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Ciona intestinalis: Chordate Development Made Simple

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Thanks to their transparent and rapidly developing mosaic embryos, ascidians (or sea squirts) have been a model system for embryological studies for over a century. Recently, ascidians have entered the postgenomic era, with the sequencing of the *Ciona intestinalis* genome and the accumulation of molecular resources that rival those available for fruit flies and mice. One strength of ascidians as a model system is their close similarity to vertebrates. Literature reporting molecular homologies between vertebrate and ascidian tissues has flourished over the past 15 years, since the first ascidian genes were cloned. However, it should not be forgotten that ascidians diverged from the lineage leading to vertebrates over 500 million years ago. Here, we review the main similarities and differences so far identified, at the molecular level, between ascidian and vertebrate tissues and discuss the evolution of the compact ascidian genome. *Developmental Dynamics 233:1–19, 2005.* © 2005 Wiley-Liss, Inc.

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INTRODUCTION

The ascidian *Ciona intestinalis* has emerged recently as a model system for understanding the evolution of chordate development and genome organization. Ascidians are members of the chordate clade Urochordata, which diverged from the last common ancestor of all chordates at least 520 million years ago (Chen et al., 2003). This divergence creates over one billion years of independent evolution between extant ascidians and modern vertebrates, such as human, mouse, chick, frog, and zebrafish. Despite this evolutionary distance, the basic features of the chordate body plan remain recognizable in ascidian larvae. Because of their relative simplicity and their position as an outgroup to the vertebrates, ascidians have unique potential to illuminate the molecular mechanisms underlying the primitive body plan from which modern chordates diversified.

The close relationship between ascidians and vertebrates was first recognized in the mid-19th century by the embryologist Alexander Kowalevsky, who noted the striking similarities between ascidian larvae and vertebrate embryos (Kowalevsky, 1866). Kowalevsky's observation of a notochord and a dorsal neural tube in the ascidian larva provided clear evidence that ascidians are, along with vertebrates and the cephalochordate amphioxus, members of the phylum Chordata. The adult ascidian also possesses recognizable chordate features, even though it is considered more divergent than the larva and is a sedentary filter-feeder (although some deep-sea ascidians are adapted to capture larger food particles; Fig. 1). The feeding basket of the adult

ABBREVIATIONS CNS central nervous system **kb** kilobase(s) **Mb** megabase(s) **bHLH** basic helix-loop-helix **FGF** fibroblast growth factor **TVC** trunk ventral cell **DCEN** dorsocaudal epidermal neuron **VCEN** ventrocaudal epidermal neuron **GFP** green fluorescent protein

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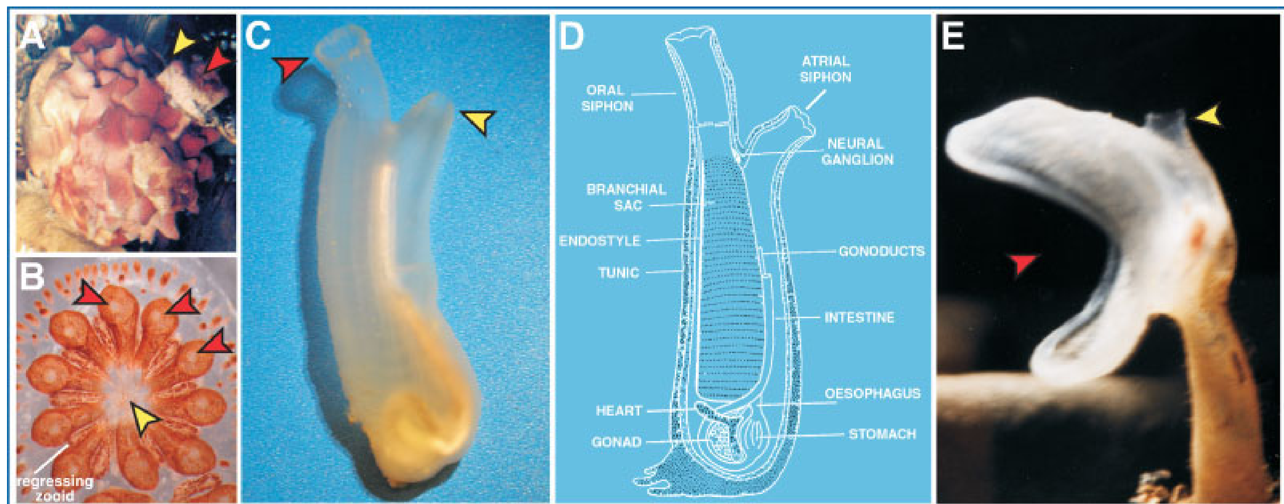

Fig. 1. Morphological diversity of adult ascidians. A,B,C,E: Red arrowheads point to the oral siphon; yellow arrowheads point to the atrial siphon. **A:** The solitary ascidian *Halocynthia roretzi*, which is used as food crop in Japan. **B:** The colonial ascidian *Botryllus schlosseri*. Colonies may be composed of up to 100 systems of 7–10 zooids produced asexually through budding. Each zooid has its own oral siphon, whereas all zooids in a system share a common atrial siphon. The colony shown is in "Stage D," or the takeover stage of colony development. Older zooids are regressing through apoptosis, whereas younger buds are preparing to open their oral siphons (photograph courtesy of Dr. Diana Laird). **C:** The solitary ascidian *Ciona intestinalis*. **D:** Schematic diagram of the adult morphology of *Ciona*. **E:** The predatory deep-sea ascidian *Megalodicopia hians*. The atrial siphon is enlarged and highly modified to close quickly and capture large food particles, including small crustaceans (cfr. Okuyama et al., 2002).

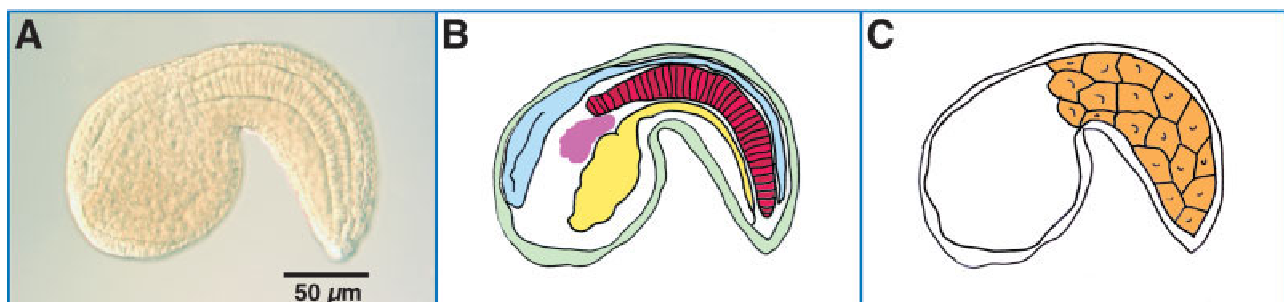

Fig. 2. Tissue organization in the ascidian larva. **A:** Photomicrograph of a *Ciona intestinalis* larva ~14 hr postfertilization, developing at 15°C. B,C: Drawings showing the six larval tissues. **B:** The central nervous system (blue), the notochord (red), the endoderm (yellow), the mesenchyme (purple), and the epidermis (green) are represented. Although the epidermis is shown only in section, it covers the entire larva. **C:** The 18 muscle cells, which flank each side of the notochord, are shown in orange.

contains gill slits that appear to share a common origin with the gill slits of other chordates (Aros and Viragh, 1969). Likewise, the endostyle of the adult ascidian is a homolog of the vertebrate thyroid gland, sequestering iodine and producing thyroid hormone (Eales, 1997).

As a model system for understanding chordate development, ascidians offer several advantages. First, the ascidian larva is relatively simple, being composed of only ~2,500 cells when fully developed (Satoh, 1994). Furthermore, the cleavage program of the embryo is invariant, and accurate fate maps have been drawn to trace the embryonic development of different ascidian species (Conklin, 1905; Ortolani, 1971; Nishida, 1987). When fully developed, the ascidian larva contains only six tissue types: nervous system, notochord, muscle, ectoderm, endoderm, and mesenchyme (Fig. 2). The notochord is composed of only 40 cells; the muscle of 36 cells (40 cells in the Japanese ascidian *Halocynthia roretzi*); and the central nervous system (CNS) of ~350 cells, of which ~100 are neurons (Nicol and Meinertzhagen, 1991; Satoh, 1994). *Ciona* embryos and larvae are transparent, allowing for direct observation of tissues without need for sectioning.

The recent sequencing and assembly of the *C. intestinalis* genome by the Joint Genome Institute (JGI; Dehal et al., 2002), greatly aids molecular studies, allowing rapid identification of genes of interest and of the noncoding regions flanking them. The ongoing sequencing of the closely related species *Ciona savignyi* (http://www.broad.mit.edu/annotation/ciona/) should provide an invaluable resource for comparative approaches, such as phylogenetic footprinting, to identify conserved *cis*-regulatory regions (e.g., Zhang and Gerstein, 2003).

Ciona embryos are readily amenable to experimentation in the laboratory. Adults are hermaphrodites, and in vitro fertilization can produce thousands of synchronously dividing embryos. In addition, the *C. intestinalis* embryo develops rapidly, with the tadpole larva completing development in 18 hr when reared at 18°C (Whittaker, 1977).

Transgenic experiments in *C. intestinalis* are greatly facilitated by the ease of introducing plasmid DNA into fertilized eggs by means of electroporation. This feature allows rapid, large-scale screening of genomic DNA fragments linked to reporter genes for the identification of *cis*-regulatory elements controlling gene expression, as well as of plasmids misexpressing tissue-specific genes to analyze their function (reviewed by Di Gregorio and Levine, 2002). These advantages have made *C. intestinalis* a valuable model for understanding the gene networks controlling chordate development.

Developmental mutants have also been generated in *C. intestinalis* and *C. savignyi* by chemical mutagenesis and, more recently, through insertional mutagenesis using the *Minos* transposable element (Moody et al., 1999; Nakatani et al., 1999; Sordino et al., 2001; Sasakura et al., 2003a, b; Matsouka et al., 2004). Stable transgenics expressing GFP have also been generated in both species, allowing enhancer-trap screens to be conducted (Deschet et al., 2003; Sasakura et al., 2003a, b; Matsuoka et al., 2004; Awazu et al., 2004). These advances make a suite of powerful techniques for germline mutagenesis finally applicable to ascidians.

In this article, we review the current understanding of ascidian development, focusing on structures that are considered to be homologs of vertebrate tissues. We will discuss the molecular mechanisms underlying the development of these structures, in regard to similarities observed across chordates as well as differences that may represent clade-specific acquisitions or losses. We will focus primarily upon evidence from *C. intestinalis* but will also discuss findings from other ascidians where appropriate.

ASCIDIAN GENOMICS

The *C. intestinalis* genome is ~155 Mb in size, or approximately one-twentieth the size of the human genome (Dehal et al., 2002). Of these, ~117 Mb are composed of nonrepetitive, euchromatic sequence, coding for ~16,000 genes, approximately half the number present in the human genome. An annotated assembly of the genome is freely available through the JGI's Web site (http://genome.jgi-psf.org/ciona/), providing a resource that greatly accelerates the identification of homologs of genes previously studied in other organisms.

In addition to the sequencing of the genome, a large-scale expressed sequence tag (EST) project has been carried out, resulting in the characterization of ~18,000 independent cDNA clones, estimated to represent ~85% of the *C. intestinalis* transcripts (Satou et al., 2002a; Satoh et al., 2003). The results of this EST survey are available through the Kyoto University Web site (http://ghost.zool.kyoto-u.ac.jp/indexr1.html), and in most cases cDNA clones are linked to their best hit in the *C. intestinalis* genome assembly. For several cDNA clones, in particular for those encoding transcription factors, expression data from *in situ* hybridizations are also available (Satoh et al., 2003; Imai et al., 2004).

The small size of the *C. intestinalis* genome provides a distinct advantage for understanding genome organization and gene function. The urochordate lineage diverged before the extensive gene duplications that have occurred in vertebrates. Therefore, in many cases, the *C. intestinalis* genome contains only a single homolog of the multiple paralogous genes present in vertebrate genomes. For example, there are only 6 fibroblast growth factor (FGF) genes in the *C. intestinalis* genome, compared with the 22 FGF genes found in vertebrates, and 5 of the 6 *C. intestinalis* FGF genes show homology with vertebrate FGF genes (Satou et al., 2002b).

However, it is important to note that the *C. intestinalis* genome is not simply a miniature model of vertebrate genomes, but has undergone considerable independent evolution. For example, there are three *Tbx6* genes in the *C. intestinalis* genome, resulting from lineage-specific duplications (Takatori et al., 2004). *Cyclooxygenase* (*COX*) also appears to have undergone independent duplication events in ascidians and vertebrates (Jarving et al., 2004). Likewise, the *C. intestinalis* genome does not contain homologs of all vertebrate genes. For instance, *C. intestinalis* appears to lack homologs of the *Hox7*, *Hox8*, *Hox9*, and *Hox14* paralog groups (Fig. 3; Dehal et al., 2002; Spagnuolo et al., 2003). The apparent absence of *Hox7* and *Hox8* genes likely represents lineage-specific losses, as members of these paralog groups are present in echinoderms, amphioxus, and vertebrates (Long and Byrne, 2001; Holland, 2003). The absence of *Hox9* is more equivocal, as *CiHox10* may represent a direct ortholog of *Hox9/10* paralog group genes in echinoderms (Long and Byrne, 2001). Similarly, two clustered posterior Hox genes, both related to *Abd-B*, have been indicated as *CiHox11/12* and *CiHox12/13* due to their ambiguous sequence homologies (Wada et al., 2003). The absence of a *Hox14* homolog suggests that no member of this paralog group was present in the last common ancestor of chordates. Although *Hox14* genes have been identified from amphioxus and gnathostomes, it remains unclear whether a *Hox14* gene was present in the common ancestor of cephalochordates and vertebrates (Powers and Amemiya, 2004; Ferrier, 2004).

Finally, lineage-specific innovations are also present in the *C. intestinalis* genome. For example, functional cellulose synthases have been identified from both *C. intestinalis* and *C. savignyi*. These enzymes, which are responsible for the synthesis of cellulose, a component of the tunic, represent the only known example of cellulose synthesis in metazoans. Cellulose synthase may have been acquired early in the evolution of the urochordate lineage through a lateral transfer of the gene from a bacterial genome into the urochordate genome (Dehal et al., 2002; Matthysse et al., 2004; Nakashima et al., 2004).

Despite its lineage-specific innovations, the *C. intestinalis* genome provides a valuable tool for understanding general principles of gene function and regulation applicable to vertebrates. The majority of genes in the *C. intestinalis* genome are homologs of vertebrate genes (Dehal et al., 2002). In addition, the relatively compact organization of the ascidian genome (~1 gene per 7.5 kb of sequence in *C. intestinalis* compared with ~1 gene per 100 kb in humans) simplifies the search for regulatory regions controlling gene expression (Dehal et al., 2002; Di Gregorio and Levine, 2002).

Fig. 3.

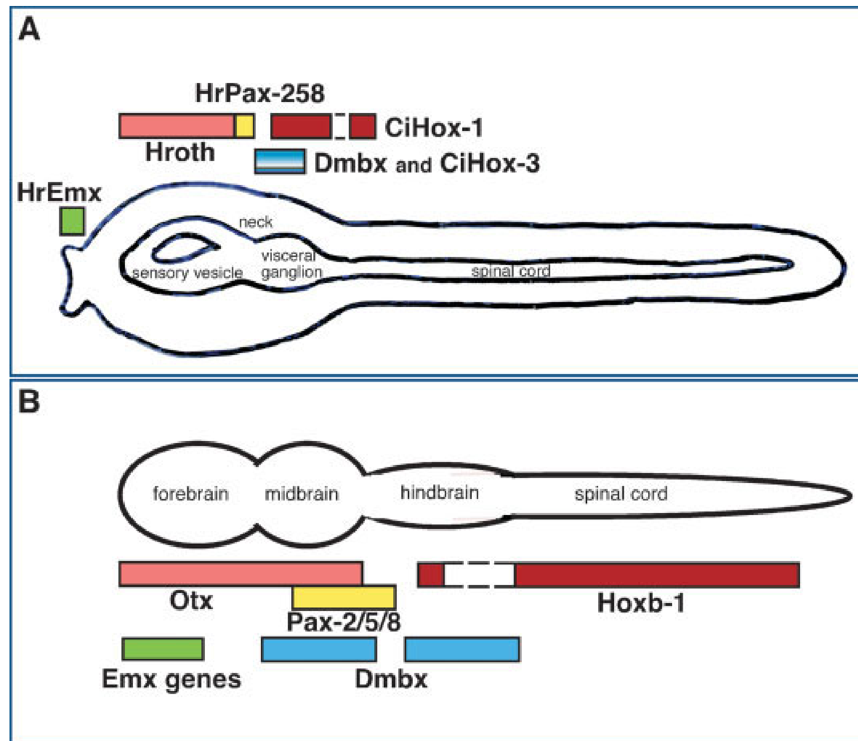

Fig. 4.

LARVAL TISSUES

Notochord

The notochord is an axial structure unique to the chordate lineage. Derived from mesodermal tissue, the notochord provides support for paraxial tissues in the embryo, being replaced by the ossified spine later in the development of vertebrate embryos. The vertebrate notochord also provides signals crucial to the patterning of surrounding tissues such as the nascent spinal cord. Whereas the notochord of vertebrates contains hundreds of cells, the fully developed notochord of the *C. intestinalis* larva is composed of only 40 cells (Satoh, 1994). The ascidian notochord arises from two cell lineages that become clonally restricted early in development. The 32 anterior and medial notochord cells, referred to as the primary or A-lineage, are derived from blastomeres in the anterior half of the embryo at the 64-cell stage. The eight posterior cells of the notochord, termed the secondary or B-lineage, are derived from blastomeres in the posterior half of the embryo at the 110-cell stage. After neurulation, these 40 cells undergo convergence and extension to form the notochord (Munro and Odell, 2002). The small number and relatively large size of these cells make the ascidian notochord particularly amenable to experimentation.

Experimental manipulations have demonstrated that specification of the primary notochord lineage of ascidians requires inductive signals from vegetal blastomeres fated to become endoderm (Nakatani and Nishida, 1994). This requirement seems to be similar to the inductive specification of mesoderm by vegetal cells in amphibians (Nieuwkoop, 1969). The factors leading to notochord specification have been well studied and have been thoroughly reviewed by Satoh (2003). Briefly, nuclear localization of maternal β-catenin in endodermal precursors before the 32-cell stage occurs in response to the FGF signal emanating from the endoderm and, in turn, directly activates expression of *FoxD*, a gene encoding a winged-helix transcription factor required for induction of both the primary and secondary notochord lineages (Imai et al., 2002a). In primary notochord precursors, the zinc finger transcription factor ZicL acts downstream of FoxD and binds sites in the *Brachyury* enhancer required for its activation (Yagi et al., 2004a). Suppressor of Hairless [Su(H)] also binds to the *Brachyury* promoter, activating expression (Corbo et al., 1998). In the secondary notochord lineage Notch signaling appears to have a role in transducing the signal from FoxD in endodermal cells to Su(H) in notochord cells (Imai et al., 2002a).

In *Xenopus*, notochord induction relies upon different inducing signals, including activin and members of the Wnt family of signaling molecules (e.g., Sokol and Melton, 1992). FGF signaling is mediated in vertebrate embryos by the Ras/MEK/MAPK cascade. In ascidians, these intermediates have been shown to play a conserved role during formation of the secondary notochord cells, as well as in the specification of the neural tissue (Kim and Nishida, 2001; Hudson et al., 2003).

Studies carried out by Nishida and colleagues show that activin does not have notochord-inducing activity in ascidians, whereas bFGF is able to induce the expression of *As-T*, the *Halocynthia* homolog of *Brachyury*, as well as notochord formation (Nakatani et al., 1996). More recent work from the Nishida group shows that a transcription factor of the Ets family, HrEts, is a key mediator of the FGF-inducing activity not only for the notochord but also for mesenchyme and CNS (Miya and Nishida, 2003).

In ascidians, as well as in vertebrates, the best characterized initiator of notochord formation is *Brachyury*, which encodes a T-box transcription factor essential for development of the notochord in all chordates where it has been studied (Herrmann and Kispert, 1994). Yasuo and Satoh (1993) demonstrated that the *Brachyury* homolog in *H. roretzi* is expressed exclusively in the notochord and its precursors, providing the first molecular evidence for the homology of ascidian and vertebrate notochords. Subsequent analysis of the *C. intestinalis Brachyury* (*Ci-Bra*) gene has shown a similar notochord-specific pattern of expression (Corbo et al., 1997a). The requirement of Brachyury for notochord formation in *C. intestinalis* has been demonstrated through the down-regulation of *Ci-Bra* expression by the action of the *Xenopus bix* gene (Di Gregorio et al., 2002). Notochord development has also been blocked in *C. savignyi* by morpholino-directed inhibition of *Brachyury* translation (Satou et al., 2001a).

Fig. 3. Hox gene complements in representative chordates. Boxes represent gene loci and are color-coded to identify paralogous groups of genes. Lines between gene boxes represent chromosomal linkage, where known. Drawings of a mouse embryo, amphioxus, and a *Ciona intestinalis* larva are shown (not to scale), with the positions of central nervous system (CNS, blue), notochord (red), endoderm (yellow), and mesodermal mesenchyme (purple; shown only in *Ciona*) depicted. **A:** *Hox* genes in the mouse. Organization of *Hox* genes in four chromosomal clusters is shown at the top of the panel. *Hox* gene expression patterns along the anteroposterior axis are shown overlying a mouse embryo. An approximate representation of gene expression domains along the anteroposterior axis is shown for members of the mouse *HoxB* cluster (adapted from Gilbert, 2003; Zeltser et al., 1996). **B:** *Hox* genes in the cephalochordate amphioxus. Expression domains of *AmphiHox-1*, *AmphiHox-3*, and *AmphiHox-4* are adapted from Wada et al. (1999a). **C:** *Hox* genes in *C. intestinalis*. Gene expression patterns in the larva are shown as colored bars. *CiHox1*, *CiHox3*, *CiHox5/7*, *CiHox10*, and *CiHox11/12* are expressed in the CNS. *CiHox5/7* is also expressed in the trunk mesenchyme, as are *CiHox2* and *CiHox4*. *CiHox10* also shows expression in the larval endoderm. In the juvenile, *CiHox3* is expressed in the neural complex and the heart, whereas *CiHox10*, *CiHox11/12*, and *CiHox12/13* are expressed in the intestine (adapted from Gionti et al., 1998; Locascio et al., 1999; Nagatomo and Fujiwara, 2003; Ikuta et al., 2004).

Fig. 4. Comparison of generalized gene expression patterns in the central nervous system (CNS) of ascidians and vertebrates. Homologous genes are coded in the same colors. *Otx* (pink), *Pax2/5/8* (yellow), *Dmbx* (blue), *Hox1* (red), and *Emx* (green) homologs are shown. **A:** Gene expression patterns along the anteroposterior axis of the ascidian larva. In *Ciona intestinalis*, the expression domains of *CiDmbx* and *CiHox-3* overlap (shaded box; Takahashi and Holland, 2004). *Hroth*, *HrPax-258*, *CiDmbx*, and *CiHox-1* are expressed in the CNS (Wada et al., 1996, 1998; Takahashi et al., 2002; Nagatomo and Fujiwara, 2003; Takahashi and Holland, 2004), whereas *HrEmx* is expressed only in the epidermis (Oda and Saiga, 2001). **B:** Gene expression patterns along the anteroposterior axis of the vertebrate CNS (Simeone et al., 1992a, b; Joyner, 1996; Marshall et al., 1994; Takahashi et al., 2002). In the mouse, *Emx1* and *Emx2* are expressed in the developing cerebral cortex and olfactory bulbs (Simeone et al., 1992a).

Expression of *Brachyury* in ascidians is distinct in that it is localized only to notochord cells (Yasuo and Satoh, 1993; Corbo et al., 1997a). In contrast, *Brachyury* expression during early vertebrate embryogenesis is pan-mesodermal, only later becoming restricted to the notochord (Herrmann and Kispert, 1994). Fujiwara et al. (1998) have demonstrated that expression of *Ci-Bra* is restricted to the notochord by the zinc-finger transcription factor Snail. *C. intestinalis* snail (Ci-sna) is expressed in muscle lineages and represses *Ci-Bra* expression there by binding sites found in the *Ci-Bra* enhancer (Fujiwara et al., 1998).

Similarities between the ascidian and vertebrate notochord have also been observed in the expression and function of *forkhead (fkh)/HNF-3*β*/FoxA2* homologs. The *C. intestinalis forkhead* gene (*Ci-fkh/HNF-3*β*/Ci-FoxA-a*) is first expressed at the 16-cell stage, in a broad domain including lineages fated to form a variety of tissues (Di Gregorio et al., 2001). However, by gastrulation, *Ci-fkh* expression is restricted to the notochord, neural tube, and endoderm lineages, a pattern very similar to that of the mouse *HNF-3*β gene, which is expressed in the node, notochord, floor plate, and gut (Ang et al., 1993; Monaghan et al., 1993; Ruiz i Altaba et al., 1993; Sasaki and Hogan, 1993).

Conservation of *fkh/HNF-3*β's role in the development of notochord and other tissues is further supported by functional analysis of *fkh/HNF-3*β in the ascidian *Molgula oculata*. Reduction of *fkh/HNF-3*β transcripts in *M. oculata* inhibits morphogenetic movements of notochord and endodermal cells, causing incomplete gastrulation (Olsen and Jeffrey, 1997). These results are similar to defects in gastrulation observed in *HNF-3*β knockout mice (Ang and Rossant, 1994; Weinstein et al., 1994). Together, these results suggest a conserved role for *fkh/HNF-3*β in controlling the morphogenetic movements of gastrulation and axis formation across chordates.

The complement of genes involved in the development of the *C. intestinalis* notochord has been further analyzed using a subtractive screen designed to identify transcriptional targets downstream of Brachyury (Takahashi et al., 1999). These experiments have identified 39 novel genes regulated by Brachyury, 19 of which are expressed exclusively in the notochord (Hotta et al., 1999; reviewed by Satoh, 2003; Showell et al., 2004). Detailed regulatory analysis of one of these genes, *Ci-tropomyosin-like*, has shown it to be a direct target of Brachyury (Di Gregorio and Levine, 1999). Further investigation into the role of each of these genes is likely to provide significant insights into the regulatory networks and morphogenetic mechanisms governing formation of the ascidian notochord. This knowledge will provide a valuable basis for studies in vertebrates, where the understanding of the molecular mechanisms underlying development of the notochord remains quite limited (Cunliffe and Ingham, 1999).

The vertebrate notochord not only serves to provide structural support, but is also an important source of signals governing the patterning of the surrounding tissues. At present, there is no direct evidence that such signals are released by the *C. intestinalis* notochord. However, one of the Brachyury targets identified by Takahashi and colleagues (1999) is a homolog of the vertebrate *netrin* genes, which code for diffusible proteins shown to be important in axonal guidance (e.g., Colamarino and Tessier-Lavigne, 1995; Culotti and Kolodkin, 1996). Although netrins are not present in the vertebrate notochord, the presence of *netrin* transcripts in the *C. intestinalis* notochord suggests that the ascidian notochord may not only play a structural role but may also be involved in axonal guidance (Hotta et al., 2000).

It should be noted that not all the genes controlling the formation of the body plan in vertebrates have been shown to have comparable patterns of expression in ascidians and vertebrates. One representative case is that of the *hedgehog* genes. The role of *Sonic hedgehog* (*Shh*) in the induction of the floor plate by the notochord appears to represent the ancestral condition for vertebrates, as the one *hedgehog* gene identified in amphioxus has an expression pattern similar to that of *Shh* in vertebrates (Shimeld, 1999). *C. intestinalis* carries two *hedgehog* genes (*Ci-hh1* and *Ci-hh2*), which have arisen from a lineage-specific duplication (Takatori et al., 2002). Neither of the *C. intestinalis hedgehog* genes shows expression in the notochord. However, *Ci-hh2* is expressed in ventral nerve cord cells, which are considered to be a homolog of the vertebrate floor plate (Corbo et al., 1997b). It, therefore, appears that *hedgehog* expression in the ventral nerve cord/floor plate may represent the ancestral condition among chordates. In contrast, it remains unclear whether *hedgehog* was also expressed in the notochord of the last common ancestor of vertebrates and urochordates. Such expression may have arisen subsequent to the divergence of the two clades or may have been lost in the ascidian lineage.

At metamorphosis, the tail is resorbed and the notochord cells enter the trunk (Satoh, 1994). In *H. roretzi*, the remnants of the notochord have been transiently observed in juveniles but did not contribute to the formation of the adult body (Hirano and Nishida, 1997).

Not all ascidian larvae develop a tail and the associated notochord and muscle cells. Anural larvae have evolved several times independently in the ascidian clade Stolidobranchia, particularly among the Molgulidae (Huber et al., 2000). In the tailless ascidians *Molgula occulta* and *Molgula tectiformis*, *Brachyury* transcripts are present only transiently in the 10 notochord precursors (Takada et al., 2002). These cells undergo one additional round of division in *M. occulta*, whereas in *M. tectiformis*, they do not divide further. In neither species do the notochord precursors converge or extend (Takada et al., 2002). Through comparison of closely related tailed and tailless species of *Molgula*, Swalla and colleagues identified two genes, *Manx* and *bobcat*, required for tail formation (Swalla and Jeffrey, 1996; Swalla et al., 1999). Neither gene is expressed during embryogenesis of the tailless species *M. occulta*. However, expression of both genes, as well as of chordate features in the larva, is restored in crosses of *M. occulta* eggs fertilized by sperm from the tailed species *M. oculta* (Swalla and Jeffrey, 1996; Swalla et al., 1999).

Muscle

Muscle cells in the larvae and adult heart of ascidians are sarcomeric, and

are considered to be most similar to the striated troponin/tropomyosin-regulated skeletal and cardiac muscles of vertebrates (Meedel and Hastings, 1993; Meedel, 1997). Although the body-wall muscle of the adult ascidian is nonsarcomeric, it is also troponin/tropomyosin-regulated. In fully developed *C. intestinalis* larvae there are 36 mononucleated muscle cells, whereas in *H. roretzi* larvae there are 40 muscle cells (Satoh, 1994). These cells are arranged in three rows flanking either side of the notochord, but are not organized into somites as is the paraxial mesoderm of vertebrates. Like the notochord, the muscle cells are derived from two independent populations of precursors. Blastomere B4.1 of the 8-cell stage embryo gives rise to the 28 anterior and medial muscle cells, termed the primary lineage, and blastomeres A4.1 and b4.1 give rise to 8 posterior cells termed the secondary lineage (Nishida and Satoh, 1983, Meedel et al., 1987).

The role of maternal determinants in muscle development of ascidians has been known since Conklin (1905) described the pigment of the "yellow crescent" at the vegetal pole of the fertilized *Styela* egg. This pigmented myoplasm segregates to the primary muscle lineage, and the blastomeres containing it, when isolated, are competent to form differentiated muscle cells autonomously (Whittaker, 1973). Recent work carried out in *H. roretzi* by Nishida and Sawada (2001) has demonstrated the role of the zinc finger protein macho-1 in the specification of the primary muscle lineage. Maternally expressed *macho-1* mRNA is segregated to the vegetal pole before the first cleavage in the same pattern as the myogenic factor of the yellow crescent. Like the classic myogenic factor, macho-1 is necessary and sufficient for muscle differentiation (Nishida and Sawada, 2001). Reduction of *macho-1* mRNA prevents differentiation of the primary muscle lineage, whereas overexpression of *macho-1* induces ectopic muscle formation. Homologs of *macho-1* have subsequently been identified in *C. intestinalis* and *C. savignyi*, and both show patterns of maternal expression and segregation similar to that of *macho-1* in *H. roretzi*. Macho-1 protein appears to be required for the initiation of muscle differentiation, as depletion of *macho-1* mRNA by antisense oligonucleotides is characterized by a concomitant reduction of *muscle actin* transcripts before gastrulation (Nishida and Sawada, 2001; Satou et al., 2002c). However, the subsequent appearance of *muscle actin* transcripts in the tailbud stage of *macho-1* depleted *C. savignyi* embryos suggests that additional muscle determinants are present in *Ciona* (Mitani et al., 2001; Satou et al., 2002c). *ZicL*, a zinc-finger transcription factor gene closely related to *macho-1*, appears to be controlled by a pathway independent of macho-1 and is required for muscle actin expression in the B6.2 muscle precursors and their daughters (Imai et al., 2002b). Coinjection of morpholinos for both *Cs-macho-1* and *Cs-ZicL* suppresses expression of *muscle actin* in all muscle cells through the early tailbud stage. Several transcription factors and components of signaling pathways have been shown recently to be downstream of Ci-macho-1 (Yagi et al., 2004b).

Other genes that may also play a role in specification of the muscle lineages in ascidian larvae have been identified recently. *CiVegTR* is a member of the T-box gene family most closely related to the vertebrate genes *Tbx15*, *Tbx18*, and *Tbx22* (Erives and Levine, 2000; renamed *Tbx15/18/22* by Dehal et al., 2002). *CiVegTR*, like *macho-1*, is maternally expressed and localizes to the myoplasm at the vegetal pole of the fertilized egg before the first cleavage (Erives and Levine, 2000). CiVegTR binds two T-box binding sites in the *Ci-sna* minimal muscle enhancer *in vitro* (Erives and Levine, 2000). Mutations in these sites reduce the binding by CiVegTR *in vitro* and also attenuate the activity of the *Ci-sna* enhancer *in vivo*. Together, these results suggest that CiVegTR represents a second myogenic factor, which is involved in the activation of *Ci-sna* and possibly other muscle-specific genes. Although there is no evidence for maternal expression of the vertebrate homologs of *CiVegTR*, the vertebrate *Tbx18* gene is expressed in the developing somites of zebrafish (Begemann et al., 2002), chicks (Tanaka and Tickle, 2004), and mice (Kraus et al., 2001).

Another maternal gene that may act as a muscle determinant is *CiMDF* (*C. intestinalis Muscle Determinant Factor*; Meedel et al., 1997). *CiMDF* is closely related to the *MyoD* family of genes, which code for cysteine-rich/basic helix-loop-helix (Cys-rich/bHLH) transcription factors essential for the development of skeletal muscles in vertebrates (Buckingham, 1996). *CiMDF* encodes two splice variants, the smaller of which is expressed maternally (Meedel et al., 1997). Unlike *macho-1* and *CiVegTR*, *CiMDF* transcripts are present in precursors of both primary and secondary muscle lineages (Meedel et al., 2002). Interestingly, the expression of *CiMDF* in the primary muscle is autonomous, while expression in the secondary muscle is conditional, correlating with the mode of muscle determination for each lineage (Meedel et al., 2002). The functional roles of *CiVegTR* and *CiMDF* in larval muscle development have not been tested yet but will likely provide valuable insights into the regulatory networks controlling muscle development in both the primary and secondary muscle lineages.

The ascidian homolog of *Tbx6*, a T-box gene, also appears to play an important role in larval muscle development. In the mouse, *Tbx6* is required for proper formation of posterior paraxial mesoderm (Chapman et al., 1996; Chapman and Papaioannou, 1998). In *H. roretzi*, the *Tbx6* homolog *As-T2* is expressed in the muscle cell precursors, and its overexpression is sufficient to induce ectopic expression of two muscle marker genes, *myosin heavy-chain* and *muscle actin* (Mitani et al., 1999). On the other hand, a dominant-negative form of As-T2, prepared by fusing the As-T2 DNA binding domain to the repression domain of the engrailed protein, reduces the expression of *myosin heavy chain* and *muscle actin* genes (Mitani et al., 2001). These results suggest that these structural muscle genes are downstream of As-T2 (Mitani et al., 2001). In *C. intestinalis*, all three *Tbx6* homologs are expressed in muscle precursors, and *CiTbx6b* has been suggested to be downstream of *Ci-macho1* (Takatori et al., 2004; Yagi et al., 2004b).

Mesenchyme

The mesenchyme is a population of approximately 900 mesodermal cells located in the larval trunk that give rise to many of the tissues of the adult after metamorphosis (Satoh, 1994). Four distinct cell lineages contribute to the mesenchyme. A-line trunk lateral cells (TLCs) are fated to become blood cells and body-wall muscle in the adult (Nishide et al., 1989). Among the three B-line lineages, two give rise to tunic cells, whereas the trunk ventral cells (TVCs) are precursors of the heart and adult body-wall muscle (see below; Hirano and Nishida, 1997).

Mesenchyme induction is controlled by a synergistic interaction between macho-1 and FGF signaling (Kobayashi et al., 2003). In the absence of FGF signaling from the presumptive endoderm, mesenchyme precursors adopt a muscle fate, expressing structural genes such as *myosin*. However, when macho-1 function is inhibited, the expression of mesenchyme markers in the presumptive mesenchyme is blocked and ectopic expression of notochord markers is observed (Kobayashi et al., 2003).

Two bHLH transcription factor genes, *Twist-like1* and *Twist-like2*, have been shown to be involved in mesenchyme formation in *C. savignyi* (Imai et al., 2003). *Cs-Twist-like1* is upstream of *Cs-Twist-like2*, as well as of the homeobox gene *Cs-Hex*, the gene *Cs-Mist*, encoding a bHLH transcription factor, and the mesenchyme-specific gene *Cs-Mech*. Overexpression and morpholino knockdown experiments have demonstrated that *Cs-Twist-like1* is necessary and sufficient for mesenchyme differentiation (Imai et al., 2003).

Endoderm

As discussed above, the endodermal precursors in the ascidian embryo are an important source of FGF, which in turn induces the formation of mesodermal derivatives. In the hatched *C. intestinalis* larva, there are ~500 endodermal cells derived from 8 blastomeres identifiable at the 64-cell stage (Satoh, 1994). The endodermal derivatives include the trunk endoderm, which will form a primordial, nonfunctional "pharynx," and the endodermal strand, which runs ventral to the notochord as a slender row of cells. The endodermal strand has been tentatively compared with a transient and poorly characterized structure found only in fish and amphibians: the hypochord.

The hypochord was believed to be a structure of mesodermal origin until 1997, when Lofberg and Collazo showed its endodermal origin by injection of vital dyes in axolotl embryos; the same authors have suggested that the hypochord might play a role in positioning the dorsal aorta (Lofberg and Collazo, 1997). Little is known about molecular markers of this structure in either model system: among the genes expressed in the zebrafish hypochord are some of the *forkhead*-related genes (Odenthal and Nusslein-Volhard, 1998); in *C. intestinalis*, expression of *Ci-fkh* has been reported in trunk endoderm and endodermal strand (Di Gregorio et al., 2001).

In *Ciona*, endoderm differentiation is dependent upon the nuclear localization of β-*catenin*, and both anterior and posterior endoderm precursors are capable of autonomous differentiation when isolated early in development (Whittaker, 1990; Nishida, 1992; Imai et al., 2000). However, in the context of the embryo, FGF signaling inhibits the muscle-inducing effect of macho-1 in posterior endoderm precursors (Kondoh et al., 2003). An additional signal, possibly bone morphogenetic protein (BMP), also appears to play a role in the inhibition of macho-1.

Several genes are reported to be expressed in the endodermal derivatives of the *C. intestinalis* larva. For example, 29 EST clones have been shown to be expressed exclusively in these structures at the tailbud stage (Satou et al., 2001b) and 108 at the tadpole stage (Kusakabe et al., 2002). Among these genes, perhaps the best characterized is the gene encoding alkaline phosphatase, which provides a convenient histological and developmental marker of the endoderm (e.g., Kumano and Nishida, 1998); this expression is reminiscent of that observed in vertebrate (including human) enterocytes (e.g., Hinnebusch et al., 2003). In *C. intestinalis*, *Cititf1*, a homolog of the vertebrate *thyroid transcription factor-1* (*TTF-1*), is expressed in endodermal precursors before gastrulation, as well as in the trunk endoderm of the larva (Ristoratore et al., 1999). After metamorphosis, the larval endodermal cells give rise to a variety of adult endodermal organs, including endostyle, branchial sac, and digestive organs (Hirano and Nishida, 2000).

CNS

The CNS of an ascidian larva, although very simple, bears striking similarities to the CNS of vertebrate embryos, both in its morphological and molecular characteristics. Structurally, the CNS is composed of four main regions along the anteroposterior axis: the sensory vesicle, the neck, the visceral ganglion, and the caudal nerve cord. The caudal nerve cord is a hollow neural tube like the spinal cord of vertebrates but is made up of only four ependymal cells in cross-section (Nicol and Meinertzhagen, 1988a, b). The morphogenetic movements of ascidian neural tube formation display considerable similarity to the process of neurulation in vertebrates (Colas and Schoenwolf, 2001). The ventral and lateral cells of the nerve cord are derived from invagination of a neural plate, whereas closure of the neural tube involves recruitment of dorsal ectoderm to form the dorsal-most cells of the nerve cord (Nicol and Meinertzhagen, 1988a, b, 1991).

Early specification of the neural lineages appears to be directed through the action of the fibroblast growth factor FGF9/16/20, one of the six FGFs identified from the *C. intestinalis* genome project (Bertrand et al., 2003; reviewed by Meinertzhagen et al., 2004). FGF9/16/20 induces transcription of the *C. intestinalis* ortholog of *Otx* in precursors of both anterior and posterior neural lineages early in development (Bertrand et al., 2003). FGF signaling also appears to play an important role in early neural induction in the chick (Streit et al., 2000; Wilson et al., 2000). However, the role of FGFs in neural induction in *Xenopus* is more controversial (Hongo et al., 1999; Holowacz and Sokol, 1999).

Gene expression patterns along the anteroposterior axis of the ascidian larval CNS show considerable similar-

ity to those of vertebrates (Fig. 4). Homologs of *Otx* are expressed in the anteriormost region of the larval CNS, the sensory vesicle, in both *C. intestinalis* and *H. roretzi* (Wada et al., 1996; Hudson and Lemaire, 2001). These patterns of expression correspond to those of *Otx1* and *Otx2* in the anterior CNS of vertebrates, and the sensory vesicle of ascidians has been suggested, thus, to be analogous to the forebrain of vertebrates (Fig. 4; Simeone et al., 1992a; Wada et al., 1998).

Pax-258, the ascidian homolog of *Pax-2*, *Pax-5*, and *Pax-8* genes, also shows apparent conservation in its expression pattern. In *H. roretzi*, *HrPax-258* is expressed in the neck region of the larval CNS (Wada et al., 1998). Expression of *Pax-2*, *Pax-5*, and *Pax-8* in the midbrain and anterior hindbrain of vertebrates suggests that the midbrain–hindbrain boundary (MHB) represents a common ancestral feature of the chordate CNS (Fig. 4; Gruss and Walther, 1992; Noll, 1993; Joyner, 1996; Wada et al., 1998). In *C. intestinalis*, the homeobox gene *Ci-Dmbx* is expressed in the CNS immediately posterior to the regions of *Pax2/5/8* expression, a pattern similar to that of *Dmbx1* expression in the hindbrain of the mouse (Fig. 4; Takahashi et al., 2002; Takahashi and Holland, 2004). However, the ascidian *Ci-Dmbx* gene is not expressed anteriorly of *Pax2/5/8*; this finding contrasts with the strong expression seen in the midbrain of vertebrates (Takahashi et al., 2002). Therefore, the midbrain appears to be a vertebrate innovation (Takahashi and Holland, 2004).

Similarities in the patterning of the ascidian and vertebrate CNS are also observed in the expression domains of Hox genes. To date, nine *Hox* genes have been identified in the *C. intestinalis* genome, representing members of the anterior, medial, and posterior paralog groups and corresponding to a single *Hox* complex (Di Gregorio et al., 1995; Dehal et al., 2002; Spagnuolo et al., 2003). *C. intestinalis Hox* genes do not appear to be arranged in a single cluster, although most members of the complex are linked (Dehal et al., 2002; Spagnuolo et al., 2003; Fig. 3). Along with the apparent loss of cluster integrity, the expression patterns of *C. intestinalis Hox* genes appear to have diverged from the well-defined colinear pattern observed in flies and vertebrates (Ikuta et al., 2004). The anterior class representative *CiHox1* shows a segmental expression in the neck region of the CNS, as well as in the adjacent epidermis (Nagatomo and Fujiwara, 2003). *CiHox2* is expressed in the trunk lateral cells of the larval mesenchyme but is not expressed in the CNS (Ikuta et al., 2004). *CiHox3* has a domain of expression restricted to the anterior portion of the visceral ganglion (Locascio et al., 1999) and is also found in the neural complex and heart of the juvenile (Ikuta et al., 2004). *CiHox4* is not expressed in the CNS but is expressed in the trunk lateral cells of the mesenchyme (see below; Ikuta et al., 2004). *CiHox5/7* is also expressed in the trunk mesenchyme, as well as in the anterior and medial portions of the nerve cord (Gionti et al., 1998). *CiHox10* is expressed in a narrow region of the anterior nerve cord, overlapping the expression domain of *CiHox5/7* (Ikuta et al., 2004). Interestingly, this pattern of expression corresponds with the chromosomal position of *CiHox10* between *CiHox4* and *CiHox5/7*. Finally, *CiHox11/12* is expressed in the posterior region of the nerve cord and in the posterior epidermis of the tail (Ikuta et al., 2004). The expression patterns of *CiHox1* and *CiHox3* are notable in that both the anterior and posterior limits of their expression domains are well defined. In contrast, the posterior limit of expression of these genes in vertebrates is generally characterized by a gradually decreasing gradient of expression (Gilbert, 2003). *CiHox5* displays a similar gradient at the posterior end of its expression domain (Gionti et al., 1998). After metamorphosis, *CiHox10*, *CiHox11/12*, and *CiHox12/13* are all expressed in the endodermal epithelial layer of the juvenile intestine (Ikuta et al., 2004).

In contrast to the genes discussed above, neural expression is not conserved for the ascidian homolog of *ems/emx*. In both vertebrates and *Drosophila*, homologs of *emx* are expressed in the anterior regions of the CNS (Dalton et al., 1989; Simeone et al., 1992b). However, the *emx* homolog in *H. roretzi* is expressed only in the anterior trunk epidermis and in the lateral tail epidermis, but not in the CNS (Oda and Saiga, 2001; Fig. 3).

Conservation in patterning of the CNS is also observed along the dorsoventral axis of the nerve cord. As described above, in *C. intestinalis* the *hedgehog* gene *Ci-hh2* is expressed in the ventralmost cells of the nerve cord, similarly to *Sonic hedgehog* in the floor plate of vertebrates and *AmphiHh* in the ventral neural tube of amphioxus (Takatori et al., 2002; Shimeld, 1999). Likewise, *fkh/HNF-3*β*/FoxA2* homologs are expressed in the ventral cells of the *Ciona* nerve cord and in the floor plate of the vertebrate spinal cord (Corbo et al., 1997b). Expression of *Ci-fkh* is restricted to the ventral cells of the nerve cord by the action of Snail, which is expressed in precursors of the lateral ependymal cells of the nerve cord. Elimination of Snail repressor sites in the *Ci-fkh* enhancer expands expression of a *Ci-fkh/lacZ* transgene into the lateral ependymal cells (Di Gregorio et al., 2001). A similar mechanism may also be present in vertebrates, where *fkh/HNF-3*β*/FoxA2* is restricted to the floor plate and a *snail* homolog is expressed at the lateral neural plate border during neurulation (Sasaki and Hogan, 1994; Dickinson et al., 1995).

Taken together, the morphological and molecular similarities between the CNS of ascidians and vertebrates suggest a common origin for this structure before the divergence of the two groups. Although the vertebrate CNS has undergone dramatic elaboration, it appears that many of the basic mechanisms involved in establishing and patterning the CNS were laid down quite early in the evolution of chordates.

Peripheral Nervous System and Epidermis

A single layer of 800 epidermal cells covers both the trunk and the tail of the ascidian larva (Satoh, 1994). The epidermis produces the larval tunic, which is composed of cuticular layers, extracellular filaments, and cellulose (Satoh, 1994; Wang et al., 2002; Nakashima et al., 2004; Matthysse et al., 2004). Epidermal cells are derived solely from blastomeres in the animal hemisphere of the embryo, but appear

to require inductive signals from vegetal blastomeres for their specification (Wada et al., 1999b). In both *C. intestinalis* and *H. roretzi*, numerous genes display regionalized expression in the epidermis, suggesting that anteroposterior patterning of the larval body affects epidermal gene expression (Wada et al., 1999b; Oda and Saiga, 2001; Satou et al., 2001b; Kusakabe et al., 2002).

Antibody staining has demonstrated that both the dorsal and ventral epidermis of the tail of *C. intestinalis* larvae are invaded by neural cells, as is the trunk epidermis (Takamura, 1998). Peripheral neural cells of the tail are termed dorsocaudal epidermal neurons (DCEN) and ventrocaudal epidermal neurons (VCEN), and have axons that extend anteriorly to the visceral ganglion (Takamura, 1998). In the trunk, apical-trunk epidermal neurons (ATEN) extend from the visceral ganglion to overlay the sensory vesicle, whereas rostral-trunk epidermal neurons and papillar neurons extend anteriorly from the sensory vesicle (Takamura, 1998). In the tail of the *C. intestinalis* larva, the DCENs and the VCENs have cilia that extend through the tunic to the external environment and may be mechanosensory (Torrence and Cloney, 1983; Crowther and Whittaker, 1994). In *H. roretzi*, epidermal neurons in the tail display expression of the sodium channel gene *TuNaI*, as do cells in the CNS (Okada et al., 1997). An antibody to the actin-binding protein gelsolin also specifically recognizes epidermal neurons in both the trunk and tail of the larva (Ohtsuka et al., 2001). In *C. intestinalis*, a study of 56 genes with neural expression identified only three genes expressed in epidermal neurons. Only one of these genes, which showed no significant similarity to known proteins and contained no known peptide motifs, was found to have expression exclusively in epidermal neurons (Mochizuki et al., 2003).

Neural Crest and Placodes

The neural crest traditionally has been viewed as a vertebrate innovation; however, neural crest-like cells have been described recently in the large larvae of the colonial ascidian *Ecteinascidia turbinata* (Jeffery et al., 2004). These cells migrate from the anterior neural tube to the body wall and siphon primordia, before differentiating into pigment cells. These cells also express HNK-1 and *EtZic*, homologs of genes involved in the formation of the vertebrate neural crest (e.g., Bronner-Fraser, 1986; Nakata et al., 1998; Elms et al., 2003). Although such migrating cells have not been described in other ascidians, it appears that neural crest cells may have been present in the common ancestor of all chordates (Jeffery et al., 2004; reviewed by Baker and Bronner-Fraser, 1997; Wada, 2001; Holland et al., 1996).

Ascidians also appear to develop structures homologous to vertebrate placodes and their sensory organ derivatives (Manni et al., 2004). In the larva of *H. roretzi*, *HrPax-258* is expressed in the atrial primordia, ectodermal thickenings that are precursors of the atrial siphon (Wada et al., 1998). This pattern of expression is reminiscent of *Pax-2* expression in the otic placode of vertebrates (Nornes et al., 1990; Krauss et al., 1991; Wada et al., 1998). Likewise, the atrial siphon of the adult ascidian possesses ciliated cupular organs similar to the acoustico-lateralis system of vertebrates (Bone and Ryan, 1978). Based upon these similarities, Wada and colleagues concluded that the atrial primordia are homologous to the otic placodes of vertebrates (Wada et al., 1998). It, therefore, appears that such placodes, and the paired sensory organs they give rise to, were already present in the last common ancestor of all chordates. However, the strict interpretation of direct homology between the cupular organ of ascidians and the vertebrate ear has been questioned by Mackie and Singla (2003), who have noted that the hair cells of the ascidian cupular organ are primary sensory cells, in contrast to the hair cells of the vertebrate ear, which are secondary sense organs.

Larval Behavior

The fully developed, hatched ascidian larva actively swims before settling on the substrate and initiating metamorphosis (Svane and Young, 1989). During this free-swimming period, the larva displays a characteristic pattern of behavior, initially swimming upward (toward more illuminated surface waters), before swimming downward (away from illuminated surface waters; Svane and Young, 1989). In *C. intestinalis*, morpholino knockdown of *Ci-opsin1* translation is sufficient to inhibit the photosensitivity responsible for downward swimming behavior (Inada et al., 2003; Tsuda et al., 2003).

ADULT TISSUES

Heart and Blood

The heart of an ascidian lies in the proximal region of the body (see Fig. 1D), close to the holdfast, which attaches the adult to the substrate. The ascidian heart was first described in juveniles by Willey in 1893 and at later stages by Damas in 1899. It is a simple V-shaped tube composed by a single layer of unicellular, striated muscle cells (Kalk, 1970) and surrounded by a single-layered pericardial epithelium, or pericardium. The connection of the heart to the pericardium by a raphe defines a pericardial cavity, the only coelomic cavity in ascidians (Millar, 1953; Hirano and Nishida, 1997).

The embryonic precursors of the heart are two vegetal blastomeres, the B7.5 pair, which will give rise to two muscle cells located in the anteriormost region of the larval tail and to two trunk cells (Satoh, 1994). At the end of neurulation, these cells will divide again and will eventually form a total of eight cells in the trunk region of the larva, known as trunk ventral cells (TVCs). The TVCs will migrate, divide again, and finally fuse into a band of cells along the midline (Davidson and Levine, 2003). In *H. roretzi*, lineage tracing studies carried out by Hirano and Nishida (1997) have shown that some of these cells will give rise to the muscles of the latitudinal mantle and of the atrial siphon and that the remaining ones will form the heart and the pericardium of the juvenile. Tracing analysis in *C. intestinalis*, however, has confirmed only the relationship between the B7.5 precursors and the heart (Satou et al., 2004). Toward the conclusion of the rotation of the body axis, a stage of metamorphosis, the heart begins beating and blood cells start flowing through it. The heart periodically reverses its beating direction, thus re-

versing the direction of the blood flow throughout the body. This reversal appears to be due to the presence of two "automatic centers," one on each side of the heart, which alternately initiate waves of contractions along the walls of the heart, thus periodically reversing its beat (Millar, 1953). Based on this description, these centers might be the equivalent of the pacemaker cells found in vertebrate hearts.

The simple ascidian heart possesses several features that can provide insight into the development of the vertebrate heart. First, the morphogenetic movements leading to the formation of the ascidian heart are comparable to those observed during the first stages of vertebrate heart development. Second, the molecular wiring and the gene regulatory cascades appear to be evolutionarily conserved between ascidian and vertebrate hearts.

For instance, recent work by Satou and colleagues (2004) in *C. savignyi* shows that *Cs-Mesp*, encoding a transcription factor of the bHLH family, is responsible for the specification of the TVCs, and, therefore, for the formation of the heart primordium. The presence in the *C. savignyi* genome of a single ortholog of vertebrate *Mesp* genes has greatly facilitated functional analyses. In *C. savignyi* embryos, *Cs-Mesp* expression begins just before gastrulation in the B7.5 blastomeres and continues in their progeny for two additional cell divisions. By neurulation, *Cs-Mesp* transcripts become undetectable (Satou et al., 2004). Embryos of *C. savignyi* in which *Cs-Mesp* translation was disrupted by morpholino oligonucleotides develop into normal larvae but fail to form a heart at the time of metamorphosis, because the TVCs are not specified (Satou et al., 2004). Similarly, double-knockouts of the mouse homologs *Mesp1* and *Mesp2* fail to form cardiac tissue (Kitajima et al., 2000).

Among the genes acting downstream of Mesp, there appear to be homologs of *Nkx2.5/tinman* and *Hand*, which play a major role in heart formation in vertebrates as well as in *Drosophila* (Satou et al., 2004; cfr. Grow and Krieg, 1998; Kolsch and Paululat, 2002). *Cs-Mesp*, in turn, appears to be downstream of macho-1 (Nishida and Sawada, 2001; Satou et al., 2004). *Cs-Mesp* expression is also dependent upon β-catenin, which in ascidians specifies endodermal cells and has been shown to be involved in cardiac myogenesis in mammals (Imai et al., 2000; Nakamura et al., 2003; Satou et al., 2004).

Work recently published by Davidson and Levine (2003) suggests an intriguing connection between cytoplasmic determinants of the germ-line and early cardiac mesoderm in *C. intestinalis*. It is known from cell-lineage studies that the germ cells derive from the blastomere B7.6 and that two tail muscle cells and all the TVCs derive from the blastomere B7.5 (see above). Both B7.5 and B7.6 derive from the blastomere B6.3, but this cell is unable to initiate zygotic transcription in response to specification signals, either emanating from adjacent tissues, such as the FGF provided by endodermal cells, or maternally inherited, such as the cytoplasmic determinants leading to the germ-line fate. A molecular explanation of this phenomenon could be the presence of "repressors of cell specification," asymmetrically localized in the B6.3 cells. When the B6.3 cells divide, the repressors are segregated only in the B7.6 daughters, which, therefore, will become germ cells. The B7.5 daughters, freed of the repressors, can now respond to the specification signals and give rise to muscle cells and TVCs (Davidson and Levine, 2003).

The EST gene expression project (http://ghost.zool.kyoto-u.ac.jp/indexr1.html) has identified 150 genes that are expressed, among other tissues, in the heart of young adults; the corresponding sequences encode, for example, putative ribosomal proteins, RNA-binding proteins, putative cytoskeletal components, and homologs of human proteins still uncharacterized. The analysis of the regulatory elements controlling the expression of such genes in the heart should provide useful information on the gene cascades controlling the differentiation of this organ and is likely to shed light on the genes involved in early stages of vertebrate heart development.

The circulatory system in ascidians is open, and the blood, propelled by the heart, flows in a series of rudimentary vessels (Millar, 1953). At least six different types of blood cells have been described in ascidians (Wright, 1981; Rowley et al., 1984). Among these are the peculiar "vanadocytes," which are able to accumulate the vanadium dispersed in the seawater by means of recently characterized vanadium-binding proteins (Michibata et al., 2002; Trivedi et al., 2003). This ability appears to be a unique feature of ascidians and, to date, has not been reported in any other system. The biological meaning of this process is still mysterious.

Endostyle

The endostyle is an endodermally derived organ of the adult ascidian that secretes mucus used for food capture by the branchial basket. The outer regions of the U-shaped ascidian endostyle traditionally have been considered to share a common evolutionary origin with the vertebrate thyroid gland, based on the finding that both organs sequester iodine and display thyroid peroxidase activity (reviewed by Eales, 1997).

Recent analyses of gene expression patterns have further supported the homology of the endostyle and the vertebrate thyroid gland. In *C. intestinalis*, homologs of the vertebrate *thyroid peroxidase* (*TPO*) are expressed specifically in the thyroid-equivalent regions of the endostyle (Ogasawara et al., 1999a). Two *Fox* class transcription factor genes, *FoxE* and *FoxQ*, are also expressed in these regions of the *Ciona* endostyle (Ogasawara and Satou, 2003). *Ci-FoxE* is a homolog of the vertebrate *thyroid transcription factor-2/FoxE1* gene, which is required for thyroid gland formation in the mouse (De Felice et al., 1998). *Cititf1* is expressed in regions of the endostyle that secrete digestive proteins and provide structural support (Ristoratore et al., 1999). Several other genes with endostyle-specific expression patterns have also been identified, including two genes related to the vertebrate *von Willebrand Factor* genes (Sasaki et al., 2003).

Gill Slits

The branchial basket, used for filter-feeding and oxygenation of the blood in the adult ascidian, is perforated by hundreds of ciliated pharyngeal gill slits (Satoh, 1994). Based upon their

morphology, these gill slits are considered homologs of the pharyngeal gill slits of amphioxus and vertebrates. This homology is further supported by the expression of *Pax1/9* in the pharyngeal gills of *C. intestinalis* and *H. roretzi* (Ogasawara et al., 1999b). These expression patterns appear to be homologous to the expression pattern of *Pax1/9* in the pharyngeal gills of amphioxus (Holland et al., 1995) and to the expression of *Pax1* and *Pax9* in the pharyngeal pouches of the mouse (Neubuser et al., 1995; Wallin et al., 1996). Similar expression of *Pax1/9* in the gills of the hemichordate *Ptychodera flava* suggests that pharyngeal gill slits may have arisen early in deuterostome evolution and are not solely a chordate innovation (Ogasawara et al., 1999b).

Sensory Organs

Both the oral and atrial siphon of the adult ascidian contain sensory structures that may be homologous to vertebrate sensory organs. In the atrial siphon, the ciliated cupular organ shows similarities with the vertebrate inner ear, based upon morphological and developmental data (discussed above; Wada et al., 1998). In the oral siphon, a sensory structure termed the coronal organ has been described recently from the colonial ascidians *Botryllus schlosseri* and *Botrylloides violaceus* (Burighel et al., 2003). The coronal organ contains ciliated receptor cells that have synaptic connections with secondary sensory neurons. On the basis of this morphology, it appears that the coronal organ shares a common evolutionary origin with the lateral line, and inner ear, of the vertebrate acoustico-lateralis system (Burighel et al., 2003).

Immunity and Allorecognition

Adaptive immunity is a vertebrate-specific innovation that is not present in ascidians (Kasahara et al., 2004). However, the *C. intestinalis* genome carries several genes involved in innate immunity, including Toll-like receptors, which have homologs in vertebrates, as well as in protostomes such as *Drosophila* (Dehal et al., 2002; Azumi et al., 2003). In addition, several genes of the complement innate immunity pathway that appear to represent chordate innovations have been identified from *C. intestinalis* and other ascidians (Fujita, 2002; Fujita et al., 2004). These genes include *mannose-binding lectins* (*MBL*), *ficolins*, and *MBL-associated serine proteases* (*MASPs*) (Fujita et al., 2004). Numerous innate immunity genes are expressed in the hemocytes of adult *C. intestinalis*, including components of the complement pathway related to genes identified in vertebrate hemocytes (Pinto et al., 2003; Shida et al., 2003). These genes may be involved in inflammatory-like reactions mediated by the blood cells, which occur in response to injuries (Di Bella and De Leo, 2000). Several innate immunity genes have also been shown to be expressed during metamorphosis of the ascidian *Boltenia villosa*, at a time when mesenchymal cells migrate throughout the body and can contact the external environment (Davidson and Swalla, 2002).

In addition to these ancestral immune responses, ascidians also display the ability to distinguish conspecifics as self/nonself. Although ascidians are simultaneous hermaphrodites, they are almost completely self-sterile (Rosati and De Santis, 1980; Murabe and Hoshi, 2002). Functionally, self-sterility is achieved by the inability of spermatozoa to bind the vitelline coat of autologous eggs, as well as by the inactivation of those spermatozoa that do bind (Rosati and De Santis, 1978; Kawamura et al., 1987). Recent work by Murabe and Hoshi (2002) has supported Morgan's (1944) hypothesis that self-sterility is determined by multiple loci with allelic diversity that display haploid expression in sperm and diploid expression in eggs.

In colonial ascidians, allorecognition provides an important barrier to colony fusion, which can lead to both somatic and germ line parasitism (Stoner et al., 1999). In *Botryllus schlosseri*, a single locus, termed *Fu/HC*, is responsible for allorecognition (Scofield et al., 1982). This locus is characterized by high levels of polymorphism compared with other regions of the genome (Scofield et al., 1982; Stoner et al., 1999). It is likely that such polymorphism confers a developmental advantage to *Fu/HC* heterozygotes, which reach sexual maturity at significantly higher frequency than *Fu/HC* homozygotes (De Tomaso and Weissman, 2004).

EVOLUTIONARY CONSIDERATIONS

Notochord

The main innovation found in ascidians with respect to other invertebrates is the presence of a notochord during the larval stages. The ascidian notochord represents one of the simplest, closest approximations of the vertebrate notochord experimentally available. But are ascidian and vertebrate notochords true homologs?

Experimental manipulations have demonstrated that specification of the primary notochord lineage of ascidians requires inductive signals from vegetal blastomeres fated to become endoderm (Nakatani and Nishida, 1994). This requirement seems to be similar to the inductive specification of mesoderm by vegetal cells in amphibians (Nieuwkoop, 1969). As discussed above, several genes, including Brachyury, appear to have evolutionarily conserved roles in notochord formation in both ascidians and vertebrates.

However, among vertebrates, the notochord not only serves to provide structural support, but is also an important source of signals governing the patterning of the surrounding tissues. During early vertebrate development, this organ plays a major role in patterning the neural tube and, in particular, in controlling the formation of the floor plate, the ventral-most region of the spinal cord, an essential source of molecules that direct the axonal paths along and across the midline. For example, in notochordless frog embryos, the floor plate is absent and its position is occupied by ectopic ventral neurons and glial cells; the number of motor neurons is reduced to 15%, and, as a result, the locomotor activity is severely affected (Clarke et al., 1991).

At present, there is no direct evidence that such signals are released by the *C. intestinalis* notochord. The absence of hedgehog from the *C. intestinalis* notochord is in clear contrast to its role in vertebrates as a signaling

molecule emanating from the notochord, bringing into question the ancestral role of hedgehog in axial patterning.

The expression of a *netrin* gene in the *Ciona* notochord might argue in favor of a role in axonal guidance, but functional studies will be necessary to support this hypothesis. From the data currently available, it would seem that the notochord first evolved as a supporting structure and subsequently has acquired a patterning role.

β-Catenin

Similar to what is seen in *C. elegans*, endoderm specification in ascidians is dependent upon the nuclear localization of β-catenin, an event that is part of the Wnt signaling cascade (Rocheleau et al., 1997; Imai et al., 2000). When ascidian embryos are depleted of β-catenin by morpholino oligo treatment (Satou et al., 2001a) or when the nuclear translocation of β-catenin is blocked by injection of cadherin mRNA (Imai et al., 2000), the differentiation of the endoderm is blocked and supernumerary epidermal cells are observed.

β-Catenin appears to have an ancient and conserved role in early embryonic patterning. For instance, in cnidarians, it is required for the formation of the entoderm (Wikramanayake et al., 2003). In echinoderms, the accumulation of β-catenin in the nuclei of the vegetal blastomeres, which include the precursors of endomesodermal tissues, is required for the establishment of the cell fate of these tissues and is also necessary for the specification of the animal–vegetal axis (Logan et al., 1999). In the case of vertebrates, such as *Xenopus*, β-catenin specifies the dorsal–ventral axis (Schneider et al., 1996). Triggered by the action of dorsal determinants, including Dishevelled and GBP (GSK-3 binding protein), the nuclear translocation of β-catenin takes place, in the frog embryo, by the blastula stage and extends progressively from the vegetal to the animal pole, i.e., from bottom to top. This phenomenon is reminiscent of the formation of a nuclear gradient of the Dorsal protein, which is necessary for the establishment of the dorsal–ventral axis in *Drosophila*, where, however, the nuclear translocation of Dorsal occurs in the cells located on the ventral side of the embryo (Rushlow et al., 1989). In frog embryos, after the onset of zygotic transcription, the interaction of β-catenin with TCF/Lef proteins leads to the activation of genes such as *Siamois*, which take part in the formation of the Spemann's organizer, the structure necessary for axis specification (reviewed by De Robertis and Kuroda, 2004).

In higher vertebrates, β-catenin retains its role in cell fate specification, as shown by numerous reports demonstrating its requirement for developmental processes ranging from cardiac myogenesis (Nakamura et al., 2003) to the specification of neural crest derivatives (Hari et al., 2002). Of interest, a targeted disruption of the β-catenin gene in mouse leads to a block in the formation of the anterior–posterior axis, as well as lack of gastrulation, mesoderm formation, and development of head structures (Huelsken et al., 2000). These results might suggest that the ancestral role seen for β-catenin in endoderm specification in worms and ascidians is not retained in vertebrates. Nonetheless, when β-catenin is conditionally inactivated in the embryonic endoderm of developing mice, a dramatic phenotype, namely the formation of multiple hearts along the AP axis, is observed. These observations suggest that, in the mouse embryo, β-catenin is not required for the specification of the endoderm but rather for the maintenance of its identity, because when this gene is inactivated, the endodermal cells switch to a precardial mesoderm fate (Lickert et al., 2002).

Organizer

Do ascidian embryos have an organizer(s)?

The ascidian egg undergoes a dramatic cytoplasmic reorganization between fertilization and the first cleavage, a phenomenon known as ooplasmic segregation. The most evident event of this process is the transient localization of the myoplasm to the vegetal pole of the zygote, where it establishes a gastrulation center, followed by its segregation to the posterior pole (Jeffery, 1992). However, the presence of a physical structure equivalent to a vertebrate organizer has never been described in ascidians. The near completion of the genome and EST projects, therefore, has raised the question of whether ascidian embryos have the molecules that characterize an organizer. We can now try to answer this question by looking at the presence and expression patterns of representative homologs of vertebrate organizer genes in *Ciona*. For example, very recent work has shown that the *Not* gene in *Ciona* has a complex expression pattern, which is only in part reminiscent of the expression seen in vertebrates and is different between *Ciona* and the distantly related species *Halocynthia* (Utsumi et al., 2004). In addition, the *Ciona* genome apparently contains most or all of the other known molecular components necessary to build an organizer (Dehal et al., 2002).

One of the main differences between chordates and, for example, fruit flies, is the so-called dorsal–ventral axis inversion that might have taken place during the evolution of the Bilateria. This hypothesis is based on the opposite orientations of the nerve cord, which is dorsal in chordates and ventral in arthropods and annelids, and of structures such as the heart, which is ventral in chordates and dorsal in flies and worms (reviewed by Gerhart, 2000).

Molecular studies have revealed that the dorsal–ventral axis is established by an evolutionarily conserved battery of signaling molecules, including Short gastrulation (Sog) in *Drosophila* and its vertebrate counterpart Chordin and their antagonists, Decapentaplegic (Dpp) and BMP-4, respectively (Holley et al., 1995).

C. intestinalis possesses a *chordin* homolog, which is expressed only from the 64-cell stage on in the precursors of the nervous system, and later on in the neural folds. In tailbud larvae, the gene is found in both the sensory vesicle and the nerve cord, as well as in a region surrounding the sensory vesicle (Imai et al., 2004). Conversely, there is not a clear homolog of BMP-4 but a gene equally related to BMP-2 and BMP-4, indicated as BMP-2/4, which is detected in ventral regions of the embryo, such as the endodermal strand and the ventral epidermis, but also in dorsal structures, such as the anterior-most regions of the CNS. However, work carried out on *Halo-*

cynthia by Darras and Nishida (2001) rules out a role for these factors in neural induction and presents evidence for their function in the specification of the pigmented sensory cells of the sensory vesicle.

The *Ciona* genome also contains a *noggin* homolog, which is expressed maternally and is subsequently found in the nervous system (Imai et al., 2004). Originally identified in *Xenopus*, this gene encodes a secreted polypeptide that is able to directly induce neural tissues and acts as a BMP inhibitor, thus promoting development of dorsal structures (reviewed by Smith, 1999). The role of noggin in ascidians as a neural inducer has not yet been described, but its conserved expression in the neural folds and in the nerve cord of *C. intestinalis* might perhaps suggest a conserved role in neural tube closure.

So why does *Ciona* lack a physical organizer? Perhaps this structure was present in the common chordate ancestor and has disintegrated in ascidians, being replaced by more maternal determinants. Or alternatively, the molecular components were recruited to form the organizer later during chordate evolution.

Muscle/Mesoderm

The main difference observed in muscle development between ascidians and vertebrates is the early, autonomous specification of the blastomeres that will give rise to the larval muscle. The role of maternal determinants in muscle development of ascidians has been known since Conklin (1905) described the pigment of the "yellow crescent" at the vegetal pole of the fertilized *Styela* egg, which segregates to the primary muscle lineage and makes the blastomeres containing it competent to form differentiated muscle cells autonomously (Whittaker, 1973). The molecular identity of the muscle determinant(s) had remained mysterious until *macho-1* was identified (Nishida and Sawada, 2001). Recent work by Yagi et al. (2004b) indicates that *Tbx6b* and *snail* expression in developing muscle cells might be controlled by macho-1, since putative binding sites for this transcription factor have been identified in their 5′-flanking regions. Conversely, studies carried out in *C. savignyi* show that the expression of *ZicL* in muscle cells, which is in turn necessary to maintain muscle differentiation, is macho-1–independent, suggesting that *ZicL* might be part of another "ascidian-specific" gene regulatory cascade (Imai et al., 2002b).

The functional potential of macho-1 to induce muscle differentiation does not appear to be conserved in vertebrates. Injection of *H. roretzi macho-1* mRNA into the animal cap of *Xenopus* results in the formation of ectopic cement glands rather than in muscle differentiation (Satou et al., 2002c). Nonetheless, other components of the muscle gene regulatory network that have been shown to be necessary for muscle formation in vertebrate embryos appear to be conserved in ascidians. This is the case, for example, of the MyoD-related transcription factor CiMDF (Meedel et al., 1997). However, other myogenic regulatory factors, such as Myf5, or the differentiation genes Mrf4 and myogenin (Kassar-Duchossoy et al., 2004) do not seem to be present in the *Ciona* genome. Also the inhibitory role exerted by *msx*-related genes on myogenesis (e.g., Lee et al., 2004) is yet to be elucidated in ascidians, where only one *msx*-related gene, *Ci-msxb*, has been fully characterized (Aniello et al., 1999).

Another crucial difference in mesodermal patterning between ascidian and vertebrate embryos is the absence of graded morphogenetic fields within the embryonic territories. It appears as if different ascidian blastomeres are able to read only "on/off" signals, as opposed to vertebrate cells that respond to different levels of morphogens. This mechanism, which has been indicated as a "digital" read-out, might be explained by the small size of the ascidian embryos and by the mosaic nature characterizing part of their development (reviewed by Nishida, 2002).

CNS

In both ascidians and vertebrates, the initiation of neural fates is dictated by inductive interactions. The crucial role of FGFs in neural induction of both ascidians and chick suggests an ancestral role for this signaling pathway. In contrast, BMP signaling, a well characterized neural inducer in *Xenopus*, does not appear to be necessary or sufficient for induction of neural fates in ascidians, suggesting this mechanism is a vertebrate innovation (Darras and Nishida, 2001). With respect to axial patterning, many markers of both anteroposterior and dorsoventral regionalization also appear to share common expression patterns in ascidians and vertebrates, likely underlying evolutionarily conserved roles.

Despite these similarities, ascidians appear to lack homologs of several genes with important roles in the vertebrate CNS. For example, the absence of neurothophins and their associated receptors (e.g., NGF and TrkA) from the *C. intestinalis* genome supports the hypothesis that these genes arose during vertebrate evolution in association with increased neuronal specialization (Hallböök, 1999; Dehal et al., 2002). Likewise, genes required for the synthesis of histamine, melatonin, and epinephrine, all of which are associated with vertebrate brain function, have not been identified in ascidians (Dehal et al., 2002). Although the ascidian CNS lacks the functional complexity and structural elaboration of the vertebrate brain, there is compelling evidence that much of the basic patterning of the CNS, at both the genetic and structural levels, arose before the diversification of chordates.

FUTURE DIRECTIONS

Despite the evolutionary distance between ascidians and vertebrates, the two groups display considerable conservation of both morphology and gene function. Therefore, ascidians can provide a valuable resource for understanding the molecular mechanisms underlying vertebrate development. Given the phylogenetic position of Urochordates, studies carried out on ascidians will continue to offer insights into the origins of chordate innovations. The sequencing of the *C. intestinalis* and *C. savignyi* genomes and the fact that ascidian embryos are amenable to studies of gene expression, function, and regulation, will rapidly provide new findings in this field of research.

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REFERENCES

Aniello F, Locascio A, Villani MG, Di Gregorio A, Fucci L, Branno M. 1999. Identification and developmental expression of *Ci-msxb*: a novel homologue of *Drosophila msh* gene in *Ciona intestinalis*. Mech Dev 88:123–126.

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

Ang SL, Wierda A, Wong D, Stevens KA, Cascio S, Rossant J, Zaret KS. 1993. The formation and maintenance of the definitive endoderm lineage in the mouse: involvement of HNF3/forkhead proteins. Development 119:1301–1315.

Aros B, Viragh S. 1969. Fine structure of the pharynx and endostyle of an ascidian (*Ciona intestinalis*). Acta Biol 20:281–297.

Awazu S, Sasaki A, Matsuoka T, Satoh N, Sasakura Y. 2004. An enhancer trap in the ascidian *Ciona intestinalis* identifies enhancers of its *Musashi* orthologous gene. Dev Biol 275:459–472.

Azumi K, De Santis R, De Tomaso A, Rigoutsos I, Yoshizaki F, Pinto MR, Marino R, Shida K, Ikeda M, Arai M, Inoue Y, Shimizu T, Satoh N, Rokhsar DS, Du Pasquier L, Kasahara M, Satake M, Nonaka M. 2003. Genomic analysis of immunity in a Urochordate and the emergence of the vertebrate immune system: "waiting for Godot". Immunogenetics 55:570–581.

Baker CV, Bronner-Fraser M. 1997. The origins of the neural crest. Part II. An evolutionary perspective. Mech Dev 69: 13–29.

Begemann G, Gibert Y, Meyer A, Ingham PW. 2002. Cloning of zebrafish T-box genes *tbx15* and *tbx18* and their expression during embryonic development. Mech Dev 114:137–141.

Bertrand V, Hudson C, Caillol D, Popovici C, Lemaire P. 2003. Neural tissue in ascidian embryos is induced by FGF9/16/20, acting via a combination of maternal GATA and Ets transcription factors. Cell 115:615–627.

Bone Q, Ryan KP. 1978. Cupular organs in *Ciona* (Tunicata: Ascidiacea). J Zool Lond 186:417–429.

Bronner-Fraser M. 1986. Analysis of the early stages of trunk neural crest migration in avian embryos using monoclonal antibody HNK-1. Dev Biol 115:44–55.

Buckingham M. 1996. Skeletal muscle development and the role of the myogenic regulatory factors. Biochem Soc Trans 24:506–509.

Burighel P, Lane NJ, Fabio G, Stefano T, Zaniolo G, Carnevali MD, Manni L. 2003. Novel, secondary sensory cell organ in ascidians: in search of the ancestor of the vertebrate lateral line. J Comp Neurol 461:236–249.

Chapman DL, Papaioannou VE. 1998. Three neural tubes in mouse embryos with mutations in the T-box gene *Tbx6*. Nature 391:695–697.

Chapman DL, Agulnik I, Hancock S, Silver LM, Papaioannou VE. 1996. *Tbx6*, a mouse T-box gene implicated in paraxial mesoderm formation at gastrulation. Dev Biol 180:534–542.

Chen JY, Huang DY, Peng QQ, Chi HM, Wang XQ, Feng M. 2003. The first tunicate from the Early Cambrian of South China. Proc Natl Acad Sci U S A 100: 8314–8318.

Clarke JD, Holder N, Soffe SR, Storm-Mathisen J. 1991. Neuroanatomical and functional analysis of neural tube formation in notochordless *Xenopus* embryos; laterality of the ventral spinal cord is lost. Development 112:499–516.

Colamarino SA, Tessier-Lavigne M. 1995. The role of the floor plate in axon guidance. Annu Rev Neurosci 18:497–529.

Colas JF, Schoenwolf GC. 2001. Towards a cellular and molecular understanding of neurulation. Dev Dyn 221:117–145.

Conklin EG. 1905. The organization and cell-lineage of the ascidian egg. Acad Nat Sci Phil 13:1–119.

Corbo JC, Levine M, Zeller RW. 1997a. Characterization of a notochord-specific enhancer from the *Brachyury* promoter region of the ascidian, *Ciona intestinalis*. Development 124:589–602.

Corbo JC, Erives A, Di Gregorio A, Chang A, Levine M. 1997b. Dorsoventral patterning of the vertebrate neural tube is conserved in a protochordate. Development 124:2335–2344.

Corbo JC, Fujiwara S, Levine M, Di Gregorio A. 1998. Suppressor of Hairless activates *Brachyury* expression in the *Ciona* embryo. Dev Biol 203:358–368.

Crowther RJ, Whittaker JR. 1994. Serial repetition of cilia pairs along the tail surface of an ascidian larva. J Exp Zool 268: 9–16.

Culotti JG, Kolodkin AL. 1996. Functions of netrins and semaphorins in axon guidance. Curr Opin Neurobiol 6:81–88.

Cunliffe VT, Ingham PW. 1999. Switching on the notochord. Genes Dev 13:1643–1646.

Dalton D, Chadwick R, McGinnis W. 1989. Expression and embryonic function of *empty spiracles*: a *Drosophila* homeo box gene with two patterning functions on the anterior-posterior axis of the embryo. Genes Dev 3:1940–1956.

Damas D. 1899. Les formations epicardiques chez *Ciona intestinalis*.

Darras S, Nishida H. 2001. The BMP/CHORDIN antagonism controls sensory pigment cell specification and differentiation in the ascidian embryo. Dev Biol 236:271–288.

Davidson B, Levine M. 2003. Evolutionary origins of the vertebrate heart: specification of the cardiac lineage in *Ciona intestinalis*. Proc Natl Acad Sci U S A 100: 11469–11473.

Davidson B, Swalla BJ. 2002. A molecular analysis of ascidian metamorphosis reveals activation of an innate immune response. Development 129:4739–4751.

De Felice M, Ovitt C, Biffali E, Rodriguez-Mallon A, Arra C, Anastassiadis K, Macchia PE, Mattei MG, Mariano A, Scholer H, Macchia V, Di Lauro R. 1998. A mouse model for hereditary thyroid dysgenesis and cleft palate. Nat Genet 19: 395–398.

De Robertis EM, Kuroda H. 2004. Dorsal-ventral patterning and neural induction in *Xenopus* embryos. Annu Rev Cell Dev Biol 20:285-308.

De Tomaso AW, Weissman IL. 2004. Evolution of a protochordate allorecognition locus. Science 303:977.

Dehal P, Satou Y, Campbell RK, Chapman J, Degnan B, De Tomaso A, Davidson B, Di Gregorio A, Gelpke M, Goodstein DM, Harafuji N, Hastings KE, Ho I, Hotta K, Huang W, Kawashima T, Lemaire P, Martinez D, Meinertzhagen IA, Necula S, Nonaka M, Putnam N, Rash S, Saiga H, Satake M, Terry A, Yamada L, Wang HG, Awazu S, Azumi K, Boore J, Branno M, Chin-Bow S, De Santis R, Doyle S, Francino P, Keys DN, Haga S, Hayashi H, Hino K, Imai KS, Inaba K, Kano S, Kobayashi K, Kobayashi M, Lee BI, Makabe KW, Manohar C, Matassi G, Medina M, Mochizuki Y, Mount S, Morishita T, Miura S, Nakayama A, Nishizaka S, Nomoto H, Ohta F, Oishi K, Rigoutsos I, Sano M, Sasaki A, Sasakura Y, Shoguchi E, Shin-i T, Spagnuolo A, Stainier D, Suzuki MM, Tassy O, Takatori N, Tokuoka M, Yagi K, Yoshizaki F, Wada S, Zhang C, Hyatt PD, Larimer F, Detter C, Doggett N, Glavina T, Hawkins T, Richardson P, Lucas S, Kohara Y, Levine M, Satoh N, Rokhsar DS. 2002. The draft genome of *Ciona intestinalis*: insights into chordate and vertebrate origins. Science 298:2157–2167.

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

Di Bella MA, De Leo G. 2000. Hemocyte migration during inflammatory-like reaction of *Ciona intestinalis* (Tunicata, Ascidiacea). J Invertebr Pathol 76:105–111.

Di Gregorio A, Levine M. 1999. Regulation of *Ci-tropomyosin-like*, a Brachyury target gene in the ascidian, *Ciona intestinalis*. Development 126:5599–5609.

Di Gregorio A, Levine M. 2002. Analyzing gene regulation in ascidian embryos: new tools for new perspectives. Differentiation 70:132–139.

Di Gregorio A, Spagnuolo A, Ristoratore F, Pischetola M, Aniello F, Branno M, Ca-

riello L, Di Lauro R. 1995. Cloning of ascidian homeobox genes provides evidence for a primordial chordate cluster. Gene 156:253–257.

Di Gregorio A, Corbo JC, Levine M. 2001. The regulation of *forkhead/HNF-3beta* expression in the *Ciona* embryo. Dev Biol 229:31–43.

Di Gregorio A, Harland RM, Levine M, Casey ES. 2002. Tail morphogenesis in the ascidian, *Ciona intestinalis*, requires cooperation between notochord and muscle. Dev Biol 244:385–395.

Dickinson ME, Selleck MA, McMahon AP, Bronner-Fraser M. 1995. Dorsalization of the neural tube by the non-neural ectoderm. Development 121:2099–2106.

Eales JG. 1997. Iodine metabolism and thyroid-related functions in organisms lacking thyroid follicles: are thyroid hormones also vitamins? Proc Soc Exp Biol Med 214:302–317.

Elms P, Siggers P, Napper D, Greenfield A, Arkell R. 2003. *Zic2* is required for neural crest formation and hindbrain patterning during mouse development. Dev Biol 264:391–406.

Erives A, Levine M. 2000. Characterization of a maternal T-box gene in *Ciona intestinalis*. Dev Biol 225:169–178.

Ferrier DE. 2004. *Hox* genes: did the vertebrate ancestor have a *Hox14*? Curr Biol 14:R210–211.

Fujita T. 2002. Evolution of the lectin-complement pathway and its role in innate immunity. Nat Rev Immunol 2:346–353.

Fujita T, Endo Y, Nonaka M. 2004. Primitive complement system-recognition and activation. Mol Immunol 41:103–111.

Fujiwara S, Corbo JC, Levine M. 1998. The snail repressor establishes a muscle/notochord boundary in the *Ciona* embryo. Development 125:2511–2520.

Gerhart J. 2000. Inversion of the chordate body axis: are there alternatives? Proc Natl Acad Sci U S A 97:4445–4448.

Gilbert SF. 2003. Developmental biology. Sunderland, MA: Sinauer Associates, Inc.

Gionti M, Ristoratore F, Di Gregorio A, Aniello F, Branno M, Di Lauro R. 1998. *Cihox5*, a new *Ciona intestinalis Hox*-related gene, is involved in regionalization of the spinal cord. Dev Genes Evol 207:515–523.

Grow MW, Krieg PA. 1998. Tinman function is essential for vertebrate heart development: elimination of cardiac differentiation by dominant inhibitory mutants of the *tinman*-related genes, *XNkx2-3* and *XNkx2-5*. Dev Biol 204:187–196.

Gruss P, Walther C. 1992. *Pax* in development. Cell 69:719–722.

Hallböök F. 1999. Evolution of the vertebrate neurotrophin and Trk receptor gene families. Curr Opin Neurobiol 9: 616–621.

Hari L, Brault V, Kleber M, Lee HY, Ille F, Leimeroth R, Paratore C, Suter U, Kemler R, Sommer L. 2002. Lineage-specific requirements of β-catenin in neural crest development. J Cell Biol 159:867–880.

Herrmann BG, Kispert A. 1994. The *T* genes in embryogenesis. Trends Genet 10:280–286.

Hinnebusch BF, Henderson JW, Siddique A, Malo MS, Zhang W, Abedrapo MA, Hodin RA. 2003. Transcriptional activation of the enterocyte differentiation marker intestinal alkaline phosphatase is associated with changes in the acetylation state of histone H3 at a specific site within its promoter region in vitro. J Gastrointest Surg 7:237–244.

Hirano T, Nishida H. 1997. Developmental fates of larval tissues after metamorphosis in ascidian *Halocynthia roretzi*. I. Origin of mesodermal tissues of the juvenile. Dev Biol 192:199–210.

Hirano T, Nishida H. 2000. Developmental fates of larval tissues after metamorphosis in the ascidian, *Halocynthia roretzi*. II. Origin of endodermal tissues of the juvenile. Dev Genes Evol 210:55–63.

Holland PW. 2003. More genes in vertebrates? J Struct Funct Genomics 3:75–84.

Holland ND, Holland LZ, Kozmik Z. 1995. An amphioxus *Pax* gene, *AmphiPax-1*, expressed in embryonic endoderm, but not in mesoderm: implications for the evolution of class I paired box genes. Mol Mar Biol Biotechnol 4:206–214.

Holland ND, Panganiban G, Henyey EL, Holland LZ. 1996. Sequence and developmental expression of *AmphiDll*, an amphioxus *Distal-less* gene transcribed in the ectoderm, epidermis and nervous system: insights into evolution of craniate forebrain and neural crest. Development 122:2911–2920.

Holley SA, Jackson PD, Sasai Y, Lu B, De Robertis EM, Hoffmann FM, Ferguson EL. 1995. A conserved system for dorsal-ventral patterning in insects and vertebrates involving sog and chordin. Nature 376:249–253.

Holowacz T, Sokol S. 1999. FGF is required for posterior neural patterning but not for neural induction. Dev Biol 205:296–308.

Hongo I, Kengaku M, Okamoto H. 1999. FGF signaling and the anterior neural induction in *Xenopus*. Dev Biol 216:561–581.

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

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

Huber JL, da Silva KB, Bates WR, Swalla BJ. 2000. The evolution of anural larvae in molgulid ascidians. Semin Cell Dev Biol 11:419–426.

Hudson C, Lemaire P. 2001. Induction of anterior neural fates in the ascidian *Ciona intestinalis*. Mech Dev 100:189–203.

Hudson C, Darras S, Caillol D, Yasuo H, Lemaire P. 2003. A conserved role for the MEK signalling pathway in neural tissue specification and posteriorisation in the invertebrate chordate, the ascidian *Ciona intestinalis*. Development 130:147–159.

Huelsken J, Vogel R, Brinkmann V, Erdmann B, Birchmeier C, Birchmeier W. 2000. Requirement for β-catenin in anterior-posterior axis formation in mice. J Cell Biol 148:567–578.

Ikuta T, Yoshida N, Satoh N, Saiga H. 2004. *Ciona intestinalis* Hox gene cluster: its dispersed structure and residual colinear expression in development. Proc Natl Acad Sci U S A 101:15118–15123.

Imai K, Takada N, Satoh N, Satou Y. 2000. β-catenin mediates the specification of endoderm cells in ascidian embryos. Development 127:3009–3020.

Imai KS, Satoh N, Satou Y. 2002a. An essential role of a *FoxD* gene in notochord induction in *Ciona* embryos. Development 129:3441–3453.

Imai KS, Satou Y, Satoh N. 2002b. Multiple functions of a Zic-like gene in the differentiation of notochord, central nervous system and muscle in *Ciona savignyi* embryos. Development 129:2723–2732.

Imai KS, Satoh N, Satou Y. 2003. A Twist-like bHLH gene is a downstream factor of an endogenous FGF and determines mesenchymal fate in the ascidian embryos. Development 130:4461–4472.

Imai KS, Hino K, Yagi K, Satoh N, Satou Y. 2004. Gene expression profiles of transcription factors and signaling molecules in the ascidian embryo: towards a comprehensive understanding of gene networks. Development 131:4047–4058.

Inada K, Horie T, Kusakabe T, Tsuda M. 2003. Targeted knockdown of an *opsin* gene inhibits the swimming behaviour photoresponse of ascidian larvae. Neurosci Lett 347:167–170.

Jarving R, Jarving I, Kurg R, Brash AR, Samel N. 2004. On the evolutionary origin of *cyclooxygenase* (*COX*) isozymes: characterization of marine invertebrate *COX* genes points to independent duplication events in vertebrate and invertebrate lineages. J Biol Chem 279:13624–13633.

Jeffery WR. 1992. A gastrulation center in the ascidian egg. Dev Suppl:53–63.

Jeffery WR, Strickler AG, Yamamoto Y. 2004. Migratory neural crest-like cells form body pigmentation in a urochordate embryo. Nature 431:696–699.

Joyner AL. 1996. *Engrailed*, *Wnt* and *Pax* genes regulate midbrain—hindbrain development. Trends Genet 12:15–20.

Kalk M. 1970. The organization of the tunicate heart. Tissue Cell 2:99–118.

Kasahara M, Suzuki T, Pasquier LD. 2004. On the origins of the adaptive immune system: novel insights from invertebrates and cold-blooded vertebrates. Trends Immunol 25:105–111.

Kassar-Duchossoy L, Gayraud-Morel B, Gomes D, Rocancourt D, Buckingham M, Shinin V, Tajbakhsh S. 2004. *Mrf4* determines skeletal muscle identity in *Myf5:Myod* double-mutant mice. Nature 431:466–471.

Kawamura K, Fujita H, Nakauchi M. 1987. Cytological characterization of self incompatibility in gametes of the ascidian, *Ciona intestinalis*. Dev Growth Differ 29: 627–642.

Kim GJ, Nishida H. 2001. Role of the FGF and MEK signaling pathway in the ascidian embryo. Dev Growth Differ 43:521–533.

Kitajima S, Takagi A, Inoue T, Saga Y. 2000. MesP1 and MesP2 are essential for the development of cardiac mesoderm. Development 127:3215–3226.

Kobayashi K, Sawada K, Yamamoto H, Wada S, Saiga H, 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 130:5179–5190.

Kolsch V, Paululat A. 2002. The highly conserved cardiogenic bHLH factor *Hand* is specifically expressed in circular visceral muscle progenitor cells and in all cell types of the dorsal vessel during *Drosophila* embryogenesis. Dev Genes Evol 212:473–485.

Kondoh K, Kobayashi K, Nishida H. 2003. Suppression of macho-1-directed muscle fate by FGF and BMP is required for formation of posterior endoderm in ascidian embryos. Development 130:3205–3216.

Kowalesky A. 1866. Enwickelungsgeischitchte der einfachen Ascidien. Mem Acad Imp Sci St Petersb 7:1–19.

Kraus F, Haenig B, Kispert A. 2001. Cloning and expression analysis of the mouse T-box gene *Tbx18*. Mech Dev 100:83–86.

Krauss S, Johansen T, Korzh V, Fjose A. 1991. Expression of the zebrafish paired box gene *pax[zf-b]* during early neurogenesis. Development 113:1193–1206.

Kumano G, Nishida H. 1998. Maternal and zygotic expression of the endoderm-specific *alkaline phosphatase* gene in embryos of the ascidian, *Halocynthia roretzi*. Dev Biol 198:245–252.

Kusakabe T, Yoshida R, Kawakami I, Kusakabe R, Mochizuki Y, Yamada L, Shin-i T, Kohara Y, Satoh N, Tsuda M, Satou Y. 2002. Gene expression profiles in tadpole larvae of *Ciona intestinalis*. Dev Biol 242:188–203.

Lee H, Habas R, Abate-Shen C. 2004. MSX1 cooperates with histone H1b for inhibition of transcription and myogenesis. Science 304:1675–1678.

Lickert H, Kutsch S, Kanzler B, Tamai Y, Taketo MM, Kemler R. 2002. Formation of multiple hearts in mice following deletion of β-catenin in the embryonic endoderm. Dev Cell 3:171–181.

Locascio A, Aniello F, Amoroso A, Manzanares M, Krumlauf R, Branno M. 1999. Patterning the ascidian nervous system: structure, expression and transgenic analysis of the *CiHox3* gene. Development 126:4737–4748.

Lofberg J, Collazo A. 1997. Hypochord, an enigmatic embryonic structure: study of the axolotl embryo. J Morphol 232:57–66.

Logan CY, Miller JR, Ferkowicz MJ, McClay DR. 1999. Nuclear beta-catenin is required to specify vegetal cell fates in the sea urchin embryo. Development 126: 345–357.

Long S, Byrne M. 2001. Evolution of the echinoderm *Hox* gene cluster. Evol Dev 3:302–311.

Mackie GO, Singla CL. 2003. The capsular organ of *Chelyosoma productum* (Ascidiacea: Corellidae): a new tunicate hydrodynamic sense organ. Brain Behav Evol 61:45–58.

Manni L, Lane NJ, Joly JS, Gasparini F, Tiozzo S, Caicci F, Zaniolo G, Burighel P. 2004. Neurogenic and non-neurogenic placodes in ascidians. J Exp Zoolog Part A Comp Exp Biol 302B:483.

Marshall H, Studer M, Popperl H, Aparicio S, Kuroiwa A, Brenner S, Krumlauf R. 1994. A conserved retinoic acid response element required for early expression of the homeobox gene *Hoxb-1*. Nature 370: 567–571.

Matsuoka T, Awazu S, Satoh N, Sasakura Y. 2004. *Minos* transposon causes germline transgenesis of the ascidian *Ciona savignyi*. Dev Growth Differ 46:249–255.

Matthysse AG, Deschet K, Williams M, Marry M, White AR, Smith WC. 2004. A functional cellulose synthase from ascidian epidermis. Proc Natl Acad Sci U S A 101:986–991.

Meedel TH. 1997. Development of ascidian muscles and their evolutionary relationship to other chordate muscle types. In: Collier JR, editor. reproductive biology of invertebrates. Vol. VII. Progress in developmental biology. New York: John Wiley and Sons. p 305–330.

Meedel TH, Hastings KE. 1993. Striated muscle-type *tropomyosin* in a chordate smooth muscle, ascidian body-wall muscle. J Biol Chem 268:6755–6764.

Meedel TH, Crowther RJ, Whittaker JR. 1987. Determinative properties of muscle lineages in ascidian embryos. Development 100:245–260.

Meedel TH, Farmer SC, Lee JJ. 1997. The single *MyoD* family gene of *Ciona intestinalis* encodes two differentially expressed proteins: implications for the evolution of chordate muscle gene regulation. Development 124:1711–1721.

Meedel TH, Lee JJ, Whittaker JR. 2002. Muscle development and lineage-specific expression of *CiMDF*, the *MyoD*-family gene of *Ciona intestinalis*. Dev Biol 241: 238–246.

Meinertzhagen IA, Lemaire P, Okamura Y. 2004. The neurobiology of the ascidian tadpole larva: recent developments in an ancient chordate. Annu Rev Neurosci 27: 453–485.

Michibata H, Uyama T, Ueki T, Kanamori K. 2002. Vanadocytes, cells hold the key to resolving the highly selective accumulation and reduction of vanadium in ascidians. Microsc Res Tech 56:421–434.

Millar RH. 1953. *Ciona*. Liverpool: University Press of Liverpool.

Mitani Y, Takahashi H, Satoh N. 1999. An ascidian T-box gene *As-T2* is related to the *Tbx6* subfamily and is associated with embryonic muscle cell differentiation. Dev Dyn 215:62–68.

Mitani Y, Takahashi H, Satoh N. 2001. Regulation of the muscle-specific expression and function of an ascidian T-box gene, *As-T2*. Development 128:3717–3728.

Miya T, 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 261:25–38.

Mochizuki Y, Satou Y, Satoh N. 2003. Large-scale characterization of genes specific to the larval nervous system in the ascidian *Ciona intestinalis*. Genesis 36:62–71.

Monaghan AP, Kaestner KH, Grau E, Schutz G. 1993. Postimplantation expression patterns indicate a role for the mouse *forkhead/HNF-3 alpha*, *beta* and *gamma* genes in determination of the definitive endoderm, chordamesoderm and neuroectoderm. Development 119:567–578.

Moody R, Davis SW, Cubas F, Smith WC. 1999. Isolation of developmental mutants of the ascidian *Ciona savignyi*. Mol Gen Genet 262:199–206.

Morgan TH. 1944. The genetic and physiological problems of self-sterility in *Ciona*. VI. Theoretical discussion of genetic data. J Exp Zool 95:37–59.

Munro EM, Odell G. 2002. Morphogenetic pattern formation during ascidian notochord formation is regulative and highly robust. Development 129:1–12.

Murabe N, Hoshi M. 2002. Re-examination of sibling cross-sterility in the ascidian, *Ciona intestinalis*: genetic background of the self-sterility. Zool Sci 19:527–538.

Nagatomo K, Fujiwara S. 2003. Expression *of* Raldh2, *Cyp26* and *Hox-1* in normal and retinoic acid-treated *Ciona intestinalis* embryos. Gene Expr Patterns 3: 273–277.

Nakamura T, Sano M, Songyang Z, Schneider MD. 2003. A Wnt- and beta-catenin-dependent pathway for mammalian cardiac myogenesis. Proc Natl Acad Sci U S A 100:5834–5839.

Nakashima K, Yamada L, Satou Y, Azuma J, Satoh N. 2004. The evolutionary origin of animal cellulose synthase. Dev Genes Evol 214:81–88.

Nakata K, Nagai T, Aruga J, Mikoshiba K. 1998. *Xenopus Zic* family and its role in neural and neural crest development. Mech Dev 75:43–51.

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

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

Nakatani Y, Moody R, Smith WC. 1999. Mutations affecting tail and notochord development in the ascidian *Ciona savignyi*. Development 126:3293–3301.

Neubuser A, Koseki H, Balling R. 1995. Characterization and developmental expression of *Pax9*, a paired-box-containing gene related to *Pax1*. Dev Biol 170: 701–716.

Nicol D, Meinertzhagen IA. 1988a. Development of the central nervous system of the larva of the ascidian, *Ciona intestinalis* L. I. The early lineages of the neural plate. Dev Biol 130:721–736.

Nicol D, Meinertzhagen IA. 1988b. Development of the central nervous system of the larva of the ascidian, *Ciona intestinalis* L. II. Neural plate morphogenesis and cell lineages during neurulation. Dev Biol 130:737–766.

Nicol D, Meinertzhagen IA. 1991. Cell counts and maps in the larval central nervous system of the ascidian *Ciona intestinalis* (L.). J Comp Neurol 309:415–429.

Nieuwkoop PD. 1969. The formation of the mesoderm in urodele amphibians. I. Induction by the endoderm. Rouxs Arch Entwicklungsmech 162:341–373.

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 121:526–541.

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

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

Nishida H, 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 99:382–394.

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

Nishide K, Nishikata T, Satoh N. 1989. A monoclonal antibody specific to embryonic trunk-lateral cells of the ascidian *Halocynthia roretzi* stains coelomic cells of juvenile and adult basophilic blood cells. Dev Growth Differ 31:595–600.

Noll M. 1993. Evolution and role of *Pax* genes. Curr Opin Genet Dev 3:595–605.

Nornes HO, Dressler GR, Knapik EW, Deutsch U, Gruss P. 1990. Spatially and temporally restricted expression of *Pax2* during murine neurogenesis. Development 109:797–809.

Oda I, Saiga H. 2001. *Hremx*, the ascidian homologue of *ems/emx*, is expressed in the anterior and posterior-lateral epidermis but not in the central nervous system during embryogenesis. Dev Genes Evol 211:291–298.

Odenthal J, Nusslein-Volhard C. 1998. *fork head* domain genes in zebrafish. Dev Genes Evol 208:245–258.

Ogasawara M, Di Lauro R, Satoh N. 1999a. Ascidian homologs of mammalian *thyroid peroxidase* genes are expressed in the thyroid-equivalent region of the endostyle. J Exp Zool 285:158–169.

Ogasawara M, Satou Y. 2003. Expression of *FoxE* and *FoxQ* genes in the endostyle of *Ciona intestinalis*. Dev Genes Evol 213:416–419.

Ogasawara M, Wada H, Peters H, Satoh N. 1999b. Developmental expression of *Pax1/9* genes in urochordate and hemichordate gills: insight into function and evolution of the pharyngeal epithelium. Development 126:2539–2550.

Ohtsuka Y, Okamura Y, Obinata T. 2001. Changes in *gelsolin* expression during ascidian metamorphosis. Dev Genes Evol 211:252–256.

Okada T, Hirano H, Takahashi K, Okamura Y. 1997. Distinct neuronal lineages of the ascidian embryo revealed by expression of a sodium channel gene. Dev Biol 190:257–272.

Okuyama M, Saito Y, Ogawa M, Takeuchi A, Jing Z, Naganuma T, Hirose E. 2002. Morphological studies on the bathyal ascidian, *Megalodicopia hians* Oka 1918 (Octacnemidae, Phlebobranchia), with remarks on feeding and tunic morphology. Zool Sci 19:1181–1189.

Olsen CL, Jeffery WR. 1997. A *forkhead* gene related to *HNF-3beta* is required for gastrulation and axis formation in the ascidian embryo. Development 124:3609–3619.

Ortolani G. 1971. Sul cell-lineage delle ascidie. Boll Zool 38:85–88.

Pinto MR, Chinnici CM, Kimura Y, Melillo D, Marino R, Spruce LA, De Santis R, Parrinello N, Lambris JD. 2003. CiC3-1a-mediated chemotaxis in the deuterostome invertebrate *Ciona intestinalis* (Urochordata). J Immunol 171:5521–5528.

Powers TP, Amemiya CT. 2004. Evidence for a Hox14 paralog group in vertebrates. Curr Biol 14:R183–R184.

Ristoratore F, Spagnuolo A, Aniello F, Branno M, Fabbrini F, Di Lauro R. 1999. Expression and functional analysis of *Cititf1*, an ascidian *NK-2* class gene, suggest its role in endoderm development. Development 126:5149–5159.

Rocheleau CE, Downs WD, Lin R, Wittmann C, Bei Y, Cha YH, Ali M, Priess JR, Mello CC. 1997. Wnt signaling and an APC-related gene specify endoderm in early *C. elegans* embryos. Cell 90:707–716.

Rosati F, De Santis R. 1978. Studies on fertilization in the Ascidians. I. Self-sterility and specific recognition between gametes of *Ciona intestinalis*. Exp Cell Res 112:111–119.

Rosati F, De Santis R. 1980. Role of the surface carbohydrates in sperm-egg interaction in *Ciona intestinalis*. Nature 283:762–764.

Rowley AF, Rhodes CP, Pratcliffe NA. 1984. Protochordate leucocytes: a review. Zool J Linnean Soc 80:283–295.

Ruiz i Altaba A, Prezioso VR, Darnell JE, Jessell TM. 1993. Sequential expression of *HNF-3 beta* and *HNF-3 alpha* by embryonic organizing centers: the dorsal lip/node, notochord and floor plate. Mech Dev 44:91–108.

Rushlow CA, Han K, Manley JL, Levine M. 1989. The graded distribution of the dorsal morphogen is initiated by selective nuclear transport in *Drosophila*. Cell 59:1165–1177.

Sasaki H, Hogan BL. 1993. Differential expression of multiple *fork head* related genes during gastrulation and axial pattern formation in the mouse embryo. Development 118:47–59.

Sasaki H, Hogan BL. 1994. *HNF-3*β as a regulator of floor plate development. Cell 76:103–115.

Sasaki A, Miyamoto Y, Satou Y, Satoh N, Ogasawara M. 2003. Novel endostyle-specific genes in the ascidian *Ciona intestinalis*. Zool Sci 20:1025–1030.

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

Sasakura Y, Awazu S, Chiba S, Kano S, Satoh N. 2003b. Application of *Minos*, one of the Tc1/*mariner* superfamily transposable elements, to ascidian embryos as a tool for insertional mutagenesis. Gene 308:11–20.

Satoh N. 1994. Developmental biology of ascidians. New York: Cambridge University Press.

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

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

Satou Y, Imai KS, Satoh N. 2001a. Action of morpholinos in *Ciona* embryos. Genesis 30:103–106.

Satou Y, Takatori N, Yamada L, Mochizuki Y, Hamaguchi M, Ishikawa H, Chiba S, Imai K, Kano S, Murakami SD, Nakayama A, Nishino A, Sasakura Y, Satoh G, Shimotori T, Shin IT, Shoguchi E, Suzuki MM, Takada N, Utsumi N, Yoshida N, Saiga H, Kohara Y, Satoh N. 2001b. Gene expression profiles in *Ciona intestinalis* tailbud embryos. Development 128:2893–2904.

Satou Y, Yamada L, Mochizuki Y, Takatori N, Kawashima T, Sasaki A, Hamaguchi M, Awazu S, Yagi K, Sasakura Y, Nakayama A, Ishikawa H, Inaba K, Satoh N. 2002a. A cDNA resource from the basal chordate *Ciona intestinalis*. Genesis 33:153–154.

Satou Y, Imai KS, Satoh N. 2002b. *Fgf* genes in the basal chordate *Ciona intestinalis*. Dev Genes Evol 212:432–438.

Satou Y, Yagi K, Imai KS, Yamada L, Nishida H, Satoh N. 2002c. *macho-1*-related genes in *Ciona* embryos. Dev Genes Evol 212:87–92.

Satou Y, Imai KS, Satoh N. 2004. The ascidian *Mesp* gene specifies heart precursor cells. Development 131:2533–2541.

Schneider S, Steinbeisser H, Warga RM, Hausen P. 1996. Beta-catenin translocation into nuclei demarcates the dorsalizing centers in frog and fish embryos. Mech Dev 57:191–198.

Scofield VL, Schlumpberger JM, West LA, Weissman IL. 1982. Protochordate allorecognition is controlled by a *MHC*-like gene system. Nature 295:499–502.

Shida K, Terajima D, Uchino R, Ikawa S, Ikeda M, Asano K, Watanabe T, Azumi K, Nonaka M, Satou Y, Satoh N, Satake M, Kawazoe Y, Kasuya A. 2003. Hemocytes of *Ciona intestinalis* express multi-

ple genes involved in innate immune host defense. Biochem Biophys Res Commun 302:207–218.

Shimeld SM. 1999. The evolution of the *hedgehog* gene family in chordates: insights from amphioxus *hedgehog*. Dev Genes Evol 209:40–47.

Showell C, Binder O, Conlon FL. 2004. T-box genes in early embryogenesis. Dev Dyn 229:201–218.

Simeone A, Gulisano M, Acampora D, Stornaiuolo A, Rambaldi M, Boncinelli E. 1992a. Two vertebrate homeobox genes related to the *Drosophila empty spiracles* gene are expressed in the embryonic cerebral cortex. EMBO J 11:2541–2550.

Simeone A, Acampora D, Gulisano M, Stornaiuolo A, Boncinelli E. 1992b. Nested expression domains of four homeobox genes in developing rostral brain. Nature 358:687–690.

Smith WC. 1999. TGF beta inhibitors. New and unexpected requirements in vertebrate development. Trends Genet 15:3–5.

Sokol SY, Melton DA. 1992. Interaction of Wnt and activin in dorsal mesoderm induction in *Xenopus*. Dev Biol 154:348–355.

Sordino P, Belluzzi L, De Santis R, Smith WC. 2001. Developmental genetics in primitive chordates. Philos Trans R Soc Lond B Biol Sci 356:1573–1582.

Spagnuolo A, Ristoratore F, Di Gregorio A, Aniello F, Branno M, Di Lauro R. 2003. Unusual number and genomic organization of *Hox* genes in the tunicate *Ciona intestinalis*. Gene 309:71–79.

Stoner DS, Rinkevich B, Weissman IL. 1999. Heritable germ and somatic cell lineage competitions in chimeric colonial protochordates. Proc Natl Acad Sci U S A 96:9148–9153.

Streit A, Berliner AJ, Papanayotou C, Sirulnik A, Stern CD. 2000. Initiation of neural induction by FGF signalling before gastrulation. Nature 406:74–78.

Svane I, Young CM. 1989. The ecology and behavior of ascidian larvae. Oceanogr Mar Biol Annu Rev 27:45–90.

Swalla BJ, Jeffery WR. 1996. Requirement of the *Manx* gene for expression of chordate features in a tailless ascidian larva. Science 274:1205–1208.

Swalla BJ, Just MA, Pederson EL, Jeffery WR. 1999. A multigene locus containing the *Manx* and *bobcat* genes is required for development of chordate features in the ascidian tadpole larva. Development 126:1643–1653.

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

Takahashi T, Holland PW. 2004. Amphioxus and ascidian *Dmbx* homeobox genes give clues to the vertebrate origins of midbrain development. Development 131:3285–3294.

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

Takahashi T, Holland PW, Cohn MJ, Shimizu K, Kurokawa M, Hirai H. 2002. An orphan PRD class homeobox gene expressed in mouse brain and limb development. Dev Genes Evol 212:293–297.

Takamura K. 1998. Nervous network in larvae of the ascidian *Ciona intestinalis*. Dev Genes Evol 208:1–8.

Takatori N, Satou Y, Satoh N. 2002. Expression of *hedgehog* genes in *Ciona intestinalis* embryos. Mech Dev 116:235–238.

Takatori N, Hotta K, Mochizuki Y, Satoh G, Mitani Y, Satoh N, Satou Y, Takahashi H. 2004. T-box genes in the ascidian *Ciona intestinalis*: characterization of cDNAs and spatial expression. Dev Dyn 230:743–753.

Tanaka M, Tickle C. 2004. *Tbx18* and boundary formation in chick somite and wing development. Dev Biol 268:470–480.

Torrence SA, Cloney RA. 1983. Nervous system of ascidian larvae: caudal primary sensory neurons. Zoomorphology 99:103–115.

Trivedi S, Ueki T, Yamaguchi N, Michibata H. 2003. Novel vanadium-binding proteins (vanabins) identified in cDNA libraries and the genome of the ascidian *Ciona intestinalis*. Biochim Biophys Acta 1630:64–70.

Tsuda M, Sakurai D, Goda M. 2003. Direct evidence for the role of pigment cells in the brain of ascidian larvae by laser ablation. J Exp Biol 206:1409–1417.

Utsumi N, Shimojima Y, Saiga H. 2004. Analysis of ascidian *Not* genes highlights their evolutionarily conserved and derived features of structure and expression in development. Dev Genes Evol 214:460–465.

Wada H. 2001. Origin and evolution of the neural crest: a hypothetical reconstruction of its evolutionary history. Dev Growth Differ 43:509–520.

Wada S, Katsuyama Y, Sato Y, Itoh C, Saiga H. 1996. *Hroth* an orthodenticle-related homeobox gene of the ascidian, *Halocynthia roretzi*: its expression and putative roles in the axis formation during embryogenesis. Mech Dev 60:59–71.

Wada H, Saiga H, Satoh N, Holland PW. 1998. Tripartite organization of the ancestral chordate brain and the antiquity of placodes: insights from ascidian *Pax-2/5/8*, *Hox* and *Otx* genes. Development 125:1113–1122.

Wada H, Garcia-Fernandez J, Holland PW. 1999a. Colinear and segmental expression of amphioxus *Hox* genes. Dev Biol 213:131–141.

Wada S, Katsuyama Y, Saiga H. 1999b. Anteroposterior patterning of the epidermis by inductive influences from the vegetal hemisphere cells in the ascidian embryo. Development 126:4955–4963.

Wada S, Tokuoka M, Shoguchi E, Kobayashi K, Di Gregorio A, Spagnuolo A, Branno M, Kohara Y, Rokhsar D, Levine M, Saiga H, Satoh N, Satou Y. 2003. A genomewide survey of developmentally relevant genes in *Ciona intestinalis*. II. Genes for homeobox transcription factors. Dev Genes Evol 213:222–234.

Wallin J, Eibel H, Neubuser A, Wilting J, Koseki H, Balling R. 1996. *Pax1* is expressed during development of the thymus epithelium and is required for normal T-cell maturation. Development 122:23–30.

Wang J, Karabinos A, Zimek A, Meyer M, Riemer D, Hudson C, Lemaire P, Weber K. 2002. Cytoplasmic intermediate filament protein expression in tunicate development: a specific marker for the test cells. Eur J Cell Biol 81:302–311.

Weinstein DC, Ruiz i Altaba A, Chen WS, Hoodless P, Prezioso VR, Jessell TM, Darnell JE Jr. 1994. The winged-helix transcription factor HNF-3 beta is required for notochord development in the mouse embryo. Cell 78:575–588.

Whittaker JR. 1973. Segregation during ascidian embryogenesis of egg cytoplasmic information for tissue-specific enzyme development. Proc Natl Acad Sci U S A 70:2096–2100.

Whittaker JR. 1977. Segregation during cleavage of a factor determining endodermal alkaline phosphatase development in ascidian embryos. J Exp Zool 202:139–153.

Whittaker JR. 1990. Determination of alkaline phosphatase expression in endodermal cell lineages of an ascidian embryo. Biol Bull 178:222–230.

Wikramanayake AH, Hong M, Lee PN, Pang K, Byrum CA, Bince JM, Xu R, Martindale MQ. 2003. An ancient role for nuclear β-catenin in the evolution of axial polarity and germ layer segregation. Nature 426:446–450.

Willey A. 1893. Studies on the Protochordata. I. On the origin of the branchial stigmata, praeoral lobe, endostyle, atrial cavities, etc. in *Ciona intestinalis*, Linn., with remarks on *Clavelina lepadiformis*. Q J Micro Sci 34:317–360.

Wilson SI, Graziano E, Harland R, Jessell TM, Edlund T. 2000. An early requirement for FGF signalling in the acquisition of neural cell fate in the chick embryo. Curr Biol 10:421–429.

Wright A. 1981. Urochordates. In: Ratcliffe NA, Rowley AF, editors. Invertebrate blood cells. New York: Academic Press. p 565–626.

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

Yagi K, Satoh N, Satou Y. 2004b. Identification of downstream genes of the ascidian muscle determinant gene *Ci-macho1*. Dev Biol 274:478–489.

Yasuo H, Satoh N. 1993. Function of vertebrate *T* gene. Nature 364:582–583.

Zeltser L, Desplan C, Heintz N. 1996. *Hoxb-13*: a new Hox gene in a distant region of the *HOXB* cluster maintains colinearity. Development 122:2475–2484.

Zhang Z, Gerstein M. 2003. Of mice and men: phylogenetic footprinting aids the discovery of regulatory elements. J Biol 2:11.