20 Solitary Ascidians

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20.1 INTRODUCTION

The tunicates present various ecological behaviors comprising sessile or pelagic adult forms in addition to colonial or solitary animals. Solitary ascidians are present in several tunicate groups, meaning that both solitary and colonial ascidians are not restrictive or typical to a given clade. Whereas distribution of solitary ascidians is scattered in the urochordate tree, they share some common features, and these can be studied in a common specific chapter. Despite the large diversity of solitary ascidians, they can be characterized by typical features such as an individual sessile adult presenting two siphons (one inhalant and one exhalant, allowing the circulation of sea water), a pharynx supported by an endostyle and a large branchial basket structure. Usually hermaphrodites, fertilization takes place in sea water after the release of gametes and gives rise to a swimming pelagic larva which will have to settle in a definitive substrate. Unlike in colonial ascidians, asexual reproduction is not documented.

Solitary ascidians have had a noticeable historical contribution to developmental and cell biology studies and include several well-established models in marine biology such as *Ciona intestinalis*. As the sister group of vertebrates, ascidians genetics data and genomics tools have opened broad perspectives to understand the development and evolution of chordates. Moreover, the financial importance of some solitary ascidians species is notable as marine alimentary resources, like *Microcosmus sabatieri* (usually named "violet" or "sea fig") in the south of France; *Styela clava* in Korea; or *Halocyntia roretzi*, which has been popular in Japan. On the contrary, negative ecological consequences can result from invasive species, like *Styela clava*. The preceding succinct presentation of solitary ascidians highlights the necessity and relevance of an overview.

20.2 HISTORY OF THE MODEL

The evolutionary history of tunicates is documented by fossil records comprising organisms attested or suggested to be solitary ascidians. The fact that the first tunicate fossil evidence seems to correspond to solitary ascidians is probably due to a typical shape presenting two siphons in a "bag-shaped" morphology characterized by a pharynx and gill slit, making fossils of solitary ascidians easier to identify than other tunicates. The oldest attested representative is *Shankouclava shankpuense*, which has an estimated age of 524 million years corresponding to the second Turgenevian stage of the Cambrian, discovered in China (Chen et al. 2003). This discovery introduced tunicates, at least solitary ascidians, as part of the high diversity explosion of the Cambrian, witnessing the emergence of several major current groups of animals. Finally, some hypothetical identifications, such as *Yarnemia ascidiformis* (Chistyakov et al. 1984) or *Burykhia hunti* (Fedonkin et al. 2012) from the Russian Ediacaran (550 and 555 million years old, respectively), suggest an older appearance of ascidians.

The current species, *Ascidiella aspersa*, was the first experimental model in developmental biology, on which Laurent Chabry studied blastomere recombination at the end of the 19th century (Chabry 1887). Chabry destroyed one of the blastomeres of two-cell embryos and found that the surviving one was able to form a half-embryo (more precisely, a dwarf malformed larva). He obtained similar results with the same kind of experiment on four-cell embryos and deduced that an amputated early embryo is unable to compensate for deleted cells during the development. Consequently, pioneer experiments made by Chabry suggested that each part of the larva came from specific cells emerging during the first divisions. Next, Edwin Grant Conklin deepened our understanding of embryogenesis by working on the lineage of embryonic cells and the segregation of the egg cytoplasm of various species of solitary ascidians such as *Styela canopus* (Conklin 1905a, 1905b). He reconstructed the lineage of cells from the first divisions to the well-developed larva and confirmed the suggestions coming from Chabry's experiments; development is characterized by cell lineages, which give specific tissues in the future larva what was called "a development in mosaic". Conklin's studies on egg cytoplasm segregation, in addition to cell lineage characterization, led to the hypothesis that female determinants are present in the eggs to drive and participate in the cell fate establishment during development. Solitary ascidians consequently allowed the discovery of two fundamental points in developmental and cell biology: the existence of maternal determinants (now known as maternal RNA) and the existence of cell lineages. In the same period as Conklin's experiments, other biologists focused on tunicate reproduction biology, such as Thomas Morgan, who demonstrated in 1904, on *Ciona intestinalis*, that self-fertilization is blocked. We currently know that this kind of biological barrier has probably been selected to prevent consanguinity and facilitate genetic mixing and increasing variability (see embryogenesis section for details). From these pioneer studies by Chabry and Conklin, interest in solitary ascidian biology crossed time, and several biologists continued descriptive works. Throughout his career, Norman John Berrill developed ascidians as biological models (Berrill and Watson 1930; Berrill 1932a; Berrill and Watson 1936; Berrill and Sheldon 1964). He described various species (Berrill 1932b) and also focused on development and organ functionality, such as the gut and stomach (Berrill 1929). He particularly took advantage of solitary ascidians as an easy model to understand seminal functionality. Importantly, Berrill participated in the validation of the mosaic development theory, in opposition to regulative development, which considers that blastomere fate can be regulated during development to be able to form a normal embryo in case of cell destruction. Next, since the 70s, a new generation of researchers from several countries have expanded our understanding of ascidian biology. As one example among others, Guisseppina Ortolani worked on cell lineage differentiation or fertilization mechanisms on *Ciona*, *Phallusia* or *Ascidia*. She notably participated in the discovery of muscle cell lineages. Richard Whitteker validated Conklin's proposition in 1973 of the presence of maternal determinants in

eggs driving cell lineage. In addition to research in Europe, strong expertise on solitary ascidians emerged in Japan, led by Noriyuki Satoh. One of Satoh's major contributions is his research on egg cytoplasmic factors establishing cell fate during embryogenesis. Thanks to horseradish peroxidase tracer techniques, he was able to follow cell lineage and identify maternal factors with monoclonal antibodies, an innovative approach at the beginning of its career. Next, he described the mechanism regulating expression of acetylcholinesterase in muscle differentiation. With his research and the formation of several future researchers, he actively participated in developing molecular techniques on ascidian species. For instance, he was at the origin of the first transcriptomic project but also on the sequencing of the *Ciona* genome. In addition, he provided, thanks to the ghost database, several molecular tools and data on *Ciona* development to the scientific community. Finally, in the 90s, the complementarity between developmental biology, genetics and incorporation of new molecular approaches opened new perspectives to discover maternal determinants, making mosaic development possible, but also on the importance of regulation between blastomeres. In 2002, the first ascidian genome, from *Ciona intestinalis*, was sequenced and annotated, opening an avenue of possibilities on embryogenesis, metamorphosis and molecular signaling pathway understanding. To date, several genomes and transcriptomes from different solitary ascidians such as *Phallusia mammilata*, *Ciona savigny*, *Molgula occulata* and *Halocynthia roretzi* have expanded the amount of molecular data on this group and contributed to easier molecular phylogenetic analysis, accessible molecular functionality comparison between chordates and experiment design. The International Tunicate Meeting (ITM), which occurs every two years, alternately in Japan, Europe and the United States, was initiated in 2001, illustrating the dynamism of research on ascidians where solitary species count as most of the biological models, in addition to a few colonial species such as *Botryllus genus* or Appendicularia such as *Oikopleura genus*.

This focus on solitary ascidian models' contribution to developmental biology is fundamental, but one must not ignore the debate on ascidian evolution and their position among the animals' phylogenetic tree in the 19th century. Ascidians have been considered close to molluscs for a long time because of their flask adult body devoid of hard structure. The first questioning of this belonging was made by Savigny in 1816, who recognized tunicates as distinct and separate from molluscs. Next, studies from Vadimir Kovalevsky during the 19th century questioned the relationship between tunicates and other animals. Indeed, Kovalevsky described the larval body plan of two species of solitary ascidians, *Ciona intestinalis* and *Phallusia mammilata*, and discovered an organization similar to chordate animals (1866). In particular, the presence of a dorsal chord in tadpole swimming larvae led to considering chordates as composed of three groups: tunicates (comprising solitary ascidians), cephalochordates (as genus *Amphioxus*) and vertebrates. Consequently, thanks to solitary ascidian larval descriptions, the phylogenetic position and evolutionary history of tunicates became better understood. From Kovalevsky's studies to the beginning of the 21st century, ascidians were considered the first divergent branch of chordates (making cephalochordates the sister-group of vertebrates). More recently, thanks to molecular phylogeny made possible by genome sequencing and statistical method development, it was established that tunicates are the sister-group of vertebrates, whereas cephalochordates are the first divergent chordate phylum, making tunicates the closest "invertebrates" to vertebrates (Delsuc et al. 2006). Consequently, ascidians became important in comparative studies from an evo-devo perspective to understand vertebrate evolution. Whereas the phylogenetic position of tunicates is now consensual and established, the relationship inside tunicates is more debated, and several phylogenies frequently emerge in the literature, although a consensus is currently appearing (Figure 20.1).

Tunicates are commonly considered to be composed of five major phyla: Appendicularia, Phlebobranchia, Aplousobranchia, Thaliacea and Stolidobranchia. Appendicularia are characterized by a pelagic lifestyle with a tadpole-shaped adult form, illustrated by the best-known species, *Oikopleura dioika*. Though Appendicularia are often positioned as the first branch separated from other tunicate groups, debate on the phylogenetic position of this group is not totally closed, and it could be the sister of the Stolidobranchia (Delsuc et al. 2006; Delsuc et al. 2018; Kocot et al. 2018; Tatián et al. 2011; Satoh 2013). The four other groups (Phlebobranchia, Aplousobranchia, Thaliacea, Stolidobranchia) are grouped together in recent phylogenetic analysis and form a monophyletic clade. Phylogeny inside this large group has been debated because of the difficulties of reconstructing the life history for several reasons: the convergent features, the secondary loss and the high evolution rate of DNA sequences, making molecular phylogeny difficult to perform. According to the current consensual phylogeny, Stolidobranchia was the first group to diverge from the others. Then, Phlebobranchia, Thaliacea and Aplousobranchia are considered monophyletic. Thaliacea diverged first, and Phlebobranchia grouped with Aplousobranchia to compose Enterogona.

Thaliacea, including salps, are pelagic only and form a planktonic colony made by the aggregation of multiple individuals. An important point to keep in mind is the presence of both solitary and colonial ascidians in Stolidobranchia and Phlebobranchia, whereas Aplousobranchia are only colonial and represent the group containing the highest number of species. In these three groups, adult forms are settled to the substrate, whereas Thaliacea are pelagic. Stolidobranchia, characterized by the presence of one gonad pair and an atrium formed from a unique indentation, is composed of colonial ascidians like *Botryllus schlosseri* as well as solitary ones such as *Molgula oculata*. Stolidobranchia are also characterized by a folded branchial sac. Phlebobranchia and Aplousobranchia, both usually grouped into Enterogona, possess an even number of gonads, and the atrium is formed by two indentations. Phlebobranchia present a branchial sac vascularized by longitudinal blood vessels, whereas Aplousobranchia have

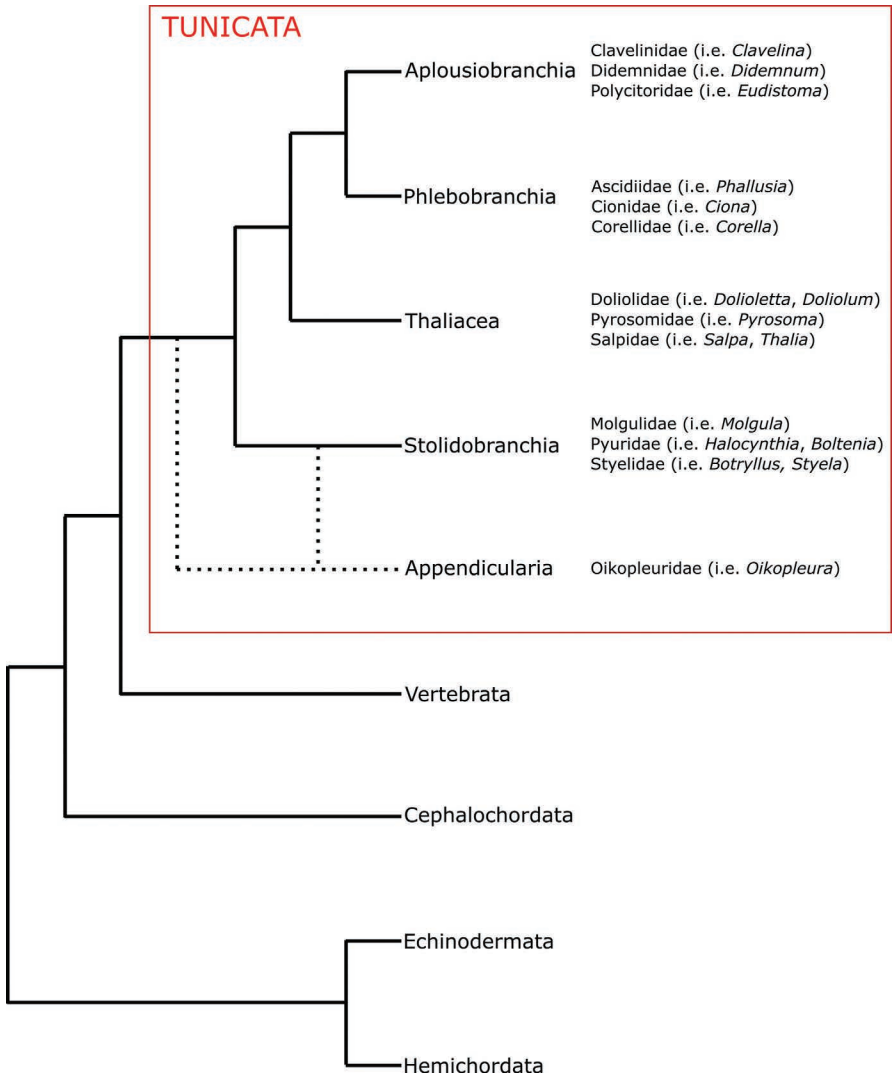

FIGURE 20.1 Consensual phylogeny of tunicates among deuterostomes. Tunicates are the sister-group of vertebrates. Among tunicates, Appendicularia are usually considered the basis of the phylogenetic tree. Solitary ascidian biological models belong mainly to the Stolidobranchia and Phlebobranchia groups.

a simple anatomy. The well-established biological models of solitary ascidians *Ciona intestinalis* and *Phallusia mammillata* belong to Phlebobranchia, a group also composed of a few colonial species such as *Perophora namei* with the particularity to present several individuals distributed along a long slender stolon. Aplousobranchia is composed of colonial species such as *Clavelina lepadiformis* or *Aplidium elegans*. Stolidobranchia and Phlebobranchia tunicates are both colonial and solitary, and this makes them ideal model animals to study in order to better understand evolution, convergence and the impact of environment to determine their lifestyle.

20.3 GEOGRAPHICAL DISTRIBUTION

Solitary ascidians are ubiquitously distributed across oceans and closed seas (Shenkar and Swalla 2011). The most-described species appear to originate from the Pacific region, possibly resulting from an artifact of sampling because taxonomists have been particularly active in this region. Solitary ascidians are marine, and no freshwater species have been reported. However, several species live in estuarine, and ascidians can usually support high variations of salinity (Lambert 2005; Shenkar and Swalla 2011). As an example, *Ciona intestinalis* can support a range of salinity from 12 to 40% and is able to survive a short bath in brackish water with a salinity less than 10% (Dybern 1967; Therriault and Herborg 2008). Solitary ascidians are also tolerant to temperatures lower than 1.9°C allowing, as we will see, survival at the poles (Primo and Vázquez 2009), but also to temperatures over 35°C, as reported in the Arabic Sea (Monniot and Monniot 1997). Resistance to variations could explain the ubiquitous repartition of ascidians. *Ciona intestinalis* is a perfect example showing the capacity of solitary

ascidians to colonize various environments, leading to a ubiquitous distribution. It has been sampled in the Pacific Ocean (east and west), in the Atlantic on both American and European coasts and in the Mediterranean Sea.

In addition to the presence of several ubiquitous species, the capacity of larvae to settle in any substrate, such as soft sediments, rocks or coral reefs, facilitates colonization and expansion. Particularly, larvae can settle on several artificial substrates such as floating dock or ship hulls, leading to an artificial geographical spreading of some species at harbors around the world. Consequently, some solitary ascidians have a current ubiquitous repartition, but this does not seem natural as resulting from a secondary colonization mediated by human activities. For example, it has been reported in the port of Salvador, which receives cargo ships from several continents, that the ascidians species inventory presents a mix between possible endogenous ones (such as *Ascidia nordestina*), introduced ones (such as *Cnemidocarpa irene*) and ubiquitous ones. Importantly, for some solitary ascidians characterized by a wide/ubiquitous distribution, it can be difficult to evaluate if the geographical distribution is natural or artificial, resulting from centuries of spreading thanks to travels and maritime trades. It is thus assumed that some ascidians can have an unknown natural repartition. On the other hand, some cases of invasion are clearly documented. *Corella eumyota*, found natively in the southern hemisphere, is now established in the north Atlantic and Mediterranean Sea (Lambert et al. 1995; Collin et al. 2010). Moreover, *Styela genus* represents a relevant example of global repartition induced artificially. *Styela clava*, although coming from the northwest Pacific, was accidentally introduced in the East Pacific, Atlantic and European coasts. In Canada, this species has been described to disturb aquaculture, probably due to a overabundant population leading to the decrease of food availability for filter animal culture such as mussels or oysters, which suffer growth delay (Bourque et al. 2007; Arsenault et al. 2009). Coupled with dispersion driven by settlement on mobile artificial supports, some solitary ascidians can extend their life area by taking advantage of artificial waterways. This is the case of the Suez Canal, which has allowed to the endemic species *Herdmania momus* to disperse from the Red Sea toward the Mediterranean Sea (Shenkar and Loya 2008). Taken together, this high tolerance of ascidians to various environments, their capacity to spread thanks to artificial support and their potential impact on food availability for other filter animals make solitary ascidians a suitable model to understand the consequences of invasive species.

In opposition to species presenting a ubiquitous geographical repartition, some ascidians exhibit a specific distribution, making them endemic to a given area. The majority of ascidian species inventories reveal, in addition to new species description, a mixed composition with both ubiquitous and endemic species. This is typically the case in the Port of Salvador or more recently in the Gulf of Mexico. The Brazilian coast is also rich in endemic tunicates, such as the solitary ascidian *Eudistoma vannamei*. Relatively "closed" environments such as the Mediterranean Sea or the Red Sea present various endemic species, likely because of the reduced dispersal capacity compared to open environments. For example, 12 species are considered endemic to the Red Sea, representing 17% of the ascidian diversity (Shenkar and Loya 2008; Shenkar 2012).

Several solitary ascidians have been discovered in low-temperature environments in both the Arctic and Antarctic. *Styela rustica* can live in the north Atlantic in the Svalbard region, a colonization which seems recent (Demarchi et al. 2008). In the southern hemisphere, a number of species have been discovered in the South Shetland Islands such as *Styela wandeli* or *Molgula pedonculata* (Tatian et al. 1998). Antarctic species seem to be particularly adapted to survive in extreme conditions, such as *Cnemidocarpa verrucose*, known to be able to filter all ranges, particularly the finest, of organic particles to get enough nutrients in a poor environment (Tatián et al. 2004).

This large repartition shows also that the majority of solitary ascidians are shallow-water species and live on the continental shelf in harbors, reefs, and various coastal environments. In addition, abyssal species are also documented thanks to several sampling campaigns in the Pacific and other deep-sea regions. Abyssal species from the Pacific are represented by *Molgula sphaeroidea* or *Adagnesia bafida*, also discovered in the Atlantic at a depth of about 3,000 m. The deepest solitary ascidian discovered was in the Pacific at 7,000 m depth. Illustrating the ubiquitous presence of deep-sea species, we can also cite *Agnezia monnioti*, discovered in the Arabian Sea at 3,162 m depth. In *Styela gagetyleri*, localized in the same region but at 368 m depth (which is already considered a deep-sea conditions), the number of folds of the branchial sac is reduced, implying a decrease of cilia quantity and thus oxygen exchange surface. This could result from an adaptation to low oxygen levels and an optimization of the capacity to capture nutrients. Observations and species descriptions have led scientists to notice that abyssal species are in the high majority of solitary ascidians and not colonial ones. It has been proposed that the column shape of the body of solitary species allows a vertical elongation, creating a distance between the siphons and deep-sea soft and muddy sediments, whereas colonial ascidians are closed to the substrate and cover it in such a way that the siphon stays close to the mud, which could be problematic to capture food in a poor environment.

All studies made on Tunicate spatial distribution brought to light that solitary ascidians composed between 20% and 40% of the diversity (others are colonial ascidians) in tropical environments, whereas solitary ascidians represent most of the species at the two poles and temperate climates, with, for example, 58% and 70% of the diversity in the Antarctic and European coasts, respectively. This distribution is explained by the lifestyle of colonial ascidians presenting an indeterminate growth allowing colonization of most biological matter support in rich tropical environments.

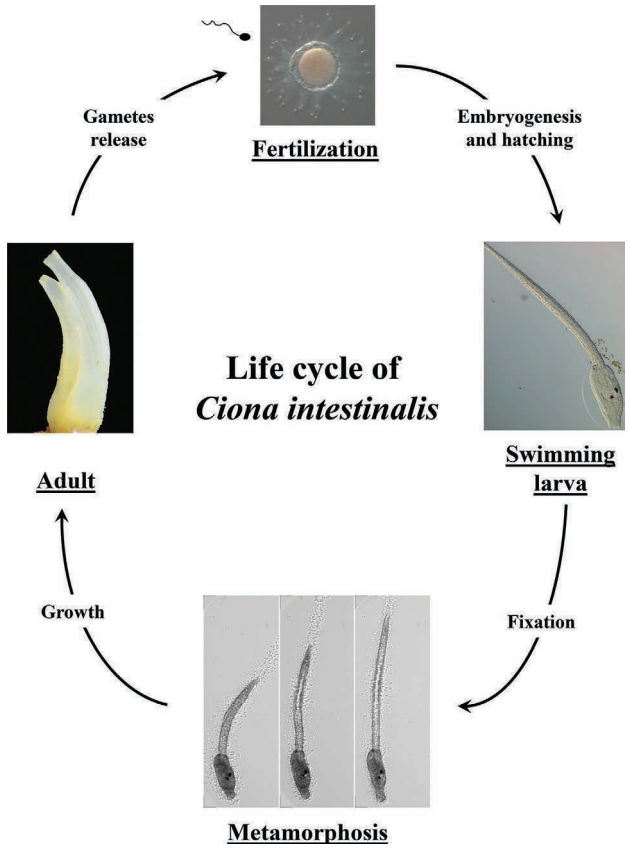

FIGURE 20.2 An example of solitary ascidian life cycle, *Ciona intestinalis*. After gametes are released, embryogenesis takes place in sea water and gives rise to a swimming larva in few hours. After a period of free swimming (four to eight hours in the case of *C. intestinalis*), the larva adheres to a substrate and starts metamorphosis, with the regression tail as the most dramatic event of this process. The pictures of the tail regression were captured from a time-lapse of *C. intestinalis* metamorphosis (Soulé and Chambon, unpublished data, photo credit Soulé and Chambon). After metamorphosis, the juvenile will give rise to a sexually mature adult in one to two months depending on the feeding conditions. (Adult picture photo courtesy of JP Chambon.)

20.4 LIFE CYCLE

Solitary ascidians are characterized by a bi-phasic life cycle (Figure 20.2), composed by a swimming larva and a sessile adult. Adults are usually hermaphrodites, producing both sperm and oocytes, accumulated in two separated gonoducts. Gamete production is controlled by a seasonal cycle and by light, and it can also be managed in culture. When gametes are mature, obscurity or light variations lead to their release in sea water, thereby inducing a synchronization of gamete release between individuals. Cross-fertilization (self-fertilization is usually blocked/sub-efficient) gives rise to a swimming tadpole larva after embryogenesis.

20.4.1 Hatching

At the end of embryogenesis, the fully formed larva is embedded in a chorion composed of a layer of maternal test cells (TCs) surrounded by a vitelline coat (VC) and at the most exterior part by follicular cells (FCs). The first tail movements appear before hatching, and these, coupled with apoptosis of test cells, contribute to the larva escaping from the chorion (Maury et al. 2006; Zega et al. 2006). Tail movements are due to muscle contractions under the control of the larval nervous system (reviewed in Meinertzhagen et al. 2004). From hatching, the larva adopts a pelagic behavior by swimming and dispersing in the environment.

20.4.2 Swimming and Pre-Metamorphic Phase

Using electrophysiological methods to record muscle tail contraction, the swimming behavior of *Ciona intestinalis* was characterized from hatching to the acquisition of metamorphic competence (Zega et al. 2006). Three different larval movements were observed: tail flicks, "spontaneous" swimming and shadow response. The *Ciona* larvae swim for longer periods and more frequently during the first hours after hatching. The swimming behavior changes during the free swimming phase and switches from photopositive to photonegative during the pre-metamorphic period. Using a Morpholino-knockdown approach against Ci-opsin1, the visual pigment expressed in the photoreceptor of the ocellus, it was observed that the *Ciona* larvae swimming behavior was affected (Inada et al. 2003), suggesting a photic control of the swimming phase. Recently, thanks to the recent completion of the *Ciona* larval central nervous system (CNS) connectome (Ryan et al. 2016), a group of photoreceptors that control the switch to the photonegative swimming behavior at the pre-metamorphic phase were identified (Salas et al. 2018). The competency for metamorphosis is acquired a few hours after hatching (8–12 hours in the case of *Ciona intestinalis*) and leads to the research of a substrate by the larvae. In its search for settlement, in addition to visual, geotactic and chemosensory inputs, the larva also exhibits strong thigmotactic behavior (Rudolf et al. 2019). These changes in behavior are probably correlated with the capacity of the larva to respond to a wide variety of external and endogenous signals (reviewed in Karaiskou et al. 2015). The settlement is the first step of metamorphosis and is mediated through the adhesive papilla, localized at the most anterior extremity of the larva. This is done preferentially on substrates (natural as well as artificial) presenting a bacterial film. The onset of metamorphosis is strictly associated with larva adhesion since papilla-cut larva are unable to fully metamorphose (Nakayama-Ishimura et al. 2009).

20.4.3 Metamorphosis

From settlement, the tadpole larva will undergo a metamorphosis characterized by a schematic sequence of events that transform a solitary ascidian larva to a juvenile one (Figure 20.3). Ascidian metamorphosis has been described by Cloney (1982), leading to characterization of ten successive steps globally shared between species despite a few variations: 1) secretion of adhesives by the anterior papilla,

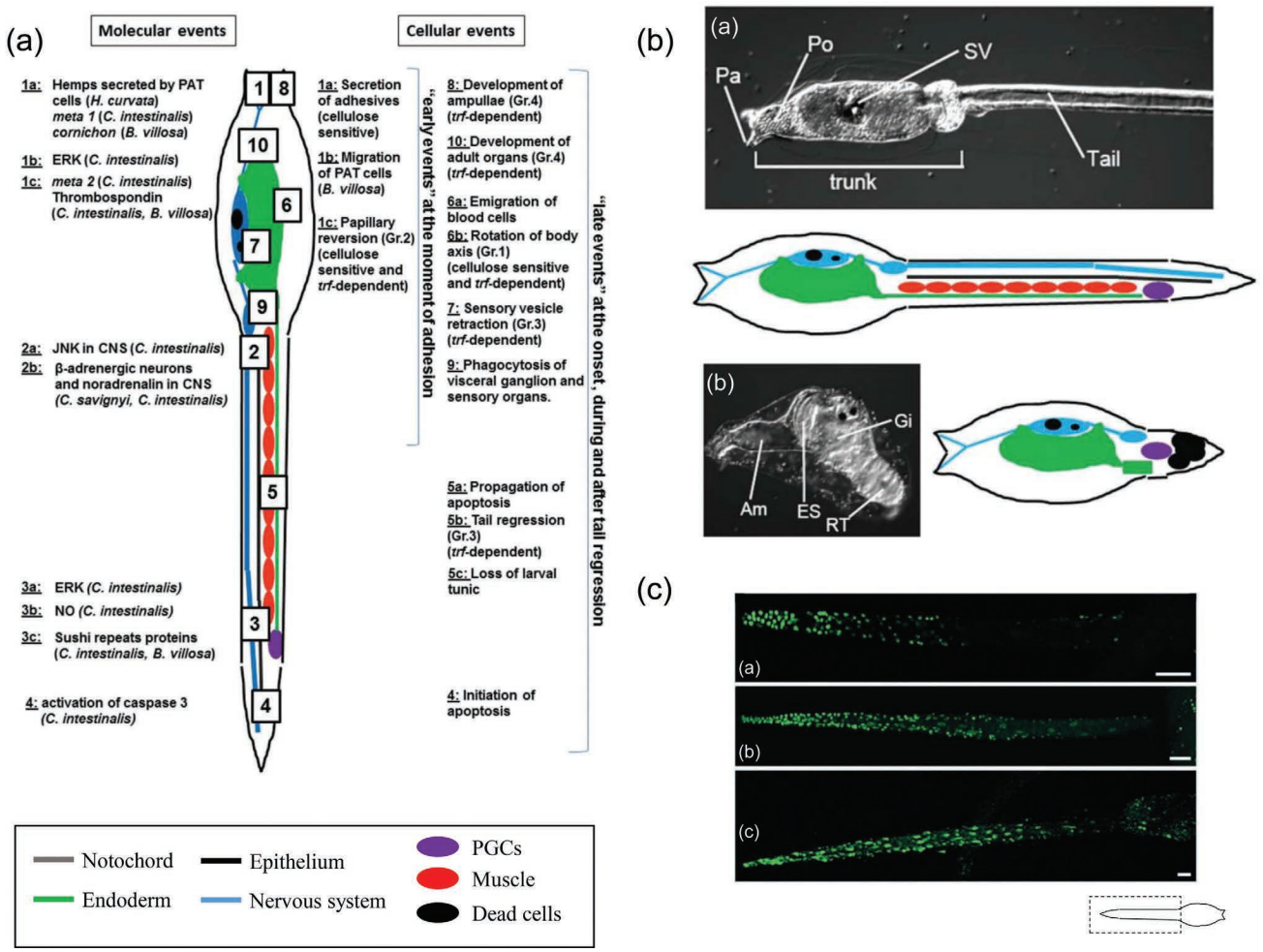

FIGURE 20.3 Metamorphosis of solitary ascidians. (a) Summary of molecular and cellular events that occur at the onset of the metamorphosis in solitary ascidians. Sequential numbers refer to the order of events. Gr.: Group according to classification in Nakayama-Ishimura *et al*. (2009). (b) Metamorphosis of the ascidian *Ciona intestinalis*. From the swimming larva and its schematic representation (a) to a juvenile soon after metamorphosis and its schematic representation (b). Pa, papilla; Po, preoral lobe; SV, sensory vesicle. The preoral lobe of larva is elongated and becomes transparent to be an ampulla (Am). Adult organs, such as endostyle (ES) and gills (Gi) start to develop in the trunk. The tail is retracted toward the trunk (RT). (c) TUNEL labeling of a metamorphic *Ciona intestinalis* larva tail at successive stages (a–c) of the tail regression. Schematic representation to show where the apoptotic cells are detected in the sequential TUNEL labeling. Apoptotic cells appear in green. Scale bars: 220 µm in (a); 140 µm in (b); 80 µm in (c). ([a] Adapted from Karaiskou et al. 2015; [b] adapted from Karaiskou et al. 2015; [c] adapted from Chambon et al. 2002.)

leading to larval settlement; 2) reversion and retraction of the papillae; 3) tail regression, also named tail resorption; 4) loss of the outer cuticle layer composing the tunic; 5) retraction of the sensory vesicles; 6) phagocytosis of sensory organs, visceral ganglion and cells of the axial complex and elimination of other specific larval structures (TLOs); 7) emigration of pigmented and blood cells from the epidermis to the external tunic; 8) digestive gut establishment by an expansion of the branchial basket in addition to visceral organ rotation through an arc of about 90°; 9) a global growth characterized by the expansion and elongation of the ampullae corresponding to the foot of the animals, allowing strong anchoring to the substrate concomitantly with tunic enlargement; and, finally, 10) total disappearance of larval rudiments, followed by the construction of adult tissues (PJOs). Next, the inhalant siphon opens first, and then the opening of the exhalant one allows the circulation of water in the pharynx, and the juvenile becomes ready to filter sea water to feed. In the past 20 years, many studies have allowed better comprehension at the molecular scale of these metamorphic events (reviewed in Karaiskou et al. 2015 and Figure 20.3).

Using gene profiling approaches, the secretion in the papillae of an EGF-like molecule named Hemps, which seems to control larva adhesion, was reported (Eri et al. 1999). The same approach in *Boltenia villosa* and *Ciona intestinalis* identified probable components of this potential adhesion regulated pathway (Davidson and Swalla. 2001; Nakayama et al. 2001). The activation of mitogen-activated protein kinase (MAPK) ERK was also reported in papillae around the time of adhesion and is a prerequisite for the subsequent tail regression event (Chambon et al. 2007).

Simultaneously, the JNK/MAPK pathway is also activated in the CNS, and similarly to the ERK pathway, it is essential for tail regression. The CNS seems to have a preponderant part in the onset of metamorphosis; expression of the β_1-adrenergic receptor was reported in this tissue in *Ciona intestinalis* and *Ciona savignyi* (Kimura et al. 2003). More recently, the neurotransmitter GABA was reported as a key regulator of *Ciona* metamorphosis (Hozumi et al. 2020), reinforcing the previous hypothesis of the preponderant role of the larval nervous system and sensory organs in selecting sites for adhesion and in the onset of metamorphosis (Cloney 1982). One of the most dramatic event of this process is the regression of the tail larva, which occurs a few hours after adhesion. Two not mutually exclusive mechanisms were reported during this event: the first involves the contractile properties of either the tail epithelial layer (observed in the solitary ascidian *Distaplia occidentalis, Aplidium constellatum, Diplosoma, Ecteinascidia turbiniata*, *C. intestinalis, Ascidia callosa, Corella willmeriana macdonaldi* and the colonial ascidian *Botryllus schlosseri*) or notochord cells (observed in *Boltenia villosa, Herdmania curvata, Styela gibbsii, Molgula mahattensis, Molgula occidentalis* and *Polycitor mutabilis*; reviewed by Cloney 1982); the second involves a massive apoptotic cell death of almost all of the cells that composed the tail and was observed in *C. intestinalis* (Chambon et al. 2002; Tarallo and Sordino 2004) and *Molgula oculata* (Jeffery 2002). Recently, using live microscopy, both mechanisms were observed during *Ciona intestinalis* tail regression, and they seem to be sequential, since initial contraction of the tip tail preceded apoptosis (Krasovec et al. 2019). Apoptosis appears to be the driving force of tail regression in solitary ascidians and affects almost all the cell types that compose the tail (the tunic, epidermal, notochord, tail muscle cells and the CNS), with two exceptions, the endodermal strand cells and the primordial germ cells (PGCs) (Figure 20.3). These two cell types escape apoptosis, the endodermal strand by migrating before the tail regression (Nakazawa et al. 2013), while the PGCs move toward the trunk at the time of tail regression in coordination with the progression of cell death (Krasovec et al. 2019). The most remarkable feature is that through sequential TUNEL pictures, it has been confirmed *in vivo* that apoptosis starts at the tail tip and continues up to the tail base by a perfect antero-posterior wave (Chambon et al. 2002; Krasovec et al. 2019). The same polarized propagation of apoptosis was reported in two other species of ascidians, *Molgula occidentalis* and *Asicidia ceratodes* (Jeffery 2002).

An arising and challenging question is the coordination mechanism of the metamorphic events. New insights were provided by the identification of the gene network downstream of the MAPK, ERK and JNK activation previously reported, respectively, in the papillae and the CNS. Among them is *Ci-sushi*, a gene under JNK control, with expression patterns at the tip of the tail, for which loss of function experiments lead to the inhibition of the initiation of apoptosis (Chambon et al. 2007). In addition, papilla and tail cut experiments on larva coupled with analyses of metamorphic mutants (*swimming juveniles* and *tail-regression fail* [*trf*]) allowed classification of metamorphic events in four groups (Nakayama-Ishimura et al. 2009). Group 1 includes a cellulose-sensitive and *trf*-independent event: body axis rotation; Group 2 encompasses a cellulose-sensitive and *trf*-dependent event: papillae retraction; Group 3 includes cellulose-independent and *trf*-dependent events, sensory vesicle retraction and tail regression; and Group 4 comprises cellulose-independent and *trf*-independent events, including ampullae formation and adult organ growth.

20.4.4 Juvenile and Adult

Metamorphosis in ascidians results in a dramatic modification of their body plan, transforming them in a few hours from swimming larva to sessile juvenile and after few months of growing to a sexually mature adult. Classically, juvenile growth timing depends on food availability and temperature. Consequently, the settled phase represents almost the entire life cycle, whereas the swimming phase is transitory and allows the dispersion of individuals.

20.5 EMBRYOGENESIS

20.5.1 Fertilization and Maternal Determinants

Ascidian embryogenesis is a rapid process involving a small number of cells (about 2,600 cells in *Ciona intestinalis*) and occurs within a chorion composed of test cells, a vitelline coat and follicular cells (Figure 20.4). It starts with fertilization, which, in solitary ascidians, occurs after the release of sperm and eggs into the surrounding seawater. To ensure fertilization, spermatozoids are activated and then attracted toward the eggs by a common factor released by mature oocytes (after germinal vesicle breakdown) called sperm-activating and sperm-attracting factor (SAAF) (Kondoh et al. 2008; Yoshida et al. 2002). The ascidians eggs are spawned embedded in a layer of follicular cells surrounding a vitelline coat, under which the test cells enclose the egg itself. In some species, such as *Styela plicata*, sperm and eggs are released at different times, while they are released simultaneously in *Ciona* and *Halocyntia*, allowing sperm to interact with self-eggs. In these latter species, which are known to be self-sterile, a self- and non-self recognition system was reported during fertilization, probably to promote outcrossing. In *Ciona*, this process is ensured by a couple of receptors expressed at the surface of the sperm (s-Themis A and B) and ligands expressed on the VC (v-Themis A and B). If a sperm containing s-Themis A and B interacts with an egg expressing both v-Themis A and B on the VC, its ability to bind the VC is reduced, and it is not able to fertilize the self-recognized egg (Harada et al. 2008). In addition to this self-recognition system, the polyspermy block involves a glycosidase enzyme released from the surface of FCs. It is interesting to notice that this enzyme activity release is not species specific, which means that sperm of a species could block the egg of an another (Lambert 2000). This sperm

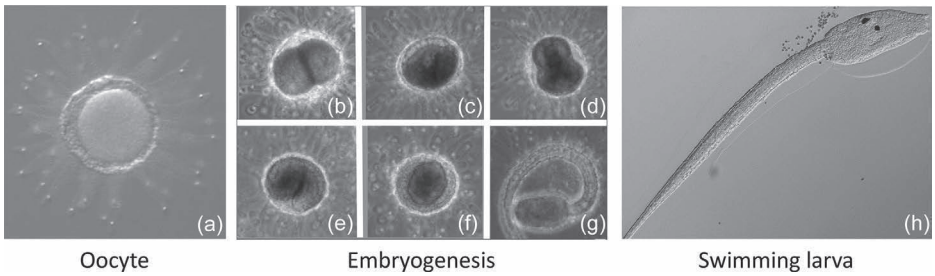

FIGURE 20.4 Embryogenesis of *Ciona intestinalis*. (a) Unfertilized oocyte in its chorion, FC (follicular cells), VC (vitelline coat), TC (test cells) (photo credit S. Darras); (b–g) capture from time-lapse microscopy of *Ciona intestinalis* embryogenesis in the chorion (photo credit J. Soule and JP Chambon); (b) two-cell stage; (c) mid-gastrula; (d) neurulae; (e) early tailbud; (f) tailbud; (g) hatching larva.; (h) swimming larva. (Photo courtesy of JP Chambon.)

competition may participate in the interspecific competition for space, leading to differential abundance of the ascidian community in natural environment.

Sperm entry into the egg results in a rise in calcium concentration through the egg, which initiates development, followed by a series of repetitive calcium waves. These waves are necessary for the completion of meiosis and initiate a signal-transduction cascade which brings about the remodeling of the male pronucleus and cytoskeletal rearrangements, as well as alterations in gene regulation at both the post-transcriptional and post-translational level (Tadros and Lipshitz 2009). The calcium waves are also responsible for the stimulation of ATP production necessary to match the energy demand associated with the onset of development (Dumollard and Sardet 2001). At this stage, the early embryo is dependent on maternal mRNAs and proteins, known as maternal factors, that are produced and stored in the egg during oogenesis to survive and develop prior to the full activation of the zygotic developmental program (Oda-Ishii et al. 2016). The transition from maternal products to zygotic factors occurs starting from the eight-cell stage and is called the maternal-to-zygotic transition (MZT) (Oda-Ishii et al. 2016; Treen et al. 2018). In ascidian embryos, four maternal factors are involved in the establishment of the first zygotic gene expression: ß-catenin, Tcf, Gata.a and Zic.r-a (also called Macho-1).

20.5.2 Ooplasmic Segregation and Establishment of Embryonic Axis

Following the completion of meiosis and the fusion of the male and female pronuclei, a series of synchronous and rapid cell divisions occur, called the cleavage stage. The first cleavage occurs 1 hr 45 min after fertilization in *Halocynthia roretzi* at 13°C and 1 hr in *Ciona intestinalis* at 18°C. Two synchronous and four asynchronous cleavages later, about 9 h later in *Halocynthia* and 5 h later in *Ciona*, the embryo will reach the 110-cell stage and the beginning of gastrulation (Figure 20.5a).

During this cleavage stage, establishment of the primary and secondary embryonic axis occurs. The primary axis, or animal–vegetal (AV) axis, of the embryo is set up during oogenesis. At fertilization, the sperm enters the egg in the animal hemisphere, defined by the position where the polar bodies form, and its nucleus is transported toward the vegetal pole by the actin-dependent contractions of the first ooplasmic segregation (Lemaire 2009; Satoh 1994). The secondary axis, or antero-posterior (AP) axis, is set up orthogonally to the AV axis following ooplasmic movements that localize asymmetric cleavage determinants to the posterior pole of the embryo. This asymmetric partitioning of determinants is responsible for the intrinsically different potentials of the anterior (so-called A- and a-line) and posterior (B- and b-line) blastomeres in response to induction (Feinberg et al. 2019).

20.5.3 Germ Layer Segregation

The 16-cell stage marks the onset of the mid-blastula transition, characterized by asynchronous cleavages, ß-catenin-dependent cell cycle asynchrony (Dumollard et al. 2013) and the appearance of the three germ layers of the embryo—endoderm, mesoderm and ectoderm. This process involves two binary fate choices coupled with the first two A-V-oriented rounds of cell divisions between the 8- and 32-cell stages. In both *Ciona* and *Halocynthia*, the first fate choice identifies the animal and vegetal destinies. It is driven by the transcriptional action of nuclear ß-catenin during the 8- and 16-cell stages, but as of today, the mechanisms responsible for the localization of ß-catenin are still unknown (Rothbächer et al. 2007; Hudson et al. 2013; Takatori et al. 2010).

In the A5.1 cell (Figure 20.5a) at the 16 cell-stage, nuclear localization of maternal ß-catenin controls the segregation of mesendoderm and ectoderm by forming a complex with TCF DNA-binding proteins to mediate the canonical Wnt signalling pathway. An active ß-catenin/TCF complex induces the mesendodermal fate by promoting the expression of notochord/neural/endodermal (NNE) factors *Foxa.a*, *Foxd* and *Fgf9/16/20* and by repressing ectoderm gene expression both directly and indirectly via NNE factors. Cells where the complex is inactive will acquire an ectodermal fate (Figure 20.5b) (Hudson et al. 2013; Hudson 2016).

The second binary fate choice takes place at the transition to the 32-cell stage and leads to the segregation of endoderm

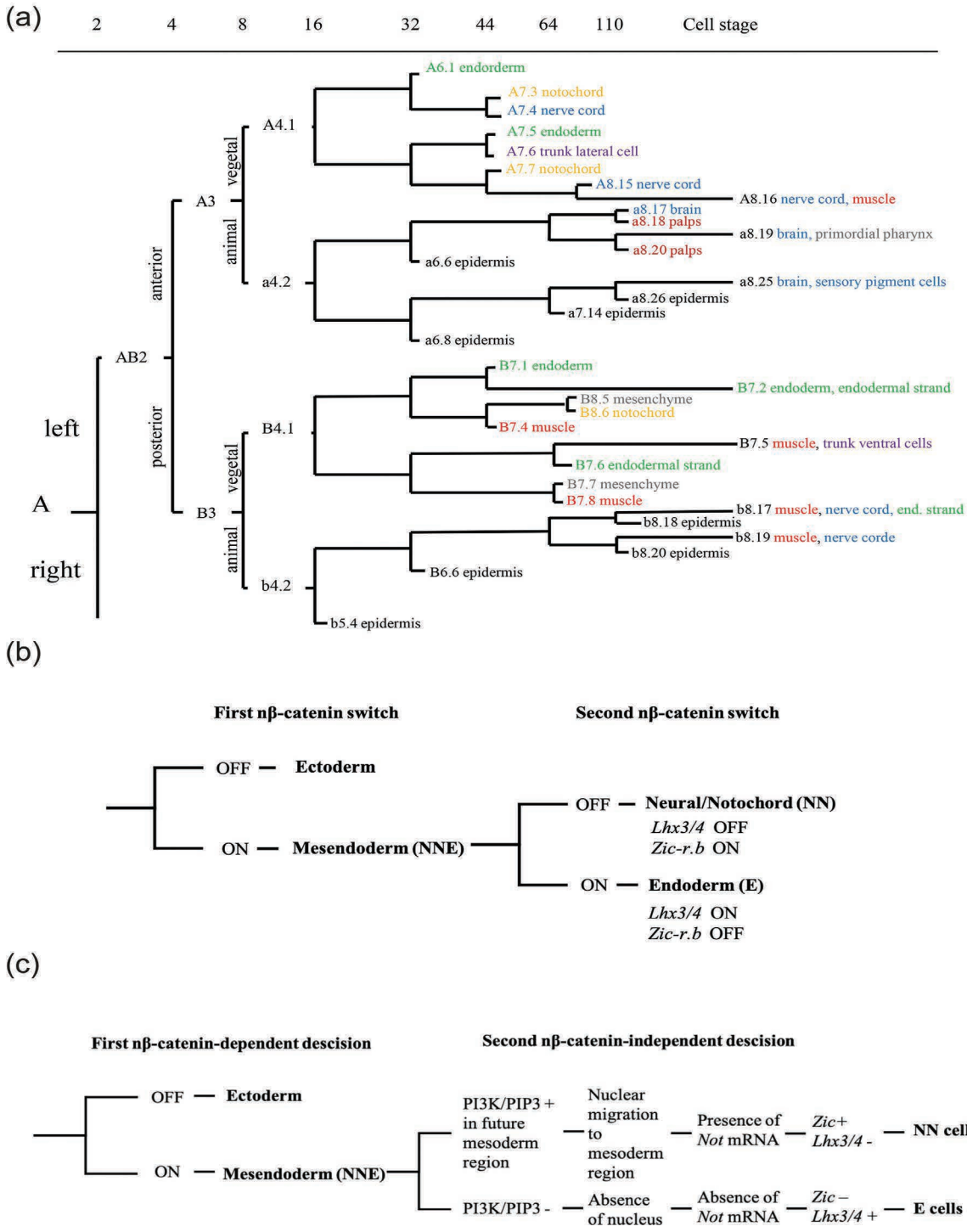

FIGURE 20.5 Cell lineage and developmental fate segregation in solitary ascidian embryos. (a) Cell lineage in ascidians. Lineage tree with the blastomere fate restriction at the successive cell divisions represented by color code (blue: nervous system, green: endoderm, red: muscle, orange: notochord, black: epidermal, gray: mesenchyme, purple: trunk lateral and ventral cells). Since ascidians are bilaterally symmetrical, only the left half of the embryo is shown. (b) Fate segregation in A-line mesendoderm lineages of *Ciona intestinalis*. Two successive rounds of nβ-catenin-driven binary fate decisions that segregate the mesendoderm lineages from the ectoderm lineages at the 16-cell stage and then the neural/notochord (NN) lineages from the endoderm (E) lineages at the 32-cell stage. (c) Fate segregation in the A-line mesendoderm lineage of *Halocynthia roretzi*. Two successive binary fate decisions that segregate the mesendoderm lineages from the ectoderm lineages at the 16-cell stage and then the neural/notochord lineages from the endoderm lineages at the 32-cell stage. The first is nβ-catenin-dependent. The second involves a ß-catenin-independent mechanism involving several Wnt pathway components, as Wnt5a and APC/GSK3 segregation of *not* mRNA transcripts. ([a] Modified from Kumano and Nishida 2007; [b] Hudson et al. 2016; [c] Takatori et al. 2010; Takatori et al. 2015.)

and notochord/neural (NN cells or mesoderm) from mesendoderm precursors. Two distinct regulatory processes have been discovered to achieve the same fate decision in the same A lineage in *Ciona* and *Halocynthia*.

In the case of *Ciona* embryos, this second fate choice involves a second ß-catenin-dependent process during the 32-cell stage. Continued activity of the ß-catenin/TCF complex in mesendodermal cells induces endoderm fate (E cells), whereas inactivation of the complex leads to the acquisition of the notochord/neural fate. During this second phase, ß-catenin/TCF works directly or indirectly in the E cells with the targets of the first phase of ß-catenin activity, *Foxa.a*, *FoxD* and *Fgf9/16/20*, to activate the E specifier *Lhx3/4* and to repress the NN specifier *Zic-r.b* (Figure 20.5B) (Hudson et al. 2016).

In *Halocynthia* embryos, a different mechanism exists. A possible explanation for this difference is the presence of nuclear ß-catenin in NN cells at the 32-cell stage (Hudson et al. 2013). Thus, *Halocynthia* NN specification depends on a Wnt-dependent but ß-catenin-independent mechanism involving *Not* mRNA transcripts. The asymmetrical partitioning of *Not* mRNA regulates the expression of transcription factors required for fate segregation. In endoderm cells, *Not* will be absent, and thus endoderm differentiation will occur. On the contrary, in NN cells, *Not* is present and will promote *Zic* expression as well as repressing *Lhx3/4* expression, thus promoting NN fate and repressing E fate (Figure 20.5c) (Hudson et al. 2016; Takatori et al. 2010; Takatori et al. 2015) (Figure 20.5c).

20.5.4 Larval Tail Muscle Formation

Muscle formation in ascidian is a well-known example of cell autonomous process first demonstrated by Conklin in 1905. However, recent studies have brought to light the importance of cell–cell interaction as another important factor.

At the larval stage, the only fully differentiated and functional muscles are those of the tail and most solitary species present between 18 and 21 muscle cells on either side of the tail. Muscle cells originated either from the primary muscle cell lineage and the B4.1 blastomeres or from the secondary lineage of A4.1 and b4.2 (Figure 20.5a) (Razy-Krajka and Stolfi 2019; Satoh 2013).

The primary lineage consists of 14 muscle cells located on either side of the tail specified following a cell autonomous specification and differentiation involving the Zic.r-a (Macho-1) maternal determinant. Zic.r-a will trigger the primary tail muscle specification regulatory network by activating the transcription of *Tbx6-related* (*Tbx6-r*) muscle determinants at the 16-cell stage and downstream factors at the 64-cell stage (Razy-Krajka and Stolfi 2019; Satoh 2013; Yagi et al. 2005). On the other hand, the secondary lineage gives rise to the muscle cells flanking the tip of the tail, whose numbers vary between species (ten cells of b4.2 origin in *Halocynthia* compared to four in *Ciona).* In the A-line, muscle potential is induced by intricate feed-forward signaling relay from the neighboring b6.5 lineage cells to A7.6 to A8.16. In *Ciona*, the Nodal and Delta/Notch signaling pathways are responsible for this, while in *Halocynthia*, a yet-unknown signal from the same b6.5 lineage induces the expression of Wnt5.a, which then promotes muscle fate in A8.16 (Figure 20.5a) (Tokuoka et al. 2007). Finally, the last muscle/neural cell fate decision in *Ciona* will see FGF/ERK signaling activating the muscle determinants *Tbx6-r.b* and *Mrf* expression. In *Halocynthia*, what regulates this final fate decision is yet another unknown parameter, but FGF/ERK signaling is not involved (Razy-Krajka and Stolfi 2019; Tokuoka et al. 2007).

20.5.5 Neural Plate Patterning

Similar to vertebrate neurulation, the ascidian neural plate is curled up dorsally to form a tube-like structure known as the neural tube. The neural plate emerges at the mid-gastrula stage and is composed of 40 cells at the neural plate stage, arranged in six rows and eight columns of cells along the A-P axis formed from posterior to anterior. The I and II rows compose the posterior neural plate and derive from the A-lineage. They will contribute to the caudal nerve cord, motor ganglions and posterior sensory vesicle. On the other hand, the a-lineage will give rise to the anterior four rows III to VI. Rows III and VI will contribute to the anterior part of the sensory vesicle, part of the oral siphon primordium and anterior brains. Finally, rows V and VI give rise to neurons of the peripheral neural system (PNS) (Hudson 2016; Imai et al. 2009; Wagner and Levine 2012). Once the neural tube is completely closed, the tail becomes distinguishable (Kumano and Nishida 2007).

Different signaling pathways are responsible for the patterning of the neural plate, such as Nodal, Nodal-dependent Snail, FGF/MEK/ERK and Delta/Notch (Hudson 2016; Hudson et al. 2007; Razy-Krajka and Stolfi 2019; Satoh 2013).

20.5.6 Neural Development

The ascidian nervous system is composed of the peripheral neural system and the central nervous system, and its development starts with neural induction at the 32-cell stage. CNS development starts in two blastomeres, pairs A6.2 and A6.4 (Figure 20.5a), which become neural fate restricted at the 64-cell stage under FGF induction (Hudson et al. 2016). It consists of approximately 330 cells and about 117 neurons and originates from three lineages: the A and a- and b-lines (Hudson et al. 2007). The CNS presents three morphologically distinct structures: the anterior-most sensory vesicle, the trunk ganglion (also called visceral ganglion) and the tail nerve cord (Hudson et al. 2016). The A-line blastomeres become fate restricted following a neuro-epidermal binary fate decision involving a β-catenin-driven binary fate switch. This lineage will give rise to the posterior part of the sensory vesicle as well as the ventral and lateral parts of both trunk ganglion and tail nerve cord (Hudson et al. 2013). The anterior part of the sensory vesicle and the dorsal part of the visceral ganglion and tail nerve cord respectively

originate from the a-line (a6.5) and b-line (b6.5) blastomeres, which become restricted to neural fate at the 112-cell stage (Hudson et al. 2016; Roure et al. 2014).

PNS development starts with the birth of the a6.5 blastomere (Figure 20.5a). It is composed of different types of epidermal sensory neurons (ESNs): the papillary neurons of the adhesive papillae, the epidermal sensory neurons and the bipolar tail neurons (BTNs) distributed in the epidermis of the trunk and tail (Hudson 2016; Meinertzhagen and Okamura 2001).

20.5.7 Cardiac Development

The adult ascidian heart consists of a one-cell-layer single myocardial tube surrounded by a pericardium. It is formed of two distinct territories: the first heart field (FHF) and the second heart field (SHF) and originates from a single pair of blastomeres in the 64-cell stage embryos, the B7.5 cells (Figure 20.5a). The first division of the cardiac founder cells is symmetric and occurs during gastrulation. It leads to the appearance of two symmetrical pairs of pre-cardiac founder cells each consisting of a B8.9 and B8.10 blastomeres (Figure 20.5a) (Cooley et al. 2011). During neurulation, in each pre-cardiac lineage, founder cells divide a second time, asymmetrically this time, and each blastomere will give rise to four cells: two small anterior cells, which will migrate to form the heart, and two large posterior B7.5 granddaughter cells, which will differentiate as anterior tail muscles in both Halocynthia and *Ciona* (Figure 20.5a) (Christiaen et al. 2010; Davidson et al. 2006).

Two maternal determinants are responsible for the specification of the blastomeres: macho-1 and β-catenin. They activate the B7.5-specific expression of the transcription factor Mesp (Christiaen et al. 2009; Stolfi et al. 2010), which determines a competence domain facilitating either pre-cardiac or pre-vascular specification (Satou et al. 2004). Within the Mesp-expressing cells, subsequent inductive signals will induce specific identities. In the future cardioblasts, Mesp, in conjunction with FGF/MAPK signaling, will activate downstream components of the core cardiac regulatory (Davidson et al. 2006). BMP and FGF signalling will then either directly or indirectly regulate cardiac target gene expression of *FoxF* and the heart determinants *Nkx2.5*, *GATAa* and *Hand-like/NoTrlc* in the anterior the trunk ventral cells (Christiaen et al. 2010).

Following the second division, a first FGF-dependent migration of the trunk ventral cells (TVCs) to the ventral trunk region occurs. There they will undergo a series of successive asymmetric divisions along the mediolateral axis, followed by a second migration that will lead to a segregation of the heart cells from the lateral TVCs, precursors of the atrial siphon muscle (ASM) cells (Stolfi et al. 2010). The TVCs migrate dorsally toward each side of the trunk, where they will settle as a ring of cells at the base of the atrial siphon primordia (Stolfi et al. 2010).

20.5.8 Notochord

The ascidian larval notochord is composed of a single row of 40 cells that form through intercalation and originate from two of the four founder cell lineages. The anterior 32 notochord cells, termed the primary notochord, derive from the A-line founder lineage, whereas the posterior eight cells, termed the secondary notochord, are generated from the B-line founder lineage.

The anterior notochord precursors originate from A6.2 and A6.4 blastomeres, which are bipotential notochord/nerve cord precursors at the 32-cell stage. They are induced at the 32-cell stage and acquire developmental autonomy at the 64-cell stage (Jiang and Smith 2007).

In *Ciona* embryos, FGF and MAPK signaling are required at the 32–64-cell stage to polarize the blastomeres, which will divide asymmetrically into the induced notochord precursors and nerve cord precursors, which are the default fates (Hashimoto et al. 2011). In the secondary notochord lineages, which become fate restricted at the 110-cell stage (Jiang and Smith 2007), FGF signaling is necessary for two processes. It is first required at the 64-cell stage to suppress muscle fate in the mother cell of the notochord and mesenchyme precursors (Darras and Nishida 2001; Imai et al. 2002; Kim and Nishida 1999; Kim et al. 2000; Kim and Nishida 2001). Second, it is required to activate expression of *Ci-Nodal* in the b6.5 blastomere at the 32-cell stage, which is required for the specification of the secondary notochord precursor (Hudson and Yasuo 2005; Hudson and Yasuo 2006).

In the primary notochord precursors of *Halocynthia*, FGF is expressed in the notochord precursor and inhibited in the nerve cord precursor cells by the *Efna.d* signal coming from the animal hemisphere (Satou and Imai 2015). FGF expression leads to activation of Hr-Ets, which, coupled with Hr-FoxA and Hr-Zic.r-d, promotes the expression of the notochord-specific gene *Brachyury* (*Hr-Bra*) at the 64-cell stage. *Bra* then activates various downstream genes that are essential for notochord formation (Hashimoto et al. 2011). BMP2/4 is, on the other hand, implicated in the secondary notochord induction in *Halocynthia*. BMP2/4 is involved in the asymmetric cleavage of the B7.3 blastomeres as well as in the specification of secondary notochord cells (Darras and Nishida 2001).

20.5.9 Primordial Germ Cells

Primordial germ cells are the founders of gametes. It has been observed in several animals that the germ line is set aside early in embryogenesis and has to be "maintained" until differentiation of gametes in the mature gonads. PGCs can be specified by either inheritance of maternal determinant (pre-formation) or by induction (epigenesis). In ascidians, PGCs are specified during embryogenesis in posterior-vegetal blastomeres by the inheritance of postplasmic/PEM mRNAs in B7.6 blastomeres (reviewed in Kawamura et al.

2011), among them *Ci-Vasa*, an ATP-dependent DEAD-box RNA helicase, and *pem1*, which have been shown to repress mRNA transcription by inhibiting activating phosphorylations on the C-terminal domain (CTD) of the RNAPII (Shirae-Kurabayashi et al. 2006; Shirae-Kurabayashi et al. 2011; Kumano et al. 2011). During gastrulation, B7.6 divides asymmetrically, giving rise notably to B8.12, the founder of the eight PGCs localized at the tip of the larva tail at the end of the embryogenesis.

These cells will remain at this localization until the tail regression at metamorphosis, during which the PGCs will reach the trunk and the presumptive gonad.

20.6 ANATOMY

20.6.1 Larva

Anatomy of the larva is fundamental to the understanding of the phylogenetic affiliation of urochordates. The characteristic chordate body plan allowed Kovalevsky to discover that ascidians are closer to vertebrates and cephalochordates. The ascidian larva presents a morphology divided in two parts: the anterior trunk and the posterior tail. The larva is usually composed of a low number of cells, 2,600 cells in the case of *Ciona* intestinalis. A typical anatomy is common to the solitary ascidian larva with some tissues present all along the larva, whereas others are specific to the tail or to the trunk (Figure 20.6a).

The totality of the larval body is surrounded by the tunic, which is composed of a cellulose derivative, the tunicine. The epidermis, under the tunic, covers the entire animal body. Two internal tissues are distributed along the entire antero-posterior axis. The central nervous system is characterized by a dorsal neural tube as in a classical deuterostomian body plan organization. In the most anterior part of the trunk, neurones of the CNS compose the adhesive papilla, a sensitive structure which interacts with the environment to find a suitable substrate. These adhesive papillae allow the fixation of the larva. From the adhesive papilla to the tip of the tail, the CNS is then composed by the brain, in the trunk, the nerve ganglion which allows the junction between the posterior trunk and the most anterior part of the tail and finally the neural tube prolonged until the tip of the tail. Additional peripheral neurons are distributed along the tail epidermis. In the brain composing the CNS, an otolith and an ocellus are present and allow analyses of gravity and luminosity, respectively. The second tissue present both in the trunk and the tail is the endoderm. Endoderm is present in the postero-ventral part of the trunk and is prolonged in the ventral side of the tail by a line of cells named the endodermal strand.

The other tissues are specific either to the tail or of the trunk. In the tail, ventrally to the CNS but dorsally to other tissues, the notochord is present in almost the total length of the tail. Note that the presence of the notochord in the larva, absent in the adult, argues in favor of this model as suitable for the study of the anatomy and development of embryos and larvae to better understand animal evolution. The notochord plays the role of support structure for the muscles distributed laterally along the tail. These muscles allow the swimming movement of the larva after hatching and research on an adapted support for the settlement. Last, in the ventral side of the tip of the tail, in the posterior prolongation of the endodermal strand, are eight localized primordial germ cells, which will give rise to the gonads and the gametes in the adult. Finally, the larval trunk houses the heart in its ventral side and a sub-developed gut with a non-functional stomach. The outline of the pharynx is also present.

After hatching, the swimming phase and settlement lead to the metamorphosis phase, which will give rise to the adult animal. Tissues have been divided in three groups by Cloney according to their fate during metamorphosis, and this classification is still used. Group 1 correspond to tissues that exclusively function in the larval stage (transitory larval organs or TLOs) and can disappear during the metamorphosis; group 2 are tissues that function in both larval and adult stages (larval-juvenile organs/tissues or LJOs), conserved during the metamorphosis transition; and group 3 includes tissues emerging during the metamorphosis and consequently exclusively functioning in juvenile and the next adult stage (prospective juvenile organs or PJOs). Adult anatomy depends on LJOs, and PJO tissues compose a typical morphotype of solitary ascidians.

20.6.2 Juvenile and Adult

The adults of solitary ascidians are characterized by a bag-shaped morphology settled by a foot and distally to the point of fixation two siphons with sensory organs (usually a paired number) distributed around their opening (Figure 20.6b). The largest siphon, farthest from the foot, is the inhalant one, which allows the entry of the sea water in a large and surdimensioned pharynx upholstered with mucus and gill slits allowing respiration and filtration of nutriments. The pharynx is supported by a developed endostyle along its height on the side of the animal carrying the inhaling siphon. At the basis of the pharynx is the esophagus, driving aliments to the stomach, localized in the foot of the ascidian proximally to the substrate. From the stomach, the intestine climbs upward and the anus opens into the peribranchial cavity, opened on the outside by the exhaling siphon. Near the stomach, the heart surrounded by a pericarp manages the circulation through a few vessels carrying blood cells through the animal via a circuit organized around the gill sac. Around the stomach and the heart are localized the gonads, one for solitary ascidians belonging to Phlebobranchia and Aplousobranchia, two for those belonging to Stolidobranchia. Gonads produce both sperm and oocytes, which accumulate in two separated gonoducts alongside to the exhalant siphon parallel to the gut. Distally to the substrate and localized between the two siphons is the nerve ganglion from which the innervation is made toward the other organs of the animal. Finally, muscles are distributed all over the animal, participating in the maintenance body shape and fundamentally in pharynx contraction, thus allowing control of the water flow and its

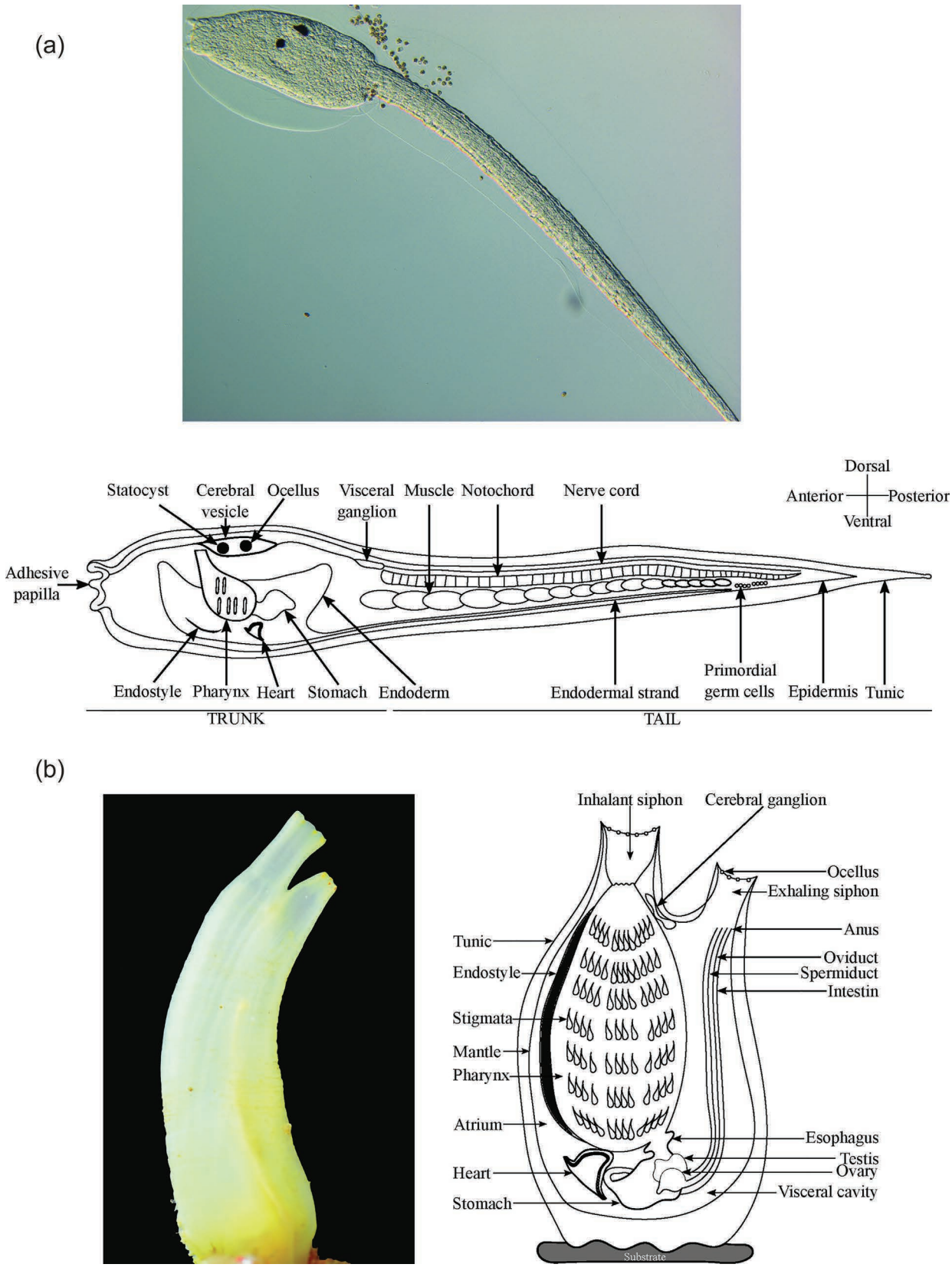

FIGURE 20.6 Classical anatomy representative of solitary ascidians. The larva, composed of a trunk and a tail, present a typical deuterostomian organization plan with a dorsal notochord. Adults are filtering individuals permanently settled to a substrate. Their body is organized around the pharynx and the two siphons, allowing circulation of water bringing food and oxygen.

brutal expulsion if necessary. In addition, muscles surround the siphons and allow them to open or close according to the animal's behavior.

20.7 GENOMIC, TRANSCRIPTOMIC, PROTEOMIC AND BIOINFORMATICS RESOURCES (DATABASES)

20.7.1 Genomics

The first solitary ascidian genome published was of *Ciona intestinalis* type A (now renamed *Ciona robusta*) in 2002, and most of the genomic DNA used for sequencing were isolated from the sperm of a single individual in Half Moon Bay, California (Dehal et al. 2002). The draft genome has been generated by the whole-genome shotgun method (WGS) with eight-fold coverage (Dehal et al. 2002). In this method, the whole genome of *Ciona* was fragmented (in around 3 kbp fragments) and cloned into plasmids (genomic library) for sequencing. In addition, two other libraries were made for this project, one with a mix of genomic DNA of three Japanese individuals cloned into bacterial artificial chromosomes (BACs) for BAC end sequencing and one from another Californian individual cloned into cosmids for cosmid library sequencing (Satoh 2004). Thanks to bioinformatic tools, all these reads were organized into overlapping contigs and then into scaffolds. The *Ciona* genome is approximatively ~159 Mb (comparable with *Drosophila*), rich in AT (65%; as a comparison, the human genome has 45%) and is composed of ~117 Mb of non-repetitive and euchromatic sequences, ~18 Mb of high-copy tandem repeats such as rRNA or tRNA and ~17 Mb of low-copy transposable elements (Satoh 2004). Like those of other invertebrates, the *Ciona* genome exhibits a very high level of allelic polymorphisms, with 1.2% of nucleotides differences between alleles. In 2008, the genome assembly was improved and led to the identification of 15,254 genes, 20% residing in operons, which contain a large majority of single-exon genes (Satou et al. 2008). Another particularity of the *Ciona* genome is its compaction, highlighted by the number of identified genes (15,254) in 117 Mb of euchromatic genome, which gives an average of a gene every 7.7 kb. Using the two-color fluorescent *in-situ* hybridization technique (FISH), a large part (around 82%) of the non-repetitive and euchromatic DNA has been mapped onto chromosomes but also a part of the rDNA and histones clusters (Shoguchi et al. 2006, 2008). *Ciona intestinalis* has 14 pairs of chromosomes, which are in majority telocentric. More recently, a new *Ciona intestinalis* type A assembled genome was published; this genome was sequenced by the Illumina technique and comes from an inbred line. This new genome suggests a previous overestimation on the genome size, since almost the entire genome was sequenced on ~123 Mb. This study also predicts a lower number of identified genes (14,072), which are all mapped on chromosomes (Satou et al. 2019).

From these genomes released, the genes involved in development are well characterized, among them transcription factors (~643), but also genes engaged in a variety of signaling and regulatory processes reported in vertebrate development, such as FGFs (Satou et al. 2002a), Smads (Yagi et al. 2003) and T-box genes (Takatori et al. 2004). Interestingly, developmental genes appear to be often a single copy in the *Ciona* genome, while they have been duplicated in vertebrates, simplifying functional studies, and they could help unravel complex developmental processes in vertebrates. In addition, some evolutionarily innovations were reported, such as a group of genes engaged in cellulose metabolism (Nakashima et al. 2004). There are also several lost genes in the *Ciona* genome, for example, several Hox genes (Hox7, 8, 9 and 11).

Taken together, these studies and the knowledge they brought (sequencing, annotation, physical map) make the *Ciona intestinalis* genome among the most useful to allow investigation at a global scale (chromosomal and genome-wide) of the regulation of gene regulatory networks during development. The *Ciona* genomic information is accessible at https://genome.jgi.doe.gov/portal/ but also in others databases (see the following for details).

Today, with the emergence of the high-throughput–next generation sequencing (reviewed in Pareek et al. 2011), genomes of several solitary but also colonial ascidians genomes have been performed. Interestingly, the choice of sequenced species is well distributed on ascidian phylogeny (Figure 20.1). Indeed, in addition to *Ciona intestinalis* type A (*Ciona robusta*), five Phlebobranchia were sequenced, two *Phallusia* (*Phallusia mammillata* and *Phallusia fumigata*), two additional *Ciona* (*Ciona savignyi, Ciona intestinalis type B*) and one *Corella* (*Corella inflata*); seven Stolidobranchia, three *Molgula* (*Molgula oculata*, *Molgula occulta*, *Molgula occidentalis*), one *Botrylloides* (*Botrylloides leachii*), one *Botryllus* (*Botryllus schlosseri*) and two *Halocyntia* (*Halocyntia roretzi*, *Halocyntia aurantum*). All these genomes and gene annotations are available in the ANISEED database (see the following for details). These genome decoding works allow comparative genomics of ascidians and promise very interesting insights into the A5.1 cell (Figure 20.5a) at the 16-cell stage for ascidian but also chordate evolution.

20.7.2 Transcriptomic

The first information about the ascidian transcriptome was obtained by express sequenced tag (EST) analyses (Satou et al. 2002b). This approach is based on the generation of cDNA clones from total mRNA purification in order to get gene expression information. The cDNA project conducted on *Ciona intestinalis* has generated gene expression information at different developmental stages of *Ciona*, such as fertilized egg, cleaving embryo, gastrulae/neurulae, tailbud embryo and tadpole larva but also in adult tissues corresponding to testis, ovary, endostyle, neural complex, heart and blood cells and whole young adults (Satoh 2013). This classification has also led to temporal and spatial information of gene expression; since the cDNA libraries used for EST analyses were not amplified or normalized, an abundance of

EST in each stage or tissue may reflect gene specific expression (EST count) (Satoh 2013).

These clones were sequenced and categorized (based on similarity to known proteins), and numbers of them were subjected to analysis by whole-mount in situ hybridization (ISH), revealing expression patterns of up to 1,000 genes during *Ciona* development and in adults (Satou et al. 2002b). Coupled with genomic information, cDNA analyses led to the identification and spatial expression profiles of almost all transcription factor genes, among them 46 basic helix-loop-helix, 26 basic leucine zipper domains, 15 E-twenty-six, 24 forkhead box, 21 high motility group, 83 homeobox family members and 17 nuclear receptor family members (Satou et al. 2003a; Wada et al. 2003; Yagi et al. 2003; Yamada et al. 2003) of genes encoding proteins involved in major signaling pathways (receptor tyrosine kinase, MAPK, Notch, Wnt, TGF-β, hedgehog, JAK/STAT) (Satou et al. 2003b, 2003c; Hino et al. 2003) but also gene encoding proteins involved in major cellular processes (cell polarity, actin dynamics, cell cycle, cell junction and extracellular matrix) (Sasakura et al. 2003b, 2003c; Kawashima et al. 2003; Chiba et al. 2003).

All the published and unpublished spatiotemporal data concerning EST included in the cDNA library and EST count are available in the GHOST and ANISEED databases.

A similar EST approach was conducted on five different developmental stages of the anural ascidian *Molgula tectiformis* and gives new insights on the molecular mechanisms of the tailless mode of development of this species (Gyoja et al. 2007).

From these initial works, different types of microarrays were prepared, coupled with cell sorting allowing the identification of the gene regulatory networks involved during heart precursor migration (Christiaen et al. 2008). Microarrays coupled with chemical inhibitors of either JNK or ERK/MAPK pathways also led to the identification of gene networks involved in the onset of metamorphosis (Chambon et al. 2007).

More recently, the recent emergence of single-cell RNA sequencing (scRNA-seq), coupled with previous genomic and transcriptomic data, revolutionized, as in other experimental models, the way to investigate cell specification during embryogenesis by allowing identification of novel cell types, or cell-state and dynamic. Applied to *Ciona* embryogenesis, from gastrulation to tadpole larva, scRNA-seq permitted the identification of 40 new cell types (40 neuronal subtypes) in the larva (Cao et al. 2019). In addition, this study also allowed a better comprehension of the evolution of vertebrate telencephalon by comparing *Ciona* larva gene expression data with other chordate animals.

In addition to EST data, new transcriptomic data coming from RNA-seq technologies and microarray are also integrated in ANISEED (see database section for details).

20.7.3 Proteomics

In addition to genomics and transcriptomics, proteomics completes the set of necessary data to address fundamental questions in developmental but also cell biology of solitary ascidians. These data were generated using the protein mass fingerprint-based method in which previous cleavage into smaller peptides of protein of interest is followed by mass spectrometry analysis (MALDI/TOF), eventually with a previous separation of proteins on 2D-gel electrophoresis.

Compared to genomics and transcriptomics, a few proteomics studies were reported, but recently this approach seems to be used as a tool to evaluate the environmental impact of ascidians. Using two conditions to rear *Ciona intestinalis*, at 18°C (the usual working temperature) and 22°C, a clear distinction in the protein expression pattern in ovaries was observed (Lopez et al. 2017). It was previously known that the reproductive capacity of this species is altered by temperature up to 20°C; in this study, a range of temperature-response proteins were identified, making proteomics on *Ciona* a good approach to evaluate the impact of global temperature change. More recently, a proteomics approach was performed on two solitary ascidians, *Microcosmus exasperatus* and *Polycarpa mytiligera*, both collected at different locations on the Mediterranean coast of Israel (five sites) and along the Red Sea coast (four sites) (Kuplik et al. 2019). Differentiated protein profiles were obtained in the two ascidians from different localities. Here again, proteomics analysis of ascidians may reflect the conditions in their environments and make this approach a potential good biomarker for monitoring coastal marine environment health.

Furthermore, proteomic methods in *Ciona* were used to investigate sperm cell components and to examine their functions (reviewed in Inaba 2007) but also to study the function and interactions of gametes (Satoh 2013). In addition, a proteomic analysis on three embryonic stages of *Ciona* intestinalis (unfertilized eggs 16-cell stage and tadpole larvae) allowed the creation of a protein expression profile and provided a dynamic overview of protein expression during embryogenesis. Interestingly, when a protein dataset was compared with mRNA levels at these same stages, nonparallel expression patterns of genes and proteins were observed (Nomura et al. 2009). In many cases, a change in protein network, protein expression, protein modification or localization is independent of gene expression or translation of new mRNA transcripts. A proteomic-based approach is capable of highlighting differential protein expression or modifications and will be essential to understand molecular mechanisms that sustain developmental process and/or cell behavior or cell fate in ascidians.

Ascidian proteomic datasets are available in the CIPRO database, which is an integrated *Ciona intestinalis* protein database (www.cipro.ibio.jp).

20.7.4 Databases

Several databases are available for the ascidian research community, and most of them emerged from ascidian laboratories. In this section, we provide a short description of the principal databases with a particular emphasis on GHOST and ANISEED, which are the main ascidian databases for the worldwide scientific community.

- The Ascidians Chemical Biology Database (ACBD) (created in 2010 in Japan) is a bibliographical database that compiles publications concerning the effect of chemical compounds on ascidian development and tends to promote ascidians as a model organism for whole-animal chemical screening.
- The Database of Tunicate Gene Regulation (DBTGR) (created in 2005 in Japan) focuses on tunicate gene regulation, including regulatory elements in the promoter region and the associated TF. In addition, it integrates a list of gene reporter constructs.
- The website of the Joint Genome Institute (JGI) (created in 1997 in United States) hosts the *Ciona intestinalis* type A genome and contains a genome browser.
- MAboya Gene Expression pattern and Sequence Tags (MAGEST) (created in 2000 in Japan) provides *Halocyntia roretzi* 3'- and 5'-tag sequences (20,000 clones) from the fertilized egg cDNA library, the amino acid fragment sequences predicted from the EST data set and the expression data from whole-mount in situ hybridization.
- *Ciona intestinalis* Adult *In Situ* hybridization Database (CiAID) (created in 2009 in Japan) gives access to gene expression patterns in adult juveniles with a body atlas.
- The *Ciona intestinalis* Protein (CIPRO) database (created in 2006 in Japan) is a *Ciona intestinalis* protein database that contains 3D expression profiling, 2D-PAGE and mass spectrometry-based large-scale analyses at various developmental stages, curated annotation data and various bioinformatic data.
- Four-Dimensional Ascidian Body Atlas (FABA) (created in 2010 in Japan) contains ascidian three-dimensional (3D) and cross-sectional images through the developmental time course (from fertilized egg to larva) to allow morphology comparison and provide a guideline for several functional studies of a body plan in chordate. Note that a second database called FABA2 (created in Japan) exists, focusing on later developmental stages, from hatching to seven-day-old juveniles.
- *Ciona intestinalis* Transgenic Line RESsources (CITRES) (created in 2012 in Japan) provides the ascidian research community with transgenic lines but also contains DNA constructs to perform transgenesis, image collections of *Ciona* GFP-expressing strains and publications.
- Ghost database (originally created in 2002 in Japan; http://ghost.zool.kyoto-u.ac.jp/) is one of the first ascidian databases available for the ascidian research community and the most useful from the beginning. This database provides all the data concerning the *Ciona intestinalis* EST project conducted by Satoh's lab (see transcriptomic section for details), such as EST count, that provide temporal expression information and published and unpublished ISH at several developmental stages. In addition to that, the database contains a genome browser, a search engine for specific expression or expression pattern of a given genes and gene annotation. At the beginning, this database represented an extraordinary source of molecular tools, since it provides a set of 13,464 unique cDNA clones available as the "*Ciona* intestinalis gene collection released" for the scientific community, ready for use in cDNA cloning, microarray analysis and other genome-wide analyses. Almost the entire database is now integrated in the ANISEED database.
- ANISEED (created in 2010 in France; www.aniseed.cnrs.fr) is the biggest and most complete database for the ascidian community (Dardaillon et al. 2019). There is a constant input of new data, and it provides functionally annotated gene and transcript models in both wild-type and experimentally manipulated conditions using formal anatomical ontologies. The advantages of this database are the extra information, going beyond genes by pointing out repeated elements and cis-regulatory modules and also providing orthology comparison within or even outside ascidians (tunicates, echinoderms, cephalochordates and vertebrates). There are enhanced functional annotations for each species, achieved by an improved orthology detection and manual curation of gene models. This database is user friendly, with three types of browsers, each offering a different but complementary point of view: a developmental browser which selects data based either on the gene expression or the territory of interest, an advanced genomic browser focusing on gene sets and gene regulation and a genomicus synteny browser that explores the conservation of local gene order across deuterostome. This later new release has a reference of the taxonomic range of 14 species, among them a non-ascidian species, the appendicularian *Oikopleura dioika*, which is a novelty. Finally, the new and powerful Morphonet morphogenetic browser enables a 4D exploration of gene expression profiles and territories.

20.8 FUNCTIONAL APPROACHES/TOOLS FOR MOLECULAR AND CELLULAR ANALYSES

In addition to classical over/ectopic expression of genes, several tools or technical approaches were developed by the ascidian community by taking advantage of biological particularities and/or experimental advantages offered by solitary ascidians.

20.8.1 Microinjection/Electroporation

To follow specific expression patterns of regulatory genes or to probe gene function, experimental biologists usually introduce reporter constructs or synthetic mRNA in

fertilized eggs. In most animal models, these approaches are usually achieved by the microinjection technique. Solitary ascidians, essentially *Ciona*, allow an alternative technique, a simple electroporation method. This permits manipulation and screening of hundreds of synchronous developing embryos, either wild type or mutant, thus allowing greater confidence in functional screening, which is not possible with most of the other animal models.

20.8.2 Reporter Gene

The efficient introduction of reporter constructs by electroporation (Corbo et al. 1997), coupled with the facility (compared to the other animal models) to identify and clone the core promoter and associated enhancers of a given gene, made the solitary ascidian *Ciona intestinalis* an excellent model to study cis-regulation. Indeed, due to the *Ciona* compact genome, the *cis*-regulatory elements (CREs) are usually located within the first 1.5 kb upstream of the transcription start site, making it relatively easy to capture significant transcriptional units and clone them upstream of a reporter gene to drive its expression. Coupled with the electroporation technique, this allows a simple and rapid generation of hundreds of transient transgenic embryos expressing fluorescent proteins, which develop quickly to the larval stage (Zeller et al. 2006). These transient assays allowed rapid identification and characterization of up to 83 *Ciona cis*-regulatory elements, almost all enhancers, which activate transcription in a more or less tissue-specific manner (reviewed in Irvine 2013).

20.8.3 Loss-of-Function Approaches

To understand the molecular basis of development, experimental biologists expect to specifically inhibit the functions of a particular gene in particular cells at particular developmental stages. The basic technologies for examining gene functions by loss of function approaches have been established in *Ciona*, such as the knockdown of genes by antisense morpholino oligonucleotides (MOs) (Satou et al. 2001), transposon-mediated germ cell transformation and mutagenesis (Sasakura et al. 2003c, Sasakura et al. 2005), zinc-finger nucleases (ZFNs) (Kawai et al. 2012), transcriptional activator-like effector nucleases (TALENs) (Treen et al. 2014) and clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) (Sasaki et al. 2014). These technologies have supported detailed and thorough analyses to reveal molecular and cellular mechanisms that underlie development of *Ciona*, since almost of them can be performed in a tissue-specific manner during embryogenesis.

20.8.3.1 MOs

The antisense morpholino oligonucleotide strategy consists of MOs that bind to the targeted mRNA and prevent translation. They were tested in a range of models, including ascidians, in which they were extensively used since they allow a rapid and high-throughput approach for functional studies. In addition, MOs are able to target maternal mRNA determinants as well as zygotic genes. The efficiency of this technique was first tested in *Ciona savignyi*, in which MOs were able to target the maternal pool of β-catenin mRNA and abolish endodermal differentiation (Satou et al. 2001). Since then, MOs were extensively used and allowed identification of key genes in tissue differentiation during embryogenesis, such as the maternal determinant Macho-1 for muscle differentiation in *Halocyntia roretzi* (Nishida and Sawada 2001) and in *Ciona savignyi* (Satou et al. 2002c); in tissue formation, for instance, chondroitin-6-O-sulfotransferase involved in *Ciona intestinalis* notochord morphogenesis (Nakamura et al. 2014); or even for cell fate, for example, Ci-Sushi, which controls the initiation of apoptosis at the onset of *Ciona intestinalis* metamorphosis (Chambon et al. 2007). However, there are several limitations to injecting MOs in solitary ascidians, notably the restricted numbers of mutants to analyze and the difficulty of interpreting some phenotypes due to off-target effects.

20.8.3.2 RNA Interference

Based on the introduction in the cells of double-strand RNA, which are converted in small interfering RNA (siRNA), causing the destruction of specific mRNA, this approach was successfully used in colonial ascidians but has had few successes with solitary ones, except the electroporation of short-hairpin RNA targeting tyrosinase-encoding gene in *Ciona* embryo leading to the absence of melanization of the tailbud pigmented cells (Nishiyama and Fujiwara 2008). To date, the use in solitary ascidians is very limited.

20.8.3.3 ZNFs and TALENs

The nuclease activity ZNFs and TALENs induces double-strand breaks (DSBs) at target sequences. In the case of ZNFs, mutations occur when DSBS are repaired by non-homologous end joining (NHEJ), which introduces insertional or deletional mutations at the target sequence. TALENs provoke mutations when the cellular DNA repair mechanisms fail. Both approaches were established in *Ciona intestinalis* by the Sasakura lab (Kawai et al. 2012; Treen et al. 2014) and are a very promising strategy to mutate endogenous genes during development. ZNFs were tested in a *Ciona* transgenic line expressing EGFP to introduce mutations in EGFP loci. When eggs were injected, it resulted in inheritable mutations with high frequency (about 100%), no toxic effect on embryogenesis and few off-target effects (Kawai et al. 2012). TALEN knockouts can be performed by electroporation and allow fast generation of mutants and a quick screening involving numbers of embryos not possible with other animals. Toxicity is a major concern with TALEN when ubiquitous knockouts are generated, but using tissue-specific promoters reduces this problem and allows mutations in a tissue-specific manner (Treen et al. 2014).

20.8.3.4 CRISPR/Cas 9

Since its discovery in 1987 (Ishino et al. 1987), CRISPR/Cas9 has become one of the most powerful tools for researchers to alter the genomes of a large range of organisms. CRISPR/Cas9 uses a short guide RNA (sgRNA) that binds to its target site; Cas9 protein is recruited to the binding site and induces a double-strand break at the target genomic region. In solitary ascidians, this technique was first successfully tested in *Ciona intestinalis* (Sasaki et al. 2014) and more recently in *Phallusia mammillata* (McDougall et al. 2021).

In *Ciona*, the most widely used application of CRISPR is for targeted mutagenesis in somatic cells of electroporated embryos. In this method established in 2014 by Sasakura lab (Sasaki et al. 2014) and recently improved by Stolfi (Gandhi et al. 2018), *in vitro* fertilized one-cell-stage embryos are electroporated with plasmids, allowing the zygotic expression of Cas9 protein and sgRNA. Interestingly, Cas9 can be expressed in a cell-specific manner, and the targeted mutations are a powerful means to dissect the tissue-specific functions of a gene during development.

20.8.4 Genetics, Mutagenesis and Transgenesis

Natural mutants often arise in wild populations, probably due to the high polymorphism between individuals within a given population (Satoh 2013). Moreover, the rapid life cycle and the possibility of self-fertilization (natural or induced with chemical or enzyme treatment), coupled with a rapid embryogenesis and a morphologically simple tadpole that allows simple phenotype detection, make both *Ciona* (*intestinalis* and *savignyi*) excellent models for mutagenesis. In addition to characterization of the *Ciona savignyi* natural mutant *frimousse* (Deschet and Smith 2004), Smith's lab took advantage of the self-fertility in this species to perform a mutagenesis screen notably using N-ethyl-N-nitrosourea (ENU)-induced mutations affecting early development. This random approach led to the isolation of a number of mutants with notochord defects such as *chongmague* and *chobi* (Nakatani et al. 1999). Since then, the transgenesis technique was established in *Ciona* using transposon-mediated transgenesis that allow creation of stable germ lines but also to use it for insertional mutagenesis and enhancer trapping.

The Tc1/mariner transposable element *Minos* (isolated from *Drosophila hydei*) is a small DNA transposon (2000 bp) activated by a "cut and paste" system in which a transposase is able to excise the transposon from the DNA and integrate it into a target sequence. When a plasmid containing *Minos* is microinjected or electroporated in *Ciona* eggs with transposase mRNA, *Minos* is excised from the vector DNA and integrated in the *Ciona* genome, and this event is observed in somatic and germ cells (Sasakura et al. 2003c). In the latter case, this insertion is inherited by the progeny, and its stability was reported over ten generations in several transgenic lines (Sasakura 2007). Insertions of *Minos* can disrupt gene function to create mutants, such as the *swimming juvenile*, which exhibits a cellulose synthesis defect and absence of tail regression during metamorphosis due to the integration of *Minos* at *Ci-CesA* promoter, a gene involved in cellulose synthesis in *Ciona intestinalis* (Sasakura et al. 2005). In addition to insertional mutagenesis, the transposon-based technique was also able to create stable marker lines when CRE of tissue-specific gene driving expression of fluorescent proteins were used with a *Minos*-based transposable element. Another potentiality of *Minos* transposons is the enhancer trapping technique. It consists of insertions using a reporter gene in a *Minos* transposons construct (GFP, for example), and if there is an enhancer close to the transposon insertions, the expression patterns of reporter genes are affected according to the enhancer. In *Ciona*, an intronic enhancer in the *Ci-Musashi* gene was identified by this approach (Awazu et al. 2004).

20.9 CHALLENGING QUESTIONS

Researchers in the ascidian field face many challenging questions. In this section, a brief overview of some of them will be given, followed by a detailed discussion of the unique opportunity provided by the ascidians to develop quantitative modeling of chordate embryos.

20.9.1 Evolution of Ascidians

As described in the genomic section, 11 ascidian genomes are now sequenced and annotated, some of them with transcriptomic data and identification of cis-regulatory modules. In addition, the compilation of these data in the ANISEED database will greatly facilitate comparative developmental genomics between ascidian species and allow new insights in ascidian evolution. Immediate application of this approach could lead to better understanding of the differences in gene-regulatory networks during embryogenesis observed between *Ciona intestinalis* and *Halocyntia roretzi* (see embryogenesis section for details). Indeed, these two species exhibit at least two differences for notochord and muscle secondary lineage which both require FGF but dependent on nodal and Delta/Notch for *Ciona* and independent of both of them for *Halocyntia*. Further analyses of the developmental genomics of these two species may allow evolutionary inference to better understand these changes.

Another example concerns the phenotypic change observed in several species that do not develop a tail during embryogenesis and do not develop notochord or tail muscles; instead, they give rise to non-motile tail-less larva without functional notochord or larval tail muscle or directly to a juvenile (Satoh 2013). Anural development occurred independently several times during ascidian evolution. Cross-fertilization approach of the tail-less *Molgula occulta*, and its close relative urodele species *Molgula oculata* gives rise to a hybrid embryo with a short tail containing a notochord. Swalla and Jeffery (1990) suggested an evolution of the anural mode of development by relatively simple genetic changes. Comparative genomics studies permitted by the release of the genome of these two

species will certainly detect key genomics changes for these different modes of embryogenesis.

20.9.2 Ascidians for Therapeutic Advances

In the last few years, several studies have been conducted on the identification and characterization of chemical diversity produced from marine ascidians (Palanisamy et al. 2017). The essential part of these chemical compounds is used by ascidian species to prevent predatory fish, as an anti-fouling and anti-microbial mechanism and to control settlement (reviewed in Watters 2018). Ascidians, like several marine organisms, produce a rich variety of secondary metabolites with potential therapeutic properties in human medicine, with a range of biological activities such as cytotoxicity, antibiotic and immunosuppressive activities, inhibition of topoisomerases and cyclin-dependent kinases (Duran et al. 1998). Most of these compounds were identified by the liquid chromatography-mass spectrometry method. Among them, Ecteinascidin was isolated from *Ecteinascidia turbinata* and is currently used as a cancer drug to treat soft-tissue sarcoma and ovarian cancer (Gordon et al. 2016); Aplidin isolated from *Aplidium albicans* has given promising results in myeloma treatment (Delgado-Calle et al. 2019). In addition, anti-malarial effects were identified from extracts coming from three ascidians, *Microcosmus goanus, Ascidia sydneiensis* and *Phallusia nigra* (Mendiola et al. 2006). Between 1994 and 2014, up to 580 compounds were isolated from ascidians and offer a wide range of opportunities to identify molecules with therapeutic properties for human diseases.

In addition to screening for molecules with potential therapeutic effects, ascidian embryos have also started to be used as an experimental model to study the neurodevelopmental toxicity of different compounds (Dumollard et al. 2017).

20.9.3 When Developmental Biology Becomes Quantitative: A Big Step toward "Computable Embryos"

The transition from a single fertilized cell to a complex organism, with various cell types that compose its tissues in the correct numbers and their fine regulation in space and time, is the question at the heart of developmental biology. Decades of research in this field have designed a broad portrait of the fundamental processes involved during embryogenesis: from the description of the genetic programs of embryonic cells and the mechanisms regulating gene transcription to how cell fates and behaviors are coordinated by cell communication and the way this translates into morphogenesis.

Developmental mechanisms have traditionally been studied at the tissue level in a qualitative manner. For example, consider the current view of the classical chemical signaling during fate specification. A surprisingly small number of signaling pathways involving cell surface receptor and activating ligands act in widely different cellular contexts to produce the diversity of fate specification events occurring during embryogenesis (Perrimon et al. 2012). Despite this, many simple questions remain unanswered, such as: "What is the mechanism regulating the dose-response to increasing concentrations of ligands or receptors?" "How are ligand concentration and time of exposure integrated by cells?" To deepen the understanding of the principles which govern embryonic development, it is important to combine quantitative experimental approaches at the cellular scale with dynamic mathematical models including mechanistic details. For example, the recent development in quantitative imaging, sequencing, proteomics and physical measurements have allowed us to refine the historical morphogen concept, in which diffusible signaling molecules are proposed to coordinate cell fate specification and tissue formation using concentration-dependent mechanisms (a static readout), because it was insufficient to describe or model the complexities of patterning observed with these techniques in developing embryos (Garcia et al. 2020; Huang and Saunders 2020; Jaeger and Verd 2020; Rogers and Müller 2020; Schloop et al. 2020).

While physical modeling of life has a long history (Thompson 1917), it has remained a theoretical exercise for a long time: insufficient measurements of physical parameters for constraining models coupled with a largely qualitative and static description of phenotypes have rendered it difficult to apply physics to developing embryos and even to single cells. The recent technological breakthrough mentioned previously, however, reduced this difficulty while making "computable embryos" through a precise physical description of embryonic development more necessary than ever to capture key developmental concepts and bridge genomic information and dynamic phenotypes (Biasuz et al. 2018). First, our brains are simply unable to cope with the large amount of data generated, much of which are unrelated to the mechanism being studied. Second, biology involves several layers of feedback, resulting in unintuitive non-linear behaviors. Third, biology is a multiscale process in which macroscopic properties of cells and tissues arise from the mesoscopic properties of molecules or subcellular structures.

Ascidians definitively constitute a model of choice to build a global computational model of embryogenesis. Embryonic development is a continuous progression in time. The "computable embryo" is based on the idea that a mathematical description of the system can predict the future state of the embryo from the knowledge of its current state. This global computational model of embryogenesis at the single-cell, genome-wide and whole-embryo level is a challenging task and will only be achieved using the most appropriate developmental systems (Biasuz et al. 2018).

Solitary ascidian embryos seem to be good candidates for this breakthrough. At first glance, one would rather think of the *Drosophila melanogaster* or vertebrate embryos for this role. Indeed, thanks to decades of research, a deep understanding of core developmental mechanisms has been achieved, and powerful genetic and cell biology tools exist.

These embryos, while remaining a significant motor for defining new concepts, may, however, be too complicated to incorporate these concepts into a global model of embryogenesis. In contrast, ascidian embryos, as nematodes, are simpler and develop stereotypically with few cells and invariant cell lineages, so that each cell can be named and found at the same position in all embryos (Lemaire 2009). Unlike those of nematodes (Goldstein 2001), ascidian embryo geometries have even remained essentially unchanged since the emergence of the group, around 400 million years ago, despite extensive genomic divergence (Delsuc et al. 2018; Lemaire 2011). The development of ascidians is also characterized by earlier fate restriction than most animal embryos: 94 of the 112 early gastrula cells in the ascidian *Ciona* are fate restricted, each contributing to a single larval tissue type (Nishida 1987). Moreover, ascidians are closely related to vertebrates, as they belong to the vertebrate sister-group, but ascidians kept their genomic simplicity. Indeed, they diverged before the two rounds of whole-genome duplication events which occurred in the vertebrate lineage leading to the apparition of multiple paralogues for each gene (Dehal and Boore 2005), with potentially slightly divergent activities. Finally, ascidian embryos are small (~130 μm) and transparent, and they develop rapidly externally in sea water up to the larval stage (~12 h), making them very easy to image. Thus, ascidian embryos provide a rigid framework that allows combination of analyses at cellular resolution with mathematical modeling.

These advantageous properties of ascidian embryos, especially *Phallusia mammillata* embryos, which are fully transparent, combined with the breakthrough development of light sheet microscopy (Power and Huisken 2017), have enabled the production of the first digitized version of a metazoan embryo (Figure 20.7a) (Guignard et al. 2020). Based on automatic whole-cell segmentation and tracking over five cell generations of membrane-labeled cells with two-minute temporal resolution, this research offers a complete description of early ascidian embryo development, accounting for each cell in the ten embryos analyzed. Moreover, this quantitative and dynamic atlas of cell positions and geometries can be associated with the known cell fates and interactively explored through the MorphoNet online morphological browser (Leggio et al. 2019). These "digital embryos" show that ascidian development is reproducible down to the scale of cell–cell contacts and, combined with modeling and experimental manipulations, it allows us to establish contact area-dependent inductions as an alternative to classical morphogen gradients. This work opens the door to quantitative single-cell morphology and mechanical morphogenesis modeling.

In parallel with this work, another group combined high-resolution single-cell transcriptomics (single-cell RNA sequencing) and light-sheet imaging to build the first full comprehensive atlas which describes the genome-wide gene expression of every single cell of an embryo in the early stages of development, showing the evolution from a single cell up to gastrulation in the ascidian *Phallusia mammillata* (Sladitschek et al. 2020). By providing a complete representation of the gene expression programs, which instruct individual cells to form the different cell types necessary to build an embryo, and therefore by allowing us to know precisely cell-specific expression of transcription factors at the single-cell level, this study will significantly enhance current single-cell-based gene regulatory network inference algorithms (Aibar et al. 2017) and will help to further develop single-cell-based physical models of the different steps of transcriptional control during development. Moreover, these single-cell gene expression data will feed several layers of physical description of biological processes. For example, identification of cell-adhesion molecules will allow the refining of morphogenetic models, such as oriented cell divisions, cell shape changes or cell neighbor exchanges models (Etournay et al. 2015), thereby linking mechanical and genetic information at the cellular resolution.

In spite of the convenient properties and the recent advances that have been realized thanks to the ascidian embryo model, there is still a long way to go to be able to "compute the embryo".

Typically, studies at the single-cell level are in their early days, as can be illustrated by signal transduction studies. The MAPK/ERK signaling pathway is one of the important embryonic signaling pathways used by vertebrates and invertebrates, controlling many physiological processes (Lavoie et al. 2020), and is the main inducing pathway in early ascidian embryos (Lemaire 2009). The signaling cascade from the activation of the transmembrane receptor to the phosphorylation of the ERK nuclear targets is well described (Figure 20.7b) (Lavoie et al. 2020). Our current knowledge of this pathway is, however, mostly static, and an integrated understanding of its spatio-temporal dynamics is lacking (Patel and Shvartsman 2018). For example, it has been shown that the ERK pathway can trigger two qualitatively different types of ERK activity: pulsatile or continuous (Aikin et al. 2019). To understand these non-intuitive results, it is important to combine quantitative experimental approaches at the cellular resolution with dynamic mathematical models including mechanistic details. Genetically encoded fluorescent activity sensors that convert kinase activity into nucleocytoplasmic events have been recently developed (Durandau et al. 2015; Regot et al. 2014), and these tools can now be coupled with optogenetic systems in order to activate the ERK pathway with high spatiotemporal accuracy at different levels (Gagliardi and Pertz 2019). However, these techniques were only used to track a single pathway component at a time. Yet they suggest that multiplexing sensors at different levels of the cascade could reveal the dynamics of information flow through the cell. Such quantitative measures are required to more realistically model the catalogue of cell-signaling modalities (Biasuz et al. 2018).

The technological breakthroughs of the last quarter of the century have brought a whole new perspective to developmental biology, which is now seen through the combined lenses of mathematical modeling and experimental biology. A major challenge for the future will now be to integrate

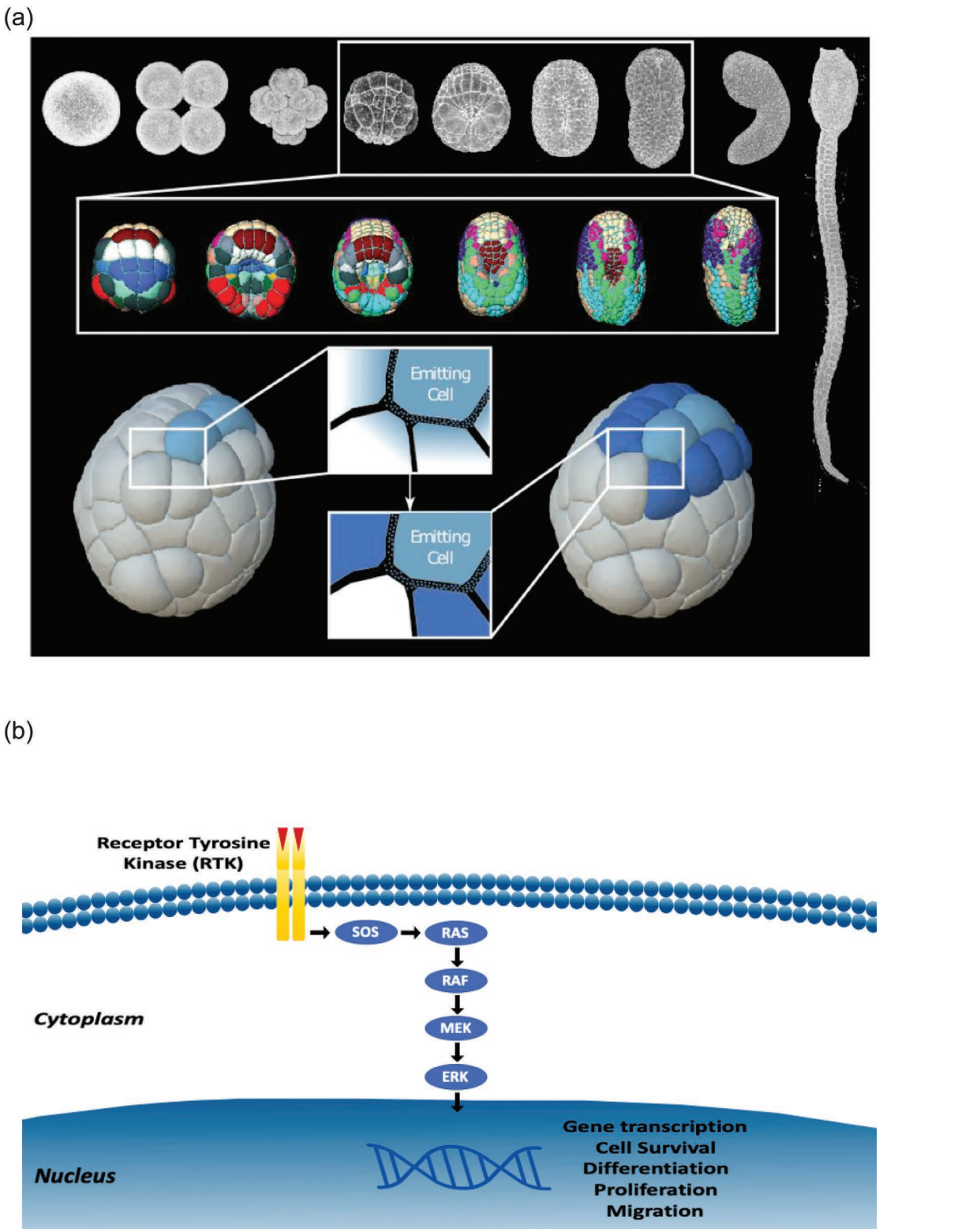

FIGURE 20.7 (a) Digitalization of *Phallusia mammillata* embryogenesis reveal contact area-dependent cell inductions. (Top) Light-sheet imaging of cell membranes (not shown) combined with automated cell segmentation and tracking allowed reconstruction of *Phallusia* embryogenesis between the 64-cell and initial tailbud stages. Digital embryos represented here are color-coded with cell fates. (Bottom) Illustration of the contact area-dependent mode of cell inductions. Light blue cells emit inducing extracellular signals (left). Among the neighbor cells which receive the signal, only the dark blue cells, which have the largest surface of contact with emitting cells, are induced (right). Digital embryos have been explored through the MorphoNet online morphological browser. (b) Simplified representation of the MAPK/ERK signaling pathway. ([a] Figure courtesy of Leo Guignard & Kilian Biasuz; [b] figure courtesy of Kilian Biasuz.)

partial models accounting for short-term activities into a global view of biological processes. Indeed, most of the modeling efforts were designed to shed light on specific processes over a short period of time. As a consequence, our physical knowledge of embryogenesis is reduced to a few unconnected kernels of insight. Increasing the number of kernels is imperative to "compute the embryo" but will not suffice: kernels will need to be incorporated into a bigger picture. The solitary ascidian embryos, which are simple and transparent and contain a relatively small number of cells and invariant cell lineages are perfect candidates to integrate these principles into a global model of embryogenesis.

20.10 GENERAL CONCLUSION

The last 20 years have been marked by extraordinary advances in the comprehensive biology of ascidians. Starting as the first experimental model organism in embryology, the ascidian embryo offers today an avenue of investigation in several biological research fields such as developmental biology, cell biology, comparative genomics, drug screening or evo-devo. The decoded genome of 13 ascidians, coupled with gene annotation, large transcriptomic data, proteomics, identification of cis-regulatory elements, large coverage of gene expression patterns by *in situ* hybridization, stereotyped and well-described cell lineages, physical maps of the genome onto chromosomes and routine generation of transgenic lines combined with cell line markers and single-cell transcriptomics (supported by FACS) render this "old" marine model one of the most promising for modern biology.

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