Development 138, 2143-2152 (2011) doi:10.1242/dev.048975

Evolutionary crossroads in developmental biology: the tunicates

Patrick Lemaire[1,2,*]

Summary

The tunicates, or urochordates, constitute a large group of marine animals whose recent common ancestry with vertebrates is reflected in the tadpole-like larvae of most tunicates. Their diversity and key phylogenetic position are enhanced, from a research viewpoint, by anatomically simple and transparent embryos, compact rapidly evolving genomes, and the availability of powerful experimental and computational tools with which to study these organisms. Tunicates are thus a powerful system for exploring chordate evolution and how extreme variation in genome sequence and gene regulatory network architecture is compatible with the preservation of an ancestral chordate body plan.

Key words: Chordates, Tunicates, Asexual development, Budding, Embryogenesis, Evolution

Introduction

Cephalochordates (including *Amphioxus*; see Glossary, Box 1), tunicates (or urochordates, see Glossary, Box 1) and vertebrates constitute the chordate phylum, which is characterized by a tadpole-like body plan at the end of embryogenesis. The rigidity of the tail of these tadpoles is ensured by a unique structure, the notochord, which gave its name to the phylum. Fossil evidence, although sometimes controversial, suggests that ancestral chordates roamed Cambrian oceans more than 550 million years ago (Morris, 1999; Shu et al., 1996).

It is now thought that cephalochordates are the most basal chordates. Tunicates and vertebrates are sister taxa (see Glossary, Box 1), which diverged more recently (Delsuc et al., 2006; Bourlat et al., 2006; Putnam et al., 2008). This molecular classification is supported at the anatomical level by the presence in tunicate larvae, but not in those of cephalochordates or of any other invertebrate, of cells similar to vertebrate migratory neural crest cells (Jeffery, 2007).

The tunicates constitute a diverse group of animals that are united by the cellulose-containing tunic that covers their body (Nakashima et al., 2004). They are traditionally split into three major classes: the ascidians, thaliaceans and appendicularians, whose phylogenetic relationships are depicted in Fig. 1 (Tsagkogeorga et al., 2009; Govindarajan et al., 2010). Ascidians, also called sea squirts, are the most diverse tunicate group and include several developmental models such as *Ciona intestinalis*, *Halocynthia roretzi* and *Botryllus schlosseri*. These vase-like benthic filter feeders (see Glossary, Box 1) ('ascidian' derives from

[1]Institut du Biologie de Développement de Marseille Luminy (IBDML, UMR 6216, CNRS, Université de la Méditerranée), Parc Scientifique de Luminy Case 907, F-13288, Marseille Cedex 9, France. [2]Centre de Recherches en Biochimie Macromoléculaire (CRBM, UMR5237, CNRS, Universités Montpellier 1 and 2), 1919 route de Mende, F-34293, Montpellier Cedex 05, France.

*Author for correspondence (patrick.lemaire@crbm.cnrs.fr)

Box 1. Glossary

Benthic. Organisms that live on the bottom of seas or lakes.

Cephalochordates. Invertebrate chordates of this class live partly buried in sand in shallow temperate or tropical waters. The class includes only two genera, one of which (*Amphioxus*) is a model organism for evo-devo studies. The adult form has a fish-like appearance but no skeleton.

Cis-regulatory modules. These are short stretches (<1 kb) of usually non-coding, genomic DNA that include clustered transcription factor binding sites and control gene expression. They can be divided into functional classes, including enhancers, insulators and silencers.

Colonial organism. An organism in which several individuals live closely associated with each other. In some tunicate colonies, different individuals are connected by root-like structures called stolons. In others, they share body parts, such as their atrial (exhaling) siphon.

Filter feeder. Organisms that feed on particulate food suspended in water. In tunicates, water enters the body via the oral siphon, is filtered on the branchial basket and expelled through an atrial siphon (in appendicularians, water is expelled through gill slits).

Hybridization. The production of offspring by interbreeding between two individuals of different species.

Kernel. A small region of a gene regulatory network that is evolutionarily highly conserved, the retention of which might underlie the conservation of body plans and the development of major body parts.

Marine snow. Small, falling organic marine detritus that plays an important role in the transport, and sequestration, of carbon to the ocean floor and constitutes an important energy source for the zooplankton in the deep, lightless zone of the ocean.

Nucleosome exclusion. The basic structural subunit of chromatin, consisting of ~200 bp of DNA and an octamer of histones. Genomic regions from which nucleosomes are excluded are more easily bound by transcription factors and are more likely to act as cis-regulatory modules.

Operon. A group of genes that functions as a single transcription unit and produces one or several polycistronic mRNAs, each encoding the protein products of several genes.

Pelagic. Pelagic organisms live in seas or lakes, far away from the shore or the bottom.

Sister taxa. Two taxa are 'sisters' if they have a last common ancestor that did not give rise to any other taxa.

Synteny. Colinearity in the order of genes (or of other DNA sequences) in a chromosomal region of two species, such as in the Hox clusters among vertebrates.

Trans-splicing. A specialized form of splicing in which exons from different primary transcripts are spliced together. In the case of tunicates, the 5′ end of the primary transcript of many coding genes is spliced out until the first splice acceptor site, and is replaced by a unique short 5′ capped RNA termed the splice leader.

Urochordates. An alternative name for tunicates. The etymology (*uro*: tail and chordates) is somewhat misleading as many tunicates have lost their tailed tadpole-like larvae. The term tunicates should therefore be preferentially used.

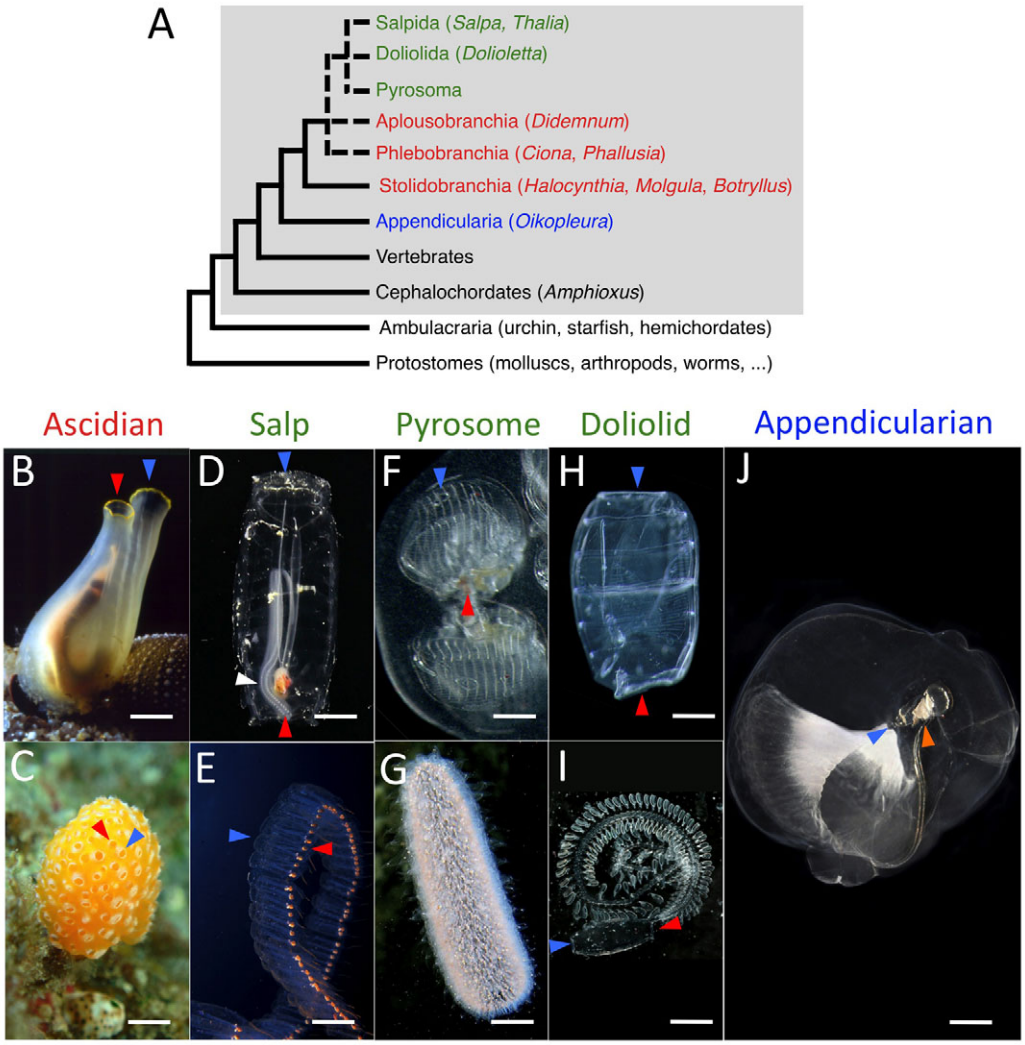

Fig. 1. Phylogeny and adult morphology of major tunicate groups. (**A**) Cladogram of phylogenetic relationships between tunicate orders/suborders and animal groups of interest (E. Douzery and F. Delsuc, Université Montpellier, France, personal communication). Ascidians, red; thaliaceans, green; larvaceans, blue; chordates, gray area. Italics highlight genera discussed in the text. Phylogenetic relationships within tunicates are inferred from 18S RNA analysis (Tsagkogeorga et al., 2009; Govindarajan et al., 2010), relationships between tunicates and other groups from genomic analyses (Delsuc et al., 2006). Dashed lines indicate uncertain relationships. (**B-J**) Typical adult morphologies in three major tunicate classes. Blue and red arrowheads indicate oral and atrial siphons, respectively. (B) Adult *Ciona intestinalis* (order, Enterogona; suborder, Phlebobranchia; genus, *Ciona*; image courtesy of T. Meedel, Rhode Island College, RI, USA). (C) *Pseudodistoma kanoko* colony (order, Enterogona; suborder, Aplousobranchia; genus, *Pseudodistoma*; image courtesy of E. Hirose, Okinawa Institute of Science and Technology, Japan). (D) Solitary *Salpa fusiformis* asexual zooid with a nascent chain of sexual zooids (white arrowhead) [order, Salpida; family, Salpidae, genus, *Salpa*; image courtesy of C. and N. Sardet, Station Biologique de Villefranche/mer (SBVM), France]. (E) Chain of *Pegea confederata* sexual zooids (order, Salpida; family, Salpidae; genus, *Pegea*; image courtesy D. Luquet, SBVM, France, copyright davidluquet.com). (F) Two *Pyrosomella verticilliata* zooids from a young colony (order, Pyrosomida; suborder, Pyrosomatidae; genus, *Pyrosomella*; image courtesy of R. Hopcroft, Alaska Fairbanks University, AK, USA). (G) A *Pyrosoma atlanticum* colony (order, Pyrosomida; family, Pyrosomatidae; genus, *Pyrosoma*; image courtesy G. Cavignaux, DORIS, France). (H) *Doliolum denticulatum* zooid (order, Doliolida; family, Doliolidae; genus, *Doliolidum*; image courtesy of R. Hopcroft). (I) A *Dolioletta gegenbauri* zooid pulling a chain of smaller asexually generated individuals (order, Doliolida; family, Doliolidae; genus, *Dolioletta*; image courtesy of L. Madin, Woods Hole Oceanic Institute, MA, USA). (J) Adult *Oikopleura dioica* (order Copelata; family, Oikopleuridae; genus, *Oikopleura*; image courtesy of E. Thompson, J.-M. Bouquet and J. Slama, SARS institute, Norway) in its food-concentrating house. The beating tail directs food (white milk powder) into the funnel leading to the oral siphon. Larvaceans have ventral gill slits (orange arrowhead) instead of an atrial siphon. Scale bars: 10 mm in B,D; 15 mm in C; 1 mm in F,H; 40 mm in E,G; 5 mm in I; 0.5 mm in J.

the Greek *askidion*: small vase) spend their adult life attached to a solid substrate (Fig. 1B,C). By contrast, thaliaceans (*thalia* means blooming in Greek, referring to their rapid proliferation under favorable environmental conditions) include three major groups, the salps, doliolids and pyrosomes (Fig. 1D-I), which are phylogenetically nested within ascidians (Fig. 1A). They swim by jet propulsion in open oceans. Finally, appendicularians include the model organism *Oikopleura dioica*. Appendicularians are also called larvaceans because they retain a larval tadpole body plan throughout their short, planktonic life. They have diverted the locomotory function of their tadpole tail to the capture of food (Bone, 1998) (Fig. 1J).

The annotated genome sequences of two ascidians (*Ciona savignyi* and *C. intestinalis*) and one appendicularian (*O. dioica*) have been published (Dehal et al., 2002; Small et al., 2007; Denoeud et al., 2010) (Table 1). Genomic sequences in *C. intestinalis* have been mapped onto its 14 chromosomes, a prerequisite to studying the global organization of the genome (Shoguchi et al., 2006). Consistent with the large population sizes of the sequenced species, sequenced tunicate genomes are highly polymorphic. These genomes are surprisingly small (*Ciona* ~160 Mb; *Oikopleura* ~70 Mb, the smallest animal genome so far), owing to several factors: their unduplicated gene complement; substantial gene loss (Holland and Gibson-Brown, 2003; Denoeud

Table 1. Features of the main tunicate model species in developmental biology

Species	Genome (Mb)	Asexual reproduction	Season of sexual reproduction in the wild	Life cycle (egg to egg)	Distribution	Eggs per adult	Egg diameter (μm)	Embryo electroporation	RNA interference
Ciona intestinalis	160	No	Most of the year‡	2-3 months	All temperate seas	Up to 10,000	140	Yes	Yes*
Ciona savignyi	190	No	Most of the year‡	2-3 months	Temperate Pacific	Up to 10,000	160	Yes	?
Phallusia mammillata	<160	No	March to December	<1 year	Europe	Up to 1 million	120	Yes	?
Halocynthia roretzi	~160	No	November to January	3 years	North East Asia	Up to 30,000	280	Yes	?
Botryllus schlosseri	~700	Yes	March to December§	<3 months	Worldwide	<5 per zooid	220-250	?	Yes†
Molgula oculata	?	No	July/August	1 year	North West Europe	Up to 5000	90	No	No
Molgula occulta	?	No	July/August	1 year	Europe	Up to 5000	110	No	No
Oikopleura dioica	70	No	All year	4 days	Warm and temperate seas	~150	65-75	?	?

*Single report.
†Only tested in adults.
‡*Ciona* reproductive season is largely determined by water temperature and so varies between regions.
§In Monterey Bay, CA, USA. Reproductive season varies with location.
?, Unknown or untested.

et al., 2010); and the compaction of intronic and intergenic sequences, possibly via a tight control of transposable elements (Denoeud et al., 2010). Ascidian and appendicularian genomes have a high prevalence of polycistronic transcripts (operons, see Glossary, Box 1) that are resolved by trans-splicing (see Glossary, Box 1) (Satou et al., 2008; Denoeud et al., 2010; Ganot et al., 2004). Frequent or infrequent trans-splicing is also found in most monocistronic genes (Matsumoto et al., 2010).

Tunicate genomes evolve rapidly. Some tunicate proteins have among the fastest evolution rates of metazoans (Denoeud et al., 2010; Putnam et al., 2008), and non-coding cis-regulatory elements of phylogenetically distant ascidians, such as *Ciona* and *Halocynthia*, can diverge up to a point at which their sequences cannot be aligned (Oda-Ishii et al., 2005). The organization of the tunicate genomes is highly dynamic, and considerable rearrangements can be observed even within the *Ciona* genus (Hill et al., 2008). Although faint traces of global synteny (see Glossary, Box 1) can be detected between vertebrates and *Ciona*, local gene order has been extensively shuffled between tunicates and vertebrates (Denoeud et al., 2010). This led, for instance, to 'exploded' tunicate Hox clusters (reviewed by Duboule, 2007).

Most tunicate developmental studies have so far focused on the embryonic development of ascidians up to the chordate tadpole larva, as this is the developmental period during which the common ancestry with vertebrates is most obvious (reviewed by Lemaire et al., 2008; Lemaire, 2009; Nishida, 2008). Ascidian embryogenesis is characterized by a stereotyped development that is based on invariant early cell lineages (see Fig. 2) and a remarkably small cell number (Kumano and Nishida, 2007). These unique, and highly derived, features make it possible to study a chordate developmental program with cellular or even subcellular resolution. As detailed below, comparisons of the embryonic strategies found in the subphylum have revealed a great diversity, which is starting to be exploited to study the complex relationships between environment, genomes and phenotypes. Some tunicates also reproduce asexually by budding and are therefore useful models in which to study regeneration and also how two parallel developmental programs that lead to the same adult forms can be encoded in a single genome.

In this article, I focus on studies carried out in a few model organisms, mostly ascidians, that have led to key insights into chordate developmental mechanisms and their evolution, with particular emphasis on the transcriptional control of development and its interface with morphogenesis and cell biology. I also highlight the diversity of tunicate developmental strategies, a richness that deserves to be explored.

Tunicate habitats and life cycles

Tunicates can be found in all marine environments. Ascidians live attached to the bottom of the seas, in both shallow waters and the deep ocean (Kurabayashi et al., 2003). In addition to their natural habitat, global shipping and global warming have led to the spread of many ascidians to non-native environments. Some invasive species, in particular the aplousobranch *Didemnum vexillum*, can strongly affect local ecosystems and the aquaculture industry (Lambert and Lambert, 2001). Thaliaceans and appendicularians are pelagic (see Glossary, Box 1) organisms that are common in most oceans. Appendicularians form a major component of the zooplankton (Bone, 1998). Their abandoned houses (Fig. 1G) contribute to marine snow (see Glossary, Box 1) and are an important food source for other pelagic organisms (Gorsky et al., 2005).

Reproductive strategies are diverse within the subphylum and include both sexual and asexual cycles. Appendicularians and solitary ascidians, such as *C. intestinalis* and *H. roretzi*, only have a sexual life cycle. With the exception of *O. dioica*, all tunicates are hermaphrodites. The duration of their sexual life cycle ranges from four days (*Oikopleura*) to several years (see Table 1). To avoid inbreeding, ascidians have developed a mechanism of self sterility, as initially studied in *Ciona* by T. H. Morgan (Morgan, 1944), similar to that developed by flowering plants and involving the interaction between highly polymorphic polycystin-1 and fibrinogen-like proteins (Harada et al., 2008). Their sexual life cycle classically produces a free-swimming tadpole larva, which

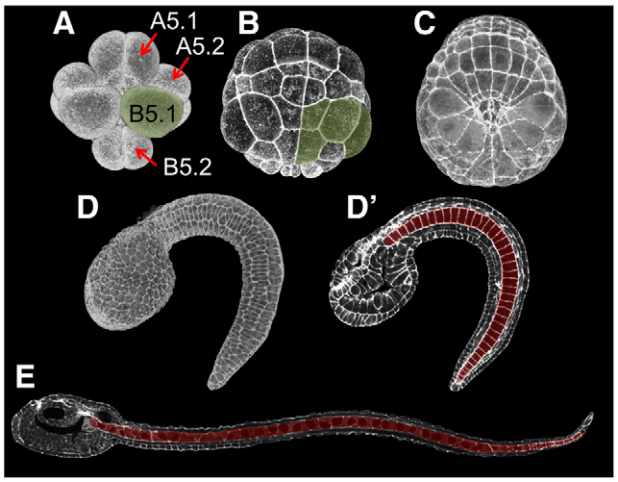

Fig. 2. Sexual and asexual life cycles in ascidians. Three-dimensional projections of confocal stacks through developing *Ciona intestinalis* embryos. (**A**) A 16-cell embryo, with the vegetal cells labeled. (**B**) A 64-cell embryo. (**C**) A mid-gastrula embryo. (**D**,**D′**) Mid-tailbud embryos. (**E**) A larva. (A,B) The B5.1 blastomere and its stereotyped progeny at the 64-cell stage are indicated in light green. (D′,E) Longitudinal sections. Note the bilateral symmetry of early embryos, and the prominent notochord (red) at the tailbud stages, which becomes vacuolated at larval stages. Images courtesy of the Four-Dimensional Ascidian Body Atlas (FABA) database (Hotta et al., 2007).

lives no more than a few days before undergoing an extensive metamorphosis during which many larval tissues, including the larval tail and most neurons, undergo apoptosis (Chambon et al., 2002). Formation of the adult organs is still imperfectly understood and may vary with the organ. Although a patterned heart field is formed during embryogenesis and survives metamorphosis (Davidson, 2007), the adult nervous system was recently proposed to originate from ependymal larval cells with neural stem-like properties (Horie et al., 2011).

In addition to their sexual life cycle, thaliaceans and colonial (see Glossary, Box 1) ascidians, such as *B. schlosseri*, reproduce asexually by budding without going through a tadpole-like developmental stage. Salps, for instance, alternate their sexual and asexual reproductive stages: solitary asexual individuals bud off a chain of sexual individuals (Fig. 1D, arrowhead; Fig. 1E), which in turn produce solitary asexual individuals (Bone et al., 1985). In colonial ascidians, the larva obtained by sexual reproduction metamorphoses into a primary adult individual, or zooid, that reproduces asexually to produce a clonal colony, such as that shown in Fig. 1C. This asexual life cycle, called blastogenesis, has evolved independently several times, as indicated by the scattering of colonial organisms in the tunicate phylogeny and by the variety of their budding strategies (e.g. Berrill, 1948). Blastogenesis has been extensively studied in the ascidian *B. schlosseri*. In a highly coordinated fashion, adult individuals from a colony undergo apoptosis every week and are replaced by new zooids derived from a population of somatic stem cells (Voskoboynik et al., 2008). An independent stem cell population regenerates the germline (Laird et al., 2005a). A distinct regenerative program, that of whole body regeneration, is activated when buds and zooids from *B. schlosseri* colonies are surgically removed, leaving only the vasculature and the tunic (Voskoboynik et al., 2007), or when small *Botrylloides leachi* blood vessel fragments are cultured (Rinkevich et al., 2007).

Experimental techniques in tunicates

The palette of experimental techniques available for each tunicate group varies greatly and reflects the popularity of the models in the scientific community. Table 1 presents the major features of popular model systems. Ascidians, in particular solitary ascidians, lead the way in both cell biology and molecular approaches. Appendicularian embryos have only recently been subjected to functional studies. No tools exist yet to study the development of thaliaceans, although at least one species can be bred in the laboratory.

The embryonic development of solitary ascidians, in particular *Ciona* and *Halocynthia*, has been studied in most detail, with *C. intestinalis* recently gaining in importance owing to its broad geographical distribution. The seasonality of these animals is an experimental limitation, which is however compensated by the very large number of synchronously developing embryos that can be reared during the reproductive season (Table 1). Mature adults can be kept in the laboratory in simple aquaria for several weeks, their gametes obtained by dissection (*Ciona*, *Phallusia*) or light-induced spawning (*Halocynthia*), and the embryos produced by in vitro fertilization. The embryos of several solitary ascidians, in particular *Phallusia mammillata*, are in the 100 μm size range and are optically transparent (see Table 1). Fluorescently labeled fixed or live embryos can be imaged with high resolution by confocal microscopy. Thanks to the invariant cell lineage, in silico segmentation and the reconstruction of individual cells from stacks of confocal images provides a quantitative, representative view of embryo geometries that can serve as a basis for the mechanical modeling of development (Tassy et al., 2006; Sherrard et al., 2010).

Solitary ascidian embryos can be surgically manipulated (reviewed by Lemaire, 2009). Thanks to their invariant cell lineage, individual blastomeres can be unambiguously identified (Fig. 2). Blastomere function can thus be assessed through their ablation by sea water microinjection (Hudson and Yasuo, 2005), by laser (Sherrard et al., 2010) or by photo-ablation (Nishida and Satoh, 1989). Conversely, individual blastomeres can also be isolated mechanically with a thin glass rod and then left to develop into partial embryos to assess their fate determination status (e.g. Nishida, 1990). Isolated blastomeres/explants and whole embryos can be treated with pharmacological inhibitors or signaling ligands (e.g. Pasini et al., 2006).

Eggs and early blastomeres of ascidian embryos can be microinjected with synthetic mRNAs or with morpholino antisense oligos (Christiaen et al., 2009b). The simplicity and efficiency of transient or stable transgenesis by in ovo electroporation is a major advantage of *C. intestinalis* (Christiaen et al., 2009a). Electroporation has been used to characterize cis-regulatory region activity, to express wild-type or mutant proteins in specific tissues, and to fluorescently mark or trace specific cell populations using standard or photo-convertible fluorescent proteins (Horie et al., 2011). The breeding of *C. intestinalis* and *C. savignyi* in the laboratory (Hendrickson et al., 2004; Joly et al., 2007) has also permitted the development of germ-line transgenesis by electroporation (Matsuoka et al., 2005), a technique that has been subsequently improved by the use of the *Minos* transposon, which opens the way to insertional mutagenesis and enhancer-trap assays (Sasakura et al., 2007). Forward genetics by chemical or insertional mutagenesis has identified interesting *Ciona* embryonic mutants (Chiba et al., 2009; Tresser et al., 2010; Deschet and Smith, 2004).

Finally, powerful computational methods and infrastructures have been developed for solitary ascidians, in particular *C. intestinalis*, which facilitate genomic data analysis and the integration of molecular and anatomical data into virtual representations of embryogenesis (Tassy et al., 2010; Endo et al., 2010).

The colonial ascidians *B. schlosseri* and *B. leachi* can be readily cultured on glass slides in the laboratory (generation time 2-3 months) (Boyd et al., 1986). These animals, which produce few embryos, are particularly adapted to the study of blastogenesis and regeneration. Thanks to the small size of *Botryllus* zooids (~1 mm), asexual development can be imaged in vivo by confocal microscopy (Voskoboynik et al., 2008). Because single zooids, or even pieces of blood vessels, extracted from a colony reconstitute a full colony, experiments employing a large number of animals of identical genetic background can be carried out. Gene function in *Botryllus* can be inhibited by injection of short interfering RNAs (siRNAs) into the blood vasculature (Laird et al., 2005b).

O. dioica is the reference species for appendicularians. It can be bred in the laboratory, has an extremely short life cycle (4 days), and is not seasonal (Nishida, 2008). Its embryos are small (65-75 μm) and transparent and can be microinjected with antisense morpholino oligonucleotides (Sagane et al., 2010).

The embryos of thaliaceans can only be obtained in small numbers and have received little attention since the 1960s. No established experimental protocols exist to collect or study the development of these embryos, and few molecular data have been collected. Yet, the doliolid *Dolioletta gegenbauri* can be cultured in the laboratory (Gibson and Paffenhöfer, 2000), and at least one salp, *Thalia democratica*, has a very short life cycle (Deibel, 1982). Such species are prime candidate model organisms for the study of thaliacean development.

Key recent findings

Recent developmental studies in tunicates have mostly focused on the transcriptional control of embryonic development, its evolution, and how transcription interfaces with the cellular bases of morphogenesis.

Transcriptional control of development

Transcription is the first output of the genome and is highly controlled during development. It is driven by cis-regulatory modules (CRMs, see Glossary, Box 1), which act as binding platforms for transcription factors (TFs). Whereas we can relatively easily identify coding and non-coding (e.g. microRNA) genes in genomes, the identification of CRMs remains a major challenge (Rister and Desplan, 2010). Recent work in tunicates has made a significant contribution to this field.

The two sequenced *Ciona* genomes are compact (the average gene size is 7.5 kb). A fraction of the non-coding sequences is conserved between these two genomes and is enriched in cis-regulatory sequences that drive gene expression (Satoh et al., 2003). Electroporation of candidate regulatory regions into fertilized eggs has led to the identification of over 500 cis-regulatory sequences, mainly for genes that encode TFs and neuronal proteins (Tassy et al., 2010). These sequences act at short range, within 3 kb of their target genes. Minimal CRMs extracted from these sequences are short (generally less than 200 bp), have binding sites for 2-4 TFs and drive expression in one or in a few cell lineages. The constraints on TF binding site order, spacing and orientation appear to be loose in these CRMs (Brown et al., 2007; Khoueiry et al., 2010; Haeussler et al., 2010). Because of this flexibility, ascidian CRMs can undergo extensive TF binding site turnover, which explains why orthologous *Ciona* and *Halocynthia* CRMs can lack detectable sequence similarity, yet display the same activity when tested in interspecies transgenesis (Oda-Ishii et al., 2005). The presence of clusters of consensus DNA sequences putatively recognized by TFs is, however, insufficient to confer cis-regulatory activity, as most such clusters in the *Ciona* genome lack CRM activity. This suggests the existence of additional cis-regulatory signatures. Indeed a di-nucleotide signature that is associated with constitutive nucleosome exclusion (see Glossary, Box 1) has been found to be statistically enriched in *Ciona* CRMs (Khoueiry et al., 2010). Conservation of this signature in *Drosophila* enhancers suggests that building rules for CRMs deduced from the analysis of tunicate genomes might have a broader relevance and application.

Gene regulatory networks (GRNs) describe the regulatory interactions that are mediated by and between TFs through direct binding to their CRMs. GRNs provide an integrated view of a developmental program, and their architecture may strongly influence the evolution of animal shape (Davidson, 2006). *Ciona* is an ideal system with which to decipher chordate developmental GRNs as it combines a simple anatomy, simple CRMs and a small repertoire of less than 700 TFs. Systematic knockdown of TFs zygotically transcribed during early embryogenesis (Imai et al., 2006), genome-wide chromatin immunoprecipitation studies (Kubo et al., 2010) and CRM analysis were recently computationally aggregated into a composite GRN of 200 genes and 500 regulatory interactions (Tassy et al., 2010), one of the largest GRNs ever reconstructed in a metazoan. This ‘regulatory blueprint for a chordate embryo’ (Imai et al., 2006) constitutes a framework by which to compare the developmental programs of different chordates and to understand how morphogenesis is transcriptionally controlled.

Comparing the developmental programs of tunicates

The similarity in embryonic lineages and larval morphologies between distantly related solitary ascidian species, such as *Ciona* and *Halocynthia* (reviewed by Lemaire, 2009), suggests that there are strong constraints for all tunicates to produce similar larvae, presumably partly because of the action of highly conserved GRNs underlying morphogenesis. Embryonic development and larval morphologies can, however, differ greatly between tunicates. Even when the morphologies are conserved the underlying networks may diverge.

First, whereas *Ciona* and *Halocynthia* larvae are very simple, with ~2500 cells and little differentiation of adult structures, the larvae of many colonial ascidians are complex, with precocious differentiation of adult structures in the tadpole head and/or multiplication of the number of larval cells (Jeffery and Swalla, 1992). Conversely, a small number of solitary ascidian species, in particular molgulids, have independently lost their larval tails (Jeffery et al., 1999). This loss is probably recent in the case of *Molgula occulta*, which can still hybridize (see Glossary, Box 1) with a closely related tailed species, *Molgula oculata* (Swalla and Jeffery, 1990). Interestingly, the early cell lineage is highly similar in all ascidians despite these differences [but the *Oikopleura* lineage differs (Stach et al., 2008; Nishida, 2008)]. The ancestral tadpole larval form has also been lost in most thaliaceans, including two entire orders, the salps and the pyrosomes (see Fig. 1A), and this loss is associated with very peculiar early developmental strategies. Salp embryos, for instance, develop within the adult, attached to it by a placenta, and in a very unusual manner (Sutton,

1960). This author described that in the embryos of *Salpa fusiformis*, early blastomeres become separated at the eight-cell stage and are individually surrounded by infiltrated follicle cells. As these latter cells degenerate, embryonic blastomeres resume contact, aggregate and seem to directly form well-organized differentiated tissues without clear gastrula or neurula stages. These tissues include, in anterior territories, a short notochord and a neural tube, which selectively lacks anterior (sensory) and posterior (nerve cord) neural territories, in agreement with the loss of the larval tadpole morphology (Lacalli and Holland, 1998). Thus, although strong constraints act on tunicates to maintain an ancestral tadpole body plan, they have frequently been overcome.

It is reasonable to expect that significant changes in embryonic strategies and larval morphologies correlate with changes in regulatory networks, as has been described in echinoderms (Hinman and Davidson, 2007). Consistent with this, *Ciona* and *Oikopleura* form morphologically distinct larvae and express divergent sets of genes in their notochords (Kugler et al., 2011). More surprisingly, tunicate regulatory networks can significantly differ even when embryonic and larval morphologies appear to be conserved. For instance, a comparison of muscle specification in *Ciona* and *Halocynthia* has revealed that inducers of secondary muscle lineages appear to differ between these two species, in spite of a conserved cell lineage (reviewed by Lemaire, 2009). A second example of the evolution of regulatory networks comes from analysis of the formation of the very similar larval tails in *M. oculata* and *C. intestinalis*. The *Manx* zinc-finger TF is expressed in the tail precursors of *M. oculata* and is required for tail development in this species (Swalla and Jeffery, 1996). This gene, however, has no detectable ortholog in *Ciona* genomes. These examples suggest that tunicate regulatory architectures can change significantly without having a major impact on developmental morphogenesis. Such regulatory changes might, however, have an impact on the evolvability of the morphogenetic processes. Tailless (anuran) ascidian larvae are preferentially found in molgulids, and expression of *Manx* is lost in one such anuran species, *M. occulta* (Swalla and Jeffery, 1996). This suggests that tail loss is easier to achieve by modification of the *Manx*-based *Molgula* network than by changes in the *Manx*-independent *Ciona* network.

In colonial tunicates, asexual reproduction by budding produces the same adult form as embryonic development without going through a tadpole-like developmental stage. Colonial tunicate genomes thus encode distinct developmental programs that give the same end product. Preliminary molecular comparisons of sexual development and blastogenesis suggest that the two pathways only converge after the establishment of the adult body plan (Tiozzo and De Tomaso, 2009; Tiozzo et al., 2005). Further comparisons of sexual and asexual programs will greatly benefit from the ongoing sequencing of the genomes of two colonial species of different orders, *B. schlosseri* (Stolidobranchia; A. Voskoboynik, personal communication) and *D. vexillum* (Aplousobranchia; A. Gittenberger, personal communication).

Conservation of developmental strategies with vertebrates

The relative phylogenetic positions of tunicates and vertebrates suggest that the simpler tunicate embryo could shed light on the more complex vertebrate developmental program. Indeed, tunicates and vertebrates share some structures and patterning mechanisms, including: a mid- to hindbrain boundary (MHB, in which FGF8 promotes hindbrain identity) (Imai et al., 2009); head placodes (Mazet and Shimeld, 2005); 'cranial' motoneurons (Dufour et al., 2006); and pigment-producing migratory neural crest-like cells (Jeffery, 2007). A detailed analysis of heart formation in *Ciona* has also revealed significant conservation of the heart GRN and has indicated that this GRN has an ancient origin, providing plausible scenarios for the evolution of the vertebrate primary heart field (which gives rise to the left ventricle and the atria) and secondary heart field (which gives rise to the right ventricle and outflow tract) (Davidson, 2007; Stolfi et al., 2010).

There are, however, also numerous examples of divergent strategies between vertebrates and ascidians. For instance, ascidians form tadpole larvae in the absence of a structure homologous to the vertebrate 'organizer', which is essential for the formation of vertebrate 'tadpoles' (Kourakis and Smith, 2005). Although the expression of a minority of genes is well conserved between zebrafish and *Ciona*, global gene expression profiles are remarkably different in these two species (Sobral et al., 2009), even for crucial developmental genes, such as the Hox genes (Ikuta et al., 2004; Ikuta et al., 2010). An extreme divergence of Hox gene expression and structure is also found between appendicularians and vertebrates (Seo et al., 2004). Even the minority of genes with conserved expression patterns between tunicates and vertebrates might have changed function. For example, secreted Nodal factors are expressed during pre-gastrula stages in a large part of the vegetal hemisphere in ascidians and vertebrates. This signaling pathway, which is required for endoderm and mesoderm formation in vertebrates, plays no role in endoderm formation in ascidians and only a minor role in mesoderm induction (Hudson and Yasuo, 2005; Hudson and Yasuo, 2006). Another example of conserved expression but divergent function comes from a comparison of the role of Sonic hedgehog and BMPs in motoneuron specification in vertebrates and *Ciona* (Hudson et al., 2011).

Overall, tunicate studies indicate that a surprisingly high level of divergence in genomes and transcriptional regulatory networks is compatible with the long-term preservation of the ancestral chordate body plan. Several theories have been put forward to explain the stability of morphologies in the context of changing regulatory networks, ranging from gradual and homogeneous neutral changes throughout the network (Ciliberti et al., 2007) to the specific conservation of small subnetworks, or kernels (see Glossary, Box 1), which are isolated in a sea of reorganized networks and are sufficient to account for body plan stability (Davidson and Erwin, 2006). Comparisons of GRN architecture within tunicates and with vertebrates will help with assessing the relative contribution of these, or other, mechanisms.

Downstream of GRNs: morphogenesis, cell biology and transcriptional control

Early tunicate embryogenesis has been extensively studied (reviewed by Kumano and Nishida, 2007; Lemaire, 2009). Recent studies have combined imaging, cell biology and computational simulations to identify a diversity of original mechanisms that control morphogenesis and cell fate via the regulation of cell division and cellular mechanics. As exemplified below, the simplicity and stereotypic development of ascidian embryos have enabled complex morphogenetic phenomena to be broken down into a few simple steps, each controlled by a small set of regulatory molecules.

The stereotypic pattern of ascidian development implies that a tight temporal and spatial control of the cell cycle and cell divisions exists. Indeed, the duration of the G2 phase in neural plate cells, regulated by CDC25 expression, coordinates neurulation in *C. intestinalis* (Ogura et al., 2011). Several mechanisms based on the

asymmetric localization of mRNAs spatially regulate cell divisions and the precise positioning of the cleavage planes in early *Halocynthia* embryogenesis (Negishi et al., 2007; Takatori et al., 2010; Nishida and Sawada, 2001), as exemplified in Fig. 3. Other ascidian unequal cleavages, however, are not controlled by localized mRNAs. At the onset of *Ciona* gastrulation, for example, several ectodermal cells located just above the equator undergo stereotyped divisions: the spindles align along the meridians of the embryo, and the more equatorial daughter cell is always larger than its more vegetal sister. Inhibition of gastrulation leads to equalization of the size of the daughters, suggesting that forces exerted by gastrulating endodermal cells create asymmetry by pulling and displacing ectodermal spindles towards the vegetal pole of the embryo (Tassy et al., 2010).

Indeed, endodermal progenitors drive the early phases of gastrulation, during which the embryo evolves from a ball to a cup geometry in two successive steps (Sherrard et al., 2010). Computational simulation of the mechanical forces acting on this simple system has revealed that a local mechanism, based on the differential regulation of cortical tensions on the apical and basolateral sides of endodermal progenitors, is sufficient to explain the observed global deformation of the embryo (Sherrard et al., 2010). Interestingly, in this system, apical constriction is not sufficient to obtain invagination, in contrast to the commonly accepted textbook view. Several signaling ligands and TFs specifically act in the endodermal GRN, and it will be interesting to dissect their role in this morphogenetic process.

How a GRN can control a specific morphogenetic process at the cellular level is best illustrated by the migration of heart precursors from the tail to the ventral part of the head in *C. intestinalis*, a process that is largely independent of heart fate specification (Beh et al., 2007). FACS sorting of wild-type or manipulated heart precursors followed by microarray analysis identified targets of the *C. intestinalis* heart GRN that controls the cellular processes involved in this migration (Christiaen et al., 2008). This study indicates that heart precursor migration can be broken down into distinct cellular processes (such as adhesion or membrane protrusion), each controlled by a module of cytoskeletal effectors and regulators. Each module includes a large set of constitutively expressed proteins, the action of which is coordinated by a small GRN subcircuit acting on a small number of cellular effectors (such as RhoDF for membrane protrusion). Thus, although both GRNs and cytoskeletal networks are large, their coupling is ensured by just a few key proteins in each type of network. This parsimonious coupling between networks that act at different organizational levels is in keeping with findings reported in *Drosophila* (Kölsch et al., 2007) and vertebrates (Chung et al., 2010).

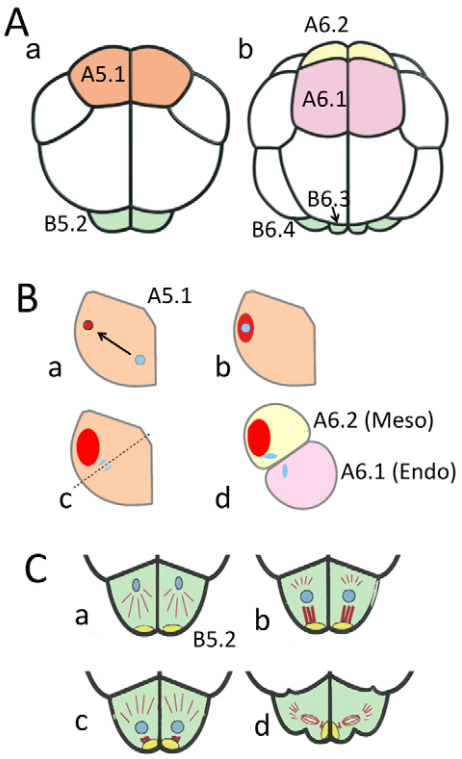

Fig. 3. Control of the geometry of cell divisions in early ascidian embryos. (**A**) (**a**) Schematized vegetal view of a 16-cell ascidian embryo showing the position of two blastomeres, A5.1 (orange) and B5.2 (green), that undergo spatially regulated divisions. (**b**) Vegetal view of a 32-cell embryo showing the descendants of A5.1 (A6.2, yellow and A6.2, pink) and of B5.2 (B6.3 and B6.4, green). (**B**) The asymmetric division of A5.1, which leads to the formation of one mesoderm precursor (A6.2, yellow) and one endoderm precursor (A6.1, pink) as a result of transient nuclear migration towards the mesoderm side. (**a**) The nucleus (blue) moves towards the future mesoderm cortex and starts producing mRNA of the mesoderm determinant (*Not* mRNA, red). (**b**) *Not* mRNA is released from the nucleus and is stabilized by Wnt signaling. (**c**) The nucleus migrates back towards the center of the cell before cleavage (dashed line) takes place. (**d**) As a result of cleavage and the segregation of *Not* mRNA into one cell, one mesoderm (A6.2, yellow) and one endoderm (A6.1, pink) precursor form. Modified with permission (Takatori et al., 2010). (**C**) The unequal division of B5.2, which is driven by cortically localized maternal mRNA inherited by an actin-rich structure called the centrosome attracting body (CAB, yellow). (**a**,**b**) The CAB attracts a centrosome, leading to asymmetric microtubule aster formation (microtubules in red, nucleus in blue). (**c**,**d**) A strong microtubule bundle forms on the CAB side, pulling on the nucleus and leading to a shift in spindle position. Modified with permission (Nishikata et al., 1999).

Limitations and future directions

The diversity of tunicates is both a richness and a source of confusion. The geographic location of a tunicate laboratory can strongly influence the choice of the species it works with, leading to a dilution of efforts on each individual species. As mentioned, it is not always clear whether the extrapolation of results between species, even those that are morphologically similar, is legitimate. One solution would be to focus studies on *C. intestinalis* because of its availability to most ascidian laboratories. *C. intestinalis*, however, shows some experimental limitations: the relatively limited volume of embryonic material that can be obtained from this species limits some biochemical studies, including chromatin assays. Also, *Ciona* eggs are not sufficiently transparent for high-resolution whole embryo imaging studies. By contrast, several species, including *P. mammillata*, produce up to a million optically transparent eggs. The ongoing sequencing of the genomes of a range of solitary (*Phallusia fumigata*, *P. mammillata*, *Halocynthia aurantium*, *H. roretzi*) and colonial (*B. schlosseri*, *D. vexillum*) ascidians will allow researchers to choose the most relevant tunicate model for a given question. The availability of these genomes is also a prerequisite to rigorously exploring the level of divergence between the developmental programs of closely or distantly related tunicates, as has been performed in drosophilids (Kalinka et al., 2010) and worms (Yanai and Hunter, 2009). Such genomic studies should be extended to thaliaceans, in particular to

those that can be bred in the laboratory, such as *D. gegenbauri*. This would resolve the phylogenetic position of this class of animals and provide tools for a molecular characterization of their peculiar embryogenesis.

Ascidians are among the most suitable metazoan systems for mid-scale overexpression studies and for morpholino-mediated gene interference, but they also present some experimental limitations. First, all solitary ascidians are seasonal, although their embryos can be obtained during a large part of the year (Table 1). Second, RNA interference (RNAi), which has become a major research tool in invertebrate and vertebrate model systems (Perrimon et al., 2010), is currently only used routinely to study blastogenesis in *B. schlosseri*. As such, the development of RNAi technology for other tunicates should be a priority. Short hairpin RNAs (shRNAs) might be a promising approach to explore, as they have been reported to be functional in one *Ciona* study (Nishiyama and Fujiwara, 2008). Finally, cell lines have been invaluable in other systems to decipher signaling pathways (e.g. Nybakken et al., 2005) or transcriptional regulatory control mechanisms (Birney et al., 2007). So far, and in spite of many attempts, not a single marine invertebrate cell line has been established, suggesting that a profound difference exists between terrestrial and marine organisms in this respect.

Conclusions

Fueled by progress in live imaging, genomics and computational approaches, developmental biology has undergone profound changes over recent years. We can now realistically aim to understand and computationally model how global organismal shape is encoded in the genome and how it can evolve. Tunicates have certain attributes that should allow them to contribute significantly to this quest. They include powerful model organisms, such as *C. intestinalis*, in which GRNs are being deciphered and linked to the cell biology and cell mechanics of development. In parallel, the tunicate subphylum offers a diversity of species, of morphologies and of developmental strategies. Very similar embryos can be produced by distantly related species, such as *Ciona* and *Halocynthia*, despite considerable genomic differences. Conversely, closely related species belonging to the *Molgula* genus produce very different larvae, and many thaliaceans have lost the ancestral chordate larval body plan. Furthermore, the coexistence in the same species of sexual and asexual reproductive cycles, the latter bypassing the tadpole stage, provides a fascinating illustration that two developmental programs that lead to the same outcome via different routes can be encoded in a single genome. Tunicates are thus ideal for an evolutionary exploration, within the chordate phylum, of the relationships between genotype, regulatory network architecture and phenotype.

Acknowledgements

I thank members of my laboratory for useful comments and suggestions, in particular S. Darras for helpful discussions. S. Tiozzo, A. Voskoboynik and H. Nishida provided feedback on the original manuscript and A. Voskoboynik, H. Nishida, W. C. Smith and W. R. Jeffery on Table 1. This work was funded by CNRS, the European Union and the Agence Nationale pour la Recherche (ANR).

Competing interests statement

The author declares no competing financial interests.

References

Beh, J., Shi, W., Levine, M., Davidson, B. and Christiaen, L. (2007). FoxF is essential for FGF-induced migration of heart progenitor cells in the ascidian Ciona intestinalis. *Development* **134**, 3297-3305.

Berrill, N. J. (1948). The gonads, larvae, and budding of the polystyelid ascidians stolonica and distomus. *J. Mar. Biol. Assoc. UK* **27**, 633-650.

Birney, E., Stamatoyannopoulos, J. A., Dutta, A., Guigó, R., Gingeras, T. R., Margulies, E. H., Weng, Z., Snyder, M., Dermitzakis, E. T., Thurman, R. E. et al. (2007). Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. *Nature* **447**, 799-816.

Bone, Q. (1998). *The Biology of Pelagic Tunicates*. Oxford, UK: Oxford University Press.

Bone, Q., Pulsford, A. L. and Amoroso, E. C. (1985). The placenta of the salp (Tunicata: Thaliacea). *Placenta* **6**, 53-63.

Bourlat, S. J., Juliusdottir, T., Lowe, C. J., Freeman, R., Aronowicz, J., Kirschner, M., Lander, E. S., Thorndyke, M., Nakano, H., Kohn, A. B. et al. (2006). Deuterostome phylogeny reveals monophyletic chordates and the new phylum Xenoturbellida. *Nature* **444**, 85-88.

Boyd, H. C., Brown, S. K., Harp, J. A. and Weissman, I. L. (1986). Growth and sexual maturation of laboratory-cultured Monterey Botryllus schlosseri. *Biol. Bull.* **170**, 91-109.

Brown, C. D., Johnson, D. S. and Sidow, A. (2007). Functional architecture and evolution of transcriptional elements that drive gene coexpression. *Science* **317**, 1557-1560.

Chambon, J., Soule, J., Pomies, P., Fort, P., Sahuquet, A., Alexandre, D., Mangeat, P. and Baghdiguian, S. (2002). Tail regression in Ciona intestinalis (Prochordate) involves a Caspase-dependent apoptosis event associated with ERK activation. *Development* **129**, 3105-3114.

Chiba, S., Jiang, D., Satoh, N. and Smith, W. C. (2009). Brachyury null mutant-induced defects in juvenile ascidian endodermal organs. *Development* **136**, 35-39.

Christiaen, L., Davidson, B., Kawashima, T., Powell, W., Nolla, H., Vranizan, K. and Levine, M. (2008). The transcription/migration interface in heart precursors of Ciona intestinalis. *Science* **320**, 1349-1352.

Christiaen, L., Wagner, E., Shi, W. and Levine, M. (2009a). Electroporation of transgenic DNAs in the sea squirt Ciona. *Cold Spring Harb. Protoc.* **2009**, pdb.prot5345.

Christiaen, L., Wagner, E., Shi, W. and Levine, M. (2009b). Microinjection of morpholino oligos and RNAs in sea squirt (Ciona) embryos. *Cold Spring Harb. Protoc.* **2009**, pdb.prot5347.

Chung, M., Nascone-Yoder, N. M., Grover, S. A., Drysdale, T. A. and Wallingford, J. B. (2010). Direct activation of Shroom3 transcription by Pitx proteins drives epithelial morphogenesis in the developing gut. *Development* **137**, 1339-1349.

Ciliberti, S., Martin, O. C. and Wagner, A. (2007). Robustness can evolve gradually in complex regulatory gene networks with varying topology. *PLoS Comput. Biol.* **3**, e15.

Davidson, B. (2007). Ciona intestinalis as a model for cardiac development. *Semin. Cell Dev. Biol.* **18**, 16-26.

Davidson, E. H. (2006). *The Regulatory Genome: Gene Regulatory Networks in Development and Evolution* (1st edn). Burlington, MA: Academic Press.

Davidson, E. H. and Erwin, D. H. (2006). Gene regulatory networks and the evolution of animal body plans. *Science* **311**, 796-800.

Dehal, P., Satou, Y., Campbell, R. K., Chapman, J., Degnan, B., De Tomaso, A., Davidson, B., Di Gregorio, A., Gelpke, M., Goodstein, D. M. et al. (2002). The draft genome of Ciona intestinalis: insights into chordate and vertebrate origins. *Science* **298**, 2157-2167.

Deibel, D. (1982). Laboratory determined mortality, fecundity and growth rates of Thalia democratica Forskal and Dolioletta gegenbauri Uljanin (Tunicata, Thaliacea). *J. Plankton Res.* **4**, 143-153.

Delsuc, F., Brinkmann, H., Chourrout, D. and Philippe, H. (2006). Tunicates and not cephalochordates are the closest living relatives of vertebrates. *Nature* **439**, 965-968.

Denoeud, F., Henriet, S., Mungpakdee, S., Aury, J., Da, Silva, C., Brinkmann, H., Mikhaleva, J., Olsen, L. C., Jubin, C., Cañestro, C. et al. (2010). Plasticity of animal genome architecture unmasked by rapid evolution of a pelagic tunicate. *Science* **330**, 1381-1385.

Deschet, K. and Smith, W. C. (2004). Frimousse-a spontaneous ascidian mutant with anterior ectodermal fate transformation. *Curr. Biol.* **14**, R408-R410.

Duboule, D. (2007). The rise and fall of Hox gene clusters. *Development* **134**, 2549-2560.

Dufour, H. D., Chettouh, Z., Deyts, C., de Rosa, R., Goridis, C., Joly, J. and Brunet, J. (2006). Precraniate origin of cranial motoneurons. *Proc. Natl. Acad. Sci. USA* **103**, 8727-8732.

Endo, T., Ueno, K., Yonezawa, K., Mineta, K., Hotta, K., Satou, Y., Yamada, L., Ogasawara, M., Takahashi, H., Nakajima, A. et al. (2010). CIPRO 2.5: Ciona intestinalis protein database, a unique integrated repository of large-scale omics data, bioinformatic analyses and curated annotation, with user rating and reviewing functionality. *Nucleic Acids Res.* **39**, D807-D814.

Ganot, P., Kallesoe, T., Reinhardt, R., Chourrout, D. and Thompson, E. M. (2004). Spliced-Leader RNA trans splicing in a chordate, Oikopleura dioica, with a compact genome. *Mol. Cell. Biol.* **24**, 7795-7805.

Gibson, D. and Paffenhöfer, G. (2000). Feeding and growth rates of the doliolid, Dolioletta gegenbauri Uljanin (Tunicata, Thaliacea). *J. Plankton Res.* **22**, 1485-1500.

Gorsky, G., Youngbluth, M. J. and Deibel, D. (2005). *Response of Marine Ecosystems to Global Change: Ecological Impact of Appendicularians*. Paris: Gordon and Breach Scientific Publishers.
Govindarajan, A. F., Bucklin, A. and Madin, L. P. (2010). A molecular phylogeny of the Thaliacea. *J. Plankton Res*. doi:10.1093/plankt/fbq157.
Haeussler, M., Jaszczyszyn, Y., Christiaen, L. and Joly, J. (2010). A cis-regulatory signature for chordate anterior neuroectodermal genes. *PLoS Genet.* **6**, e1000912.
Harada, Y., Takagaki, Y., Sunagawa, M., Saito, T., Yamada, L., Taniguchi, H., Shoguchi, E. and Sawada, H. (2008). Mechanism of self-sterility in a hermaphroditic chordate. *Science* **320**, 548-550.
Hendrickson, C., Christiaen, L., Deschet, K., Jiang, D., Joly, J., Legendre, L., Nakatani, Y., Tresser, J. and Smith, W. C. (2004). Culture of adult ascidians and ascidian genetics. *Methods Cell Biol*. **74**, 143-170.
Hill, M. M., Broman, K. W., Stupka, E., Smith, W. C., Jiang, D. and Sidow, A. (2008). The C. savignyi genetic map and its integration with the reference sequence facilitates insights into chordate genome evolution. *Genome Res*. **18**, 1369-1379.
Hinman, V. F. and Davidson, E. H. (2007). Evolutionary plasticity of developmental gene regulatory network architecture. *Proc. Natl. Acad. Sci. USA*, **104**, 19404-19409.
Holland, L. Z. and Gibson-Brown, J. J. (2003). The Ciona intestinalis genome: when the constraints are off. *BioEssays* **25**, 529-532.
Horie, T., Shinki, R., Ogura, Y., Kusakabe, T. G., Satoh, N. and Sasakura, Y. (2011). Ependymal cells of chordate larvae are stem-like cells that form the adult nervous system. *Nature* **469**, 525-528.
Hotta, K., Mitsuhara, K., Takahashi, H., Inaba, K., Oka, K., Gojobori, T. and Ikeo, K. (2007). A web-based interactive developmental table for the ascidian Ciona intestinalis, including 3D real-image embryo reconstructions: I. From fertilized egg to hatching larva. *Dev. Dyn*. **236**, 1790-1805.
Hudson, C. and Yasuo, H. (2005). Patterning across the ascidian neural plate by lateral Nodal signalling sources. *Development* **132**, 1199-1210.
Hudson, C. and Yasuo, H. (2006). A signalling relay involving Nodal and Delta ligands acts during secondary notochord induction in Ciona embryos. *Development* **133**, 2855-2864.
Hudson, C., Ba, M., Rouvière, C. and Yasuo, H. (2011). Divergent mechanisms specify chordate motoneurons: evidence from ascidians. *Development* **138**, 1643-1652.
Ikuta, T., Yoshida, N., Satoh, N. and Saiga, H. (2004). Ciona intestinalis Hox gene cluster: Its dispersed structure and residual colinear expression in development. *Proc. Natl. Acad. Sci. USA* **101**, 15118-15123.
Ikuta, T., Satoh, N. and Saiga, H. (2010). Limited functions of Hox genes in the larval development of the ascidian Ciona intestinalis. *Development* **137**, 1505-1513.
Imai, K. S., Levine, M., Satoh, N. and Satou, Y. (2006). Regulatory blueprint for a chordate embryo. *Science* **312**, 1183-1187.
Imai, K. S., Stolfi, A., Levine, M. and Satou, Y. (2009). Gene regulatory networks underlying the compartmentalization of the Ciona central nervous system. *Development* **136**, 285-293.
Jeffery, W. R. (2007). Chordate ancestry of the neural crest: new insights from ascidians. *Semin. Cell Dev. Biol*. **18**, 481-491.
Jeffery, W. R. and Swalla, B. J. (1992). Evolution of alternate modes of development in ascidians. *BioEssays* **14**, 219-226.
Jeffery, W. R., Swalla, B. J., Ewing, N. and Kusakabe, T. (1999). Evolution of the ascidian anural larva: evidence from embryos and molecules. *Mol. Biol. Evol*. **16**, 646-654.
Joly, J., Kano, S., Matsuoka, T., Auger, H., Hirayama, K., Satoh, N., Awazu, S., Legendre, L. and Sasakura, Y. (2007). Culture of Ciona intestinalis in closed systems. *Dev. Dyn*. **236**, 1832-1840.
Kalinka, A. T., Varga, K. M., Gerrard, D. T., Preibisch, S., Corcoran, D. L., Jarrells, J., Ohler, U., Bergman, C. M. and Tomancak, P. (2010). Gene expression divergence recapitulates the developmental hourglass model. *Nature* **468**, 811-814.
Khoueiry, P., Rothbächer, U., Ohtsuka, Y., Daian, F., Frangulian, E., Roure, A., Dubchak, I. and Lemaire, P. (2010). A cis-regulatory signature in ascidians and flies, independent of transcription factor binding sites. *Curr. Biol*. **20**, 792-802.
Kölsch, V., Seher, T., Fernandez-Ballester, G. J., Serrano, L. and Leptin, M. (2007). Control of Drosophila gastrulation by apical localization of adherens junctions and RhoGEF2. *Science* **315**, 384-386.
Kourakis, M. J. and Smith, W. C. (2005). Did the first chordates organize without the organizer? *Trends Genet*. **21**, 506-510.
Kubo, A., Suzuki, N., Yuan, X., Nakai, K., Satoh, N., Imai, K. S. and Satou, Y. (2010). Genomic cis-regulatory networks in the early Ciona intestinalis embryo. *Development* **137**, 1613-1623.
Kugler, J. E., Kerner, P., Bouquet, J., Jiang, D. and Di Gregorio, A. (2011). Evolutionary changes in the notochord genetic toolkit: a comparative analysis of notochord genes in the ascidian Ciona and the larvacean Oikopleura. *BMC Evol. Biol*. **11**, 21.
Kumano, G. and Nishida, H. (2007). Ascidian embryonic development: an emerging model system for the study of cell fate specification in chordates. *Dev. Dyn*. **236**, 1732-1747.
Kurabayashi, A., Okuyama, M., Ogawa, M., Takeuchi, A., Jing, Z., Naganuma, T. and Saito, Y. (2003). Phylogenetic position of a deep-sea ascidian, Megalodicopia hians, inferred from the molecular data. *Zool. Sci*. **20**, 1243-1247.
Lacalli, T. C. and Holland, L. Z. (1998). The developing dorsal ganglion of the salp Thalia democratica, and the nature of the ancestral chordate brain. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **353**, 1943-1967.
Laird, D. J., De Tomaso, A. W. and Weissman, I. L. (2005a). Stem cells are units of natural selection in a colonial ascidian. *Cell* **123**, 1351-1360.
Laird, D. J., Chang, W., Weissman, I. L. and Lauzon, R. J. (2005b). Identification of a novel gene involved in asexual organogenesis in the budding ascidian Botryllus schlosseri. *Dev. Dyn*. **234**, 997-1005.
Lambert, C. C. and Lambert, G. (2001). A global overview of ascidian introductions and their possible impact on the endemic fauna. In *The Biology of Ascidians* (ed. H. Sawada, H. Yokosawa and C. C. Lambert), pp. 249-257. Tokyo: Springer-Verlag.
Lemaire, P. (2009). Unfolding a chordate developmental program, one cell at a time: invariant cell lineages, short-range inductions and evolutionary plasticity in ascidians. *Dev. Biol*. **332**, 48-60.
Lemaire, P., Smith, W. C. and Nishida, H. (2008). Ascidians and the plasticity of the chordate developmental program. *Curr. Biol*. **18**, R620-R631.
Matsumoto, J., Dewar, K., Wasserscheid, J., Wiley, G. B., Macmil, S. L., Roe, B. A., Zeller, R. W., Satou, Y. and Hastings, K. E. M. (2010). High-throughput sequence analysis of Ciona intestinalis SL trans-spliced mRNAs: alternative expression modes and gene function correlates. *Genome Res*. **20**, 636-645.
Matsuoka, T., Awazu, S., Shoguchi, E., Satoh, N. and Sasakura, Y. (2005). Germline transgenesis of the ascidian Ciona intestinalis by electroporation. *Genesis* **41**, 67-72.
Mazet, F. and Shimeld, S. M. (2005). Molecular evidence from ascidians for the evolutionary origin of vertebrate cranial sensory placodes. *J. Exp. Zool. B Mol. Dev. Evol*. **304**, 340-346.
Morgan, T. H. (1944). Some further data on self fertilization in Ciona. *J. Exp. Zool.* **97**, 231-248.
Morris, S. C. (1999). *The Crucible of Creation: The Burgess Shale and the Rise of Animals*. Oxford, UK: Oxford University Press.
Nakashima, K., Yamada, L., Satou, Y., Azuma, J. and Satoh, N. (2004). The evolutionary origin of animal cellulose synthase. *Dev. Genes Evol.* **214**, 81-88.
Negishi, T., Takada, T., Kawai, N. and Nishida, H. (2007). Localized PEM mRNA and protein are involved in cleavage-plane orientation and unequal cell divisions in ascidians. *Curr. Biol*. **17**, 1014-1025.
Nishida, H. (1990). Determinative mechanisms in secondary muscle lineages of ascidian embryos: development of muscle-specific features in isolated muscle progenitor cells. *Development* **108**, 559-568.
Nishida, H. (2008). Development of the appendicularian Oikopleura dioica: culture, genome, and cell lineages. *Dev. Growth Differ.* **50 Suppl. 1**, S239-S256.
Nishida, H. and Satoh, N. (1989). Determination and regulation in the pigment cell lineage of the ascidian embryo. *Dev. Biol*. **132**, 355-367.
Nishida, H. and Sawada, K. (2001). macho-1 encodes a localized mRNA in ascidian eggs that specifies muscle fate during embryogenesis. *Nature* **409**, 724-729.
Nishikata, T., Hibino, T. and Nishida, H. (1999). The centrosome-attracting body, microtubule system, and posterior egg cytoplasm are involved in positioning of cleavage planes in the ascidian embryo. *Dev. Biol*. **209**, 72-85.
Nishiyama, A. and Fujiwara, S. (2008). RNA interference by expressing short hairpin RNA in the Ciona intestinalis embryo. *Dev. Growth Differ*. **50**, 521-529.
Nybakken, K., Vokes, S. A., Lin, T., McMahon, A. P. and Perrimon, N. (2005). A genome-wide RNA interference screen in Drosophila melanogaster cells for new components of the Hh signaling pathway. *Nat. Genet*. **37**, 1323-1332.
Oda-Ishii, I., Bertrand, V., Matsuo, I., Lemaire, P. and Saiga, H. (2005). Making very similar embryos with divergent genomes: conservation of regulatory mechanisms of Otx between the ascidians Halocynthia roretzi and Ciona intestinalis. *Development* **132**, 1663-1674.
Ogura, Y., Sakaue-Sawano, A., Nakagawa, M., Satoh, N., Miyawaki, A. and Sasakura, Y. (2011). Coordination of mitosis and morphogenesis: role of a prolonged G2 phase during chordate neurulation. *Development* **138**, 577-587.
Pasini, A., Amiel, A., Rothbächer, U., Roure, A., Lemaire, P. and Darras, S. (2006). Formation of the ascidian epidermal sensory neurons: insights into the origin of the chordate peripheral nervous system. *PLoS Biol*. **4**, e225.
Perrimon, N., Ni, J. and Perkins, L. (2010). In vivo RNAi: today and tomorrow. *Cold Spring Harb. Perspect. Biol*. **2**, a003640.
Putnam, N. H., Butts, T., Ferrier, D. E. K., Furlong, R. F., Hellsten, U., Kawashima, T., Robinson-Rechavi, M., Shoguchi, E., Terry, A., Yu, J. et al. (2008). The amphioxus genome and the evolution of the chordate karyotype. *Nature* **453** 1064-1071.
Rinkevich, Y., Paz, G., Rinkevich, B. and Reshef, R. (2007). Systemic bud induction and retinoic acid signaling underlie whole body regeneration in the urochordate Botrylloides leachi. *PLoS Biol*. **5**, e71.

Rister, J. and Desplan, C. (2010). Deciphering the genome's regulatory code: the many languages of DNA. *BioEssays* **32**, 381-384.
Sagane, Y., Zech, K., Bouquet, J., Schmid, M., Bal, U. and Thompson, E. M. (2010). Functional specialization of cellulose synthase genes of prokaryotic origin in chordate larvaceans. *Development* **137**, 1483-1492.
Sasakura, Y., Oogai, Y., Matsuoka, T., Satoh, N. and Awazu, S. (2007). Transposon mediated transgenesis in a marine invertebrate chordate: Ciona intestinalis. *Genome Biol*. **8 Suppl. 1**, S3.
Satoh, N., Satou, Y., Davidson, B. and Levine, M. (2003). Ciona intestinalis: an emerging model for whole-genome analyses. *Trends Genet*. **19**, 376-381.
Satou, Y., Mineta, K., Ogasawara, M., Sasakura, Y., Shoguchi, E., Ueno, K., Yamada, L., Matsumoto, J., Wasserscheid, J., Dewar, K. et al. (2008). Improved genome assembly and evidence-based global gene model set for the chordate Ciona intestinalis: new insight into intron and operon populations. *Genome Biol*. **9**, R152.
Seo, H., Edvardsen, R. B., Maeland, A. D., Bjordal, M., Jensen, M. F., Hansen, A., Flaat, M., Weissenbach, J., Lehrach, H., Wincker, P. et al. (2004). Hox cluster disintegration with persistent anteroposterior order of expression in Oikopleura dioica. *Nature* **431**, 67-71.
Sherrard, K., Robin, F., Lemaire, P. and Munro, E. (2010). Sequential activation of apical and basolateral contractility drives ascidian endoderm invagination. *Curr. Biol*. **20**, 1499-1510.
Shoguchi, E., Kawashima, T., Satou, Y., Hamaguchi, M., Sin-I, T., Kohara, Y., Putnam, N., Rokhsar, D. S. and Satoh, N. (2006). Chromosomal mapping of 170 BAC clones in the ascidian Ciona intestinalis. *Genome Res*. **16**, 297-303.
Shu, D., Morris, S. C. and Zhang, X. (1996). A Pikaia-like chordate from the Lower Cambrian of China. *Nature* **384**, 157-158.
Small, K. S., Brudno, M., Hill, M. M. and Sidow, A. (2007). A haplome alignment and reference sequence of the highly polymorphic Ciona savignyi genome. *Genome Biol*. **8**, R41.
Sobral, D., Tassy, O. and Lemaire, P. (2009). Highly divergent gene expression programs can lead to similar chordate larval body plans. *Curr. Biol.* **19**, 2014-2019.
Stach, T., Winter, J., Bouquet, J., Chourrout, D. and Schnabel, R. (2008). Embryology of a planktonic tunicate reveals traces of sessility. *Proc. Natl. Acad. Sci. USA* **105**, 7229-7234.
Stolfi, A., Gainous, T. B., Young, J. J., Mori, A., Levine, M. and Christiaen, L. (2010). Early chordate origins of the vertebrate second heart field. *Science* **329**, 565-568.
Sutton, M. F. (1960). The sexual development of Salpa fusiformis (Cuvier). *J. Embryol. Exp. Morphol.* **8**, 268-290.
Swalla, B. J. and Jeffery, W. R. (1990). Interspecific hybridization between an anural and urodele ascidian: differential expression of urodele features suggests multiple mechanisms control anural development. *Dev. Biol*. **142**, 319-334.
Swalla, B. J. and Jeffery, W. R. (1996). Requirement of the Manx gene for expression of chordate features in a tailless ascidian larva. *Science* **274**, 1205-1208.
Takatori, N., Kumano, G., Saiga, H. and Nishida, H. (2010). Segregation of germ layer fates by nuclear migration-dependent localization of Not mRNA. *Dev. Cell* **19**, 589-598.
Tassy, O., Daian, F., Hudson, C., Bertrand, V. and Lemaire, P. (2006). A quantitative approach to the study of cell shapes and interactions during early chordate embryogenesis. *Curr. Biol*. **16**, 345-358.
Tassy, O., Dauga, D., Daian, F., Sobral, D., Robin, F., Khoueiry, P., Salgado, D., Fox, V., Caillol, D., Schiappa, R. et al. (2010). The ANISEED database: digital representation, formalization, and elucidation of a chordate developmental program. *Genome Res.* **20**, 1459-1468.
Tiozzo, S. and De Tomaso, A. W. (2009). Functional analysis of Pitx during asexual regeneration in a basal chordate. *Evol. Dev*. **11**, 152-162.
Tiozzo, S., Christiaen, L., Deyts, C., Manni, L., Joly, J. and Burighel, P. (2005). Embryonic versus blastogenetic development in the compound ascidian Botryllus schlosseri: insights from Pitx expression patterns. *Dev. Dyn*. **232**, 468-478.
Tresser, J., Chiba, S., Veeman, M., El-Nachef, D., Newman-Smith, E., Horie, T., Tsuda, M. and Smith, W. C. (2010). doublesex/mab3 related-1 (dmrt1) is essential for development of anterior neural plate derivatives in Ciona. *Development* **137**, 2197-2203.
Tsagkogeorga, G., Turon, X., Hopcroft, R. R., Tilak, M., Feldstein, T., Shenkar, N., Loya, Y., Huchon, D., Douzery, E. J. P. and Delsuc, F. (2009). An updated 18S rRNA phylogeny of tunicates based on mixture and secondary structure models. *BMC Evol. Biol*. **9**, 187.
Voskoboynik, A., Simon-Blecher, N., Soen, Y., Rinkevich, B., De Tomaso, A. W., Ishizuka, K. J. and Weissman, I. L. (2007). Striving for normality: whole body regeneration through a series of abnormal generations. *FASEB J.* **21**, 1335-1344.
Voskoboynik, A., Soen, Y., Rinkevich, Y., Rosner, A., Ueno, H., Reshef, R., Ishizuka, K. J., Palmeri, K. J., Moiseeva, E., Rinkevich, B. et al. (2008). Identification of the endostyle as a stem cell niche in a colonial chordate. *Cell Stem Cell* **3**, 456-464.
Yanai, I. and Hunter, C. P. (2009). Comparison of diverse developmental transcriptomes reveals that coexpression of gene neighbors is not evolutionarily conserved. *Genome Res*. **19**, 2214-2220.