTAG-3’ and 5’-CCAGCAGATTCCATACAAAG-3’ for cytoplasmic actin.

Tail ablation

The larvae (3 to 6 hours post-hatching) were bisected at the neck using tungsten needles. The resulting tailless trunks were cultured at 18°C in seawater with 50 mg/l streptomycin that was changed daily. Four days after attachment (equivalent to juvenile stage 5), the juveniles were fed a microalgae mixture to label the digestive tract. On the following day, the animals were observed to determine whether a complete digestive tract was present, as would be indicated by the presence of food in the lumen and feces.

RESULTS AND DISCUSSION

Isolation of an ENU-induced Ciona intestinalis brachyury mutant

Five hundred and twenty-nine F1 adults were screened and 14 mutants in nine complementation groups were isolated. Several of the mutants had short tail phenotypes, including mutant 411 shown in Fig. 1A. At a gross level, the head and trunk of the mutant 411 larvae appeared largely normal, while the tail appeared to lack vacuolated notochord cells. Single nucleotide polymorphism (SNP)-based linkage analysis using 46 sets of PCR primers targeting the various chromosome arms was used to link mutant 411 to chromosome arm 12p (Fig. 1B). Chromosome arm 12p contains at least three notochord genes (data not shown), including brachyury (Shoguchi et al., 2008). The brachyury gene from homoygous 411 mutants was found to have six nucleotide substitutions in exons compared with the published C. intestinalis cDNA sequence (NM_001078478). The most significant of these substitutions was a cytosine for thymine in exon 2 that changed amino acid 49 from an arginine to a stop codon (Fig. 1C). The predicted protein from this mutant gene would be truncated at the beginning of the DNA-binding T-box (Fig. 1C). The fact that the mutant 411 phenotype is similar to previous reports for Ciona embryos with downregulated or knocked-down brachyury (Di Gregorio et al., 2002; Satou et al., 2001; Yamada et al., 2003) allowed us to confidently conclude that the mutant phenotype was due to a loss-of-function brachyury allele. In addition, we observed that four genes previously shown to be downstream of brachyury (noto4, noto8, prickle (pk) and tropomyosin (Hotta et al., 2000)) and laminin alpha 3/4/5 were not expressed in homozygous mutant 411 embryos (data not shown). Because the predicted protein is so severely truncated, the mutation is likely to be a null. In the remainder of this manuscript we will refer to this mutant as brachyury−/− (‘bra−’).

brachyury mutant at tailbud stages

To investigate the morphology and fates of the notochord lineage in the homozygous mutants (bra−/−), the line was crossed to a stable line expressing GFP under the control of the C. intestinalis brachyury 5′ regulatory region (Joly et al., 2007). At the early tailbud stage (Fig. 1D,E), the GFP-fluorescing cells in bra−/− embryos were organized into a mass of tissue that was nearly as long and narrow as the wild-type notochord, and the overall anterior/posterior (A/P) length of the embryo was similar to wild type. Despite this, the convergent extension (C/E) of the notochord lineage was severely disrupted. In embryos double stained with DAPI and phallacidin (Fig. 1F,G), the disrupted morphology of the notochord lineage was evident and, most noticeably, the cells of the notochord lineage failed to intercalate into a single column, unlike in wild-type embryos (Fig. 1G, arrow).
mutation reported here provides a new tool for investigating \textit{brachyury} function. Microarray analysis was used to identify three new notochord genes of unknown function that are downregulated in the homozygous \textit{bra}^{−/−} mutant larvae (see Fig. S1 in the supplementary material).

**Notochord cell fate transformation in \textit{brachyury} mutants**

The manipulation of factors upstream of notochord specification, including bFGF, β-catenin, ZicN and FoxD, demonstrates the potential for presumptive notochord cells to assume neural and endodermal fates in ascidians (Imai et al., 2000; Imai et al., 2002a; Kumano et al., 2006; Minokawa et al., 2001). However, morpholino knockdown of \textit{brachyury} in \textit{Ciona savignyi} resulted in a short tail phenotype, but with no reported ectopic endoderm (Satou et al., 2001). The mutant line described here offers a stable, non-mosaic background in which to study the effects of loss of \textit{brachyury} function. On examination of the pan-neural gene \textit{ETR-1} and muscle actin, there was no indication of notochord cells transiating to neural or endodermal tissues in \textit{bra}^{−/−} larvae (data not shown). By contrast, the examination of alkaline phosphatase (AP) activity, as a marker of endoderm, revealed definitive, but highly variable, transfating of notochord to endoderm-like cells in the \textit{bra}^{−/−} larvae. Approximately one-half of the \textit{bra}^{−/−} larvae had ectopic AP staining in the core of the tail (Fig. 2B,C). Because of the variability in AP staining, the genotypes of individual larvae were determined by genomic PCR and dideoxy sequencing (Fig. 2B,C, lower panels). Identical results were obtained by in situ hybridization using an endoderm-specific marker, kyotograil2005.572.7.1 (Fig. 2E,F). Finally, AP activity colocalized with \textit{brachyury} promoter-driven GFP fluorescence in \textit{bra}^{−/−} larvae, but not in wild-type larvae, indicating that the notochord cells were transfated to endoderm in the homozygous mutant (Fig. 2G,H).

The fate of cells in the notochord lineage of \textit{bra}^{−/−} embryos not expressing ectopic endoderm markers is unknown, although by morphology and gene expression they do not become notochord. Previous reports show that ascidian Zic can prevent notochord cells from becoming endoderm, but Zic is not expressed in the notochord until after gastrulation (Imai et al., 2002b; Kumano et al., 2006). Thus, one possibility is that transfating of notochord cells in \textit{bra}^{−/−} embryos is repressed late in the process of determination, and that only a fraction of the embryos are able to overcome the repression and express ectopic endoderm markers.

Although genes required for tail elongation, including \textit{pk} and \textit{laminin alpha 3/4/5} are not expressed in \textit{bra}^{−/−} mutants, tail elongation was not completely disrupted, as was observed in \textit{aimless/chongmague} double homozygous mutants in \textit{C. savignyi} (Veeman et al., 2008). In fact, the A/P axis in \textit{bra}^{−/−} mutants was similar to that of wild-type embryos at early tailbud stage. One possibility is that the transfated notochord cells are following the morphogenetic pathway of endoderm in \textit{bra}^{−/−} mutants. Cells of the endodermal strand do form a single-file row underlying the notochord in the wild type, although the morphogenetic mechanisms are unknown. Alternatively, there may be an intrinsic and cell fate-independent program in the notochord lineage to undergo at least the initial steps of C/E.

**Transfated notochord lineage contributes to definitive endoderm**

Following the larval stage, ascidians undergo metamorphosis. In wild-type ascidians, the tail is largely reabsorbed and most adult organs are present by juvenile stage 4 (Fig. 3A,D) (Chiba et al., 2004). \textit{bra}^{−/−} larvae also undergo metamorphosis, but the resulting juveniles are highly abnormal (Fig. 3B,C). As with larvae, \textit{bra}^{−/−} juveniles were variable (Table 1), with some showing a more moderate phenotype.

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**Fig. 2. Notochord cells express endoderm markers in \textit{bra}^{−/−} mutant.**

(A-C) Alkaline phosphatase (AP) activity in wild-type (A) and \textit{bra}^{−/+} (B,C) larvae. The two \textit{bra}^{−/−} larvae are representative of the range of ectopic AP activity observed. (D-F) Expression of the endoderm/endodermal strand marker \textit{Kyotograil2005.572.7.1} in wild-type (D) and \textit{bra}^{−/−} (E,F) larvae. The black arrowheads indicate ectopic expression in the mutant larva. Genotypes were confirmed by PCR. The red arrowheads in the lower panels indicate the mutation that results in a premature stop codon in the \textit{bra}^{−} mutant. (G,H) Ectopic AP activity in the notochord lineage of wild-type and mutant larvae. The notochord lineage is indicated by GFP fluorescence. (G) Merged image for a wild-type larva. No colocalization of AP and GFP expression was found in anterior (A-line) notochord cells. (H) Merged image for a \textit{bra}^{−/−} larva. Ectopic AP activity and GFP fluorescence are colocalized (arrow) in the mutant. Scale bars: 100 μm.
with well-developed adult organs, such as endostyle and protostigmata (e.g. Fig. 3B), while others were more severely disrupted (e.g. Fig. 3C). Even in the less severe examples the digestive tracts were abnormal and did not appear to make a complete tract (e.g. Fig. 3E). Nearly all bra−/− juveniles arrested at this stage, presumably because of these defects in the digestive system.

The failure of the bra−/− juveniles to make a functional digestive tract could have several underlying causes. One possibility is that brachyury is needed in the endoderm lineage at metamorphosis for its proper development. However, brachyury transcript could not be detected by RT-PCR in wild-type larvae and stage 3 juveniles, making this unlikely (Fig. 3K).

Despite the fact that the brachyury gene is not transcribed past the tailbud stage, the perdurance of GFP allows us to follow the notochord lineage well into the juvenile stage (Fig. 3F-J) (Deschet et al., 2003). As has been described previously (Cloney, 1978), the remnants of the notochord are coiled at one end of the developing juvenile at stage 3 (Fig. 3F). By stage 4, the remaining notochord debris is greatly reduced in wild-type (H), but not in bra−/− (I,J) juveniles. In the stage 4 bra−/− juveniles, the GFP-expressing cells have incorporated into definitive endodermal structures, such as the stomach (arrow in I) or intestine (arrow in J).

Table 1. Tail ablation

<table>
<thead>
<tr>
<th>Swimming larvae</th>
<th>Good</th>
<th>Moderate</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT with tail</td>
<td>195</td>
<td>0 (0%)</td>
<td>31 (14%)</td>
</tr>
<tr>
<td>WT without tail</td>
<td>80</td>
<td>1 (1%)</td>
<td>36 (31%)</td>
</tr>
<tr>
<td>bra−/− with tail</td>
<td>2 (1%)†</td>
<td>174 (70%)</td>
<td>74 (30%)</td>
</tr>
<tr>
<td>bra−/− without tail</td>
<td>30 (11%)</td>
<td>167 (60%)</td>
<td>86 (30%)</td>
</tr>
</tbody>
</table>

*Good means the juvenile had a complete digestive tract. Moderate means the juvenile had a recognizable, but incomplete, digestive tract. Severe means the juvenile had no recognizable digestive tract. Typical phenotypes of these are shown in Fig. 3.
†This juvenile completed tail resorption.

Table 1. Tail ablation

The above observations that transfated notochord cells in bra−/− animals could contribute to definitive digestive organs suggested that gut development was abnormal due to an excess of cells. As a possible mechanism to correct this defect, the tails of larvae were removed and the remaining trunks were allowed to settle and undergo metamorphosis. Tail ablation resulted in an increase from 1% to 11% of bra−/− animals that had well-formed digestive tracts, indicating that the persistence of the tail remnants in the juvenile disrupts normal development (Table 1).
In conclusion, the mutant line reported here will provide a useful tool for future studies on notochord specification and \textit{brachyury} function. Among the unresolved issues is the variability in the expression of endoderm markers in \textit{bra}–/– mutants. Investigation in this area may provide an insight into the quantitative effects of other loci and mechanisms of embryonic robustness.

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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/135/DC1

References