Tunicata

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Chapter vignette artwork by Brigitte Baldrian.

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Above all, perhaps, I am indebted to a decidedly vegetative, often beautiful, and generally obscure group of marine animals, both for their intrinsic interest and for the enjoyment I have had in searching for them. N. J. Berrill (1955)

INTRODUCTION

Tunicates are a group of marine filter-feeding animals[1] that have been traditionally divided into three classes: (1) Appendicularia, also known as larvaceans because their free-swimming and pelagic adult stage resembles a larva; (2) Thaliacea, which includes three orders of free-swimming and pelagic adult forms with complex life cycles (Salpida, Pyrosomida, and Doliolida); and (3) Ascidiacea, colloquially referred to as sea squirts,[2] which is comprised of diverse sessile solitary and colonial species and includes some of the most extensively studied tunicates. It was mainly the ascidians that served as the inspiration for seminal studies in developmental biology and evolution conducted last century by British zoologist and prolific writer N. J. Berrill, whose quote appears above.

Evolutionary Relationships

Although Tunicata is well established as a monophyletic group and united by their indisputably synapomorphic ability to synthesize cellulose, the phylogenetic relationships between the three classes and many orders and families have yet to be satisfactorily settled. Appendicularia, Thaliacea, and Ascidiacea remain broadly used in textbooks and scientific literature as the three classes of tunicates; however, recent molecular phylogenies have provided support for the monophyly of only Appendicularia and Thaliacea, but not of Ascidiacea (Swalla et al. 2000; Tsagkogeorga et al. 2009; Wada 1998). A paraphyletic Ascidiacea calls for a reevaluation of tunicate relationships. The most up-to-date phylogeny with a comprehensive taxonomic sampling of tunicates using 18S rRNA supports three clades for the Tunicata (Fig. 4.1; Tsagkogeorga et al. 2009): (1) Appendicularia, (2) Stolidobranch ascidians, and (3) Aplousobranch ascidians+ Phlebobranch ascidians+Thaliacea. All three groups of the latter clade show resemblance in association of the gonads to the gut in concordance with the Enterogona classification (Perrier 1898). However, the precise position of the Appendicularia and the Thaliacea remains unresolved. Appendicularia has traditionally been considered to be at the base of the Tunicata (Wada 1998; Swalla et al. 2000), but recent molecular phylogenies place them as sister group to Stolidobranchia (Zeng et al. 2006; Tsagkogeorga et al. 2009). On the other hand, Thaliacea generally groups closer to Phlebobranchia than to Aplousobranchia using ribosomal molecular phylogenies (Zeng et al. 2006; Tsagkogeorga et al. 2009), but the low taxonomic sampling and a high 18S evolutionary rate of thaliaceans and aplousobranchs bring some uncertainty to this grouping. In an attempt to provide an evolutionary framework of the developmental mechanisms in Tunicata, developmental studies of several species of tunicates are reviewed here in a comparative manner with respect to our understanding of tunicate relationships.

Relationships to Other Chordates

For several centuries after Linnaeus first established the classification system for living organisms, ascidians were grouped together based on adult morphological features. This group contained a stereotypical adult body plan including

[1] Actually, there are specialized predatory ascidians in the Molgulidae and Octacnemidae families that do not filter feed but rather swallow whole crustaceans and other invertebrates (Tatián et al. 2011).

[2] The term "sea squirt" has been adopted for these animals, as seawater can be strongly propelled through the oral siphon upon physical perturbation. Other colloquial terms apply to certain species or genera ("sea peach" for *Halocynthia aurantium*, "sea grape" for *Molgula* spp.). The literate translation for "sea squirt" is used in Spanish (*chorro de mar*), Finnish (*meritupet*), Swedish (*Sjöpungar*), or Polish (*Żachwy*); more externally descriptive terms are also used, such as "sea bag" (*Sekkedyr* in Norwegian or *Søpunge* in Danish), "sack pipe" (*Zakpijpen* in Dutch), or "sea sheath" or "sea vagina" (*Seescheide* in German). Humorous, if crude, terms abound especially in Portuguese, like *mija-mija* ("piss-piss"), *Maria Mijona* ("Pissing Mary"), or *mijão* ("big pisser").

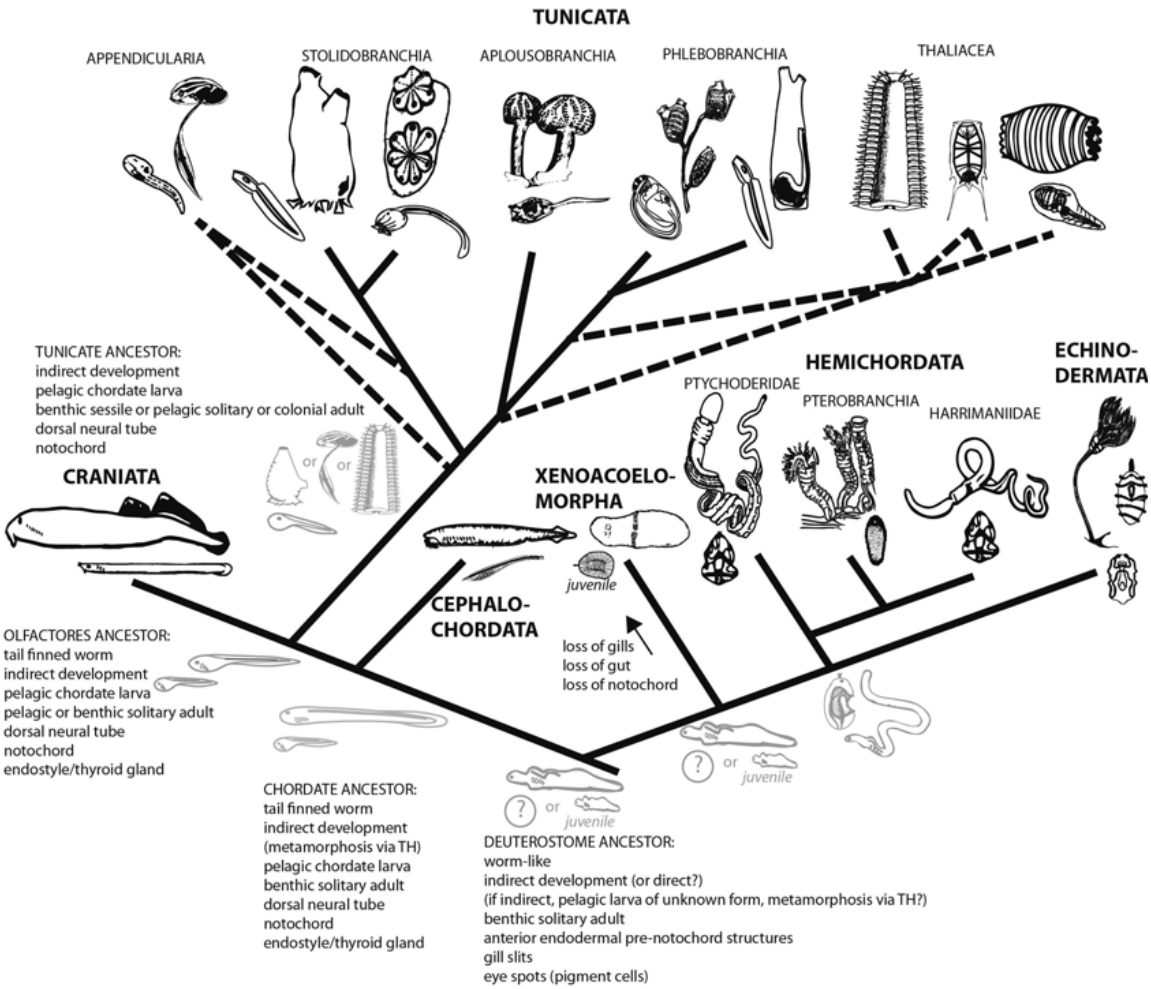


Fig. 4.1 Current understanding of tunicate relationships within the deuterostomes. *Dashed lines* represent possible relationships in uncertain branches (see text for details). Larval (*below*) and adult (*above*) forms have been included at each node to facilitate understanding of distinct arguments discussed in the text of the major evolutionary transitions of extant clades and their ancestors (in *gray*). Descriptions of the most parsimonious ancestral characteristics are shown at every node representing possible tunicate ancestors. Cases of multiple larval or adult ancestral forms are separated by "or", direct development (no larva) is shown by "juvenile," and unknown forms are presented as a question mark (?) in a *circle*

(1) a U-shaped gut delimited by an incurrent and an excurrent siphon, (2) a large branchial cavity for filter feeding, and (3) the tunic, a characteristic mantle later discovered to be made of animal cellulose. Already by the turn of the nineteenth century, Jean-Baptiste de Lamarck realized that ascidians and thaliaceans could be grouped as Tunicata (Lamarck 1816). However, based strictly on the adult morphological features of solitary or colonial forms, these animals remained difficult to place in broader evolutionary animal groups; for example, in the mid-nineteenth century, tunicates were grouped together with ectoprocts and brachiopods within the mollusks (Fig. 4.2A) (Milne-Edwards 1843; Haeckel 1866). A unified Tunicata as we consider them today was first proposed by Huxley (1851), who included Appendicularia in the Tunicata proposed by Lamarck.

Only after Kowalevsky had described the tailed larval form of ascidians (Kowalewski 1866), containing a dorsal neural tube, a typical chordate notochord, and lateral muscle cells, did zoologists begin to realize that tunicates should be

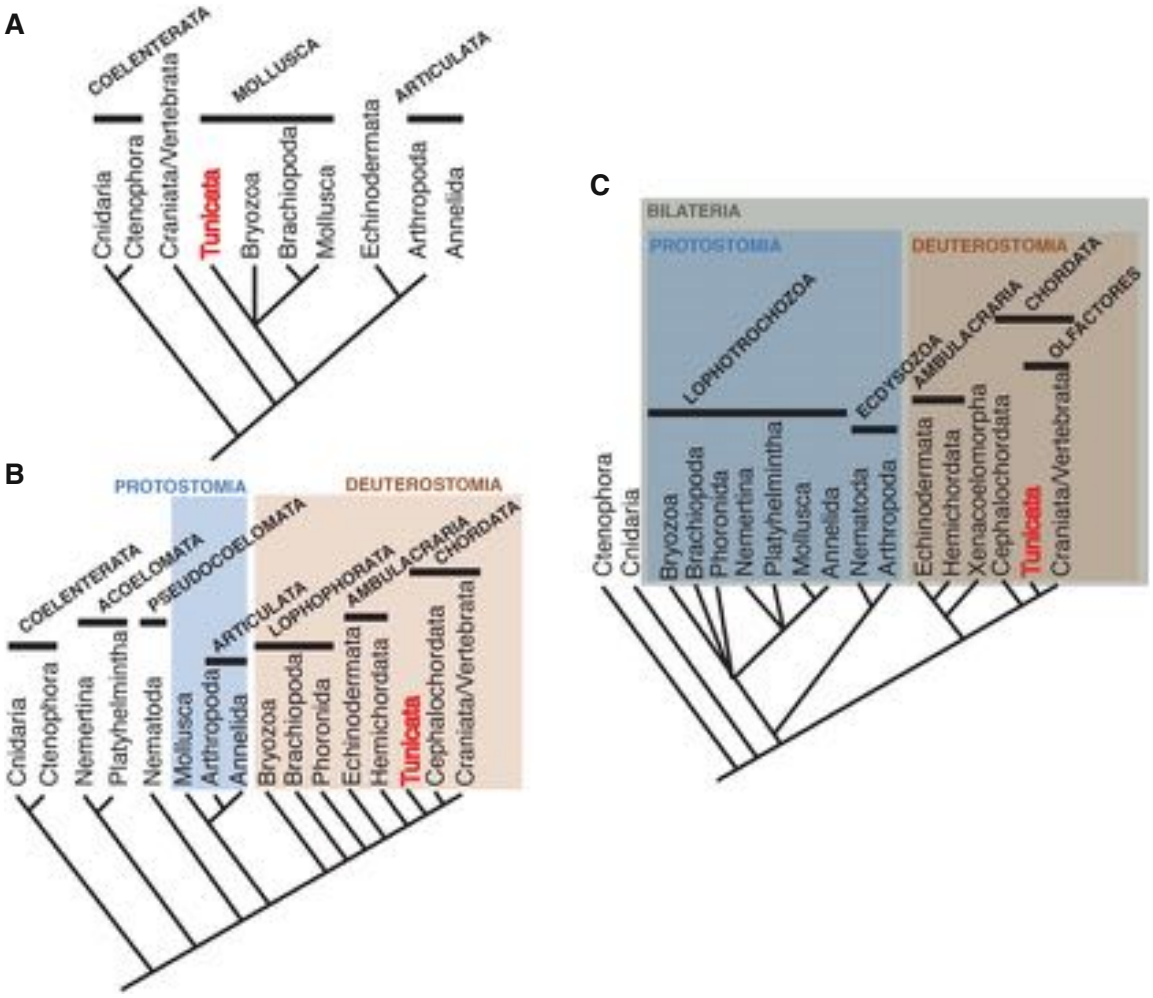


Fig. 4.2 Historic overview of the position of Tunicata within the Metazoa. (**A**) View of animals relationships through the nineteenth century (Milne-Edwards 1843; Haeckel 1866), highlighting the position of the Tunicata as a chordate due to the presence of the notochord (in *red*). (**B**) Popular view of animal relationships throughout the twentieth century with the lophophorates (Hyman 1959; Zimmer and Larwood 1973; Nielsen 2002) basal to the tunicates. (**C**) Current view using molecular phylogenies (Field et al. 1988; Cameron et al. 2000; Swalla et al. 2000; Winchell et al. 2002)

placed within the chordates. In the late nineteenth century, zoologists such as Haeckel (1874), Bateson (1884, 1886), Brooks (1893), Willey (1894), and Garstang (1894) supported Chordata and Hemichordata as sister phyla based on notochord-like structures as the main synapomorphy. Within Chordata three subphyla were recognized, Vertebrata, Cephalochordata, and Tunicata, which Lankester (1877) attempted to rename as Urochordata, to highlight the evolutionary importance of this character within the phylum. Because cephalochordates shared more morphological features with the vertebrates, including a metameric muscle segmentation pattern, the tunicates were placed at the base of Chordata. Because of the unique body plan of adult tunicates, it was even proposed that they be raised to phylum status (Kozloff 1990; Cameron et al. 2000; Zeng and Swalla 2005).

At the turn of the twentieth century, zoologists realized that chordates, hemichordates, and echinoderms share a common embryological trait: the blastopore, the first opening that forms in the gastrulating embryo, developed into the anus of the adult. Thus, they were classified within the superphylum Deuterostomia (Grobben 1908), opposite to Protostomia, or animals whose blastopores developed into the mouth instead. A morphological

feature that generated confusion for almost the entire twentieth century but that was relevant to the general debate of early deuterostome evolution was the lophophore of brachiopods, ectoprocts, and phoronids (see Vol. 2, Chapters 10, 11, and 12), a crown-like feeding apparatus composed of numerous tentacles. In spite of convincing arguments for nearly half a century that supported a monophyletic Lophophorata (those animals bearing a lophophore) as being part of or closely related to Deuterostomia (Fig. 4.2B; Hyman 1959; Zimmer and Larwood 1973; Nielsen 2002), molecular phylogenetic work contested their deuterostome affinities and instead grouped them together with annelids, mollusks, and other diverse phyla in Lophotrochozoa (Halanych 2004; Helmkampf et al. 2008).

Conserved gene sequences, such as large and small subunit ribosomal rDNAs, were the first molecular characters used for testing phylogenies of deuterostomes and other phyla (Field et al. 1988; Cameron et al. 2000; Swalla et al. 2000; Winchell et al. 2002). Additional protein-coding gene sequences (both mitochondrial and nuclear), intron-exon boundaries, miRNAs, and other empirical evidence rendered additional support for the monophyly of Deuterostomia and Ambulacraria (Echinodermata + Hemichordata) and also resulted in new and previously unexplored hypothetical relationships such as the placement of *Xenoturbella* together with acoel worms as the sister group to the Ambulacraria (see Vol. 1, Chapter 9 for discussion) or the inversion of Tunicata and Cephalochordata positions within Chordata (Delsuc et al. 2006; Telford and Copley 2011). The latter proposed relationship for Chordata suggests that Tunicata is the sister group to Craniata/Vertebrata within a group named Olfactores (reviewed in Delsuc et al. 2006), to the exclusion of Cephalochordata (Fig. 4.2C). Some of the implications of this new rearrangement for our understanding of chordate evolution will be discussed in the following.

Tunicate and Chordate Origins

For nearly 200 years, zoologists have wondered about the origins of our own phylum, and the literature is rife with heated debates on the origins of chordates (Fig. 4.3). In this section the main hypotheses that have been proposed and how more recent views of deuterostome relationships have affected our understanding of the chordate ancestor are reviewed.

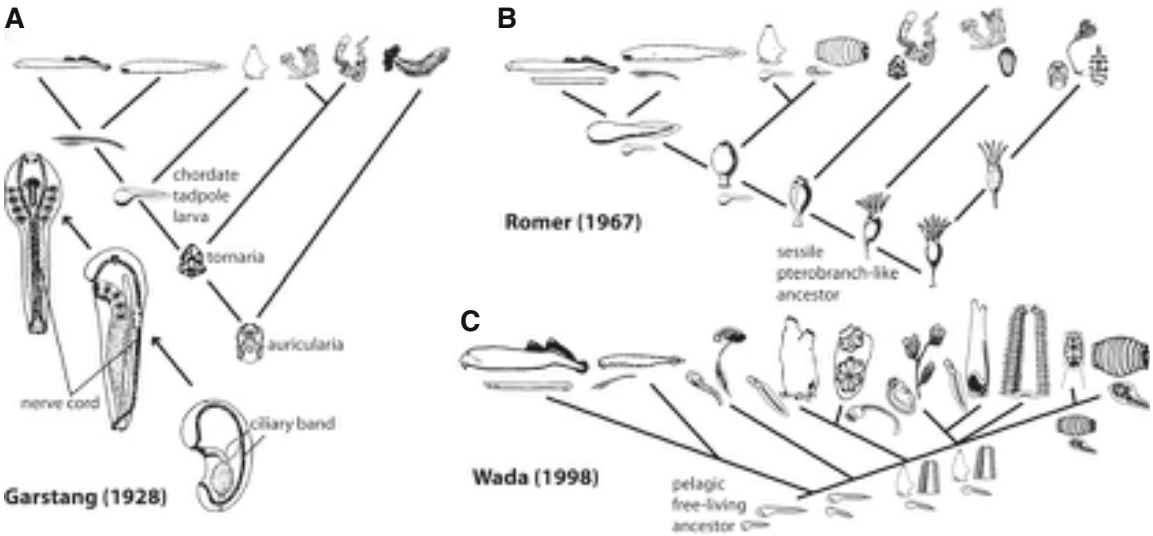


Fig. 4.3 Chordate origins hypotheses. (**A**) Garstang's hypothesis suggested that chordates evolved from larval pelagic forms; a scenario representing the evolutionary transition from a dipleurula-like ancestor that modified the ciliary band into the nerve cord of the chordate larva is shown. (**B**) Romer hypothesized the evolution of chordates from a colonial pterobranch-like ancestor; the ancestor eventually transitioned to a free-living life history and modified the tentacles into gills. (**C**) Wada's hypothesis was formulated according to the phylogenetic understanding of chordate relationships by the turn of the century; the basal position of the larvaceans among the tunicates and the paraphyly of ascidians suggested an ancestor with both a pelagic larva and adult

Garstang Hypothesis (1928): Walter Garstang proposed that chordates evolved through a series of modifications to the ancestral larva, independently from changes in the adult form (Fig. 4.3A), and therefore retraced the evolutionary steps back in time starting from (1) a permanently free-swimming chordate larva to (2) a protochordate-like larva (i.e., cephalochordate or ascidian), to (3) a tornaria-like larva (i.e., hemichordate), to (4) an auricularia-like larva (i.e., echinoderm). From this ancestral reconstruction, it may be extrapolated that the free-swimming larval and adult stages of Craniata/Vertebrata as well as Cephalochordata evolved from an ascidian-like ancestor that suppressed metamorphosis. The latter, in turn, evolved from plankton-feeding benthic organisms (similar to extant hemichordates and echinoderms) with external ciliated tentacles and food groves that contained pelagic larvae (tornaria and auricularia, respectively) (reviewed in Berrill 1955; Brown et al. 2008). Garstang also observed similarities between the development of the neural tube and endostyle (i.e., the thyroid gland precursor; see Metamorphosis section below for more details) of chordate larvae and the formation of the circumoral and adoral ciliary bands of echinoderm larvae. He assumed these processes were homologous, which would support his hypothesis. However, expression patterns of several key developmental genes in the echinoderm larva do not resemble the expected expression patterns of either invertebrate or vertebrate embryos and generally show echinoderm-specific patterns. These results have caused some difficulties in inferring homologies between chordate and echinoderm larvae and have been used to support the view of a secondarily acquired indirect-developing larva in the Ambulacraria (Echinodermata + Hemichordata) (reviewed in Swalla 2006; Brown et al. 2008).

Garstang was highly influenced at the time by work and ideas previously developed by Arthur Willey (1894). Willey had already noted that larval forms could recapitulate ancestral forms since the discovery of the chordate nature of the ascidian larva (Kowalewski 1866) and had also proposed the close relationship of the hemichordates and echinoderms due to the similarities of the tornaria and auricularia larvae. Ideas of recapitulation prevailed in the nineteenth century (Haeckel 1869) and set the stage for twentieth century ideas of heterochrony in development as an important factor in the evolution of animals.

Romer Hypothesis (1967): Alfred Romer was a vertebrate paleontologist and comparative morphologist who came to address the question of chordate evolution from a vertebrate point of view (Fig. 4.3B). In his hypothesis, two major evolutionary novelties were necessary for the evolution of the chordate body plan: (1) the evolution of pharyngeal slits from feeding tentacles (lophophores) and (2) the evolution of a free-living stage in the life cycle of an ancestral sessile, filter-feeding organism. As Romer explained in his departing American Association for the Advancement of Science (AAAS) presidential address published in Science (1967): "Instead of passively waiting for food to come to it, the animal could go in search of food and could explore new areas or new habitats in which it might exist," thus leading to the evolution of the cephalochordates and the vertebrates. A recurrent idea previously postulated by Garstang was that of a pedomorphic event in an ancestral ascidian-like larva that evolved into a free-living form in the cephalochordate and craniate/vertebrate ancestor.

The Romer hypothesis of chordate evolution became popular in the twentieth century and prevailed in many Zoology textbooks. Phylogenies at the time supported the pterobranchs and crinoids at the base of Deuterostomia, with the lophophorates as the sister group to the deuterostomes. However, phylogenetic data no longer support this hypothesis (Fig. 4.3B), and the sessile pterobranchs are likely derived from a free-living wormlike ancestor within the hemichordates (Fig. 4.1; Brown et al. 2008).

Wada Hypothesis (Wada 1998): Based on phylogenetic understanding of Tunicata relationships using 18S rDNA, Hiroshi Wada proposed a pelagic free-living ancestor of the chordates. The relationship generally accepted at the time was as follows: Cephalochordates were the sister taxon to the Craniata/Vertebrata; larvaceans were at the

base of the tunicates; ascidians were shown to be paraphyletic (Fig. 4.3C). Therefore, the most parsimonious reconstruction scenario of the chordate ancestor was that of a pelagic noncolonial and indirect-developing free-swimming form resembling the life cycles of lampreys, cephalochordates, and larvaceans that were placed at the base of the Tunicata. Since then, phylogenetic relationships of the chordates have gained from new analysis methods, additional gene sequences, and new genomes and transcriptomes that have substantially changed our view of tunicate relationships. In particular, a new placement of Tunicata as sister group of the Craniata/Vertebrata and genome-wide sequence analyses and developmental studies in larvaceans have begun to change our views of chordate ancestry. These will be discussed next.

The Wormlike or Vermiform Deuterostome Ancestor Hypothesis: Although references to a wormlike ancestor of the chordates date back to A. Willey (1894),[3] later work by morphologists focused mostly on macroevolutionary reconstructions based solely on ancestral pelagic larval forms (Garstang 1928; Nielsen 1995). Only recently has this hypothesis been revisited, thanks to recent developmental studies and a new understanding of deuterostome relationships. More specifically, gene expression patterns have emerged as new characters that can be evaluated as shared novelties (synapomorphies) among the various groups within the deuterostomes (Nielsen 1995; Cameron et al. 2000; Wicht and Lacalli 2005; Gudo and Syed 2008).

The vermiform ancestor hypothesis postulates that chordates and deuterostomes evolved from a solitary, sexually reproducing, free-living wormlike ancestor with pharyngeal slits, i.e., similar to an enteropneust worm (Fig. 4.1; Cameron et al. 2000; Gerhart et al. 2005; Brown et al. 2008). Initially, a benthic vermiform deuterostome ancestor with a pelagic dipleurula-like larva, termed the "notoneuron," was discussed by Nielsen (1995) in his Trochaea hypothesis. However, the evolutionary sequence that gave rise to the notoneuron assumed the existence of ancestral holopelagic forms, which very much resembles the recapitulation hypothesis of the nineteenth century (Fig. 4.3A; Haeckel 1869; reviewed in Garstang 1928; Collins and Valentine 2001).

In contrast to the previous hypotheses, the most complete and parsimonious scenario based on current phylogenetic evidence (Fig. 4.1) is presented here. Within the Chordata, three hypothetical ancestors are discussed: chordate, olfactorean (tunicate + vertebrate), and tunicate.

The chordate and olfactorean ancestors may be assumed as being very similar in development and form: indirect-developing vermiform organisms with gill slits, dorsal neural tube, notochord, and a pigmented sensory organ throughout all stages of their life cycle (Fig. 4.1). Whether the adult of the olfactorean ancestor was pelagic or benthic is difficult to predict with confidence, but the chordate ancestor was very likely benthic, with a lifestyle resembling that of the cephalochordate adult. This view can be supported as cephalochordates are now placed basal to Olfactores. The parsimonious conclusion is that the olfactorean ancestor was also benthic.

The tunicate ancestor was likely an indirect developer that had a pelagic free-swimming larva with a notochord, a dorsal neural tube, and asymmetric pigmented sensory organs, traits that are found in most tunicate clades (Fig. 4.1). This ancestral larva probably did not have any gills, as most larvae in extant tunicates do not develop or utilize their gills until after metamorphosis. Due to the unresolved phylogenetic positions of Appendicularia, Thaliacea, and several species of phlebobranch and aplousobranch ascidians, there are few confident predictions we can make on the adult form of the tunicate ancestor, other than to say it was likely a filter feeder. While the first tunicate ancestor was likely very similar to its vermiform olfactorean forebear, we cannot rule out a scenario in which the transition to sessility occurred in the stem leading to the last common ancestor of all extant tunicates.

[3] A. Willey wrote in his treaty on "Amphioxus and the Ancestry of the Vertebrates" (1894): "The ultimate or primordial ancestor of the vertebrates would, on the contrary, be a wormlike animal whose organization was approximately on a level with that of the bilateral ancestors of the Echinoderms."

Historical Milestones of Tunicate Developmental Biology

The chordate nature of ascidians was first recognized by Russian zoologist Alexander Kowalevsky (1866). He generated detailed descriptions of embryogenesis and the anatomy and general organization of the chordate larva in two species of ascidians, *Phallusia mammillata* and *Ciona intestinalis*. As mentioned above, his original descriptions suggested that these animals were closer to the vertebrates rather than to their previous classification as mollusks. His discovery set the stage for renewed discussions of the centuries-old questions about the evolution of animal form (Raff and Love 2004).

Kowalevsky's careful anatomical descriptions of the ascidian embryo and larva served as one of the inspirations for an attempt to unravel the evolutionary history of animals by analyzing the features that appeared transiently during particular developmental stages. The ascidian embryo was thus held up in support of a recapitulatory view of evolution. Although such views have been largely superseded, it is imperative to contextualize them. At that time, these studies were among the first to suggest that the development of an organism could reveal information about its evolutionary origins. A modern-day analogy would be the "hidden" evolutionary history that is stored in morphogenesis as well as the genes and genomes of organisms, which has revolutionized our understanding of phylogeny and evolution in the last decades.

Near the end of the nineteenth century, the French embryologist Laurent Chabry, working on *Ascidiella aspersa*, performed the first experiments on animal embryos at the single-cell level, manipulating individual blastomeres with the micromanipulator he had invented and built himself (Fig. 4.4A; Chabry 1887; reviewed in Fischer 1992; Sander and Fischer 1992). This was a radically new approach at the time and generated much discussion in those days of wildly differing theories

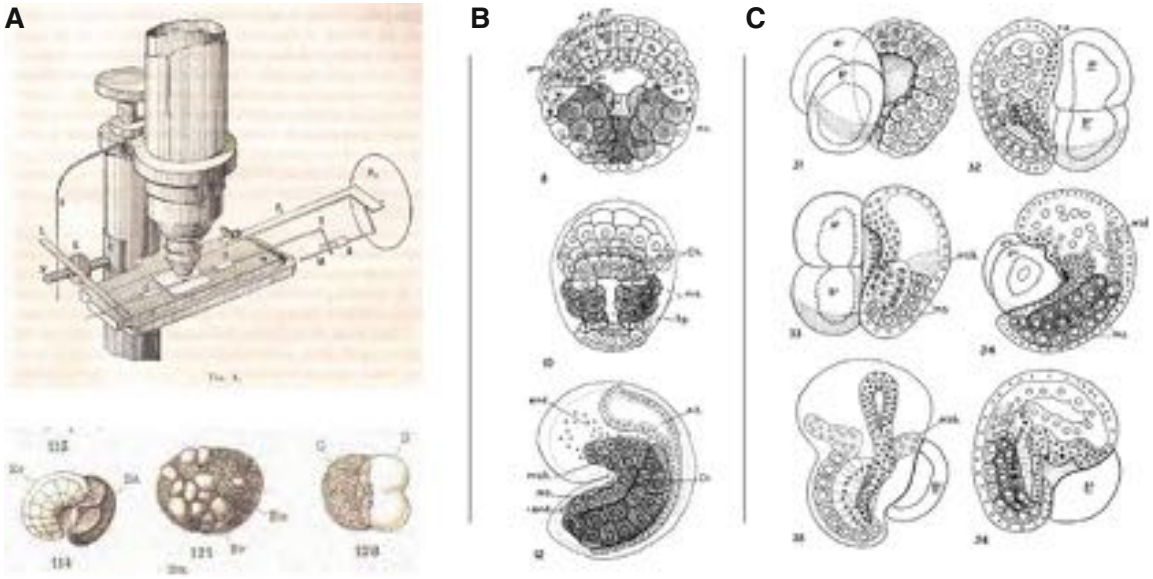


Fig. 4.4 Classic works of ascidian embryology. (**A**) *Top*: Laurent Chabry's micromanipulator, of his own invention and construction. *Bottom row*: some of the "monsters" (damaged or naturally defective embryos) whose abnormal development he characterized. The illustrations are from his thesis (Chabry 1887). (**B**) Illustrations by Conklin of a *Styela canopus* embryo, showcasing two of his greatest discoveries: the posterior vegetal myoplasm, or "organ-forming substance" (Conklin 1905a), and the invariant cell lineages of the early embryo (Conklin 1905b). (**C**) Conklin's illustrations of the embryos whose blastomeres he managed to damage through swirling or suction. His documentation of these half or quarter embryos helped establish a role for mosaic, as opposed to regulative, mechanisms of cell fate specification (Conklin 1905c)

on the very essence of embryogenesis. Chabry's work was eagerly followed up by other luminaries of those early days of experimental embryology, such as Roux, Driesch, Spemann, and Mangold.

In the history of ascidian embryology, we skip some 20 years forward to describe the work of American embryologist Edwin G. Conklin, who conducted both detailed descriptive and ingenious experimental research on the ascidian embryo. At the Marine Biological Laboratories at Woods Hole, Conklin used a local species, *Styela canopus*,[4] to ask basic questions about cell fates during embryonic development. This species was particularly suited for this line of inquiry because its eggs contained a naturally occurring yellow-orange pigment in the cytoplasm that became progressively restricted after each round of cell division until it was ultimately inherited by the larval muscles (Fig. 4.4B). Conklin therefore hypothesized, correctly, that this cytoplasm contained an "organ-forming substance" that was sufficient and necessary for larval muscle specification and differentiation (Conklin 1905a).

The cellular simplicity of the ascidian embryo also allowed Conklin to follow embryonic cells during development up to their final fate. The cell lineages he described for *Styela* became the most complete embryonic lineage mapping ever done for any animal at the time (Conklin 1905b). Moreover, he could manipulate early embryos en masse by spurting and shaking and assess the results of injuring particular blastomeres in this way (Conklin 1905c). This allowed him to demonstrate the highly deterministic mode of development of ascidians, which contrasted abruptly with the highly regulative mode of development that was discovered in sea urchins around the same time (Fig. 4.4C). He called this highly deterministic mode of development in ascidians "mosaic." He and others also noted similar patterns of embryogenesis and highly stereotypical cell numbers and lineages in embryos of other species of solitary ascidians. This was followed by several decades of comparative works describing the diversity and evolution of developmental modes found in ascidians and tunicates, most notably by Berrill (1930, 1931, 1932, 1935a, b, 1936, 1947a, b, c, 1948a, b, c, d, e, 1950a, b, 1951).

As the broader field of developmental genetics matured and incorporated the techniques and approaches of molecular biology and genomics, ascidian embryogenesis quickly came to be understood and interpreted in the light of gene expression and function. Gone were the sectarian squabbles of regulative vs. mosaic development: conserved intercellular signaling pathways were found to regulate the induction of the majority of cell fates in the ascidian embryo. A new picture emerged, in which mechanisms of "mosaic" development (e.g., localized maternal determinants) critically set the stage for the intricate and invariant unfolding of events that nonetheless require the constant instructions and affirmations of cell-cell communication, until all progenitors are specified and their derivatives fully differentiated.

Many of the first discoveries in this new molecular age of ascidian biology were achieved by researchers working on *Halocynthia roretzi*, but *Ciona intestinalis* quickly rose to prominence[5] when its genome was sequenced in 2002 (Dehal et al. 2002). Researchers working on ascidians were privileged to be among the first to lay eyes on the genomic underpinnings of animal development, as *C. intestinalis* was only the seventh animal to have its genome sequenced at the time. The adaptation of an electroporation-based high-throughput transgenesis protocol also boosted the popularity of *C. intestinalis* among developmental biologists (Corbo et al. 1997).

[4] There appears to be some confusion as to which species was originally used in Conklin's experiments. Although in his original studies the species was named as *Cynthia* (*Styela*) *partita*, this name is no longer valid, having been synonymized with *Styela canopus*. However, the ecologically dominant *Styela* species around Woods Hole nowadays is the invasive *S. clava*.

[5] There are approximately 70 laboratories in the world currently using *Ciona* as a model species; for further information on *Ciona* and other ascidian online resources, visit http://www.aniseed.cnrs.fr/aniseed/.

Tunicata: Experimental Models for EvoDevo Research

There has been a variety of work on many different species representing all orders in the Tunicata, and it is with some injustice that the four species below have been highlighted above all the others, solely based on the relative importance attached to them by a handful of bipedal terrestrial olfactoreans. However, they represent a good sampling of different traits, historical backgrounds, and other points of interest to the experimentalist.

Ciona intestinalis (*Linnaeus*, *1767*), like its close relative *C. savignyi*, is a rather unspecialized solitary phlebobranch ascidian. A common invasive species in temperate waters around the globe, it is easily obtainable from the wild. In fact, this was the major reason *C. intestinalis* gained traction with developmental biologists (culminating in the sequencing of its genome in 2002): researchers in Japan, North America, and Northern and Southern Europe could work on the same species… or so they thought! It is now understood that the same name has been used for two closely related but genetically distinct and reproductively isolated species, currently referred to as "Type A" and "Type B." The *Ciona* community awaits official publication of the bombshell revelation (Lucia Manni, personal communication) that the specimen originally described by Linnaeus, and thus the specific epithet *intestinalis*, most likely belonged to the "Type B" species of Northern Europe. This would mean that the "Type A," studied by the majority of labs around the world, actually corresponds to *Ciona robusta*, currently a junior synonym that nonetheless describes the type specimen for "Type A."

Halocynthia roretzi (*Drasche*, *1884*), also known as the sea pineapple, is a prized culinary delicacy in parts of Japan and Korea. A large solitary stolidobranch ascidian, its eggs and larvae are roughly twice as large as those of *Ciona* spp., but develop according to the same cell lineages, with roughly the same number of cells as *Ciona* and other smaller solitary species. Many of the modern-day classics in ascidian developmental biology were performed on *H. roretzi* by Hiroki Nishida in Japan. However, its limited distribution has prevented its adoption as a model organism by researchers outside East Asia. Other obstacles to widespread use as a model organism include its inaptitude for the electroporation protocol so useful for transient transfections in *Ciona*, and deadly outbreaks of the insidious soft tunic syndrome disease (caused by a species of kinetoplastid flagellate) among sea pineapples under intense cultivation. However, important insights into developmental biology continue to be regularly generated by work on this venerable model, and the community eagerly awaits the results of the ongoing efforts to sequence and annotate its genome and that of its North American close relative, the sea peach *Halocynthia aurantium*.

Botryllus schlosseri (*Pallas*, *1766*) is the leading model colonial species. Research in *Botryllus schlosseri* has focused mainly in the study of budding, i.e., blastogenesis, and in studies of self- and nonself recognition of adult colonies. Also known as the star tunicate due to the star-shaped arrangement of individual zooids within the colony, *B. schlosseri* develops synchronously and undergoes weekly developmental cycles that generate new zooids at the same time as old zooids regress. A relatively fast turnover of zooids and simplicity in the maintenance of colonies in the laboratory have made this species a suitable model organism for studies on stem cells, differentiation, apoptosis, and sexual vs. asexual development. Stages of blastogenic development were described by Sabbadin as well as Watanabe, and its complete genome was finally published in 2013. Upon contact of two adult colonies, an allorecognition reaction occurs, which results in either fusion of the colonies forming a larger chimeric colony (parabiont) or

rejection of the two colonies, revealing a histocompatibility locus with important evolutionary implications for the evolution of adaptive immunity of vertebrates.

Oikopleura dioica (*Fol*, *1872*) is an emerging model organism for larvacean biology. Their small size and rapid generation cycle mean they have to be cultured in suspensions of large numbers of individuals. Nonetheless, this allows for inland culturing of different strains, enabling classical genetic analyses. Furthermore, the female germ line and ovary develop as a single, multinucleated syncytium called a coenocyst, allowing for targeted genetic manipulations in ovo by injecting the coenocyst. Larvaceans hold many of the keys to understanding the evolution of tunicates and chordates, and work on *O. dioica* will figure heavily into understanding the biology of these amazing creatures.

Soon thereafter the genome of the related *Ciona savignyi* was sequenced (Vinson et al. 2005; Small et al. 2007). This was followed by the genome sequence of the larvacean *Oikopleura dioica* (Denoeud et al. 2010) and the colonial stolidobranch ascidian *Botryllus schlosseri* (Voskoboynik et al. 2013), which was already the established colonial tunicate model for studies on blastogenesis and histocompatibility. Other tunicate genomes currently in the sequencing pipeline include those of the phlebobranch ascidians *Phallusia mammillata*, *Phallusia fumigata* (Tassy et al. 2010; Roure et al. 2014), and *Didemnum vexillum* (Abbott et al. 2011; Stefaniak et al. 2012), as well as the stolidobranch ascidians *Halocynthia roretzi*, *Halocynthia aurantium* (Tassy et al. 2010), *Molgula occulta*, *Molgula oculata*, and *Molgula occidentalis* (Stolfi et al. 2014).

Tunicate genomes are rapidly evolving (Denoeud et al. 2010; Tsagkogeorga et al. 2012) and quite compact relative to the genomes of their sister taxa, the vertebrates. The *Ciona* genomes are approx. 150–170 Mb in length, while the *Oikopleura dioica* genome is the smallest chordate genome sequenced to date, at 70 Mb. The *Botryllus schlosseri* genome is the largest tunicate genome sequenced thus far, at an estimated 580 Mb. The sizes of the tunicate genomes currently being sequenced appear to fall somewhere in the range of those already sequenced. Protein-coding genes are estimated to number between 15.000 in *Ciona* and 27.000 in *Botryllus*.

Thanks to the *Ciona intestinalis* sequencing efforts, a large amount of gene expression data has been generated for this species, including spatiotemporal expression pattern profiles for the vast majority of the approx. 700 transcriptional regulators (i.e., sequence-specific transcription factors and major cell signaling molecules) during embryonic development (Satou et al. 2001, 2002, 2003; Kusakabe et al. 2002; Mochizuki et al. 2003; Imai et al. 2004). Concurrent to the genomic revolution, the adaptation and refinement of techniques for molecular manipulation of ascidian embryos (Corbo et al. 1997; reviewed in Stolfi and Christiaen 2012; Treen et al. 2014) have allowed researchers to characterize the functions of many developmentally important genes in ascidians in quite some detail. The ease of manipulation of great numbers of synchronized embryos allows for large-scale analyses of gene function, a prime example of which was the construction of a provisional "blueprint" of regulatory networks at single-cell resolution for every cell of the *C. intestinalis* early embryo and larval nervous system (Fig. 4.5; Imai et al. 2006, 2009).

EARLY DEVELOPMENT

Tunicate development is marked by the contrast between the simple and the complex and between the conservative and the innovative. We use the ascidian embryo as a starting point for understanding the development of tunicates as a group,

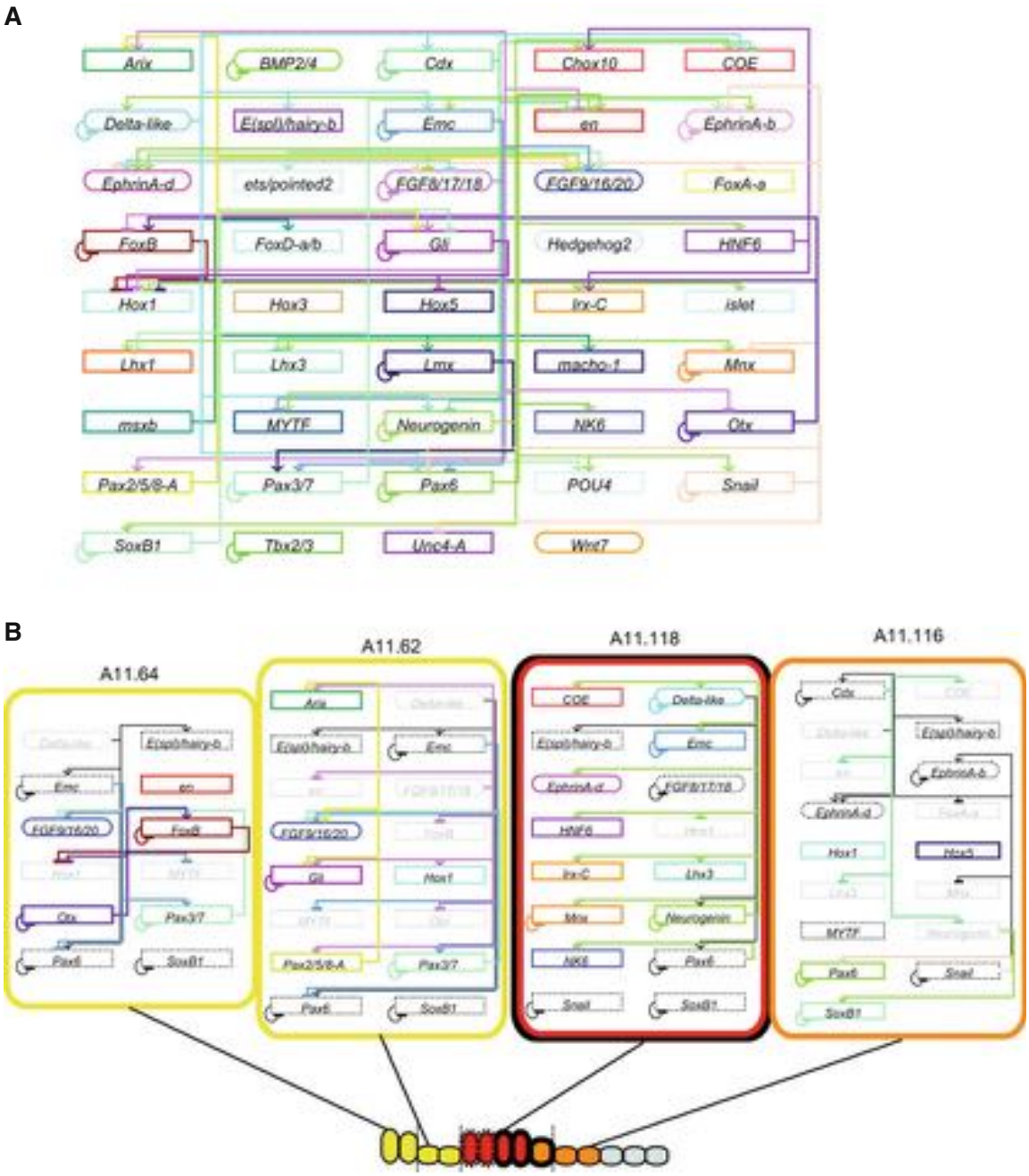


Fig. 4.5 Example of cell-specific gene regulatory networks in *Ciona intestinalis*. (**A**) Circuit diagram of overall regulatory connections between a subset of regulatory genes (transcription factors and intercellular signaling pathway components) expressed in the developing central nervous system of the *Ciona intestinalis* larva. (**B**) Cell-specific circuit diagrams of regulatory connections operating in selected cells of the posterior sensory vesicle (A11.64), neck (A11.62), motor ganglion (A11.118), and nerve cord (A11.116) (Figures adapted with permission of the authors from Imai et al. (2009))

as phylogenetic and embryological evidence suggest all extant tunicates descend from an ascidian-like common ancestor. Much is known about the early development of ascidians, which have been the intense focus of over 100 years' worth of developmental genetic studies. The vast

knowledge on the subject generated over the years can only be done justice by an extended format review, as has been elegantly done most recently by Satoh (2013). Here, we attempt to summarize a summary of what is known.

The ascidian life cycle may be separated into three or four important developmental periods: (1) embryogenesis (i.e., the formation of the larva), (2) larval development (i.e., settlement and metamorphosis), (3) growth and maturation (i.e., transition from juvenile to sexually mature adult), and (4), only for colonial species, blastogenesis (i.e., development of asexually propagated clonal generations). Considerable interspecific variation is found in egg/embryo size, reproductive strategies, and adult zooidal size (Fig. 4.6). The relative timing of the different stages of the ascidian life cycle also varies substantially from species to species, which highlights the importance of heterochrony and modularity in the evolution of developmental processes.

The Ascidian Embryo

Although ascidian embryos are greatly reduced in cell number and highly derived relative to vertebrates in strategies for cell fate specification, they are profoundly steeped in their chordate pedigree, as evidenced by highly conserved chordate-specific embryonic fate maps and gene regulatory networks. Within the ascidians, one finds great variation in the mode of development: viviparity, asexual reproduction, direct development, and adultation, i.e., the precocious differentiation of adult structures prior to hatching of the swimming larva. However, even distantly related ascidians share the same streamlined, invariant embryonic cell lineages. Therefore, phylogenetic evidence suggests that the simple, solitary ascidian embryo likely represents the ancestral condition and that all the other strikingly different modes are variations on this basic theme.

Embryonic development has been characterized in great detail in a number of indirectly developing, solitary ascidian species (reviewed in Lemaire 2009, 2011). In all species, the bilaterally symmetric embryos develop in an invariant manner, and cell division rates are tightly regulated. Unlike certain species of nematodes in which apoptosis, or programmed cell death, plays a large role in development by culling unused cells, there is no evidence that apoptosis is involved in normal embryogenesis in ascidians. All the cells that are born during the development of a typical ascidian embryo seem to be accounted for upon hatching (Hotta et al. 2007).

Quite remarkably, embryogenesis of the various solitary ascidians is perfectly conserved at least up until the neurula stage (and probably well beyond that) as far as cell lineages, divisions, and arrangements are concerned. The embryos may differ in size, developmental rate, and even gene expression, but there is a 1:1 correspondence between each and every cell in *Ciona intestinalis* and *Molgula occidentalis* gastrula embryos, in spite of the estimated 500 million years of evolutionary divergence between the two species (Fig. 4.7).

That species on opposite branches of the tunicate phylogeny have the same exact cell lineages would seem to imply that genetically they must be very conservative as well and that the *cis-regulatory* elements (or "enhancers") orchestrating the gene regulatory networks responsible for embryonic development must be highly conserved. In fact, the exact opposite is true. There is virtually no conservation between *Ciona intestinalis* and *Halocynthia roretzi* noncoding sequences (Oda-Ishii et al. 2005) nor between certain congeneric species, like *Molgula occidentalis* and *Molgula oculata* (Stolfi et al. 2014). This is consistent with the observations that the genomes of ascidians, and tunicates as a whole, are rapidly evolving (Seo et al. 2001; Tsagkogeorga et al. 2012). In some cases, a lack of sequence conservation does not necessarily imply divergent regulatory mechanisms, as some enhancers are fully compatible in cross-species transgenic assays (Johnson et al. 2004; Oda-Ishii et al. 2005; Roure et al. 2014). However, there is mounting evidence for great variation in regulatory strategies controlling identical developmental processes

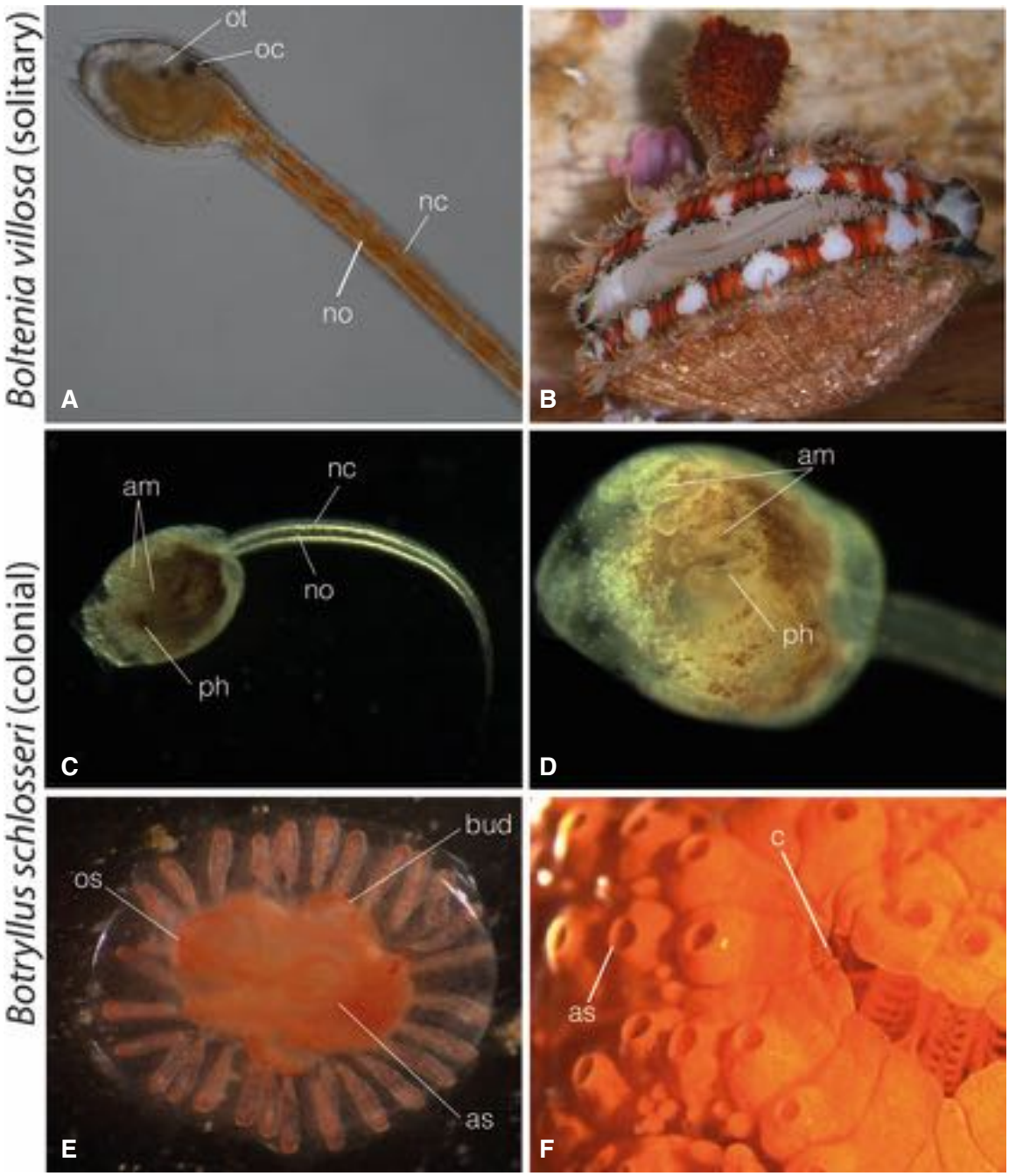


Fig. 4.6 Two styelid ascidians with different life histories: solitary *Boltenia villosa* (**A**, **B**) and colonial *Botrylloides violaceus* (**C**–**F**). (**A**) *B. villosa* orange solitary larva shows chordate features including the notochord (*no*) and the dorsal nerve cord (*nc*); these larvae present light and gravity sensing organs, i.e., ocellus (*oc*) and otolith (*ot*). Palps or adhesive papillae can be seen in the anterior (left). (**B**) An adult *Boltenia villosa* "hitchin' a ride" on a *Chlamys* scallop at Friday Harbor Laboratories. (**C**) *B. violaceus* larva also shows the characteristic chordate features in the tail, i.e., notochord (*no*) and nerve cord (*nc*), as well as a single sensory organ in the head that serves for light and gravity sensing, i.e., photolith (*ph*), and anterior protrusions known as ampullae (*am*). (**D**) Anterior magnified view of *B. violaceus* larval head, which shows the prominent ampullae and some pigmented cells before settlement of the larva. (**E**) The oozoid with open oral (*os*) and atrial (*as*) siphons after settlement shows lateral buds that will develop into the next asexual generation of blastozooids; note extended ampullae around the edges of the newly settled colony. (**F**) Magnification of a *B. violaceus* colony shows the oral openings of each zooid and the common cloacal aperture (*c*) that allows internal visualization of branchial sacs (Courtesy of J. Greer)

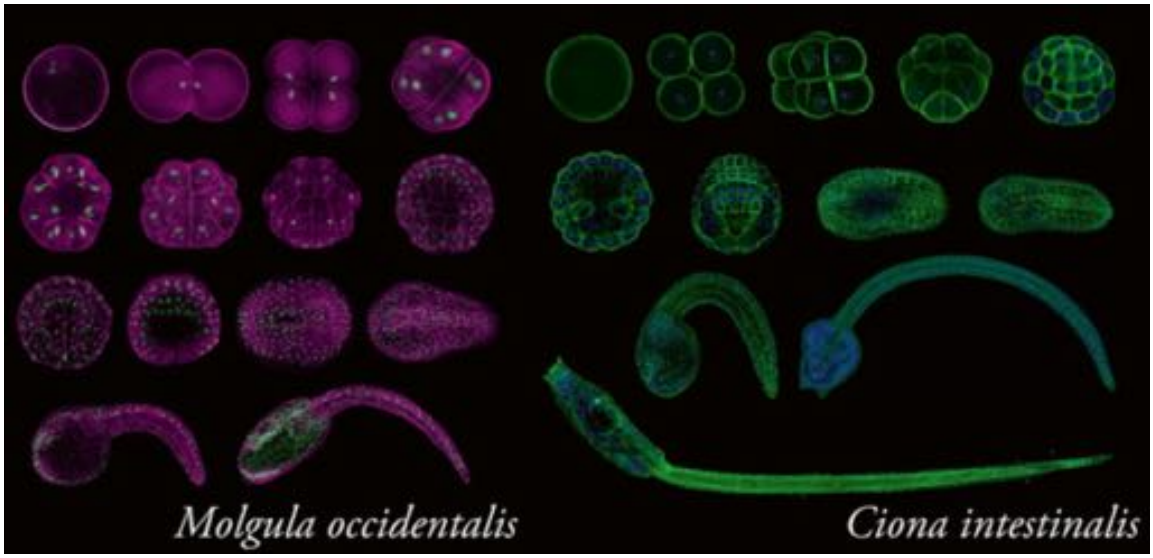


Fig. 4.7 Comparison of *Molgula occidentalis* (Stolidobranchia) and *Ciona intestinalis* (Phlebobranchia) embryogenesis. Comparative diagram of embryonic stages of *Molgula occidentalis* (*left*) and *Ciona intestinalis* (*right*), two distantly related species of solitary ascidians that share near-identical cell lineages and fate maps, in spite of negligible conservation of noncoding genomic sequences. Embryos were stained with DAPI and fluorescent phalloidin conjugates and are all at roughly the same scale (Images were adapted from Stolfi et al. (2014))

in distantly related ascidians[6] (Takahashi et al. 1999; Hudson et al. 2007, 2013; Tokuoka et al. 2007; Takatori et al. 2010).

This remarkably minimalist embryo must have evolved at a bottleneck – the last common ancestor of all extant ascidians and possibly all extant tunicates. However, the incredible derivations seen in the more unusual ascidian embryos demonstrate that, far from being an evolutionary dead end, the simplified ascidian embryo has been elaborated and reconfigured many times over. Later we will discuss the exceptional examples of derivation from this typical embryo and their impact on our understanding of the evolution of tunicate development. For now, it is this "typical" solitary ascidian embryo that will serve heavily as a reference for the general description of embryogenesis in this chapter, as it has been the workhorse of most genetic and molecular studies in the tunicates.

[6] Both examples given above are referred to as developmental system drift or DSD (see Vol. 1, Chapter 1) This refers to the divergence in molecular mechanisms governing the development of identical homologous characters in different species (True and Haag 2001). As outlined above, this can refer to the divergence in primary nucleotide sequence of orthologous enhancers that are functionally interchangeable in cross-species transgenic assays but also to the *functional* divergence of orthologous enhancers that do not drive identical gene expression patterns in their respective species of origin but are not interchangeable in cross-species assays.

The Typical Ascidian Larva

Before describing the embryonic development of ascidians, it is crucial to explain its end product: the typical solitary ascidian larva. The chordate nature of ascidians is most obvious during the larval stage (Figs. 4.6, 4.7, and 4.8). The ascidian larva is roughly divided into two parts, frequently but misleadingly termed the "trunk" and the "tail." The former is mostly composed of the anterior central nervous system (CNS), peripheral nervous system (PNS), and undifferentiated mesoderm and endoderm. From this mesodermal component are derived the adult branchiomeric muscles, heart, blood, tunic cells, and other cell types. The endoderm gives rise to the pharyngeal gill slits (later, the branchial basket), the endostyle, and the anterior digestive tract. Given the homology of these tissues and progenitor types to

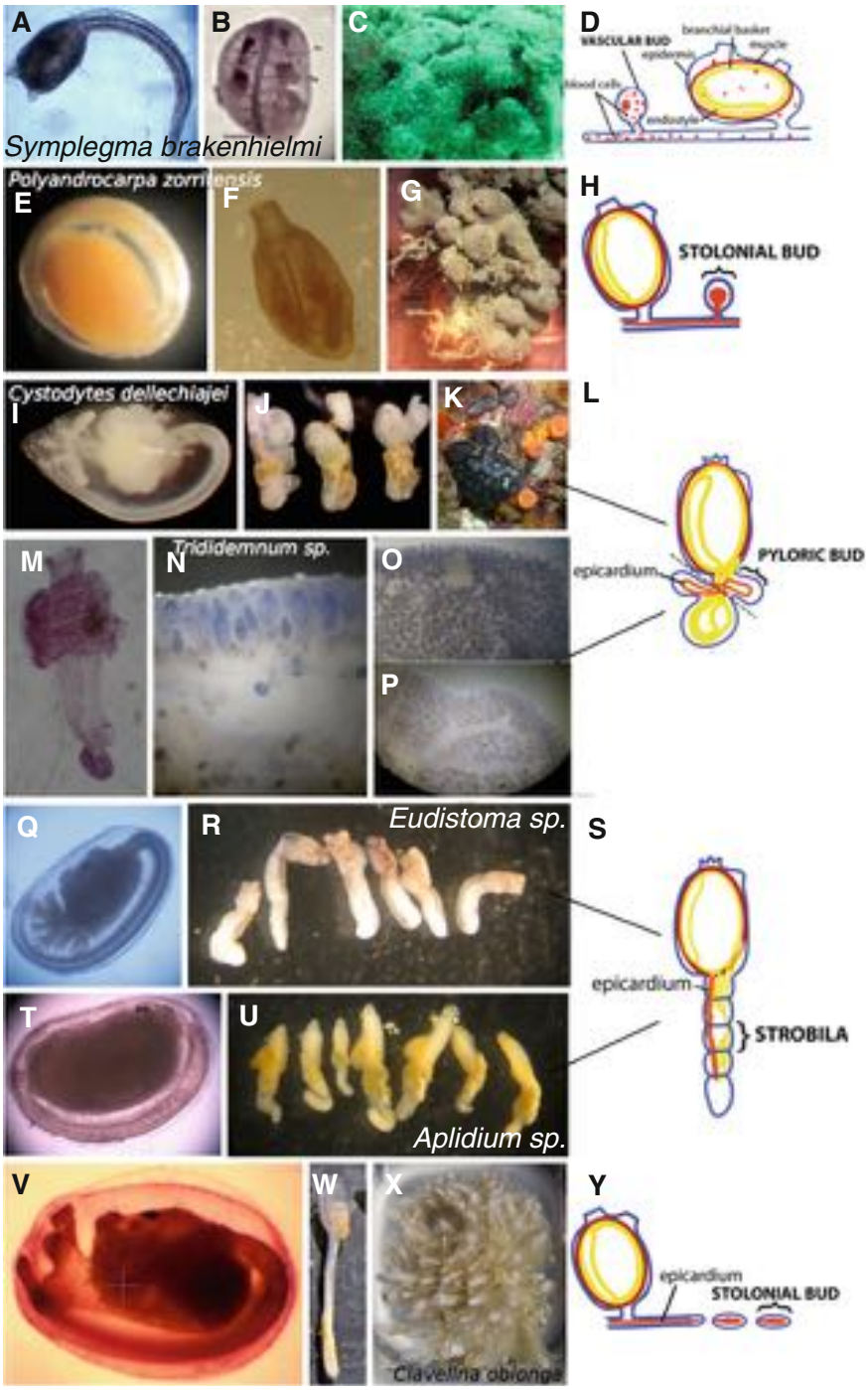


Fig. 4.8 Diversity of colonial forms and budding modes of Ecuadorean ascidians. (**A**–**D**) *Symplegma brakenhielmi* larva (**A**), zooid (**B**), and live colony (**C**) buds mainly by vascular budding (**D**). (**E**–**H**) *Polyandrocarpa zorritensis* larva (**E**), zooid (**F**), and live colony (**G**) buds mainly by stolonial budding (**H**). (**I**–**L**) *Cystodytes dellechiajei* larva (**I**), brooding zooids (**J**), and live colony (**K**) buds mainly by pyloric budding (**L**). (**M**–**P**) *Trididemnum* sp. pyloric bud during the formation of a new abdomen (**M**), lateral view of zooids and buds (**N**); dorsal view of the colony shows branchial regions of the zooids in blue and a clear common protruding cloaca (**O**), whereas a ventral view of the colony reveals the cloacal canals (**P**). (**Q**–**S**) *Eudistoma* sp. larva (**Q**) and zooids (**R**) bud by abdominal strobilation (**S**). (**T**, **U**) *Aplidium sp*. larva (**T**) and zooids (**U**) bud by strobilation (**S**). (**V**–**Y**) *Clavelina oblonga* larva (**V**), zooid (**W**), and colony (**X**) bud by stolonial budding from the abdominal region (**Y**). (**D**, **H**, **L**, **S**, **Y**) represent only symbolic/generic drawings of the distinct budding modes (*capital and bold letters*) in ascidians, but are not meant to illustrate the specific modes of budding observed for the species shown (Modified from Brown and Swalla (2012), and photo courtesy from G. Agurto (CENAIM-Ecuador))

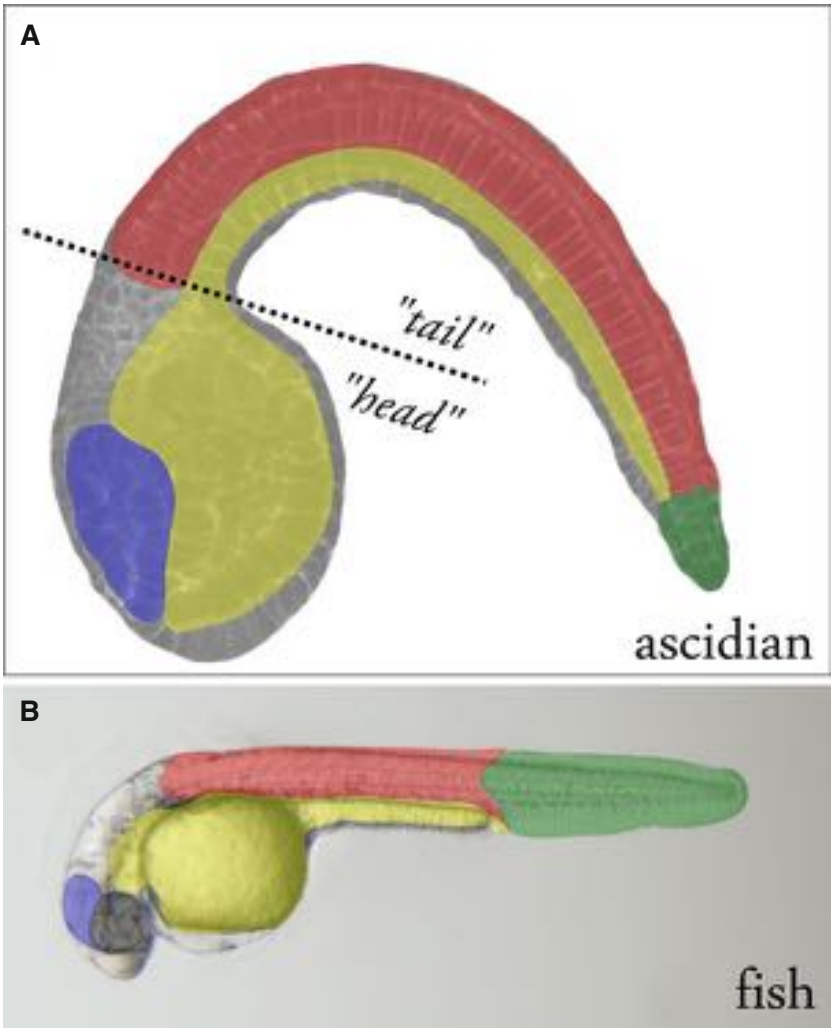


Fig. 4.9 Comparison of ascidian and vertebrate embryo anatomies. (**A**) *Ciona intestinalis* mid-tailbud stage embryo, with major tissues/territories color coded: *blue*, sensory vesicle + brain; *yellow*, endoderm; *red*, axial + paraxial mesoderm (notochord and muscle, respectively); *green*, tailbud (© Alberto Stolfi, 2015. All Rights Reserved). (**B**) Zebrafish embryo with tissues color-coded according to their homologs in *Ciona*: *blue*, forebrain; *yellow*, endoderm + yolk sac; red, trunk (notochord + paraxial muscles); *green*, postanal tail. Comparison between the two suggests the "tail" of ascidian larvae is homologous to the trunk + tail of vertebrates, and the ascidian larval "head" (formerly known as the "trunk") contains tissues and precursor cells homologous to those located in the vertebrate head (brain, endostyle/thyroid, pharyngeal endoderm). Zebrafish image is from Okuda et al. (2010)

their counterparts in vertebrates, mostly located in the head or just posterior to it, it would be more accurate to refer to this region as a "head." Therefore, this term is preferentially used herein.

The posterior half of the larva is composed mostly of caudal CNS and PNS, notochord (axial mesoderm), larval muscles (paraxial mesoderm), and a strand of undifferentiated endoderm that extends posteriorly almost to the very end of the larva. The presence of this gut rudiment, which gives rise to the adult intestine (Nakazawa et al. 2013), suggests that this "tail" is not entirely equivalent to the defining chordate postanal tail, but rather to the trunk (cervical + thoracic regions) of vertebrates, with perhaps a vestigial postanal tail at its very posterior end (Fig. 4.9). Although it would be more appropriate to refer to this structure as a "trunk-tail," it is here called "tail" for simplicity.

Thus, although the larvae of ascidians and amphibians indeed share a basic chordate body plan, as defined by the unequivocal presence of chordate-specific body structures such as the dorsal hollow neural tube and a notochord, their "tadpole" characteristics may only be superficially similar.

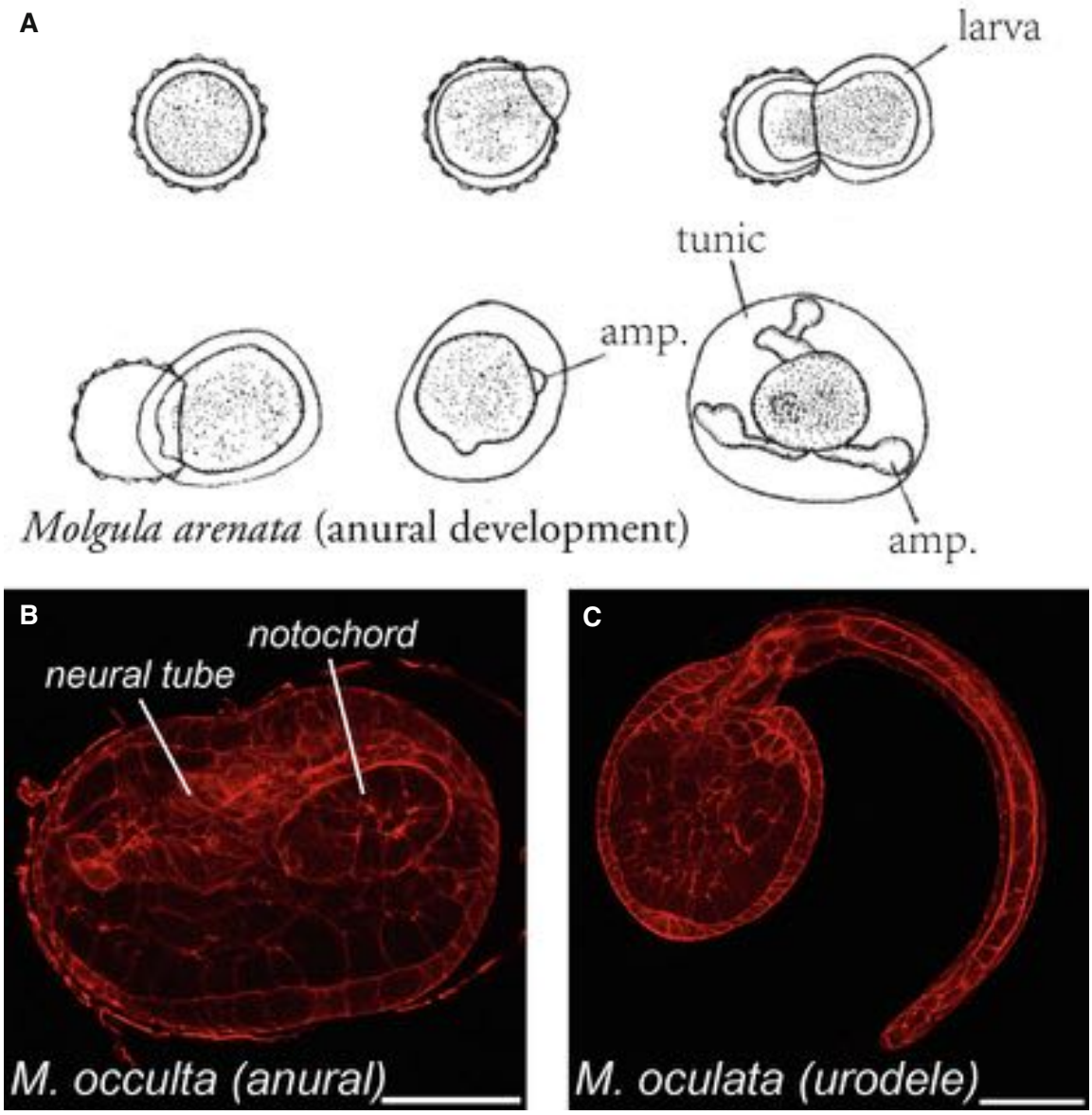


Fig. 4.10 Anural development in the Molgulidae. (**A**) Illustrations by Berrill (1931) of the development, hatching, and metamorphosis of *Molgula arenata*, an indirect-developing tailless (*anural*) species. *Amp.* epidermal ampulla. (**B**) Confocal microscopy image of a hatching larva of the anural species *Molgula occulta*. Note the presence of notochord cells that have failed to proliferate and intercalate like their counterparts in the embryos of urodele species (© Alberto Stolfi, 2015. All Rights Reserved). (**C**) Confocal image of a hatched larva of the urodele (tailed) species *Molgula oculata. M. occulta* and *M. oculata* are closely related and sympatric off the coast around Roscoff, in northern Brittany (France). Embryos were stained with a fluorescent phalloidin conjugate. Anterior is to the right in (**B**, **C**). Scale bars = 50 μm (© Alberto Stolfi, 2015. All Rights Reserved)

Anural and Direct Development

Within the ascidians, there are some curious departures from the typical tadpole-shaped larva. The incredible diversity of developmental modes was studied in detail by Berrill (1928, 1930, 1931, 1935a, 1936) and more recently reviewed by Jeffery and Swalla (1992). The best-studied case is the loss of the familiar tadpole form by larvae of diverse stolidobranchs, notably in the Molgulidae and Styelidae families (Fig. 4.10). In many of these cases, the embryo develops like a typical solitary ascidian embryo, but morphogen-

esis of the tail structures – the notochord, larval muscles, and nerve chord – is severely impaired (Fig. 4.10A). These embryos are said to be "anural" or "tailless," which are misnomers if one considers that these lost or degenerate structures correspond mostly to the axial and paraxial mesoderm of the vertebrate thoracic or cervical segments. However, in this chapter we will continue to use these terms and their opposites, "urodele" or "tailed," for lack of better words.

Anural development may occur due to improper cell fate specification, arrested differentiation, impaired morphogenetic movements, acceleration of apoptosis, or any combination of these. In the anural species *Molgula occulta*, there are fewer notochord cells (Swalla and Jeffery 1990), which remain relatively amorphous and do not extend a functional notochord through convergence and intercalation (Fig. 4.10B, C; Berrill 1931; Swalla and Jeffery 1990). Furthermore, the larval muscles of *M. occulta* fail to differentiate, in part due to recent loss of certain larval muscle-specific "structural" genes such as muscle actin (Kusakabe et al. 1996). The anural condition has independently evolved in different clades (Berrill 1931; Hadfield et al. 1995; Huber et al. 2000), although the developmental causes underlying the condition in these other species have not been extensively studied. Vestigial acetylcholinesterase activity in the muscles of some larvae but not others (Bates and Mallett 1991; Swalla and Jeffery 1992; Bates 1995; Tagawa et al. 1997) and distinct inactivating mutations in larval muscle actin pseudogenes in different anural species (Jeffery et al. 1999) suggest that the tailless conditions of different anural species are not identical.

Berrill associated the anural condition with mud- or sand-flat habitat in which larval motility may be dispensible (offering no adaptive value) and, as a result, easily lost (Berrill 1931). However, the finding of anural *Molgula* species that preferentially attach to rocky substrates along rough coastlines challenged this view (Table 4.1; Young et al. 1988; Bates and Mallett 1991; Nishikawa 1991; Bates 1995). In these cases, there is even perhaps positive selection for direct development from adhesive eggs, without the need for hatching prior to settlement. The extreme tidal changes of high latitudes have also been proposed as a factor that could make swimming at best redundant as a dispersal mechanism (Huber et al. 2000). It is likely that a combination of such biogeographical and ecological factors may have been permissive for the multiple examples of evolution of tailless larvae in the Molgulidae. Whether the molgulids are genetically predisposed to generate anural larvae is a tantalizing hypothesis that remains to be answered.

Table 4.1 Berrill's survey of development in the Molgulidae

Species	Depth found (in fathoms)
Oviparous, urodele	
Attached	
Molgula tubifera	0–3
Molgula manhattensis	0–3
Molgula simplex	20
Molgula socialis	20
Unattached	
Molgula oculata	40
Oviparous, anural	
Attached	
Molgula retortiformis	2–50
Unattached	
Molgula occulta	0–70
Molgula solenata	0–5
Molgula provisionalis	15
Molgula arenata	8
Bostrichobranchus pilularis	8
Eugyra arenosa	5–20
Ovoviviparous, urodele	
Attached	
Molgula complanata	2–10
Molgula cooperi	0–1
Molgula verrucifera	0–5
Molgula citrina	2–5
Molgula platei	–
Ovoviviparous, anural	
Attached	
Molgula bleizi	0–1

Berrill (1931) lists a detailed account of the development (urodele, anural), reproductive (oviparous, ovoviviparous), or ecological (range of depth of occurrence) strategies of each species in the Molgulidae family. Adapted from Berrill (1931)

A closely related condition found in some ascidians is direct development (Young et al. 1988; Bates and Mallett 1991; Swalla and Jeffery 1992; Tagawa et al. 1997). So similar are the two conditions that in different studies the distinction is made using different criteria and often the terms made interchangeable. In this review we distinguish direct development from anural development only by the fact that direct developers will not hatch or break through the chorion prior to the extension of the ampullae. In direct developers, it is these epidermal protrusions that penetrate the chorion and allow the juvenile to "hatch." In many of these species, the eggs themselves are adhesive, allowing for settlement prior to hatching (Young et al. 1988). In both anural and direct development, individuals are presumed to follow the normal course of embryogenesis, with specification of vestigial larval structures that may or may not differentiate. Other criteria for distinguishing direct vs. indirect development of anural species (e.g., differentiation of larval muscles) are difficult to assay and may be ambiguous depending on the assay. Further detailed characterization of the embryogenesis and morphogenesis in the Molgulidae will be needed to classify the potentially multiple gradations of anural and indirect development.

From Fertilization to Gastrulation

Fertilization and Embryonic Axis Determination

During oogenesis, the egg is polarized along an animal-vegetal (AV) axis. Unfertilized eggs are arrested in metaphase of meiosis I, with the meiotic spindle localized at the animal pole, where the polar bodies will form following fertilization. Endoplasmic reticulum-rich cortex (cER), associated maternal "postplasmic" mRNAs, and mitochondria are largely excluded from the animal pole in unfertilized eggs, but only after sperm entry the trigger of a calcium wave leads to actomyosin contraction and concentration of these maternal determinants at the vegetal pole (Prodon et al. 2008). A second phase of ooplasmic segregation occurs after meiosis is complete, shunting the cER and mitochondria toward a presumptive posterior pole, giving rise to a posterior/vegetal cytoplasmic complex (Sardet et al. 1989, 2007; Roegiers et al. 1999). Thus, the anterior-posterior (AP) axis is established roughly perpendicular to the AV axis.

Early Cleavages

The first cleavage occurs along the AP axis, equally partitioning posterior/vegetal cytoplasm into bilaterally symmetric left and right blastomeres. The second cleavage occurs perpendicular to the first, and the third cleavage occurs perpendicular to the second, resulting in a bilaterally symmetric eight-cell embryo in which each half is partitioned into four major quadrants: anterior animal ("a-line," pronounced "small a-line"), posterior animal ("b-line" or "small b-line"), anterior vegetal ("A-line" or "big/large A-line"), and posterior vegetal ("B-line" or "big/large B-line") (Fig. 4.11). Starting from these eight major octants (two halves divided into four quadrants each), the cell lineages of the embryo can be traced throughout development using Conklin's supremely elegant nomenclature system that allows for easy inference of the mitotic history of any given cell[7] (Conklin 1905b).

[7] Conklin's nomenclature system hinges on a tripartite name for each and every cell. This name is composed of a letter and two integers. Let us consider an example, the B7.5 cell. The letter indicates it is derived from the posterior vegetal ("B") blastomere of the right half (indicated by underlining) of the eight-cell embryo. The first number ("7") indicates the mitotic generation to which the cell belongs (the seventh generation, defining the first generation as the single-cell zygote). The second number is the cell's unique identifier, calculated by simple arithmetic from the identifier of its mother cell, B6.3. To derive the identifiers of its daughter cells, the mother cell's identifier ("3") is first multiplied by two ($3 \times 2 = 6$). The daughter cell closest to the original sperm-entry point receives this even number ("6") as its identifier (B7.6). The other daughter cell is then given this number minus 1 ($6 - 1 = 5$). Hence, its name is B7.5. There are some limitations to applying this nomenclature to later development. For instance, sperm-entry point becomes an untenable landmark as cells rearrange and alter their positioning relative to one another. Furthermore, all lineages eventually cease to develop in a stereotypic manner. Therefore, it is not advisable to insist upon Conklin's nomenclature beyond a certain time point in some lineages.

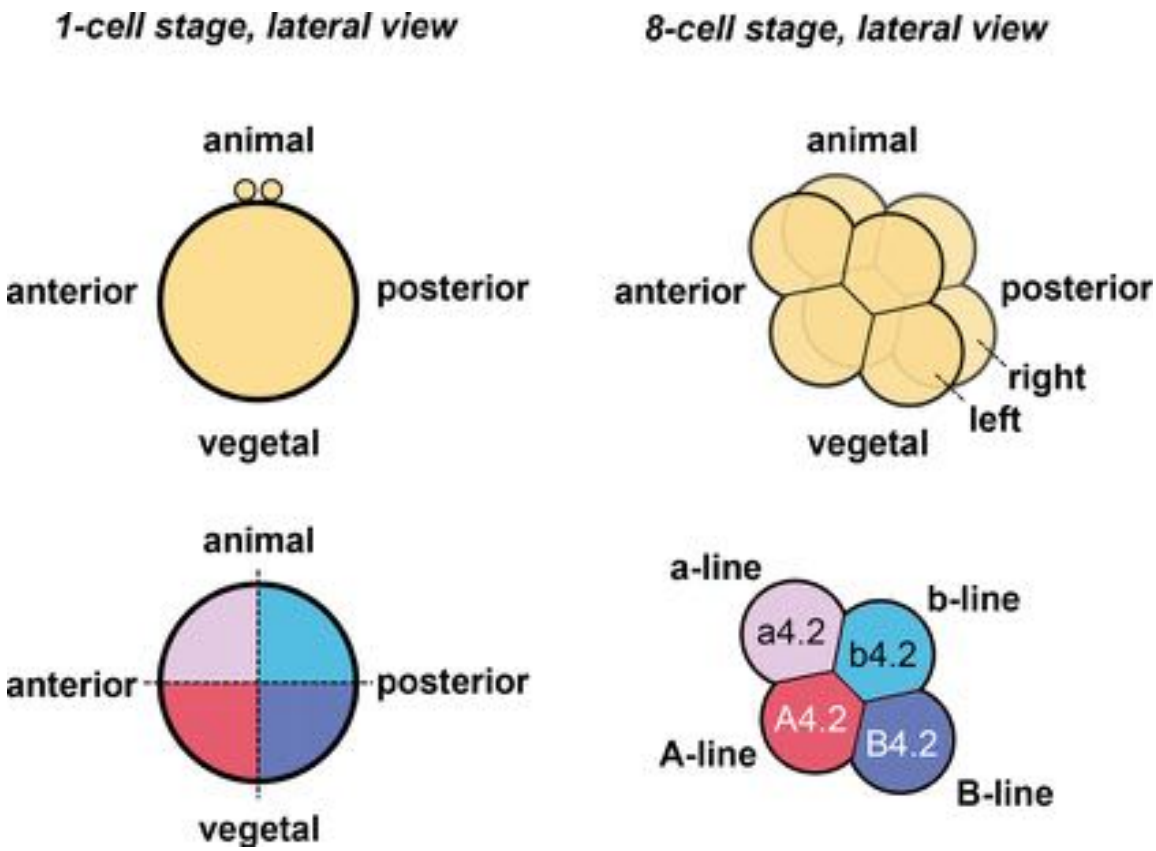


Fig. 4.11 The major cell lineages of the ascidian embryo. *Top left*: diagram of an ascidian one-cell stage embryo (zygote) viewed laterally, showing the major embryonic axes and the polar bodies at the animal pole. *Bottom left*: division of this zygote into the major quadrants of the embryo, as defined by the major axes, animal-vegetal and anterior-posterior. *Top right*: lateral view of an eight-cell stage embryo, showing *left* and *right* hemispheres of the bilaterally symmetric embryo. *Bottom right*: lateral view of only the left hemisphere of the embryo shown at top, with each blastomere labeled with its unique identifier and color-coded according to the quadrants of the one-cell stage embryo. These are the founder cells of the major lineages of the ascidian embryo: a-line (anterior/animal), b-line (posterior/animal), A-line (anterior/vegetal), and B-line (posterior/vegetal). The cells on the *right* hemisphere have the same identifiers, which are *underlined* to indicate their origin from the right hemisphere (e.g., a4.2, b4.2, A4.2, B4.2) ()

Each quadrant gives rise to a multitude of cell lineages that develop according to distinct, but invariant, cleavage patterns and orientations. The only symmetry is bilateral, with right-side and left-side lineages being identical until neurulation is complete. The observations and classic experiments of Conklin (1905a, c), Whittaker (1973, 1977, 1982), and Nishida (1992, 1993, 1994a, b, 1996) indicated the presence of maternal determinants that patterned the ascidian embryo not only molecularly, through the specification of the various cell lineages and their generation of the various differentiated larval tissues, but also physically, by controlling the asymmetric cleavage patterns of the early embryos. The molecular nature of several such determinants was soon to be revealed, heralded initially by the discovery of *Macho1*, the maternal gene at the top of the regulatory cascade for the autonomous specification of primary larval muscles (Nishida and Sawada 2001).

Additional proteins were subsequently identified whose mRNAs are localized to the cER/centrosome-attracting body (CAB), comprising the postplasmic/PEM (posterior end mark) genes (Satou and Satoh 1997; Satou 1999; Sardet et al. 2003). These encode a wide variety of proteins, most of which have not been extensively studied

in ascidians. One exception is the original PEM protein, Pem1, a fascinating molecule that executes multiple functions critical for early development in multiple ascidian species. First, Pem1 is involved in the control of unequal cell cleavages through asymmetric mitotic spindle positioning (Negishi et al. 2007). Remarkably, Pem1 is also a transcriptional regulator, globally repressing transcription in early posterior blastomeres and in the germ line (Kumano et al. 2011). While the first function requires Pem1 localization to the CAB (independently of *Pem1* mRNA localization to the same structure), the second depends on Pem1 nuclear localization. These distinct functions also depend on different domains within Pem1. Furthermore, *Pem1* mRNA localization to the CAB is another important property, as it ensures an AP "gradient" of cell size and transcriptional activity, with more posterior cells being progressively smaller and transcriptionally silenced for longer, due to this asymmetric inheritance of *Pem1* mRNA at each round of cell division. The stepwise release from transcriptional silencing constitutes a "timing mechanism" that is critical for the patterning of the early ascidian embryo along the AP axis (Kumano and Nishida 2009).

Germ Layer Specification

At the vegetal pole, nuclear accumulation of maternally provided Catenin Beta-1 (Beta-catenin) helps these cells maintain mesendoderm potential and suppresses their specification as ectoderm (Imai et al. 2000), although the positional cue for the accumulation itself is not known. In *Ciona intestinalis*, *s*uppression of ectoderm fates involves antagonism of maternal Gata4/5/6 function, which is required for "naïve" ectoderm specification and later for neural induction (Rothbächer et al. 2007).

The vegetal half of the embryo at the 16-cell stage, having repressed an ectodermal fate, is then further divided into vegetal mesendoderm and a "marginal zone" by the 32-cell stage. The marginal zone refers to a ring of cells surrounding the mesendoderm proper and abutting the animal pole (ectoderm). This marginal zone does not correspond to a specific germ layer, as it includes the A-line neural/notochord progenitors. The vegetal mesendoderm in turn is largely fated to give rise to endoderm, except for A6.3, which will also give rise to the A7.6 mesenchymal lineage.

In *Ciona intestinalis*, binary fate choice between vegetal mesendoderm and marginal zone involves sustained nuclear Beta-catenin accumulation. While nuclear accumulation of maternally deposited Beta-catenin drives specification of the entire vegetal half of the embryo between the eight- and 16-cell stages, Beta-catenin nuclear localization is rapidly downregulated in the marginal zone, being restricted to their more vegetal sister cells. This differential Beta-catenin signaling is sufficient and necessary to distinguish the two layers (Hudson et al. 2013).

Surprisingly, this mechanism is not conserved in *Halocynthia roretzi*, in which marginal zone cells also show sustained nuclear Beta-catenin staining. Instead, a peculiar mechanism for asymmetric inheritance of mRNA of the marginal zone determinant *Not* seems to drive mesendoderm patterning. This mechanism involves nuclear migration, retention of locally transcribed *Not* mRNA in a Wnt5-dependent manner, and later repositioning of the nucleus at mitosis, resulting in one daughter cell inheriting all the *Not* mRNAs, which are sufficient and necessary for marginal zone specification (Takatori et al. 2010). This is a remarkable example of developmental system drift between *Ciona* and *Halocynthia*.

Gastrulation

By the 110-cell stage, the germ layers have segregated, the fate map has been established, and gastrulation commences. The endoderm cells drive gastrulation by invagination, which occurs in a two-step process in which the cells undergo first apical constriction then basolateral shortening (Sherrard et al. 2010). This is immediately followed by the involution of the cells of the marginal zone (mesoderm). Since this marginal zone is only one to two cell diameters wide, its ingression is swift and nearly concurrent with epiboly of ectodermal cells. Although this epiboly was hypothesized to push the marginal zone cells as

they ingressed, experiments revealed that the ectoderm cells were not required for proper gastrulation (Sherrard et al. 2010). Once ingressed, mesodermal cells do not immediately move great distances, the most dramatic being the inversion of the larval muscle precursors along the AP axis. Subsequent neurulation and epiboly of the epidermis effectively seal in the endoderm, mesoderm, and neural tube.

Less is known about how the different germ layers and tissues remain physically compartmentalized, though evidence from other organisms suggests that differential contractility and cortical tensions may be involved (Krieg et al. 2008). Additionally, cadherin family gene expression patterns are dynamically regulated during *Ciona intestinalis* development and may play a role in the adhesive properties of the different cell lineages of the embryo (Noda and Satoh 2008). It remains to be seen how the general principles of germ layer organization and cell sorting in larger embryos apply to the much smaller, invariant embryos of solitary ascidians.

Development of the Ectoderm

Neural Induction

The CNS is derived from two of the major quadrants of the embryo: anterior/animal (a-line) and anterior/vegetal (A-line). Either lineage gives rise to both neural and nonneural lineages. The basic mechanisms by which this induction occurs in ascidian embryos are known and differ slightly between the two lineages. In *Ciona intestinalis*, neural induction of a-line occurs at the 32-cell stage and was shown to be carried out by FGF signaling. More specifically, Fgf9/16/20 ligand expressed in vegetal-pole cells is sufficient and necessary to induce neurogenic ectoderm specifically in those a-line cells in contact with the Fgf9/16/20-expressing cells (Hudson and Lemaire 2001; Bertrand et al. 2003). Those cells that do not receive this signal go on to become epidermis and components of the PNS. While this induction still requires Gata4/5/6 activity, like earlier induction of basic ectoderm, the instructive cue is nonetheless the spatially restricted Fgf9/16/20 ligand. This induction is dependent on direct contact of inducing and induced cells, as is the case for almost every other documented FGF-dependent induction event in the *C. intestinalis* embryo.

Induction of CNS from A-line progenitors also involves FGF signaling. Surprisingly, in this lineage FGF signaling inhibits CNS specification. A-line neural precursors are specified by the absence of FGF signaling, while their sister cells are induced by FGF signaling to become notochord precursors (Minokawa et al. 2001; Yasuo and Hudson 2007). The availability of FGF ligands is not limiting, as these cells *are* the source of Fgf9/16/20 and are thus surrounded by ligand. Instead, the localized cue for differential activation cells was found to be Ephrin, which is expressed in a-line cells and antagonizes FGF-dependent ERK signaling in A-line neural progenitors in a contact-dependent manner (Picco et al. 2007). It was shown that this occurs through Ephrin/Eph-dependent localized recruitment of Rasa1 (p120 RasGAP) protein, which is an inhibitor of the Ras/ERK pathway (Haupaix et al. 2013).

Given the requirement for FGF in induction of a-line neural tissue and the conserved role for FGF in neural induction in vertebrates, this inversion in the outcomes of induction seems quite puzzling. However, FGF has since been shown to be repeatedly employed as a simple binary switch to decide between two opposing cell fates in sister cell pairs throughout ascidian embryogenesis (Davidson et al. 2006; Hudson et al. 2007; Shi and Levine 2008; Squarzoni et al. 2011; Stolfi et al. 2011; Wagner and Levine 2012). Accordingly, the role of FGF in the A-line notochord/neural decision is likely to be an ascidian-specific regulatory motif, although the induction of a-line neural progenitors may indeed be a manifestation of an ancestral FGF-dependent mechanism for neural induction.

Neurulation

Neurulation refers to the chordate-specific morphogenesis of a dorsal, hollow neural tube starting from a flat neural plate. In the typical ascidian larva, the neural plate is a flat epithelium of

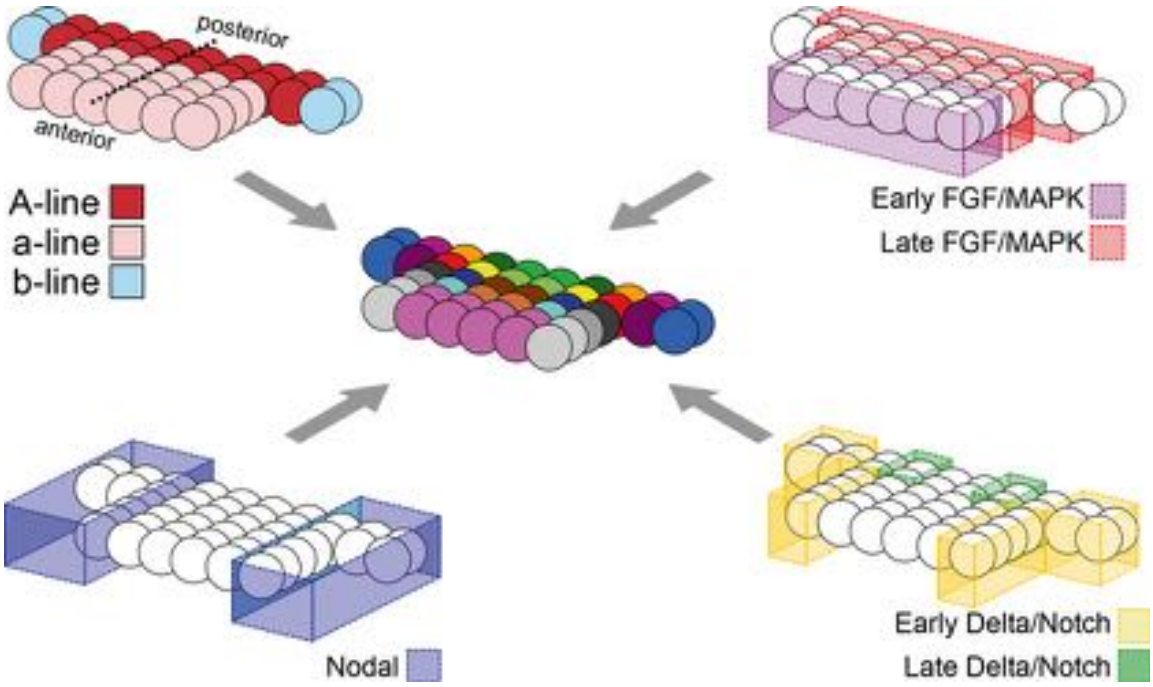


Fig. 4.12 Neural plate patterning in *Ciona*. Cartoon diagram summarizing the findings of Hudson et al. (2007) on the grid-like patterning of the *C. intestinalis* neural plate by FGF/MAPK(ERK), Nodal, and Delta-Notch signaling pathways. *Boxed cells* are under the direct influence of the indicated signals. Further input into the regulatory mechanisms for cell fate specification come from the cell lineage histories of each cell, divided into the three major lineages of the neural plate (A-line, a-line, and b-line). *Dashed line* indicates the dorsal midline. *Color coding* in the neural plate represents unique cell identities as assayed by gene expression profiles. Cell numbers and identities are accurately and realistically portrayed, but the relative sizes and positions of the cells are slightly modified for clarity. Anterior is to the *bottom left* (© Alberto Stolfi, 2015. All Rights Reserved)

neurogenic a- and A-line-derived ectodermal cells that stretch over the dorsal surface of the embryo by epiboly. It forms a highly unusual grid of rectangular cells, an organization that is critical for its proper patterning into rows and columns (Hudson et al. 2007). In *Ciona intestinalis*, this plate was shown to be under the influence of several inductive cues that pattern it into rows and columns of differently fated cells (Fig. 4.12). Namely, Delta and Nodal signals emanate from regions just lateral to the neural plate, while an intricate interplay of FGF and ephrin signals reiteratively determine the binary cell fate choices of anterior/posterior sister cell pairs, by their opposing actions on ERK pathway activation (Hudson et al. 2007; Haupaix et al. 2014).

Upon neurulation (Fig. 4.13), the rows and columns of the neural plate are precisely reorganized into the simplest of neural tubes: four AP columns or stacks of cells (one dorsal column (the neural tube roof plate, formed by the lateral-most cells of the neural plate), one ventral column (the floor plate, formed by the medial-most columns of the neural plate), and two bilaterally symmetric lateral columns, formed from the remainder of neural plate cells) (Nicol and Meinertzhagen 1988a, b; Cole and Meinertzhagen 2004). Thus, the patterning of the neural plate is critical not only for later compartmentalization of the CNS but for the actual morphogenesis of the neural tube. Neurulation is incomplete at the anterior terminus of the neural tube where it remains open, giving rise to the neurogenic portion of the larval stomodeum (Veeman et al. 2010). The neural tube is eventually internalized, after being completely covered dorsally by the epidermis.

Like in most of the embryo, the cell lineages of the neural tube are invariant through neurulation, except for the exact AP order of intercalation of the dorsal and ventral cells. Neural tube

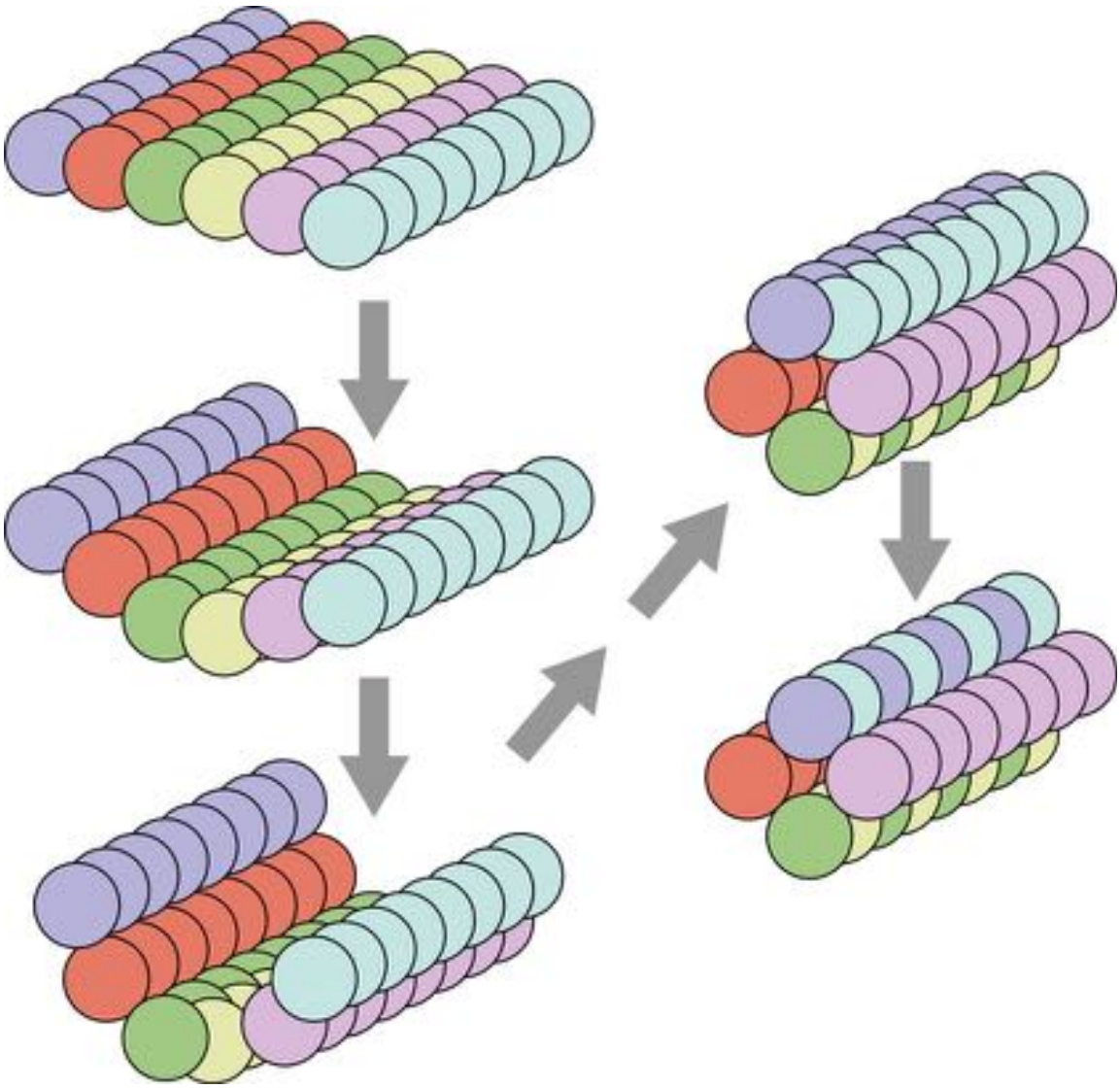


Fig. 4.13 Neurulation. Cartoon diagram depicting the mode of neurulation in solitary ascidian embryos. The flat, dorsal neural plate is a grid-like sheet of cells. The lateral and medial columns are displaced in opposite directions (dorsal and ventral, respectively) by as of yet unknown forces. The cells of the lateral columns meet at the dorsal midline and intercalate to form a single column of cells, the neural tube "roof." The cells of the medial columns also intercalate, forming a single column of cells (the "floor"). Thus, the ascidian neural tube is a simple bundle of four single-cell columns (one dorsal, one ventral and two lateral). A small lumen forms along the inside of the bundle, from the apical surfaces of the neural tube cells (© Alberto Stolfi, 2015. All Rights Reserved)

progenitors proliferate during neurulation, but it was shown that a prolonged G2 phase in dorsal epidermal midline cells is essential for their intercalation (Ogura et al. 2011). Thus, there is a tight coordination between cell division and morphogenesis of the neural tube.

Further elaboration of the neural tube is uneven along the AP axis, foreshadowing the regionalization of the larval central nervous system. At the anterior, the neural tube lumen is engorged (Marino et al. 2007) and eventually becomes the sensory vesicle (SV), which houses the larval melanocytes and associated sensory (light- and acceleration-sensing cells). Distinct rates of proliferation and differentiation occur along the length of the AP axis, resulting in an irregularly shaped neural tube and discontinuous neural tube lumen (Cole and Meinertzhagen 2004).

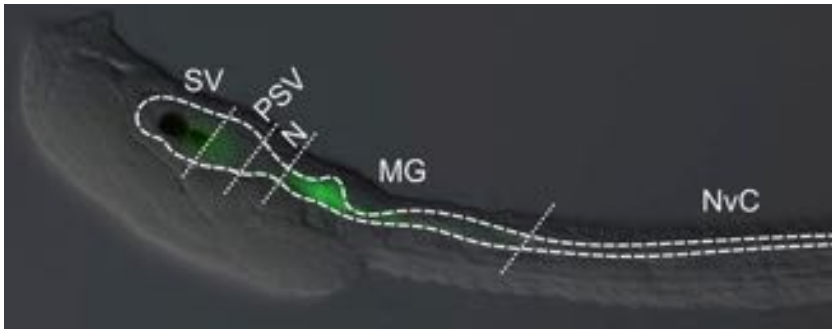


Fig. 4.14 The ascidian larval central nervous system (CNS). *Ciona intestinalis* larva electroporated with the *Vesicular acetylcholinesterase transporter* (*Vacht*)>*unc-76*::*eGFP* plasmid, fluorescently labeling the major neuronal centers of the larval CNS. The five major anatomical subdivisions of the CNS are demarcated, from anterior to posterior: sensory vesicle, posterior sensory vesicle (*PSV*, also known as the brain), neck (*N*), motor ganglion (*MG*), and nerve cord (*NvC*) (Adapted with permission from Stolfi et al. (2011))

Central Nervous System Patterning

At the end of larval development, the central nervous system (CNS) contains approximately 100 neurons (Imai and Meinertzhagen 2007a) and is divided into at least five anatomical compartments along the AP axis (Fig. 4.14). At the anterior end lies the sensory vesicle (SV). Immediately posterior to that is the larval "brain" or posterior sensory vesicle (PSV): a tightly bundled cluster of neurons, photoreceptors, and other putative sensory cells associated with the SV. Posterior to the brain is the neck, an undifferentiated mass of neural progenitors that will give rise to certain branchiomeric neurons of the adult (Dufour et al. 2006). Next is the motor ganglion (MG), comprised of motor neurons and interneurons that drive the swimming behavior of the larva (Bone 1992; Horie et al. 2010). Most posteriorly, along the length of the tail lies the nerve cord. Other than a few scattered neurons of uncertain function, the nerve cord is composed chiefly of elongated ependymal cells and the axons of MG neurons that project posteriorly toward the tailbud. The clonal subdivisions of the neural tube (a-line and A-line) together with cell signaling are responsible for the establishment of regionalized patterns of gene expression. These molecular domains are assumed to then specify and drive the differentiation of the anatomical subdivisions.

Gene expression patterns support a fundamentally bipartite organization of the ascidian CNS: *Otx* expression marks the larval brain and adult anterior CNS progenitors, while absence of *Otx* expression characterizes the remainder (Ikuta and Saiga 2007). However, a tripartite organization emerges when one looks at several genes expressed in the neck. Among these, *Pax2/5/8* and *Phox2* suggest homology of the neck to the vertebrate hindbrain, a claim strongly supported by the fact that these progenitors give rise to *Tbx20*$^{+}$ motor neurons innervating the pharyngeal muscles of the adult (Dufour et al. 2006). The motor ganglion (MG) progenitors express several sequence-specific transcription factor (TF) genes involved in spinal cord motor neuron specification in vertebrates such as *Pax6*, *Lhx3/4*, *Islet*, *Onecut*, and *Nk6*, such that homology of this region to the vertebrate spinal cord is hard to refute. Not enough is known about the adult anterior CNS progenitors, and whether they constitute a molecularly or physically distinct compartment apart from the larval brain, leaving us three functionally distinct regions: larval/adult brain, adult branchiomeric motor neurons, and larval tail motor neurons.

The correspondences to the major divisions of the vertebrate CNS are somewhat tenuous and controversial. There are at least three major factors that make these comparisons a dicey proposition. First, there is a drastic alteration in the positioning of CNS neural precursors and differentiated neurons. For instance, the motor ganglion, argued to be homologous to the vertebrate spinal cord based on molecular, embryological, and functional grounds, is arrayed as only a single cluster of several bilateral pairs of cells. There is no dorsoventral axis to speak of and no serial

repetition of this structure, as there is only one muscle to innervate on either side of the larva. Posterior to the motor ganglion, the nerve cord over the majority of its length contains no neurons of its own, supporting instead the axon bundles of the motor ganglion neurons. This drastic reconfiguration is no doubt a result of the extreme reduction in cell numbers and size of the larva.

Another issue complicating comparative studies is the heterochrony of larval and adult CNS differentiation. Popularly, the ascidian larval CNS is thought to simply degenerate and the adult CNS thought to be formed de novo from an unorganized mass of naïve progenitors. This view has been convincingly refuted, with lineage tracing experiments revealing the contribution to the adult CNS of progenitors from specific regions of the embryonic neural tube (Dufour et al. 2006; Horie et al. 2011). The different larval and adult-differentiating portions of the neural tube will naturally be in different genetic regulatory states, compromising the simple one-to-one pairing of static "snapshots" of gene expression patterns that tend to be emphasized.

Related to this is a third complication, namely, that many of the genes used in comparing the regionalization of the CNS are reiteratively used in different cell types at different stages, carrying out different functions. For example, although Fgf8/17/18 was shown to act as an organizing molecule in the motor ganglion and neck regions as well as at the vertebrate midbrain/hindbrain boundary (Imai et al. 2009), the repeated use of FGF signaling for cell fate decisions in *Ciona* leaves open the question as to whether this organizer is a conserved or convergent feature. Similarly, *Otx* is also a critical regulator of posterior PNS fate (Roure et al. 2014), muddling its intimate association with anterior CNS specification. Finally, given the rapid pace of development of ascidian embryos, where every cell division is potentially a binary fate choice between two completely different lineages, gene expression patterns are highly dynamic and rapidly changing over the course of embryogenesis (Ikuta and Saiga 2007). Great care must be taken to ascertain the temporal and cellular context in which gene expression is taking place before seizing these to support claims of homology.

That said, it seems that the embryological and molecular evidence thus far presented best supports a tripartite nature of the ascidian CNS (Wada et al. 1998). When comparing to vertebrates, the motor ganglion and nerve cord may correspond to the spinal cord, the neck may be homologous to the hindbrain, and the anterior *Otx*-expressing domain would encompass everything anterior to the hindbrain. The finer subdivisions seen both in ascidians and vertebrates (midbrain, midbrain/hindbrain boundary, etc.) would be lineage-specific elaborations of this basic frame, adapted to their respective specialized needs.

Motor Neuron Specification

The gene regulatory networks and inductive events governing neuronal specification in the ascidian CNS have best been studied in motor ganglion neurons (MGNs). In the *Ciona intestinalis* MG, four of the five pairs of MGNs are specified from the A9.30 blastomere (Fig. 4.15A; Cole and Meinertzhagen 2004). Each pair corresponds to a molecularly and morphologically distinct subtype (Stolfi and Levine 2011) and is born and specified in an invariant manner by a series of short-range signaling events (Fig. 4.15B, C; Stolfi et al. 2011). This is in stark contrast to subtype specification in the vertebrate spinal cord, which is patterned along the DV axis by opposing BMP and Shh morphogen gradients. It was shown that in *C. intestinalis*, although the neural tube roof plate expresses BMP and the floor plate expresses Hedgehog, these molecules have no bearing on patterning the MG (Hudson et al. 2011). This is not entirely surprising, given that all MGNs are at the same DV position and thus cannot be easily patterned by DV morphogen gradients.

The A9.29 lineage, just posterior to the A9.30 lineage, gives rise to the fifth MGN, the A10.57 motor neuron, and to the decussating GABAergic interneurons that lie at the base of the tail and presumably play a role in the left-right alternation of muscle contraction that drive swimming behavior (Nishino et al. 2010; Nishitsuji et al. 2012). Although physically removed from the rest of the MGNs, these neurons are likely an integral component of the ascidian larval motor system and should be considered as belonging to the MG.

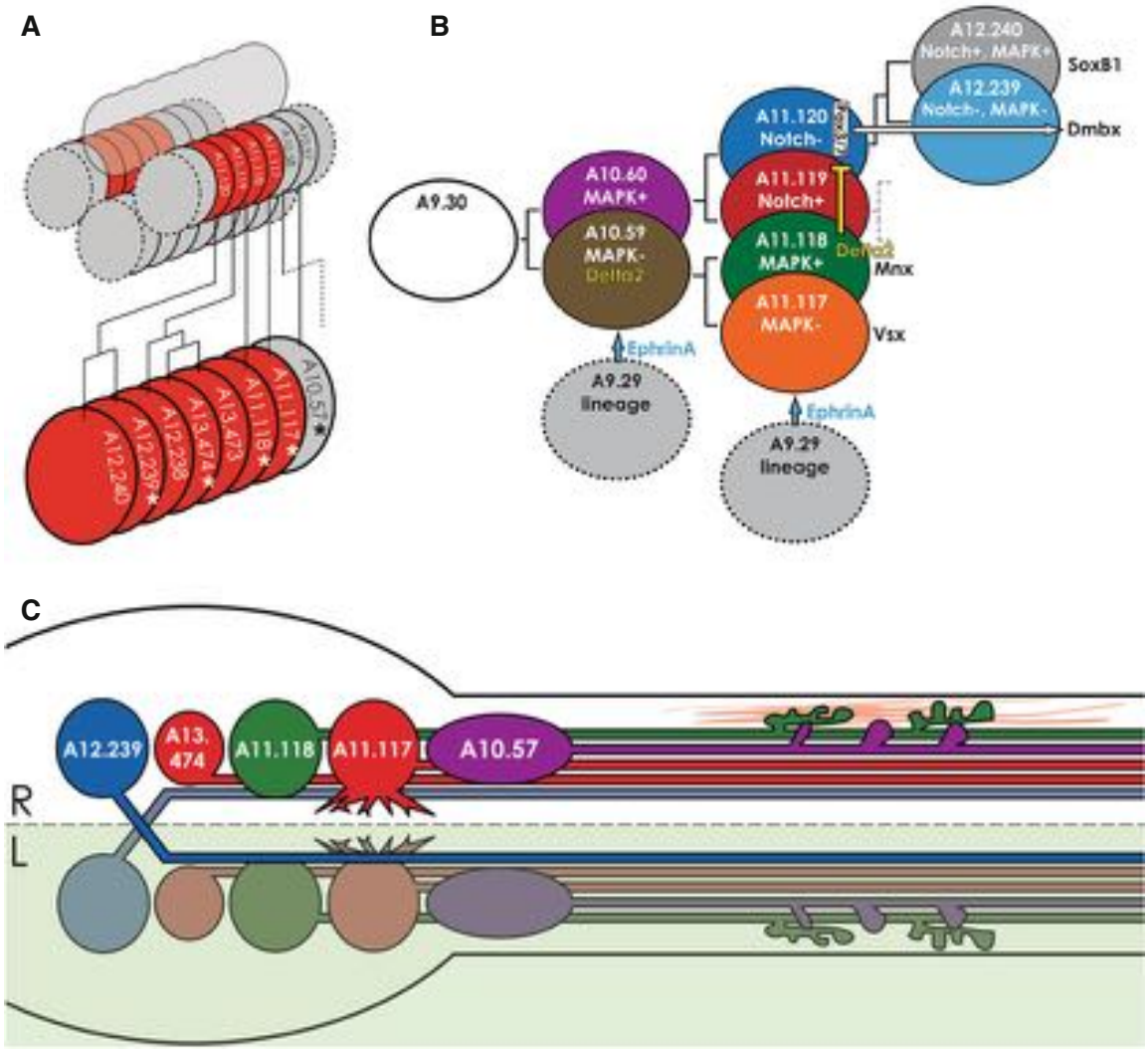


Fig. 4.15 The cell lineages, patterning, and neuronal subtypes of the *Ciona* motor ganglion. (**A**) The cell lineage of the motor ganglion. Cells in *red* are those descended from the A9.30 blastomeres of the neural plate, which gives rise to four of the five pairs of motor ganglion neurons (indicated by *asterisks*). Anterior is to the *left* (Diagram adapted from Stolfi and Levine (2011). Lineage information is from Cole and Meinertzhagen (2004)). (**B**) Summary diagram adapted from Stolfi et al. (2011) depicting the major cell signaling events that pattern the motor ganglion into specific progenitor and neuronal subtypes. Ephrin-A signals from the adjacent A9.29 lineage suppress MAPK(ERK) signaling in the posterior half of the A9.30 lineage and then in the A11.117 cell, which is specified as a *Vsx*+ interneuron. Delta2 ligand expressed in the posterior half of the lineage signals to A11.119 cell, limiting the expression of *Pax3/7* to the anterior-most cell of the lineage, which gives rise to the *Dmbx*+ interneuron (A12.239). The *Mnx*+/*Nk6*+ motor neuron (A11.118) is specified by later activation of ERK signaling, and neuronal differentiation of A12.239 is driven by stereotyped downregulation of ERK and Notch signaling in this cell by unknown means. Anterior is to the top. (**C**) Summary diagram from Stolfi and Levine (2011) of the different neuronal subtypes of the motor ganglion of *C. intestinalis*, depicting their most salient morphological features, such as decussating axons of A12.239, frondose motor end plates of A11.118, dendritic arborizations of A11.117, and elongated cell body and en passant motor end plates of A10.57. Anterior is to the left

Surprisingly, the Hox cluster genes appear to play limited functions in the larval CNS of *Ciona intestinalis*, with no obvious CNS patterning defects observed in loss-of-function experiments (Ikuta et al. 2010). Nine Hox genes have been found in *C. intestinalis* and *Oikopleura dioica*, with a nearly complete set of anterior and posterior Hox genes, but lacking a number of central Hox genes (Fig. 4.16) found in vertebrates and cephalochordates (Seo et al. 2004; Brown et al.

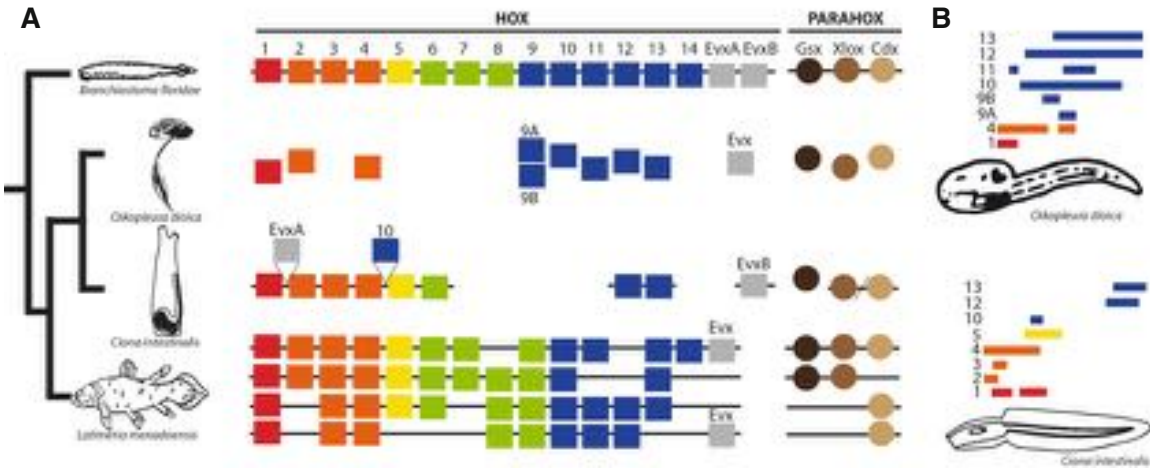


Fig. 4.16 Chordate Hox and ParaHox genes. (**A**) Evolutionary reconstruction of the Hox gene complex in chordates and cephalochordates. The complete Hox and ParaHox clusters are shown for the cephalochordate (*Branchiostoma floridae*). A disintegration in the order of the clusters and the lack of central Hox genes can be seen in the tunicates (*Oikopleura dioica* and *Ciona intestinalis*). The coelacanth (*Latimeria menadoensis*) Hox and ParaHox clusters are also shown (*dark colored circles*). Hox genes are color-coded by their relative position of expression: anterior genes, *red* and *orange boxes*; central genes, *yellow* and *green boxes*; and posterior genes, *blue boxes*. *Evx* duplications and transpositions are shown in *gray boxes*. *Dashed lines* indicate large gaps between Hox genes that remain on the same chromosome but have been dispersed. (**B**) Hox gene expression pattern relative to the anterior-posterior anatomical position of the larva of *O. dioica*. (**C**). Hox gene expression pattern relative to the anterior-posterior anatomical position of the larva of *C. intestinalis* (Figures are based on data from Ikuta et al. (2004), Seo et al. (2004), Brown et al. (2008), Amemiya et al. (2010), David and Mooi (2014), Mulley and Holland (2014))

2008; Amemiya et al. 2010; Ikuta et al. 2004; Ikuta 2011; David and Mooi 2014). The Hox complex itself is fragmented and eroded in the genomes of tunicates (Spagnuolo et al. 2003; Ikuta et al. 2004; Seo et al. 2004), with partial loss of collinearity of gene expression and cluster organization (Fig. 4.16). The same is observed for the ParaHox genes (Ferrier and Holland 2002). Those Hox cluster genes that remain are expressed in the nerve cord, notochord, and muscle of the larva (Ikuta et al. 2004; Seo et al. 2004) and undoubtedly play indispensable biological roles that have yet to be fully elucidated, but it is nonetheless stunning to see the rock stars of bilaterian embryonic patterning remain behind the scenes in the tunicates.

Neural Plate Borders, Placodes, and Neural Crest

From the lateral borders of the neural plate arise a handful of different cell types, mostly associated with the PNS. The most conspicuous of these are the melanin-containing pigment cells associated with the sensory vesicle (Fig. 4.17). In the basic larva of species covering diverse families, there are only two pigment cells. One is an otolith (also known as the statocyst or statocyte) that is required for geotaxis of the larva, presumably by acting as a weight that can activate associated mechanosensory cells, depending on the relative orientation of the larva in the water column (Tsuda et al. 2003; Jiang et al. 2005). The other is an ocellus, associated with a photoreceptor complex (Tsuda et al. 2003; Horie et al. 2008). In some species, the otolith or the ocellus may be missing. In other species (e.g., *Botryllus schlosseri* and other botryllids; Fig. 4.6C), both have been combined into a dual function pigment cell termed the "photolith" (Sorrentino et al. 2000).

In species with separate otolith and ocellus, both cell types are derived from left-right equivalent cells in the neural plate, and their differentiation into one or the other occurs mainly through relative positioning along the anterior-posterior axis after intercalation at the dorsal midline (Darras and Nishida 2001; Abitua et al. 2012). The cell that ends up more anterior invariably becomes the otolith, and the one that ends up

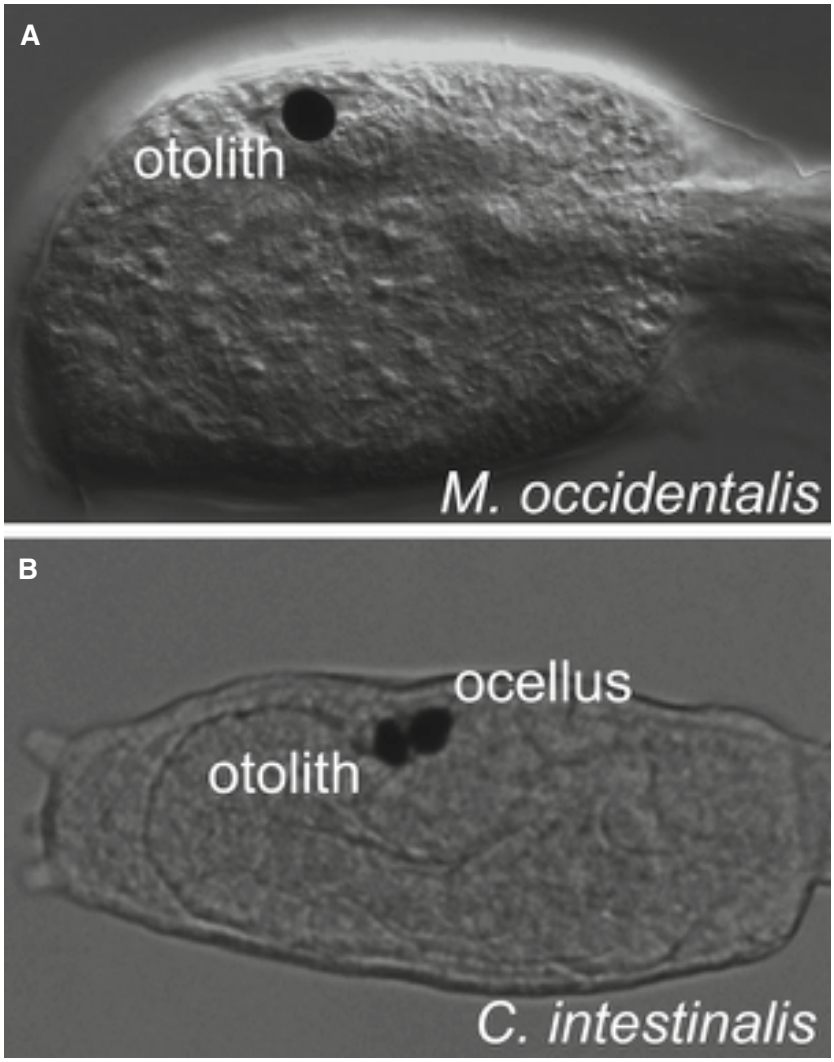


Fig. 4.17 The pigment cells of ascidian larvae. (**A**) Larva of *Molgula occidentalis*, showing a single melanocyte in the sensory vesicle, an otolith (also known as a statocyte or statocyst) associated with putative geotactic mechanoreceptory cells. (**B**) Larva of *Ciona intestinalis*, showing two melanocytes in the sensory vesicle: one otolith and one ocellus pigment cell (© Alberto Stolfi, 2015. All Rights Reserved)

more posterior is specified to become an ocellus. Both subsequently undergo an epithelial-to-mesenchymal transition as they delaminate from the neural tube epithelium and enter the sensory vesicle lumen.

Wada was the first to note that the specification of otolith/ocellus precursors from the neural plate borders closely resembles the development of neural crest-derived melanocytes in vertebrates (Wada et al. 1997; Wada 2001). Interestingly, neural plate border cells (NPBCs) express combinations of transcription factors that in vertebrates participate in the specification of neural crest cells. These include orthologs of *Pax3/7*, *Msx*, *Dlx*, *Zic*, *Snail*, *Id*, and others (Wada et al. 1997; Abitua et al. 2012). Molecularly, the expression of common neural plate border specifying genes like the ones listed above further supports the claims for homology. Later, neural plate border descendants express genes associated with more advanced neural crest developmental processes, including *Foxd* and *Mitf* orthologs (Abitua et al. 2012).

Now, are the NPBCs in fact neural crest cells? That is a trickier question to answer. Since neural crest cells were initially identified in vertebrates, they were defined by those criteria that described them in vertebrates. And there are more than a few such criteria, given the myriad embryonic origins, molecular signatures, and differentiated fates of all those cells collectively referred to as the "neural crest." As such, it is a tall order to

expect to find cells outside the vertebrates that strictly fit those multiple criteria.[8] Taking this into consideration, we venture that the answer is then ascidian NPBCs are homologous to neural crest cells, but are *not* neural crest cells.

We have seen that ascidian NPBCs share an embryological origin and certain regulatory signatures with vertebrate neural crest cells. Furthermore, like their craniate counterparts, the NPBCs give rise to melanocytes and sensory neurons. What are obviously missing are two things: (1) the capacity/propensity for long-range migration and (2) the ability to give rise to mesoderm-like differentiated cell types.

Along the descent of vertebrates, a rudimentary neural crest similar to the ascidian NPBCs must have come to express genes that conferred on them the entire suite of neural crest properties we have come to recognize. In tunicates, we find other cells that display some of these neural crest-like properties. Pigment cell precursors capable of migrating long distances were identified in the complex adultative larvae of *Ecteinascidia turbinata* (Jeffery et al. 2004). These were proposed as homologous to the A7.6-derived mesenchymal cells of *Ciona intestinalis* and *Halocynthia roretzi*, which do express some neural crest factors such as *Twist-related* genes (Jeffery et al. 2008). However, the embryological origin of the A7.6 lineage is not from the neural plate borders, being of clear endomesodermal origin. Interestingly, the forced expression of *Twist-related* genes in neural plate border cells of *C. intestinalis* was found to confer migratory properties to them, resulting in a long-range migration of pigment cell precursors, mimicking the behavior of *E. turbinata* pigment cell precursors (Abitua et al. 2012). Such an experiment may represent a very simplified reenactment of the evolutionary co-option that gave rise to *bona fide* vertebrate neural crest cells.

[8] If the forelimbs had been initially identified in birds and been strictly defined as feathery appendages that bear three digits, then one would be infallibly correct in claiming that only birds have forelimbs and that the homologous structures found in all non-avian vertebrates are *like* forelimbs, but *not* forelimbs.

Epidermis and Epidermal Sensory Neurons

The entire larva is covered by an epithelium composed of epidermal cells and related cell types that share a common clonal origin. Neurons comprising the PNS are embedded throughout the epidermis in both the head and tail regions, and other specialized structures arise at the border regions between the epidermis and neurogenic ectoderm. All these epidermis derivatives are descended from the four animal-pole octants of the eight-cell embryo. The major roles of the larval epidermis are to seal in the different tissues and organ primordia and to synthesize the tunic. The tunic, from which the tunicates derive their name, is made of cellulose. Tunicates are the only metazoans capable of synthesizing cellulose. This is made possible by epidermal expression of the cellulose synthase enzyme, thought to be derived from a gene that was horizontally transferred from an unidentified prokaryotic organism to the last common ancestor of all tunicates (Nakashima et al. 2004; Matthysse et al. 2004; Sasakura et al. 2005).

In the head, PNS neurons (including those derived from the neural plate borders) are dispersed along the anterior and dorsal regions, being absent from the ventral side of the epidermis (Imai and Meinertzhagen 2007b). In the tail, regularly interspaced epidermal cells at the dorsal and ventral midlines differentiate into caudal epidermal sensory neurons (CESNs) that form thin projections into the tunic (Fig. 4.18) and are thought to serve a mechanosensory role (Pasini et al. 2006; Imai and Meinertzhagen 2007b; Terakubo et al. 2010; Yokoyama et al. 2014).

Caudal CESNs are induced from neurogenic epidermis of the tail, which is induced at the dorsal and ventral midlines. The dorsal midline neurogenic epidermis is induced by FGF signaling, while the ventral midline neurogenic epidermis is induced by ADMP/BMP signaling (Pasini et al. 2006). Within these median stripes of neurogenic epidermis, putative mechanosensory neurons are specified through Delta-Notch lateral inhibitory signaling (Pasini et al. 2006), which controls the deployment of a core PNS neurogenic code consisting of the transcription factors Mytf,

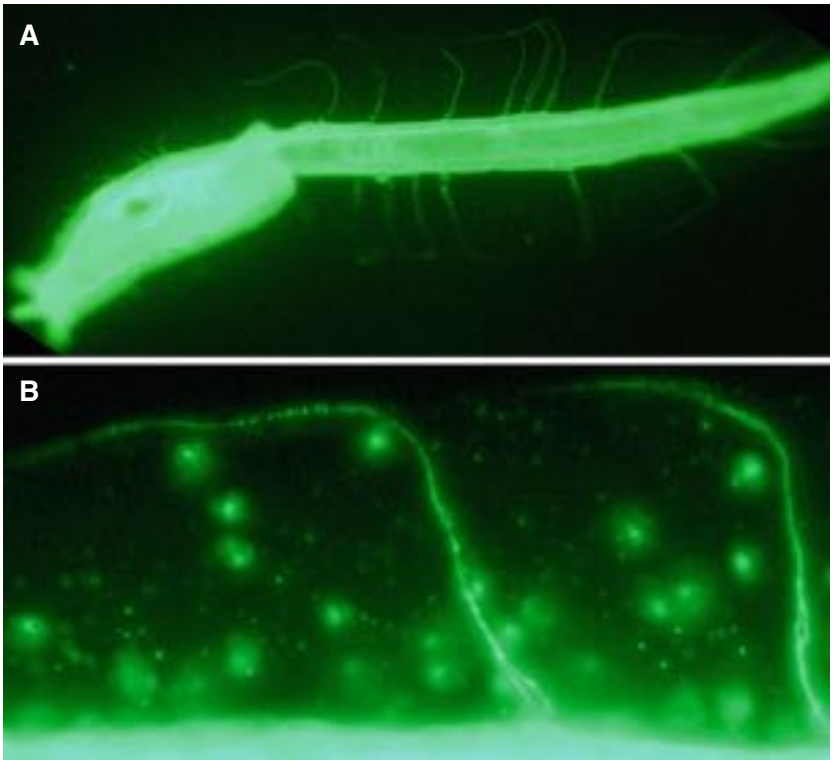


Fig. 4.18 Caudal epidermal sensory neurons of *Ciona*. (**A**) The thin ciliary projections of the caudal epidermal sensory neurons into the tunic fin revealed by beta-tubulin immunostaining. (**B**) Higher magnification view of tunic fin sensory projections (Figure is adapted from Pasini et al. (2006))

NeuroD-related, Pou4, and Atoh and the microRNA *miR-124* (Chen et al. 2011; Tang et al. 2013). Further AP patterning of these sensory neurons is achieved through antagonistic retinoic acid and FGF signals emanating from the anterior larval muscles and tailbud, respectively (Pasini et al. 2012).

Adult Siphon Primordia

Also arising from the head epidermis are the siphon primordia that give rise to the oral and aboral (or incurrent and excurrent) openings, or siphons, of the adult. The excurrent siphon is also known as the "atrial" siphon, due to its association with the peribranchial atrium or chamber. These are observed late in embryogenesis as rosettes of morphologically distinct epidermal cells. In phlebobranchs, one incurrent and two excurrent siphon primordia are specified, and initially the juveniles, or young adults, have three siphons each. Later in adult life, the two excurrent siphons fuse into a single siphon in a poorly studied process. In contrast, the excurrent siphon of stolidobranch ascidians arises from a single primordium in the larva or young juvenile.

While part of the incurrent siphon primordium is determined in a lineage-dependent manner from cells forming the neuropore[9] (Veeman et al. 2010), the excurrent siphon primordia are induced de novo from naïve epidermis by retinoic acid-dependent expression of *Hox1* in combination with later induction by FGF/ERK signaling (Kourakis and Smith 2007; Sasakura et al. 2012).

Adhesive Organ

At the most anterior end of the head is the adhesive organ, which in the majority of ascidians (one notable exception being the Molgulidae) consists of three protruding clusters of putative sensory and adhesive-secreting cells. These cell clusters are commonly referred to as "palps." The palps are remarkably elaborated in phlebobranch ascidians that undergo adultation (discussed below), perhaps because their larger, heavier larvae need to be more securely fastened to the substrate.

The adhesive organ primordium arises from the anterior borders of the neural plate and is

[9] The opening formed by the incomplete closure of the neural tube

specified in part by Foxc (Wagner and Levine 2012). Later adhesive organ development appears to require FGF/ERK signaling and an intricate interplay between the transcription factors Sp8, Emx, and Islet to specify the different cell types composing the palps and to regulate their morphogenesis (Wagner et al. 2014).

Development of the Mesoderm

Larval Muscles

The ascidian larva is specialized for dispersal, this being in fact its only role in the life cycle. Indispensable to this function are the larval muscles, arranged as bands of striated, mononucleated cells on either side of the tail. These muscle cells are divided into primary and secondary muscle lineages, according to their mode of specification. In *Ciona intestinalis*, there are 18 muscle cells per side and 21 cells per side in the slightly larger *Halocynthia roretzi* embryo. This difference in muscle cell number is entirely due to variation in the secondary lineage, while the primary lineage is invariant between the two species. It does not appear that these numbers deviate substantially among the larvae of the various solitary species. However, in those species showing extreme adultation and caudalization (explained later), larval muscles are greatly elaborated, both in terms of number of rows of cells and total numbers of cells (Cavey and Cloney 1976; Cavey 1982).

Primary muscle cells are autonomously specified, through inheritance of posterior/vegetal mitochondria-rich cytoplasm (Conklin's famous orange-yellow myogenic cytoplasm or "myoplasm"), shown by Whittaker to be sufficient and necessary for larval muscle formation (Whittaker 1973, 1982). The critical molecular component of the myoplasm was identified as maternally deposited *Macho1* mRNA, which is localized to the cER and translated into Macho1 protein in the primary larval muscle precursors (Nishida and Sawada 2001). Macho1 protein in turn activates its targets, among them the *Tbx6-related* family of genes (Yagi et al. 2005). Tbx6-related proteins then activate the myogenic regulatory factor gene *Mrf*, also known as *Myod* (Meedel et al. 1997, 2002, 2007; Imai et al. 2006). Mrf, Tbx6, and other transcription factors then cooperate to activate the expression of a battery of "differentiation" genes such as myosins, muscle actins, troponins, tropomyosins, etc. (Johnson et al. 2004, 2005; Brown et al. 2007; Izzi et al. 2013).

In contrast, secondary muscle cells are induced by extracellular signals from A- and b-line progenitors that do not inherit any maternal *Macho1*. Cell-cell signaling results in activation of *Tbx6-r* and *Mrf* independent of Macho1 (Kim and Nishida 2001; Hudson et al. 2007; Tokuoka et al. 2007). However, the exact identity of the signaling pathways used for the same inductive event differs between *Ciona intestinalis* and *Halocynthia roretzi*, representing another example of developmental system drift in ascidian evolution (Hudson et al. 2007; Tokuoka et al. 2007; Hudson and Yasuo 2008).

The variation in the *number* of secondary muscle cells being specified between *Ciona* and *Halocynthia* appears to be due to slight differences in cell positioning. In *C. intestinalis*, muscle-inducing cells are in contact with only the b7.9 blastomere, whereas in *H. roretzi* contact is made with both b7.9 and b7.10, resulting in induction of extra muscle progenitors (Kim and Nishida 2001; Hudson et al. 2007; Tokuoka et al. 2007).

Cardiopharyngeal Mesoderm

The term "cardiopharyngeal mesoderm" refers to multipotent progenitors that give rise to heart precursors and muscles of the peribranchial chamber and excurrent siphon of the adult (collectively referred to as "pharyngeal muscles"). In the embryo, four cardiopharyngeal progenitors (CPPs), two on either side of the embryo, are specified from the B7.5/B7.5 pair of blastomeres, which also give rise to anterior larval muscle cells (Fig. 4.19).

Investigations into marginal zone specification have largely ignored the B7.5 pair of blastomeres, which are situated at the posterior end of the marginal zone. Embryologically, the B7.5 cells are a confluence of vegetal and posterior endoderm and mesoderm, and this "hybrid" nature holds also true at the molecular level. In *Ciona intestinalis*, the B7.5 cells are delineated

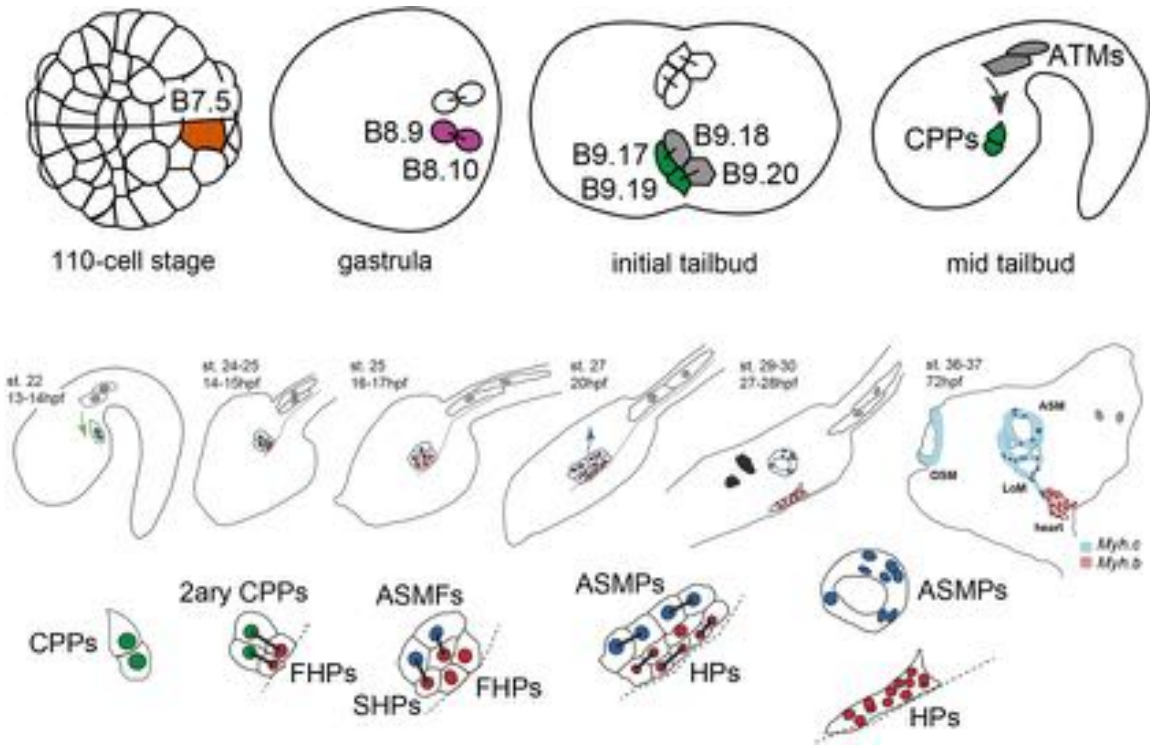


Fig. 4.19 The B7.5 lineage and cardiopharyngeal mesoderm. Diagram of the cell division and morphogenetic events of the B7.5 lineage, which gives rise to the cardiopharyngeal progenitor cells (*CPPs*) that will generate the heart and atrial siphon muscles (*ASM*) of the juvenile/adult. Refer to text for details. *ATMs* anterior tail muscles, *FHPs* first heart precursors, *SHPs* second heart precursors, *ASMFs* atrial siphon muscle founder cells, *ASMPs* atrial siphon muscle precursors, *HPs* heart precursors, *OSM* oral siphon muscles, *ASM* atrial siphon muscles, *LoM* longitudinal body wall muscles, *Myh.c* Myosin heavy chain.c (also known as *MHC3*), *Myh.b* Myosin heavy chain.b (also known as *MHC2*) (Figure is adapted from Wang et al. (2013))

by expression of the *Mesogenin/MesP* homolog *Mesp*, which is exclusively expressed in these cells and is necessary for the subsequent ontogenesis of lineage and its derivatives including the adult heart and pharyngeal muscles (Davidson et al. 2005; Satou et al. 2004). In *Ciona intestinalis*, *Mesp* is activated by Tbx6.b and Lhx3/4, zygotically expressed transcription factors under the direct regulatory control of beta-catenin and Macho1 maternal determinants, respectively. The expression territories of Tbx6.b (posterior) and Lhx3/4 (vegetal) overlap precisely in the B7.5, resulting in synergistic activation of *Mesp* in these cells alone (Christiaen et al. 2009). A Pem1-dependent timing mechanism has been proposed to help coordinate the precise overlap in time of these transcriptional cascades in the B7.5 (Tolkin and Christiaen 2012).

Experiments carried out in *Ciona intestinalis* and later extended to other ascidian species revealed that the CPPs are specified from the B7.5 lineage by FGF signaling (Davidson et al. 2006; Cooley et al. 2011). FGF-dependent activation of the ERK pathway and its effector, the transcription factor Ets.b, results in upregulation of a core of cardiac regulatory network genes and cell migration effector genes (Davidson and Levine 2003; Davidson et al. 2006; Beh et al. 2007; Christiaen et al. 2008). In contrast, their sister cells do not activate the ERK pathway and the cardiopharyngeal program and are instead specified as anterior primary larval muscle cells, as they inherit some myoplasm.

The CPPs detach from their sister cells and migrate anteriorly into the head (Hirano and Nishida 1997). In *Ciona intestinalis*, they migrate

along a ventral path and are thus called trunk ventral cells (TVCs), but in other species such as *Molgula* spp., these cells undertake a more lateral migration (Stolfi et al. 2014).

In the head, the CPPs undergo two rounds of asymmetric cell division to give rise to ventral/medial heart progenitors and dorsal/lateral pharyngeal muscle progenitors. The pharyngeal muscle progenitors are specified by a Tbx1/10-Ebf cascade (Stolfi et al. 2010; Wang et al. 2013; Razy-Krajka et al. 2014), which is antagonized by Nk4 in the heart precursors. The heart progenitors remain quiescent in the larva until needed for heart organogenesis during metamorphosis. On the other hand, the adult muscle progenitors migrate to dorsal regions and surround the excurrent siphon primordia while the larva is still swimming (Fig. 4.19).

During metamorphosis, cardiac and pharyngeal muscles differentiate, each expressing a unique suite of muscle structural genes (Stolfi et al. 2010; Razy-Krajka et al. 2014). The pharyngeal muscle progenitors will further migrate and proliferate to elaborate the musculatures of the excurrent siphon and the peribranchial chamber, which comprises essentially the entire body wall of the adult (Hirano and Nishida 1997; Razy-Krajka et al. 2014). Given the striking parallels in ontogeny and clonal topology between the cardiopharyngeal mesoderm in ascidians and vertebrates, it has been proposed that ascidian pharyngeal muscles are homologous to certain craniofacial muscles in vertebrates (Stolfi et al. 2010; Tzahor and Evans 2011; Tolkin and Christiaen 2012).

Notochord Formation

The notochord, one of the defining chordate traits, is the most salient feature of the ascidian larva and serves primarily as a hydrostatic skeleton required for the biomechanics of swimming (Jiang and Smith 2007). It is a single column of 40 cells, which are vacuolated late in larval development to form a hollow tube. Embryologically, the notochord has two origins (Fig. 4.20). The 32 primary notochord cells are derived from the A-line, specifically the A7.3 and A7.7 blastomeres. The remaining eight secondary notochord cells, occupying the posterior tip of the notochord, are derived from the B8.6 blastomere.

The primary notochord precursors are induced by an FGF signal and activate transcription of *Brachyury* (*Bra*). *Bra* is sufficient and necessary for notochord fate in *Ciona* and *Halocynthia* (Yasuo and Satoh 1998; Takahashi et al. 1999). Later, the secondary notochord precursors are induced by Delta-Notch signaling to also activate *Bra* (Hudson and Yasuo 2006). Upstream factors also regulating *Bra* activation in both lineages include Foxa and Foxd, while Zic-related appears to be required for *Bra* only in the primary notochord precursors (Imai et al. 2002a, b, 2006; Wada and Saiga 2002; Yagi et al. 2004; Kumano et al. 2006).

At the moment of gastrulation, notochord precursors are fate restricted and begin to ingress, prior to dividing twice to give rise to the final number of notochord cells. The cells form a notochord plate, which undergoes convergent extension, contributing to the elongation of the tail (reviewed in Jiang and Smith 2007). Final differentiation of the notochord involves the deposition of extracellular luminal matrix by each notochord cell. These lumens then fuse with each other to generate a single tube extending along the length of tail (Dong et al. 2009).

Downstream of Bra, approximately 450 genes have been identified as being upregulated in the notochord (Hotta et al. 1999; Takahashi et al. 1999). Of these, some are direct targets of Bra, with earlier-activated targets containing more Bra binding sites in upstream cis-regulatory sequences than targets activated later, which typically contain only one binding site (Katikala et al. 2013). There are also indirect targets of Bra, mediated by transcription factors that are direct Bra targets (Katikala et al. 2013). All in all, the notochord genes constitute a diverse group of proteins involved in several steps of notochord morphogenesis and differentiation.

Mesenchyme

With the exception of the anterior portion of the notochord, mesodermal cells in the head of the larva are undifferentiated and have been collectively referred to as "mesenchyme" due to their

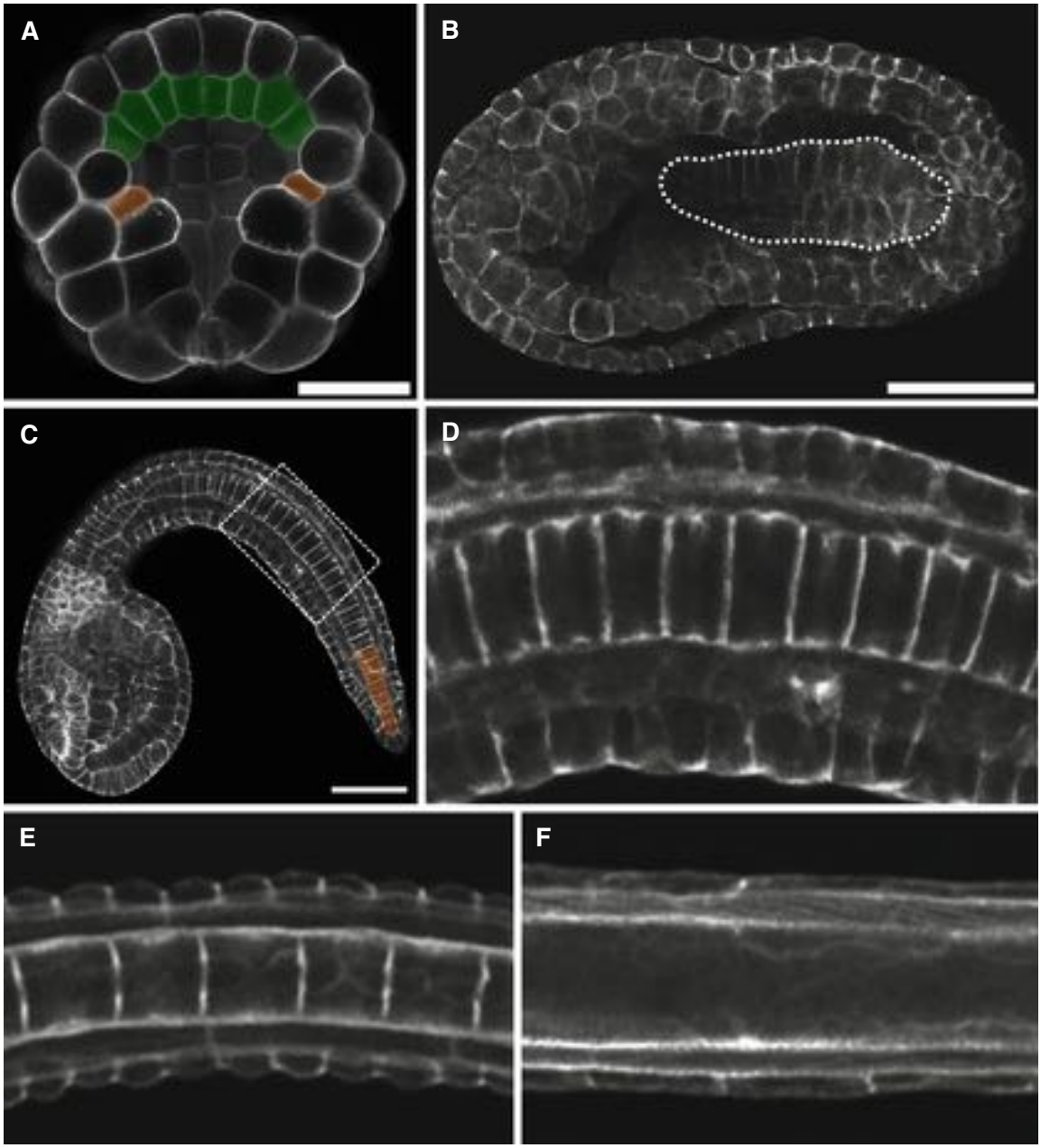


Fig. 4.20 The ascidian notochord. (**A**) 110-cell stage *Ciona intestinalis* embryo. The primary notochord precursors are false colored in *green*; the secondary notochord precursors are colored in *orange*. (**B**) Early tailbud stage embryo, with the cluster of intercalating notochord cells outlined by the *dotted line*. (**C**) Notochord in a mid-tailbud stage embryo, showing a single row of cells in the "stack of coins" configuration. Secondary notochord lineage colored in *orange*. (**D**) Magnified view of boxed area in (**C**). (**E**) Notochord cells in late tailbud stage embryo, already elongated and undergoing vacuolization. (**F**) Vacuolization of notochord cells is complete in the larva, with the unified lumens resulting in one long hollow tube running the entire length of the notochord. Scale bars in all panels equal 50 μm (© Alberto Stolfi, 2015. All Rights Reserved)

loose, seemingly disorganized distribution. These cells undergo an epithelial-to-mesenchymal transition after gastrulation and engage in proliferation and migration to varying degrees. Among these, there is usually a strong distinction made between the B7.5-derived cardiopharyngeal progenitors and mesenchyme derived from the A7.6, B7.7, and B8.5 pairs of blastomeres (Tokuoka et al. 2004).

The most anterior mesodermal lineage, the A7.6, has been reported to give rise to diverse

adult tissues including hematopoietic (blood) cells, gill slit lining, part of the stomach and peribranchial chamber, and incurrent (oral) siphon muscles (Hirano and Nishida 1997; Tokuoka et al. 2005). There were discrepancies between A7.6 tracing in different species, and new tools like photoconvertible intracellular fluorescent proteins may be needed to ascertain whether these represent true species-specific differences. The remaining mesenchymal lineages, B7.7 and B8.5, have been reported in *Ciona* as giving rise to blood cells as well as diverse cells that come to populate the adult tunic, whereas in *Halocynthia roretzi* they are described as giving rise to tunic cells only (Hirano and Nishida 1997; Tokuoka et al. 2005).

FGF signaling and the basic helix-loop-helix (bHLH) factor Twist-related were shown to be required for specification of mesenchyme (Imai et al. 2002a, 2003; Tokuoka et al. 2005), but little else is known about the developmental events and processes involved in specification and differentiation of A7.6, B7.7, and B8.5 mesenchymal derivatives of the adult.

Development of the Endoderm

Endoderm specification occurs immediately downstream of vegetally localized, stabilized maternal Beta-catenin, in part by activation of Lhx3/4 (Imai et al. 2000; Satou et al. 2001). In the *Ciona intestinalis* larva, there are approx. 500 endodermal cells, the majority of these residing in the head while a minority comprises the endodermal strand, a single column of cells that runs underneath the notochord to the posterior tip of the animal. The endoderm is largely undifferentiated upon hatching, but undergoes a process of differentiation while the larva is still swimming, in preparation for settlement and metamorphosis (Nakazawa et al. 2013).

Cell tracing experiments in *Halocynthia roretzi* revealed that the clonal boundaries of these cells in the larva were invariant and could be traced to blastomeres in the pre-gastrula embryo (Hirano and Nishida 2000). Furthermore, differentiated adult organs could be traced to specific regions of the endoderm fate map in the larva, indicating that the "anlagen" of these organs are laid out in the larva and are not significantly rearranged during metamorphosis. However, there was variation among individuals in the contributions of each blastomere to the final differentiated organs, suggesting that the endoderm is patterned at the larval stage in a position-dependent manner by extrinsic cues, and not according to deterministic, lineage-dependent processes. Late in larval development, the digestive tract rudiment can be seen to undergo tubular morphogenesis (Nakazawa et al. 2013), while in those species that show adultation, these and other organs are fully formed at the moment of larval hatching.

Primordial Germ Cells

The highly asymmetric divisions of the posterior vegetal end of the embryo eventually give rise to the B7.6/B7.6 pair of blastomeres, the smallest and most posterior cells in the pre-gastrula embryo. They are kept transcriptionally silent through inheritance of *Pem1* mRNAs and protein. They also inherit other postplasm components including the homolog of the major metazoan germ cell marker Vasa (Fig. 4.21; Takamura et al. 2002; Brown and Swalla 2007; Brown et al. 2009; Shirae-Kurabayashi et al. 2006).

After gastrulation, these cells end up in the ventroposterior portion of the larval tail. Each B7.6 cell divides and gives rise to two daughter cells: B8.11 and B8.12. *Vasa* mRNAs and protein are asymmetrically inherited by the more posterior B8.12 cell, whose descendants are eventually incorporated into the adult gonad and give rise to the animal's germ line (Takamura et al. 2002; Shirae-Kurabayashi et al. 2006). Thus, the B8.12 cells are the primordial germ cells (PGCs). Curiously, *Pem1* is asymmetrically inherited by B8.11, suggesting the PGCs are released from global transcriptional repression around this stage.

It was found that germ cells could be secondarily induced in adults generated from larvae in which the tail, and therefore the B8.12-derived primary germ cells, had been severed. This suggests that there may be mechanisms that allow for induction of secondary germ cells in case the primary germ line is somehow lost (Takamura et al. 2002).

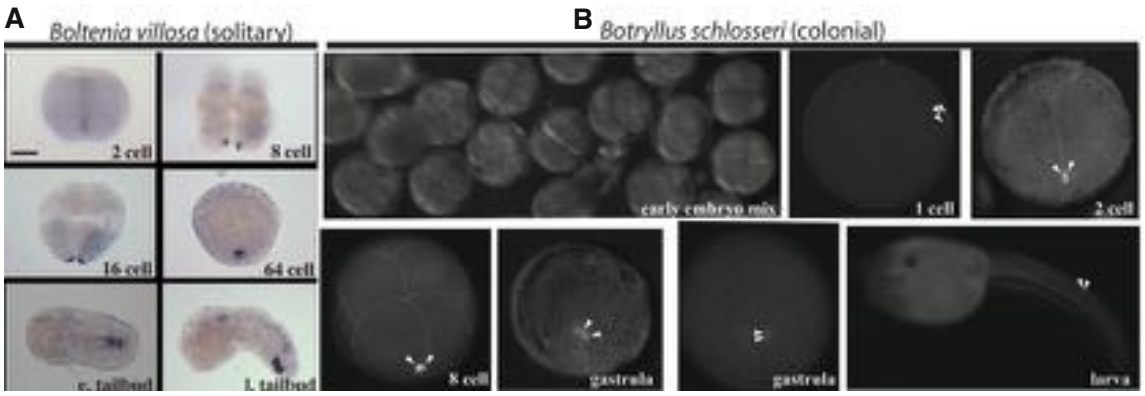


Fig. 4.21 The primordial germ cells (*PGCs*) of solitary and colonial styelid ascidians. RNA-dependent helicase *Vasa* transcripts are shown as early PGC marker. (**A**) *Vasa* transcripts in blue for the solitary *Boltenia villosa*. (**B**) *Vasa* transcripts marked by *white arrowheads* in the closely related colonial *Botrylloides violaceus*. Note that in both cases *Vasa-positive* cells are localized to a few posterior cells of the early embryos. Developmental stages are noted below each panel. Scale bar in (**A**) equals 50 μm (From Brown et al. (2007, 2009))

Taken as a whole, ascidian embryos are excellent organisms in which to observe cellular processes in vivo and investigate the function of genes involved in such processes. The gene regulatory networks of the ascidian embryo (Fig. 4.5) have been considerably annotated, but these networks so far have mostly concerned the connections among the transcription factors and signaling pathways. More recently, our knowledge of these networks has started to extend to their interface with the realm of effector genes – those that do not directly regulate transcription but rather interact with other proteins to carry out mechanical or enzymatic functions. The best-studied effector genes are those that are responsible for the unique properties of differentiated cells, also known as terminal differentiation genes. More challenging genes to study are those that are involved in transient cellular properties and behaviors like polarity, motility, adhesion, contractility, and other components of morphogenesis. There have been some notable examples where the genes controlling these processes in the ascidian embryo were discovered. For instance, forward genetic screens for notochord morphogenesis defects in *Ciona* identified genes required for the convergent extension of this tissue (Jiang et al. 2005; Veeman et al. 2008). A later phase of notochord morphogenesis, namely, the formation of the notochord tube, has been studied using a combination of sophisticated imaging of fluorescently tagged proteins and targeted disruption of candidate genes (Dong et al. 2009, 2011; Denker et al. 2013; Deng et al. 2013). Finally, cell-specific transcriptome profiling can reveal those genes upregulated during morphogenesis, which was done for the migrating CPPs and revealed several candidate migration genes (Christiaen et al. 2008). Although the tissues and cell types investigated thus far are not many, the future for research on cellular morphogenesis in ascidian embryos holds great promise.

Early Development of Other Tunicates

Recently acquired evidence suggests that the thaliaceans and larvaceans, once thought to be distinct from ascidians, appear to group within the paraphyletic Ascidiacea. Phylogenetic trees strongly support the placement of a monophyletic Thaliacea squarely within the phlebobranch ascidians, i.e., Enterogona (Tsagkogeorga et al. 2009). Phylogenetic support for a revised classification of the larvaceans as the sister group to the stolidobranch ascidians is not as strong, but embryological data suggest they may be highly derived ascidians (Stach et al 2008; Fujii et al. 2008). Here, we discuss what is known about developmental processes in these less-frequently studied groups.

Larvaceans: Reduction and Neoteny

In the larvaceans, as the name implies, there is no morphological contrast between the embryo/larva and the adult. Larvaceans are planktonic tunicates that retain a functional notochord and paraxial musculature throughout their entire adult lives. These constitute a true postanal tail that has been adapted to beat vigorously and generate water currents through the "house," an intricately sculpted cellulosic filtration apparatus (Fig. 4.22). This house is shaped through the patterning of the head epidermis into a complex landscape of specialized cells that secrete specific subsets of oikosins, the major protein component of the house (Fig. 4.22; Spada et al. 2001; Thompson et al. 2001; Hosp et al. 2012). The house is many times the size of the actual animal and utilizes a series of canals and filters to trap

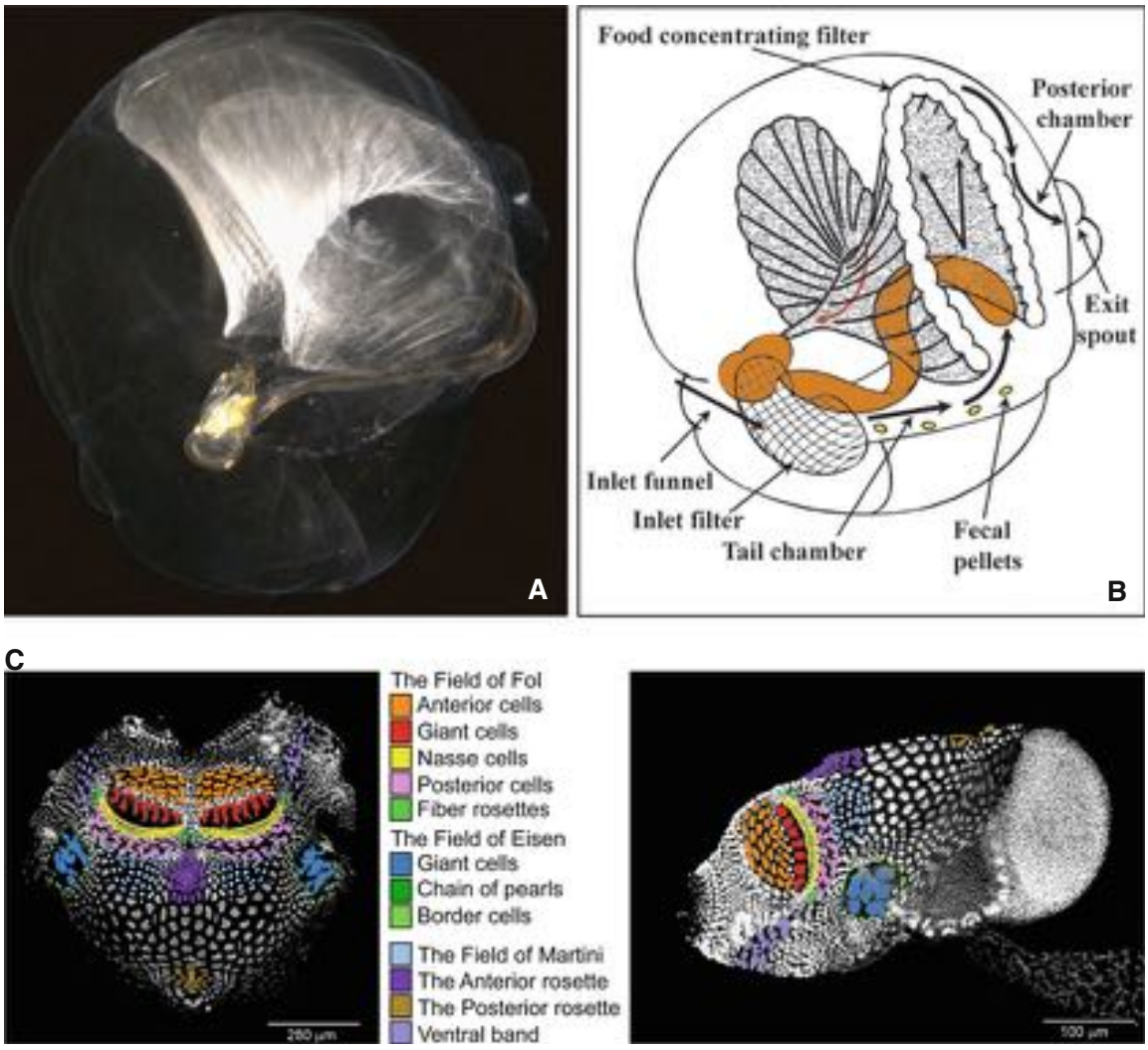


Fig. 4.22 The larvacean house. (**A**) Lateral view of an *Oikopleura dioica* individual and surrounding house. Anterior is to the *top right*. (**B**) Illustration of the animal and house depicted in (**A**). Animal is colored in *orange*. *Black arrows* indicate the direction of water currents that are swept into the house by beating of the animal's tail and through the filter apparatus of the house. *Red arrow* indicates movement of food particles toward the animal's mouth. (**C**) Oikoplastic epidermis of *O. dioica* stained with Hoechst (*left*) and To-Pro3 (*right*), revealing cell nuclei. Different fields of cells have had their nuclei false colored to highlight the different fields that shape the house through synthesis and secretion of various proteins, including oikosins (Panels (**A**, **B**) were adapted from Bouquet et al. (2009); (panel **C**) was adapted from Hosp et al. (2012))

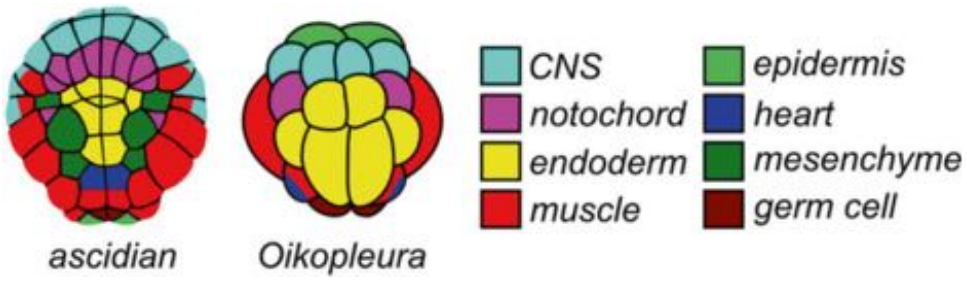


Fig. 4.23 The ascidian and larvacean fate maps. Comparison between the typical solitary ascidian and *Oikopleura dioica* (larvacean) embryonic fate maps. The ascidian fate map is largely based on the work of Nishida (1987) and the *Oikopleura* map based primarily on Stach et al. (2008) and Fujii et al. (2008)

particle food. The animal can eject from its house and does so with some regularity, some five to ten times per day, synthesizing a new one in just 10 minutes (Alldredge 1977).

Occasionally imagined as representing the common ancestor of all tunicates, these remarkable creatures likely evolved from an ascidian-like ancestor instead (Stach et al. 2008). Secondarily evolved heterochronies superimposed on the biphasic development of ascidians would have resulted in neoteny. This view is supported by recent tunicate phylogenies that place larvaceans within the ascidian tree, although these analyses are hampered by long-branch attraction problems (Tsagkogeorga et al. 2009). On the other hand, other analyses have placed the larvaceans as the sister group to all other tunicates (Delsuc et al. 2006, 2008).

The larvacean embryo and developmental times are even more reduced than those of solitary ascidians. The complete life cycle of the species *Oikopleura dioica* is only 5 days at 20 °C. Gastrulation occurs at the 32-cell stage, as opposed to gastrulation in ascidians which happens at the 110-cell stage (Fujii et al 2008). The embryo is also mostly fate restricted at this stage, and this map is very similar to the ascidian fate map, in spite of the fewer cells (Nishida 2008; Stach et al. 2008). Each cell lineage develops in a way that is very reminiscent of the homolog in ascidian embryos, albeit clearly reduced in cell numbers (Fig. 4.23). For instance, there are 20 notochord cells at the end of embryogenesis, as opposed to 40 in most solitary ascidians (Nishino et al. 2001).

Strikingly, only a fraction of the *Ciona* notochord toolkit genes are expressed in the *Oikopleura dioica* notochord (13/50), and half of them are completely absent from the *O. dioica* genome (Kugler et al. 2011). How larvaceans cope with a reduced number of genes to build a notochord is not yet clear. Since the larvacean tail is used to draw the water current through the animal's house, as opposed to a larval dispersal mechanism, it is highly elaborated during the larval and adult stages, as notochord cells proliferate to generate over 100 cells arranged into four longitudinal columns (Søviknes and Glover 2008). It is possible that this difference in gene expression underlies the adaptation of the larvacean notochord for this purpose.

Another clear example of cell count reduction during embryogenesis is seen in the neural plate, which is comprised of just two columns of four cells each when neurulation begins (Fujii et al. 2008), as opposed to the eight columns and six cells of the *Ciona* and *Halocynthia* neural plates. Additionally, larvacean tail muscles are made of only a single row of ten cells on either side of the notochord (Nishino et al. 2000), in contrast to the 18 or 21 cells per side seen in typical ascidian larvae.

Given these striking parallels with the slightly more complex embryos of solitary ascidians, it seems reasonable to conclude that larvaceans may be descended from a sessile, solitary ascidian-like ancestor, but have specialized in part through reduction in cell number, basic toolkit gene number, and acceleration of development.

Embryonic Development of Thaliaceans

Although the thaliaceans are pelagic and free swimming in their adult phase, they are very distinct from the larvaceans and represent an alternative evolutionary trajectory from an ascidian-like ancestor. They resemble pelagic ascidians, filter-feeding as they float in the water column. Salps use muscular contractions of the pharyngeal cavity to generate locomotion by jet propulsion. They constantly produce and consume mucous nets that trap particles passing through the pharyngeal cavity, which allows for simultaneous filter feeding and locomotion. As a result, they can filter a prodigious amount of water. Doliolids also move about through jet propulsion, but their feeding and swimming are separate functions, as they use cilia to draw particles through the mucous feeding net, being similar in this manner to ascidians. Pyrosome colonies move through the combined flow of each minuscule zooid, which is generated by cilia as well (reviewed in Alldredge and Madin 1982).

Pyrosomes and salps lack a caudate larval stage and both have direct developing embryos (Julin 1912; Berrill 1950a, b; Sutton 1960). Of the three main orders in Thaliacea, only certain species of the doliolids have a tadpole-like larva (Godeaux 1955), which has been used as a reminder of their ascidian pedigree and to support evidence for phylogenies that place them as the sister group to the salps + pyrosomes (Tsagkogeorga et al. 2009). However, a more recent and complete phylogeny that includes a higher number of thaliacean species supports a salp + doliolid clade with the pyrosomes in a basal position (Govindarajan et al. 2010), suggesting independent losses of the tadpole stage in pyrosomes and salps (Fig. 4.1).

A complete review of thaliacean development was last compiled by Bone (1998). Pyrosome embryogenesis is direct, giving rise to a peculiar embryo termed "cyathozooid" (Huxley 1851). The cyathozooid differentiates on top of a yolk droplet and sprouts a stolon under the droplet, which forms four buds by constriction. These buds will develop into the four initial blastozooids (called tetrazooids in this specialized case) that develop around the cyathozooid, which quickly degenerates afterward.

Perhaps the most specialized developmental mode observed in all of the tunicates occurs in the salps. In early embryogenesis, invading follicle cells separate the blastomeres, which come back together again later in development (Todaro 1880; Sutton 1960). The function of this deliberate fragmentation of the early embryo has never been ascertained.

Doliolid embryonic development occurs from internally fertilized eggs and generates a pelagic chordate-like larva, which will eventually differentiate the typical barrel-shaped form of doliolid zooids in its head, while the tail regresses to form the oozooid of the asexually reproducing generation.

Relatively little else is known about thaliacean development, and much less is known about the underlying molecular mechanisms. All three orders are capable of asexual reproduction, and salps and doliolids have complex alternation of sexual and asexual generations (see below). We can only hope that major developmental studies in the thaliaceans will be revived quite soon, in time for the next round of reviews and book chapters.

LATE DEVELOPMENT

Ascidian embryonic development, as reviewed in the previous section, is highly conserved, being nearly indistinguishable between some distantly related species. In contrast, there is a greater diversity observed in later stages of development. The following section will focus on some of these differences in late larval development, such as heterochronic shifts, the distinct modes of asexual reproduction, and blastogenesis in the colonial ascidians. Therefore, while the previous section relied heavily on studies using *Ciona intestinalis* and *Halocynthia roretzi*, the following section will mostly focus on comparative work done across several species to provide a comprehensive overview of late larval, early adult, and blastogenic development in the Tunicata.

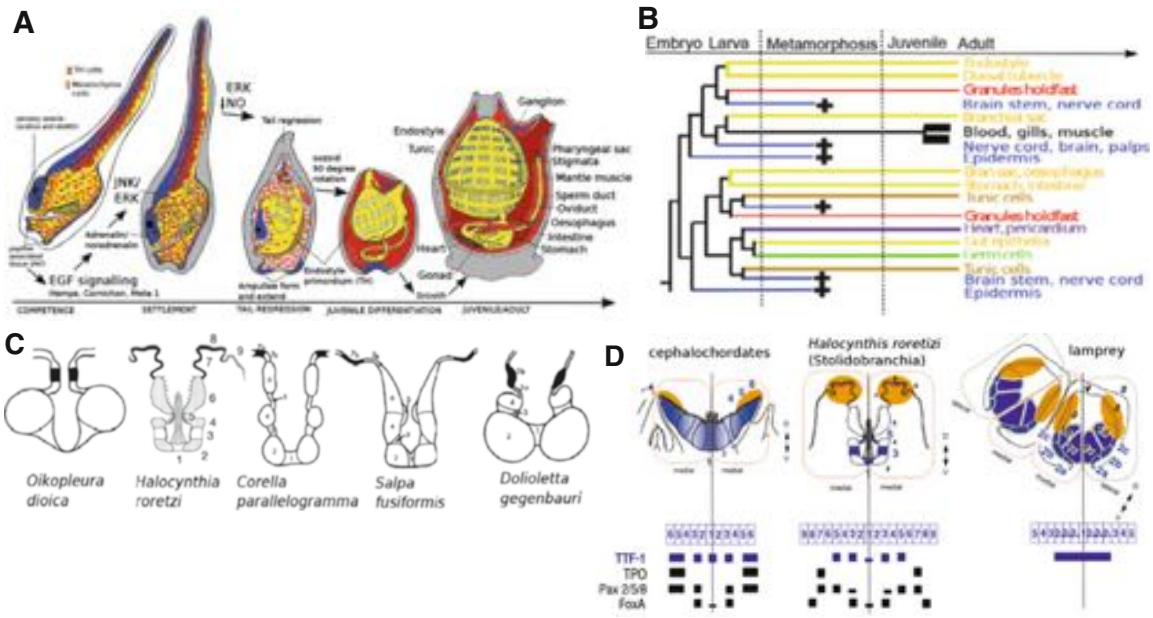


Fig. 4.24 Metamorphosis in ascidians. (**A**) Events of metamorphosis highlighting players and pathways involved (© Biodidac, 2015. All Rights Reserved). (**B**) Representation of embryonic cell lineages that contribute to the juvenile and adult; note that many cells undergo cell death (*black crosses*) and that mostly endoderm and mesoderm contribute to the adult (From Brown et al. (2012)). (**C**) Comparison of the endostyle of different tunicates shows resemblance in their organization and dorsolateral iodine-containing and peroxidase activity regions of the thyroid gland (in *black*) (Adapted from Fredriksson et al. (1988)). (**D**) Gene expression pattern supports homology between the endostyle of cephalochordates and ascidians with the endostyle of the lamprey (Adapted from Ogasawara et al. (1999, 2001), Hiruta et al. (2005), Kluge et al. (2005))

Late Larval Development

Developmental timing of embryogenesis and larval development differs between solitary and colonial ascidians. Solitary free-spawning ascidians develop rapidly: embryogenesis occurs within 8–48 h of fertilization (e.g., 15 h in *Ciona intestinalis*, 9 h in *Molgula occidentalis*), and hatched larva then swims freely for several hours or even days before settlement. In contrast, colonial species that brood have relatively longer periods of embryogenesis that can take anywhere from days to weeks (e.g., 5–6 days in *Botryllus schlosseri* or 4–6 weeks in *Botrylloides violaceus*), but present shorter larval periods that only last between several minutes to a couple of hours after they have been released (e.g., 1–2 h in *Botryllus schlosseri*). However, in both solitary and colonial ascidians, the larval period is nonfeeding and serves primarily for dispersion.

A few hours after hatching (or immediately after hatching, in the case of some colonial species), ascidian larvae acquire the ability (or "competency") to respond to settlement cues from the environment (Figs. 4.24 and 4.25). Competent larvae actively seek suitable locations and substrates on which to settle. The preferred cues will obviously vary according to the ecological niche of the species in question. For many solitary species, competent larvae seek protected and dark places, often settling on rocks or hard substrates with indirect sunlight. Biological cues, such as naturally occurring bacteria (Roberts et al. 2007), and physical cues such as trauma, crowding, or stress (Degnan et al. 1997; Davidson and Swalla 2002) have been used to artificially induce metamorphosis in different ascidian species.

Billie Swalla and collaborators found that immune genes are highly expressed at the early onset of metamorphosis in ascidians, suggesting

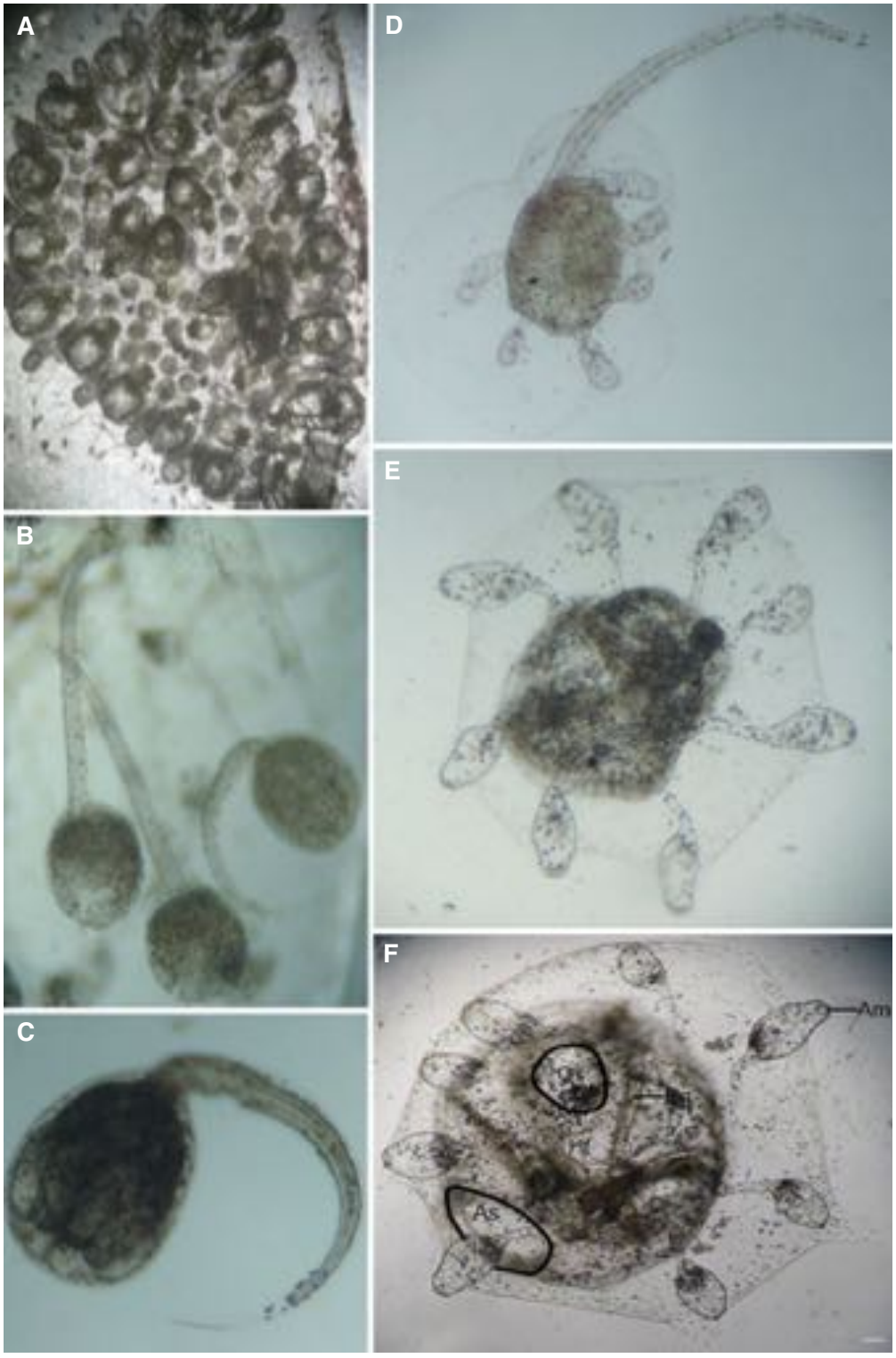


Fig. 4.25 Larval release and settlement (metamorphosis) of an unidentified *Botryllus* sp. from Santa Marta, Colombia. (**A**) Ventral view of the brooding colony hours before larval release; many round larvae can be seen in between the zooids. (**B**) Larvae right after hatching and release. (**C**) Larval head that shows the protruding ampullae (or papillae) in the anterior region of the head. (**D**) Attaching larva with extended ampullae; eye spot and tail still present. (**E**) Oozooid just before the opening of the siphons; almost regressed tail and eye spot can still be observed. (**F**) Completely differentiated oozooid; *Am* ampulla, *As* atrial siphon, *En* endostyle, *Ht* heart, *Os* oral siphon. Scale bar equals 100 µm (Courtesy of L. Restrepo (Universidad de los Andes))

that the activation of the innate immune system may occur at this stage. One intriguing explanation for this is that the innate immune system is deployed in sensing microbiota or bacterial biofilm as cues for settlement. Another possibility may be a direct involvement of immune cells in later metamorphic events including remodeling or phagocytosis of larval tissues (Davidson and Swalla 2002). One question that remains to be answered is whether the immune system response observed in these cases is specific to ascidian metamorphosis or whether it also plays a role in the metamorphic events of other tunicates or chordates.

Most solitary species' larvae have three adhesive papillae (palps) in the anterior end of the head that function in adhesion to the substrate and in responding to settlement cues. In response to environmental stimuli, the papilla-associated tissues (PATs) activate the expression of EGF signaling components (e.g., Hemps, Cornichon, Meta1) and thrombospondins. Thrombospondins are secreted extracellular proteins known to bind several ligands and are involved in many cellular processes, including tissue formation or repair (Eri et al. 1999; Davidson and Swalla 2001; Nakayama et al. 2001).

The activation and regulation of signaling pathways in PATs is followed by the release of noradrenaline/adrenaline (Kimura et al. 2003) and the activation of transduction pathways such as JNK and ERK in the larval CNS (Tarallo and Sordino 2004; Chambon et al. 2007). Further evidence supporting signaling from the PATs to the CNS comes from the disruption of sensory vesicle retraction (a key step in metamorphosis) in papilla-cut larvae of *Ciona intestinalis* (Nakayama-Ishimura et al. 2009). In summary, environmental stimuli are sensed by the PAT, which then signals to the nervous system to trigger the onset of metamorphosis.

Metamorphosis

Metamorphosis involves a radical transition in the life cycle of indirect-developing organisms. Larval body and behavior are remodeled into their final adult forms (Fig. 4.24), in order for the individual to occupy a different ecological niche. Tissue-remodeling processes during animal metamorphosis are known to occur mainly through the activation of programmed cell death in larval tissues and the differentiation of juvenile and adult tissues.

There is variation in the degree of change that results from metamorphosis among the different chordate taxa: it may be absent or minimal as in mammals or birds, subtle as in fish that undergo slight changes in fins and gut, more pronounced as in flatfish or cephalochordates that undergo rotation of parts of their bodies, or extreme as in frogs or tunicates that can lose entire larval structures and rapidly generate adult tissues and organs. However, this is not the same "catastrophic" metamorphosis of some animals such as echinoderms (cf. Chapter 1), ectoprocts (cf. Vol. 2, Chapter 11), or others, in which an entire adult body is practically generated de novo from a few cells, with little input from embryonic patterning. Even in tunicates, the majority of adult structures arise from primordia that have been specified, patterned, and primed for differentiation during the course of embryogenesis and larval development.

Because of the great diversity of metamorphic transformations observed in the chordates, these were long thought to have evolved independently. In the following, studies of metamorphosis are described for several tunicates including those on ascidian model species, focusing on how such studies have contributed to our understanding of the origins of metamorphosis in the chordates and deuterostomes in general.

Due to the dramatic transformation of the non-feeding larvae into the sessile feeding juveniles and adults of ascidians, these have been used primarily as experimental study models for metamorphosis in the tunicates. Few studies on metamorphosis have been carried out in larvaceans, which do not feature a strong contrast between the larval and adult forms, and, to our knowledge, no work has been done on metamorphosis in Thaliacea. While some species of doliolid thaliaceans present tailed larvae, all species of salps and pyrosomes have dispensed their larval

stage entirely and instead directly develop into an oozooid from the sexual cycle to reproduce asexually (Bone 1998).

Key Steps in Ascidian Metamorphosis

A rapid regression of the larval tail marks the onset of metamorphosis in the ascidians. We have already discussed the key events that precede metamorphosis, including the activation of a sensitive period that allows the larva to respond to external stimuli (i.e., competency), the secretion of adhesives by the palps to facilitate attachment to the substrate and signaling by the larval PAT and CNS (Fig. 4.24A). Upon settlement, rapid regression starting at the most posterior tip of the larval tail is mediated by a few distinct mechanisms. These include apoptosis of tunic cells, epidermis, notochord, tail muscle, and dorsal neural tube (Chambon et al. 2002), contractile morphogenetic events of either epidermal or muscle cells in the tail that generate the forces for resorption (Cloney 1982), or migration of cells of the tail (e.g., endodermal strand and PGCs) into the head, which has now become the entire body of the animal (Shirae-Kurabayashi et al. 2006; Nakazawa et al. 2013).

The relative modularity of these different steps in metamorphosis is demonstrated by the striking phenotypes of certain genetic mutants. In *Ciona intestinalis*, two mutants showing metamorphic defects have been studied: (1) *swimming juvenile* (*sj*), a transposon-generated cellulose synthase mutant strain, and (2) *tail regression failed* (*trf*), a naturally isolated mutant strain. Both mutants show that several key steps in metamorphosis can occur even in the absence of larval tail regression (Nakayama-Ishimura et al. 2009). These mutants clearly show that the developmental processes of metamorphosis are indeed decoupled from each other.

In *Ciona intestinalis*, tail regression is preceded by a decrease in endogenous nitric oxide (NO) signaling (Fig. 4.24A). While NO was found to delay metamorphosis, in part through inhibition of caspase-dependent apoptosis (Comes et al. 2007), it was also found to promote ERK signaling during the acquisition of competency (Bishop et al. 2001; Comes et al. 2007; Castellano et al. 2014). Although the same inhibitory effect of NO on metamorphosis was observed in *Boltenia villosa* and *Cnemidocarpa finmarkiensis*, the opposite was found in *Herdmania momus*, in which NO appears to promote metamorphosis (Ueda and Degnan 2013). These results point to complex roles for NO immediately prior to and after the onset of metamorphosis, which may vary from species to species.

Remodeling of the Larval Body

Metamorphosis proceeds with the remodeling of the larval body and differentiation of the juvenile tissues and organs for another 1 or 2 days (approx. 36 h in *Ciona intestinalis* or 24–36 h in *Botryllus schlosseri*) (Fig. 4.25). Some of the major events of remodeling in the larval head concern the regression of the papillae (extended during settlement), the release of the larval tunic, the formation of epidermal extensions termed ampullae, and a 90° rotation of the larval AP body axis so that the oral and atrial siphons of the juvenile come to lie opposite to the site of attachment to the substrate and sensory vesicle regression (Cloney 1982; Nakayama-Ishimura et al. 2009). Mesenchyme cells begin substantial migration within and across the epidermis of the differentiating juvenile, giving rise to their various derivatives including blood and tunic cells. Additional undifferentiated endoderm and mesoderm precursors develop into the organs and musculature of the adult.

In late embryonic and early larval development, apoptosis appears to occur in many mesenchymal and CNS progenitors (Tarallo and Sordino 2004), but these findings are in stark contrast to lineage tracing experiments that find invariant contributions of specific embryonic blastomeres to specific juvenile tissues and organs. Apoptosis is observed in mesenchymal cells that generally give rise to blood and muscle in the juvenile, although a role for programmed cell death in these progenitors is unclear (Fig. 4.24B). In the larval CNS, two waves of apoptosis have been reported, one pre-metamorphic anterior-posterior wave that progresses from the sensory vesicle in the head along

the nerve cord to the tip of the tail and a second metamorphic wave from posterior to anterior that follows regression (Tarallo and Sordino 2004).

In contrast to the extensive cell death observed in the nerve cord, the brain, and the motor ganglion, most of the anterior sensory vesicle and the neck region actually contribute to the adult CNS (Tarallo and Sordino 2004; Horie et al. 2011).

In summary, developmental processes of ascidian metamorphosis largely redeploy the cellular processes that are universal for any developing system, such as apoptosis, proliferation, migration, and differentiation. However, more work will be needed to understand exactly how they are regulated in the metamorphosing ascidian juvenile.

Thyroid Hormone Metabolism in Ascidian Metamorphosis

The series of events that occur during metamorphosis require regionalized responses to systemic signals. Therefore, one can hypothesize that a common and plesiomorphic endocrine mechanism evolved to regulate metamorphosis in all chordates. Triiodothyronine (T3) and its prohormone, thyroxine (T4), collectively referred to as thyroid hormones (THs), regulate basal metabolism in vertebrates and serve as the trigger of metamorphosis in amphibians and fish (Furlow and Neff 2006; Gomes et al. 2014). In these vertebrates, a peak in the level of T3 in the blood is associated with the onset of metamorphosis. Ever since the discovery that the thyroid gland, specifically the THs produced by its follicular cells, triggers metamorphosis in frogs (Gudernatsch 1912; Allen 1925), much research has been carried out to unravel the mechanism of TH action in other vertebrates (Holzer and Laudet 2013). However, the role of THs in the metamorphosis of invertebrates is not well understood.

Studies in ascidians and cephalochordates have revealed TH synthesis activity during metamorphosis, and T4 inhibitor treatments were found to suppress metamorphosis of competent larvae (D'Agati and Cammarata 2006). Furthermore, thyroid hormone receptor (TR) homologs have been identified both in *Ciona intestinalis* and the cephalochordate *Branchiostoma floridae*. Therefore, there is evidence to support the plesiomorphic nature of thyroid hormone metabolism in chordates. However, it was found that ascidian and cephalochordate TRs are mainly activated by TRIAC, a TH derivative that is also present in mammals but at much lower concentrations. Furthermore, these invertebrate chordate receptors cannot bind T3, the most active vertebrate thyroid hormone (Carosa et al. 1998; Paris et al. 2008). In contrast to the high sequence similarity between the DNA-binding domains of all chordate TRs, there is poor sequence conservation in the ligand-binding domains of ascidian and cephalochordate TRs. This further suggests that TRIAC, and not canonical THs, is the active ligand of basal chordate TRs. Surprisingly, recent evidence suggests that both T3 and T4 regulate the metamorphosis of sand dollars (Saito et al. 1998; Heyland and Hodin 2004), which raises the possibility that TH metabolism was involved in metamorphosis even in the last common deuterostome ancestor. Further studies are needed in the protostomes to test for TH involvement in metamorphosis of earlier bilaterians or even metazoans.

The Endostyle: Precursor to the Thyroid Gland

The endostyles of tunicates and cephalochordates have long been recognized as homologous to the thyroid gland of vertebrates, a connection first suggested by A. Dohrn at the Stazione Zoologica in Naples, Italy (Dohrn 1886).[10] In both ascidians and cephalochordates, the endostyle forms a groove along the ventral edge of the pharynx and contains distinct functional "zones" of specialized cells. The general organization of functional zones in the endostyles of ascidians and cephalochordates is essentially the same, although the overall number of zones may vary (Fig. 4.24C,

[10] Curiously, the Stazione Zoologica has an old tradition of TH research. It was the same place where in 1910 J.F. Gudernatsch made the initial observations that the thyroid gland acted on the induction of metamorphosis, but as he did not trust his original results because the organs he used were not fresh, he published his findings a couple of years later from research done in Prague (Gudernatsch 1912; Brown and Cai 2007).

D): the zones in the bilateral dorsal-most crest-like region of the endostyle closer to the pharynx concentrate iodine and contain peroxidase activity essential for thyroid-like activity, whereas the ventral-most inner groove region contains alternating zones that serve either as support elements or secrete mucoproteins that coat the pharynx to facilitate capture of food particles (Bone et al. 2003; Hiruta et al. 2005; Sasaki et al. 2003). Physiological similarities that support homology of the endostyle and the thyroid gland are backed by conserved gene expression patterns in the developing endostyle. Orthologs of the vertebrate thyroid markers TPO, TTF-1, Pax2/5/8, FoxE4, FoxQ1, and FoxA have been shown to be expressed in the endostyles of both ascidians and cephalochordates (Fig. 4.24D; for details, see Hiruta et al. 2005).

The adult solitary ascidian endostyle of *Ciona intestinalis* derives from an endostyle primordium formed by anterior and ventral endodermal cells in the head of the larva. Within a day of metamorphosis, this primordium elongates and differentiates (Jacobs et al. 2008). The rapid and anticipatory differentiation of the endostyle observed in *C. intestinalis* and other phlebobranchs that show a relatively delayed onset of metamorphosis suggests that TH production may be crucial for the completion of metamorphic differentiation. However, in stolidobranchs (e.g., *Herdmania momus*), the endostyle differentiates only at the end of metamorphosis along with all other juvenile structures, arguing against an important role for the endostyle in metamorphosis.

This apparent incongruence might be disentangled if other cell types, before endostyle differentiation, produced THs. Seemingly resolving this issue, D'Agati and Cammarata (2006) reported that THs are produced by mesenchyme cells of mesodermal origin, completely unrelated to the endostyle. These cells were found scattered throughout the head, particularly behind the papillae and at the basal and posterior region of the head of several phlebobranch ascidian species.

Although further characterization of the specific roles and targets of THs is necessary, a comparative study of TH larvae of different species may reveal whether endocrine mechanisms are employed to modulate heterochronic development in ascidians (i.e., adultation). In summary, ascidian metamorphosis relies on information-carrying systems such as the nervous system, the endocrine system, and particularly the immune system, in order to coordinate a response to environmental cues, which involves an intricate and highly regulated redeployment of developmental processes to drastically reconfigure the individual's body.

Asexual Reproduction: Modes of Budding (Blastogenesis)

The considerable variation observed in the developmental modes of budding in the tunicates (ascidians and thaliaceans) is indicative of the independent evolution of asexual reproduction and coloniality. Several evolutionary transitions to colonialism have been identified in the tunicates, which are accompanied by several morphological, developmental, and reproductive changes, such as miniaturization of individual body size, primarily asexual reproduction by budding and brooding (Davidson et al. 2004). Fully developed larvae are released from the colony to rapidly metamorphose and begin the asexual cycle of development, otherwise known as budding or blastogenesis. In all modes of budding, the epidermis of new individuals derives from preexisting epidermal progenitors, suggesting that mechanisms of epidermal cell replenishment may act locally at the sites of budding and may not differ all that much between solitary and colonial species. In contrast, the endodermal (pharynx, gut, etc.) and mesodermal (muscle, heart, etc.) derivatives originate from distinct precursor tissues in different species. In an attempt to identify homologous developmental mechanisms of budding, a comparison of bud precursor tissues and cells involved in this process will be reviewed next for several species of ascidians.

Budding in Ascidians

In colonial stolidobranchs (specifically in *Polyandrocarpa misakiensis*, *Botryllus schlosseri*, and *Symplegma reptans*), the endoderm-derived

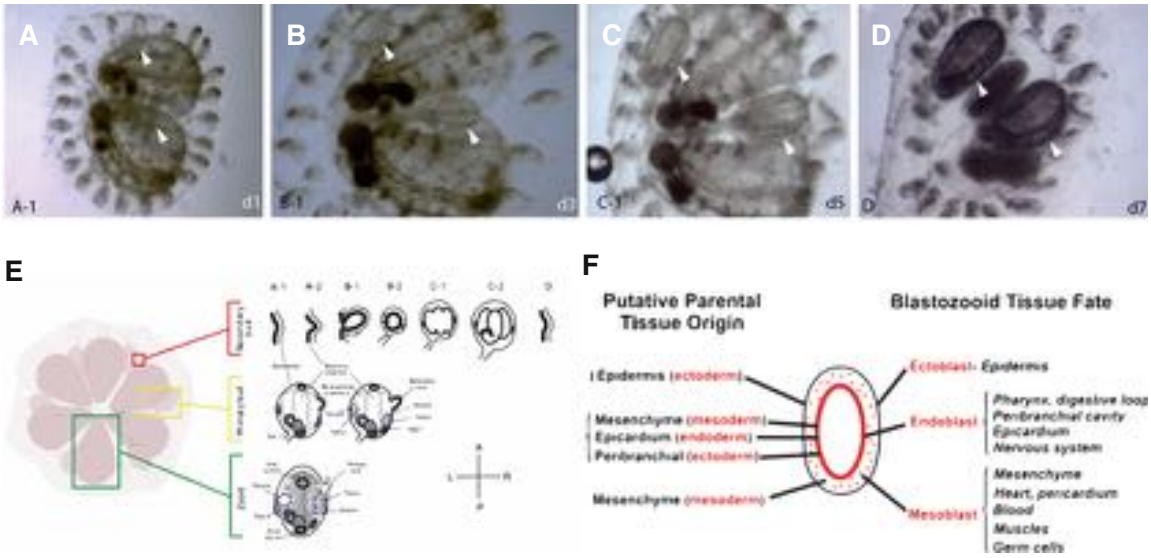


Fig. 4.26 Blastogenic stages in the botryllid ascidians. (**A**–**D**) Developmental events of budding in *Botrylloides violaceus*, stage is shown below to the *left*, and the time in days is below to the *right*. (**E**) Representation of botryllid development. *Arrowheads* point to primary buds. (**F**) Representation of the origin and contribution of the double vesicle stage that is recapitulated during embryogenesis and blastogenesis of botryllids (Adapted from Brown et al. (2007, 2009) and Tiozzo et al. (2008))

outer epithelium surrounding the branchial sac (known as the peribranchial epithelium) contributes to the formation of mesendodermal tissues of the peribranchial buds. Circulatory mesenchymal cells or hemoblasts also contribute to these buds. Evaginations of the lateral peribranchial epithelium and epidermis form an early bud that resembles a double vesicle (Fig. 4.26). The inner vesicle derived from the peribranchial epithelium undergoes a series of folds that will generate most organ primordia. In between the double vesicle, mesenchymal progenitors or hemoblasts are thought to contribute to the differentiation of several tissues and organs, including the pharynx, muscles, and mantle (Sabbadin et al. 1975). One well-documented example includes the participation of hemoblasts in the differentiation of muscle cells near the oral siphon primordium, presumably induced by the oral epidermis (Sugino et al. 2007).

An extensive amount of research demonstrating somatic stem cell exchange in chimeric colonies of *Botryllus schlosseri* suggests that circulatory mesenchymal progenitors are responsible for the differentiation of zooids with distinct genotypes in the same colony (Pancer et al. 1995; Stoner and Weissman 1996). Serial transplantations of circulatory stem cells between different colonies showed that at least two distinct populations of progenitors occur in the circulatory cells of the colony, including germ line and at least one somatic stem cell lines. It was also shown that a minimum of 5.000 transplanted circulatory mesenchyme cells was sufficient to establish a new genotype in the chimera. From this we can estimate the occurrence of one stem cell for every 5.000 mesenchymal cells in the blood[11] (Laird et al. 2005). In certain species of stolidobranchs such as *Botryllus* and *Botrylloides* spp., the double vesicle stage is also observed in vascular buds. In these buds, the outer vesicle is formed from the evagination of the ectodermal vasculature (Fig. 4.26), which connects the zooids of the colony, whereas the inner vesicle is formed from the accumulation and differentiation of circulatory mesenchymal cells within the outer vesicle instead originating from an epithelium (Brown

[11] A stem cell ratio of 1:5.000 in the blood is a relatively high number of circulatory stem cells; for comparison, in humans 1:5.000–10.000 bone marrow cells are stem cells and 1:100.000 nucleated blood cells are stem cells.

et al. 2009). Three possible mechanisms have been suggested for the origin of circulatory mesenchymal progenitors in the stolidobranchs: (1) dedifferentiation of the atrial epithelium and transdifferentiation of these cells into new epithelia, e.g., intestinal epithelial cells (Kawamura and Fujiwara 1995; Kawamura et al. 2008), (2) self-renewal of pluri- or multipotent cells in the circulatory system that may differentiate into distinct cell fates (Freeman 1964; Laird and Weissman 2004; Laird et al. 2005; Kürn et al. 2011; Brown and Swalla 2012), and (3) self-renewal of pluri- or multipotent cells in stem cell niches that circulate and differentiate into distinct cell fates (Voskoboynik et al. 2008; Rinkevich et al. 2013; Jeffery 2014).

In aplousobranchs that are exclusively colonial, bud development is strictly associated with the presence of the epicardium, an endoderm-derived epithelium that extends from the base of the pharynx to the pericardium, covering the entire abdominal or postabdominal regions of the zooid. Thus, colony propagation by budding is generally activated after settlement of the larva and specifically when the oozooid has differentiated. However, the most precocious case of budding in colonial ascidians has been documented in the Holozoidae (i.e., *Distaplia* and *Hypsistozoa*), in which zooids can already differentiate in the larval head of the swimming larva (see below) and also generate larval buds or probuds that can self-replicate or differentiate into new zooids. Other mechanisms of bud formation documented in the aplousobranchs include strobilation (specifically in *Aplidium* spp., *Eudistoma* spp., and *Pycnoclavella* spp.), stolonial budding (specifically in *Clavelina lepadiformis*), and pyloric budding (specifically in *Diplosoma listerianum* and *Didemnum* spp.).

Strobilation occurs by the constriction of abdominal or postabdominal segments (Nakauchi 1982, 1986; reviewed in Brown and Swalla 2012). Each segment generates an independently developing bud that detaches from the parental zooid and begins to differentiate in neighboring areas of the tunic. As each segment develops from distinct regions along the length of the abdomen or postabdomen, bud primordia may vary according to the amount of initial tissue and cell types they inherit. In stolonial budding of *Clavelina lepadiformis* (Brien and Brien-Gavage 1927), budding stolons sprout radially from the base of the zooid, specifically from the post-thoracic region containing the epicardium. Each of the stolons undergoes an enlargement of the tip, which later detach from the parental zooid to form independently developing stolonial buds (Berrill 1951). In contrast, pyloric buds form between the thoracic and abdominal regions of the zooid where the esophagus, stomach, and pyloric gland are located. This corresponds to the site where the epicardium of the zooid prevails (reviewed in Brown and Swalla 2012; Nakauchi 1982). The thoracic region of the parental zooid develops a new abdomen, whereas the parental abdomen develops a new thoracic region. Therefore, this budding mode forms two daughter zooids from one single parental zooid.

In colonial phlebobranchs (specifically *Perophora viridis* and *P. japonica*), buds form at regular intervals along tubular structures also known as stolons, which connect the zooids of the colony. The stolons consist of epidermal diverticula that project from the base of each zooid and spread through the substrate, thus expanding the reach of the colony. Both the stolons of *Perophora* and the colonial vasculature of *Botryllus* are ectodermally derived but differ greatly in form and function. In *Perophora*, stolons are covered by a thin tunic and grow external tubular networks in direct contact with the substrata, whereas the vasculature of *Botryllus* generates a network of canals within a common and more solid tunic of the colony. Within the lumen of a stolon, there is a single-layered epithelium derived from mesenchyme or from the septum that partitions the stolon, allowing for blood flow in opposite directions (Freeman 1964; Mukai et al. 1983).

Since the earliest observations of budding in *Perophora*, Kowalevsky (1874) already noted the contribution of the septum for the formation of stolonial buds; he wrote: "… die innere Haut der Knospe durch eine Verdickung der Scheidewand der Wurzeln …", i.e., "… the inner layer of the bud is formed from a thickening of the stolonial

septum ….” In fact, cuboidal cells in the budding zone of the septum acquire a columnar shape and form a small vesicle on the upper part of the septum at a close distance (approx. 100–200 μm) to the tips of the stolons; these are the earliest recognizable structures of a stolonial bud (Deviney 1934; Kawamura et al. 2008).

As the vesicle grows, several circulatory mesenchymal cells are incorporated, the epidermis of the stolon protrudes, and organ primordia continue to differentiate (Deviney 1934). Among the circulatory mesenchymal cells that were observed to integrate in the developing bud, a lymphocyte-like undifferentiated cell type – similar in cytological characteristics to the hemoblasts in botryllids – was also seen. Some cellular characteristics of these undifferentiated lymphocyte-like cells have also been observed in the budding zone cells of the septum, including a large nucleolus and a mitochondria-rich cytoplasm, demonstrating the possible mesenchymal origin of bud progenitor cells (Kawamura et al. 2008). Furthermore, the stem cell potential of these cells alone was demonstrated by the rescue of irradiated colonies after transplantation of isolated lymphocytes (Freeman 1964). Therefore, this experiment unequivocally demonstrates that circulatory lymphocytes in phlebobranchs are necessary and sufficient for budding. Attempts to rescue irradiated botryllids after hemoblast transplants have not been successfully achieved (Laird and Weissman 2004), which may be suggestive of distinct stem cell potentiality in these two populations of undifferentiated circulatory mesenchymal cells. The homology between these two stem cell types remains to be tested.

Another mode of budding known as “terminal budding” has been reported in *Perophora japonica* and includes the participation of the septum and the incorporation of many circulatory mesenchymal cells at the tip of the stolon to form a terminal stellate bud that serves as a propagule (Mukai et al. 1983). The stellate buds develop three to six epidermal projections, detach from the stolon, and drift away by currents. These pelagic buds will eventually settle again; the stellate projections of the pelagic bud turn into stolonial primordia that grow and begin to form new zooids by stolonial budding. Therefore, a limited input of circulatory mesenchymal cells into the bud occurs at a very early stage before detachment cuts off this mesenchymal cell source, in contrast to constant contribution of mesenchymal cells to the stolonial buds.

Budding in Thaliaceans

In colonial, pelagic thaliaceans, stolonial budding is used to rapidly multiply when environmental conditions are optimal for growth. Salps form chains of buds that emerge by terminal constriction of internal stolons in the oozooid, which undergo differentiation into long chains of blastozooids that often remain attached. The stolons specifically form from the pericardium and are organized into epidermis, endoderm derived from tissues of the endostyle, and mesodermal tissues surrounding the heart. As the stolon grows, constriction occurs in several terminal segments forming a series of blastozooids that will develop and mature synchronously. As chains of blastozooids differentiate, older segments of blastozooids in the most distal parts of the chain release sperm and fertilize the eggs of the younger, more proximal blastozooids. However, blastozooids can survive either as chains or as solitary forms when detached from the chains (Brooks 1893).

In contrast, pyrosomes begin to bud by stolonial budding during embryonic development, when the developing embryo (or cyathozooid, see above) gives rise to an initial colony of four blastozooids (the tetrazooids) (Bone 1998). These blastozooids continue to generate stolons, which undergo constriction to form additional blastozooids, thus expanding the colony. There is no solitary phase, as pyrosomes are obligate colonials.

Doliolids have the most complex life cycles of all thaliaceans. Fertilization and embryonic development occur internally in the gonozooid. After the larvae are released, a metamorphic-like event results in the development of the oozooid. The oozooid next forms a posterior-dorsal stolonial primordium that will continue to grow and undergo stolonial budding by terminal constriction. These buds then migrate independently to the posterior ventral edge of the doliolid and align

to form three rows of buds, which will eventually develop into specialized blastozooids. These include trophozooids, specialized for feeding and responsible for nurturing the entire colony, and phorozooids which develop into the free-living hermaphroditic sexual gonozooids. At this stage, the original oozooid ceases feeding, being nurtured by its trophozooids, and becomes specialized for navigating and transporting the colony (Bone 1998; Paffenhöfer and Köster 2011).

Life History Evolution

On the Biphasic Nature of Ascidian Development

Of the major chordate-specific traits, the notochord, dorsal hollow nerve cord, and somites (represented as a single iteration of paraxial mesoderm that gives rise to the larval muscles) are only present at the larval stage. In contrast, other chordate characters such as the endostyle, the pharyngeal gill slits, and the branchiomeric muscles are fully formed only in the juvenile. Thus, the claim that metamorphosis "replaces" the chordate body plan of the larva with an ascidian-specific adult plan is not accurate at a detailed anatomical level. Either stage features a unique subset of chordate-specific anatomical structures. In this way, the chordate body plan in ascidians is biphasic in time, but ever (partially) present through the life of the individual. The continuity of the chordate body plan in tunicates is clearest in larvaceans, for obvious reasons, and in those ascidian species that undergo anural development or adultation (discussed below).

From an ontological point of view, this biphasic nature does not mean the larval and adult phases are discontinuous. On the contrary, there is a clear linear ontological progression of all the major adult structures from primordia that are specified and patterned during the course of embryogenesis and undergo morphogenesis and differentiation even as the larva is swimming. As such, it is more akin to a "swimming embryo" than a true larva.

The contrast between motile, nonfeeding larva and sessile, filter-feeding adult is likely a result of heterochrony relative to the ancestral chordate, with larval structures quickly differentiating and adult structures being slower to differentiate. Even this heterochrony is not absolute. As will be explained below, in perhaps the majority of ascidian species, this boundary between larva and adult is not clearly defined (due to widespread occurrence of adultation) and in some cases does not exist at all (i.e., in direct developing species).

Ultimately, the evolution of the tunicates from a vermiform ancestor must have involved the acquisition of a biphasic developmental mode through heterochrony, with later secondarily derived heterochronies (adultation and direct development). With the exception of the germ cells, the intestine, and the nervous system, this partitioning between adult and larval components correlates with their location in either the head (mostly adult) or tail (mostly larval). The germ cells are set aside prior to gastrulation and are physically contained within the tail during the larval phase. Immediately upon settlement, the germ cells and surrounding endoderm cells migrate into the head during tail retraction, which precedes all other events that occur during metamorphosis. Within the nervous system, the fractured rudiment of the adult CNS can be found interspersed with the fully differentiated components of the larval CNS along the AP axis (Dufour et al. 2006; Horie et al. 2011). This is probably due to historical contingency, the result of descent from an ancestor with a brain-like structure anterior to those compartments controlling homochronic feeding and locomotion functions.

In contrast, the separation of adult feeding/respiration and larval locomotor functions in time mirrors the strict separation of the mesoderm into anterior (head: branchial/pharyngeal muscles, vasculature, heart, blood cells) and posterior (tail: swimming muscles, notochord) compartments. Whether or not this spatial compartmentalization was already partially completed in the last common ancestor of tunicates and vertebrates and whether this could have represented a precondition for additional temporal compartmentalization are two of the more intriguing questions surrounding the origins of tunicates.

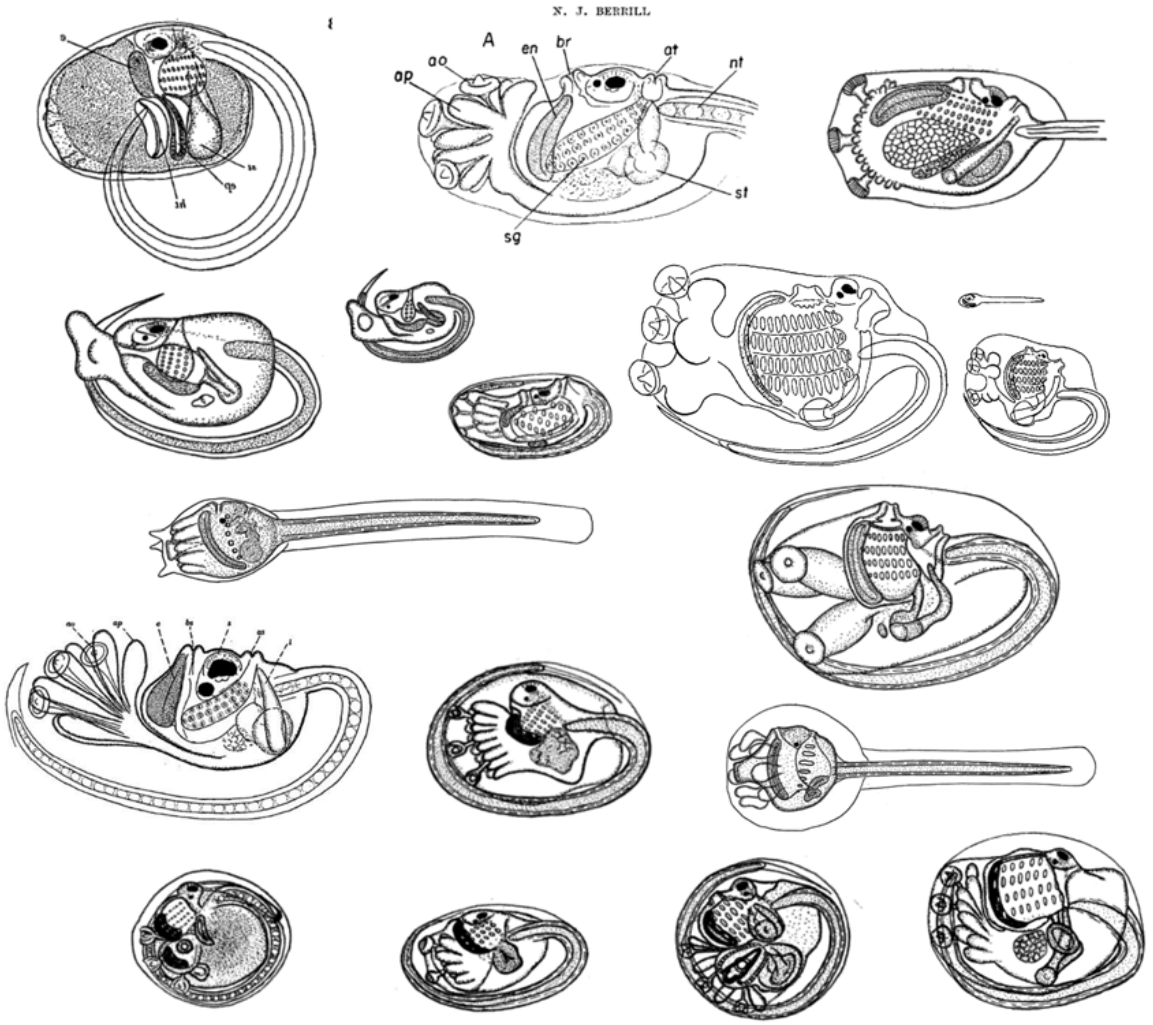
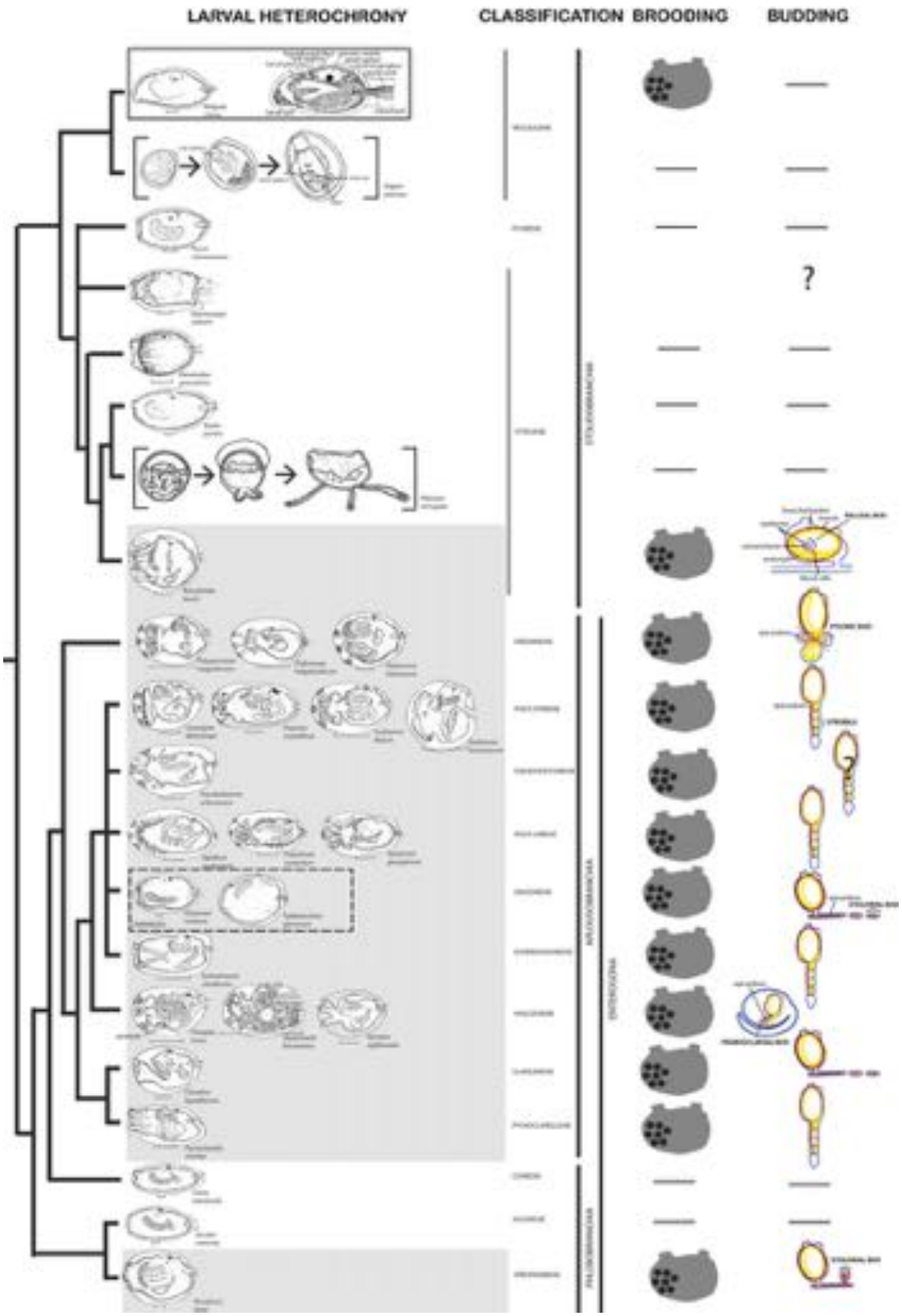


Fig. 4.27 Adultation of ascidian larvae. A composite of several illustrations of works by N. J. Berrill on larvae of several species of ascidians showing varying degrees of adultation or premature differentiation of the juvenile stage while the larva is still swimming (From Berrill, various works)

Fig. 4.28 Evolutionary reconstruction of ascidian adultation, brooding, and budding modes. *Left*: head anatomy of ascidian swimming larvae shows different degrees of adultation (see text for details). With some variation, solitary ascidian larvae show a lower degree of adult differentiation in the head compared to colonial forms (*shaded box*). Exceptional cases include (1) two cases of anural development (direct development) in the stolidobranchs (*squared brackets*); (2) *Molgula citrina* that presents the highest degree of adultation among solitary species (*solid rectangle*), a labeled cross section of the head that has been included to show detail of the internal anatomy (Adapted from Grave 1926); (3) the Diazonidae that present the lowest degree of larval adultation among colonial species (*dashed rectangle*). *Center*: species that brood their embryos irrespective of species-specific differences are marked with the "brood" symbol (*gray* individual with *black* spots representing the embryos); note that *Molgula citrina* and other solitary styelids can brood in the peribranchial cavity. *Right*: schematic representation of the distinct budding modes (*bold* and *capital letters*) is shown; color code represents tissues of endodermal (*yellow*), mesodermal (*red*), and ectodermal origins (*blue*); the epicardium (important tissue for budding, see text for details) of the Enterogona is shown in *orange*; germ layer derivatives shown for every case of budding have only been labeled in *Botrylloides leachi*. *Minus* (−) signs represent absence of that character, and *question marks* (*?*) show cases in which some controversy occurs in the literature regarding the trait (Modified from Millar (1971) and Brown and Swalla (2012))


LARVAL HETEROCHRONY
CLASSIFICATION
BROODING
BUDDING
?

Adultation and Heterochrony in Development

The precocious differentiation of adult structures during the embryonic or larval stages is known as adultation (Fig. 4.27). This heterochrony in development, which blurs the line between larva and adult, can be observed in different ascidian species, having likely evolved independently multiple times.

Among ascidians, one sees variation in the extent of adultation (Fig. 4.28), with some species, like *Molgula citrina*, showing limited adultation, while others, like *Ecteinascidia turbinata*, showing extensive growth and differentiation of adult structures, supported by highly elaborated larval structures such as a tail with increased number of muscle cells, required for the larva to swim in spite of its greater mass. This increase in size and complexity of larval structures (muscle, notochord, motor ganglion) is termed "caudalization." In some species of colonial ascidians that undergo adultation, larvae may already be carrying asexually reproducing buds, or blastozooids, and thus constitute a "swimming colony." It is tempting to speculate that this odd configuration of an oozooid larva carrying a blastozooid bud might be an evolutionary forerunner to the acquisition of "catastrophic" metamorphosis. A simple heterochrony could result in the senescence of the larva/oozooid before the initiation of metamorphosis and in the initiation of the adult stage by the blastozooid.[12] This would lead to the discontinuity between embryonic patterning and adult body seen in echinoderms, entoprocts, and other taxa.

In one recent study, three non-adultative phlebobranch ascidian species were found to be prone to slight adultation if metamorphosis was artificially delayed, while there was no such regulative adultation seen in the three stolidobranch ascidians assayed (Jacobs et al. 2008). This suggests that adultation may be dynamically regulated.

[12] This is technically what occurs in pyrosomes, in which the oozooid generates a few blastozooids and immediately senesces. However, because pyrosomes are direct developers and the oozooids and blastozooids share a common body plan, this is not seen as a catastrophic metamorphosis.

Table 4.2 Egg sizes of primitive (non-adultative) and adultative species of ascidians

Primitive (non-adultative) developers	
Species	Size of egg (μm)
Ascidiella aspersa	170
Boltenia echinata	170
Ciona intestinalis	160
Diazona violacea	160
Halocynthia pyriformis	260
Molgula manhattensis	110
Phallusia mammillata	160
Styela canopus	150

Adultative developers	
Species	Size of egg (μm)
Aplidium nordmanni	380
Clavelina lepadiformis	260
Dendrodoa grossularia	480
Distomus variolosus	590
Hypsistozoa fasmeriana	25*
Molgula citrina	200
Stolonica socialis	720

Modified from Berrill (1930). Top: egg size of species that develop into "primitive"-type larvae that do not undergo adultation. This is considered the ancestral condition, due to the nearly identical embryos of distantly related species that develop in this manner (e.g., *Ciona* vs. *Halocynthia*). Bottom: egg sizes and species whose larvae show adultation. Most adultative species develop from larger and more yolk-laden eggs. Asterisk (*) indicates the exception to this, the viviparous *Hypsistozoa fasmeriana*, whose eggs are entirely devoid of yolk and are nurtured by the parental colony through a placenta-like structure (see text, Fig. 4.29, and Brewin (1956) for more details

Such a capacity to "anticipate" metamorphosis could compensate for the negative consequences of delayed metamorphosis, which might happen if the right environmental cues for settlement are not present. While this conclusion was supported by the correlation between length of competency period and tendency toward adultation, the phylogenetic significance of this finding awaits a larger sampling of species.

Adultation goes hand in hand with increased egg size and ovoviviparity, in which eggs, embryos, and hatchlings are brooded inside the parental individual or colony (Table 4.2). This is probably due to an increased threat of predation on the energetically rich, yolky eggs that are required to sustain the development of adultative larvae. There is also a strong association between adultation and coloniality, which has also arisen

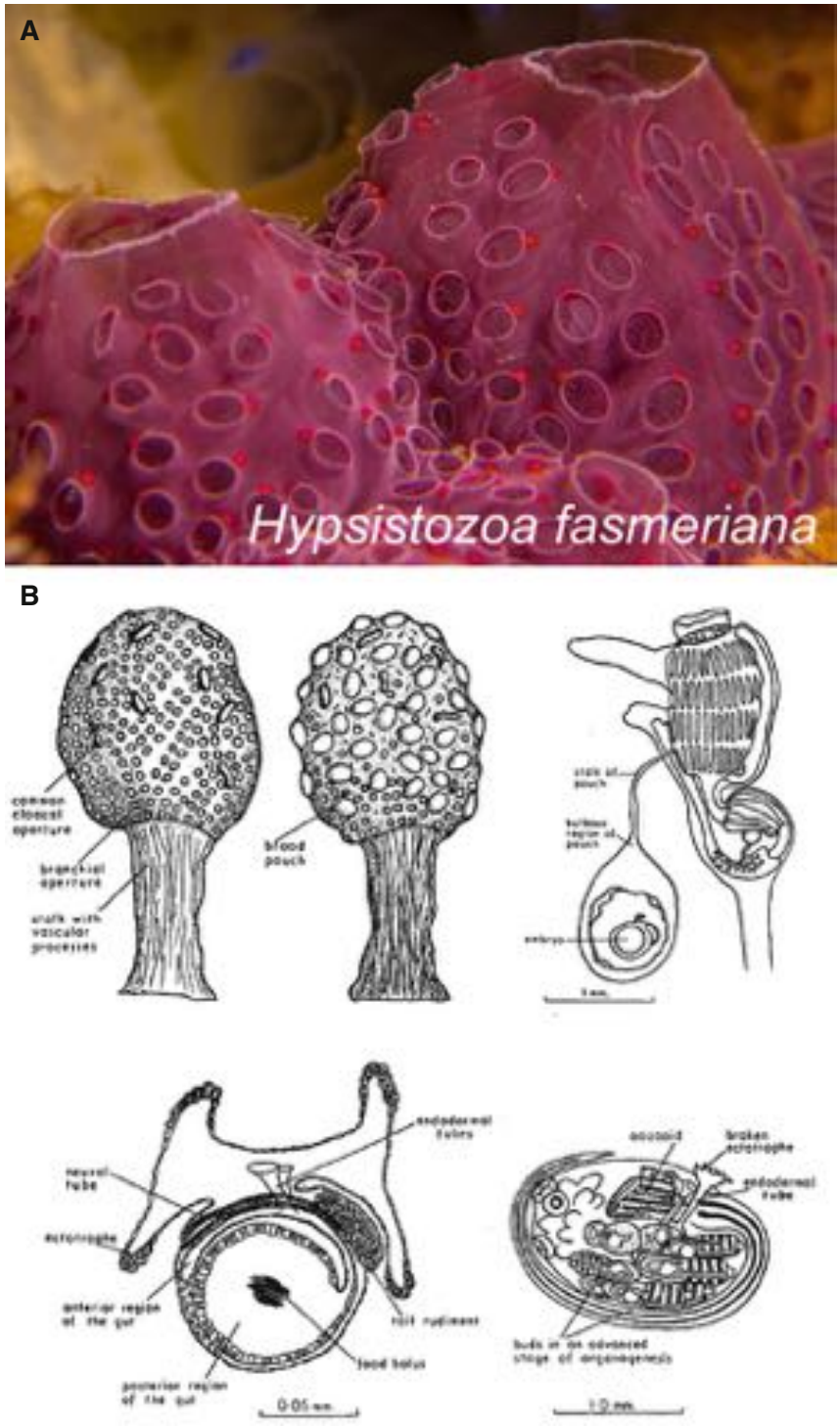


Fig. 4.29 True viviparity in *Hypsistozoa fasmeriana*. (**A**) Colonies of *Hypsistozoa fasmeriana* (Photograph by Paul Caiger) (© Paul Caiger, 2015. All Rights Reserved). (**B**) Illustrations of *H. fasmeriana* colonial and embryonic development. *Top left*: young colony. *Top middle*: senescing colony, preparing for shedding of larvae. *Top right*: anatomy of *H. fasmeriana* zooid showing embryo being brooded in attached brood pouch. *Bottom left*: structure of *H. fasmeriana* embryo and associated ectotroph, an extra-embryonic tissue. Endodermal tubes connect the brood pouch with the lumen of the gut of the embryo, presumably to transfer food and nutrients from the parent to the embryo. *Bottom right*: large larva with numerous buds in an advanced stage of differentiation (a swimming colony) (Illustrations in (**B**) are reproduced with permission from Brewin (1956): http://jcs.biologists.org/content/s3-97/39/435.short)

independently in different ascidian families (Fig. 4.28). There are, however, some notable exceptions, such as the adultative solitary *Molgula citrina* or non-adultative colonial *Diazona* spp. Possible explanations for the association between coloniality and adultation are discussed below.

Some adultative species appear to have evolved true viviparity, with secondary reduction in egg size since nutrients now can be passed from parent to daughter during development. This appears to be the case between the closely related *Botryllus* and *Botrylloides* genera (Berrill 1935). A more extreme example of viviparity is found in *Hypsistozoa fasmeriana*, which has extremely small eggs (approx. 25 μm diameter), whose growth into large "swimming colonies" or compound larvae, each comprising an oozooid plus several blastozooids in various stages of development, depends on transfer of food through a connection between the colony's brood pouch and the embryo's gut (Fig. 4.29; Brewin 1956).

Maximum Direct Development

There are some very rare examples in which direct development and adultation have been combined in a process of "direct adultation," also called "maximum direct development," in which early embryonic development may truly bypass the formation of a larva (urodele or anural) prior to differentiation of adult structures. The maximum direct developers potentially include *Polycarpa tinctor* (Millar 1962), *Pelonaia corrugata* (Fig. 4.28; Millar 1954), and *Molgula pacifica* (Young et al. 1988; Bates 2002), although in all these cases a detailed stage-by-stage description of development is lacking and we are left without any certainty about how development in these species occurs. It is hypothesized that the various species with anural development find themselves at different stages of an evolutionary transition toward maximum direct development.

Embryogenesis vs. Blastogenesis

Only after metamorphosis do ascidians begin to feed and grow. In solitary species, the settled juvenile grows and matures as a single individual. In colonial species, the settled juvenile is called the oozooid (Fig. 4.25), i.e., the founding individual of a colony derived from sexual reproduction. The oozooid then activates asexual budding cycles (i.e., blastogenesis) to generate blastozooids, individuals derived from asexual generations or blastogenesis (Fig. 4.26). Although oozooids and blastozooids show certain anatomical differences, such as different arrangement of the pharyngeal slits of the branchial sac, the general organization and body plan are maintained. Also, expression patterns of many developmental genes that are expressed in embryogenesis are redeployed during the development of the bud. For example, *Botryllus schlosseri* orthologs of *Six*, *Eya*, and *FoxI* (transcription factors associated with the vertebrate placode network) expressed in anterior and posterior placodal regions in the metamorphosing larva are also expressed in corresponding anterior and posterior regions of the developing bud (Tiozzo et al. 2005; Gasparini et al 2013). The anterior placode region will eventually differentiate into cells of the oral siphon, ciliated duct, and cerebral ganglion, whereas the posterior placode region will differentiate into cells of the atrial siphon of both oozooid and blastozooid, similar to their development in solitary ascidians (Mazet et al. 2005) and larvaceans (Bassham and Postlethwait 2005).

Contrary to the reported effects of thyroid hormone inhibitors on *Ciona intestinalis* juveniles during metamorphosis, the thyroid hormone inhibitor thiourea was found to accelerate bud development and inhibit stolon growth in the colonial phlebobranch *Perophora orientalis*. Conversely, the thyroid derivative thyroxine inhibited bud development but induced stolon growth (Fukumoto 1971). These results provide an exciting opportunity to compare modular effects of endocrine signaling during zooid differentiation between metamorphosis and blastogenesis. However, the expression patterns of the main thyroid pathway genes remain to be elucidated in ascidians.

Future work is needed to compare developmental mechanisms of the earliest stages of embryogenesis vs. blastogenesis, in order to identify similarities and differences in patterning events between bud progenitors and early blastomeres. In sum, the existence of dual developmental

trajectories (sexual vs. asexual) to generate the same body form in a single species provides an excellent opportunity to address questions about developmental pathway co-option in evolution.

Evolution of Coloniality

Within the deuterostomes, the hemichordate pterobranchs (Chapter 2) and many species of tunicates have a colonial stage in their life cycle. Colonies are defined as aggregates of individuals that are "connected together, either by living extensions of their bodies, or by material that they have secreted" (Barrington 1967). In the tunicate literature, colonies with living extensions that interconnect the individuals have been referred to as "social forms," whereas colonies that secrete material to embed the individuals have been referred to as compound forms (Milne-Edwards 1841; Pérez-Portela et al. 2009). In deuterostomes, the colonial life stage is generally initiated after metamorphosis. Asexual reproduction of the colony occurs by clonally generating new individuals from preexisting juvenile or adult tissues. Curiously, individuals of most species that form colonies are (1) smaller in size than their closely related solitary species clades, (2) able to brood and release larvae with a high degree of adultation (see above), and (3) imbued with highly regenerative and propagative potentials (Davidson et al. 2004; Brown and Swalla 2012).

The current phylogeny of Deuterostomia supports the idea that coloniality in this group has evolved independently multiple times but that the ability to regenerate was likely present in the ancestral deuterostome. In the clade comprised by the questionable Xenacoelomorpha + Ambulacraria (Fig. 4.1), coloniality only occurs in the pterobranchs, but regenerative potential has also been documented for the harrimaniids, ptychoderids, echinoderms, and xenacoelomorphs (see Chapters 1 and 2; Vol. 1, Chapter 9). Among the Chordata, ascidians are the only subphylum that show convergent evolution of coloniality in several species that also have full regenerative potential (i.e., whole body regeneration). Regenerative potential, albeit much more modest (i.e., tissue, organ, and structure regeneration), has also been reported in solitary ascidians, cephalochordates (Somorjai et al. 2012), some Craniata/Vertebrata, and, most remarkably, platyhelminths (see Vol. 2, Chapter 4 for a detailed account on regeneration in the latter). Therefore, we can speculate that developmental mechanisms of asexual reproduction and regeneration preceded the establishment of colonial lifestyles.

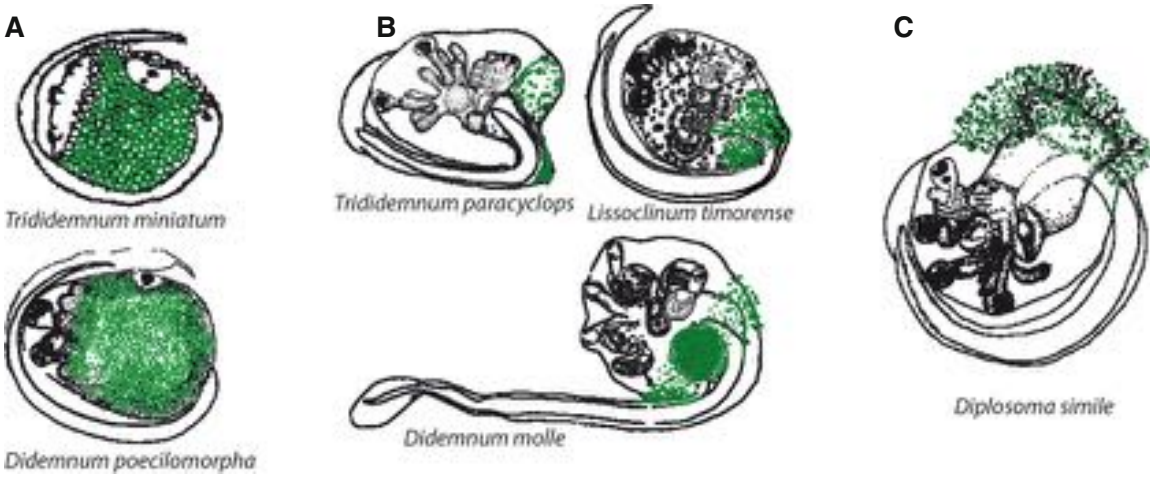


Fig. 4.30 Vertical transmission mechanisms of photosymbionts in didemnid ascidian larvae. (**A**) Prochloron cyanobacteria (*green*) embedded within the larval tunic. (**B**) Prochloron attached to hairlike projections and tunic folds in the posterior region of the larval head that does not contain larval tunic. (**C**) Prochloron attached and inserted into the rastrum, a T-shaped extension of larval ectoderm in the posterior-dorsal region of the larval head (Modified from Kott (2001))

Conceptual arguments on life history trade-offs between individual forms and cooperative entities such as colonies are starting to emerge (see recent published books edited by Calcott and Sterelny 2011; {Inserting} Bouchard and Huneman 2013). However, we are far from understanding how selective forces act to co-opt developmental modules in the evolution of such higher-level organized forms. To begin to address this question, it is necessary to first identify the developmental processes associated with coloniality.

Photosymbiosis in Colonial Didemnid Ascidians

Last, but not least, symbiosis occurs between an oxygenic photosynthetic cyanobacteria and several tropical colonial ascidian species of the Didemnidae. The symbiont *Prochloron* spp. (Kühl et al. 2012) is found associated, almost exclusively, with four different genera of didemnids: *Diplosoma*, *Lissoclinum*, *Didemnum*, and *Tridemnum* (Yokobori et al. 2006). *Prochloron* is usually present in the outer surface of the tunic, within the common cloacal system of the colony, or embedded in the tunic as either free-living cells or intracellularly in tunic cells. *Prochloron* is transmitted vertically from the mother colony to the daughter colonies by three distinct mechanisms: (1) by direct attachment into a sticky posterior region of the larval head before the larvae are released, (2) by encapsulating into a specialized pouch-like organ in the posterior head of the larvae, or (3) by the transmission of *Prochloron-containing* tunic cells with intracellular *Prochloron* from the mother colony to the developing larval tunics while brooding (Fig. 4.30; Kott 2001; Yokobori et al. 2006; Hirose and Hirose 2007).

The evolution of vertical transmission mechanisms of the host species suggests an obligate symbiotic interaction in which the didemnid ascidians provide shelter for the symbiont and the prochloron provides protection. Two protection mechanisms have been suggested: (1) UV screening mechanisms by producing mycosporine-like amino acids (MAAs) (Kühl et al. 2012) and (2) cyanobactins (toxins) to protect the ascidians from predators or parasites (Donia et al. 2011). In sum, didemnid ascidians provide an opportunity to study the effects of symbiosis in development and the recurrence and particular adaptations of this biological interaction across several species.

OPEN QUESTIONS

Tunicate/Chordate Origins

- What was the tunicate ancestor like?
- How did it evolve from the olfactorean ancestor?
- Which (if existing) are the autapomorphies of Olfactores (Tunicata + Vertebrata)?

Tail Loss in the Molgulids

- How much do the multiple, independent cases of evolutionary loss of the larval tail structures in the Molgulidae have in common at the molecular or cellular levels?
- Is there a common point of evolutionary convergence for the different anural species?
- Did those species exhibiting direct development evolve from indirectly developing tailless species?

Adultation of Colonial Ascidian Larvae

- How are the much bigger larvae of certain colonial ascidians formed?
- How have they evolved presumably from a simple larva of the solitary kind?
- Do the mechanisms involved in patterning and growth of these larger embryos resemble in any way perhaps those lost in the, presumably, drastically reduced ascidian ancestor?

Evolution of Larvaceans

- Did Appendicularia evolve from an ascidian-like stock or did they evolve directly from free-swimming, pelagic ancestors?
- If they evolved from a sessile ascidian ancestor, what were the embryological and gene

regulatory hurdles that needed to be overcome in order for larvaceans to break free from the constraints of the typical solitary ascidian embryo archetype?
- What are the mechanisms that regulate larvacean metamorphosis, and how conserved are these with the rest of the tunicates?

Evolution and Development of Thaliaceans
- What are the relationships among the thaliacean orders (pyrosomes, salps, and doliolids?
- How did complex life cycles evolve in this class?
- How far do metamorphic events in the thaliaceans resemble those of other tunicates?
- What are the underlying molecular and genetic mechanisms of thaliacean development?

Regeneration and Coloniality
- How closely do the mechanisms of blastozooid patterning resemble those that pattern the embryo/oozooid?
- How is the formation and regression of zooids modulated in colonial species?
- What are the signaling mechanisms that orchestrate the synchronized differentiation of blastozooids in certain species?
- When do bud progenitors first reorganize themselves into a new individual with a body plan that corresponds to an embryonic stage, and which stage would that be?
- Is there an analogous "phylotypic" stage for blastogenesis?
- How closely do the mechanisms of blastozooid patterning resemble those that pattern the embryo/oozooid?
- Given that of embryonic patterning are set up by localized maternal determinants that presumably would be very difficult to redeploy in a blastogenic bud, at what point(s) in the gene regulatory network(s) do the embryonic and blastozooid programs converge?
- Was the last common ancestor of all tunicates imbued with the remarkable regenerative potential of colonial tunicates and subsequently lost from the less regenerative solitary ascidians?

Gene Expression and Function
- Why has the Hox complex fragmented or its regulation become less cohesive in tunicates?
- Has the presumed loss of segmentation in tunicate body plans relaxed the constraints on Hox cluster integrity?
- Do the Hox genes play a more peripheral role in gene regulatory networks governing embryonic development in the tunicates, relative to their role in other phyla?
- What were the genes potentially co-opted by the neural plate border cells in the craniate ancestor that might have modified these into *bona fide* neural crest cells?
- How are metamorphic processes of apoptosis, proliferation, migration, and differentiation regulated in the tunicates, and to what extent are these regulatory mechanisms redeployed in ascidians, larvaceans, and thaliaceans?

Developmental System Drift
- How pervasive is developmental system drift among the tunicates, especially those with very conservative, simplified embryos like most solitary ascidians?
- Are the genomes of tunicates rapidly evolving in order to adapt to the various other environmental and ecological concerns faced by them, such as feeding, temperature, salinity, predation, reproduction, etc.?
- If so, do species-specific *cis*/*trans* compensatory mechanisms evolve in order to maintain the same exact embryo?
- Is this invariant embryo required due to the lack of regulative developmental processes that might allow for the ontogenesis of a viable sea squirt via a very different embryo?
- Is embryonic development more variable among the colonial species (not related to the separate issue of adultation) because these have evolved/retained greater developmental plasticity, as evidenced by their blastogenic mode of reproduction?

Prochloron Photosymbiosis

- Why is prochloron present only in some species of didemnids?
- How did host-symbiont interaction become obligatory?
- How does obligate symbiosis trigger the evolution of specific mechanisms and organs for vertical transmission in the host?
- Does intracellular prochloron within tunic cells represent a causative event of an older symbiotic interaction of particular lineages?
- Did prochloron diversify after symbiosis with the host?

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