REVIEW / SYNTHÈSE

Using ascidian embryos to study the evolution of developmental gene regulatory networks[1]

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Abstract: Ascidians are ideally positioned taxonomically at the base of the chordate tree to provide a point of comparison for developmental regulatory mechanisms that operate among protostomes, non-chordate deuterostomes, invertebrate chordates, and vertebrates. In this review, we propose a model for the gene regulatory network that gives rise to the ascidian notochord. The purpose of this model is not to clarify all of the interactions between molecules of this network, but to provide a working schematic of the regulatory architecture that leads to the specification of endoderm and the patterning of mesoderm in ascidian embryos. We describe a series of approaches, both computational and biological, that are currently being used, or are in development, for the study of ascidian embryo gene regulatory networks. It is our belief that the tools now available to ascidian biologists, in combination with a streamlined mode of development and small genome size, will allow for more rapid dissection of developmental gene regulatory networks than in more complex organisms such as vertebrates. It is our hope that the analysis of gene regulatory networks in ascidians can provide a basic template which will allow developmental biologists to superimpose the modifications and novelties that have arisen during deuterostome evolution.

Résumé : Les ascidiens occupent une position taxonomique idéale à la base de l'arbre évolutif des chordés pour fournir un point de comparaison des mécanismes régulateurs du développement qui sont en opération chez les protostomiens, les deutérostomiens non chordés, les chordés invertébrés et les vertébrés. Nous proposons dans notre rétrospective un modèle du réseau génique de régulation qui mène à la formation de la notochorde des ascidiens. Le but du modèle n'est pas de faire la lumière sur l'ensemble des interactions entre les molécules du réseau, mais plutôt de fournir un schéma de travail de l'architecture régulatrice qui mène à la spécification de l'endoderme et à la structuration du mésoderme chez les embryons d'ascidiens. Nous décrivons une séries de méthodologies, tant informatiques que biologiques, d'usage courant ou en développement, pour l'étude des réseaux géniques de régulation chez les embryons d'ascidiens. Nous croyons que, compte tenu du mode simplifié de développement et de la taille réduite du génome chez les ascidiens, les outils actuellement disponibles aux chercheurs intéressés à la biologie des ascidiens permettront une dissection accélérée des réseaux géniques de régulation du développement par comparaison à ce qui se passe chez les organismes plus complexes, tels que les vertébrés. Nous espérons que l'analyse des réseaux géniques de régulation chez les ascidiens fournira une maquette de base sur laquelle les biologistes du développement pourront surimposer les modifications et les nouveautés qui sont apparues durant l'évolution des deutérostomiens.

[Traduit par la Rédaction]

Introduction

Ascidians are marine invertebrate chordates and are the largest class within the subphylum Urochordata. Commonly called sea squirts, these bilaterian animals have been studied by developmental biologists since their chordate affinity was first described in the late-19th century (Kowalevsky 1866; Kowalevsky 1871). Adult ascidians are sessile filter feeders that reproduce via motile tadpole larvae which possess key features of the chordate body plan such as notochord flanked by muscles and a dorsal, hollow neural tube (Satoh 1994). There has been a renewed interest in recent years to exploit the ascidian embryo as a means to understand the molecular mechanisms underlying the developmental programs of the

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ancestral chordate, as well as modern chordates such as humans (for recent reviews see Di Gregorio and Levine 1998, 2002; Satou and Satoh 1999; Satoh 2001, 2003; Satoh et al. 2003).

From an embryological perspective, the ascidian is an ideal experimental system in which to decipher cell fate specification mechanisms at the molecular level. The ascidian tadpole larva consists of approximately 2500 cells that form a limited set of tissues including epidermis, central and peripheral nervous systems, notochord, muscle, endoderm, and mesoderm (Satoh 1994). This simple configuration of the tadpole larva represents the basic chordate body plan. Development in ascidians is extremely rapid — about a dozen cell divisions produces a fully developed *Ciona intestinalis* (L., 1767) larva that hatches 18 h post fertilization when reared at 18 °C. Extensive research over the last century has defined the nearly complete cell lineage of the ascidian embryo up to the gastrula stage and most of the major cell lineages are well characterized (Conklin 1905; Ortolani 1955, 1957, 1962; Nishida and Satoh 1983; Nishida and Satoh 1985; Nishida 1987). The early blastomeres of the embryo are uniquely shaped and positioned, and are thus amenable to experimental manipulation. In situ hybridization may be used to visualize the differential patterns of gene expression patterns with high cellular resolution. Two commonly studied ascidians, *C. intestinalis* and *Ciona savignyi* Herdman, 1882, have relatively short life cycles of about 3 months that have facilitated the implementation of culturing techniques and mutagenesis screens (Moody et al. 1999; Nakatani et al. 1999; Sordino et al. 2000, 2001; Deschet et al. 2003).

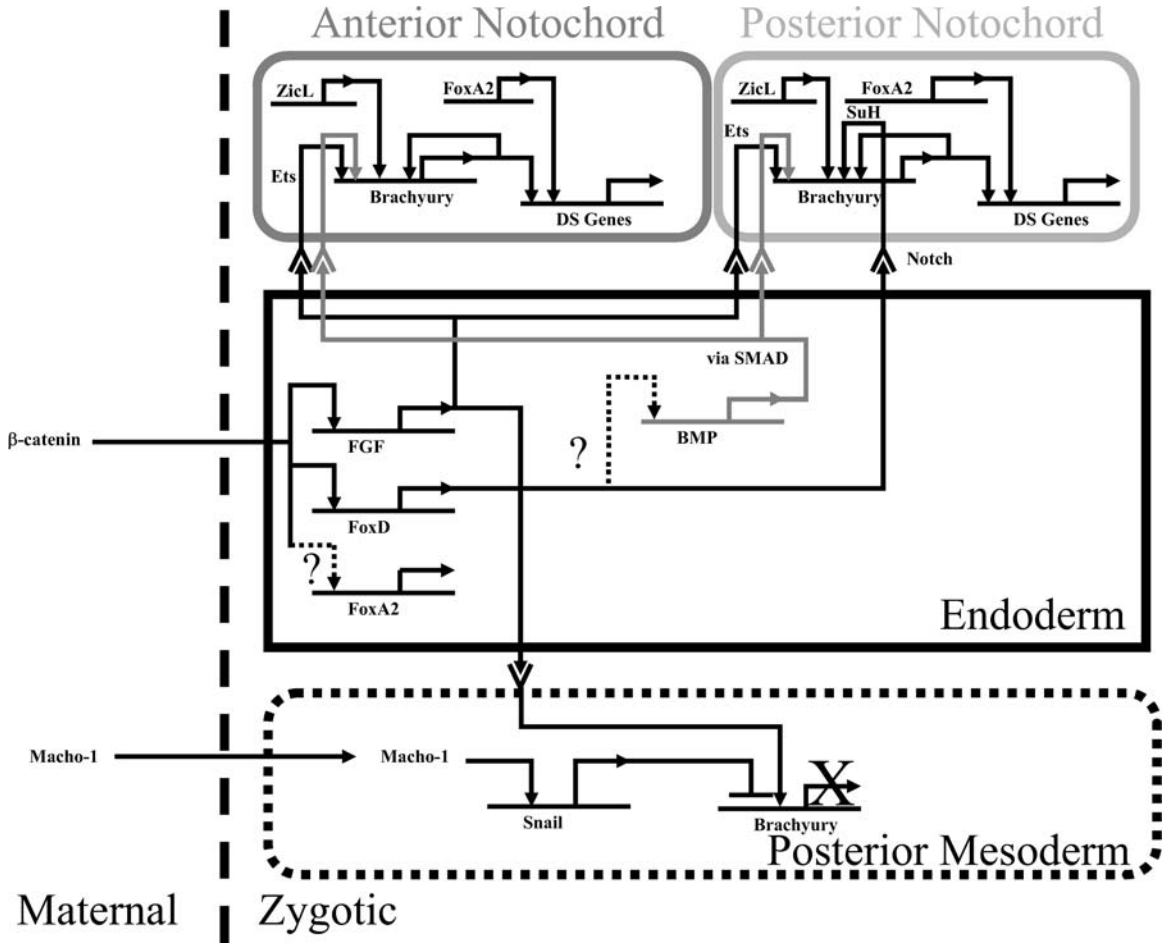
From a molecular perspective, ascidians provide an ideal platform with which to examine developmental gene regulation. A key feature is the ability to generate hundreds, or even thousands, of transgenic embryos using a simple electroporation technique (Corbo et al. 1997*b*; Di Gregorio and Levine 2002; Zeller 2004). Although these embryos often express transgenes mosaically, they have proved invaluable for examining the expression of tissue-specific regulatory modules that function during embryogenesis. Recently, a simple to build electroporator was reported to allow the precise creation of transgenic embryos with predictable levels of mosaic transgene expression, thus controlling the extent of mosaic expression. The functions of developmentally important genes may be assayed by misexpressing genes under the control of tissue-specific regulatory modules via electroporation or by microinjection of RNA (e.g., Corbo et al. 1998; Takahashi et al. 1999; Imai et al. 2000; Di Gregorio and Levine 2002). Recently, the use of morpholino antisense oligonucleotides has been demonstrated to effectively suppress gene expression during ascidian development (Satou et al. 2001*a*, 2001*b*; Imai et al. 2002*a*, 2002*c*; Wada and Saiga 2002; Miya and Nishida 2003) and has even been used in a large-scale screen to analyze genes of unknown function that are conserved between ascidians and vertebrates (Yamada et al. 2003). It is possible to generate stable, transgenic lines of ascidians (Deschet et al. 2003), and recently, a transposon system has been demonstrated to function in ascidians (Sasakura et al. 2003) that will compliment traditional chemically induced mutant screening strategies.

From a genomics perspective, ascidians provide an extensive collection of sequence resources that include two draft genome sequences, a collection of nearly 500 000 expressed sequence tag (EST) sequences deposited in GenBank, and an extensive set of in situ hybridization-based expression data of over 5000 genes. The draft genome of *C. intestinalis* was reported in 2002 (Dehal et al. 2002) and the draft sequence of *C. savignyi* was recently released by the Whitehead Center for Genome Research (http://www.broad.mit.edu/annotation/*Ciona*/). The *C. intestinalis* genome is predicted to express nearly 16 000 genes and there are few examples of gene duplication events, suggesting that gene regulatory analysis in ascidians should be relatively simple compared with vertebrates. An extensive set of EST sequence data and in situ hybridization data is available on the Web (http://ghost.zool.kyoto-u.ac.jp/indexr1.html) and has been published in a series of papers on both embryonic ESTs (Nishikata et al. 2001; Satou et al. 2001*c*; Fujiwara et al. 2002; Kusakabe et al. 2002) and adult/juvenile tissue ESTs (Takamura et al. 2001; Inaba et al. 2002; Ogasawara et al. 2002). Comparative genomics-based methods for gene regulatory analysis, such as phylogenetic footprinting techniques (e.g., VISTA analysis (Mayor et al. 2000; Loots et al. 2002)), promise to accelerate the rate at which gene regulatory research may be pursued in ascidians. At the present time, ascidians are currently the only deuterostome group in which the genomes from related species within the same genus have been sequenced.

A proposed model of the ascidian notochord gene regulatory network

We propose a working model of the gene regulatory network (GRN) that specifies notochord cell fate in ascidian embryos. This model, shown in Fig. 1, is based on the experimental results from a number of laboratories and will be briefly described here. A detailed accounting of the key experiments used to construct this model will be discussed later in this review. During early embryogenesis, the differential nuclear localization of β-catenin in the endoderm lineage is believed to activate the transcription of several key genes including a fibroblast growth factor (FGF) ligand (FGF9/16/20) and the transcription factor FoxD (Imai et al. 2002*a*, 2002*b*). This FGF ligand, and possibly other FGF ligands as well, produced from the endoderm cells signals the surrounding mesoderm and patterns this mesoderm along the anteroposterior axis (Kim et al. 2000; Kim and Nishida 2001; Nishida 2002). An Ets class transcription factor has recently been shown to mediate this signal (Miya and Nishida 2003). A maternally inherited transcription factor called Macho-1 that localizes in the posterior mesoderm serves as a "molecular switch" to determine if mesoderm receiving the FGF signal will adopt an anterior (notochord) or posterior (mesenchyme and muscle) fate (Nishida and Sawada 2001; Kobayashi et al. 2003). In addition to FGF signaling, recent experiments have suggested that bone morphogenic protein (BMP) signaling also plays an important role in notochord induction (Darras and Nishida 2001). One of the genes downstream of this FGF-signaling event is the gene encoding the conserved transcription factor Brachyury (Yasuo and Satoh 1994; Nakatani et al. 1996; Corbo et al. 1997*b*). The synergistic activity of Brachyury together

Fig. 1. A working model of the gene regulatory network that gives rise to the ascidian notochord. Maternal determinants are shown on the left of the broken vertical line, while zygotically expressed genes are shown on the right, within a respective tissue type. Solid lines and arrows represent direct or indirect interactions within the network that are supported by experimental evidence as detailed in the text. The dotted line progressing from FoxD to BMP indicates a hypothetical regulatory interaction between FoxD and BMP as described in the text.

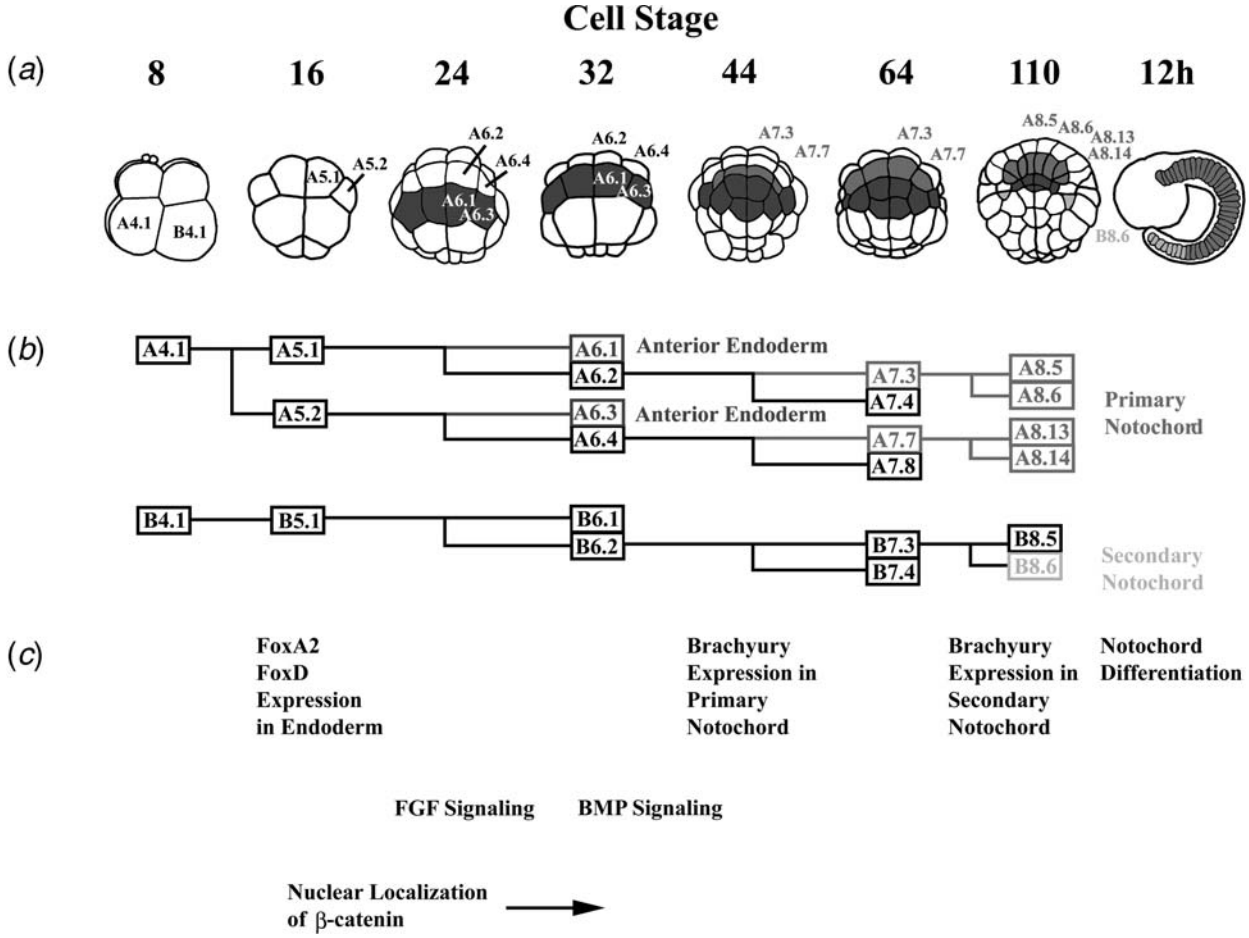


with the transcription factor FoxA2 specifies a notochord cell and presumably activates a series of genes that are required in the differentiation of the notochord cells (Yasuo and Satoh 1998; Di Gregorio and Levine 1999; Hotta et al. 1999, 2000; Takahashi et al. 1999; Shimauchi et al. 2001*a*). It is unclear how the *FoxA2* gene is regulated, but it is known to be expressed in the endoderm, ventral cells of the neural tube, and the notochord (Corbo et al. 1997*a*; Shimauchi et al. 1997). In addition to these key regulatory genes, several other regulatory genes have also been implicated in the formation of the ascidian notochord including a Zic-type transcription factor (Imai et al. 2002*c*; Yagi et al. 2004), a winged-helix transcription factor called FoxD, and Notch signaling (Imai et al. 2002*b*). Additional details of this model will be provided in the following discussion of the key experiments supporting this GRN. Readers should continue to consult Fig. 1, as details of the experiments implicating the various regulatory genes are described.

The cell lineage of the notochord and inductive events

The notochord of the ascidian tadpole larva is composed of exactly 40 cells in a single file "stack-of-coins" arrangement as shown in Fig. 2. The cell lineage of the ascidian embryo, first described by Conklin (Conklin 1905) with subsequent refinements by Ortolani (Ortolani 1955, 1957, 1962), was verified with modern cell lineage analysis techniques by Nishida and Satoh (Nishida and Satoh 1983, 1985; Nishida 1987). The notochord cells are derived from two cell lineages that arise in the early embryo. The anterior 32 cells are descendents of the A4.1 blastomere pair (anterior-vegetal blastomeres) of the 8-cell-stage embryo and are referred to as the primary lineage. The secondary lineage, consisting of the 8 posterior notochord cells, is derived from the B4.1 descendents (posterior-vegetal) of the 8-cell-stage embryo. The notochord cells are specified during early embryogenesis and later will undergo convergent extension movements to arrange their final axial orientation along the anteroposterior axis (Miyamoto and Crowther 1985; Cloney 1990; Munro and Odell 2002*a*, 2002*b*). These movements are completed at about 12 h post fertilization in the *C. intestinalis* embryo. From this point forward the notochord cells differentiate by forming an extracellular component called the notochordal sheath and produce numerous vacuoles that eventually will form the lumen of the notochord (Cloney

Fig. 2. The cell lineage of the endoderm and the primary and secondary notochord cells of ascidians. (*a*) Patterns of cell divisions during ascidian embryogenesis from the 8-cell stage to the tadpole larva stage (~12 h post fertilization for *C. intestinalis*), indicating the spatial relationships between notochord and endoderm (dark gray) precursors. (*b*) Cell lineages of the endoderm, the primary (A-lineage, medium gray) and secondary (B-lineage, light gray) notochord cells. The pattern of cell divisions depicted here can be compared with the spatial locations of the cells within the actual embryo as shown in part A. (*c*) Key events in the specification of endoderm and notochord during ascidian development. Cell-lineage nomenclature is after Conklin (1905).



1964; Miyamoto and Crowther 1985; Crowther and Whittaker 1986; Cloney 1990).

There are three pairs of blastomeres at the 16-cell stage that are fated to give rise to both notochord and endoderm (A5.1, A5.2, and B5.1). At this time, transcripts of the transcription factors FoxA2 in *Halocynthia roretzi* (von Drasche, 1884) (Shimauchi et al. 1997) and FoxD in *C. savignyi* (Imai et al. 2002*b*) are detected in these blastomeres, but the early embryonic expression pattern of *Ciona* FoxA2 gene has not been reported. In the ascidian *Molgula oculata* Forbes, 1848, expression of the *FoxA* gene begins around the 44- to 64-cell stage and inhibiting the function of this gene with antisense oligonucleotide treatment disrupts migration of both endoderm and notochord cells (Olsen and Jeffery 1997). At this time, maternally supplied β-catenin protein is probably beginning to differentially localize to the nuclei of endodermal cells, a process that appears to be complete by the 32-cell stage (Imai et al. 2000). By the 32-cell stage, the endoderm (A6.1, A6.3, and B6.1) and notochord (A6.2, A6.4, and B6.2) fates have separated in the A- and B-lineages (Fig. 2). The three pairs of notochord precursors are situated at the vegetal-marginal zone immediately flanking the three pairs of endoderm precursors. The A-lineage notochord founder cells (A7.3 and A7.7) are born at the 44-cell stage (Darras and Nishida 2001), although in most reports these cells are reported as appearing at the 64-cell stage. These two pairs of the primary lineage founder cells become fate restricted at this time and divide three more times to form the 32 primary notochord cells. The secondary notochord precursors (B8.6) do not become restricted to notochord fate until the 110-cell stage. These B8.6 blastomeres will divide two more times to form the last 8 cells (Nishida 1987).

Early experiments with partial ascidian embryos suggested that the notochord formed autonomously, consistent with the notion that ascidian cell specification was mosaic in nature. In embryos of *C. intestinalis* and *H. roretzi*, B4.1 and A4.1 partial embryos produced notochord cells (Crowther and Whittaker 1986; Nishikata and Satoh 1990) Early experiments with *H. roretzi* showed that isolated blastomeres from 110-cell embryos could autonomously differentiate into notochord cells. We now know that both the primary lineage and the secondary lineage of the notochord have already been specified by the 110-cell stage, allowing for autonomous differentiation from this point forward. With the

notochord cell lineage well characterized, Nishida's laboratory examined notochord cell specification on a much finer scale. When grown in a state of continuous dissociation until the 110-cell stage, embryonic cells failed to differentiate into notochord (Nishida 1992). This not only revealed that inductive interactions between cells must be necessary for ascidian notochord formation, as in the development of the vertebrate notochord, but that the induction occurs early. Complementing this study, Nishida's laboratory conducted a more refined embryo dissociation experiment in which embryos were dissociated at different cleavages from egg to the 64-cell stage, and then cultured as partial embryos in normal seawater from the 110-cell stage onward (Nakatani and Nishida 1994). The results from this series of experiments indicated that embryos which dissociated around the 32-cell stage failed to produce notochord cells, but notochord cells were produced in embryos that dissociated after this time or when the dissociation was stopped prior to the 32-cell stage.

The preceding sets of experiments established the 32-cell stage as a critical time in ascidian notochord induction but did not identify which cells in the early embryo produced notochord. Therefore, Nishida's laboratory proceeded to carry out a series of elegant experiments using partial ascidian embryos (Nakatani and Nishida 1994). Partial embryos consisting of presumptive A-lineage notochord cells generated at the 8-, 16-, 64-, or 110-cell stage produced notochord cells, while partial embryos created at the 32-cell stage did not. At the 32-cell stage, presumptive A-line notochord precursors would not produce notochord cells unless cocultured with a presumptive endoderm cell or with a second presumptive notochord cell. This indicated that a notochord cell needs a signal from a neighboring cell to be specified. It also explains why any partial embryo resulting from a blastomere isolated at other than the early 32-cell stage can signal itself and form notochord, especially because the notochord and endoderm share a cell lineage up until this stage (see Fig. 2). Similar experiments were performed with partial embryos of B-lineage presumptive notochord cells. Notochord cells could not be produced from B-lineage partial embryos produced prior to the 64-cell stage, suggesting that an induction takes place around this time in development. Similar types of partial embryo experiments examining notochord specification, other than creating A- or B-lineage quarter embryos, have not yet been reported in embryos of *C. intestinalis*.

To examine which molecules might confer the notochord-inducing signal in ascidians, factors implicated in vertebrate embryo mesoderm induction were tested for their ability to induce notochord fate in isolated presumptive notochord blastomeres from *H. roretzi*. When applied exogenously to isolated presumptive notochord blastomeres, low levels of recombinant human bFGF, but not activin, successfully induced notochord features in these cells (Nakatani et al. 1996). Additional experiments demonstrated that this FGF treatment was also sufficient for activating the expression of the notochord-specific transcription factor Brachyury less than one cell division later at the 64-cell stage (Nakatani et al. 1996). Six FGF genes have been identified in the recently released genome of *C. intestinalis* (Satou et al. 2002), an FGF receptor has been characterized from *H. roretzi* (Kamei et al. 2000; Shimauchi et al. 2001*a*), and experiments inhibiting FGF signaling in embryos of *H. roretzi* (Kim and Nishida 2001; Minokawa et al. 2001) have all implicated endogenous FGF signaling in the induction of notochord cells. The function of one of the FGF ligand genes, *FGF9/16/20*, has been more thoroughly examined in *C. savignyi* embryos (Imai et al. 2002*a*). When an antisense morpholino oligonucleotide was used to perform a gene "knock-down" of the *FGF9/16/20* gene, the initial stages of notochord induction were blocked, but notochord gene expression was observed later in development, suggesting that perhaps additional FGF ligands are present. The *FGF9/16/20* gene is thought to be a downstream target of the maternal β-catenin that localizes to the nuclei of endoderm cells (Imai et al. 2002*a*). We should note that these FGF signals are involved in the general patterning of the mesoderm along the anteroposterior axis of the ascidian embryo (reviewed by Nishida 2002) and the notochord represents the anterior mesoderm in the ascidian embryo.

Unlike the A-lineage notochord cells, the B-lineage notochord precursor cells did not respond to induction by exogenously applied bFGF (Nakatani et al. 1996). In the search for additional signaling molecules, the Nishida laboratory discovered that BMP is likely the second signaling molecule (Darras and Nishida 2001). In their model, beginning at the 24-cell stage, an FGF signal from the anterior and posterior endoderm cells initiates the first phase of notochord induction. At the 44-cell stage, BMP signaling only from the anterior endoderm cells, and not the posterior endoderm cells, completes the induction of the notochord precursors. This observation may also explain why only the combination of an anterior endoderm precursor, but not a posterior endoderm precursor, with a B-line notochord precursor will result in specification of notochord (Kim et al. 2000). These experiments also demonstrated that inhibition of BMP signaling, by overexpression of its antagonist chordin, resulted in the reduced formation of both primary and secondary notochord cells, indicating the importance of FGF and BMP signals for the induction of both notochord lineages.

Transcriptional activation of Brachyury, a notochord-specific transcription factor

In ascidians, the transcription factor Brachyury is the earliest expressed notochord-specific gene. The *Brachyury* gene has been identified and at least partially characterized in a number of different ascidian species including *C. intestinalis* (Corbo et al. 1997*b*), *C. savignyi* (Imai et al. 2000), *H. roretzi* (Yasuo and Satoh 1993), several *Molgula* species (Takada et al. 2002), and several larvacean (pelagic urochordates) species (Bassham and Postlethwait 2000; Nishino et al. 2001). In all cases examined, *Brachyury* is expressed in the notochord lineage. Within the last few years, our understanding of *Brachyury* gene regulation has dramatically improved, as results from many experiments have linked several signal transduction pathways and transcription factors to *Brachyury*. There are at least three different pathways that positively regulate *Brachyury* expression: (1) FGF signaling, (2) BMP signaling, and (3) Notch signaling. There is also an interaction with the Snail repressor protein that mediates transcriptional repression (Fig. 1). In this section, we

will summarize the relevant experiments that led us to the GRN model we presented in Fig. 1.

We have previously mentioned that in early ascidian embryos between the 24- and 44-cell stages, FGF and BMP signaling is required to induce the formation of both primary and secondary lineage notochord. When bFGF is exogenously applied to isolated notochord precursor cells, these cells were induced to form notochord and they activated the expression of the *Brachyury* gene (Nakatani et al. 1996). One possible FGF ligand is encoded by the *FGF9/16/20* gene isolated from *C. savignyi*. The *Ciona FGF9/16/20* gene is a downstream target of β-catenin and when the function of *FGF9/16/20* is knocked-down by injecting early embryos with a specific morpholino oligonucleotide, early *Brachyury* expression is inhibited. However, *Brachyury* expression in the notochord will begin later in development (Imai et al. 2002*a*). Consistent with this data, when endogenous FGF signaling was chemically inhibited in *H. roretzi* embryos or by the overexpression of a dominant-negative ras, which is a component of the FGF signaling pathway, *Brachyury* was not expressed and the notochord was not formed (Nakatani and Nishida 1997; Kim et al. 2000). Finally, a recent study has demonstrated that an Ets class transcription factor in *H. roretzi* mediates FGF signaling in ascidian embryos. Interfering with the function of this transcription factor prevented the expression of *Brachyury* and the formation of the notochord (Miya and Nishida 2003). Similarly, when endogenous BMP signaling was inhibited with overexpression of ascidian chordin or *Xenopus* noggin, both BMP antagonists, *Brachyury* expression and notochord formation were abrogated (Darras and Nishida 2001). These results clearly demonstrate that *Brachyury* expression is dependent on both FGF and BMP signaling pathways, as indicated in Fig. 1, although it is unclear if transcription factors that mediate these signaling events, namely Ets (for FGF) and SMADs (for BMP), directly bind to the *Brachyury cis*-regulatory domain.

A third signaling pathway has been implicated in the activation of the *Brachyury* gene — Notch signaling. There are several lines of evidence that support this hypothesis. An analysis of the *Brachyury cis*-regulatory domain demonstrated that several putative binding sites for the Suppressor of Hairless (Su(H)) transcription factor, a mediator of Notch signaling (reviewed by Artavanis-Tsakonas et al. 1995), was required for transcriptional activation of the gene (Corbo et al. 1997*b*). Subsequent experiments demonstrated that a *C. intestinalis* Su(H) orthologue bound to these sites to transcriptionally regulate the *Brachyury* gene (Corbo et al. 1998). So what regulates Notch signaling in notochord induction? One clue comes from the analysis of the *C. savignyi FoxD* gene, which encodes a winged helix class transcription factor. Like *FGF9/16/20* gene, this gene is a downstream target of β-catenin and is transiently expressed in the early endoderm lineage (Imai et al. 2002*b*). Inhibition of the function of *FoxD* with specific morpholinos prevented the expression of *Brachyury* and the formation of the notochord — both primary and secondary lineages. This phenotype could be partially rescued by the co-injection of an activated form of the *C. savignyi* Notch receptor, but this would only occur in the B-lineage secondary notochord lineage (Imai et al. 2002*b*). These results suggest that *FoxD* may be regulating notochord specification through at least two mechanisms: (1) Notch signaling in the secondary lineages and (2) some other signaling pathway in the primary and (or) secondary lineages. Since *FoxD* knock-downs prevent notochord formation in both lineages, some common signaling pathway is likely to mediate this interaction. By itself, activated Notch rescues only the B-lineage notochord fate; therefore, Notch and this unknown signaling pathway may be functionally redundant in this lineage. Perhaps this second signal could be BMP signaling (indicated with a dotted line in Fig. 1), although in vertebrates, FoxD3 is a transcriptional repressor that inhibits BMP expression (Sasai et al. 2001).

In addition to inducing the fate of the anterior mesoderm (notochord), FGF signaling also patterns the posterior mesoderm (mesenchyme and muscle) in ascidian embryos (Nishida 2002). If FGF signaling activates *Brachyury* expression, then how is *Brachyury* transcriptionally repressed in the posterior mesoderm? The Nishida laboratory has recently reported that a maternal mRNA encoding the transcription factor Macho-1 in *H. roretzi* is selectively sequestered to the posterior embryo as development proceeds (Nishida and Sawada 2001). Recent experiments have demonstrated that Macho-1 function, which is normally only present in the posterior mesoderm, acts as a switch to modulate the actions of FGF signaling in the mesoderm (Kobayashi et al. 2003). Notochord cells do not normally inherit Macho-1 mRNA or protein, so they express *Brachyury* in response to FGF signaling. In the posterior mesoderm, Macho-1 activates the expression of the ascidian *Snail* gene that encodes a transcriptional repressor (Kobayashi et al. 2003). Previous experiments in *C. intestinalis* have demonstrated that *Snail* mediates transcriptional repression of the *Brachyury* gene in the posterior mesoderm (Fujiwara et al. 1998). In these experiments, *Snail* was shown to bind to specific target sequences located in the *Brachyury cis*-regulatory domain.

We have now accounted for three activating interactions (FGF, BMP, and Notch signaling) and the single repressive interaction (*Snail*) that regulate *Brachyury* expression (Fig. 1). There is a fourth positively acting interaction that has been recently defined. In both *H. roretzi* and the two *Ciona* species, genes encoding a Zic-type zinc finger transcription factor have been shown to regulate a variety of developmental processes, including notochord specification (Imai et al. 2002*c*; Wada and Saiga 2002; Yagi et al. 2004). When the function of either gene is inhibited by morpholino injection, notochord development is abrogated and *Brachyury* is not expressed. In both *Ciona* species, a series of functional experiments have demonstrated that *ZicL* is downstream of both β-catenin and *FoxD* function (Imai et al. 2002*c*; Yagi et al. 2004), although it is unclear if β-catenin activates *ZicL* directly or indirectly via *FoxD*. *ZicL* is first expressed at the 32-cell stage in the A-lineage notochord precursor cells (A6.2 and A6.4, Fig. 2). It is unclear if *ZicL* expression in these cells is mediated by a *FoxD*-regulated signaling pathway such as Notch. It is possible that the expression of *FoxD* at the 16-cell stage in A5.1 and A5.2, the cells that will give rise to A6.2 and A6.4, is sufficient to directly activate *ZicL* in A6.2 and A6.4 when the 32-cell stage is reached. We have now accounted for all of the early interactions, depicted in Fig. 1, that lead to *Brachyury* expression.

Identification of down stream Brachyury target genes

Experiments in *Xenopus* had previously demonstrated that the function of *Brachyury* alone was not sufficient to promote notochord fate in animal cap assays. Only when *Brachyury* and the *FoxA* orthologue *Pintallavis* were injected together in animal caps did those cells transform to notochord cells (O'Reilly et al. 1995). Ascidians also have a *FoxA* orthologue that has been characterized from both *H. roretzi* and *C. intestinalis*. This gene begins expression at the 16-cell stage (Shimauchi et al. 1997) and is expressed in tissues that include the endoderm, notochord, and ventral neural tube (Corbo et al. 1997*a*; Shimauchi et al. 1997). It is unclear how the ascidian *FoxA2* gene is regulated, but an analysis of the *C. intestinalis* FoxA2 *cis*-regulatory domain has implicated the *Snail* repressor and a possible T-box protein like Ci-VegTR (which is expressed in early vegetal blastomeres) as possible transcriptional regulators (Di Gregorio et al. 2001). To test whether the synergistic functions of *Brachyury* and *FoxA2* were required to specify ascidian notochord cells, a transgene was constructed that expressed the *Brachyury* cDNA under the control of the FoxA2 promoter. When this construct was electroporated into fertilized eggs, the resulting embryos displayed a phenotype consistent with the ectopic production of notochord cells (Takahashi et al. 1999). Essentially, all cells that expressed both *Brachyury* and *FoxA2* were at least partially converted to a notochord fate. This experiment has also been repeated using mRNA injections of both transcription factors and the same results were obtained (Yasuo and Satoh 1998; Shimauchi et al. 2001*a*).

Because great quantities of transgenic embryos may be obtained via electroporation (Corbo et al. 1997*b*; Di Gregorio and Levine 2002; Zeller 2004; Zeller et al. 2004), large numbers of transgenic embryos ectopically expressing *Brachyury* were generated and used in a subtractive hybridization screen to identify potential downstream targets of *Brachyury* (Takahashi et al. 1999). Nearly 40 genes were identified that were predominately expressed in the notochord and subsequent experiments characterized the temporal and spatial expression patterns of these genes (Hotta et al. 1999, 2000). As might be expected, these genes are expressed after *Brachyury* begins to be expressed in the notochord founder cells of the early embryo. Some of the target genes initiate expression about a cleavage after *Brachyury*, while others initiate expression much later in development. These varied temporal expression patterns suggest that some genes may be direct targets of *Brachyury* function, while others may be indirect targets. As expected, many of these genes encode the types of molecules that would be expected to be produced in the differentiated notochord.

Validating the ascidian notochord GRN

The summary of experimental data in the previous section supports the GRN model that we have presented in this review. The goal in designing this model is to determine the overall regulatory architecture of the network, without necessarily knowing all of the minute details. In the case of the ascidian notochord GRN, we now have a reasonable view of the overall regulatory architecture. However, further experimentation is required to validate this model and expand it to encompass additional regulatory factors and downstream targets as these molecules are identified. The GRN provides a framework for designing experiments that can determine the functional relationships between the molecules portrayed in the network, as well as in identifying additional molecules that play important roles in the development of the notochord in chordates.

In the last few years, advances in gene regulatory experimentation in ascidians have reached a point where GRN models may now be rigorously tested. To test GRNs, the functions of key regulatory molecules must be modulated both positively and negatively, and the results of these manipulations monitored by observing the resulting embryonic phenotypes as well as by identifying changes in the expression of downstream genes. With the completion of both *C. intestinalis* and *C. savignyi* genomes, microarrays have begun to be used to assay global gene expression during ascidian embryogenesis (Azumi et al. 2003; Ishibashi et al. 2003). Once readily available, microarrays may be used to examine the expression of target genes in embryos in which the GRNs have been experimentally manipulated. It will be necessary to manipulate GRNs at different times and in different cell types during embryonic development, necessitating the use of appropriate methods for misexpressing genes. In addition to misexpressing genes with wild-type functions, it will be necessary to express genes that have been mutated to have positively or negatively acting functions. For example, the DNA-binding domain of a transcription factor may be fused to either an activation or a repression domain to alter the function of the protein. Similar types of modifications and (or) truncations may be made to receptors to alter their functions as well. These types of approaches, first developed in other systems and used extensively in embryos such as the sea urchin embryo (Davidson et al. 2002*a*, 2002*b*; Oliveri et al. 2002; Rast et al. 2002), have now begun to be employed in ascidians. The next key step in the analysis of ascidian GRNs will be to combine these genetic manipulations with microarray analysis to examine changes in the global patterns of gene expression during embryonic development.

Two strategies are currently used in ascidians to mis- or over-express genes: mRNA injections and transgene-based expression. Microinjection in ascidians is relatively simple to perform, although the throughput is quite small compared with producing transgenic embryos with electroporation. One of the advantages of mRNA injection is that there is very little lag time for producing protein off of the mRNA template; protein is likely produced prior to first cleavage and may be produced in all blastomeres that inherit the injected mRNA. This is an important consideration when trying to manipulate GRN function in early development. Although transgenes have been used to ectopically express genes in ascidian embryos, those genes are expressed with the temporal, spatial, and quantitative regulation provided by the *cis*-regulatory DNA used to create the transgene. The earliest reported zygotic transcription occurs at the 16-cell stage in ascidians (Shimauchi et al. 1997; (Imai et al. 2002*b*), and transgene-based expression will not be suitable for early GRN manipulations unless *cis*-regulatory DNAs are identified that confer earlier zygotic expression. A drawback of mRNA injections is that the injected mRNAs are di-

luted during embryogenesis as blastomeres divide, mRNAs are degraded during this time, and there is no spatial control. For manipulating later aspects of GRN function, transgene-based methods are ideal. Large numbers of transgenic embryos may be produced via electroporation (Di Gregorio and Levine 2002; Zeller 2004) and a variety of *cis*-regulatory DNAs have been identified that will confer temporally and spatially restricted patterns of expression on targeted genes (e.g., Harafuji et al. 2002).

To complement ectopic expression experiments, targeted gene knock-down using morpholino antisense oligonucleotides have proven to function well in ascidians (Satou et al. 2001*a*). Morpholinos, designed against specific mRNAs, are microinjected into fertilized eggs where they effectively prevent the translation of the targeted mRNA. Although designed to be resistant to degradation, morpholinos tend to lose effectiveness as development proceeds. Because ascidian embryogenesis is rapid, this potential problem is minimized. As with mRNA injections, injections of morpholinos provide no means to control the spatial or temporal distribution of the knock-down effect. Morpholinos should prove effective at preventing the translation of maternal as well as zygotically transcribed genes. To date, morpholinos have been used to knock-down the functions of the following genes relevant to the development of the ascidian notochord: *Brachyury* (Yamada et al. 2003), *FoxD* (Sasai et al. 2001), *HrZicN* (Wada and Saiga 2002), *FGF9/16/20* (Imai et al. 2002*a*), *Macho-1* (Nishida and Sawada 2001; Kobayashi et al. 2003), *HrEts* (Miya and Nishida 2003), and *CsZicL* (Imai et al. 2002*c*). Although RNA interference (RNAi) techniques have been successfully employed to knock-down gene function in other invertebrate embryonic systems such as *Drosophila melanogaster* Meigen, 1830 and *Caenorhabditis elegans* (Maupas, 1900) Dougherty, 1955, (e.g., Bargmann 2001; Schmid et al. 2002), there have been no published reports of this technique being successfully used in ascidian embryos.

Manipulations of *Brachyury* expression during ascidian embryogenesis have been accomplished by both ectopic expression, using both mRNA injections (Yasuo and Satoh 1998; Shimauchi et al. 2001*a*) and transgene expression (Takahashi et al. 1999), as well as by gene knock-down approaches using morpholinos (Yamada et al. 2003). Results from these experiments are consistent with *Brachyury* acting as a transcriptional activator on the downstream notochord target genes; however, these experiments do not address if these are direct or indirect targets. The *Brachyury* target genes initiate expression at several different times during development, suggesting that there are both direct and indirect targets (Hotta et al. 1999, 2000). Some initiate expression one cleavage after the *Brachyury* gene begins to be expressed, indicating that these may be direct transcriptional targets, while others initiate expression much later in embryogenesis, which is consistent with the idea that they may represent indirect targets. Experiments on a single *Brachyury* target gene, tropomyosin-like, have identified putative Brachyury-binding sites in the *cis*-regulatory domain and have demonstrated that these sites are required for proper gene expression (Di Gregorio and Levine 1999). Similar types of experiments should be conducted on the remaining sets of notochord genes, although this will require a significant labor investment.

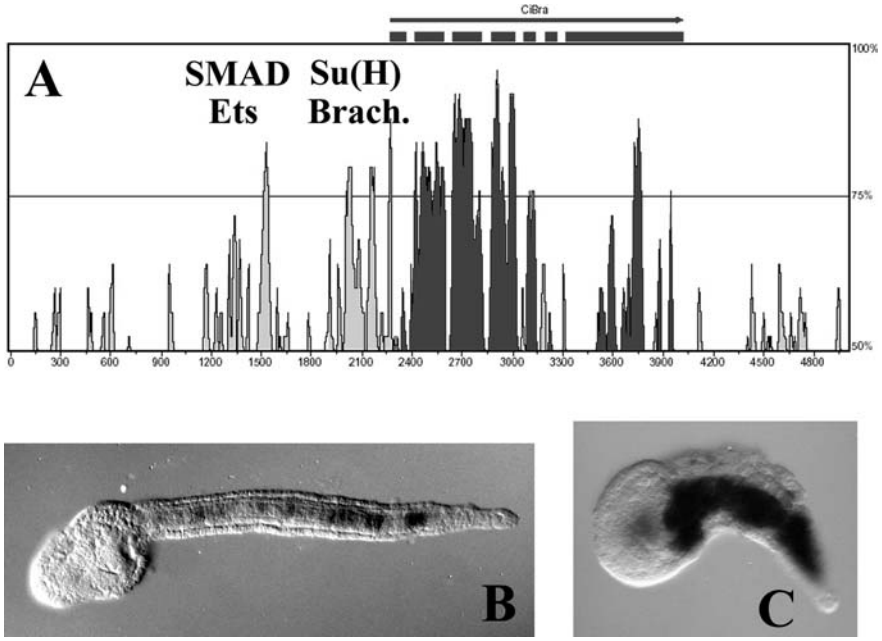
Given that the genomes of the related *Ciona* species are now available, comparative genomics and bioinformatics approaches promise to expedite the analysis of the *Brachyury* target genes and should prove invaluable for the analysis of genes in other ascidian GRNs. Reports from a number of different laboratories, using a variety of bilaterian embryo models, have demonstrated that comparative genomics methods are useful for identifying potential *cis*-regulatory modules from genomic sequences (e.g., Blanchette et al. 2002; Blanchette and Tompa 2002; Halfon et al. 2002). Although several different methods have been reported, they all essentially identify what are called "phylogenetic footprints" — sequence regions that have been constrained during evolution. The coding regions of a pair of homologous genes would be expected to be present within a phylogenetic footprint analysis, since the exons encode similar proteins like the *Brachyury* gene from *C. intestinalis* and *C. savignyi*. An interesting observation from these types of analyses is that there are often extensive regions of non-coding sequence that are also present within phylogenetic footprints. It is these non-coding regions that are often found to identify important *cis*-regulatory DNAs, when tested using appropriate experimental procedures.

Our laboratory has used phylogenetic footprinting techniques with the two *Ciona* genome sequences to identify putative *cis*-regulatory domains for a variety of ascidian genes. We have found that these computational methods are useful for identifying where to design primers to amplify by polymerase chain reaction the 5′-*cis*-regulatory domains directly from genomic sequence. When assembled into a transgene to drive green fluorescent protein (GFP) expression, these *cis*-regulatory regions quite often express GFP with the expected temporal and spatial patterns. We have successfully designed a number of transgenes in this manner, including genes that are expressed in the central nervous system, epidermis, muscle, and endoderm of the ascidian embryo.

A phylogenetic footprint analysis of a 5-kb region of the *Brachyury* gene, using the VISTA web-based service (Mayor et al. 2000; Loots et al. 2002), is displayed in Fig. 3A. Peaks indicate the positions of conserved sequences, dark gray peaks denote exons, and light gray peaks denote non-coding regions. The "full length" *cis*-regulatory region from the *Brachyury* gene, as originally reported (Corbo et al. 1997*b*), extends about 3.5 kb upstream of the start of transcription (left-most dark gray peak in Fig. 3). From the first draft of the *C. intestinalis* genome, we have determined that about 1.2 kb of this region actually extends into an additional gene near the *Brachyury* gene and has been removed from the analysis shown in Fig. 3A. The set of light gray peaks immediately adjacent to the first exon detected by the VISTA analysis roughly corresponds to the 434-bp minimal Brachyury enhancer, and the inclusion of the second set of light gray peaks corresponds to the 790-bp enhancer, both of which have been extensively tested experimentally (Corbo et al. 1997*b*). Phylogenetic footprinting thus promises to expedite the identification of *cis*-regulatory DNAs and should prove useful in the analysis of developmental gene regulation.

Phylogenetic footprinting techniques will probably be most useful for identifying *cis*-regulatory DNAs on the level of a regulatory module. To computationally identify actual transcription factor binding sites, additional bioinformatics

Fig. 3. Computational analyses can predict molecular interactions within ascidian gene regulatory networks. (A) VISTA analysis (Mayor et al. 2000) comparing the *Brachyury* genes from *Ciona intestinalis* and *Ciona savignyi*. Exons are shaded dark gray and highly conserved non-coding regions (putative regulatory modules) are shaded light gray. Transcription factor binding sites within these light gray peaks were predicted with MatInspector (Quandt et al. 1995), and are indicated as SMAD, Ets, Su(H), or Brach. (B) *Brachyury::lacZ* transgene expression in normal *C. intestinalis* embryos. Fertilized egg embryos were electroporated with a *Ci*Bra::LacZ reporter construct and stained for LacZ expression, which is only present in the notochord. (C) The *Brachyury::lacZ* transgene is misexpressed in embryos expressing ectopic *Brachyury.* Fertilized eggs were co-electroporated with *Ci*FoxA2::Brachyury and the *C*-Bra::LacZ reporter construct. Ectopic expression of *Brachyury* activates ectopic expression of the *CiBra::LacZ* transgene consistent with the hypothesis that *Brachyury* may positively autoregulate its expression.



approaches are becoming available, but there are a number of hurdles that must be overcome before these methods will become more generally useful. Transcription factors tend to bind to short degenerate sequences, so simple sequence searches are not always useful for identifying biologically meaningful regulatory sites. Additionally, only a small number of the transcription factors encoded in animal genomes have characterized binding sites, most sites are of unknown composition. Other computational approaches, beyond the scope of this review, are currently being developed to identify over-represented motifs that may prove useful for identifying the binding sites of uncharacterized transcription factors (e.g., Guhathakurta et al. 2002*a*, 2002*b*; Stormo and Tan 2002; Wang and Stormo 2003).

Despite these drawbacks, potentially useful gene regulatory predictions may be obtained by combining phylogenetic footprint analysis with the search for characterized transcription factor binding sites (e.g., Wang and Stormo 2003). Since the DNA-binding domains of homologous transcription factor proteins are conserved, these proteins are liable to bind to similar DNA target sequences. This has been demonstrated extensively with the Brachyury proteins of many different animals in both ascidians (Satoh et al. 2000) and vertebrates (Marcellini et al. 2003). Since the Brachyury-binding site has been well characterized (Kispert and Herrmann 1993), we performed a search for putative Brachyury-binding sites, using the Transfac database (Quandt et al. 1995; Wingender et al. 1996), within the phylogenetically footprinted regions of the *Brachyury* gene. As shown in Fig. 3A, our analysis detected the presence of putative Brachyury-binding sites within the 434 minimal enhancer element, suggesting that the *Brachyury* gene may regulate its own expression. In an initial analysis of this hypothesis, we electroporated *C. intestinalis* embryos with either the *Brachyury::lacZ* reporter gene alone or together with the *FoxA2::Brachyury* transgene that drives expression of *Brachyury* in embryonic cells that normally express *FoxA2*. As shown in Fig. 3B, the *Brachyury::lacZ* reporter gene is correctly expressed only in the notochord cells, while ectopic expression of this reporter gene is evident in embryos ectopically expressing *Brachyury* (Fig. 3C), suggesting that *Brachyury* may regulate its own expression.

Using a similar computational approach, we have searched the *Brachyury cis*-regulatory domain for the presence of binding sites for Suppressor of Hairless (Su(H)), Ets, and SMAD proteins that are downstream effectors of the Notch, FGF, and BMP signal transduction pathways. The Su(H)-binding sites identified in this computational analysis are the same Su(H) sites that have been experimentally demonstrated to regulate *Brachyury* expression in ascidian embryos (Corbo et al. 1997*b*). As described earlier, both FGF and BMP signaling regulate notochord specification in early ascidian embryos (Nakatani et al. 1996; Darras and Nishida 2001). Recently, the Ets protein has been shown to mediate the FGF-inducing signal in ascidians (Miya and Nishida 2003). The computational analysis has also identified puta-

tive binding sites for factors that mediate the three signal transduction pathways involved in notochord specification, although it is not known if these sites functionally regulate *Brachyury* gene expression (R.W. Zeller, unpublished observations). Thus, phylogenetic footprinting, coupled with searching for characterized transcription factor binding sites, should prove to be a powerful tool for deciphering gene regulation during development.

Comparing notochord gene expression between ascidians and other deuterostome embryos

The analysis of developmental GRNs in ascidians will provide an important data set that may be used to understand how gene regulation has changed during evolution. As an example, we will discuss the evolution of the notochord within the deuterostomes. The animals within the deuterostomes are either chordates (like ascidians, amphioxus, and vertebrates) or non-chordates (like the sister-groups of echinoderms and hemichordates). Neither echinoderms, like the sea urchin, nor hemichordates have a notochord, but both sea urchin and hemichordate embryos express *Brachyury* in endodermal tissues (Tagawa et al. 1998; Peterson et al. 1999*a*, 1999*b*). In these organisms, *Brachyury* functions control cell movements during gastrulation and endoderm development, which is believed to be the ancestral role of *Brachyury* function (Rast et al. 2002). In chordates, *Brachyury* regulation of notochord genes is believed to be a co-opted function. Therefore, in chordates, *Brachyury* would likely regulate multiple sets of targets: one group of genes that regulate cell movement during gastrulation and another of genes that make the notochord (Rast et al. 2002). A screen to identify sea urchin embryo *Brachyury* target genes has also been performed, but there is little overlap between these genes and the *Brachyury* targets identified in the *Ciona* screen (Rast et al. 2002). In the sea urchin screen, many of the *Brachyury* targets encode genes that are involved in cell movement or in the differentiation of the endoderm, while the *Ciona* screen primarily identified genes involved with notochord differentiation. These results support the hypothesis that *Brachyury* function was co-opted in chordates to make the notochord and suggest that the *Ciona* screen may have missed *Brachyury* targets that regulate cell movements. Future experiments could address this issue by characterizing putative *Brachyury* target genes that regulate cell movements.

Within the chordates, in addition to the ascidian embryo, there exists extensive research detailing the development of the notochord in amphioxus (e.g., Holland et al. 1995; Shimeld 1997), as well as in representative vertebrates such as *Xenopus*, zebrafish, chickens, and mice. The expression patterns and functions of signal transduction pathway genes, such as *BMP*, *FGF*, and Wnt (e.g., Isaacs 1997; Casey et al. 1998; Dickmeis et al. 2001; Yasuo and Lemaire 2001; Faure et al. 2002), as well as several transcription factors, including Brachyury (Wilkinson et al. 1990; Smith et al. 1991; Kispert et al. 1994; Schulte-Merker et al. 1994), FoxA2 (Ruiz i Altaba and Jessell 1992; Ang et al. 1993; Kaestner et al. 1993; Strahle et al. 1993; Ang and Rossant 1994), and Xnot or floating head (von Dassow et al. 1993; Stein and Kessel 1995; Talbot et al. 1995; Gont et al. 1996; Stein et al. 1996), that are involved in notochord development are well described in many vertebrate species. Significant research has also focused on the regulation of key notochord genes such as *Brachyury* (e.g., Schulte-Merker et al. 1994; Griffin et al. 1995; O'Reilly et al. 1995; Schulte-Merker and Smith 1995; Clements et al. 1996; Casey et al. 1998) and mutant screens in zebrafish have identified numerous alleles that effect notochord development (Odenthal et al. 1996; Stemple et al. 1996), although the molecular identity of many of these genes remains unknown. In vertebrates, the notochord arises from the chordamesoderm of the Organizer, a specialized group of cells that defines the dorsal-most region of the embryo (recently reviewed by De Robertis et al. 2000). The Organizer forms in response to several different signaling pathways, including β-catenin, Wnt, and TGFβ family members. *Brachyury* is expressed in the prospective mesoderm of the early vertebrate embryo and is involved in the development of the notochord as well as in regulating cell movements during gastrulation (reviewed by Herrmann and Kispert 1994; Smith 1997, 1999; Papaioannou and Silver 1998; Technau 2001).

Ascidians are not known to possess a vertebrate Organizer, so early specification events regulating the expression of *Brachyury* may not be completely conserved between ascidians and vertebrates. Although there are a number of Organizer genes that are present in the ascidian genome, few have been characterized. One of the genes that has been characterized is the *chordin* gene from *H. roretzi*. It is expressed in the primary lineage notochord cells at the 64-cell stage and overexpression of this gene, or the *Xenopus* noggin gene, early in embryogenesis disrupts notochord formation in ascidians (Darras and Nishida 2001). Despite the fact that ascidians do not have an Organizer, many of the signaling pathways that are required for Organizer function in vertebrates, as mentioned above, may also be required for *Brachyury* expression in ascidians. Interestingly, *Brachyury* indirectly autoregulates its own expression in *Xenopus* embryos by activating the expression of embryonic FGF (Casey et al. 1998). Recall that our computational analysis and preliminary experiment suggests that *Ciona Brachyury* also regulates its own expression (Fig. 3). The computational analysis suggests that this interaction is direct, but we cannot rule out indirect regulation via FGF signaling, as in *Xenopus*. Future experiments should address these questions.

Brachyury function in ascidians and vertebrates is likely to be conserved, although at the present time there is limited molecular evidence to support this hypothesis. The large-scale mutant zebrafish embryo screens identified a number of loci that are involved in the specification, formation, and maintenance of the notochord (Odenthal et al. 1996; Stemple et al. 1996). Unfortunately, the identities of the genes corresponding to most of these mutants are unknown. Some vertebrate *Brachyury* targets, such as eFGF and the Bix transcription factors, identified from screens in *Xenopus* embryos (Tada et al. 1997; Casey et al. 1998; Tada and Smith 2001) have not been analyzed in ascidians. Clearly, the screens in zebrafish, *Xenopus*, and ascidians have failed to identify all of the loci regulating notochord development. We know the identities of most of the characterized *Brachyury* target genes from ascidians. Perhaps the identities of some of the

remaining zebrafish mutants correspond to some of the *Ciona Brachyury* targets, although we are unsure if this analysis has been reported.

Some genes implicated in the development of the vertebrate notochord have not been examined in ascidian embryos. It is likely that some of these genes will not have ascidian orthologues and will represent vertebrate novelties. For example, the zebrafish one-eyed pinhead (*oep*) gene is required for pre-chordal plate and endoderm formation and is also expressed in the notochord (Zhang et al. 1998; Feldman and Stemple 2001; Griffin and Kimelman 2003), but there is no *oep* orthologue present in the current release of the *C. intestinalis* genome (R.W. Zeller, unpublished observations). In other cases, ascidians will have orthologues to vertebrate genes required for notochord development. For example, the *Xenopus Xnot* gene (floating head in zebrafish) encodes a homeodomain transcription factor that is required for early notochord specification (von Dassow et al. 1993; Talbot et al. 1995; Gont et al. 1996). This gene has been isolated and characterized in sea urchins (Peterson et al. 1999*b*) and is present in the ascidian genome, but it has not been characterized (R.W. Zeller, unpublished results). As more notochord genes are identified in vertebrates, their ascidian orthologues should be characterized as well, to develop a more complete model of the notochord GRN operating in the ascidian embryo.

The *Brachyury* gene is central to notochord formation in chordates, although it is also expressed in non-chordate deuterostome and protostome embryos. Because ascidians are situated at the base of the chordate tree, they are ideally suited for comparisons among "higher" chordates (vertebrates), as well as non-chordate deuterostomes. To understand how gene regulatory mechanisms have evolved over time, we must be able to identify the large sets of genes that function together in a developmental process and then construct and test a GRN model. The large-scale efforts to understand gene regulation in the sea urchin embryo (Davidson et al. 2002*a*, 2002*b*; Oliveri et al. 2002; Rast et al. 2002) will provide us with a detailed example of developmental mechanisms in a non-chordate deuterostome. Ascidians are now poised to exploit the recent advances in large-scale gene regulatory analysis to provide us with a detailed understanding of developmental gene regulation in a basal chordate. Compared to vertebrates, gene regulatory analysis in ascidians is relatively straightforward. By assembling the basic framework of important developmental GRNs in ascidians, we may be able to provide researchers with a "blueprint" with which to model vertebrate GRNs. Comparisons of the developmental mechanisms between ascidians and sea urchins may shed light on the evolution of chordates from a deuterostome ancestor. Similarly, comparisons between ascidian and vertebrates will provide clues to the evolution of vertebrates from a common chordate ancestor.

Summary

Ascidians are ideally suited for gene regulatory analysis. Development is rapid, producing a 2500-cell tadpole larva composed of few tissue types. The availability of sequenced genomes from two closely related species will accelerate research efforts as computational genomics methods are improved. The ability to easily create transgenic embryos, as well as the ability to modulate the expression of genes, are all key requirements for analyzing gene regulation on a global scale. Ascidian researchers are now poised to exploit this embryo for defining and testing developmental GRNs that may provide "blueprints" for examining gene regulation in vertebrate embryos. Because ascidians are basal chordates, comparisons between developmental gene regulatory mechanisms in ascidians, vertebrates, and non-chordate deuterostomes will provide us with the data required to investigate not only how chordates evolved, but how vertebrates evolved from a basal chordate ancestor. This new era of gene regulatory experimentation in ascidian embryos promises to unify the efforts of the disciplines of evolution, development, genomics, and genetics towards understanding and deciphering the gene regulatory code.

References

Ang, S.L., and Rossant, J. 1994. HNF-3 beta is essential for node and notochord formation in mouse development. Cell, **78**(4): 561–574.

Ang, S.L., Wierda, A., Wong, D., Stevens, K.A., Cascio, S., Rossant, J., and Zaret, K.S. 1993. The formation and maintenance of the definitive endoderm lineage in the mouse: involvement of *HNF3/forkhead* proteins. Development (Camb.), **119**(4): 1301–1315.

Artavanis-Tsakonas, S., Matsuno, K., and Fortini, M. E. 1995. Notch signaling. Science (Wash., D.C.), **268**(5208): 225–232.

Azumi, K., Takahashi, H., Miki, Y., Fujie, M., Usami, T., Ishikawa, H., et al. 2003. Construction of a cDNA microarray derived from the ascidian *Ciona intestinalis*. Zool. Sci. (Tokyo), **20**(10): 1223–1229.

Bargmann, C.I. 2001. High-throughput reverse genetics: RNAi screens in *Caenorhabditis elegans*. Genome Biol. **2**(2): reviews1005.1–1005.3. Available from http://genomebiology.com/2001/2/2/reviews/1005 [published 31 January 2001]. doi:10.1186/gb-2001-2-2-reviews1005

Bassham, S., and Postlethwait, J. 2000. *Brachyury* (*T*) expression in embryos of a larvacean urochordate, *Oikopleura dioica*, and the ancestral role of *T*. Dev. Biol. **220**(2): 322–332.

Blanchette, M., and Tompa, M. 2002. Discovery of regulatory elements by a computational method for phylogenetic footprinting. Genome Res. **12**(5): 739–748.

Blanchette, M., Schwikowski, B., and Tompa, M. 2002. Algorithms for phylogenetic footprinting. J. Comput. Biol. **9**(2): 211–223.

Casey, E.S., O'Reilly, M.A., Conlon, F.L., and Smith, J.C. 1998. The T-box transcription factor *Brachyury* regulates expression of *eFGF* through binding to a non-palindromic response element. Development (Camb.), **125**(19): 3887–3894.

Clements, D., Taylor, H.C., Herrmann, B.G., and Stott, D. 1996. Distinct regulatory control of the *Brachyury* gene in axial and non-axial mesoderm suggests separation of mesoderm lineages early in mouse gastrulation. Mech. Dev. **56**(1–2): 139–149.

Cloney, R. 1964. Development of the ascidian notochord. Acta Embryol. Morphol. Exp. **7**: 111–130.

Cloney, R. 1990. Urochordata — Ascidiacea. *In* Reproductive biology of marine invertebrates. *Edited by* K.G. Adiyodi and R.G. Adiyodi. IBH Publishing Co. Pvt. Ltd., New Delhi and Oxford. pp. 391–451.

Conklin, E.G. 1905. The organization and cell lineage of the ascidian egg. J. Acad. Nat. Sci. Phila. **13**: 1–119.

Corbo, J.C., Erives, A., Di Gregorio, A., Chang, A., and Levine, M. 1997*a*. Dorsoventral patterning of the vertebrate neural tube is conserved in a protochordate. Development (Camb.), **124**(12): 2335–2344.

Corbo, J.C., Levine, M., and Zeller, R.W. 1997*b*. Characterization of a notochord-specific enhancer from the *Brachyury* promoter region of the ascidian, *Ciona intestinalis*. Development (Camb.), **124**(3): 589–602.

Corbo, J.C., Fujiwara, S., Levine, M., and Di Gregorio, A. 1998. Suppressor of hairless activates *Brachyury* expression in the *Ciona* embryo. Dev. Biol. **203**(2): 358–368.

Crowther, R.J., and Whittaker, J.R. 1986. Developmental autonomy of presumptive notochord cells in partial embryos of an ascidian *Ciona intestinalis*. Int. J. Invertebr. Reprod. Dev. **9**: 253–261.

Darras, S., and Nishida, H. 2001. The BMP signaling pathway is required together with the FGF pathway for notochord induction in the ascidian embryo. Development (Camb.), **128**(14): 2629–2638.

Davidson, E.H., Rast, J.P., Oliveri, P., Ransick, A., Calestani, C., Yuh, C.H., et al. 2002*a*. A genomic regulatory network for development. Development (Camb.), **295**(5560): 1669–1678.

Davidson, E.H., Rast, J.P., Oliveri, P., Ransick, A., Calestani, C., Yuh, C.H., et al. 2002*b*. A provisional regulatory gene network for specification of endomesoderm in the sea urchin embryo. Dev. Biol. **246**(1): 162–190.

Dehal, P., Satou, Y., Campbell, R.K., Chapman, J., Degnan, B., De Tomaso, A., et al. 2002. The draft genome of *Ciona intestinalis*: insights into chordate and vertebrate origins. Science (Wash., D.C.), **298**(5601): 2157–2167.

De Robertis, E.M., Larrain, J., Oelgeschlager, M., and Wessely, O. 2000. The establishment of Spemann's organizer and patterning of the vertebrate embryo. Nat. Rev. Genet. **1**(3): 171–181.

Deschet, K., Nakatani, Y., and Smith, W.C. 2003. Generation of *Ci-Brachyury-GFP* stable transgenic lines in the ascidian *Ciona savignyi*. Genesis, **35**(4): 248–259.

Dickmeis, T., Rastegar, S., Aanstad, P., Clark, M., Fischer, N., Korzh, V., and Strahle, U. 2001. Expression of the anti-dorsalizing morphogenetic protein gene in the zebrafish embryo. Dev. Genes Evol. **211**(11): 568–572.

Di Gregorio, A., and Levine, M. 1998. Ascidian embryogenesis and the origins of the chordate body plan. Curr. Opin. Genet. Dev. **8**(4): 457–463.

Di Gregorio, A., and Levine, M. 1999. Regulation of Citropomyosin-like, a *Brachyury* target gene in the ascidian, *Ciona intestinalis*. Development (Camb.), **126**(24): 5599–5609.

Di Gregorio, A., Corbo, J.C., and Levine, M. 2001. The regulation of *forkhead/HNF-3*β expression in the *Ciona* embryo. Dev. Biol. **229**(1): 31–43.

Di Gregorio, A.D., and Levine, M. 2002. Analyzing gene regulation in ascidian embryos: new tools for new perspectives. Differentiation, **70**(4–5): 132–139.

Faure, S., de Santa Barbara, P., Roberts, D.J., and Whitman, M. 2002. Endogenous patterns of BMP signaling during early chick development. Development (Camb.), **244**(1): 44–65.

Feldman, B., and Stemple, D.L. 2001. Morpholino phenocopies of *sqt*, *oep*, and *ntl* mutations. Genesis, **30**(3): 175–177.

Fujiwara, S., Corbo, J.C., and Levine, M. 1998. The snail repressor establishes a muscle/notochord boundary in the *Ciona* embryo. Development (Camb.), **125**(13): 2511–2520.

Fujiwara, S., Maeda, Y., Shin, I.T., Kohara, Y., Takatori, N., Satou, Y., and Satoh, N. 2002. Gene expression profiles in *Ciona intestinalis* cleavage-stage embryos. Mech. Dev. **112**(1–2): 115–127.

Gont, L.K., Fainsod, A., Kim, S.H., and De Robertis, E.M. 1996. Overexpression of the homeobox gene *Xnot-2* leads to notochord formation in *Xenopus*. Dev. Biol. **174**(1): 174–178.

Griffin, K., Patient, R., and Holder, N. 1995. Analysis of FGF function in normal and no tail zebrafish embryos reveals separate mechanisms for formation of the trunk and the tail. Development (Camb.), **121**(9): 2983–2994.

Griffin, K.J., and Kimelman, D. 2003. Interplay between FGF, *one-eyed pinhead*, and T-box transcription factors during zebrafish posterior development. Development (Camb.), **264**(2): 456–466.

Guhathakurta, D., Palomar, L., Stormo, G.D., Tedesco, P., Johnson, T.E., Walker, D.W., Lithgow, G., Kim, S., and Link, C.D. 2002*a*. Identification of a novel cis-regulatory element involved in the heat shock response in *Caenorhabditis elegans* using microarray gene expression and computational methods. Genome Res. **12**(5): 701–712.

Guhathakurta, D., Schriefer, L.A., Hresko, M.C., Waterston, R.H., and Stormo, G.D. 2002*b*. Identifying muscle regulatory elements and genes in the nematode *Caenorhabditis elegans*. Pac. Symp. Biocomput. 2002: 425–436.

Halfon, M.S., Grad, Y., Church, G.M., and Michelson, A.M. 2002. Computation-based discovery of related transcriptional regulatory modules and motifs using an experimentally validated combinatorial model. Genome Res. **12**(7): 1019–1028.

Harafuji, N., Keys, D.N., and Levine, M. 2002. Genome-wide identification of tissue-specific enhancers in the *Ciona* tadpole. Proc. Natl. Acad. Sci. U.S.A. **99**(10): 6802–6805.

Herrmann, B.G., and Kispert, A. 1994. The *T* genes in embryogenesis. Trends Genet. **10**(8): 280–286.

Holland, P.W., Koschorz, B., Holland, L.Z., and Herrmann, B.G. 1995. Conservation of *Brachyury* (*T*) genes in amphioxus and vertebrates: developmental and evolutionary implications. Development (Camb.), **121**(12): 4283–4291.

Hotta, K., Takahashi, H., Erives, A., Levine, M., and Satoh, N. 1999. Temporal expression patterns of 39 *Brachyury*-downstream genes associated with notochord formation in the *Ciona intestinalis* embryo. Dev. Growth Differ. **41**(6): 657–664.

Hotta, K., Takahashi, H., Asakura, T., Saitoh, B., Takatori, N., Satou, Y., and Satoh, N. 2000. Characterization of *Brachyury*-downstream notochord genes in the *Ciona intestinalis* embryo. Dev. Biol. **224**(1): 69–80.

Imai, K., Takada, N., Satoh, N., and Satou, Y. 2000. (beta)-Catenin mediates the specification of endoderm cells in ascidian embryos. Development (Camb.), **127**(14): 3009–3020.

Imai, K.S., Satoh, N., and Satou, Y. 2002*a*. Early embryonic expression of *FGF4/6/9* gene and its role in the induction of mesenchyme and notochord in *Ciona savignyi* embryos. Development (Camb.), **129**(7): 1729–1738.

Imai, K.S., Satoh, N., and Satou, Y. 2002*b*. An essential role of a *FoxD* gene in notochord induction in *Ciona* embryos. Development (Camb.), **129**(14): 3441–3453.

Imai, K.S., Satou, Y., and Satoh, N. 2002*c*. Multiple functions of a *Zic*-like gene in the differentiation of notochord, central nervous system and muscle in *Ciona savignyi* embryos. Development (Camb.), **129**(11): 2723–2732.

Inaba, K., Padma, P., Satouh, Y., Shin, I.T., Kohara, Y., Satoh, N., and Satou, Y. 2002. EST analysis of gene expression in testis of the ascidian *Ciona intestinalis*. Mol. Reprod. Dev. **62**(4): 431–445.

Isaacs, H.V. 1997. New perspectives on the role of the fibroblast growth factor family in amphibian development. Cell. Mol. Life Sci. **53**(4): 350–361.

Ishibashi, T., Nakazawa, M., Ono, H., Satoh, N., Gojobori, T., and Fujiwara, S. 2003. Microarray analysis of embryonic retinoic

acid target genes in the ascidian *Ciona intestinalis*. Dev. Growth Differ. **45**(3): 249–259.

Kaestner, K.H., Lee, K.H., Schlondorff, J., Hiemisch, H., Monaghan, A.P., and Schutz, G. 1993. Six members of the mouse forkhead gene family are developmentally regulated. Proc. Natl. Acad. Sci. U.S.A. **90**(16): 7628–7631.

Kamei, S., Yajima, I., Yamamoto, H., Kobayashi, A., Makabe, K.W., Yamazaki, H., et al. 2000. Characterization of a novel member of the FGFR family, *HrFGFR*, in *Halocynthia roretzi*. Biochem. Biophys. Res. Commun. **275**(2): 503–508.

Kim, G.J., and Nishida, H. 2001. Role of the FGF and MEK signaling pathway in the ascidian embryo. Dev. Growth Differ. **43**(5): 521–533.

Kim, G.J., Yamada, A., and Nishida, H. 2000. An FGF signal from endoderm and localized factors in the posterior-vegetal egg cytoplasm pattern the mesodermal tissues in the ascidian embryo. Development (Camb.), **127**(13): 2853–2862.

Kispert, A., and Herrmann, B.G. 1993. The Brachyury gene encodes a novel DNA binding protein. EMBO J. **12**(8): 3211–3220.

Kispert, A., Herrmann, B.G., Leptin, M., and Reuter, R. 1994. Homologs of the mouse Brachyury gene are involved in the specification of posterior terminal structures in *Drosophila*, *Tribolium*, and *Locusta*. Genes Dev. **8**(18): 2137–2150.

Kobayashi, K., Sawada, K., Yamamoto, H., Wada, S., Saiga, H., and Nishida, H. 2003. Maternal macho-1 is an intrinsic factor that makes cell response to the same FGF signal differ between mesenchyme and notochord induction in ascidian embryos. Development (Camb.), **130**(21): 5179–5190.

Kowalevsky, A. 1866. Entwickelungsgeschichte der einfachen Ascidien. Mem. Acad. Imp. Sci. St. Petersbourg, 10 Ser. **7**: 1–19.

Kowalevsky, A. 1871. Weitere Studien uber die Entwicklung der einfachen Ascidien. Arch. Mikrosk. Anat. **5**: 459–463.

Kusakabe, T., Yoshida, R., Kawakami, I., Kusakabe, R., Mochizuki, Y., Yamada, L., et al. 2002. Gene expression profiles in tadpole larvae of *Ciona intestinalis*. Dev. Biol. **242**(2): 188–203.

Loots, G.G., Ovcharenko, I., Pachter, L., Dubchak, I., and Rubin, E.M. 2002. rVista for comparative sequence-based discovery of functional transcription factor binding sites. Genome Res. **12**(5): 832–839.

Marcellini, S., Technau, U., Smith, J.C., and Lemaire, P. 2003. Evolution of Brachyury proteins: identification of a novel regulatory domain conserved within Bilateria. Dev. Biol. **260**(2): 352–361.

Mayor, C., Brudno, M., Schwartz, J.R., Poliakov, A., Rubin, E.M., Frazer, K.A., et al. 2000. VISTA : visualizing global DNA sequence alignments of arbitrary length. Bioinformatics, **16**(11): 1046–1047.

Minokawa, T., Yagi, K., Makabe, K.W., and Nishida, H. 2001. Binary specification of nerve cord and notochord cell fates in ascidian embryos. Development (Camb.), **128**(11): 2007–2017.

Miya, T., and Nishida, H. 2003. An Ets transcription factor, *HrEts*, is target of FGF signaling and involved in induction of notochord, mesenchyme, and brain in ascidian embryos. Dev. Biol. **261**(1): 25–38.

Miyamoto, D.M., and Crowther, R.J. 1985. Formation of the notochord in living ascidian embryos. J. Embryol. Exp. Morphol. **86**: 1–17.

Moody, R., Davis, S.W., Cubas, F., and Smith, W.C. 1999. Isolation of developmental mutants of the ascidian *Ciona savignyi*. Mol. Gen. Genet. **262**(1): 199–206.

Munro, E.M., and Odell, G.M. 2002*a*. Morphogenetic pattern formation during ascidian notochord formation is regulative and highly robust. Development (Camb.), **129**(1): 1–12.

Munro, E.M., and Odell, G.M. 2002*b*. Polarized basolateral cell motility underlies invagination and convergent extension of the ascidian notochord. Development (Camb.), **129**(1): 13–24.

Nakatani, Y., and Nishida, H. 1994. Induction of notochord during ascidian embryogenesis. Dev. Biol. **166**(1): 289–299.

Nakatani, Y., and Nishida, H. 1997. Ras is an essential component for notochord formation during ascidian embryogenesis. Mech. Dev. **68**(1–2): 81–89.

Nakatani, Y., Yasuo, H., Satoh, N., and Nishida, H. 1996. Basic fibroblast growth factor induces notochord formation and the expression of As-T, a Brachyury homolog, during ascidian embryogenesis. Development (Camb.), **122**(7): 2023–2031.

Nakatani, Y., Moody, R., and Smith, W.C. 1999. Mutations affecting tail and notochord development in the ascidian *Ciona savignyi*. Development (Camb.), **126**(15): 3293–3301.

Nishida, H. 1987. Cell lineage analysis in ascidian embryos by intracellular injection of a tracer enzyme. III. Up to the tissue restricted stage. Dev. Biol. **121**(2): 526–541.

Nishida, H. 1992. Developmental potential for tissue differentiation of fully dissociated cells of the ascidian embryo. Roux's Arch. Dev. Biol. **201**: 81–87.

Nishida, H. 2002. Patterning the marginal zone of early ascidian embryos: localized maternal mRNA and inductive interactions. Bioessays, **24**(7): 613–624.

Nishida, H., and Satoh, N. 1983. Cell lineage analysis in ascidian embryos by intracellular injection of a tracer enzyme. I. Up to the eight-cell stage. Dev. Biol. **99**(2): 382–394.

Nishida, H., and Satoh, N. 1985. Cell lineage analysis in ascidian embryos by intracellular injection of a tracer enzyme. II. The 16- and 32-cell stages. Dev. Biol. **110**(2): 440–454.

Nishida, H., and Sawada, K. 2001. macho-1 encodes a localized mRNA in ascidian eggs that specifies muscle fate during embryogenesis. Nature (Lond.), **409**(6821): 724–729.

Nishikata, T., and Satoh, N. 1990. Specification of notochord cells in the ascidian embryo analysed with a specific monoclonal antibody. Cell Differ. Dev. **30**(1): 43–53.

Nishikata, T., Yamada, L., Mochizuki, Y., Satou, Y., Shin-i, T., Kohara, Y., and Satoh, N. 2001. Profiles of maternally expressed genes in fertilized eggs of *Ciona intestinalis*. Dev. Biol. **238**(2): 315–331.

Nishino, A., Satou, Y., Morisawa, M., and Satoh, N. 2001. Brachyury (T) gene expression and notochord development in *Oikopleura longicauda* (Appendicularia, Urochordata). Dev. Genes Evol. **211**(5): 219–231.

Odenthal, J., Haffter, P., Vogelsang, E., Brand, M., van Eeden, F.J., Furutani-Seiki, M., et al. 1996. Mutations affecting the formation of the notochord in the zebrafish, *Danio rerio*. Development (Camb.), **123**: 103–115.

Ogasawara, M., Sasaki, A., Metoki, H., Shin-i, T., Kohara, Y., Satoh, N., and Satou, Y. 2002. Gene expression profiles in young adult *Ciona intestinalis*. Dev. Genes Evol. **212**(4): 173–185.

Oliveri, P., Carrick, D.M., and Davidson, E.H. 2002. A regulatory gene network that directs micromere specification in the sea urchin embryo. Dev. Biol. **246**(1): 209–228.

Olsen, C.L., and Jeffery, W.R. 1997. A forkhead gene related to HNF-3beta is required for gastrulation and axis formation in the ascidian embryo. Development (Camb.) **124**(18): 3609–3619.

O'Reilly, M.A., Smith, J.C., and Cunliffe, V. 1995. Patterning of the mesoderm in *Xenopus*: dose-dependent and synergistic ef-

fects of *Brachyury* and *Pintallavis*. Development (Camb.), **121**(5): 1351–1359.

Ortolani, G. 1955. The presumptive territory of the mesoderm in the ascidian germ. Experientia (Basel), **11**: 445–446.

Ortolani, G. 1957. Il territorio precoce della corda nelle ascidie. Acta. Embryol. Morphol. Exp. 1: 33–36.

Ortolani, G. 1962. Territorio presuntivo del sistema nervoso nelle larve di ascidie. Acta. Embryol. Morphol. Exp. **5**: 189–198.

Papaioannou, V.E., and Silver, L.M. 1998. The T-box gene family. Bioessays, **20**(1): 9–19.

Peterson, K.J., Cameron, R.A., Tagawa, K., Satoh, N., and Davidson, E.H. 1999*a*. A comparative molecular approach to mesodermal patterning in basal deuterostomes: the expression pattern of *Brachyury* in the enteropneust hemichordate *Ptychodera flava*. Dev. Biol. **126**(1): 85–95.

Peterson, K.J., Harada, Y., Cameron, R.A., and Davidson, E.H. 1999*b*. Expression pattern of *Brachyury* and *Not* in the sea urchin: comparative implications for the origins of mesoderm in the basal deuterostomes. Dev. Biol. **207**(2): 419–431.

Quandt, K., Frech, K., Karas, H., Wingender, E., and Werner, T. 1995. MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. Nucleic Acids Res. **23**(23): 4878–4884.

Rast, J.P., Cameron, R.A., Poustka, A.J., and Davidson, E.H. 2002. brachyury Target genes in the early sea urchin embryo isolated by differential macroarray screening. Dev. Biol. **246**(1): 191–208.

Ruiz i Altaba, A., and Jessell, T.M. 1992. *Pintallavis*, a gene expressed in the organizer and midline cells of frog embryos: involvement in the development of the neural axis. Development (Camb.), **116**(1): 81–93.

Sasai, N., Mizuseki, K., and Sasai, Y. 2001. Requirement of *FoxD3*-class signaling for neural crest determination in *Xenopus*. Development (Camb.), **128**(13): 2525–2536.

Sasakura, Y., Awazu, S., Chiba, S., and Satoh, N. 2003. Germ-line transgenesis of the Tc1/*mariner* superfamily transposon *Minos* in *Ciona intestinalis*. Proc. Natl. Acad. Sci. U.S.A. **100**(13): 7726–7730.

Satoh, G., Harada, Y., and Satoh, N. 2000. The expression of nonchordate deuterostome *Brachyury* genes in the ascidian *Ciona* embryo can promote the differentiation of extra notochord cells. Mech. Dev. **96**(2): 155–163.

Satoh, N. 1994. Developmental biology of ascidians. Cambridge University Press, Cambridge, UK.

Satoh, N. 2001. Ascidian embryos as a model system to analyze expression and function of developmental genes. Differentiation, **68**(1): 1–12.

Satoh, N. 2003. The ascidian tadpole larva: comparative molecular development and genomics. Nat. Rev. Genet. **4**(4): 285–295.

Satoh, N., Satou, Y., Davidson, B., and Levine, M. 2003. *Ciona intestinalis*: an emerging model for whole-genome analyses. Trends Genet. **19**(7): 376–381.

Satou, Y., and Satoh, N. 1999. Developmental gene activities in ascidian embryos. Curr. Opin. Genet. Dev. **9**(5): 542–547.

Satou, Y., Imai, K.S., and Satoh, N. 2001*a*. Action of morpholinos in *Ciona* embryos. Genesis, **30**(3): 103–106.

Satou, Y., Imai, K.S., and Satoh, N. 2001*b*. Early embryonic expression of a LIM-homeobox gene *Cs-lhx3* is downstream of β-catenin and responsible for the endoderm differentiation in *Ciona savignyi* embryos. Development (Camb.), **128**(18): 3559–3570.

Satou, Y., Takatori, N., Yamada, L., Mochizuki, Y., Hamaguchi, M., Ishikawa, H., et al. 2001*c*. Gene expression profiles in *Ciona intestinalis* tailbud embryos. Development (Camb.), **128**(15): 2893–2904.

Satou, Y., Imai, K.S., and Satoh, N. 2002. Fgf genes in the basal chordate *Ciona intestinalis*. Dev. Genes Evol. **212**(9): 432–438.

Schmid, A., Schindelholz, B., and Zinn, K. 2002. Combinatorial RNAi: a method for evaluating the functions of gene families in *Drosophila*. Trends Neurosci. **25**(2): 71–74.

Schulte-Merker, S., and Smith, J.C. 1995. Mesoderm formation in response to Brachyury requires FGF signalling. Curr. Biol. **5**(1): 62–67.

Schulte-Merker, S., van Eeden, F.J., Halpern, M.E., Kimmel, C.B., and Neusslein-Volhard, C. 1994. *no tail* (*ntl*) is the zebrafish homologue of the mouse *T* (*Brachyury*) gene. Development (Camb.), **120**(4): 1009–1015.

Shimauchi, Y., Yasuo, H., and Satoh, N. 1997. Autonomy of ascidian *fork head/HNF-3* gene expression. Mech. Dev. **69**(1–2): 143–54.

Shimauchi, Y., Chiba, S., and Satoh, N. 2001*a*. Synergistic action of *HNF-3* and *Brachyury* in the notochord differentiation of ascidian embryos. Int. J. Dev. Biol. **45**(4): 643–652.

Shimauchi, Y., Murakami, S.D., and Satoh, N. 2001*b*. FGF signals are involved in the differentiation of notochord cells and mesenchyme cells of the ascidian *Halocynthia roretzi*. Development (Camb.), **128**(14): 2711–2721.

Shimeld, S.M. 1997. Characterisation of amphioxus *HNF-3* genes: conserved expression in the notochord and floor plate. Dev. Biol. **183**(1): 74–85.

Smith, J. 1997. *Brachyury* and the *T-box* genes. Curr. Opin. Genet. Dev. **7**(4): 474–480.

Smith, J. 1999. *T-box* genes: what they do and how they do it. Trends Genet. **15**(4): 154–158.

Smith, J.C., Price, B.M., Green, J.B., Weigel, D., and Herrmann, B.G. 1991. Expression of a *Xenopus* homolog of *Brachyury* (*T*) is an immediate-early response to mesoderm induction. Cell, **67**(1): 79–87.

Sordino, P., Heisenberg, C.-P., Cirino, P., Toscano, A., Giuliano, P., Marino, R., Pinto, M.R., and DeSantis, R. 2000. A mutational approach to the study of development of the protochordate *Ciona intestinalis* (Tunicata, Chordata). Sarsia, **85**: 173–176.

Sordino, P., Belluzzi, L., De Santis, R., and Smith, W.C. 2001. Developmental genetics in primitive chordates. Philos. Trans. R. Soc. Lond. B Biol. Sci. **356**(1414): 1573–1582.

Stein, S., and Kessel, M. 1995. A homeobox gene involved in node, notochord and neural plate formation of chick embryos. Mech. Dev. **49**(1–2): 37–48.

Stein, S., Niss, K., and Kessel, M. 1996. Differential activation of the clustered homeobox genes *CNOT2* and *CNOT1* during notogenesis in the chick. Dev. Biol. **180**(2): 519–533.

Stemple, D.L., Solnica-Krezel, L., Zwartkruis, F., Neuhauss, S.C., Schier, A.F., Malicki, J., et al. 1996. Mutations affecting development of the notochord in zebrafish. Development (Camb.), **123**: 117–128.

Stormo, G.D., and Tan, K. 2002. Mining genome databases to identify and understand new gene regulatory systems. Curr. Opin. Microbiol. **5**(2): 149–153.

Strahle, U., Blader, P., Henrique, D., and Ingham, P.W. 1993. Axial, a zebrafish gene expressed along the developing body axis, shows altered expression in cyclops mutant embryos. Genes Dev. **7**: 1436–1446.

Tada, M., O'Reilly, M.A., and Smith, J.C. 1997. Analysis of competence and of *Brachyury* autoinduction by use of hormone-inducible *Xbra*. Development (Camb.), **124**(11): 2225–2234.

Tada, M., and Smith, J.C. 2001. T-targets: clues to understanding

the functions of T-box proteins. Dev. Growth Differ. **43**(1): 1–11.

Tagawa, K., Humphreys, T., and Satoh, N. 1998. Novel pattern of *Brachyury* gene expression in hemichordate embryos. Mech. Dev. **75**(1–2): 139–143.

Takada, N., York, J., Davis, J.M., Schumpert, B., Yasuo, H., Satoh, N., and Swalla, B.J. 2002. *Brachyury* expression in tailless Molgulid ascidian embryos. Evol. Dev. **4**(3): 205–211.

Takahashi, H., Hotta, K., Erives, A., Di Gregorio, A., Zeller, R.W., Levine, M., and Satoh, N. 1999. *Brachyury* downstream notochord differentiation in the ascidian embryo. Genes Dev. **13**(12): 1519–1523.

Takamura, K., Oka, N., Akagi, A., Okamoto, K., Okada, T., Fukuoka, T., et al. 2001. EST analysis of genes that are expressed in the neural complex of *Ciona intestinalis* adults. Zool. Sci. (Tokyo), **18**(9): 1231–1236.

Talbot, W.S., Trevarrow, B., Halpern, M.E., Melby, A.E., Farr, G., Postlethwait, J.H., et al. 1995. A homeobox gene essential for zebrafish notochord development. Nature (Lond.), **378**(6553): 150–157.

Technau, U. 2001. *Brachyury*, the blastopore and the evolution of the mesoderm. Bioessays, **23**(9): 788–794.

von Dassow, G., Schmidt, J.E., and Kimelman, D. 1993. Induction of the *Xenopus* organizer: expression and regulation of Xnot, a novel FGF and activin-regulated homeo box gene. Genes Dev. **7**(3): 355–366.

Wada, S., and Saiga, H. 2002. *HrzicN*, a new Zic family gene of ascidians, plays essential roles in the neural tube and notochord development. Development (Camb.), **129**(24): 5597–5608.

Wang, T., and Stormo, G.D. 2003. Combining phylogenetic data with co-regulated genes to identify regulatory motifs. Bioinformatics, **19**(18): 2369–2380.

Wilkinson, D.G., Bhatt, S., and Herrmann, B.G. 1990. Expression pattern of the mouse *T* gene and its role in mesoderm formation. Nature (Lond.), **343**(6259): 657–659.

Wingender, E., Dietze, P., Karas, H., and Knuppel, R. 1996. TRANSFAC: a database on transcription factors and their DNA binding sites. Nucleic Acids Res. **24**(1): 238–241.

Yagi, K., Satou, Y., and Satoh, N. 2004. A zinc finger transcription factor, ZicL, is a direct activator of *Brachyury* in the notochord specification of *Ciona intestinalis*. Development (Camb.), **131**(6): 1279–1288.

Yamada, L., Shoguchi, E., Wada, S., Kobayashi, K., Mochizuki, Y., Satou, Y., and Satoh, N. 2003. Morpholino-based gene knockdown screen of novel genes with developmental function in *Ciona intestinalis*. Development (Camb.), **130**(26): 6485–6495.

Yasuo, H., and Lemaire, P. 2001. Role of Goosecoid, Xnot and Wnt antagonists in the maintenance of the notochord genetic programme in *Xenopus* gastrulae. Development (Camb.), **128**(19): 3783–3793.

Yasuo, H., and Satoh, N. 1993. Function of vertebrate *T* gene. Nature (Lond.), **364**(6438): 582–583.

Yasuo, H., and Satoh, N. 1994. An ascidian homolog of the mouse brachyury T gene is expressed exclusively in notochord cells at the fate restricted stage. Dev. Growth Differ. **36**: 9–18.

Yasuo, H., and Satoh, N. 1998. Conservation of the developmental role of *Brachyury* in notochord formation in a urochordate, the ascidian *Halocynthia roretzi*. Dev. Biol. **200**(2): 158–170.

Zeller, R.W. 2004. Generation and use of transgenic ascidians. *In* Experimental analysis of the development of sea urchins and other non-vertebrate deuterostomes. Methods Cell Biol. **74**: 713–730.

Zhang, J., Talbot, W.S., and Schier, A.F. 1998. Positional cloning identifies zebrafish one-eyed pinhead as a permissive EGF-related ligand required during gastrulation. Cell, **92**(2): 241–251.