EVOLUTIONARY CELL BIOLOGY

EVOLUTION OF THE DIGESTIVE SYSTEM

CELL TYPES AND CROSSTALKS, DEVELOPMENT AND REGENERATION



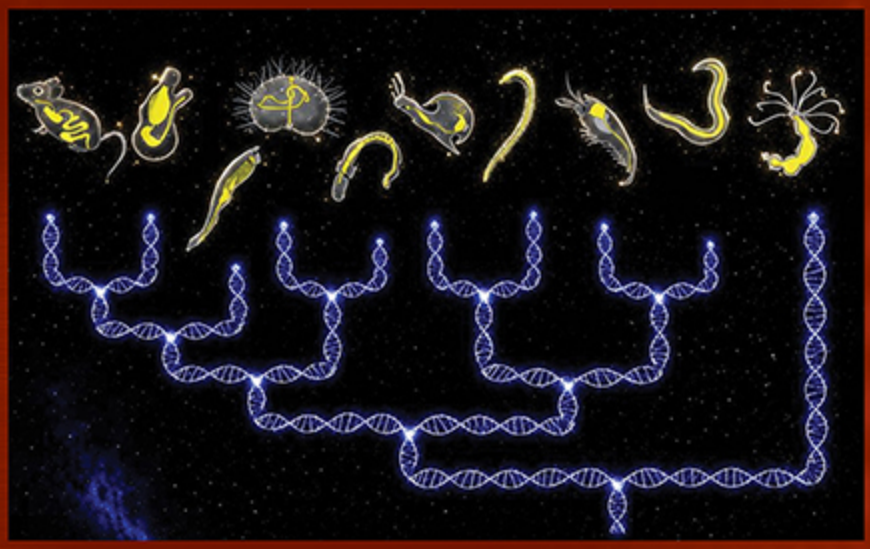
EDITED BY
ROSSELLA ANNUNZIATA
AND MARIA I. ARNONE

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Evolution of the Digestive System

The capability of cells to uptake and digest molecules to acquire energy is essential for all life forms. During evolution, with the emergence of multicellularity, specialized cell types and organs became responsible for acquiring food particles, breaking them down and absorbing nutrients. This innovation had a profound impact on animal evolution: the emergence of extracellular digestion released organisms from size constraints and enabled the diversification of complex body plans. The complexity and morphologies of guts vary among animals, yet they all form through a highly conserved developmental process known as gastrulation. During gastrulation, germ layers are specified and a through-gut forms and differentiates into a fore-, a mid- and a hind-gut, each one dedicated to specific functions in digestion. This book explores the evolutionary history of animal digestive systems, highlighting both their extraordinary diversification and their conserved features across distant branches of the tree of life. An international team of contributors offers a close look at the molecular and developmental mechanisms underlying gut formation and evolution, as well as the origins and diversification of endocrine and exocrine gut functions, dig into the function and evolution of the gut microbiome and describe the regenerative capacities of the gastrointestinal tract.

Rossella Annunziata is a Researcher in the Department of Biology and Evolution of Marine Organisms - Stazione Zoologica Anton Dohrn in Napoli, Italy. She received her PhD in Molecular Biology from the Open University of London. She has authored or co-authored numerous peer review scholarly articles and protocols.

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Evolution of the Digestive System Cell Types and Crosstalks, Development and Regeneration

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Contents

Preface

List of Contributors

Chapter 1 Evolution of Gut Patterning in Bilateria

Rossella Annunziata, Pedro Martínez, and Carmen Andrikou

Chapter 2 Distribution and Evolution of Digestive Exocrine Cells and Organs

Periklis Paganos, Maria Lorenza Rusciano, and Maria Ina Arnone

Chapter 3 Evolution and Development of the Gut Endocrine System

Maria Lorenza Rusciano, Periklis Paganos, and Maria Ina Arnone

Chapter 4 Animals Without Through-Guts

Andreas Schmidt-Rhaesa

Chapter 5 Evolution of Immunity: The Gut as an Interface with the Environment

Assunta Liberti and Larry J. Dishaw

Chapter 6 Comparative Study of Cell Types and Their Regeneration in Echinoderm Digestive Systems

José E. García-Arrarás, Julienn Torres-Rodriguez, Evelyn Avilés-Ríos, Alejandra Beltrán-Rivera, Alexis Ruiz-Hernández, Anna Márquez-Santiago, Diego Villafañe-Ortiz, Kiara Luna-Martínez, and Katherine Rivera-Bermúdez

Index

Preface

The digestive system stands as one of the most fundamental innovations in the history of animal life. Rooted in the universal need to acquire and process energy, its evolution profoundly reshaped how organisms interact with their environment. As multicellularity emerged, digestion became the function of specialized cells and organs, releasing animals from the constraints imposed by small body size and enabling the rise of internal complexity. In fact, from its origins as a simple cellular interface to its modern form as a highly regionalized, multifunctional system, the gut has played a central role in shaping the evolutionary trajectory of metazoans.

The complexity and shapes of guts vary among animals, but they all form through a highly conserved mechanism in development called gastrulation. During gastrulation, germ layers are specified and a gut tube forms and differentiates into a fore-, a mid-, and a hind-gut, each one dedicated to specific functions in digestion. The development of the gut is controlled by an intricate network of gene regulatory systems and morphogenetic processes that direct the differentiation of each gut compartment. These processes display a significant level of conservation across animals, which is beautifully confirmed by the recent high-resolution omics approaches, indicating a common origin of the different gut cell types.

Digestive systems are not “isolated” but exchange information with other parts of the body. For example, their function is controlled by the nervous system through the action of hormones (brain–gut axis), which translate environmental signals to the digestive system and, on the other side, guts control energy and metabolic balance through the release of hormones directed to the peripheral and central nervous systems (gut–brain axis). The

gut contains not just eukaryotic cells, but also a large quantity of microorganisms, which make up the gut's microbiome. This represents a complex ecosystem inhabiting all gut compartments, it influences the health of their hosts and also their adaptation capacities to different food sources. Beyond its role in food processing, the gut is thus increasingly recognized as a dynamic system where development, metabolism, physiology, immunity, and interactions with the environment converge.

This volume brings together six chapters that collectively explore the gut across levels of biological organization, from genes and cells to tissues, organs, and whole-organism systems, while placing these insights within a broad evolutionary framework.

The opening chapter examines the evolutionary and developmental logic underlying the organization of animal digestive systems. By focusing on the molecular and developmental mechanisms underlying gut regionalization, it describes the molecular mechanisms that pattern the gut along its main axis, highlighting the conserved transcription factors, signaling pathways, and gene regulatory networks that specify discrete digestive domains in nephrozoan animals. The chapter further explores how these patterned regions are integrated with gut musculature and the nervous system to ensure coordinated function, particularly the directional transport of food, offering a unified view of gut architecture as an outcome of both developmental programs and physiological integration.

Building on this structural and developmental perspective, the second chapter shifts attention to the cellular foundations of digestion. It traces the evolutionary history of digestive exocrine cells across Metazoa, underscoring their putative deep evolutionary conservation and widespread distribution. By exploring the evolutionary origins of these cell types and the specialized organs they give rise to, such as the pancreas, the chapter

reinforces a central evolutionary principle: that complex organs arise through the modification and redeployment of pre-existing cellular building blocks rather than through the de novo emergence of entirely new structures.

The third chapter expands the functional scope of the gut by focusing on its endocrine dimension. It explores how gut-derived hormonal systems regulate organismal homeostasis, metabolism, and behavior, and how these systems are integrated with the nervous system through the brain–gut axis. Comparative evidence across metazoans reveals that gut endocrine cells are evolutionarily ancient and share molecular and functional features with neurons, pointing to a deep evolutionary connection between neurosecretory and endocrine systems.

Complementing these perspectives, the fourth chapter revisits digestive system diversity by examining sac-shaped digestive tracts. These systems provide critical insights into the origins and transitions of digestive tract architectures. By surveying a wide range of taxa, this chapter highlights the diversity of solutions to nutrient acquisition and processing, as well as the evolutionary flexibility of digestive designs.

The fifth chapter broadens the conceptualization of the gut by framing it as an ecological and symbiotic interface. It explores the emergence of the gut microbiota as a key component of animal biology, shaping development, immunity, metabolism, and behavior. By introducing the concept of the holobiont, this chapter emphasizes that the gut is not solely an animal organ but a complex ecosystem, where host and microbial interactions are fundamental to physiological function and evolutionary innovation.

Finally, the sixth chapter provides a focused case study on echinoderms, highlighting the importance of integrating classical morphology with

modern molecular approaches. Emphasizing on the sea cucumber *Holothuria glaberrima* as an experimental system, it examines intestinal cell types, their molecular characterization, and their roles in regeneration. This chapter underscores the importance of comparative and integrative approaches in understanding cell identity, plasticity, and evolutionary relationships across metazoans.

Together, these chapters present a unifying view of the gut as a central axis of animal evolution, an organ system built upon ancient cellular origins, shaped by conserved developmental mechanisms, diversified through evolutionary innovation, and deeply integrated with physiological regulation and environmental interactions. By bridging disciplines and scales of analysis, this volume aims to provide both a synthesis of current knowledge and a framework for future research into one of the most fundamental systems in biology.

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1 Evolution of Gut Patterning in Bilateria

Rossella Annunziata, Pedro Martínez, and Carmen Andrikou

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1.1 INTRODUCTION

Within animals, there is a strong degree of variation in gastrointestinal (GI) tract anatomy. It can range from the complex vertebrate tripartite gut comprising the esophagus, stomach, and intestine along with domain-associated organs (e.g. pancreas, liver), to the simple sac-shaped gut with only one opening, characteristic of Cnidaria, Platyhelminthes, and Xenacoelomorpha (Schmidt-Rhaesa, 2007). Cases of complete loss of the digestive system are also reported, for example in deep-sea Vestimentiferan tubeworms (Bright & Lallie, 2010) and bivalve species (Gustafson & Reid, 1988). Other invertebrates entirely missing the digestive tract are sponges and placozoans.

Gut development has been widely studied in metazoans, especially in Bilateria, which include protostomes and deuterostomes (McGhee, 2007; Sheaffer & Kaestner, 2012; Stainier, 2002, 2005; Zorn & Wells, 2009). These two groups (introduced by Karl Grobben in 1908) were initially characterized by their differences in early embryonic features: in protostomes, the blastopore generally forms the mouth, and the body cavity

or coelom forms by schizocoely, a process in which solid masses of mesodermal origin gradually split apart to create internal space. In contrast, in deuterostomes the blastopore becomes the anus, and the coelom develops by enterocoely, where coelomic pouches bud off from the embryonic gut, known as the archenteron. Variations on these embryological features often occur in these two major clades (Grapin-Botton & Constam, 2007; Stern, 2004). In fact, it has been suggested that the developmental relationships of mouth and anus openings are secondary by-products of variations in axial and mesoderm development, thus challenging the traditional taxonomic assignment of animals as protostomes or deuterostomes (Martín-Durán et al., 2016).

The GI tract forms through gastrulation, which leads to the formation of the archenteron, a primitive gut tube that initially shows no obvious morphological or functional differentiation. Nonetheless, even when morphological differences are not yet visible, distinct or partially overlapping expression domains of signaling molecules and transcription factors (TFs) can be detected along the archenteron's antero-posterior (A-P) axis, pointing to an initial regionalization process. The combination of cell autonomous specification together with external signaling events establishes molecularly distinct regions with sharply defined gene expression boundaries, marking the primordia of different digestive organs. These molecular components form complex gene regulatory networks (GRNs) which mechanistically explain the link between embryonic development and the genomic programs that encode it (Davidson & Peter, 2015). Decoding the GRNs and the morphogenetic mechanisms that generate the distinct domains of the GI tract during development in animals occupying different phylogenetic positions is essential to reconstruct how digestive structures and functions have evolved. In this context, the

evolution of cells specialized for extracellular digestion can be considered a key innovation that provided animals with different, spatially separated digestive functions. This, in turn, released multicellular organisms from the physical constraints associated with small body sizes, and likely facilitated the subsequent evolution of complex internal structures. It can thus be argued that the molecular networks orchestrating digestive development were among the first patterning systems to evolve, guiding the transition from collections of phagocyte cells (as in sponges) to single blind guts (as in cnidarians) and, later on, the bilaterians' throughout guts.

Despite their gut diversity, bilaterians share a broadly conserved set of regulatory genes for controlling gut formation and patterning. In addition, they also share a common set of digestive cell types including secretory (glandular) cells, absorptive columnar cells, enteroendocrine cells, and phagocytic cells. These facts point to deep homologies shared by a "digestive tube" in Bilateria. Whether the homology extends to cnidarians is still a matter of debate. In fact, although the gut-like structure of anthozoan cnidarians (like the sea anemone *Nematostella*) and bilaterian guts are functionally similar, they are not straightforward, direct homologues (Steinmetz et al., 2017). And it is for this very reason that our chapter focuses just on bilaterian structures and limits the scope of comparisons to Bilateria.

1.2 GUT PATTERNING IN DEUTEROSTOMES

Deuterostomes form one of the two major branches of bilaterian animals and include two monophyletic groups: chordates, comprising vertebrates and protochordates (cephalochordates, or lancelets, and tunicates), and ambulacrarians, to which echinoderms and hemichordates belong. The word deuterostome means "second mouth" and refers to the fact that,

during early embryogenesis, the blastopore becomes the anus, while the mouth forms secondarily (as described above). A summary of the main GI structures in deuterostome representatives can be found in **Figure 1.1**.

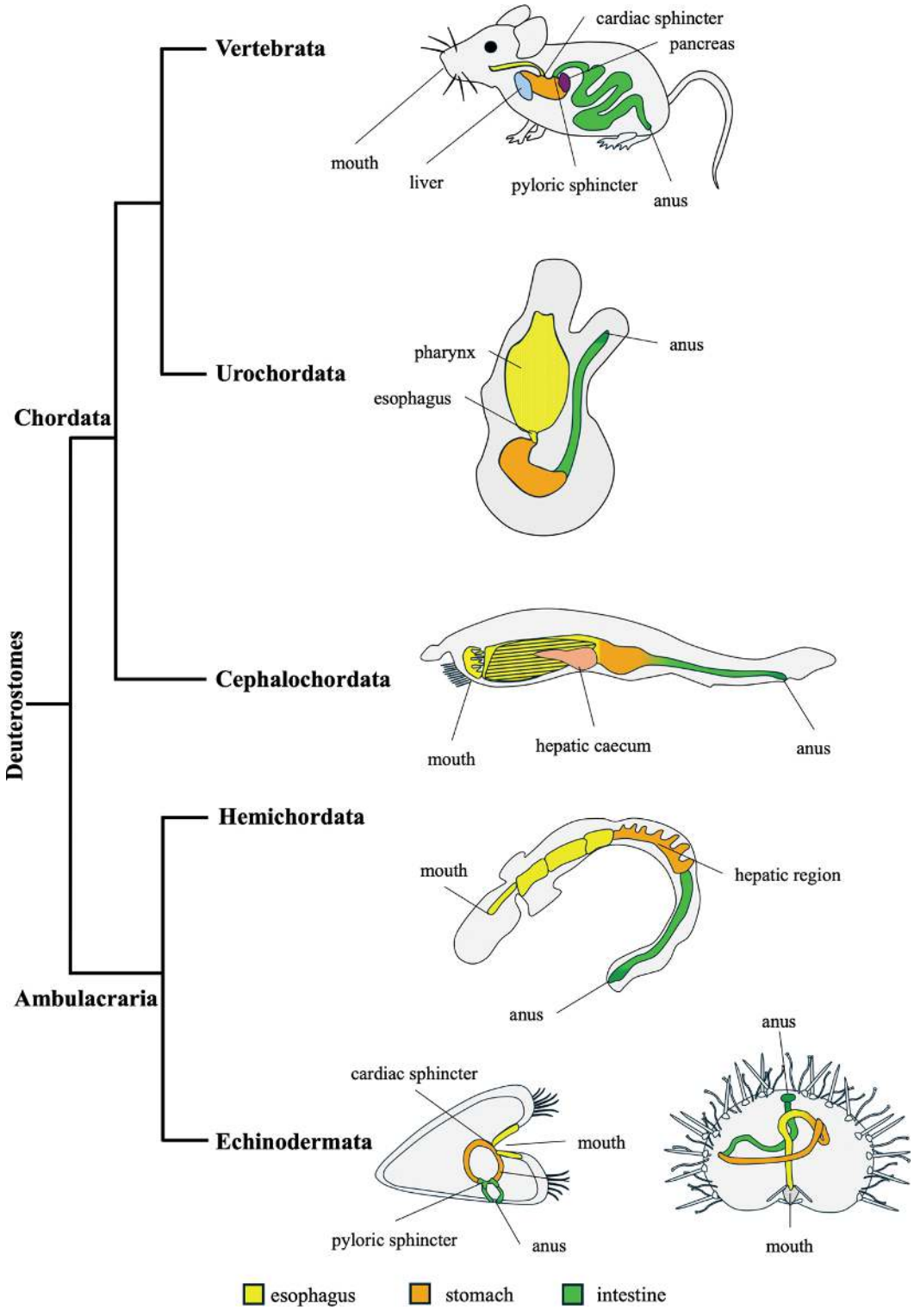


FIGURE 1.1 Overview of the general anatomy of the GI system in representative deuterostome taxa. For most groups, the adult body plan and digestive tract organization are shown. In

echinoderms, both the larval (left) and adult (right) stages are depicted to highlight the major anatomical reorganization of the gastrointestinal system occurring during metamorphosis. ⏎

1.2.1 Gut Patterning in Chordates

Gut development and patterning have been extensively examined across vertebrate models, including mouse, chicken, frog, and fish (Chalmers & Slack, 2000; Wallace & Pack, 2003; Zorn & Wells, 2009). In these animals the gut forms through similar but distinct morphogenetic strategies that reflect their specific embryonic architectures: in amphibians, endodermal cells are internalized by invagination through the blastopore, forming the archenteron in a relatively direct process; in birds and mammals, endodermal progenitors ingress individually through the primitive streak and undergo involution and extensive migration before reassembling into the definitive gut tube, all this in coordination with axial elongation and mesoderm formation; fishes display an intermediate strategy, in which endodermal precursors are specified at the blastoderm margin and internalized through coordinated involution and convergence movements, giving rise to the gut tube. In all these animals, the earliest endodermal cells to exit the blastopore or the primitive streak give rise to the anterior regions of the gut, whereas cells internalized later contribute progressively to more posterior territories.

Despite differences in early embryonic morphogenesis, the overall organization of the vertebrate gut is remarkably conserved, with only minor lineage-specific variations (Smith et al., 2000). Across vertebrates, the foregut gives rise to organs such as the thyroid, lungs, esophagus, liver, stomach, and pancreas; the midgut forms the small intestine; and the hindgut develops into the large intestine. (McGrath & Wells, 2015; Zorn & Wells, 2009). In addition, different cellular layers can be identified in the

vertebrate alimentary canal: the mucosa, submucosa, and muscularis layers, which are derived from the endoderm, mesoderm, and ectoderm, respectively.

Vertebrate gut development and patterning has been deeply studied in mouse embryos (Zorn & Wells, 2009). In mouse, endodermal cells are generated twice, at two different developmental times: a first population, which is the primitive endoderm, is specified in the preimplantation blastocyst, and during gastrulation these cells intercalate with incoming definitive endoderm originating in the anterior primitive streak; finally, the two populations of endodermal cells form a mosaic layer known as the gut endoderm (Nowotschin & Hadjantonakis, 2020; Zorn & Wells, 2009).

After gastrulation, the definitive endoderm is patterned along the A-P axis and undergoes complex morphogenetic events transitioning from a two-dimensional sheet to a three-dimensional tube surrounded by a primitive mesenchyme. A-P patterning of the mouse gut tube is driven by reciprocal signaling with the surrounding mesenchyme, involving signaling pathways such as Wnt, Fgf, Nodal, and Bmp, which drive posterior identity while suppressing anterior fates (Arnold & Robertson, 2009). In addition, the two TFs *sim2* and *rfx2* are involved in the establishment of the left/right axis in the stomach and gut looping, respectively (Chung et al., 2012; Wyatt et al., 2021).

The earliest signs of organ specification appear in the nascent gut tube through the localized activation of TFs that define future organ territories/domains. These include *nkx2.1* in the respiratory primordium, Hex in the liver field, *sox2* and *pdx1* in the presumptive stomach, *sox17* in the extrahepatic biliary region, *foxI3* in the pharyngeal pouch, *pdx1*, *pax6*, and *ptf1a* in the pancreas, *pdx1* and *cdx2* in the duodenum, and *sox2* and *cdx2* in the intestine (Duque et al., 2022; Hart et al., 2013; Hasten &

Morrow, 2019; Ikonomou et al., 2020; Keng et al., 2000; Shih et al., 2015; Uemura et al., 2013). Gene expression analyses have also defined the murine TF toolkit operating later on in distinct gut territories and associated organs including *ttf1* in the distal esophagus; *osr2* in the stomach; *brn4, mnx, nkx6.1* in endocrine pancreatic cells; *nkx2.2, rbpj, mist1*, and *xbp1* in acinar pancreatic cells; *tgif2, rfx6, isx, tcf, hnf1, hnf4,* and *runx* in the intestine and *foxd4* in the rectum and the anus (Bate et al., 2026; Chen et al., 2021; Choi et al., 2006; Desai et al., 2008; Fijneman et al., 2012; Hussain et al., 2002; Jin et al., 2005; Kaser et al., 2008; Lan et al., 2001; Masui et al., 2007; Nelson et al., 2005; Pan et al., 2015; Wang et al., 2018).

Among these regulatory genes, the two ParaHox *pdx1* and *cdx2* have crucial roles in the specification of regional identities and the correct functioning of the vertebrate gut. *pdx1* shows a high A-P gradient of expression along the mouse intestinal tract, and it is essential for pancreas development and β-cell maintenance (Monaghan et al., 1993; Offield et al., 1996; Stoffers et al., 1997). It is expressed in the antrum, pylorus, and duodenum, marking the region of the early gut tube from which several accessory digestive organs such as the liver, gallbladder, and both dorsal and ventral pancreas emerge. In *pdx1*-null embryos, the pancreas is severely reduced and lacks insulin-producing β-cells (Offield et al., 1996). Loss of *pdx1* also disrupts morphogenesis of the proximal duodenum, producing an abnormal pyloric sphincter, impaired gastric emptying, and a duodenum that resembles a posterior extension of the stomach (Jonsson et al., 1994; Offield et al., 1996). *cdx2* is initially required for establishing and maintaining posterior identity and, at later stages, it is required for maintaining A-P identity. The regulation of *cdx2* expression in the developing gut is a point of convergence by which signaling pathways regulate intestinal development also by acting through Hox genes which are

expressed in an A-P sequence along both the endodermal and mesodermal components of the developing gut (Beck et al., 2000). However, experimental evidence shows that Hox factors do not play major roles in endodermal gut differentiation as disrupting individual Hox genes has minor effects on gut endoderm formation (Aubin et al., 1997; Boulet & Capecchi, 1996; Manley & Capecchi, 1995; Warot et al., 1997; Zacchetti et al., 2007). A recent study in chick showed that rather than acting only as regional identity markers, Hox genes directly influence tissue tension, epithelial remodeling, and epithelial-mesenchymal interactions, producing the characteristic villous architecture of the small intestine and the smoother structure of the large intestine. This reveals a direct link between Hox-mediated patterning and biomechanical processes, demonstrating that regional gut morphology arises as a direct consequence of genetically encoded differences in tissue mechanics (Gill et al., 2024).

The recent generation of scRNA-seq data (Chan et al., 2019; Nowotschin et al., 2019; Peng et al., 2019; Pijuan-Sala et al., 2019) and their combination together with gene expression data and bulk RNA-seq confirmed with unprecedented resolution the establishment of distinct territories along the A-P axis of the mouse embryonic gut tube, prior to the appearance of its organs. The analysis revealed a core set of ~20 TFs defining A-P domains (*nkx2-1, pax8, nkx2-5, nkx2-3, isl1, otx2, prrx2, six1, foxg1, irx3, hob1, meis2, gata6, foxa3, cdx2, hoxa7, hoxb8, hoxc8, hoxc9, tlx2*). In this analysis, only a subset of Hox genes appear expressed in the gut, and most of these appear in posterior regions suggesting that A-P patterning of the gut tube and the emergence of organ domains occur before or independently from a classical Hox code, confirming the results of the functional assays. They confirmed active Fgf and Wnt signaling, and identified Notch activity in posterior intestinal clusters. Bmp, Hedgehog,

Jak/Stat, and Hippo signals were found active in multiple regions, while Nodal was inactive. Retinoic Acid (RA) signaling showed both activating and repressive outputs restricted to posterior domains. These studies also supported the dual origin of the gut endoderm (made of primitive endoderm and definitive endoderm) and that both descendants become comparably patterned along the A-P axis of the gut tube while retaining aspects of their lineage history through expression of a core set of genes.

Additional advanced approaches to studying gut development have led to the introduction of vitro culture systems that generate three-dimensional epithelial "mini-guts" from single mouse stem cells, known as 3D intestinal epithelial organoids (Sato & Clevers, 2013). These systems enable controlled in vitro analyses of gut cell-fate determination, stem cell dynamics, gene function, and disease models. More recently, Li and colleagues developed human "gastroids," self-organized multilayer gastric organoids derived from human pluripotent stem cells, providing an in vitro model to study stomach patterning (Li et al., 2025). In parallel, another recent study (Xu et al., 2025) integrated single-cell transcriptomes from 218 organoid and tissue-model samples to generate the first human endoderm-derived organoid cell atlas, capturing the diversity of endodermal lineages across organoids generated from multiple stem-cell sources and under different experimental conditions. Together, these innovative approaches promise to explain gut patterning with unprecedented cellular and molecular resolution, opening new avenues for dissecting the development of digestive systems and their associated diseases.

1.2.2 Gut Patterning in Protochordates

Protochordates represent the closest extant outgroups to vertebrates and therefore provide a crucial comparative framework for reconstructing the

ancestral organization, developmental logic, and molecular architecture of endoderm-derived organs in chordates. Their phylogenetic position, predating both the whole genome duplications and the extensive morphological diversification that characterize early vertebrate evolution, allows the protochordate digestive system to be viewed as a functional and regulatory baseline against which vertebrate innovations can be assessed. Protochordates include lancelets and tunicates which are both suspension feeders and share a common sequence of digestive compartments along the A-P axis (mouth, pharynx, esophagus, stomach/chamber, intestine, and anus).

Although it lacks several vertebrate innovations, such as complex foregut derivatives, liver, and pancreas, the protochordate digestive tract preserves the core regulatory networks governing endoderm formation and gut patterning combining canonical Wnt, Bmp, Nodal, and Fgf signaling in a manner broadly comparable to the early steps of vertebrate gut formation (Imai et al., 2000; Lemaire, 2009). In fact, molecular studies, single-cell transcriptomics, and morphological analysis show that protochordates possess precursors to vertebrate digestive organs and functions. This suggests that there is significant evidence for homologies between the protochordate and vertebrate digestive tracts, confirming that the basic chordate gut plan originated in the common ancestor of both groups (D'Aniello et al., 2023).

Tunicates, or urochordates, include three distinct lineages: ascidians, salps, and larvaceans. As a group, they exhibit notable variation in body architecture, developmental trajectories, and reproductive modes, and their adult forms span a broad ecological spectrum, ranging from permanently attached filter feeders to fully pelagic, free-moving organisms. After gastrulation, the primitive gut tube elongates and folds into the

characteristic U-shape in which the anterior region differentiates into the pharynx with its branchial sac and endostyle, while the posterior part gives rise to the esophagus, stomach, and intestine. By metamorphosis, these regions are fully patterned and functional, and histological analyses show that the tunicate gut is made of a single endodermal epithelial layer, lacking both muscle and other tissue layers, including sphincters (Budelmann et al., 1997a; Satoh, 1994).

Most information about gut patterning in tunicates is derived from studies on the ascidian *Ciona intestinalis*. Ascidians are sessile tunicates that typically feature a U-shaped digestive tract made of pharynx, esophagus, stomach, and intestine (Ruppert et al., 2003). They develop through a larval stage that lacks specialized organs, with the digestive system forming later in the juvenile stage following metamorphosis. The pharynx forms a large branchial chamber with numerous gill slits and an endostyle, and connects posteriorly to the esophagus. Ascidians also develop a hepatopancreas (or pyloric gland), a branched organ arising at the junction between stomach and intestine (Hirano & Nishida, 2000; Orito et al., 2015).

All nine Hox genes encoded in the Ciona genome are expressed along the juvenile gut in a clear A-P sequence (Ikuta et al., 2004; Nakayama et al., 2016). *pdx1* and *cdx* show region-specific expression in the juvenile posterior stomach and anterior intestine (just posterior to the stomach), respectively (Nakayama et al., 2016). Functional studies indicate that *hox-10* knockout eliminates the posterior intestine, while loss of *hox-12* disrupts its proper formation (Kawai et al., 2015; Sasakura & Hozumi, 2018). These posterior intestinal domains, marked by *hox-10* and *hox-12*, originate from the larval endodermal strand (Nakazawa et al., 2013), suggesting that both

genes contribute to endodermal-strand migration and/or intestinal morphogenesis (Kawai et al., 2015).

soxB1, tfl, ptf1a, and *mnx* are also expressed in the Ciona digestive tract, showing patterns that parallel those seen in vertebrates. *soxB1*, the tunicate ortholog of vertebrate *sox2*, is active from the pharynx through the stomach (Nakayama & Ogasawara, 2017); *ttf1* is restricted to the endostyle (Ogasawara et al., 1999); *ptf1a* and *mnx* are expressed in the stomach in a domain reminiscent of the position of the vertebrate pancreas (Nakayama & Ogasawara, 2017). Notably, the *ptf1a* domain overlaps with regions expressing exocrine digestive enzyme genes, mirroring, as detailed in Chapter 2, its regulatory role in vertebrates. Together, these expression patterns suggest that parts of the GRN operating in the tunicate stomach are evolutionarily related to those that pattern the vertebrate pancreas. This is thoroughly discussed in Chapter 2.

A recent study provided a transcriptional landscape of three distinct regions of the adult Ciona gut, the stomach, proximal intestine, and distal intestine (Gerdol et al., 2024), revealing that key components of major signaling pathways involved in vertebrate gut development, including Wnt, Notch, and Tgfβ/Bmp, are robustly expressed throughout the Ciona intestine.

Lancelets, of which amphioxus is the best-studied representative, are fish-like marine chordates, and their phylogenetic position makes them well suited for providing insights into the ancestral organization and patterning of the chordate digestive system. In these animals, gastrulation leads to the formation of a straight, unsegmented gut tube which progressively regionalizes into a pharynx, an esophagus, and an intestine subdivided into an anterior midgut and a posterior hindgut. Within the midgut lies an

endoderm-derived pouch, called the hepatic caecum, which has been proposed to function as a proto-liver or proto-pancreas.

Unlike vertebrates, which remodel the gut through extensive morphogenetic bending and looping after the ingression of the archenteron, the amphioxus gut largely retains a simple, straight tubular shape though it exhibits rich molecular patterning. Like in tunicates, the alimentary canal of lancelets is composed of a single layer of endoderm-derived epithelium, but sphincter-like structures are present at the velum, a valve-like structure located at the posterior end of the pharynx, separating the pharyngeal chamber from the midgut, and at the anus (Budelmann et al., 1997a).

The expression profiles of several developmental TFs have been described in early embryos. Among the 15 Hox genes encoded in their genomes, only *hox-1* and *hox-14* show endodermal expression (*hox-1* in the pharyngeal region and *hox-14* in the hindgut) (Holland & Holland, 1996; Schubert et al., 2005; Pascual-Anaya et al., 2012). Amphioxus larvae express *pdx1* in the midgut and *cdx* in the hindgut (Brooke et al., 1998). Functional studies have also demonstrated that *pdx1* is required for midgut specification, while *cdx* plays a key role in anus formation during amphioxus gut development (Zhong et al., 2020). Other TFs include *soxB1c*, the amphioxus ortholog of vertebrate *sox2*, which marks the pharyngeal endoderm (Meulemans & Bronner-Fraser, 2007), and *ttf1*, expressed in the endostyle (Ogasawara, 2000).

Recent scRNAseq analysis of the amphioxus adult digestive tract (Dai et al., 2024) revealed a sophisticated spatial organization of the gut. Different clusters of cells resulted specialized for intracellular digestion, lipid metabolism, and immune defense, mirroring the functional segmentation seen in vertebrates. In more detail, clustering analyses showed that the digestive tract is divided into four main regions: midgut 1 and midgut 2

cluster together, while midgut 3, hindgut, and the hepatic diverticulum form distinct groups. Marker gene expression supports clear regionalization: Hox gene expression shows collinearity along the A-P axis of the gut, with distinct Hox groups marking distinct regions: *hox3-7* are enriched in midgut 1, *hox-8* in midgut 2, and *hox9-12* in the hindgut. *pdx1* is strongly enriched only in midgut 3, while *cdx* is specific to the hindgut. In this study, the authors also performed bulk and single-cell transcriptomic analyses of wild-type and *pdx1*-mutant amphioxus and showed that Insulin-like peptide gene expression is independent of *pdx1* and that it primarily controls body growth. They propose that *pdx1*-dependent regulation of insulin genes and the clustering of insulin-secreting gut endocrine cells likely originated in the vertebrate lineage.

1.2.3 Gut Patterning in Ambulacraria

Ambulacraria is a monophyletic clade within Deuterostomia that includes two, exclusively marine, sister clades: the echinoderms (including sea urchins, sea stars, sea cucumbers, sea lilies, feather stars, and brittle stars) and the hemichordates (acorn worms and pterobranchs). Members of both groups produce bilaterally symmetric, ciliated, free-swimming dipleurula-type larvae, which metamorphose into pentaradial adults in echinoderms and worm-like forms in hemichordates (Arnone et al., 2015).

Echinoderms have long served as versatile research organisms in developmental, cellular, and molecular biology thanks to their transparent embryos, external development, and ease of experimental manipulation (Arnone et al., 2015). Their divergence before the vertebrate genome-duplication events also offers a simplified comparative context and the availability of genomic and transcriptomic resources (Magri et al., 2026; Marlétaz et al., 2023; Parey et al., 2024) together with the application of

high-throughput assays for mapping GRNs, such as ATAC-seq and scRNA-seq (Massri et al., 2025; Meyer et al., 2023; Paganos et al., 2021; Paganos et al., 2025; Tominaga et al., 2023; Voronov et al., 2024), enable detailed analysis of developmental regulation. The sea urchin embryo hosts the most comprehensively resolved GRN available, explaining the regulatory logic underlying endomesoderm specification (Croce & McClay, 2010; Croce et al., 2011; Davidson et al., 2002; Peter & Davidson, 2010). In sea urchins, gut morphogenesis initiates at the onset of gastrulation, when endodermal precursors at the vegetal pole undergo a coordinated invagination to form the archenteron (Lyons et al., 2012). Once elongated, the archenteron reaches the oral ectoderm and attaches to it, establishing the site of future mouth opening. Subsequent regionalization along the A-P axis partitions the gut into three morphologically and molecularly distinct domains: the foregut (prospective esophagus), the midgut (stomach), and the hindgut (intestine and anus). The functional larval gut is made of a single epithelium of cells and consists of a muscular esophagus, a large spherical stomach, and a tubular intestine and it is composed of cell types with highly specialized morphologies and functions. Two sphincters made of myoepithelial cells, the cardiac sphincter and the pyloric sphincter, separate the esophagus from the stomach and the stomach from the intestine, respectively (Annunziata et al., 2014; Burke & Chia, 1980; Paganos et al., 2021). Myoepithelial cells are also present in the anal sphincter.

While the foregut and midgut are already molecularly specified by the late gastrula stage and undergo mainly morphological changes thereafter, the hindgut remains a major site of cell specification throughout late gastrulation. Several aspects of intestinal differentiation during this period have been elucidated. *Pdx1* (previously named *xlox* in *Xenopus* and in most invertebrates) and *cdx* play central roles in intestine formation. Both

ParaHox genes are expressed in nested domains in the posterior region of the invaginating archenteron, with *pdx1* positioned anterior to *cdx* (Cole et al., 2009). By the end of gastrulation, these domains segregate, with *pdx1* becoming restricted to the pyloric sphincter and *cdx* marking the entire intestine (Cole et al., 2009). Resembling the vertebrate phenotype, knockdown of *pdx1* results in a malformed gut lacking the pyloric sphincter and showing reduced digestive capacity (Cole et al., 2009). Pdx1 in sea urchin is also required for *cdx* activation, while Cdx subsequently represses it in intestinal cells (Cole et al., 2009) through the action of a Wnt10 signaling event (Annunziata & Arnone, 2014).

In late gastrula embryos, cells expressing *pdx1* and *cdx* also co-express *hox11/13b*, *brachyury* (*bra*), *blimp1a*, and *foxA*, together defining a regulatory state specific to the most posterior region of the archenteron. *cdx* expression is regulated by Hox11/13b, Bra, FoxA, and Pdx1, with Pdx1 likely acting as a temporal switch triggering *cdx* activation. In the larval intestine, Cdx also becomes auto-regulatory, reinforcing the intestinal regulatory state. Consistent with this, Cdx protein localization matches its mRNA domain throughout intestinal development (Annunziata & Arnone, 2014). Pdx1 is essential not only for activating stomach terminal differentiation genes, but also for inducing myosin heavy chain (*Mhc*) expression in the endodermal cells that will form the pyloric sphincter.

The resolution of this sea urchin posterior gut GRN has been further expanded using an integrative multi-omics approach integrating single-cell RNA-seq, ATAC-seq, and differential bulk RNA-seq following *pdx1* knockdown (Annunziata & Arnone, 2014; Voronov et al., 2024). This approach recapitulates the previously reported hindgut interactions, revealing that many are likely indirect, and provides higher-resolution definition of regulatory nodes. The updated GRN shows a complex and

highly interconnected architecture, confirming the conservation of key regulators shared with the vertebrates (*pdx1, cdx, foxA, blimp1*) while implicating additional TFs (*osr, runt, hnf4, irf*) absent from earlier models. Although Pdx1 influences several vertebrate homologs involved in posterior gut patterning, further comparative analyses will be required to assess how the network has been rewired around Pdx1 across echinoderms and in vertebrates. Recent studies reconstructing single-cell transcriptomic atlases of sea urchin embryonic and larval stages have defined comprehensive molecular inventories of the TF toolkit utilized during gut development and subsequent gut cell type specialization (**Figure 1.2A**). Such inventories allow the dissection of TF dynamics involved in gut patterning for development and evolution (**Figure 1.2A,B**).

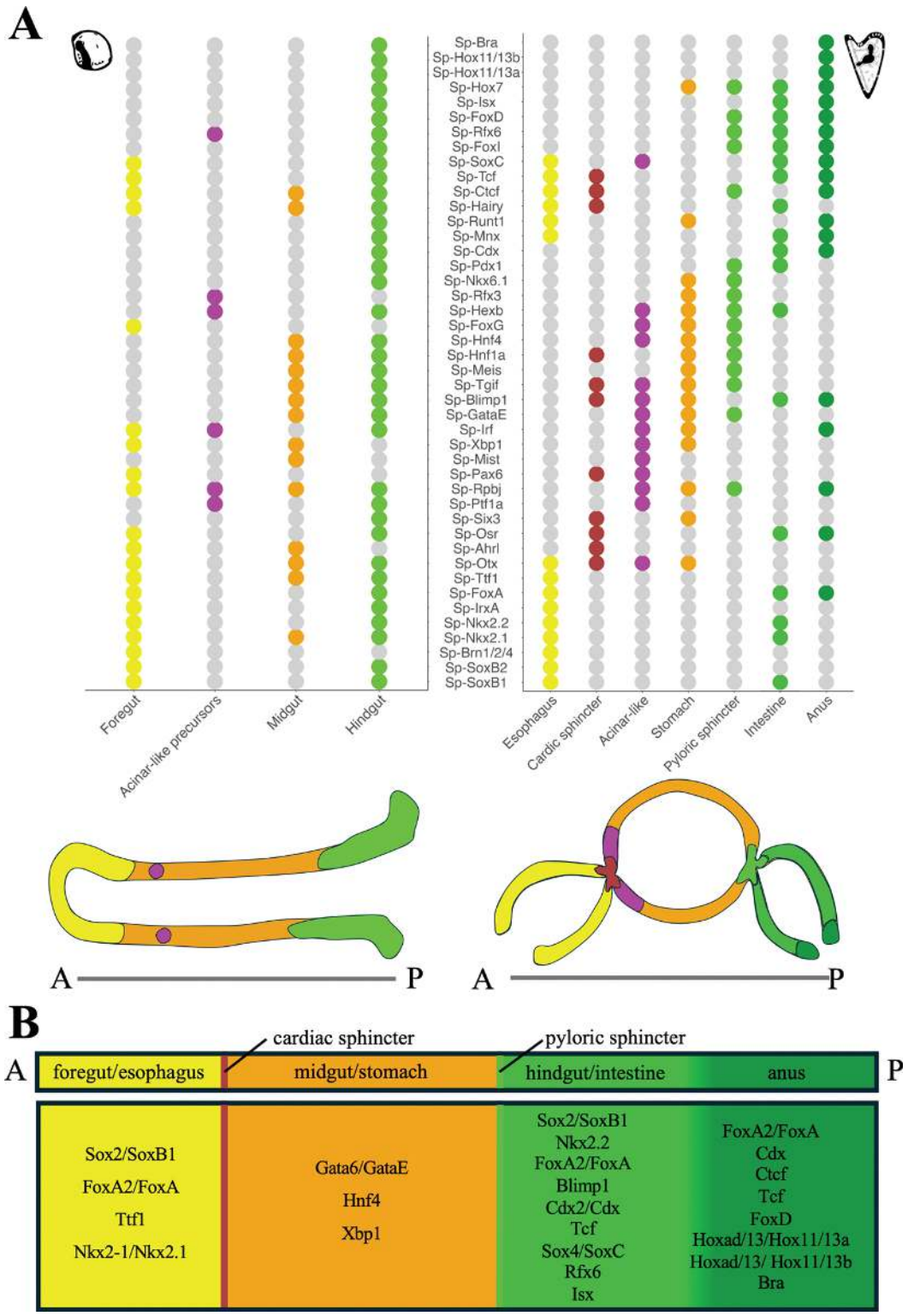


FIGURE 1.2 Gut patterning TF dynamics during development and evolution. A) Summary of TF expression across embryonic (left) and larval (right) gut domains in the sea urchin *Strongylocentrotus*

purpuratus, as revealed by single-cell transcriptomic analyses (Paganos et al., 2021, 2022; Voronov et al., 2024). Colored dots indicate TF expression within a given gut domain, whereas grey dots denote absence of expression. Dot colors correspond to specific gut domains. B) Schematic representation of an abstract gut model, illustrating shared features in the organization of mouse and sea urchin digestive systems. The diagram highlights examples of evolutionarily conserved patterning TFs along the A-P axis of the gut. ⏎

Recent studies report that early gut-patterning markers including Hox and ParaHox genes, remain expressed along the sea urchin juvenile (Paganos et al., 2025) and the adult gut (Vianello et al., 2026), suggesting that the AP molecular signature established during larval development is either maintained or re-established after metamorphosis, despite the profound reorganization of the gut that occurs during this transition (Vianello et al., 2026). Such continuity highlights a surprising stability of the larval A-P program well into adulthood.

Hemichordates are bilaterally symmetrical animals with a distinctive tripartite body organization consisting of a proboscis, collar, and trunk. Like echinoderms, gut development begins during gastrulation, which proceeds via invagination at the vegetal pole, producing the archenteron. In species with indirect development (e.g. *Ptychodera flava*), the embryo develops into a tornaria larva, which possesses a functional, through-gut (Lowe et al., 2003; Röttinger & Lowe, 2012). The tornaria larval gut typically consists of a mouth located between the developing pharynx, an esophagus, a midgut/intestine, and a terminal anus. The ParaHox gene expression has been studied in *P. flava* larva (Ikuta et al., 2013). *Cdx* is the first to appear, encircling the blastopore in early gastrulae and then extending into the posterior archenteron as gastrulation progresses. This domain persists into the tornaria larva, where *cdx* marks the hindgut and extends anteriorly up to the midgut-hindgut boundary. *Pdx1* emerges slightly later, sharing the same

blastoporal territory but remaining confined to the most posterior endoderm as the embryo transitions to the larval stage. Interestingly, this relationship shifts during metamorphic transformation. As the competent larva begins to reorganize, the relative arrangement of *cdx* and *pdx1* conforms once again to the conserved ParaHox order. Thus, spatial collinearity, the correspondence between the linear order of *pdx1* and *cdx* on the chromosome and their spatial domains of expression along the A-P axis during development, appears disrupted during early embryogenesis, but it is re-established in the transforming larva, revealing a dynamic reorganization of ParaHox domains across development.

Interestingly, the gene modules found to conserve relative A-P information between the larval and adult sea urchin guts show a comparable A-P pattern in the *P. flava* adult gut as well as in the gut of a protostome (the annelid *Platynereis dumerilii*), the amphioxus, and the clownfish (Vianello et al., 2026). This analysis revealed five recurring TF modules that map onto conserved A-P gut territories across all species (including Hox, ParaHox and other TFs involved in gut patterning). These modules can be shifted, expanded, or compressed in individual lineages, reflecting evolutionary gut morphological remodeling while representing an underlying common logic for A-P gut patterning across Bilateria.

1.3 MUSCLES ASSOCIATED WITH GUT

1.3.1 The Anatomical Composition and Distribution of GI Muscles

The anatomical and functional difference of GI tracts mainly depends on (i) the phylogeny and (ii) the lifestyle/dietary habits of the organisms (Schmidt-Rhaesa, 2007). For example, some herbivorous animals (e.g. ruminants) need severe mechanical breakdown of their food particles and

therefore their digestive tract is strongly compartmentalized to regulate the flow of food substances (Lentle & Janssen, 2014). In contrast, some suspension feeders possess a simplified digestive tube without major anatomical differences and constrictions (Hamann & Blanke, 2022). Moreover, cases where one or more gut domains are clearly absent are reported; for instance isopods miss a midgut (Hames & Hopkin, 1989), gnathostomulids lack a hindgut (Knauss, 1979), and rotifers have a cloaca – defined as a common opening for the digestive, reproductive, and urinary products – instead of a hindgut (Clément & Wurdak, 1991). Finally, species-specific gut-associated structures and regions are often found, such as the hepatopancreas in crustaceans (Vogt, 1994), the cecum in cephalopods (Bidder, 1950) and the pyloric gland in tunicates (Burighel & Cloney, 1997).

The arrangement and sub-functionalization of GI tract into specialized domains can occur via the GI musculature: the smooth muscles, which surround the digestive tube, and the sphincters that divide the tube into different compartments. The alternating waves of GI muscle contraction and relaxation (peristalsis) are therefore used for the transport of food particles along the digestive tract for ingestion (via esophagus), digestion (via stomach), and defecation (via intestine) to take place, as well as to prevent the reflux and the uncoordinated expulsion of food particles (Golenhofen & Mandrek, 1991).

Within vertebrates, the segments of the GI wall are divided into four distinct layers: the folded mucosa surrounding the gut lumen and its epithelium, the connective tissue of submucosa, the muscularis propria [consisting of an inner circular muscle layer, intermuscular space, and an outer longitudinal muscle layer (as well as an additional inner oblique muscular layer found only in the stomach)], and the serosa (comprised of

external epithelium and connective tissue) (Shen, 2009) **(Figure 1.3A)**. The coordinated contractions of the muscularis propria propel the food along the GI tract. In particular, the circular muscle layer prevents the food from traveling backwards, and the longitudinal layer shortens the digestive tube. The inner circular layer also displays some thickened portions called sphincters, whose main function is to prevent the reflux or restrict the gut contents to certain compartments (Welcome, 2019). Three main sphincters are found in vertebrates: the esophageal sphincter (upper and lower (named cardiac) parts) situated at the upper portion of the stomach, the pyloric sphincter found at the lower end of the stomach, and the anal sphincter (consists of an internal and an external layer) positioned at the posterior end of the digestive tube (**Figure 1.3B**). Additionally, an ileocecal sphincter is visible at the junction of the small and large intestine. Importantly, the esophageal muscle is unique due to its composition of both skeletal and smooth musculature. In particular, the upper part is entirely skeletal, followed by a short domain, which is a mixture of skeletal and smooth muscle, and the lower part is entirely smooth.

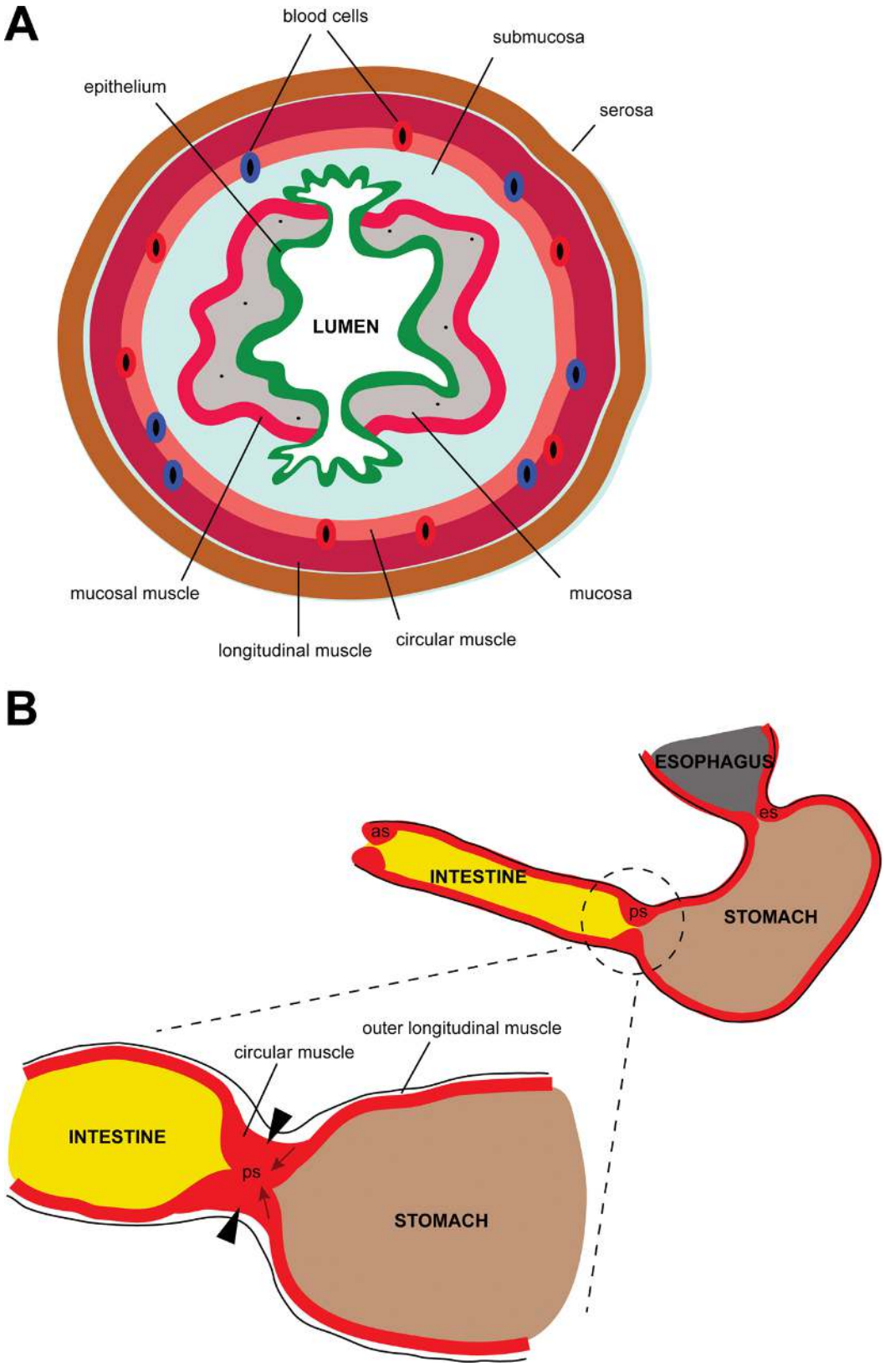

A
blood cells
submucosa
epithelium
serosa
LUMEN
mucosal muscle
mucosa
longitudinal muscle
circular muscle
B
ESOPHAGUS
es
as
INTESTINE
ps
STOMACH
outer longitudinal muscle
circular muscle
INTESTINE
ps
STOMACH

FIGURE 1.3 Architecture and function of the GI tract musculature. A) The different layers of smooth musculature surrounding the gut lumen. B) Function of the pyloric sphincter. cs, cardiac sphincter; ps, pyloric sphincter. ⏎

Invertebrates also possess gut-associated muscular constrictions to efficiently control the direction of food flow, which can however vary in their position. Some examples follow. Arthropods exhibit a valve separating their foregut and midgut (cardia) and another between the midgut and the hindgut (Snodgrass, 1935). Xiphosurans use a pyloric valve to separate their gizzard (anterior stomach) from their midgut (Owen, 1873). Decapod crustaceans have a constriction between the two domains of their foregut; the larger cardiac chamber and the smaller pyloric one, through which a bidirectional flow of food particles takes place (Kaestner, 1970). Cephalopods have a system of valves between their stomach and intestine (Budelmann et al., 1997b). Gastrotrichs have a valve between their pharynx and midgut (Harrison, 1991). Bryozoans have two gut sphincters: one between their esophagus and anterior stomach (cardia) and one between their posterior stomach (pylorus) and intestine (Gordon, 1975). In nematodes, valves are seen in each end of their intestine to prevent food backflow (Lee & Atkinson, 1976). Finally, in amphioxus, a muscle sphincter is reported between their midgut and iliocolon (anterior intestine) junction (Barrington, 1937). Interestingly, even within members of the same clade/phyla, major anatomical differences are seen. For instance, whilst all echinoderm larvae exhibit cardiac sphincters between their foregut and midgut; only echinoids, ophiuroids and holothuroids possess an additional pyloric sphincter between their midgut and hindgut compartments (Annunziata et al., 2014; Gliznutsa & Dautov, 2011; Perillo et al., 2024). Crinoid larvae, on the other hand, lack a functional gut (McEdward & Miner, 2001).

Furthermore, some invertebrates with indirect development and dramatic metamorphosis show differences in their gut compartmentalization degree between their larva and adult forms, such as echinoderms and phoronids (Hyman, 1955; Temereva, 2010). Brachiopods, on the other hand, are characterized by a blind gut in their larval form, while adults exhibit through-guts (James, 1997). This major architectural transition of their GI reflects the different lifestyles that characterize the two developmental stages (e.g. pelagic versus benthic).

1.3.2 The Molecular Regulation of GI Muscle Formation and Function

There are two main motor patterns of the GI tract; the peristalsis for propulsion and the segmentation for mixing and absorbing. A third mechanism, named migrating motor complex, is also found and refers to a cyclic, recurring motility pattern occurring during fasting. To perform these motor patterns, the gut motility is highly regulated (Sanders et al., 2012). Except for the outer anal sphincter, all the other sphincters of the GI tract as well as the smooth musculature lining the GI tract are involuntary. Therefore, most of the regulation of gut motility is tightly connected to the nervous system. Several studies have demonstrated not only the presence of extensive GI innervations by the enteric nervous system (ENS) in a number of organisms (Copenhaver, 2007; French et al., 2014; Lange & Chan, 2008; Martinez-Pereira et al., 2013; Nakajima et al., 2004; O'Gara et al., 1999; Temereva, 2010), but also the function of neurotransmitters and enteroendocrine cells in modulating GI muscle activity (García-Arrarás et al., 2019; Spencer & Hu, 2020; Wegener & Veenstra, 2015). The control systems appear to be similar between species, including both intrinsic and extrinsic regulation.

In vertebrates, the ENS is the largest unit of the peripheral nervous system. The intrinsic regulation includes a well-developed, ganglionated nervous plexus, the myenteric plexus, situated between the longitudinal and circular muscle layers of the muscularis propria and extending from the esophagus to the anus (Furness, 2005; Gabella, 1979; Hansen, 2003). A second, less defined ganglionated nerve plexus, the submucous plexus, is located in the submucosa and is predominantly found in the intestine (Gershon, 1999). Sensory nerve axons from the nerve cells of both plexuses innervate the mucosa throughout the GI tract (Furness et al., 2004). Moreover, on the lumen epithelium and the mucosa several enteroendocrine cell populations are found, which also influence the ENS function regarding GI motility (Furness, 2000). Finally, specialized mesenchymal cells, such as the interstitial cells of Cajal (ICCs), are placed in the muscularis propria and serve as pacemakers for GI smooth muscles driving the segmentation motor pattern (Dickens et al., 1999).

The ENS can also act in coordination to the central nervous system (CNS) via the vagus and pelvic nerves, and the circulatory system (Bayliss & Starling, 1901; Furness et al., 2014). This extrinsic regulation includes parasympathetic and sympathetic branches of the autonomic nervous system transmitting information to ENS, by forming synapses onto the myenteric ganglia, smooth muscle, and mucosa, and in turn the ENS is providing input to the CNS (Hansen et al., 1999). This bidirectional communication system is called the brain–gut axis.

Different types of intrinsic neuronal cells are involved in the motor patterns of the GI tract and can be classified according to their neurotransmitters (Furness, 2000). The cells that innervate the circular and longitudinal muscle layers of the GI tract and the epithelium are mainly motor neurons and enteroendocrine cells, which respond to chemical and

mechanical stimuli from the lumen (e.g. dietary signals, microbes) and/or the mucosa (neural and paracrine signals). The major excitatory neurotransmitters of these motor neurons are Acetylcholine (Ach) and tachykinins, which stimulate smooth muscle contraction, while catecholamines (e.g. epinephrine, dopamine), nitric oxide (NO), and Vasoactive Intestinal Polypeptide (VIP) are the main inhibitory neurotransmitters (Brookes et al., 1991; Costa et al., 1992; Fahrenkrug et al., 1978; Holzer & Holzer-Petsche, 1997; Xue et al., 2000). As mentioned already, the effect of both excitatory and inhibitory motor neurons can be mediated by the ICCs, which also possess NO and tachykinin transmitter receptors (Portbury et al., 1996; Young et al., 1993). The enteroendocrine cells transmit mainly cholecystokinin (CCK), secretin, somatostatin, serotonin (5-HT), corticotrophin-releasing factor, gastrin, leptin, ghrelin, and glucagon-like peptide 2 (GLP-2) (Latorre et al., 2016). Additional information on the enteroendocrine molecular profile and function can be found in Chapter 3.

Although invertebrates possess relatively simpler GI architectures, they are also equipped with enteroendocrine and nervous cells regulating gut motor patterns (García-Arrarás et al., 2019; Tierney, 2020; Wegener & Veenstra, 2015). Further details on this can be found in Chapter 3. Also, the function of some neurotransmitters seems to be conserved in regulating gut motility, as shown in few studies. For example, the role of NO in relaxing the pyloric sphincter (Mashimo et al., 2000; Sobchak et al., 2016) extends beyond the vertebrate group, since echinoderm larvae also utilize endoderm-derived neuronal NO synthase (nNOS)-positive cells to control their pyloric sphincter relaxation (Yaguchi & Yaguchi, 2019). Interestingly, recent observations on early sea urchin larvae suggest that serotonin released from apical organ neurons into the coelomic cavity controls NO

signaling in response to light (Yaguchi & Yaguchi, 2021). The pathway from photoreception to sphincter modulation has been recently reconstructed, with Opsin3.2 responding to blue light and inducing serotonin and NO pathways. On the other hand, the anal sphincter opens through the inhibitory action of Opsin2 on dopamine and Ach pathways, after responding to blue and longer wavelengths of light, to prevent the simultaneous opening of both sphincters (Yaguchi et al., 2024). Moreover, in flies serotonergic and acetylcholinergic pathways may also be involved in coordinating motor and endocrine activities of the GI tract (Fujita et al., 2024; Schoofs et al., 2014). Other neurotransmitters involved in regulating gut muscle contraction include the myoinhibitory peptide (MIP) (Williams, 2020). MIPs inhibit muscle contraction in the hindgut and pyloric junctions in arthropods (Aguilar et al., 2006; Szabo et al., 2011), but in annelid they work the opposite way by promoting gut muscle contractions (O'Gara et al., 1999; Williams et al., 2015). Interestingly, the participation of these neuropeptides in sphincter contraction seems to be used also in non-bilaterians, since in cnidarians they promote muscle contractions (Takahashi et al., 1997; Takahashi et al., 2003). The development of GI musculature occurs in parallel to the formation of the archenteron. In vertebrates, the smooth muscle that surrounds the digestive tube derives from the lateral plate splanchnic mesoderm, which undergoes differentiation and finally surrounds the developing GI tract. An important cascade mediating this differentiation is *Shh,* which signals from endoderm to mesoderm (Marigo et al., 1996; Roberts et al., 1998; Sukegawa et al., 2000). As the lateral plate mesoderm differentiates into smooth muscle, muscular constrictions (sphincters) appear along the developing tube resulting in compartmentalization along the A-P axis. Hox genes play a pivotal role in demarcating these morphological and functional boundaries between the

resulting gut compartments (Roberts et al., 1995; Yokouchi et al., 1995; Zákány & Duboule, 1999). In the chick hindgut, the expression of *abd-B*-like *Hox* genes of the A and D cluster demarcates morphological boundaries of the mid- and hindgut regions (Roberts et al., 1995; Yokouchi et al., 1995; Zákány & Duboule, 1999). In mice, HoxD contributes to the formation of the ileocaecal sphincter, which divides the small intestine from the large bowel (Roberts et al., 1995; Yokouchi et al., 1995; Zákány & Duboule, 1999), as well as the anal one (Kondo et al., 1996).

The pyloric sphincter in vertebrates is specified by *nkx2-5*, *sox9*, and *gremlin,* which are specifically expressed in the pyloric mesoderm and involved in the formation of the pyloric epithelium (Moniot et al., 2004; Nagura et al., 2025; Theodosiou & Tabin, 2005; Udager et al., 2014). Expression of *nkx2-5* and *sox9* is induced by the Bmp signal, which is downstream of the Shh signal (Roberts et al., 1995, 1998; Smith & Tabin, 1999; Theodosiou & Tabin, 2005). Other genes involved in the pyloric sphincter formation are *bapx1* (a synonym of *nkx3-2*) and *gata3 (*Nagura et al., 2025; Udager et al., 2014). In mice, *six2* also participates in the specification of the pyloric sphincter (Self et al., 2009). Additional genes found to be expressed in the forming pyloric sphincter of metamorphic tadpoles are *pax6.L, foxa1.L*, *rxrb.L/S*, etc. (Nagura et al., 2025). Finally, signaling cascades with a putative involvement are the Wnt signaling, since overexpression of Wnt produces oversized stomach and lack of pyloric sphincter (Heller et al., 2002) and RA, since inhibition of RA signaling transforms the stomach of the tadpole into a stomach with a pyloric sphincter (Bloom et al., 2013; Ruibal & Thomas, 1988).

Invertebrates also utilize Hox and ParaHox genes to form muscular constrictions within their GI, for instance annelids (Irvine & Martindale, 2000), flies *(*Pankratz & Hoch, 1995; Tremml & Bienz, 1989*)*, and sea

urchin larvae (Annunziata & Arnone, 2014), suggesting that the function of Hox genes in sphincter formation is conserved across species. In sea urchin larvae, the key gene responsible for the pyloric sphincter formation is *pdx1*. In fact, *pdx1* morphants lack the pyloric sphincter and the musculature is not differentiated (as witnessed by the lack of myosin heavy chain expression) (Annunziata & Arnone, 2014; Cole et al., 2009). Moreover, the anal sphincter of the sea urchin larva expresses *cdx*, *foxA*, *brachyury,* and *hox11/13b* genes (Paganos et al., 2021).

1.4 GUT PATTERNING IN PROTOSTOMES

As in deuterostomes, the development of the archenteron in protostomes occurs during gastrulation, giving rise to compartmentalized GI. An overview of the gut anatomy of some protostome representatives can be found in Chapter 2. During this process, the endodermal precursors get internalized into the embryo through the blastopore, a transient opening, which will later form the mouth or the anus (Lankester, 1877). The fate of the blastopore, the morphogenetic movements, and the cell lineage that will lead to archenteron formation vary significantly within protostomes. However, despite the embryological, lineage-specific, and morphological differences encountered, the molecular program for gut development is highly conserved also among protostomes and comprises orthologous genes that act not only during the specification of endoderm but also during its compartmentalization along the A-P axis (**Figure 1.4**). In this context, the incorporation of cutting-edge technologies, such as single cell sequencing, has allowed the integration and further comparison of single cell profiles from a variety of organisms, such as arthropods, Platyhelminthes, annelids and molluscs, which largely supports and enhances the extensive molecular similarities reported previously among gut compartments within the

Protostomia (Achim et al., 2018; Fincher et al., 2018; Piovani et al., 2023; Plass et al., 2018; Salamanca-Díaz et al., 2022; Wendt et al., 2020).

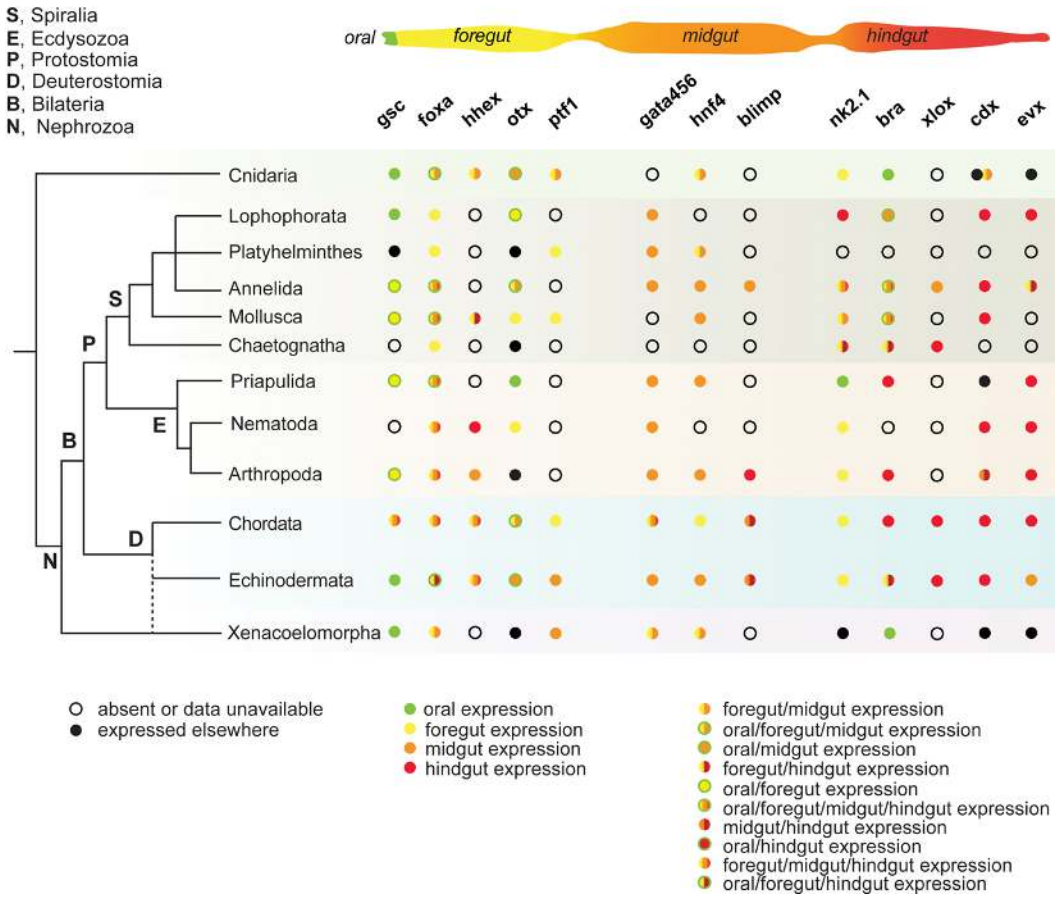


FIGURE 1.4 Phylogeny-based analysis of digestive system patterning. References can be found in the text. Information for cnidarian gene expression can be found in Steinmenz et al. (2017). ⏎

Protostomes consist of two major groups: the Spiralia and the Ecdysozoa (**Figure 1.4**). Regarding Spiralia, except for chaetognaths (John, 1933) and lophophorates (Zimmer, 1997), the remaining spiralian groups (e.g. molluscs, annelids, Platyhelminthes, nemerteans, etc.) are characterized by a highly conserved, stereotypic program of spiral cleavage (Costello & Henley, 1976; Hejnol, 2010; Henry & Martindale, 1999). In a typical spiralian embryo, the archenteron forms from one endomesodermal

precursor cell, the 4d micromere (Lambert, 2010). Lophophorates, however, exhibit radial development and develop their gut through invagination of endomesoderm at the vegetal pole (Zimmer, 1997). Also, the enigmatic chaetognaths, characterized by a total and equal cleavage, form their primitive gut via an invagination of the blastopore and subsequently a second opening (mouth) opposite to the blastopore (John, 1933). Other lineage-specific variations of the stereotypic spiral mode of development are also encountered, such as the cephalopod molluscs (Wadeson & Crawford, 2003) and parasitic Platyhelminthes (Thomas, 1986).

In stark contrast with the Spiralia, strong variations in archenteron development exist within Ecdysozoa. As an example, the two widely used model systems, the *C. elegans* and *D. melanogaster,* exhibit highly adaptive modes of development and therefore a variety of the ontogenetic programs leading to the specification and differentiation of the archenteron. In *C. elegans,* the archenteron is derived from the E lineage, descendent of the endomesodermal EMS cell (Sulston et al., 1983), while in *Drosophila* it arises from two endodermal clefts localized at either end of the embryo: the anterior and posterior primordia (Sweeton et al., 1991). Other studied ecdysozoan groups, such as the slowly evolving group of Priapulida and the Onychophora, form their archenteron using a more conserved ecdysozoan gut-patterning program including endomesodermal cells that ingress from the posterior/vegetal pole (Janssen et al., 2014; Martín-Durán & Hejnol, 2015).

After the protostomian endoderm gets specified, its different compartments start to form, namely the foregut, midgut, and hindgut. Conserved TFs in the formation of the anterior region of the alimentary canal – the foregut – are *goosecoid, foxa, hhex, otx,* and *ptf;* though with clade-specific expression particularities (**Figure 1.4**). Specifically, the

homeobox gene *goosecoid* is expressed in the developing foregut and stomodeum of priapulids, arthropods, molluscs and annelids (Arendt et al., 2001; Boyle et al., 2014; Goriely et al., 1996; Lartillot et al., 2002a; Martín-Durán et al., 2012). In lophophorates, however, it only marks the oral opening (Andrikou et al., 2019; Martín-Durán et al., 2016). The forkhead-box containing gene *foxa/hnf3* seems to have a highly conserved role in bilaterian gut patterning as it is expressed in the entire gut of several protostome species investigated, namely in nematodes, arthropods, priapulids, annelids, and molluscs, and additionally the stomodeum of annelids, molluscs, and priapulids (Arenas-Mena, 2006; Boyle & Seaver, 2008; Christodoulou et al., 2010; Horner et al., 1998; Lartillot et al., 2002a; Martín-Durán et al., 2012; Perry et al., 2015; Weigel et al., 1989). In lophophorates, chaetognaths, and Platyhelminthes, however, *foxa/hnf3* demarcates only the foregut region (Andrikou et al., 2019; Koinuma et al., 2000; Martín-Durán et al., 2016; Ordoñez & Wollesen, 2024; Vellutini et al., 2017). A recent study showed that in nematodes, *pha-4/foxA* also controls the function of pharyngeal neurons, participating therefore in the digestion process (Walker et al., 2026). The deuterostome gut marker *hhex* exhibits a more diverse pattern in protostomes: it is expressed in the foregut and hindgut in molluscs (Perry et al., 2015), in *C.elegans* it patterns the pharyngeal isthmus and the hindgut (Mörck et al., 2004) and in arthropods *hhex* expression is confined to the midgut (Tomancak et al., 2002). *otx* is expressed in the future mouth of annelids, priapulids, and lophophorates (Andrikou et al., 2019; Arenas-Mena & Wong, 2007; Boyle et al., 2014; Martín-Durán et al., 2012; Martín-Durán et al., 2016). Additionally, in annelids, *otx* expression extends to foregut and midgut regions (Boyle et al., 2014) and in lophophorates just to the foregut (Andrikou et al., 2019; Martín-Durán et al., 2016). In molluscs, *otx* expression is restricted to the

foregut region (Nederbragt et al., 2002). Lastly, the *ptf1* gene is expressed in the foregut of Platyhelminthes and molluscs (Salamanca-Díaz et al., 2022).

The TFs *gata456, hnf4,* and *blimp* are frequently involved in patterning the midgut region in protostomes (**Figure 1.4**). In particular, the *gata456* gene has a conserved expression in the midgut of ecdysozoan groups of priapulids, onychophorans, nematodes, and arthropods (Janssen & Budd, 2017; Martín-Durán & Hejnol, 2015; Okumura et al., 2005; Zhu et al., 1997), as well as in the lophophorates, Platyhelminthes and annelids (Andrikou et al., 2019; Boyle & Seaver, 2008; Boyle & Seaver, 2010; Gillis et al., 2007; Martín-Durán & Romero, 2011; Martín-Durán et al., 2016; Vellutini et al., 2017). The expression of *hnf4* gene is also conserved in patterning the protostomian midgut since it is expressed in the midgut of arthropods, onychophorans, Platyhelminthes, priapulids, molluscs and annelids (Achim et al., 2018; Janssen & Budd, 2017; Martín-Durán & Hejnol, 2015; Piovani et al., 2023; Zhong et al., 1993). Moreover, functional data has shown that in *Drosophila hnf4* regulates lipid trafficking (Vonolfen et al., 2024), while in Platyhelminthes maintains gut function (Wendt et al., 2020). Last, the *Blimp* gene is expressed in the midgut of annelids (Arenas-Mena, 2008; Boyle et al., 2014) but in arthropods is instead confined to the hindgut (Ng et al., 2006).

Finally, orthologs of *nk2.1, brachyury, pdx1/cdx*, and *evx* genes are expressed in the most posterior domain of the alimentary canal -the hindgut- in several studied protostome clades (**Figure 1.4**). For example, the T-box gene *brachyury* is expressed in the hindgut region of all ecdysozoan groups studied, namely, arthropods and priapulids (Kispert et al., 1994; Martín-Durán et al., 2012; Singer et al., 1996). Within spiralians, *brachyury* can be expressed in all gut compartments including the mouth, as

reported in members of annelids, molluscs (Arenas-Mena, 2013; Arendt et al., 2001; Boyle et al., 2014; Koop et al., 2007; Lartillot et al., 2002b). Also, in chaetognaths, *brachyury* is restricted to the mouth region (Takada et al., 2002), as it does in lophophorates (Andrikou et al., 2019; Martín-Durán et al., 2016). Interestingly, in phoronids, *brachyury* is also expressed in the ventral midgut. The expression of the ParaHox genes *cdx/caudal* and *pdx1,* which encode homeodomain TFs, is highly conserved in patterning the hindgut region. In fact, *caudal* expression is linked to hindgut formation in several organisms, such as members of nematodes, priapulids, arthropods (Martín-Durán et al., 2012; Schulz et al., 1998; Wu & Lengyel, 1998), but also annelids, molluscs, and lophophorates (Andrikou et al., 2019; Fröbius & Seaver, 2006; Johnson & Lambert, 2021; Le Gouar et al., 2003; Martín-Durán et al., 2016; Rosa et al., 2005; Vellutini et al., 2017). Pdx1 is expressed in the midgut of annelids and the hindgut of chaetognaths (Fröbius & Seaver, 2006; Hui et al., 2009; Ordoñez & Wollesen, 2025). The NK gene *nk2.1* exhibits a more variable expression pattern in protostomes. In particular, *nk2.1* is expressed in the foregut of nematodes, molluscs, and arthropods (Dunn et al., 2007; Harfe & Fire, 1998; Zaffran et al., 2000), but in priapulids transcripts of the gene are found in the oral ectoderm (Martín-Durán & Hejnol, 2015). In molluscs and chaetognaths, however, the expression is extended to the midgut (in the case of molluscs) and hindgut (in the case of chaetognaths) domains (Ordoñez & Wollesen, 2025). Finally, in annelids, *nk2.1* is expressed in the entire developing gut (Boyle et al., 2014; Tessmar-Raible et al., 2007) but in lophophorates it demarcates the hindgut (Andrikou et al., 2019). The last gene associated with hindgut development is the homeobox gene *evx*, which patterns the hindgut in priapulids, nematodes, arthropods, annelids, and lophophorates (Ahringer, 1996; Andrikou et al., 2019; Kozin et al., 2016; Martín-Durán & Hejnol,

2015; Rosa et al., 2005; Seaver et al., 2012; Tomancak et al., 2002; Vellutini et al., 2017). Additionally in annelids *evx* is transiently expressed in the developing foregut (Seaver et al., 2012).

1.5 ANCESTRAL GUT PATTERNING AND EVOLUTION OF THE THROUGH-GUT

The evolution of the through-gut in bilaterians was an important innovation as it allowed not only higher nutrient absorption efficiency but also the prevention of uncoordinated expulsion of food particles due to their unidirectional movement. How and when the through-gut emerged during evolution is still a debated subject (Andrikou et al., 2025; Hejnol & Martindale, 2008; Sedgwick, 1884; Steinmetz, 2019). Is the through-gut homologous within protostomes and deuterostomes? Did the Last Common Metazoan Ancestor already possessed a through-gut or a through-gut evolved after the split of Cnidaria/Bilateria?

To answer these questions, we need to explore gut development under a phylogenetic framework. Firstly, a through-gut can arise both by protostomy and deuterostomy, regardless of the traditional phylogenetic assignment of animals to Protostomia or Deuterostomia. Even within a certain group (e.g. brachiopods, annelids) both modes of development can be observed (Martín-Durán et al., 2016). Therefore, just the fate of the embryonic blastopore, from which the endodermal layer develops, cannot be used as a taxonomic criterion to infer independent evolution of the through-gut within nephrozoans. However, the extensive molecular similarities of GI tract patterning in both protostome and deuterostome groups do support the common ancestry of the nephrozoan gut (Arendt et al., 2001; Wu & Lengyel, 1998). Moreover, the cellular composition of

nephrozoan (and even cnidarian) guts points to a similar, ancestral, functionality (Hartenstein & Martinez, 2019).

Discussing the emergence of through-guts within metazoans, however, is more challenging, as it heavily depends on xenacoelomorph phylogenetic position (Cannon et al., 2016; Philippe et al., 2011). Xenacoelomorpha possesses a blind gut, which expresses all the known mouth/foregut markers, therefore it is homologous to the bilaterian mouth and foregut/midgut (Andrikou et al., 2025; Hejnol & Martindale, 2008) (**Figure 1.4**). Similarly, cnidarians, which also possess a blind gut, have a single opening that likely corresponds to the bilaterian mouth (Arendt, 2004; Steinmetz et al., 2017) (**Figure 1.4**). If xenacoelomorphs are really the sister group to nephrozoans (Cannon et al., 2016), the fact that they don't express orthologs of hindgut markers in their gut suggests that the assignment of these genes in hindgut patterning likely occurred after the Xenacoelomorpha/Nephrozoa split. In fact, these animals utilize hindgut markers to pattern their male gonopore (Andrikou et al., 2025; Hejnol & Martindale, 2008; Moreno et al., 2011). Therefore, it has been proposed that the last common Bilaterian ancestor likely possessed a blind digestive tract with the single opening/mouth expressing *gsc*, *foxa, gata456*, and *hfn4* and that the acquisition of the hindgut, thus the emergence of through-gut, evolved in the lineage of nephrozoans, possibly though a gonadal opening, which may have been later co-opted as a second gut opening (hindgut) (Andrikou et al., 2025; Hejnol & Martindale, 2008). The alternative scenario would be that the last common ancestor possessed a through-gut, and the hindgut with the anal opening expressing hindgut-related genes were secondarily lost in the lineage of xenacoelomorphs, regardless of their phylogenetic position. A third hypothesis is that in the last common ancestor the elongation of the blastopore along the A-P axis, followed by a

lateral closure, left the two ends open and subsequently gave rise to both mouth and anus (amphistomy) (Nielsen et al., 2018). However, so far, no description of a "clear" amphistomy exists for any of the investigated species.

Better phylogenies and in-depth analysis of the genetic interactions underlying the formation of the gut in more animals, including non-nephrozoans, are needed in order to shed light to this long-lasting problem.

1.6 CONCLUSIONS

As we have seen in the previous sections, the development and functional integration of the gut in bilaterian (nephrozoan) animals is a process that is, relatively, well understood in different animal phyla. The process underlying the construction of the digestive system starts with the specification of blastomeres in early embryos, and it is followed by the segregation and amalgamation of cells into a tubular structure, which eventually is functionally regionalized. This last process is guided by the distributed expression of TFs and signaling molecules mutually interacting in GRNs. While "traditional" deciphering of GRNs relied on a long analysis of the molecular players using a complex mix of technologies, such as cis-regulatory analysis, gene knockdowns, expression analysis (in situ hybridization), and bioinformatic integration of the data into specific (and predictive) models, nowadays, the whole process can be done much faster by the introduction of state of the art genomic technologies (i.e. single cell sequencing, spatial transcriptomics, ATAC-seq, Chip-seq, etc.) who allows bulk analysis of data in a high throughput manner, including faster but computationally demanding software packages. All the above technologies are now allowing us to follow up the development of the gut in many animals, giving us unprecedented details of the gene networks that regulate

its development. Cross-comparisons of genomic and single-cell data across species are allowing a further characterization of gene interaction and cell populations participating in the formation of the gut, at any developmental time (i.e. following active chromatin domains or cellular trajectories during embryogenesis and later developmental stages). An in-depth understanding of interspecies developmental programs is currently being achieved in many animal groups, a task that needs to be extended to less-known animal groups.

The through-gut of nephrozoans wouldn't be such an efficient system for processing food if it wasn't because of the interaction of this tissue with the musculature and the nervous system. Integration of different cell types allows the food to flow through the intestinal tube in a directional fashion, also helping in the task of contracting the gut walls as the food passes. The integrative function of tissues in different guts should be further investigated in many groups, where histology, cell type analysis, and mutual signaling still need to be uncovered. An avenue with enormous relevance to understand the functional activities of the digestive system.

A further subject that needs attention in the future is the homeostatic regulation of the animal guts. Several animals across the phylogenetic tree replace cells in their gut using stem cells, a process essential for maintaining the digestive tract's function, especially given the high-stress environment of digestion. In many of these organisms, these stem cells are “turned over” (proliferate and differentiate) in response to food intake, environmental stressors, or injury, allowing the gut to constantly renew itself. How do the stem cells function, what signals they receive, and how do they interact with their specific cellular niches needs to be much better understood. Obviously, the pioneer work done in the vertebrate intestinal crypts or in the *Drosophila* basal side of the epithelial gut monolayer should

be extended further, mostly dealing with the molecular players that control their maintenance and eventual differentiation.

All in all, the understanding of developmental and functional aspects of the animal guts has been a very active field over the last decade, but needs to be extended to multiple systems so we can find shared (and unique) properties that characterize this important system in all animals. The above could probe fruitful avenues to understand how guts develop and have changed architectures and functions over evolutionary time.

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2 Distribution and Evolution of Digestive Exocrine Cells and Organs

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2.1 INTRODUCTION

The growth and survival of all metazoans depend on the successful breakdown of food particles into nutrients and their efficient absorption, a process known as digestion. Subsequently, the absorbed nutrients are reused to form the biomolecules of the organism. Digestion is universal and predates the formation of sophisticated digestive systems, or in some cases even of specialized cell types. For instance, digestion and nutrient absorption are reported in unicellular organisms that obtain energy-rich molecules through membrane transport of small molecules or endocytic uptake of large particles that undergo lysosomal degradation. This mode of digestion is known as intracellular digestion, and it is the main mode reported in unicellular organisms and early metazoans (Opie, 1922).

The emergence of multicellularity represents a fundamental step in the history of metazoans, as division of labor dictated the emergence of functionally diverse cell types (Arendt, 2008; Brunet & King, 2017). The specialization of cell types had a pivotal impact on the development of

sophisticated digestive systems, as it led to the functional and spatial separation of digestive and absorptive processes. This allowed the establishment of more efficient digestive systems, progressing from the emergence of specialized cellular units in early metazoans to the formation of complex organs and glands such as the liver, pancreas, and salivary glands in mammals (Hartenstein & Martinez, 2019; Sensoy, 2021; Steinmetz, 2019).

Decades of research on digestive system evolution have revealed that digestive cells are among the oldest cell types, from an evolutionary point of view. In early metazoans, which were equipped with a limited number of cell types (including contractile, sensory, and epithelial ones), digestion is executed by specialized epithelial cells that engulf food particles and degrade them within lysosomal compartments (Romanova et al., 2021). Over time, as organismal complexity increased, selective pressures, including larger body size and higher mobility, and thus higher metabolic demand, created the need for diverse and energy-rich food sources, as well as more efficient digestive mechanisms (Bonner, 2024). This led to the emergence of extracellular digestion, which allowed organisms to break down large, complex food particles into smaller molecules outside the cell, within cavities, thereby reducing the risk of internal cellular damage (Hartenstein & Martinez, 2019; Steinmetz, 2019). As a consequence, digestive and absorptive functions were gradually delegated to distinct cell types (Brunet & King, 2017; Nielsen, 2019; Smith & Mayorova, 2019).

Over the past decades, significant advances in molecular biology and microscopy have enabled the comprehensive mapping of digestive cell types across the animal tree. Historically, identification of digestive cell types was initially carried out using histological approaches (e.g., using chemical stains such as hematoxylin and eosin), which differentially label

cells depending on their cytoplasmic content (Fischer et al., 2008). Later on, immunohistochemistry, a technique based on the detection of a given protein using a specific antibody (Ramos-Vara & Miller, 2014), together with *in situ* hybridization to detect specific mRNAs (Thisse & Thisse, 2008), enabled the identification of cell type-specific markers and reconstruction of molecular profiles. On the other hand, electron microscopy (EM) further enabled the characterization of digestive cell ultrastructure (Koster & Klumperman, 2003).

Nowadays, single-cell and single-nucleus transcriptomics have revolutionized the mapping of cell states and types. These methods are based on the dissociation of a multicellular specimen into individual cells (or nuclei) and subsequently sequencing of their mRNA content, generating cell-specific transcriptomic inventories (Kolodziejczyk et al., 2015; Tang et al., 2009). Computational analysis of such data enables the reconstruction of maps (single-cell/single-nucleus atlases) in which cells with similar transcriptomic profiles are grouped together, representing distinct cell states or types (Zeng, 2022).

More recently, the combination of such gene expression data with morphological and functional approaches has enabled the profiling of cell types at an unprecedented level (Camunas-Soler, 2024; Paganos et al., 2022; Paganos et al., 2025; Vergara et al., 2021; Yuste et al., 2020). These technological breakthroughs have allowed researchers to re-evaluate hypotheses regarding cell type and organ evolution, including the hypothesis that complex organs emerged through spatial organization of cell-type units that predate a given organ (Arendt et al., 2016; Brunet & King, 2017; Pavlicev et al., 2024; Wagner et al., 2016).

However, the evolutionary origins of digestive cell types still remain unclear. Comparative analyses across animal taxa have shown that complex

extracellular digestion is highly derived, and that the mode of digestion varies tremendously between taxa and across life stages, reflecting both evolutionary history and developmental constraints (Arendt et al., 2015; Hartenstein & Martinez, 2019; Steinmetz, 2019). Within this chapter, we will attempt to trace the origins and provide an overview of a cell type dedicated to digestion: the digestive exocrine cell. In most multicellular animals studied to date, digestive exocrine cells are typically epithelial in origin. These cells are able of producing and secreting digestive enzymes (else known as zymogens), acids, bile, or mucus that act locally (Arendt et al., 2015; Johansson et al., 2013; Sebé-Pedrós et al., 2018b; Smith et al., 2015). In most metazoans, these cells can be found lining an internal cavity or within the digestive tract and/or grouped in specialized organs and glands, where they secrete their products into the digestive cavity or gut lumen (Arendt et al., 2015; Karpińska & Czauderna, 2022; Paganos et al., 2022; Steinmetz, 2019).

All metazoans can be divided into two main categories: Bilateria and non-Bilateria **(Figure 2.1)**. Bilateria representatives feature bilateral symmetry, with a body organization along left–right and anterior–posterior axes. They are triploblastic, meaning that three major germ layers (ectoderm, endoderm, mesoderm) form during gastrulation and give rise to distinct cell types and organs (Barresi & Gilbert, 2023a; Brusca et al., 2022). Typically, ectoderm gives rise to the epidermis and nervous system; endoderm to the digestive tract and associated organs; and mesoderm to muscles, connective tissues, gonads, and the circulatory system (Barresi & Gilbert, 2023a; Brusca et al., 2022). In contrast, non-bilaterians diverged early in evolution and lack defining bilaterian traits such as bilateral symmetry and true mesoderm (Barresi & Gilbert, 2023a; Brusca et al., 2022). Most non-bilaterians are diploblastic, forming solely ectoderm and

endoderm during gastrulation, with the exception of Porifera and Placozoa, that lack true germ layers (Barresi & Gilbert, 2023a; Brusca et al., 2022), and ctenophores, which possess a mesoderm-like layer (Nielsen, 2019; Ryan et al., 2013). Are these substantial developmental differences among these animal groups reflected in the molecular fingerprint and function of digestive exocrine cells?

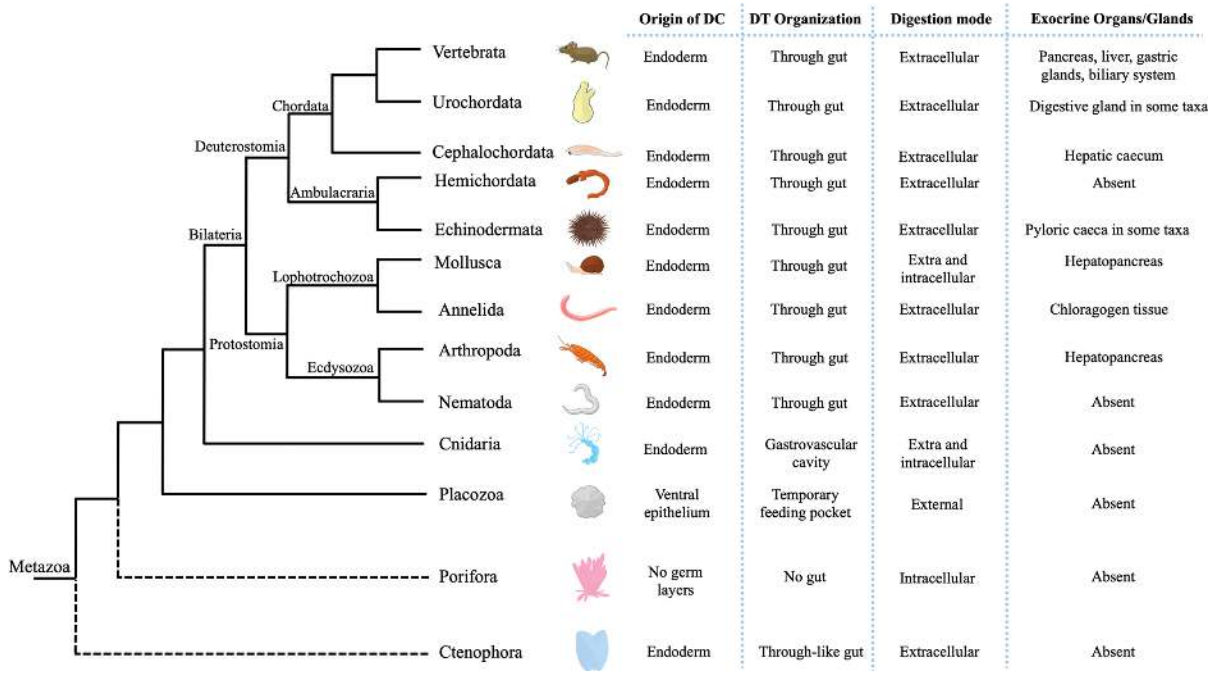


FIGURE 2.1 Phylogenetic tree of representative metazoan non-Bilateria and Bilateria lineages combined with an overview of the developmental origins of digestive cells in relation to the digestive tract organization, the mode of digestion and the presence of exocrine organs/glands across metazoans. Dotted lines indicate currently disputed phylogenetic positions. DC, digestive cells; DT, digestive tract. ⏎

Decades of research indicate that early animals lacked specialized anatomical structures and organs, yet possessed multifunctional epithelial cells capable of producing digestive enzymes. Therefore, digestive exocrine cells represent a foundation from which specialized organs might have

evolved during animal evolution. Together with the evolution of a through-gut, digestive organs and glands represent a major innovation, enabling the transition from intracellular digestion to increasingly efficient extracellular digestion and highly regionalized digestive systems supported by accessory organs. Available data suggest that the emergence of such specialized organs was gradual and involved multiple rounds of cell diversification, tissue regionalization, and increasing functional compartmentalization (Arendt et al., 2016; Brunet & King, 2017). Overall, recent advances in genomics, single-cell omics, imaging, and phylogenetics have enabled comparative analyses of digestive exocrine cell types, their gene regulatory networks (GRNs), and gut architectures across taxa, revealing molecular and morphological conservation of many digestive exocrine cells regardless of whether they are organized into discrete organs. For this reason, it is important to understand the distribution of digestive exocrine systems across animals through an integrative perspective combining comparative anatomy, developmental biology, physiology, and evolutionary history.

2.2 CELLULAR FOUNDATIONS OF DIGESTIVE EXOCRINE SYSTEMS IN NON-BILATERIAN ANIMALS

2.2.1 Porifera

Traditionally, Porifera, a phylogenetic group that includes mainly sponges, is considered the sister group to all metazoans, based on phylogenetic, molecular, and morphological data (Redmond & McLysaght, 2021). One of the main features supporting this hypothesis is the absence of specialized tissues corresponding to nervous, muscular, and digestive systems. Over the last decade, a swathe of contradictory phylogenetic studies have instead supported ctenophores as the earliest-branching lineage (Schultz et al.,

2023). However, to this day, their phylogenetic position is still disputed (Whelan & Halanych, 2023). Regardless of their phylogenetic position with respect to the other animals, Porifera are among the earliest-diverging animals that lack digestive systems and exocrine organs, and they utilize an intracellular digestion mode (Brusca et al., 2022; Leys & Hill, 2012; Steinmetz, 2019).

Fertilization in sponges can be both internal and external, and early embryonic development occurs within the adult sponge body or in the plankton, respectively (Brusca et al., 2022; Leys & Hill, 2012; Maldonado, 2006). Sponge larvae are non-feeding, and their development relies solely on the absorption of maternal nutrient reserves, a developmental strategy known as lecithotrophy. Therefore, despite being planktonic, sponge larvae lack structures related to feeding such as a mouth, a gut, or any digestive organs or systems (Brusca et al., 2022; Leys & Hill, 2012; Maldonado, 2006). In contrast, while adult sponges also lack a digestive tract, they are still capable of performing filter feeding with intracellular digestion that is partitioned between two diversified cell types: choanocytes and archeocytes (Brusca et al., 2022; Leys & Hill, 2012; Maldonado, 2006; Musser et al., 2021).

Filter feeding in sponges is initiated by pumping water containing food particles (bacteria, microalgae, small organic particles) into their body through small pores known as ostia (Brusca et al., 2022; Leys & Hill, 2012; Maldonado, 2006) and exits their body through a large excurrent opening, the osculum **(Figure 2.2A)**. Once inside the sponge body cavity, the water flows into internal canals and reaches chambers containing choanocytes, which are a specialized type of peptidocytes. Choanocytes engulf food particles by phagocytosis, and digestion takes place inside lysosomes (Brusca et al., 2022; Godefroy et al., 2019; Leys & Hill, 2012; Maldonado,

2006). Partially digested particles are further transported to archeocytes for lysosomal digestion (Brusca et al., 2022; Godefroy et al., 2019; Leys & Eerkes-Medrano, 2006; Leys & Hill, 2012; Maldonado, 2006). Nutrients are subsequently actively transported by archeocytes, as well as by diffusion, to the rest of the sponge cell types.

Despite the absence of digestive organs and glands, recent advances in genomics and transcriptomics have advanced our understanding of the molecular fingerprint of sponge digestive cells. Transcriptomic analyses in various sponge species have defined choanocytes as specialized digestive cells **(Figure 2.2A)** capable of producing proteins involved in phagocytosis, endosome–lysosome formation, as well as lysosomal proteases such as cathepsins, thus confirming their specialized role in particle capture, vesicular trafficking, and lysosomal digestion (Musser et al., 2021; Riesgo et al., 2014; Sebé-Pedrós et al., 2018a). Additionally, further studies have demonstrated that archeocytes express genes encoding lysosomal hydrolases, glycosidases, amino-acid and sugar transporters, and lipases. Interestingly, the digestive enzyme repertoire of the sponge digestive cell types includes chitinases, phospholipases, carboxypeptidases, subtilisins, cathepsins, chymotrypsins, trypsins and proteases, which are all present in Bilateria proper digestive cells (Godefroy et al., 2019; Musser et al., 2021; Rojas & Doolittle, 2006; Srivastava et al., 2010). Furthermore, combination of single-cell transcriptomics, gene expression analysis, and high-resolution imaging highlighted the transcriptional similarity of sponge digestive populations with Bilaterian digestive cells, as they were shown to express the transcription factors (TFs) *gata* and *rbpj*, as well as the signaling receptor Notch, which are known to be involved in the specification of pancreatic cells in mammals (Musser et al., 2021). Altogether, data on Porifera digestive cells suggest that sponge digestive cell types represent a

putative ancestral state predating gut formation and extracellular digestion, and that, possibly, metazoan ancestors possessed peptidocyte- or archeocyte-like cells that during evolution acquired an additional role in external digestion and were incorporated into the gut.

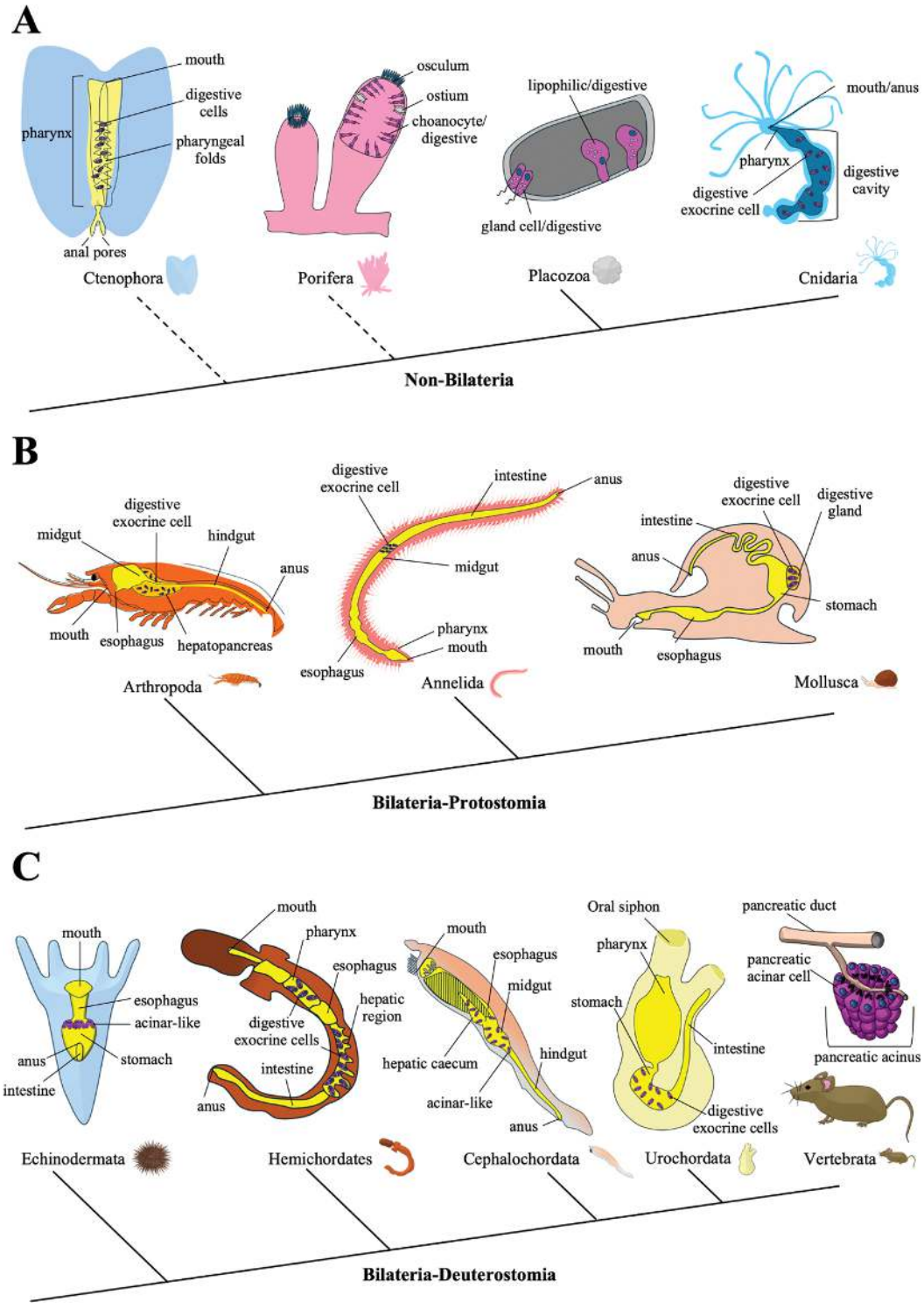


FIGURE 2.2 Overview of the spatial distribution of digestive cells across Metazoa. A) Presence and localization of digestive cells in non-Bilateria representatives. B) Distribution of digestive exocrine cells in protostome representatives. C) Spatial mapping of digestive exocrine cells across

the deuterostome lineage. For all animal representatives, the adult form is depicted, with the exception of echinoderms, for which the larval form is shown, as most available data on the distribution of digestive exocrine cells pertain to this life stage. In all animals depicted, digestive cells are shown in magenta, and, where present, the digestive tract is shown in yellow. ⏎

2.2.2 Placozoa

Placozoa is an early branching non-Bilateria lineage that emerged approximately 750–800 million years ago (Najle et al., 2023). These animals lack any body cavity, and similar to sponges they do not possess specialized organs, musculature, and nervous system (Brusca et al., 2022; Schierwater et al., 2009; Srivastava et al., 2008). However, recent data suggest that these animals are composed of approximately seven diversified cell types (Mayorova et al., 2025; Najle et al., 2023; Sebé-Pedrós et al., 2018a). Despite the absence of a digestive tract, Placozoa feed on microalgae and cyanobacteria that are digested extracellularly within a structure known as the feeding pocket (Mayorova et al., 2025). The feeding pocket is a temporary, digestive compartment formed externally during feeding, which creates a sealed extracellular space between the ventral epithelium and the substrate (Mayorova et al., 2025).

Molecular studies have shown that despite the relatively low cell type diversity in this group, Placozoa possess at least two cell types dedicated to digestion; the lipophil cells and digestive gland cells **(Figure 2.2A)** (Mayorova et al., 2025; Najle et al., 2023; Sebé-Pedrós et al., 2018a). Lipophil cells are spatially organized between ciliated ventral epithelial cells, express low levels of digestive enzymes, and contain large lipophilic secretory granules, which are released into the transient feeding pocket to lyse the prey (Mayorova et al., 2025; Najle et al., 2023; Sebé-Pedrós et al., 2018a). In contrast, digestive gland cells are small, cylindrical ventral

epithelial cells that possess a single cilium **(Figure 2.2A)** and secrete zymogens including proteases, trypsins, chymotrypsins, cathepsins, phospholipases, and carboxypeptidases into the feeding pocket (Fuzita et al., 2015b; Mayorova et al., 2025; Najle et al., 2023; Sebé-Pedrós et al., 2018a). Afterwards, cells of the ventral epithelium take up the broken-down nutrients through membrane transporters, diffusion (in the case of small molecules), and endocytosis (in the case of larger particles). Taken together, the unique mode of feeding that Placozoa utilize, using a temporary feeding pocket within which specialized cells secrete an evolutionarily conserved zymogen cocktail-mix, is thought to represent a transitional stage from intracellular toward extracellular digestion.

2.2.3 Cnidarians and Ctenophores

Cnidaria and Ctenophora are non-Bilateria animal groups that display some of the earliest examples of extracellular digestion strategy and digestive enzyme secretion (Brusca et al., 2022; Steinmetz, 2019). Cnidaria is a diverse group that includes jellyfish, sea anemones, corals, and hydroids (Brusca et al., 2022). Typically, Cnidaria are carnivorous predators, feeding on live prey that they capture using tentacles containing specialized stinging organelles within cnidocytes (nematocysts) (Kass-Simon & Scappaticci, 2002). Cnidaria possess an incomplete digestive system that arises during gastrulation and consists of a single opening surrounded by tentacles that serves both as mouth and anus, a gastrovascular cavity that represents the central digestive chamber **(Figure 2.2A)**, and the gastrodermal epithelium, which contains digestive and absorptive cell types, lining the digestive cavity (Brusca et al., 2022; Sebé-Pedrós et al., 2018b).

Most Cnidaria larvae, known as planula, are planktonic, lecithotrophic, non-feeding organisms, and the onset of feeding is traced to the adult stages

(Brusca et al., 2022). Adult Cnidaria feeding process is initiated when the prey (plankton, copepods, small crustaceans), in a coordinated process involving tentacles, nematocysts, cilia, and muscular contractions, enters the mouth (Kass-Simon & Scappaticci, 2002). The prey is then transferred to the gastrovascular cavity, where gland-like gastrodermal cells secrete digestive enzymes **(Figure 2.2A)**, including lipases and proteases, into the digestive cavity (Brusca et al., 2022).

Molecular profiling of Cnidaria representatives has defined two broad gastrodermal cell types corresponding to mucous gland cells and zymogen gland cells (Steinmetz et al., 2017). Mucous gland cells are densely packed gastrodermal cells that secrete mucus and are marked by the expression of *mucin-5AC*, while zymogen gland cells have variable morphology and secrete proteolytic enzymes (Steinmetz et al., 2017). Single-cell transcriptomics profiling of a variety of Cnidaria species revealed heterogeneous populations of gland and secretory cells, with multiple cell populations expressing unique combinations of digestive enzymes such as proteases, trypsins, chymotrypsins, chitinases, phospholipases, subtilisins, cathepsins, carboxypeptidases, and pancreatic lipases (Alama-Bermejo et al., 2021; Jouiaei et al., 2015; Molinari et al., 2025; Rojas & Doolittle, 2006; Steinmetz, 2019; Steinmetz et al., 2017). From a TF point of view, Cnidaria digestive cell types also express *gata* orthologs, as well as *hnf1* and *hnf4*, which in mammals are known to be essential for pancreatic development and function (Sebé-Pedrós et al., 2018b; Steinmetz et al., 2017). Taken together, current data suggest that Cnidaria clearly exhibit functional exocrine digestion within a cavity, despite the absence of discrete organs or glands. This organization represents a condition in which digestive secretion is a property of individual epithelial cells rather than a specialized organ system.

Ctenophora is a phylogenetic group composed primarily of comb jellies and constitutes a unique case: although they are non-Bilateria, they possess a through-like gut (Presnell et al., 2016; Tamm, 2019; Vianello et al., 2026). However, whether this structure is homologous to the true through-gut found in Bilateria remains an open question. The digestive tract in comb jellies consists of a mouth, pharynx, stomach, intestine, and specialized openings (anal pores) that resemble a Bilaterian anus **(Figure 2.2A)** (Presnell et al., 2016). Similar to Cnidaria, Ctenophora capture prey using tentacles; however, their tentacles lack nematocysts. Instead, they are equipped with adhesive cells known as colloblasts (Nielsen, 2019).

The prey is transported to the mouth via tentacle contraction and ciliary currents, then passes through the pharynx, stomach, and intestine, and the food waste is expelled through the anal pores (Presnell et al., 2016). Ctenophora are capable of performing extracellular digestion that involves a plethora of functionally and topologically diversified cell types. The pharynx is the primary digestion site, where digestive enzymes are secreted from ciliated longitudinal ridges of the pharyngeal epithelium known as the pharyngeal folds **(Figure 2.2A)** (Presnell et al., 2016). Digestion is then continued and completed in the stomach, where nutrients are taken up and distributed across the animal body by the gastrovascular canal system (Presnell et al., 2016).

Recently, single-cell transcriptomic analyses of the comb jelly *Mnemiopsis leidyi* confirmed at a molecular level the presence of distinct digestive cell states as well as of the molecular identities of ctenophore digestive enzymes. These includes chitinases, phospholipases, lipases, carboxypeptidases, subtilisins, cathepsins, chymotrypsins, trypsins and proteases (Fankboner & Reid, 1978; Mitchell et al., 2024; Sebé-Pedrós et al., 2018a; Smith & Mayorova, 2019; Steinmetz et al., 2017). Importantly,

ctenophore digestive cells were also shown to express the pancreatic TF *hnf4 (*Sebé-Pedrós et al., 2018a; Steinmetz et al., 2017*)*, highlighting once more that the TF toolkit of digestive cells is potentially reused across Metazoa. Taken together, the case of Ctenophora demonstrates that complex compartmentalized digestive systems, with regional specialization and extracellular digestion using a conserved set of digestive enzymes, are not a Biletaria innovation, but a feature linked to the emergence of a through-gut.

2.3 FROM THROUGH-GUT EMERGENCE TO DIGESTIVE EXOCRINE ORGAN DIVERSIFICATION

The emergence of Bilateria marks a major transition in animal evolution, characterized by profound changes in body plan symmetry and the evolution of a true through-gut, composed of a mouth, foregut, midgut, hindgut, and anus. This event led to the functional diversification of the mouth and anus, with the mouth being the entry point for the food and the anus the exit point for food waste. In most Bilateria animals, foregut cell types are involved in the mechanical breakdown and initial processing of food, midgut cell types are involved in digestion and nutrient absorption, and, lastly, cell types spatially located in the hindgut region are responsible for water and ion reabsorption, as well as the formation of feces (Brusca et al., 2022; Stevens & Hume, 2004).

Bilateria is a diverse animal group that can be subdivided into two major groups: Protostomia and Deuterostomia (Holland, 2000). The primary diversification of these two animal groups lies in the sequential developmental morphogenetic events leading to the formation of the mouth. In Protostomia (from the Greek πρώτο- meaning “first” and στόμα meaning “mouth”), the first opening formed during gastrulation corresponds to the mouth, whereas in Deuterostomia (from the Greek δεύτερο- meaning

"second" and στόμα meaning "mouth"), it corresponds to the anus, and the mouth forms after gastrulation is completed. The increased feeding and metabolic needs of Bilateria, together with the sophisticated compartmentalization of the digestive tract, coincide with the emergence of novel specialized digestive cell types, organs, and glands.

In most Bilateria representatives, the digestive tract derives from the endoderm and is patterned along the anterior–posterior axis through conserved signaling pathways such as Wnt/β-catenin, Hedgehog, BMP, and FGF (Dessimoz et al., 2006; McClay et al., 2020; Thompson et al., 2018; Verzi & Shivdasani, 2008). Additionally, in Bilateria representatives whose lifecycle includes a feeding larval stage, larval digestive systems are present and functional, but relatively simple, often lacking distinct accessory glands. Adult forms, by contrast, commonly exhibit increased compartmentalization or complex digestive organs. This ontogenetic pattern reflects a broader evolutionary trajectory in which digestive exocrine tissues initially evolve as diffuse epithelial domains and only later become spatially segregated into discrete organs.

2.3.1 Digestive Cell Types and Organs in Protostomia

The Protostomia clade is a large group composed of animals that show extensive morphological and cellular diversity, including the digestive exocrine organization. Despite these anatomical variations, protostomes share common mechanisms of action that rely on the secretion of digestive enzymes into the gut cavity/lumen. Protostomia is further subdivided into two major groups: Ecdysozoa and Spiralia/Lophotrochozoa (Aguinaldo et al., 1997; Brusca et al., 2022; Dunn et al., 2014). A defining characteristic of Ecdysozoa is the presence of a cuticle covering their bodies, which must be periodically shed, molted, and replaced in order for the animal to grow, a

process known as ecdysis. Representative members of this group include Arthropoda (insects, chelicerates, crustaceans, and myriapods) and nematodes.

2.3.1.1 Ecdysozoa

Arthropoda are the largest metazoan phylum, accounting for more than 80% of all described animal species and estimated to contain millions of yet uncharacterized species. Digestion in arthropods is extracellular and is executed by distinct digestive glands such as the hepatopancreas and the gastric ceca, which are endodermally derived and are responsible for the production of digestive enzymes **(Figure 2.2B)** (Brusca et al., 2022; Ceccaldi, 1989). Molecular studies have defined the zymogen repertoire produced by the digestive cells of this group that includes chitinases, amylases, phospholipases, lipases, carboxypeptidases, subtilisins, cathepsins, chymotrypsins, trypsins and proteases (Botos et al., 2000; Ferreira et al., 2015; Fuzita et al., 2015a; Hamid et al., 2013; Rawlings et al., 2018; Santiago et al., 2017; Stanley, 2006; Wang et al., 2020; Wang et al., 2023). In addition to the zymogens produced, TF orthologs of *gatae* and *hnf4* have been shown to be expressed in the same digestive domains (Okumura et al., 2007; Zhong et al., 1993). Among arthropods, the function and cell type repertoire of hepatopancreas have been extensively described mostly in crustaceans. The crustacean hepatopancreas is responsible for the synthesis and secretion of zymogens, nutrient absorption, energy storage, detoxification, as well as support of reproduction through the synthesis of yolk and ovarian maturation (Du et al., 2025). Extensive work on the crustacean hepatopancreas has identified diversified cell populations such as the resorptive/absorptive R-cells, blister-like secretory B-cells, enzyme-producing F-cells, embryonic/stem-like E-cells, and the endocrine-like M-

cells as the main cell types constituting this organ (Ceccaldi, 1989; Vogt, 2019).

In contrast, other arthropods, such as insects, lack such specialized digestive organs and glands. In the case of the fruit fly *Drosophila melanogaster* and the silk moth *Bombyx mori*, digestive function is mediated by specialized endodermally derived cell types in the midgut compartment. Typically, the insect midgut consists of regenerative cells involved in tissue renewal, known as intestinal stem cells, enteroendocrine cells that mediate hormonal regulation, and columnar cells/enterocytes for digestion and nutrient absorption (Awais et al., 2024; Hakim et al., 2010; Lemaitre & Miguel-Aliaga, 2013; Miguel-Aliaga et al., 2018). Out of all these cell types, enterocytes are the most abundant midgut cell type, are equipped with apical microvilli, and are capable of producing a wide variety of digestive enzymes.

Lastly, in other Ecdysozoa such as nematodes, in contrast to arthropods, the production of digestive enzymes (chitinases, amylases, phospholipases, lipases, carboxypeptidases, subtilisins, cathepsins, chymotrypsins, trypsins, and proteases) and nutrient absorption take place mainly in the intestine rather than in the midgut, and are mediated by intestinal epithelial cells (Bahr et al., 2025; Balasubramanian et al., 2009; Caffrey et al., 2018; Chen et al., 2020; Cooper & Eleftherianos, 2016; Huang et al., 2017; Malagón et al., 2010; Parks et al., 2023). Notably, these digestive cell populations also express the gene *xbp1,* a TF also known to be expressed in mammalian pancreatic acinar cells (Imanikia et al., 2019).

2.3.1.2 Spiralia/Lophotrochozoa

The second major Protostomia group corresponds to Spiralia/Lophotrochozoa. This group comprises diverse animal

representatives and includes mollusks (gastropods, bivalves, chitons, and cephalopods), annelids (earthworms and polychaetes), bryozoans, brachiopods, and platyhelminths. Early embryos belonging to this clade develop through a characteristic developmental program defined by diagonal cleavage planes that produce a spiral-like arrangement of blastomeres, known as spiral cleavage (Barresi & Gilbert, 2023b; Brusca et al., 2022). Moreover, taxa within this group produce a distinctive ciliated, free-swimming larval form known as the trochophore larva, which possesses a specialized feeding structure composed of ciliated tentacles called the lophophore (Barresi & Gilbert, 2023b; Brusca et al., 2022). These two characteristic features are the basis for the name of this clade.

The digestive anatomy of Annelida features a compartmentalized gut divided into foregut, midgut, and hindgut domains **(Figure 2.2B)** (Vianello et al., 2026). However, annelid worms are equipped with additional digestive accessory tissues, such as esophageal glands, which regulate blood pH and homeostasis, as well as chloragogen tissue, which functions analogously to the vertebrate liver (Jamieson, 1981; Ruppert et al., 2003). As in other Bilateria, the midgut is the primary domain involved in digestion and nutrient absorption. An important feature of annelid digestive system organization is the spatial separation of function within the midgut: digestive enzyme secretion is carried out by cells located in the anterior region **(Figure 2.2B)**, whereas nutrient absorption occurs predominantly in the posterior midgut (Edwards & Fletcher, 1988). Anterior midgut cells produce a variety of digestive enzymes, including amylases, phospholipases, lipases, carboxypeptidases, subtilisins, cathepsins, chymotrypsins, trypsins, and proteases (Alonso-del-Rivero et al., 2009; Cichocka et al., 2021; Kvist et al., 2014; Pan et al., 2010; Peaucellier, 1983; Vianello et al., 2026; Williams et al., 2015). Moreover, molecular studies

have identified the exocrine pancreas TF orthologs *gata4*, *blimp1*, *ptf1a*, *mist/bhlha15,* and *hnf4* as part of the molecular signature of those cells (Álvarez-Campos et al., 2024; Vianello et al., 2026).

Mollusks represent the second largest metazoan phylum after arthropods. The digestive system in mollusks includes a large digestive gland known as the midgut gland or hepatopancreas **(Figure 2.2B)**. In most mollusks, this gland forms as evaginations of the embryonic endodermal midgut and is present throughout larval development, although it becomes predominantly functional in adult stages (Giribet, 2013; Page, 2009; Raven, 1966). The hepatopancreas is a tubular structure composed of multiple specialized cell types dedicated to various functions, such as the secretion of digestive enzymes (basophilic cells), nutrient absorption (absorptive cells), nutrient storage (storage cells), and detoxification processes (detoxification cells) (Knauer et al., 1996; Lobo-da-Cunha, 2019; Pechenik, 2014). Molecular studies have identified chitinases, amylases, phospholipases, lipases, carboxypeptidases, cathepsins, chymotrypsins, trypsins and proteases as the principal digestive enzymes secreted by basophilic cells (Amara et al., 2010; Dennis et al., 2011; Groppe & Morse, 1993; Guionie et al., 2003; Juvvadi et al., 1997; Le Moine et al., 1997; Liang et al., 2022; Stradine & Whitaker, 1963; Yi et al., 2018). Mollusc digestive exocrine cells also express the pancreatic TF *xbp1* (Huang et al., 2018). Notably, mollusks are capable of following an intermediate strategy between extracellular and intracellular digestion, as they perform both (Morton, 1983). Moreover, in cephalopods digestion appears partitioned between the endodermally derived hepatopancreas and an additional ectodermally derived gland, the posterior salivary gland, which contains digestive cells producing proteolytic enzymes and, in some taxa, toxins (Andrews et al., 2022;

Boucaud-Camou & Boucher-Rodoni, 1983). This feature highlights a more complex organization and regionalization of digestion.

Among Protostomia, flatworms represent an exception, as the secretion of digestive enzymes is carried out by scattered endodermally derived cells within the gut epithelium, known as goblet-like secretory cells. These cells produce cysteine proteases, such as cathepsins, as well as other hydrolases and proteins involved in intracellular digestion, which is performed by adjacent phagocytic intestinal cells within the gut epithelium (Fincher et al., 2018; Goupil et al., 2016). Recent single-cell transcriptomic analyses in Müller's larvae have confirmed the presence of such specialized digestive cell populations that express an extensive repertoire of pancreatic-like enzymes (carboxypeptidases, trypsins, and pancreatic lipase-related enzymes) and the pancreatic TF *ptf1a* (Piovani et al., 2023).

Taken together, these facts lead to several conclusions. First, across Protostomia, extracellular enzyme secretion into the gut lumen represents the predominant mode of digestion, while nutrient absorption is performed by distinct absorptive epithelial cells of the midgut. Hepatopancreas-like structures constitute a major evolutionary innovation in mollusks and arthropods, whereas flatworms retain a simpler, and possibly more ancestral, epithelial digestive strategy. Finally, Protostomia exhibit increasing digestive complexity, ranging from relatively simple epithelial digestion to the emergence of specialized glands and organs, including secretory exocrine tissues and dedicated absorptive cell types.

2.3.2 Evolutionary Emergence of Complex Digestive Organs in Deuterostomia: Insights from Invertebrates & Vertebrates

Deuterostomia constitutes the second major lineage of Bilateria and comprises animal groups that display considerable morphological, developmental, and cellular diversity. Despite this diversity, many deuterostomes share common developmental features such as radial cleavage and enterocoelic coelom formation (Barresi & Gilbert, 2023b; Brusca et al., 2022). Moreover, they share conserved developmental programs, most notably regarding the formation of the anus from the blastopore, as well as common endodermal patterning mechanisms that control gut regionalization and function as described in Chapter 1. Deuterostomes rely on extracellular digestion executed by exocrine epithelial cells embedded in the gut lining or, in more derived groups, by specialized and discrete exocrine organs that release zymogens into the gut lumen or cavity. The Deuterostomia lineage is subdivided into two main groups: Ambulacraria and Chordata.

2.3.2.1 Ambulacraria

Ambulacraria is a monophyletic group originating from a common deuterostome ancestor that contains two phenotypically diverse and exclusively marine sister groups; the Echinodermata (that include sea urchins, sea stars, sea cucumbers, sea lilies, feather stars, and brittle stars) and Hemichordata (including acorn worms and pterobranchs) (Arnone et al., 2015; Hyman, 1955). Several lines of molecular, developmental, and morphological evidence unify these two clades, including canonical deuterostome developmental characteristics such as the blastopore-to-anus development, radial cleavage, and coelom formation, as well as shared genomic features, gene family histories, and aspects of body plan symmetry (Arnone et al., 2015; Hyman, 1955).

Representatives of both groups possess bilaterally symmetric, ciliated, free-swimming larvae known as dipleurula-type larvae, which post-metamorphosis give rise to adults that are pentaradially symmetric in echinoderms and vermiform (worm-like) in hemichordates (Arnone et al., 2015; Kaul-Strehlow & Röttinger, 2015). Additionally, both groups display tripartite coelomic organization, conserved GRNs controlling the anterior–posterior axial patterning of their body and endoderm specification (Arnone et al., 2015; Kaul-Strehlow & Röttinger, 2015).

Echinoderms are marine benthic invertebrates, and the embryonic development of most echinoderm species studied is indirect, producing a planktonic larval stage (Arnone et al., 2015). The free-swimming and feeding larva spends from weeks to several months, depending on the species, in the water column and eventually settles, undergoes metamorphosis, and produces the juvenile and adult body plans (Arnone et al., 2015). Importantly, a highly compartmentalized and fully functional digestive tract is already present at the larval stage (Annunziata et al., 2014; Arnone et al., 2015). Both pre-metamorphic (embryonic/larval) and post-metamorphic (juvenile/adult) stages have been extensively used as experimental systems to study developmental processes and the evolution of cell types, organs, and body plans (Dylus et al., 2018; Ernst, 2011; Formery et al., 2023; García-Arrarás et al., 1999; Paganos et al., 2025).

The echinoderm digestive tract, as highlighted in Chapter 1, arises during embryogenesis from the archenteron, and its patterning is controlled by conserved GRNs. (Annunziata & Arnone, 2014; Davidson et al., 2002; Paganos et al., 2022; Voronov et al., 2024). The larval digestive system consists of a mouth, foregut (esophagus), midgut (stomach), hindgut (intestine), and anus (**Figure 2.2C**).

In sea urchins, it has been demonstrated that at the juvenile stage dramatic cell type repertoire reorganization takes place. Juvenile individuals retain certain larval cell types such as the stomach and partially the intestine, which are used as developmental scaffolds to rebuild the adult digestive system, that will eventually be composed of a mouth, pharynx, esophagus, stomach, intestine, and anus (Hyman, 1955). According to current literature, most echinoderms lack specialized digestive organs both as larvae and adults, with the exception of adult sea stars, which possess a prominent digestive gland known as the pyloric caeca (Martinez et al., 1989). This highly branched gland, located in each arm of the adult and connected to the pyloric stomach, is the major site of digestive enzyme secretion and nutrient absorption and is shown to have a pancreatic-like cell signature (Martinez et al., 1989).

Most studies regarding digestion have been carried out in sea urchins. Interestingly, these studies have demonstrated that sea urchins possess digestive exocrine pancreatic-like cells, also known as acinar-like cells **(Figure 2.2C)**, that are molecularly and morphologically homologous to mammalian pancreatic acinar cells (Paganos et al., 2022; Perillo et al., 2016). These cells arise as two bilaterally symmetric cells during gastrulation in the most anterior portion of the midgut and express a conserved pancreatic gene regulatory module including the TFs *ptf1a* and *hnf4*, and their specification depends on the patterning TF *hnf1* and Notch signaling (Paganos et al., 2022; Perillo et al., 2016). At the early larval stage, these cells are located in the upper part of the larval stomach, forming a belt-like structure that expands into multiple bands as larval development proceeds, while remaining restricted to the upper stomach in most sea urchin species studied **(Figure 2.3A)**. In contrast, in sea stars, acinar-like

cells have been found scattered throughout the larval midgut/stomach region **(Figure 2.3B,C)**.

Single-cell profiling of pre-metamorphic sea urchin larvae confirmed the pancreatic molecular signature of acinar-like cells, showing expression of a variety of exocrine pancreas TF orthologs such as *gata6*, *blimp1*, *xbp1*, *ptf1a*, *mist/bhlha15*, *rbpj* and *hnf4* (Paganos et al., 2022). These cells are also capable of producing and secreting an extensive repertoire of zymogens including chitinases, amylases, phospholipases, lipases, carboxypeptidases, subtilisins, cathepsins, chymotrypsins, trypsins and proteases, the expression of many of which is regulated by the *ptf1a* TF as well as by the feeding conditions, with the presence of food acting as a stimulus for the upregulation of digestive enzyme expression in the *Strongylocentrotus purpuratus* sea urchin larva (Paganos et al., 2022; Perillo et al., 2016).

Single-nucleus transcriptomics further confirmed the pancreatic acinar-like signature in post-metamorphic sea urchins (Paganos et al., 2025), although the spatial organization of these cells, the similarity of the sea star pyloric caeca to pancreas or whether any hepatopancreas-like structures similar to the protostomes ones exist, remain unknown. Beyond the molecular conservation with mammalian pancreatic acinar cells, the ultrastructure of sea urchin acinar-like cells, being highly polarized, with secretory vesicles concentrated at the apical side facing the gut lumen, closely mirrors that of mammalian pancreatic acinar cells confirming that sea urchin acinar-like cells are homologous to the mammalian ones (Paganos et al., 2022).

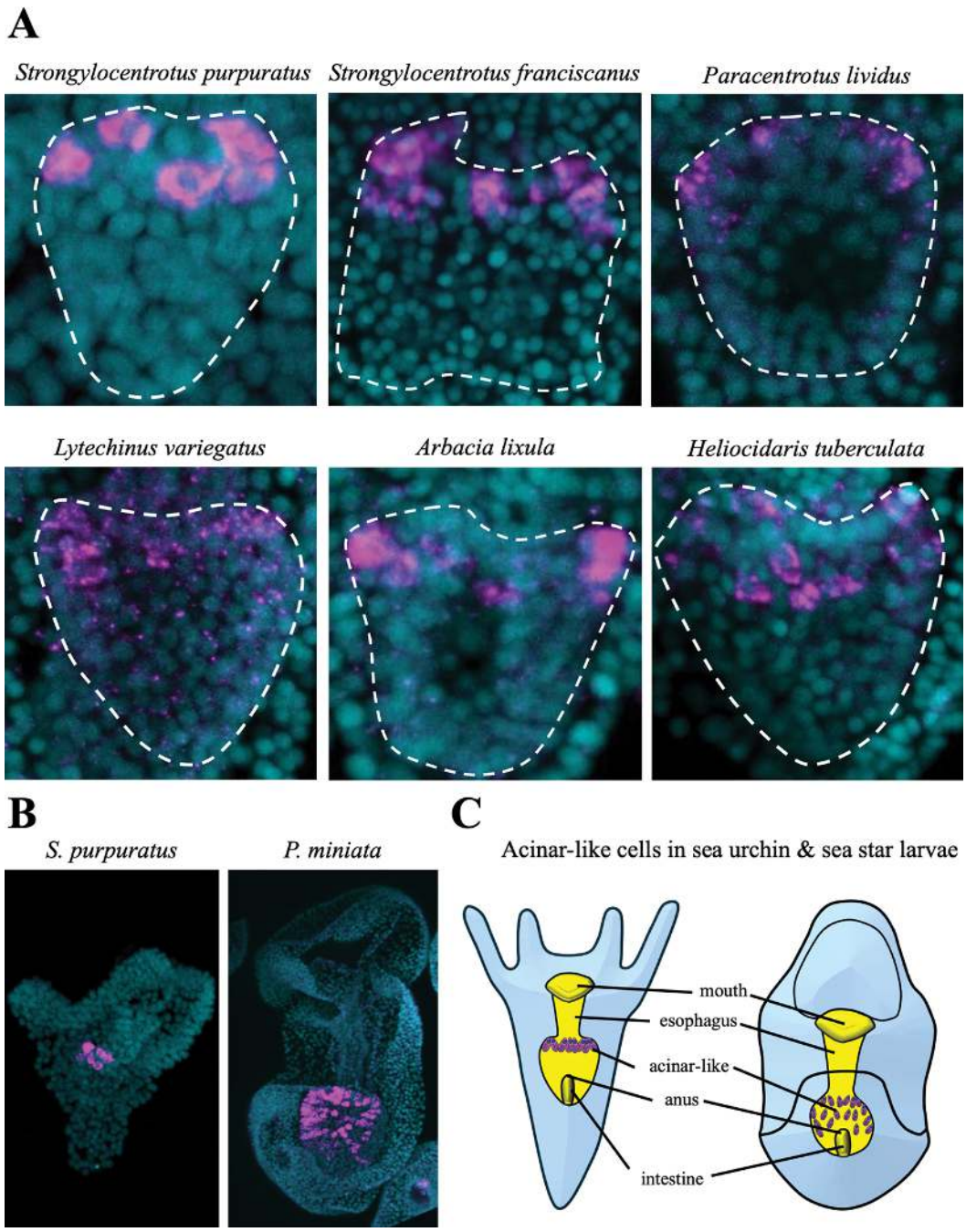


FIGURE 2.3 Distribution of acinar-like cells in sea urchin and sea star early larvae. A) Fluorescent *in situ* hybridization for *carboxypeptidase A2* (*cpa2*) carried out on six sea urchin species larvae at the early pluteus stage. All images focus on the stomach/midgut region of the larval digestive tract. Dotted lines are used to highlight the stomach/midgut region. B) Fluorescent *in situ* hybridization for *carboxypeptidase A2* (*cpa2*) in the pluteus larva of the sea urchin *S. purpuratus* and the bipinnaria

larva of the sea star *Patiria miniata*. C) Cartoons are used to summarize the position of the acinar-like cells in respect to the general body architecture and the digestive tract in the sea urchin and sea star larva. ⏎

In contrast to echinoderm body plan organization, the vermiform body plan of Hemichordata can be divided along the anterior–posterior axis into three regions: the proboscis, the collar, and the trunk. The endodermally derived digestive system is organized along the same anterior–posterior axis (Arnone et al., 2015; Kaul-Strehlow & Röttinger, 2015). The hemichordate digestive system is composed of a mouth, pharynx, esophagus, hepatic region, intestine, and anus **(Figure 2.2C)** (Vianello et al., 2026). Hemichordates lack discrete digestive exocrine glands such as a pancreas or liver; instead, digestive exocrine function is mediated by regionally specialized epithelial cells within the gut lining (Arnone et al., 2015; Kaul-Strehlow & Röttinger, 2015). These epithelial cells are localized within the pharyngeal region and, most prominently, in the hepatic region **(Figure 2.2C)**. The hemichordate digestive exocrine cells express a conserved pancreatic TF-toolkit that includes *gata4, blimp1, ptf1a, mist/bhlha15, hnf1,* and *hnf4* and are capable of producing digestive enzymes (Vianello et al., 2026). For instance, in acorn worms, specialized glandular cells in the hepatic region secrete phospholipase A2 and trypsin, while neighboring absorptive cells internalize digested nutrients (Ponczek et al., 2012; Vianello et al., 2026). Therefore, digestion is localized in the more anterior segments of the digestive tract, whereas nutrient absorption takes place in more posterior regions such as the posterior intestine.

2.3.2.2 Chordata

Chordata constitute the second monophyletic deuterostome group and include animals with remarkable morphological and ecological diversity.

The three main representatives of this group are Cephalochordata (e.g., lancelet), Urochordata (e.g., ascidians, salps), and Vertebrata, including fish, amphibians, reptiles, birds, and mammals (Barresi & Gilbert, 2023b; Brusca et al., 2022). All members of this group share distinct morphological, molecular, and developmental characteristics, foremost among them the presence of a notochord at some stage of the life cycle. Additional unifying features include a dorsal hollow nerve cord, pharyngeal slits, endocrine organs such as the endostyle or thyroid-like organs, and a post-anal tail.

2.3.2.2.1 Cephalochordata

Cephalochordata and Urochordata possess relatively simple body plans while retaining key chordate features, such as the presence of a notochord (maintained throughout the life cycle in Cephalochordata and present only during the larval stage in Urochordata). The digestive tract in Cephalochordata, such as lancelet, consists of a mouth, pharynx, esophagus, intestine, and anus (Barrington, 1937; Vianello et al., 2026). Digestion and nutrient absorption mainly take place in the intestine, which is subdivided into the anterior intestine (also known as the midgut) and the posterior intestine (hindgut) (Barrington, 1937; Vianello et al., 2026). The midgut region contains an endodermal outpocketing that is functional in both larvae and adults, known as the hepatic caecum. Both the midgut and the hepatic caecum are involved in digestion (Barrington, 1937; Wu et al., 2024). Notably, the hepatic caecum has been proposed to function as a proto-liver or proto-pancreas due to its role as a major digestive, metabolic, and absorptive center (Barrington, 1937; Wu et al., 2024). In contrast, the hindgut region is primarily dedicated to nutrient absorption and the transport of undigested material **(Figure 2.2C)**.

Recent single-cell transcriptomic studies have shed light on the cellular diversity of digestive cell types in the lancelet intestine, particularly within the hepatic caecum **(Figure 2.2C)**. These studies have identified acinar-like cells scattered within the midgut region that produce zymogens including chitinases, amylases, phospholipases, lipases, carboxypeptidases, subtilisins, cathepsins, chymotrypsins, trypsins, proteases and express orthologs of pancreatic exocrine TFs such as *gata4, blimp1, ptf1a, mist/bhlha15*, *hnf1* and *hnf4* (Dai et al., 2024; He et al., 2018; Li et al., 2022; Vianello et al., 2026). Additional digestive populations include hepatocyte-like cells that were found expressing genes involved in lipid metabolism, detoxification pathways, and amino acid processing, and endocrine-like cells scattered along the gut epithelium that express insulin-like peptides and other hormone-related genes (Dai et al., 2024). Notably, the transcriptional profiles of the endocrine-like cell types resemble those of zebrafish pancreatic endocrine islet cells (Dai et al., 2024). In summary, cephalochordates represent an additional example of increasing cellular specialization and organization that has not yet culminated in the formation of a distinct multifunctional organ such as the vertebrate pancreas.

2.3.2.2.2 Urochordata

Urochordates are marine chordates that exhibit a biphasic life cycle and body plan organization. The tunicate larva is free-swimming and equipped with a notochord, a tail, and segmented musculature. During metamorphosis, most tunicates lose structures such as the notochord and the tail and transform into sessile, filter-feeding individuals. Their digestive system consists of an oral siphon, pharynx, esophagus, stomach, intestine, and anus **(Figure 2.2C)** (Barresi & Gilbert, 2023b; Brusca et al., 2022; Holland, 2016).

Digestion primarily takes place in the stomach, where exocrine cells release zymogens (amylases, phospholipases, lipases, carboxypeptidases, chymotrypsins, proteases) into the lumen, while nutrient absorption occurs in the intestine (Berrill, 1929; Iguchi et al., 2023a; Iguchi et al., 2023b; Koyama et al., 2012; Nakayama & Ogasawara, 2017; Nevalainen et al., 2013; Oren et al., 2008). The exocrine TF profile in the tunicate exocrine cells include orthologs of the TFs *gata4*, *xbp1,* and *ptf1a* (Iguchi et al., 2023a; Ricci et al., 2016; Zhang et al., 2023). In some ascidian taxa, such as *Halocynthia roretzi*, the digestive tract is also equipped with an outpocketing that forms a distinct hepatopancreas-like region, known as the digestive gland, proposed to be involved in the secretion of digestive enzymes into the stomach, although the precise anatomical definition and role of this gland remain unclear (Shin et al., 2014).

2.3.2.2.3 Vertebrata

Vertebrata represent the third subphylum constituting Chordata. Vertebrata representatives include fish, amphibians, reptiles, birds, and mammals (Barresi & Gilbert, 2023a; Kershaw, 1983). All vertebrates bear the typical chordate characteristics, including the presence of a notochord, which in mammals is replaced during development by the vertebral column that protects the spinal cord, a dorsal hollow nerve cord, a thyroid gland or analogous organs, pharyngeal slits (also known as gill slits in some taxa), and a post-anal tail (Kardong, 2011). However, Vertebrata are characterized by key innovations, including the presence of a well-developed brain protected by a cranium (skull), an endoskeleton made of bone and cartilage, a closed blood vessel–based circulatory system, paired appendages, and advanced respiratory organs (Kardong, 2011).

A key vertebrate feature is the emergence of specialized digestive organs that integrate the regionalized gut functions observed in other taxa. The major accessory digestive organs that became highly integrated in vertebrates include the pancreas, liver, gallbladder, and gastric glands, all of which arise from foregut endodermal budding (McCracken & Wells, 2017; Pan & Wright, 2011; Zorn & Wells, 2009). Additional accessory glands, such as the salivary glands (primarily ectodermal in origin), the gastric glands, and the mammal-specific mucus-producing Brunner glands (duodenal endoderm), represent further specializations of epithelial secretory tissues (McCracken & Wells, 2017; Pan & Wright, 2011; Zorn & Wells, 2009).

2.3.2.2.3.1 Pancreas The vertebrate pancreas is a multifunctional organ that contains specialized digestive exocrine and endocrine cell types responsible for food digestion and homeostatic regulation, respectively. Thus, the vertebrate pancreas has a unique dual role as both an exocrine and an endocrine gland, and these two compartments exhibit functional crosstalk. Within this chapter, we will focus on the pancreatic exocrine cells partition, and more details on the endocrine one are available in Chapter 3.

The exocrine pancreatic compartment consists of digestive cells that are spatially organized into acini and are responsible for the production of a variety of zymogens **(Figure 2.2C)**, including chitinases, amylases, phospholipases, carboxypeptidases, subtilisins, cathepsins, chymotrypsins, trypsins and proteases (Goettig et al., 2019; Lim et al., 2022; Lyo et al., 2012; Mosztbacher et al., 2020; Oshima et al., 2002; Sapio & Fricker, 2014; Vianello et al., 2026; Zendzian & Barnard, 1967). In contrast, the endocrine compartment, composed of diverse cell types grouped into islets (islets of Langerhans), is involved in the production of hormones that regulate digestion and homeostasis, such as insulin, glucagon, and pancreatic

polypeptide (Pan & Wright, 2011). Notably, both the exocrine and endocrine compartments are in direct contact with a ductal epithelium, which contributes to enzyme transport and neutralization (Pan & Wright, 2011).

Decades of research have shown that while the cellular composition of the vertebrate pancreas appears widely conserved across vertebrates, the spatial distribution of pancreatic cell types varies among taxa. For instance, fish (jawed and jawless), reptiles, amphibians, birds, and mammals all possess a pancreas composed of endocrine islets and exocrine acini. The islets contain homologous endocrine cell lineages (including α-cells, β-cells, and δ-cells, with ε-cells and pancreatic polypeptide–producing cells variably represented across taxa), while the acini produce digestive enzymes. However, whereas in mammals the islets are embedded within the exocrine pancreatic tissue, in amphibians, reptiles, and birds they exhibit a different and often less intermingled spatial organization (with α- and β-cells in birds forming clearly distinct islet types); in jawless fishes, exocrine pancreatic cells are scattered and poorly organized with little or no distinct duct system, whereas in jawed fishes they form a well-developed, specialized, and branching exocrine gland clearly separated from the islets (Chanet et al., 2023; Kowalska & Rupik, 2021; Lee et al., 2003; Ma et al., 2025; Steiner et al., 2010; Youson & Al-Mahrouki, 1999).

Pancreatic development has been extensively studied in mammals, and it has been shown that it is initiated upon stimulation of embryonic foregut endoderm by morphogenetic signals (Hedgehog, Fgf, Notch, Wnt, and Tgf-β) originating from surrounding mesodermal and ectodermal tissues (Pan & Wright, 2011; Shih et al., 2013). This interaction leads to the formation of pancreatic buds from the endoderm, which later on fuse into an epithelial network organized into tip and trunk domains. During this process,

multipotent pancreatic progenitors proliferate and begin to differentiate, while *pdx1* and *ptf1a* are among the first TFs to be activated and are essential for pancreas formation, as disruption of the expression of either gene leads to pancreatic agenesis (Ahlgren et al., 1998; Burlison et al., 2008; Kawaguchi et al., 2002; Marty-Santos & Cleaver, 2016). Interestingly, both TFs contribute not only to early pancreatic progenitor specification but also to cell differentiation. While *pdx1* is essential for both exocrine and endocrine pancreas differentiation, *ptf1a* is crucial only for the differentiation of the exocrine acinar cell lineage (Afelik et al., 2006; Zecchin et al., 2004). Additionally, the regulatory signature of acinar cells includes TFs such as *gata4*, *blimp1*, *xbp1*, *mist/bhlha15*, *rbpj*, *hnf1* and *hnf4* (Arda et al., 2013; Chiou et al., 2017; Duque et al., 2022; Hess et al., 2011; Karlsson et al., 2021; Masui et al., 2010; Vianello et al., 2026).

2.3.2.2.3.2 Biliary System and Gallbladder The vertebrate liver consists of specialized cell populations known as hepatocytes, which produce and secrete a digestive fluid composed of water, bile acids, cholesterol, and salts, known as bile. Bile is primarily used to emulsify dietary lipids, thereby promoting pancreatic lipase activity and fat absorption (Boyer, 2013; Chiang, 2009). Moreover, the liver functions as the main detoxification and filtering center, converting lipid-soluble toxins and drugs into more soluble forms for excretion, neutralizing ammonia through its conversion to urea, and breaking down or inactivating hormones (Trefts et al., 2017). Additionally, the liver is involved in nutrient storage (such as vitamins, minerals, and glycogen), glucose homeostasis, and the production of proteins including albumin, clotting factors, cholesterol-associated proteins, and transport proteins (Rui, 2014). In most vertebrates, bile is transported through intrahepatic and extrahepatic ducts and is temporarily

stored and concentrated in a small organ, the gallbladder, prior to its release into the duodenum (Boyer, 2013).

2.3.2.2.3.3 Gastric Glands Beyond the emergence of the pancreas and liver, another major exocrine specialization in Vertebrata is the evolution of gastric glands and acid-secreting epithelial cells derived from the foregut region. In jawed vertebrates, the stomach mucosa is spatially organized into glandular units composed of mucous cells, enzyme-secreting chief cells, and acid-producing parietal cells (Yao & Forte, 2003). Gastric acid secretion is mediated by H^+/K^+-ATPase proton pumps, which represent a vertebrate innovation enabling the production of highly acidic luminal microenvironments (Koelz, 1992; Yao & Forte, 2003). This feature promotes protein digestion, activates zymogens, and contributes to antimicrobial immunity (Schubert, 2017).

2.3.2.2.3.4 Intestinal Glands and Goblet Cell Mucus Systems The vertebrate intestinal epithelium also exhibits specialized exocrine cell types that contribute to digestion and mucus-based epithelial protection. Intestinal glands (such as the crypts of Lieberkühn in mammals) contain various secretory lineages, including goblet cells that produce mucus facilitating lubrication and acting as a mechanical and microbial protective gel barrier, antimicrobial peptide–secreting Paneth cells (in mammals), and enteroendocrine cells (Clevers & Bevins, 2013; Johansson et al., 2011; Lang et al., 2007; Noah et al., 2011). Similar to the gastric glands, despite variability in the spatial organization of intestinal glands across vertebrates, mucus-secreting epithelial cells are broadly conserved. Mucin gene families exhibit deep evolutionary conservation, as they were already present in early bilaterians (Lang et al., 2007).

2.4 EVOLUTIONARY CONSIDERATIONS

2.4.1 Germ Layer Origin and Developmental Stability

In Bilateria, and also in non-Bilateria such as cnidarians and ctenophores, digestive exocrine cells are endodermally derived. This suggests that during evolution the endoderm became a stable developmental niche for digestive cell specialization. Thus, it is possible that evolutionary forces dictated the transition from digestion mediated by dispersed specialized epithelial cells to digestion coordinated by complex organs in vertebrates.

2.4.2 A Conserved Core of Regulatory and Terminal Differentiation Genes

From a GRN perspective, a conserved core of TFs has been identified across animal lineages and shown to be part of the digestive cell molecular identity. This putative regulatory kernel includes the TFs *hnf4* as well as *gata* orthologs. Notably, *hnf4* is consistently associated with digestive exocrine cells across multiple taxa, including Cnidaria, Ctenophora, Arthropoda, Annelida, Hemichordata, Echinodermata, Cephalochordata, and Vertebrata. On the other hand, lineage-specific expression of *gata* genes is shown in Deuterostomia digestive exocrine cells as well as in digestive cells in non-Bilateria and Protostomia representatives such as Porifera, Cnidaria, Arthropoda, and Annelida. Additionally, the TF *hnf1* is widely expressed in deuterostome digestive exocrine cells in some non-Bilateria and protostome taxa. Overall, this putative regulatory core predates the emergence of organ-level exocrine systems.

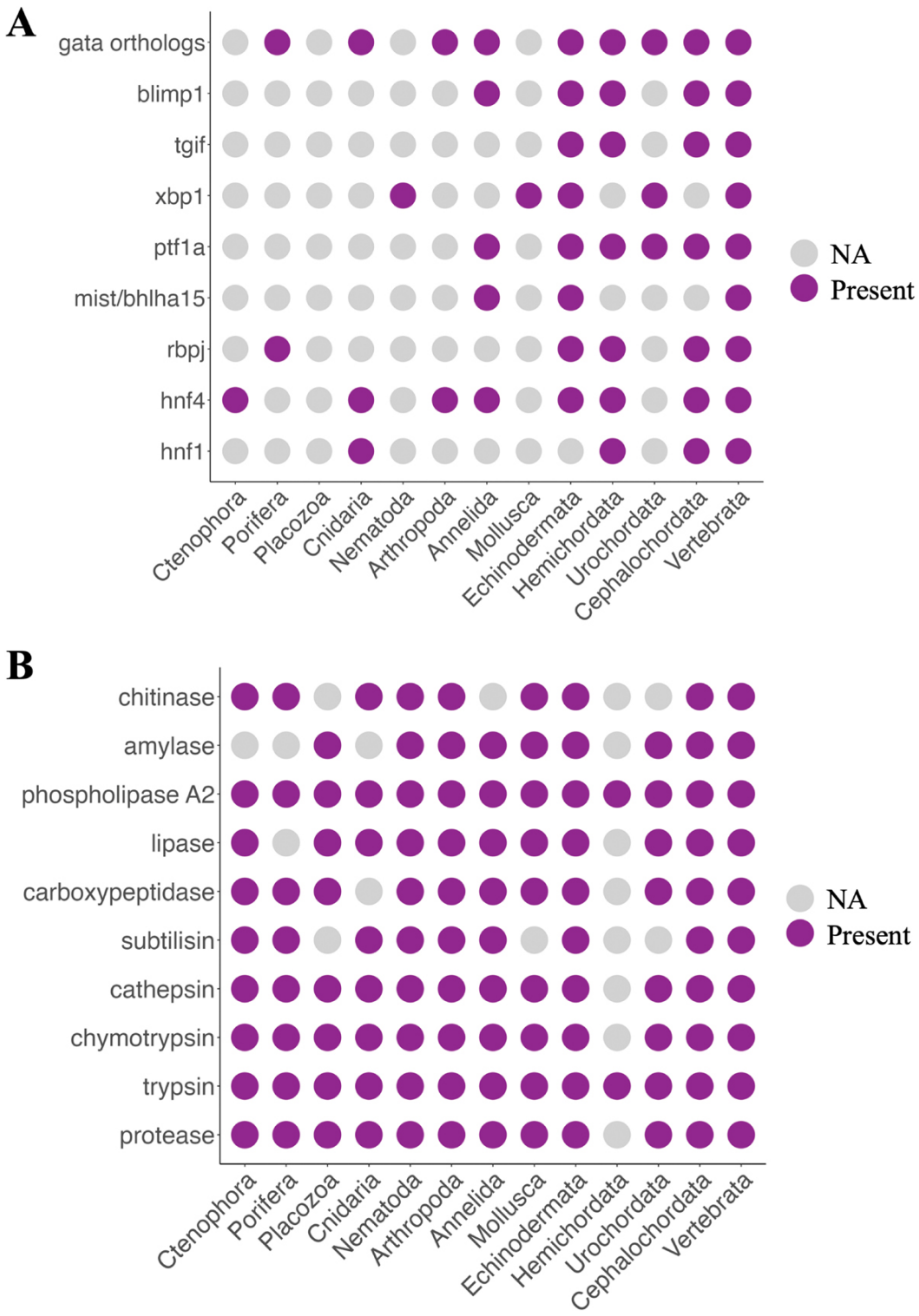


FIGURE 2.4 Summary of the expression data available on TFs associated with digestive exocrine cells and the zymogens produced by them. Present indicates that these genes are expressed in

digestive cells, while NA indicates absence either of expression or of available data. A) Conserved transcription factors. B) Conserved zymogens. ⏎

In most deuterostome species studied, the TF gene *ptf1a* is expressed in acinar-like/acinar cells. *ptf1a* transcripts consistently co-localize with those of TFs *rbpj*, *mist* and *hnf4* in echinoderms, hemichordates and vertebrates **(Figure 2.4)**. Notably, it is likely that this putative regulatory module also includes the TF *xbp1*; however, additional data are needed to fill in the missing gaps in regard to urochordates and cephalochordates **(Figure 2.4)**. In a large number of species investigated, the expression of *ptf1a* has been found to be essential for the specification and differentiation of the pancreatic acinar-like and acinar cells. This suggests the existence of an evolutionarily conserved regulatory module controlling zymogen production and exocrine cell fate. Importantly, this GRN appears to predate the formation of the pancreas as a distinct organ. Furthermore, components of this putative module are found outside deuterostomes such as in annelids, whose digestive exocrine cells express *ptf1a* and *mist/bhlha15* **(Figure 2.4)**. Notably, *ptf1a* orthologs are expressed in digestive exocrine cells of flatworms, while sponge choanocytes express *rbpj*, suggesting a putatively deep ancestral genetic program of the digestive regulatory program **(Figure 2.4)**.

Lastly, the data reviewed within this chapter clearly suggest that independently of the digestion mode used, digestive cells across metazoans utilize a core of proteolytic enzymes (proteases, trypsin, chymotrypsin, and cathepsins) and phospholipase A2 that are present in nearly all metazoan lineages from non-Bilateria to Bilateria and mammals **(Figure 2.4)**. Additionally, enzymes such as lipases, carboxypeptidases, amylases, subtilisins, and chitinases show broad digestive cell-specific expression, strengthening the hypothesis that the fundamental zymogen repertoire of

digestive exocrine cells predates the emergence of complex digestive organs and was largely retained throughout animal evolution **(Figure 2.4)**.

2.4.3 Do Cellular Building Blocks Predate Organs?

How do organs emerge during evolution? Do the cellular building blocks of organs predate the appearance of specialized organs themselves? As proposed by several authors, tissue-level organization requires functional specialization and finely tuned crosstalk among various cell types (Arendt, 2008; Arendt et al., 2016; Brunet & King, 2017). Increasing organismal complexity and greater physical distances between digestive cell types during evolution likely created selective pressure for more efficient, system-level coordination. The examples regarding digestive exocrine cells discussed in this chapter highlight a clear evolutionary pattern: digestive cells predate not only the emergence of distinct digestive organs, but also specialized feeding modes such as extracellular digestion. Therefore, it is plausible that organs emerge through the integration of pre-existing cellular programs. From sponges to vertebrates, the fundamental unit of digestion is not the organ itself, but rather the specialized epithelial digestive cell. In Porifera and Placozoa, digestion is mediated by epithelial cells. In Cnidaria and Ctenophora, digestion is performed by epithelial cells lining a digestive cavity. In Bilateria, digestion is still executed by epithelial cells, but becomes spatially regionalized along the gut. Only later in evolution, particularly in Deuterostomia and especially Vertebrata, digestive exocrine cells do coalesce into distinct organs such as the pancreas.

Evolution of Digestive Exocrine Systems Across Metazoa



Intracellular Digestion
- No gut
- Phagocytic cells
- Lysosomal digestion
- No regionalization of digestion

Cavity-Based Digestion
- Extracellular enzyme secretion
- Initial digestion in temporary cavities or proper digestive cavities
- Separation of digestive and absorptive processes

Through-Gut
- Mouth-anus separation
- Directional food flow
- Gut compartimentalization & division of labor

- Intermediate glands emerge
- Gut outpocketings
- Increasing structural specialization

- Distinct pancreatic cellular building blocks are present predating the emergence of the individual organ
- Pancreatic GRN operates in acinar-like cells

Emergence of specialized and compact organs in vertebrates and especially in mammals

Increasing complexity and subfunctionalization

FIGURE 2.5 Schematic overview of the evolutionary transitions from intracellular digestion to the emergence of extracellular digestion, digestive tract-based systems and specialized organs. ⏎

The evolution of a through-gut and subsequent regionalization enabled spatial separation of ingestion, digestion, absorption, and waste elimination. Over time, exocrine digestive cells formed digestive tissues along the digestive tract, allowing localized but coordinated secretion of zymogens. Eventually, endocrine and exocrine components coalesced into compact, distinct organs. It is possible that digestive exocrine cells shifted from being scattered throughout the digestive tract or part of multifunctional organs in invertebrates to forming simple pancreatic structures in early vertebrates, and finally compact multifunctional pancreatic organs in mammals **(Figure 2.5)**. However, the ancestry of digestive exocrine cells remains unclear. While it seems appealing to consider them ancient and evolutionarily conserved rather than the result of repeated co-option or independent evolution of ancestral exocrine programs, further research is required to trace their origins.

2.4.4 Sea Urchin Acinar-Like Cells Represent an Evolutionary Snapshot into Pancreas Evolution

Sea urchin acinar-like cells provide a compelling example. These cells are molecularly and morphologically homologous to mammalian pancreatic acinar cells, despite the absence of a proper pancreas organ in sea urchins. Remarkably, the molecular machinery of sea urchin acinar-like cells closely resembles that of mammalian acinar cells, including the gene regulatory landscape, the genes encoding digestive enzymes, as well as those encoding for receptors such as insulin receptors, which in mammals mediate crosstalk between endocrine and exocrine pancreatic compartments.

Along these lines, it has been hypothesized that acinar-like cells in the upper midgut of sea urchin larvae interact with endocrine-like cells scattered throughout the digestive tract, thereby suggesting that endocrine–exocrine communication predates organ integration. Such an example favors the hypothesis that, as vertebrate digestive systems became more complex and cell types more dispersed, selective pressures likely promoted the coalescence of pancreatic cell types into a distinct, autonomous unit. This consolidation would have reduced physical distances between interacting cells, promoted paracrine signaling and regulation, and improved coordination and responsiveness to feeding.

Therefore, it is likely that the pancreatic-like organization reported in sea urchins represents an intermediate stage of pancreas evolution. This, in turn, would suggest that most complex digestive organs, and possibly most vertebrate organs, do not arise *de novo*. Rather, they emerge through the progressive diversification, spatial reorganization, regulatory rewiring, and functional integration of already present cellular building blocks.

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3 Evolution and Development of the Gut Endocrine System

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3.1 INTRODUCTION

Multicellular organisms are equipped with specialized organs that support essential body functions. Proper organ function is regulated through constant and dynamic communication across their cell types as well as of other organs. This ensures the maintenance of organismal homeostasis and allows the organisms to adapt appropriately to any changes related to the internal or external environment. This communication is coordinated by two major systems: the nervous system and the endocrine system. Each system employs specific signaling molecules for communication. For instance, the nervous system communicates using typically rapid and short-range-acting transmission mode through cell-to-cell connections, the synaptic junctions, by releasing neurotransmitters and neuropeptides. On the other hand, the endocrine system communicates over longer distances using molecules called “hormones” that are produced and secreted by specific endocrine cells or organs. These hormones carry information and signals through the bloodstream to reach a target location (such as other

organs, muscles, skin) in which their effect takes place. These two systems must interact with each other in a complementary manner.

3.1.1 Endocrine Systems

Endocrine systems have been reported across Metazoa, and they have been best studied in mammals, especially humans and mice. For instance, the human endocrine system consists of cells, tissues, and organs (mainly glands) that produce and release hormones. These products are secreted directly into the blood or lymph, in contrast to the ones produced by exocrine glands that act locally. Examples of human endocrine glands are the hypothalamus, the pituitary gland, the adrenal glands, the gonads, the thyroid, the parathyroid, and the pancreas. In most cases, hormones are released by an endocrine gland into the bloodstream, and their actions can affect every tissue of the body with longer-lasting effects. The target cells possess hormone-specific intramembrane or intracellular receptors, and the interaction between the hormone and the receptors results in a biochemical response within the target cell that ultimately leads to a modification of its activity. The cellular responses to hormonal actions can be divided in two broad categories: (a) a fast, swift response resulting from signaling events mediated by the binding of the hormones to their respective receptor, usually G-protein-coupled membrane receptors (GPCRs), which then activate an intracellular cascade involving second messengers (like protein kinases or calcium signaling); (b) a “genomic” response, triggered by their interaction with nuclear receptors, causing the alteration of gene expression, a process that usually requires hours or even days (Cheung & Kraus, 2010).

Endocrine systems regulate many processes that are fundamental for the proper function of the organismal body, such as the regulation of metabolism and metabolic homeostasis, reproduction, sleep cycles, growth,

development, and mood. Generally, the chemical structure of hormones is highly variable, and a given chemical structure defines specific properties as well as the way they are transported and "processed" into the extracellular and intracellular fluids.

3.1.2 Hormones

Hormones can be classified into steroid hormones, peptide hormones, and amino acid-derived hormones (Hiller-Sturmhöfel & Bartke, 1998). Steroid hormones are derived from cholesterol, a hydrophobic molecule, and thus are poorly soluble in water. Therefore, they can reversibly bind to carrier proteins in the aqueous environment of the extracellular fluid. The two modes of steroid hormone binding to their target include the binding to either receptors on the cell surface or to intracellular receptors, which they reach through diffusion across the cell membrane.

Peptide hormones are often synthesized as larger protein molecules, termed pre-hormones, and then processed by post-translational cleavages or modifications such as glycosylation. They are highly polarized and freely soluble in the extracellular fluid and bind receptors on the plasma membrane of cells. The receptors of the target cells possess both an extracellular ligand domain to bind the hormone and an intracellular domain for signal transduction.

Lastly, amino acid-derived hormones or else known as "amines" are enzymatically synthesized from tyrosine or tryptophan, which are used as substrates for the enzymatic conversion. They feature a dual mechanism of action that resembles both the steroid and peptide hormone ones (Leblebicioglu et al., 2013). As aforementioned, digestive systems are not separate entities from the rest of the body, and they actively and continuously crosstalk with other organs. For instance, the functions of the

digestive system are controlled by the nervous system. Similarly, metabolites and hormones produced by the digestive system cells are directed to the peripheral and central nervous system, which receives those signals and uses them as an input to fine-tune processes in regards to energy storage and metabolic balance. This intricate crosstalk between the digestive tract and the nervous system constitutes a regulatory axis, termed "brain–gut axis". Within this chapter, we provide an overview of the distribution, evolution and development of the gut endocrine system across metazoans.

3.2 THE GUT ENDOCRINE SYSTEM IN VERTEBRATES

The textbook functions of the digestive system and gastrointestinal (GI) organs are typically food intake, digestion, and nutrient absorption, but nowadays it has been discovered that the GI system constitutes the largest endocrine organ in the mammalian body. The GI system, in cooperation with the nervous system, tightly controls energy levels and glucose homeostasis. Moreover, it is responsible for the detection and response to environmental stimuli, microbial challenges as well as sensing the presence of nutrients (Meyer & Duca, 2023). These functions are essential for the survival of all Metazoa, and any impairment of those functions can lead to disease, pathogenesis (e.g., diabetes and obesity for humans), and even death. The whole-body energy balance requires fine coordination between diverse processes including nutrient sensing, metabolism, and food intake. This is achieved through the crosstalk of the nervous and endocrine systems, that is mediated in most cases by hormones.

The organs and tissues of the GI tract (stomach, duodenum, ileum, colon and pancreas) are not primary endocrine organs of the mammalian endocrine system; however, they produce a plethora of hormones that are

crucial for the survival of the organism. For instance, amongst the GI organs, there are at least sixteen diversified cell types which synthesize and secrete different combinations of hormones. For instance, some of these hormones were shown to interact and "influence" the function of each other, in a regulatory endocrine loop.

One of the best characterized endocrine cell types of the GI tract corresponds to enteroendocrine cells (EECs). EECs are specialized endoderm-derived epithelial cells, scattered through the entire GI, while being enriched in the stomach and small intestine regions. EECs derive from intestinal stem cells (ISCs), and they can sense the microenvironmental changes. EECs rapidly respond to a given change by secreting neurotransmitters or neuropeptides that allow them to regulate diverse biological and physiological processes. EECs are highly diversified, with each EEC subtype showing specialization to diverse processes, a feature that makes their classification difficult.

However, their precise location within the GI tract can be used as a reliable classifying approach. For instance, it was demonstrated that EECs localized in diverse regions of the GI can produce diverse gut peptides and metabolites, and are involved in aiding digestion and glucose homeostasis maintenance (Beumer et al., 2018). A general classical EECs model describes them as polarized cells in the mucosa of different parts of the digestive tract, with the apical surface projected into the lumen space, while the basal part is attached to the basal lamina and it is in contact with blood capillaries where their products are secreted. Generally, the EECs bear morphological features that make them easily recognizable from other GI cell types. Such features include their elongated form and the enrichment of the basal cell body with dense vesicles **(Figure 3.1A')**. Notably, EECs can be further subdivided into two types: the "open type" and the "closed type".

In detail, “open type” EECs are cells that are in contact with the intestine or stomach lumen, whilst “closed type” EECs refer to the ones that are not in contact with the lumen. In open-type cells, the apical surface presents microvilli that differ from those of typical enterocytes in both height and density, suggesting a “receptor” function. Numerous secretory granules are primarily located in the basal half of the cell, and their contents are released by exocytosis at the basal and lateral surfaces, close to the basal membrane. Closed-type cells share a generally similar structure but lack pronounced polarity in their organization. Some endocrine cells extend basal processes that may contact other endocrine cells (Nabeyama, 1975).

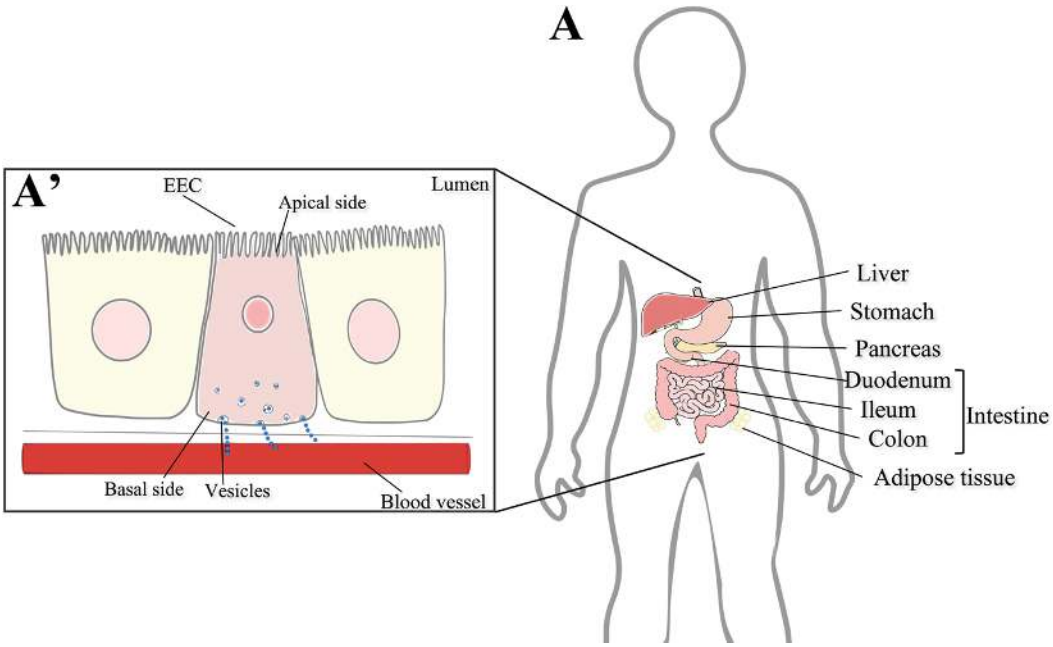


FIGURE 3.1 A) Diagram illustrating the main organs that make up the human gut endocrine system; A') representation of the classical model of EECs in mammals. ⏎

3.2.1 INTESTINAL ENTEROENDOCRINE CELLS

In the intestine, the EECs are located in the epithelium of the villi and crypts. Most of them are highly polarized and are spatially oriented with the

apical region facing the intestinal lumen. This cellular apical region is equipped with microvilli that differ in height, distribution, and density, a distinguishing feature from the surrounding "typical" enterocytes. On the other hand, the basal part of EECs generally faces the base of the intestinal epithelium and has no contact with the lumen. This basal region, together with the lateral surface of the EECs, is mediating the secretion of hormones and metabolites, directed to blood capillaries located under the epithelium (the so-called "endocrine secretion") and into the extracellular space, often distributed to the sensory nervous system ("paracrine secretion").

The intestinal EECs are located in both duodenum and ileum, where they are usually found at the basis of the intestinal villi in the crypts of Lieberkühn **(Figure 3.1A)**. The EECs in the ileum are specialized in synthesizing glicentin, neurotensins, and somatostatin. In contrast, the duodenum harbors diversified EECs producing cholecystokinin, gastric inhibitory polypeptide, somatostatin, and secretin. Secretin is a peptide hormone released by the duodenum, upon the detection of partially digested food from the stomach. Secretin functions through the stimulation of the release of bicarbonate from the pancreas that eventually neutralizes and inhibits the secretion of gastric (hydrochloric) acid, creating the right pH for digestive enzymes to work properly in the small intestine.

3.2.2 Stomach Enteroendocrine Cells

Notably, EECs located in the stomach region (stomach EECs) are highly polarized on their basal side, with numerous secretory granules that contain and release an extensive repertoire of neuromodulators and hormones including serotonin, histamine, somatostatin and gastrin **(Figure 3.1A)**. For instance, the peptide hormone gastrin is used to stimulate the release of

gastric acid and pepsinogen in the stomach, promoting the process of digestion.

3.2.3 Pancreatic Endocrine Cell Types

In humans, the pancreas is located in the abdominal region, behind the stomach, is connected to the duodenum through the pancreatic duct, and it acts as both an exocrine and endocrine gland **(Figure 3.1A)**. As previously mentioned, many hormones secreted by the intestinal EECs can also stimulate the release of pancreatic hormones. For example, the gastric inhibitory polypeptide can directly regulate the energy reservoir and metabolism of sugars by stimulating the release of insulin and decreasing glucagon production.

As illustrated in Chapter 2, the pancreas consists of specialized cell types involved in the production of zymogens (exocrine function) and hormones that regulate many metabolic processes and glucose levels (endocrine function). Based on its dual endocrine and exocrine features, the pancreas constitutes one of the best-studied organs, and especially its endocrine partition in the gut endocrine system field. The developmental cascades leading to the formation of pancreas have been extensively described in mammals. The formation of the vertebrate pancreas, and especially of the endocrine pancreas partition, can be divided into two different cell differentiation phases. During mice embryogenesis, the pancreas originates as outgrowths of the foregut, and morphogenetic signals (such as Hedgehog, FGF, Notch, Wnt, and TGF-β) from neighboring mesodermal and ectodermal cells (Edlund, 2002) lead to the formation of two endodermal buds, one on the dorsal and one on the ventral side. In the first phase, epithelial buds from the dorsal and ventral foregut grow, giving rise to post-mitotic endocrine cells. The second phase is defined by the

expansion of the pancreatic buds of the duodenum that elongate, fuse and eventually form the definitive pancreas (Slack, 1995). During this phase, cells differentiate and generate the main cell type components of the exocrine and endocrine pancreas (Pan & Wright, 2011). For instance, the resulting pancreatic progenitor cells are capable of giving rise to both endocrine and exocrine (acinar) cell types, with their multipotent state regulated by specific transcription factors. Among the earliest transcription factors expressed in the primitive gut tube are *pdx1* (pancreatic and duodenal homeobox 1) and *ptf1a* (pancreas-specific transcription factor 1a). Loss of function of either gene leads to pancreatic agenesis in mice. Both transcription factors have been shown to play a dual role, contributing both to the early specification of pancreatic progenitors and to their subsequent differentiation into distinct cell types (Li et al., 1999).

Pancreatic cell types display a high degree of complexity, that is the result of the cell type specialization, the byproduct of division of labor. As illustrated in great detail in Chapter 2 (Paganos P. and collaborators), a single cell type, the acinar cells, constitutes the entire exocrine pancreas partition. However, the endocrine partition includes an extensive repertoire of diversified endocrine cell types. The pancreatic endocrine cells are spatially organized in groups, which are known as “islets of Langerhans”. Islets are widely disseminated in the pancreas, occupying approximately 1% of the total mass of the organ (Dolenšek et al., 2015). In mice, β-cells make up roughly 75-80% of islet cells, while α-cells account for about 20% and δ-cells represent less than 10%. In humans, however, the proportion of β-cells is lower, typically ranging between 55 and 70%, whereas α-cells are more abundant reaching 35–40% and γ-cells each constitute less than 10% of the islet population. Human islets also show considerable variability in size, with diameters spanning approximately 50–500 μm and containing on

average around 1500 cells per islet. Differences in the relative proportions of endocrine cell types between individuals are associated with variations in β-cell mass, while the regional distribution of islet cells within the pancreas shows only minor variation between individuals (Aldous et al., 2023).

Decades of research have defined and characterized the different types of pancreatic islets, based on their capability of producing different hormones. Hormones produced by the various islet cells are taken up by capillaries and distributed to the bloodstream. The endocrine peptides that are active in both the stomach and pancreatic islets have been described as part of the "gastroenteropancreatic" (GEP) system (Jönsson, 1991). Pancreatic α cells produce glucagon, which antagonizes the effects of insulin and in turn stimulates gluconeogenesis in the liver. Pancreatic β cells produce and secrete insulin that maintains low levels of glucose in the bloodstream, and amylin while pancreatic δ cells secrete somatostatin regulating the activities of insulin as well as glucagon. This is also the reason for somatostatin being called the "general endocrine inhibitor". Pancreatic ε cells are able to secrete ghrelin that stimulates food intake, influences appetite, and regulates fat storage, while the PP-cells produce the pancreatic polypeptide (PP), involved in blocking zymogen secretion by the exocrine acinar cells. All these hormones display diverse functions related to metabolic homeostasis, which they execute through the deployment of different mechanisms. For instance, pancreatic hormones contribute to the homeostasis maintenance by controlling blood pressure, blood sugar levels, fluids and electrolyte balance as well as body temperature. An example highlighting the importance of the regulation of body homeostasis is that even the brain, and generally the nervous system, mainly rely on glucose as the principal metabolic source.

An interesting case of pancreatic endocrine function is in regard to the hormone insulin, which is secreted upon food intake. In the human pancreas, the insulin-producing cells constitute the majority of islet mass, followed by somatostatin-, glucagon- and PP-producing cell (Falkmer et al., 1985). Insulin is secreted by pancreatic β-cells after a rise in the blood glucose levels, in a process called "glucose-stimulated insulin secretion" (GSIS). This process represses the production of hepatic glucose, facilitating glucose uptake in the adipose tissue and skeletal muscle (Campbell & Newgard, 2021).

3.2.4 Liver Endocrine Cell Types

The liver is also considered to be a digestive endocrine organ because it is responsible for secreting at least five hormones or hormone precursors that enter the blood circulation and reach distant organs **(Figure 3.1A)**. These hormones include insulin-like growth factors (somatomedin), angiotensinogen, thrombopoietin, hepcidin, and hepatokines, the hepatocytes-derived cytokines (Meex et al., 2017). All of these were shown to have important roles in both physiological and pathological conditions, such as obesity. Insulin-like growth factors (IGFs) produced by the liver have both anabolic and metabolic functions involved in the normal organismal development and growth. For example, low production of Igf-1 (insulin-like growth factor 1) can compromise proper liver function, and lead to malnutrition and renal impairment due to its effect on growth hormone (Møller & Becker, 1992).

In recent years, several novel endocrine functions of the liver have been discovered, especially its involvement in regards to pancreatic activity regulation. The crosstalk and the regulatory interaction between the pancreas and the liver constitute the "pancreatic-liver axis". Along this axis,

amino-acid derived hormones produced by the liver act upon the pancreatic α cells leading to an increase of their mass by stimulating cell proliferation and glucagon production (Rhyu & Yu, 2021).

3.2.5 The Adipose Tissue as an Endocrine Organ

The adipose tissue can be considered as a digestive endocrine organ due to its capability of secreting hormones involved in lipid metabolism **(Figure 3.1A).** For example, leptin is a protein secreted in the blood circulation by adipose cells. The adipose cells release leptin in response to food intake and depending on the levels of body fat. Leptin induces satiation and reduces appetite; therefore, it has a contrasting effect compared to other GI and pancreatic hormones, providing feedback and acting as a mediator between the food intake and energy storage (Kleine & Rossmanith, 2016).

3.2.6 Endocrine Cells in Non-Mammalian Vertebrates

As aforementioned, a high number of EECs can be found in the mammalian digestive tract, either scattered or associated with an organ **(Figure 3.1)**. Considering other vertebrates, the endocrine GI system in birds, reptiles, amphibians, and fish does not differ much from the one described in mammals. However, the mammalian pancreas constitutes a unique case in which specialized populations of endocrine cells are spatially organized together embedded within an exocrine tissue, and constitute a compact organ fulfilling functions related to digestion and metabolic homeostasis. Regardless of the absence of a proper organ, in non-mammalian lineages such as in jawless vertebrates (hagfish and lamprey), different types of endocrine cells have been found within the epithelial layer of the midgut, producing peptides that are considered homologous to glucagon, gastrin,

PP, neuropeptide Y (NPY) and peptide Y (Falkmer et al., 1985; Youson et al., 2006).

3.3 DISTRIBUTION, LOCALIZATION AND FUNCTION OF GUT-ENDOCRINE CELLS IN INVERTEBRATES

In Bilateria and non-Bilateria (Ctenophora, Cnidaria), the food passes through the mouth and goes into an internal digestive system, where there are endocrine/sensory cells secreting chemical signals such as peptides and hormones. These sensory cells interact with a variety of cell types such as exocrine cells, thoroughly described in Chapter 2, as well as smooth muscle cells, contractile cells, and neurons, that all participate in the digestion process (Smith & Mayorova, 2019). As aforementioned, the GI endocrine system has been extensively studied in vertebrates, especially in mammals, where numerous gut endocrine cells have been identified, and their molecular, morphological, and functional aspects characterized. However, vertebrates constitute less than 5% of the total described animal species. How do the "rest" (the vast majority) of animals, especially invertebrates, cope with such processes? For instance, are there endocrine systems present in animal clades such as Cnidaria and Poriphera that do not have proper digestive organs and ingest unicellular food through phagocytosis and intracellular digestion? (Lunger, 1963) What is the presence and the role of gut endocrine cells in invertebrates? How did such complex endocrine functions emerge during evolution?

One of the best ways to trace back the evolution of the gut endocrine system is to investigate and compare its morphological organization and molecular features across a wide range of phylogenetically distant species. Such an approach allows for the identification of common mechanistic and molecular similarities as well as for underlining key differences. Cross-

species comparative analyses have shown that regardless of the high diversity amongst animals, they all use hormones and “regulatory” molecules to communicate and finely regulate digestive processes.

Hormones play a key role in understanding the life history of animals. The conservation of the structure of a hormone can be clade-dependent, with some hormones having highly conserved structures, while others showing subtle variability across species. Based on their broad role in a multitude of physiological and behavioral processes that are strictly necessary for animals' life, understanding hormone and hormone-producing cell evolution is fundamental. Since the discovery of secretin in 1902 by Bayliss and Starling, the GI tract has surpassed its typical role of digestion and food absorption, being now viewed as a multifunctional endocrine organ, able of producing more than 30 hormones by specialized EECs or by organs associated with the digestive tract, such as the pancreas.

Over the past 50 decades, histological, histochemical, ultrastructural (via electron microscopy), and immunohistochemical studies have demonstrated the presence of specialized epithelial endocrine cells in the digestive tract of numerous metazoan taxa beyond vertebrates and mammals. Taken together, these cells can be described as intestinal regulatory cells, constituting diffuse regulatory systems comparable to the enteric endocrine system of vertebrates. Moreover, the ability of endocrine cells to synthesize, secrete, and respond to neuromodulators led researchers to hypothesize a common origin between endocrine cells and elements of the nervous system (Pearse, 1969, 1973).

In the past, the immunoreactivity to a peptide was evaluated using anti-sera (even though the specific nature of such immunoreactivity, e.g., the peptide sequence was uncertain) as well as additional protein isolation techniques. Characterization of endocrine cells through ultrastructural

analysis was adopted to analyze features such as cell morphology, and the distribution, structure, and density of villi and granules. This approach led to the discovery that endocrine cells' features are highly variable in different animal species, presenting a variety of structures, cytological organizations, and molecular content produced (e.g., monoamines, peptide hormones, and other compounds).

Additionally, thorough characterization and observation of the digestive tract organization and cell type repertoire of several invertebrate species revealed many similarities in the organization and function of gut endocrine cell types. Comparative studies across metazoans showed that gut endocrine cells share many important characteristics. For instance, it was discovered that these cells are not involved in any type of digestion process (membrane or intracellular digestion, absorption, secretion of digestive enzymes, etc.). Instead, gut endocrine cells have an important regulatory role. In recent years, these methods have been complemented by the massive sequencing of animal genomes and transcriptomes, which facilitated the detection of hormones and neuropeptides with greater accuracy and allowed researchers to reconstruct their expression patterns at unprecedented levels.

Since research on gut endocrine cells has largely focused on vertebrates, particularly due to their relevance in disease, less attention has been given to other animal groups. In the following sections, we will examine the composition of gut endocrine cells across a range of bilaterian taxa (both deuterostomes and protostomes) as well as non-bilaterian groups. The aim is to present the available data for these organisms and ultimately discuss the evolutionary origins of these cell types. The nomenclature of peptides with gut endocrine functions is highly heterogeneous across species. Accordingly, throughout the different sections, we have retained the terminology used in the respective cited studies. Moreover, peptide names

are presented in regular font, whereas the names of the genes or precursors are italicized.

3.3.1 DEUTEROSTOMES

3.3.1.1 Cephalochordates

In cephalochordates, such as the lancelets *Branchiostoma lanceolatum, B. japonicus*, and *B. floridae*, different types of gut-related endocrine cells (immunoreactive to different types of hormones such as insulin, PP, somatostatin, secretin, glucagon, and gastrin) have been found within the digestive tract. Morphologically, these cells have a cylindrical shape with many secretory granules located in the basal part of the cell **(Figure 3.2D)**. These cells are not organized in clusters but are rather scattered along the entire digestive tract epithelium (Reinecke et al., 1993). Subsequent phylogenetic investigations in *B. lanceolatum* revealed that the lancelot insulin-like peptide (ILP) features structural characteristics of both vertebrate insulin and insulin-growth factors, constituting the sister group of both. Interestingly, detailed sequence analysis suggested that ILP is not only similar to vertebrate insulin but is also cleaved similarly to a pro-insulin, by removing the connecting c-peptide. In this species, *ilp* is expressed in mesodermally derived cell types during early embryogenesis, while later on its expression is restricted to the gut in both embryo and adult lancelets. Based on all these findings, it was hypothesized that this peptide has a role in both developmental processes and metabolism, displaying a mixed "function" from vertebrates' IGFs and insulin (Yu et al., 2015). More recently, single-cell transcriptomic profiling of *B. floridae* digestive tract that included cells from the hepatic diverticulum, midgut, and hindgut, identified functional segmentation in the lancelot digestive tract as well as distinct cell clusters, including epithelial-like and ciliated cells in the

midgut. Among the findings, it was shown that three types of endocrine-like cells are present in the digestive tract. These cells were scattered along the digestive tract, and they were defined as: insulin-like secreting cells (*ilp1* and *ilp2*), somatostatin-like, and glucagon-like producing cells. Knockout experiments carried out on the same species showed that the transcription factor *pdx1*, crucial for vertebrate pancreas development and insulin production by the pancreatic β cells (Jonsson et al., 1994), does not regulate *ilp* expression, and it is not essential for the development of endocrine cells in lancelets. Notably, the same authors postulate that amphioxus Ilp1 structurally and functionally resembles more to the vertebrate insulin-like growth factor 1 (Igf1) than insulin, and that it is likely it predominantly influences body growth and amino acid metabolism, rather than glucose levels and general metabolic homeostasis (Dai et al., 2024).

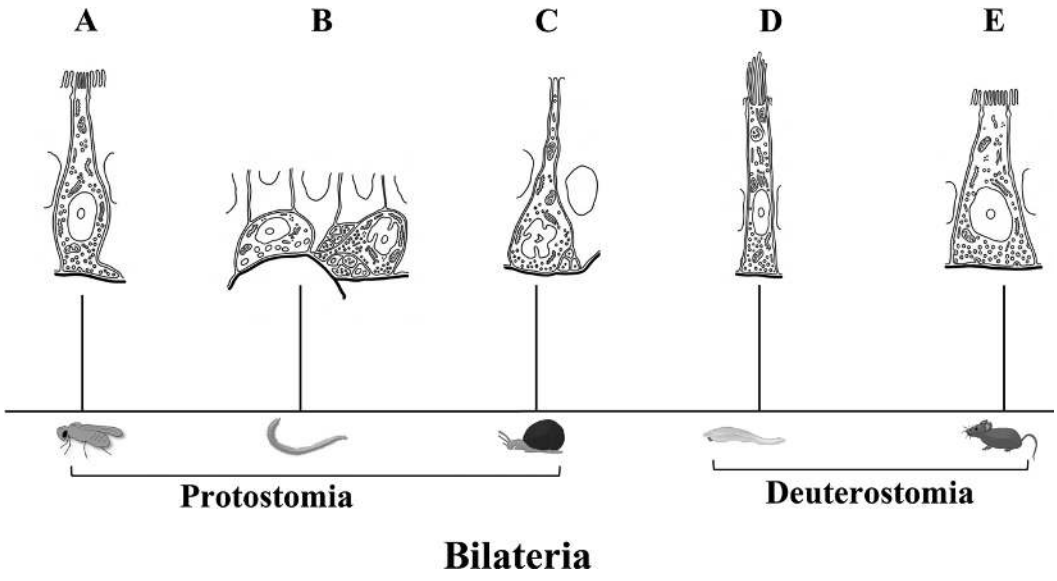


FIGURE 3.2 Schematic representations of the morphology of some endocrine gut cells described across various Bilaterian lineages. A) scheme of the open-type endocrine cell structure of the insect midgut; B) large- and small-granular basal cells in the polychaete *Nereis virens* (from (Punin & Lukyanova, 1984)); C) endocrine cell in the gut of the snail *Helix aspera* (from (Alba et al., 1988));

D) intestinal endocrine cell in the lancelet *Branchiostoma lanceolatum*; and E) endocrine cell from the mammalian small intestine. Compiled from multiple sources, particularly from (Punin, 2000). ⏎

3.3.1.2 Urochordates

In urochordate representatives, such as *Styela clava* and *Ciona intestinalis*, endocrine cells are found in the esophagus, stomach and intestine, and are able of producing hormones, including somatostatin, gastrin, PP, calcitonin and insulin-like factors. Urochordate gut endocrine cells are embedded in the epithelial layer and are in close contact to networks of cavities, vessels and blood lacunae, indicating that it is likely their secreted products enter the circulation directly. Apart from being scattered along the digestive tract, urochordate gut endocrine cells, such as the PP-producing cells in *Ciona*, are predominantly located in the stomach wall, which is indicative of functional specialization of the *Ciona* PP-producing cells (Fritsch & Sprang, 1977). Interestingly, the urochordate homolog of *pdx* is primarily expressed at the posterior end of the stomach, with weaker expression extending slightly toward the anterior. Moreover, the transcription factor *nr5a2*, a key regulator of liver, pancreas, and intestine functions in mammals, has also been identified as an important regional marker in the juvenile gut of *C. intestinalis*, where it is specifically expressed posteriorly to the *pdx* domain (Hale et al., 2014; Nakayama & Ogasawara, 2017). However, it remains a mystery whether the cells expressing these transcription factors correspond to the gut endocrine cells that had been morphologically identified in the past.

3.3.1.3 Echinoderms

A great number of endocrine cells scattered within the digestive tract has been also found in non-chordate deuterostome representatives such as

echinoderms. The ultrastructural and immunohistochemical characterization of intestinal cells in different echinoderm species, especially in Holothuroid and Asteroid species, revealed that their adult gut endocrine system consists of both neuronal cells producing diverse neuromodulators and endocrine cells with secretory granules. For instance, immunohistochemical detection of somatostatin, glucagon, pancreatic polypeptide, peptide YY, and a-MSH has been reported in the pyloric caeca, cardiac stomach and rectal caeca in the adult sea star *Marthasterias glacialis* (Martinez et al., 1989). More recently, a bombesin (BN)-type neuropeptide, key regulator of feeding and digestion, was identified and characterized in the radial nerve cord, cardiac stomach, and pyloric stomach of the starfish *Asterias rubens*. This finding demonstrates that BN-type signaling originated early within the deuterostome lineage and suggests that its role in the regulation of feeding behavior is evolutionarily ancient (Huang et al., 2025).

Genomic studies on another Echinoderm class, sea urchins, identified two members of the insulin/IGF/INSL/RLN superfamily in the Strongylocentrotus purpuratus genome, namely *Sp-igf1* and *Sp-igf2* (Burke et al., 2006).

However, as illustrated in Chapters 1 and 2 (Annuziata R. and collaborators and Paganos P. and collaborators respectively), most echinoderm species develop indirectly, with their life cycle being interrupted by a larval life phase, thus making the picture even broader. Decades of research on the molecular and functional characterization of the sea urchin larva digestive cell types have revealed the presence of numerous gut endocrine cells. For instance, such studies revealed the presence of gut endocrine cell populations, located in the larval stomach and intestine and produce *insulin-like peptide 1* (Igf1 or Ilp1), in a feeding-dependent manner. The gene coding for that peptide was shown to be structurally

organized similar to the lancelot insulin-like gene. Two other components of the insulin family have been found in this species: an insulin receptor (*insR*) and the insulin-like growth factor 2 (*igf2*). The gene structure of *igf2* is highly divergent from the vertebrate insulin, and notably, from the sea urchin *ilp1* as well, and its expression has been detected in different larval territories (Perillo & Arnone, 2014). Therefore, it has been postulated in sea urchins that Ilp1 has a role in food assimilation and energy balance due to its differential expression and localization in different food availability conditions. For instance, in late *S. purpuratus* larvae (14 days post-fertilization), *ilp1* transcript and protein levels increase during fasting, localizing primarily to the intestine, while feeding further enhances expression in the stomach (Perillo & Arnone, 2014), thus supporting a putative role of Sp-Ilp1 in nutrient assimilation and lipid storage, probably useful to prepare for metamorphosis. Based on these findings, it has been hypothesized that Ilp1 has retained the original gut endocrine functions, while Igf2 has been co-opted for other purposes, e.g., acting as a growth factor during embryogenesis. Interestingly, single-cell transcriptomics carried out on the free-swimming and feeding early larval stage of the same species revealed the presence of a distinct exocrine pancreas molecular signature in cells located in the larval upper stomach and various cells showing an endocrine-like molecular signature scattered across the larval digestive tract (Paganos et al., 2022). In detail, it was shown that distinct endocrine pancreas-associated transcription factor combinations are present in cells in the esophageal (*foxA2, nkx2.2* and *brn4*), intestinal (*foxA2, pdx1, neuroD1, mnx, nkx2.2*, and *rfx6*), and pyloric sphincter (*gata6, pdx1, nkx6.1, rfx3,* and *rfx6*) regions. Taken together, these findings pointed to the existence of at least two distinct pancreatic endodermal cell fates in the sea urchin larva digestive tract; endocrine and exocrine [details on the latter can

be found in Chapter 2 (Paganos P. and collaborators)]. Therefore, the endocrine fate appears in endoderm-derived cell lineages also in sea urchins, with Ilp1 appearing later during larval development.

Strikingly, an endocrine pancreatic regulatory molecular signature was also detected in sea urchin larvae's neurons (*foxA2, ngn3, pdx1, neuroD1, mnx, islet, rfx3, brn4,* and *mist*). Interestingly, a subset of such "endocrine pancreatic-like" neurons were double positive for the *pdx1* and *brn4*, expressed numerous genes and modulators linked to an endocrine function, while their specification and differentiation were shown to be *pdx1*-dependent (Paganos et al., 2021; Paganos et al., 2022).

3.3.1.4 Hemichordates

Hemichordates, together with echinoderms, form the Ambulacraria clade. Very recently, through bulk RNA-sequencing of the adult digestive tract of the acorn worm *Ptychodera flava,* an endocrine "module" (based on gene expression profiles) has been identified in this species. This module is present in cells spatially localized in the most posterior portion of the midgut, anteriorly to the hindgut. This region was found to be marked by high expression of *nr5a2*, which, as already mentioned, is an essential transcription factor for embryonic development, bile acid synthesis, cholesterol biosynthesis, and stem cell pluripotency (Hale et al., 2014). Moreover, other genes that have conserved roles related to metabolic sensing and regulation were detected in this region. For instance, among the genes reported were the glucose sensor *mlxip/mondoA*, ketohexokinases, and several transcription factors such as *foxO3, ncoa2,* and *pknox1.1,* the expression of which is linked to both liver and pancreatic development, as well as to endocrine functions in adults. Considering the critical roles of *nr5a2* in endocrine and pancreatic development and function of in

vertebrates, together with the mixed metabolic/endocrine molecular signature of this region, the authors postulated a possible existence of endocrine pancreatic-like cells in *P. flava.* This hypothesis is further supported by the conserved position of this "module", posteriorly to the *pdx1* domain of expression (Vianello et al., 2026).

3.3.2 PROTOSTOMES

3.3.2.1 Arthropods

Nearly 93% of all animal species are protostomes, and amongst them, arthropods are the largest phylum of invertebrates and also the largest phylum in the entire animal kingdom. One of the most studied classes of arthropods is insects. Several endocrine cell types have been identified in the gut of diverse insect species based on morphological features. To some extent, these cells appear to be similar to endocrine cells of vertebrates, as they are covered in microvilli and have common characteristics such as their organization in "open" and "closed" types **(Figure 3.2A,E)**. On the other hand, several insect species-specific features of the endocrine cells have been reported, including different degrees of development of the smooth and rough endoplasmic reticulum and Golgi apparatus, as well as the structure, size, and shape of secretory granules. Such differences indicate diverse functions and modes of action involved in the exocytosis of the endocrine products. The high diversity of the gut endocrine cells in insects has also been confirmed through molecular studies that documented response to a variety of different types of peptides, such as PP, enteroglucagon, somatostatin, insulin, bombesin, and gastrin (Brown et al., 1985). For instance, in more recent years, different EECs have been identified in *Drosophila melanogaster* midgut through single-cell RNA sequencing. In *Drosophila*, EECs function as chemosensory cells capable of

producing and releasing hormones in response to the contents of the midgut lumen, spanning from nutrients, metabolites, and even bacterial byproducts. Through these activities, *Drosophila* EECs play key roles in the regulation of gut physiology, feeding behavior, and glucose homeostasis. Typically, EECs are known to produce a maximum combination of two or three gut hormones. Interestingly, in *Drosophila* midgut, each type of EEC is found to produce at least five different gut hormones (some examples are: *Allatostatins: astA, mip, astC; tachykinin (tk), neuropeptide F (NPF), dh31, ccha1, ccha2, orcokinin B, and bursicon*), along with enzymes important for hormone biosynthesis, processing, and secretion (Hung et al., 2020). Noteworthy, in this organism, enterocytes were shown to perform both absorptive and zymogenic functions by secreting digestive enzymes. Another interesting feature is that some specialized gland cells are relatively rare and confined to a small region of the midgut, while, in contrast, endocrine cells are distributed across the entire digestive tract (Veenstra et al., 2008). In the same organism, eight insulin-like peptides have been identified, called *dilp* (1-8), *Drosophila insulin-like peptides*. Dilp 1 to 6 bind to the only insulin receptor (*dInR*), while *Dilp 7* and *Dilp 8* bind to leucine-rich G-protein-coupled receptors (Lgr) 3 and 4, respectively. The expression patterns of such *Dilp* genes differ much between embryonic and larval stages. During embryogenesis, *dilp1*, *dilp3*, *dilp5*, and *dilp6* show no detectable expression, whereas *Dilp2* is strongly expressed in the midgut region and weakly in the mesoderm. On the other hand, *dilp4* is expressed at high levels in the mesoderm and in the anterior midgut rudiment, while *dilp7* displays low ubiquitous expression with moderate levels in the midgut. In larvae, expression becomes more diversified. *dilp2* and *dilp3* show strong expression in seven cells of each brain hemisphere and in the salivary glands, *dilp4* is highly expressed in the midgut, *dilp5* shows strong

expression in seven cells of each brain hemisphere and moderate expression in the gut, *dilp6* is weakly expressed in the gut, while *dilp7* is strongly expressed in ten cells of the ventral nerve cord. Moreover, it has been shown that modifications in the expression of the *dilp2* gene, as well as altering the activity of the *dInR*, influences both cell size and cell number within organs, ultimately affecting overall body size. Considering the highly tissue-specific expression of *dilp* genes in the central nervous system, along with their broader expression in primordia of adult organs, it has been proposed that a nutritionally regulated mechanism exists in *Drosophila* in which insulin-like peptides coordinate growth through both neurosecretory and local signaling pathways (Brogiolo et al., 2001).

In other arthropods, such as chelicerates and crustaceans, some endocrine-like cells have been described, although the organization appears less uniform than in insects. For instance, in the crab *Carcinus maenas,* endocrine cells that produce the hyperglycaemic hormone (Chh) and its precursor peptide (Cprp) have been found to be in the foregut and hindgut, especially during the premoult (Webster et al., 2000).

3.3.2.2 Molluscs

The second largest invertebrate phylum is Mollusca. A great amount of data regarding endocrine cells was obtained through microscopic analysis and immunocytochemical techniques in the 70's and 80's. These studies described the presence of endocrine cells in the digestive tract of both gastropods and bivalves. For instance, in the snail *Helix aspersa,* endocrine cells were found by electron microscopy to possess a clear cytoplasm containing variable numbers of small secretory granules with various electron densities. These cells were ovoidal or rounded, with apical processes that extended toward the intestinal lumen. Their nucleus was

located in the basal portion of the cell (**Figure 3.2C**). In addition, intraepithelial nerve bundles were found present in contact with these endocrine cells. Notably, gut endocrine cells appear to be extremely rare in this species (Alba et al., 1988).

The occurrence of endocrine cells was also found in the gut of *Viviparus ater*, a freshwater gastropod. Also in this case, endocrine gut cells were rare but included both open- and closed-types. Most of them were observed in the esophagus, where they showed immunoreactivity to antisera against gastrin, insulin, serotonin, and substance P. Only a few cells were detected in the stomach, where they reacted exclusively with anti-gastrin antibodies, and in the proximal intestine, where they were immunoreactive to anti-serotonin and anti-substance P antibodies. In the salivary glands, endocrine cells scattered among the glandular cells were identified, showing immunoreactivity to antisera against neuropeptide Y, pancreatic polypeptide, and somatostatin (Franchini et al., 1994).

Within the intestinal mucosa of the gastropod mollusc *Buccinum undatum*, epithelial cells containing small, round, electron-dense cytoplasmic granules have been described. These granules resemble the secretory granules typically observed in endocrine cells of vertebrates. These cells have therefore been defined as “granular cells” that exhibit certain staining patterns and histochemical characteristics similar to those of mammalian pancreatic β-cells. On this basis, it has been proposed, as a working hypothesis for further investigation, that these cells may represent endocrine elements and could be the site of insulin production in *B. undatum* (Boquist et al., 1971). In the giant African snail *Achatina fulica,* carbohydrate metabolism has been investigated through extracts obtained from the digestive glands and the pyloric intestine, both of which were found to be able to stimulate glycogen deposition in rats. The results

suggested that *A. fulica* gut cells produce an insulin-like substance capable of regulating glycogen metabolism in the tissues of the animal (Gomih & Grillo, 1976).

The data reporting the presence of endocrine gut cells in bivalves are more scarce than in gastropods. However, ultrastructural-based evidence indicated that endocrine gut cells in the bivalve *Mytilus edulis* are scattered in the intestine and the hepatopancreas of the mussel, and likely produce an insulin-like molecule. These cells are rare and unevenly distributed in the gut of the mussel, scattered as single elements among the big prevailing population of epithelial cells. Additionally, their granules vary in size, suggesting the existence of putative different types of endocrine cells (Fritsch & Sprang, 1977). In this species, the cells immunoreactive to mammalian insulin and insulin-like molluscan substances were described, and similar immunoreactivity was also reported in several other bivalve species (Fritsch et al., 1976). In fact, the presence of insulin-like peptides in the bivalve mollusc *Mya arenaria* was immediately reported soon after the discovery of insulin. Just like in the gastropod snails, the identified insulin-like peptide was found to be expressed in neurons. Later on, advances in genome sequencing revealed that insulin-related genes vary across molluscs in a lineage-dependent manner. The insulin-related gene repertoire also includes an insulin-like peptide displaying a conserved structural organization similar to vertebrate insulin and IGFs. More recent studies have demonstrated that in the Pacific oyster *Crassostrea gigas,* Ilp is expressed in the digestive gland (Cherif-Feildel et al., 2019). Further analysis of the expression pattern of this gene was conducted in adult oysters, demonstrating that *ilp* expression in the digestive gland overlaps with that of *pdx* within the endodermal cell layer of the hepatopancreas and other oyster gut tissues (Xu et al., 2021).

3.3.2.3 Nematodes

In nematodes, the endocrine system regulates a wide range of processes, including molting, development, energy metabolism, reproduction, neural development, and behaviors such as feeding, defecation, and mating (Antebi, 2006). In addition to neurons, other cell types such as muscle and intestinal cells produce signaling molecules that act as modulators (Shi et al., 2022). In response to environmental cues, steroid and peptide hormones are synthesized and released, binding to membrane or nuclear receptors to trigger physiological responses. Because *Caenorhabditis elegans* lacks a circulatory system, hormonal signals are transmitted intercellularly through gap junctions, local diffusion, or neuronal signaling to adjacent cells. Although this mode of communication is slower than circulation-based systems, it provides a stable and coordinated mode of signaling (Nurrish, 2014).

Insulin-like peptides have been extensively studied in nematodes. For instance, *C. elegans* is capable of producing 40 insulin-like peptides (INS, in this species), but only one insulin-like receptor called *daf-2* has been reported so far (abnormal Dauer Formation protein 2) (reviewed in Zhu & Chin-Sang, 2024). Despite the fact that most *C. elegans* INS peptides show little sequence similarity to human insulin or IGFs, they retain the insulin characteristic structural fold and can function as insulin-like molecules (Pierce et al., 2001). For instance, Ins-6 has been shown to bind to and activate the human insulin receptor (Hua et al., 2003). INS peptides are mainly expressed in the nervous system, muscle, reproductive tissues, epithelia, coelomocytes and the digestive system. Most tissues express multiple INS peptides. For most INS peptides the nervous system is the major site of expression, with the exception of certain peptides such as Ins-2, Ins-27, and Ins-33 (Pierce et al., 2001; Ritter et al., 2013). Analogously

to the function of human insulin, in *C. elegans* high glucose intake induces the expression of Ins-1 in neurons, leading to the activation of the insulin-like growth factor signaling pathway and subsequent mobilization of intestinal fat. In contrast, *ins-1* mutants accumulate more fat when exposed to excess glucose (Handley et al., 2022). Elevated glucose levels also stimulate Ins-7 activity, which promotes mitochondrial ROS production and glycation, ultimately causing neuronal damage, reduced motility, and a shortened lifespan. Recent findings show that Ins-7 is also an enteroendocrine factor secreted by specialized intestinal cells upon stimuli from food deprivation, while its levels increase during fasting. Ins-7 also functions as a gut-to-brain signal that suppresses the release of a neuropeptide responsible for promoting fat loss in peripheral tissues. In this way, it acts as a homeostatic regulator, modulating neuronal signals that control energy balance during nutrient scarcity. Phylogenetic analyses indicate that *ins-7* is more closely related to members of the broader insulin/relaxin superfamily than to classical mammalian insulin or IGFs. The identification of this endogenous insulin antagonist, produced by intestinal EECs, highlights an unexpected and significant role of the intestine in regulating neuronal activity (Liu et al., 2024).

3.3.2.4 Annelids

Annelids have a well-developed neuroendocrine system (NES) in which neurosecretory cells, mainly located in the central nervous system, including the supraesophageal ganglia and ventral nerve cord, produce hormones that control processes such as regeneration, growth, and sexual maturation (Clark, 1969).

Although gut-associated endocrine cells comparable to vertebrate EECs are present, a large portion of endocrine regulation in annelids is primarily

mediated by these neurosecretory cells. Ultrastructural analysis revealed the presence of a complex intestinal "regulatory" system composed of both neural and endocrine cells, and this system displays class-specific variation. For instance, in polychaetes, the endocrine gut cells were reported to have both small (80-130 nm) and large (300–500 nm) granules, always located in the basal epithelium, but without any intestinal luminal contact ("closed" type) **(Figure 3.2B)** (Punin & Lukyanova, 1984). Furthermore, the presence of pores in the membranes of these cells suggests the release of molecules produced by them into circulation. The endocrine gut cells in earthworms (or oligochaetes) have granules and small vesicles at the basal membrane, possibly used for exocytosis. Moreover, they have been found to be immunoreactive to the pancreatic polypeptide and peptide YY (Punin, 2000).

3.3.3 Non-Bilateria

Information on the gut endocrine cells of non-Bilateria is in most cases scarce. In the cnidarian *Hydra*, endocrine cells and sensory neurons were found as an integral part of the gut (Westfall et al., 1971). The pharynx and the cnido-glandular tract regions of the sea anemone *Nematostella vectensis* were shown to contain insulin-producing gland cells, together with additional cell types that are not yet fully characterized. For instance, in *Nematostella* gastrodermal septal filaments, the ectoderm generates at least seven exocrine or insulinergic specialized cell types. At least three insulin-like peptides (Ilp1, Ilp2, Ilp3) have been identified, and one insulin receptor. All the insulin-like peptides are expressed among exocrine gland cells of the pharynx and gastrodermal septal filaments; with the addition that *ilp1* is also expressed in neurons in the parietal muscle region (Sebé-Pedrós et al., 2018; Steinmetz et al., 2017). In Placozoa, recent single-cell transcriptomics

studies have revealed a substantial diversity of cellular states, supporting the idea that additional cell types or subtypes may exist (Sebé-Pedrós et al., 2018). As described in Chapter 2, and consistent with this recent finding, the lower epithelium, specialized for extracellular digestion, has been shown to contain multiple types of gland cells as well as mucin-secreting cells (Mayorova et al., 2018; Smith & Mayorova, 2019; Smith et al., 2014). Furthermore, the organism's complex behaviors such as rapid movements and coordinated feeding are regulated by integrative systems that include several distinct types of endocrine cells. These findings indicate that the organism possesses a higher level of biological complexity than was previously recognized (Romanova et al., 2021).

3.4 SUMMARY & EVOLUTIONARY CONSIDERATIONS: THE PUZZLING EVOLUTION OF ENDOCRINE GUT CELLS

Scientific interest in the distribution, structure, and function of endocrine cells tissues, and organs associated with the alimentary canal of different animals dates back at least to the early 19th century and has been growing ever since. Despite nearly 200 years of research, the dual focus on understanding both the function and role of such cells in individual species and how these tissues evolved remains. Therefore, still open and intriguing questions in biology are the evolutionary origins of the gut endocrine cells and the subsequent emergence of endocrine systems and organs. One appealing feature of gut endocrine cells is their high molecular similarity to neurons. In the past, researchers proposed that GI endocrine cells derive from migrating neuroectodermal precursors (Pearse, 1973). However, later studies demonstrated that at least a subset of these cells originates from the endoderm (Pictet et al., 1976). Taken together, it is likely that the intestinal

endocrine cells have multiple developmental origins, while new theories propose that endocrine functions may have arisen repeatedly and independently in tissues of different origins (Rosvall, 2022).

How can cells with diverse embryological origins, such as gut endocrine cells derived from the endoderm and neurons derived from the ectoderm, perform similar functions and utilize similar molecular toolkits? This observation led to formulating the hypothesis that these two diverse cell type groups may have evolved through co-option of neuronal regulatory programs, or even that endocrine cells and neurons could have an ancient shared evolutionary origin.

Available evidence indicates that endocrine gut cells, or at least the molecular set of expressed genes that is useful to characterize such cells, occur in representatives of most major taxa across the Metazoa. Notably, only a few groups appear to lack such elements, which could reflect either true absence or limited available information on their digestive systems. Importantly, these regulatory elements are found in organisms exhibiting diverse modes of digestion, including luminal, intracellular, and mixed types and present in various regions of the digestive tract.

As already discussed in the previous sections, endocrine cells and neurons are found in the GI epithelium of many animal taxa, and both are important for the regulation and coordination of digestive and homeostatic functions, forming the so called “intestinal regulatory system” (Punin, 2000). In an attempt to trace back the evolutionary trajectory of gut endocrine cells, one must combine the available ultrastructural and physiological data with molecular and developmental data. Nowadays, a key topic highlighted in research concerns how different neuropeptides can regulate the digestive processes. Several observations support this principle. In 1975, the concept of " paraneurons " was introduced by Tsuneo Fujita,

used to describe a wide array of cells that can be inside or outside the central nervous system, and show no morphological similarity to neurons but produce neurotransmitter- or neuropeptide-like substances in response to specific stimuli and contain granules or vesicles resembling those of neurosecretory cells (Hobert, 2025). In this view, these cells may have different embryonic origins, retain the capacity to divide, and share structural and functional similarities due to convergent differentiation. Many intestinal cells can, to varying extent, be considered within this category.

The rapid expansion of genome sequencing across animal groups has generated extensive inventories of neuropeptides predicted to be expressed in many clades, some of which are likely involved in GI regulation. Overall, cells producing insulin and other hormones are components of the NES and form an essential part of the brain–gut axis (Konturek et al., 2004). In chordates, such as the lancelot *B. floridae*, neuropeptide expression has also been detected in the digestive tract (Nakayama et al., 2019), along with expression of insulin-like peptides, as already described. However, these peptides are never found coexpressed in the lancelot midgut with the transcription factor *pdx1*, suggesting clustering of endocrine cells into a pancreas and the positive regulation of insulin by *pdx1* evolved in vertebrates, highlighting conserved yet divergent features of chordate digestive systems (Dai et al., 2024). Similarly, in the urochordate *C. intestinalis*, numerous peptides and their receptors have been identified in the alimentary canal (Satake et al., 2019). In echinoderms, non-chordate deuterostomes, immunolocalization and scRNA-seq-based studies have revealed cells expressing insulin-related peptides including the presence of insulin receptor in acinar-like cells within the gut of the pluteus larva (de Pablo et al.; Paganos et al., 2022; Perillo & Arnone, 2014). Comparable

findings in another echinoderm, the sea star *Pisaster ochraceous*, also indicate the presence of insulin-related gene expression in cells of the developing gut (Wilson & Falkmer, 1965). Therefore, some echinoderms express (neuro)peptides within EECs, although it remains under investigation whether these cells directly interact with or respond to the nervous system (Garcia-Arraras et al., 2019; Hartenstein & Martinez, 2019). Furthermore, in sea urchins, a particular subtype of neurons has been identified, which displays a "pancreatic-like" molecular signature, marked by the combined expression of *pdx1 and brn1/2/4* (Cole & Arnone, 2009). *pdx1*, the pancreatic and duodenal transcription factor-1 is involved in the mammalian pancreatic cell differentiation, promotes β-cells differentiation and maintenance, and insulin transcription in these cells (Macfarlane et al., 1999).*brn1/2/4* is the sea urchin homolog of the mammalian α-cells differentiation marker *brn4*. This indicates the presence of a shared molecular program between sea urchin neurons and vertebrate pancreatic endocrine cells and suggests that vertebrate *pdx*-positive endocrine pancreas cells could either have risen through large-scale co-option of a pre-existing neuronal gene regulatory network (including factors like *pdx1, pax6, nkx6.1,* and *hb9*/*mnx*) into the pancreas lineage, or diverged from a common ancestral neuroendocrine-like precursor cell (reviewed in (Annunziata et al., 2019)).

In protostome invertebrates, the neuronal component of the NES typically dominates over the endocrine one, and insulin-producing cells are mainly located in the brain, as observed in insects and molluscs (Luo et al., 2012). Additional studies in Arthropoda have found gut-associated neuropeptides, though detailed information is currently missing (Štrus et al., 2019). Moreover, in the fruit fly *D. melanogaster* proneural genes, such as the bHLH transcription factor *lethal of scute* (*l'sc*) are initially expressed in

the embryonic endoderm and later become limited to endocrine cells, in a transition similar to the one reported in regard to vertebrate transcription factors during embryogenesis. Notch signaling is highly active in *Drosophila*'s enterocytes, and when the expression of *notch* or its ligand *delta* is altered, the endoderm is entirely transformed into endocrine cells. Conversely, increased *notch* activity leads to a reduction in the number of EECs. Taken together, the conservation across deuterostomes (including humans, mice, and zebrafish) and ecdysozoans like *Drosophila* of the genetic mechanism controlling midgut cell type selection is indicative of ancient origins, that are likely shared by all metazoans (Takashima et al., 2011; Thompson & Di Gregorio, 2014). Corroborating the shared ancestry of gut endocrine and neuronal types, five neuropeptides (sNPF, ion transporter peptide (ITP), neuropeptide-like precursor 2 (Nplp2), crustacean cardioactive peptide (Ccap), and Cnma), which were previously thought to be produced by neurons only, are also expressed in a subset of *Drosophila*'s midgut EECs (Hung et al., 2020).

In *N. vectensis* and *Aurelia aurita*, exocrine-like digestive cells and insulin-like peptide–producing cells are found within tissues expressing the transcription factor *foxA*, a conserved regulator associated with endodermal gut development in bilaterians. Although some of these tissues in *Nematostella* may derive from ectodermal lineages, the coexistence of these cell types within *foxA*-positive domains is reminiscent of the functional organization of the bilaterian gut, particularly the vertebrate pancreas. Hence, these tissues display both a cellular composition (zymogen and insulinergic cells) and physiological roles (digestive enzyme secretion and amino acid absorption) that are indeed similar to the vertebrate pancreas ones. These parallels are further supported at the molecular level. *Nematostella* gene orthologs that are typically expressed in bilaterian

midgut tissues (such as *foxA* and *hex*) and in vertebrate pancreatic development (including *nkx2.2, tbx2/3, islet, nkx6, hlxb9,* and *soxB1*) consistently co-localize within the larval pharyngeal ectoderm of *Nematostella* (Sebé-Pedrós et al., 2018; Steinmetz et al., 2017). In *Hydra*, peptide synthesis is normally associated with gastrodermal neurons, but interestingly, in individuals that are experimentally lacking neurons, ordinary gastrodermal cells can take over this function (Schaller et al., 1979).

Other non-bilaterians, such as Porifera, lack genes encoding secretory peptides, while ctenophores utilize a distinct repertoire of peptides that are not related to those found in other metazoan groups (Moroz et al., 2014). However, the ability to produce peptides likely evolved prior to the emergence of metazoans, and their role in regulating digestive and gut-homeostatic processes appears to have emerged with the evolution of the "Planulozoa" which includes Bilateria and Cnidaria (Hartenstein & Martinez, 2019).

Taken together, these findings suggest that as the complexity of the GI system increased, specialized endocrine cells emerged, deriving directly from epithelial tissues. The appearance (and possibly loss) of endocrine cells seems to have occurred multiple times independently across different taxa and likely evolved in parallel in both protostome and deuterostome lineages (**Figure 3.3**).

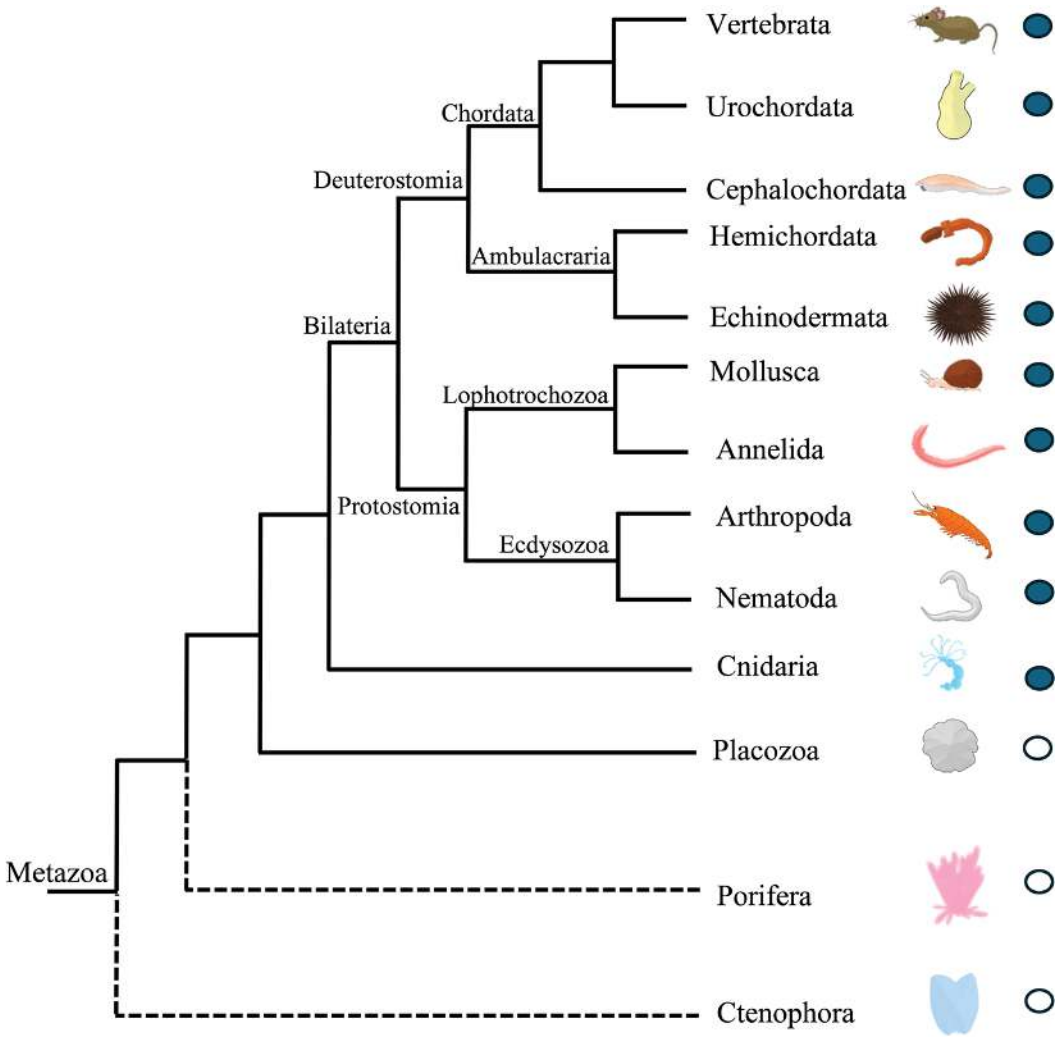


FIGURE 3.3 Phylogenetic tree of representative lineages described in this chapter. Filled circles indicate instances in which the presence of endocrine gut cells has been described in the lineage, either from morphological data or if it can be inferred by molecular data. ⏎

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4 Animals Without Through-Guts

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4.1 INTRODUCTION

The digestive system is the direct outcome of the process of internalization of cells in early development, the gastrulation. Cells become internalized in different ways, but the invagination of the blastula epithelium at one site, the blastopore, is the most typical and abundant mode and leads to an internal epithelialized cavity, the archenteron, and a connection to the external, the blastopore. During further development, the digestive system becomes modified in many ways.

Most animals have a digestive system with two openings, in which food enters at one end (the mouth), is crushed, processed and digested along its way, and the indigestible remains leave the body at the other end (the anus). Ontogenetically, both openings of this so-called “through-gut” (or one-way-gut) originate in different ways. One opening is the site of invagination of the archenteron during gastrulation, the blastopore; the other is a secondary opening. The blastopore can either become the mouth or the anus (protostomy and deuterostomy), which is name-giving for two main branches among bilaterian animals, Protostomia and Deuterostomia (Hejnol & Martindale 2009, Nielsen et al. 2018 and references therein). A process

where the slit-like blastopore becomes both mouth and anus (amphistomy) is also possible, but rare.

An intestinal tract with a single opening occurs earlier in ontogeny than the breakthrough of a second opening or the median closure (amphistomy). This sequence is also believed to have happened during evolution, and some animals have preserved the primary state of having just one opening (Schmidt-Rhaesa 2007). In general, we name such digestive systems sac-shaped. The sac-shaped condition of the digestive tract can be a primary or a secondary phenomenon. This chapter deals with taxa that primarily have a sac-shaped digestive tract; these are Ctenophora, Cnidaria, Acoelomorpha, Platyhelminthes, Gnathostomulida and Micrognathozoa. Restrictions and modifications are described below. There are a number of further animals with blindly ending guts from a variety of phyla (e.g., articulate brachiopods, species of the monogonont rotifer genus *Asplanchna*, the marine horsehair worm genus *Nectonema*; see Hejnol & Martín-Durán 2015 for more examples), but these are species or groups of species within taxa that likely go back to ancestors with a through-gut.

Through-guts seem to have clear advantages over sac-shaped guts and were a major step in evolution. The division of mouth and anus allows the ectodermally derived mouth region to become modified in multiple ways, often with the help of cuticular hard structures, to capture, crush, and swallow or pierce and suck in food items. This allows to ingest a broad variety of food items, both in size and diversity. The fact that food is processed along a linear path allows the formation of specialized regions for different kinds of food treatment and digestion. Nevertheless, it would be wrong to regard sac-shaped guts as a “primitive” feature. For the animals wearing them, they are fully functional and did not prevent amazing

radiations, like for example in cnidarians or flatworms. Let´s have a closer look.

Digestive systems are composed of regions derived from two germ layers, because the ectoderm usually forms the anterior part (and, when an anus is present, also the posterior part) of the digestive system. The main difference between endodermally and ectodermally derived tissue is that epidermal cells are capable of secretion of extracellular material to the outside. Originally, this is in the form of a glycocalyx, but several animals have evolved a cuticle. Cuticles become thicker and denser than the glycocalyx, and cuticular material can be shaped into a variety of hard structures. In the ectodermal mouth or foregut region, such hard structures abundantly form structures aiding in food capture, uptake or maceration. Cuticular hard structures are very rare in taxa with a sac-shaped gut and occur only in gnathostomulids and micrognathozoans.

Sac-shaped guts are rarely just sac-shaped. They occur in animals that do not possess body cavities and therefore never have transport systems for body fluids, which are always based on the presence of body cavities. When present, transport systems (especially circulatory systems) distribute, among others, nutrients from the digestive system into the entire body. In the absence of transport systems, the digestive system takes over this function and distributes nutrients into the body. This is especially challenging when animals become larger, and in such cases, digestive systems may develop into complexly branching systems.

The advantage of having a digestive system is that digestion can be subdivided into extracellular and intracellular digestion. In general, cells take up food particles into small vesicles by endocytosis and fuse these with lysosomes that contain digestive enzymes. The possession of a digestive cavity allows the breakdown of larger food into particles that are small

enough for endocytosis. This is performed by the presence of gland cells that secrete enzymes into the digestive cavity. The presence of extra- and intracellular digestion allows for spatial segregation of both processes, or at least a regionalization, in which the relative amount of extra- and intracellular digestion varies. In animals with a sac-shaped digestive system, we know comparably little about the food flow, with ctenophores being a fascinating exception. In most cases there are central and peripheral regions, and it is likely that extracellular digestion dominates in the central parts and nutrient uptake and intracellular digestion dominate in peripheral regions.

All taxa treated in this review have a more or less basal position in the animal tree of life. The phylogenetic position is mentioned in the sections below.

4.2 CTENOPHORA (COMB JELLIES)

Ctenophores or comb jellies are marine planktonic or benthic organisms of various shapes, from roundish to belt-shaped and with or without tentacles. Characteristic are eight rows with repeated plates made up of interconnected cilia see (**Figure 4.3A**).

Ctenophores feed on a diversity of planktonic organisms. They capture prey with special sticky structures, the colloblasts, by mucus or mechanically by holding them with lobes or engulfing them with their mouth (Haddock 2007, Purcell 1991, Tamm 2014). *Beroe*, which can swallow gelatinous prey twice its own size has macrocilia, which are amazing ciliary derivatives that can be used like teeth to hold prey or even bite it into pieces (Swanberg 1974).

Their digestive system is very complex and unique (**Figure 4.1**). The mouth opening is followed by an elongate ectodermal pharynx or

stomodaeum, which leads into an esophagus and into a central stomach, from which canals lead to the comb rows and (when present) the tentacles (Hernandez-Nicaise 1991, Tamm 2018). A further, aboral canal runs in the direction of the apical organ and branches into short canals, one or two of which can form temporary pores for defecation (Tamm 2016).

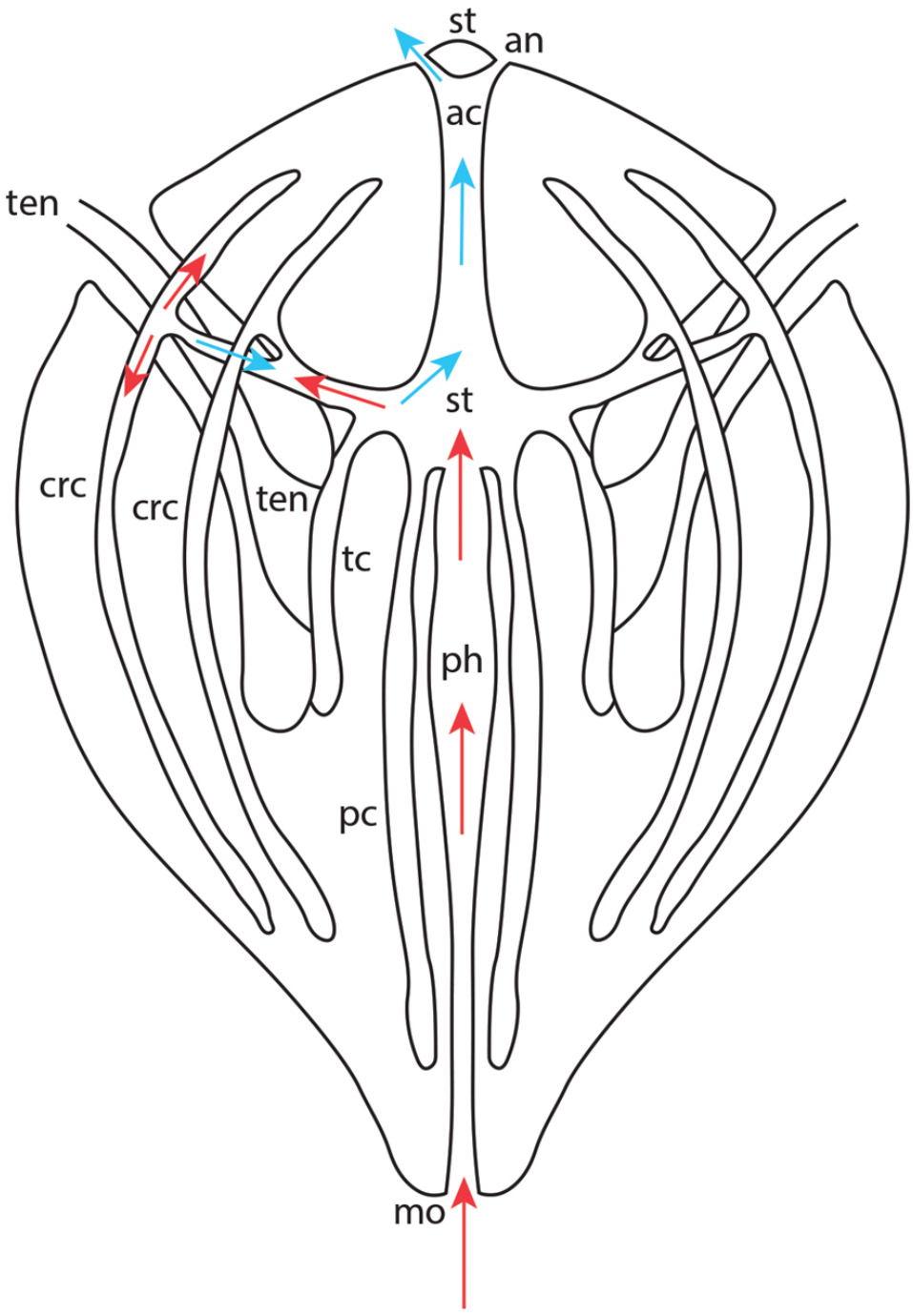


FIGURE 4.1 Schematic representation of the digestive system of ctenophores with arrows showing the food flow. Red arrows show food uptake through the mouth opening (mo), its passage through pharynx (ph) and stomach (st) into the pharnyngeal canal (pc), tentacle canal (tc) and comb row canals (crc). Blue arrows show the reverse flow from the periphery through the stomach and anal canal (ac) with exit through the anal pores (an). st = statocyst, ten = tentacle. Drawn after different sources. ⏎

The muscular and folded pharynx is expansive and breaks down prey by extracellular digestion. Its wall is composed of gland and digestive cells with microvilli and occasionally cilia (Hernandez-Nicaise 1991). It also sorts out larger indigestible particles, which are expelled through the mouth opening. Food is further broken down mechanically by the so-called gastric mill in the esophagus by the action of specially shaped cilia. The esophagus is separated from the stomach by valve-like structures. From the stomach, the partly digested food is distributed into the canal system. It has been shown that there is a strongly coordinated directional flow in the channels, which distributes nutrients in the body and supplies the energy-consuming structures such as the comb rows and the tentacles (Tamm 2018). The direction of flow can also change, bringing indigestible particles back to the stomach and from there into the aboral canal, where they are expelled through one or two pores (Tamm 2018, 2022; **Figure 4.1**). The pores open in a regular and repeated way. In a closed state they are not visible from the outside, and their opening mode remains not completely understood.

The phylogenetic position of ctenophores recently became subject of discussion, because several molecular analyses favor ctenophores as the first offshoot in the metazoan tree of live (e.g., Schultz et al. 2023, Whelan et al. 2017). Regardless of their phylogenetic position, the digestive system of ctenophores is unique and complex. It combines characteristics of sac-shaped digestive systems (such as the expulsion of indigestible particles

through the mouth) and of through-guts (the expulsion of indigestible particles through one (or more) anal pore(s), but the non-linear flow through the digestive system is peculiar for ctenophores. An interpretation as a "functionally tripartite through-gut" (Presnell et al. 2016) is probably going too far, especially, as several marker genes for regions of the through-gut are lacking in ctenophores (Steinmetz 2019).

4.3 CNIDARIA (THE STINGING ANIMALS)

This is a large taxon with approximately 14.000 described species in the subtaxa Anthozoa, Endocnidozoa, Hydrozoa, Staurozoa, Scyphozoa and Cubozoa. There are three life cycle stages: the microscopically small planula larva, the sessile polyp, and the pelagic medusa. Variations in this life cycle are possible, but most significant is that anthozoans lack the medusa stage. Endocnidozoa are strongly reduced parasites, and only one species, *Polypodium hydriforme*, has a simple digestive tract (Raikova 1994). All cnidarians are epithelially organized, which means that there are two epithelia, epidermis and gastrodermis, but no compact tissue between them. Both layers are separated by an extracellular matrix (called mesoglea in Cnidaria), which can become very voluminous in large medusae (**Figure 4.2**). The epithelially organized cells perform different functions; most significantly they have basal myofibrils and are therefore called myoepithelial cells. Nerve cells are a separate cell type and meander basally between epithelial cells of both epidermis and gastrodermis.

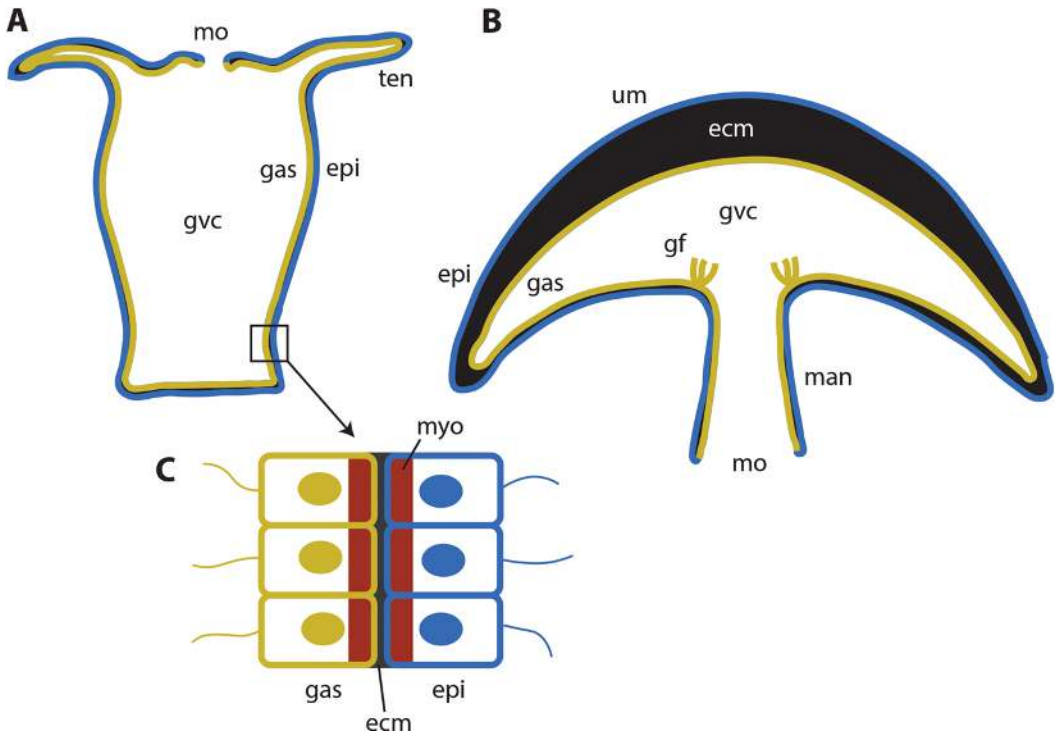


FIGURE 4.2 Schematics of cnidarian life stages and epithelia. (A) Schematic representation of a polyp. Two epithelia, epidermis (epi, in blue) and gastrodermis (gas, in yellow) surround the gastrovascular cavity (gvc), which has only one opening, the mouth (mo). In some cases, the gastrovascular cavity extends into the tentacles (ten). (B) Schematic representation of a medusa, the epithelia are labelled as in A. The extracellular matrix (ecm) is voluminous (in black). gf = gastric filaments, man = manubrium, um = umbrella. (C) Magnification of epithelia showing that they are separated by extracellular matrix (ecm, mesoglea). The epithelial cells have myofilaments in their basal part (myo). ⏎

The digestive tract, which is often called gastrovascular cavity, differs among the life cycle stages, but is always sac-shaped with only one opening. The majority of cnidarians feed on small planktonic organisms, which are caught by random contact with subsequent explosion of cnidocytes, paralysing the prey and fixing it to the cnidarian body. Contact with prey is supported by water currents from swimming movements and ciliary beating (Jordano et al. 2024). Food is primarily caught with the

tentacles and stuffed into the mouth opening. Food item size primarily depends on the size of the predator and the water currents created by it. Small medusae such as hydromedusae prey on smaller items such as protozoans (Colin et al. 2005), while larger medusae feed on larger planktonic prey (Costello & Colin 1994). Larger prey such as other medusae, shrimps, or small fishes is ingested by sea anemones or some box jellyfish such as *Chironex fleckeri* (Hamner et al. 1995). Polyps are often capable of ingesting prey that is large compared to their body size, and in some corals it has been observed that prey larger than individual polyps, for example medusae, are caught by several neighboring polyps (Gregorin et al. 2024). Energy can also be taken from symbiotic zooxanthellae, in particular in corals, but also in several other cnidarians.

Planulae of anthozoans are often larger than the ones of the remaining taxa; they have a mouth and a functional digestive tract and feed on other planktonic organisms (planktotrophy) (Martin & Koss 2002). Planulae of medusozoans (Hydrozoa, Cubozoa and Scyphozoa) are smaller and do not feed during early development. Instead, they use yolk for nutrition (lecithotrophy). Their digestive system is present, but the mouth is closed, and gastrodermal cells lie densely together and form no lumen (Martin & Koss 2002).

In polyps, the gastrovascular cavity differs in the subgroups. In general, it is a sac-shaped cavity following the mouth opening, which is surrounded by the tentacles. The gastrovascular cavity is either undivided (in Hydrozoa and Cubozoa) or is divided by a number of septae. The septae originate by infoldings of the gastrodermis. In scyphozoan polyps, four such septae are present, while in anthozoans, in which the polyp is the only life stage except for the planula, six, eight or more septae are present. In contrast to the polyps of most cnidarian taxa, anthozoan polyps have a much more

complex digestive system (Fautin & Mariscal 1991). Behind the mouth opening is an ectodermal tube-shaped region called pharynx or stomodeum. It is slit-like in cross-section and can have one or two ciliated grooves, the siphonoglyphs, running along the edges. They produce a water current into the gastrovascular cavity. This cavity is divided by gastrodermal septae into chambers (**Figure 4.3D**). In the upper region, where septae are connected to the stomodaeum, communication pores may exist between the chambers. The number of septae in anthozoan polyps is eight in octocorals and six or a multitude of six in hexacorals. In anthozoan septae, the muscular part of certain gastrodermal cells is strongly developed and forms longitudinal muscles, which can contract the entire polyp (**Figure 4.3D**). Toward the central lumen of the gastrovascular cavity the septae have gastric filaments (**Figure 4.3D**) which consist of cnidocytes, ciliary and glandular cells that create a water current and digest ingested prey. The gastrovascular cavity runs into the tentacles in anthozoans. In polyps of medusozoans, tentacles may be hollow, i.e., have a gastrovascular canal or are solid and then contain a rod of endodermal cells (Thomas & Edwards 1991; **Figure 4.3E** for a modified medusa). These cells have vacuoles and perform, in cooperation with the surrounding extracellular matrix, a skeletal function.

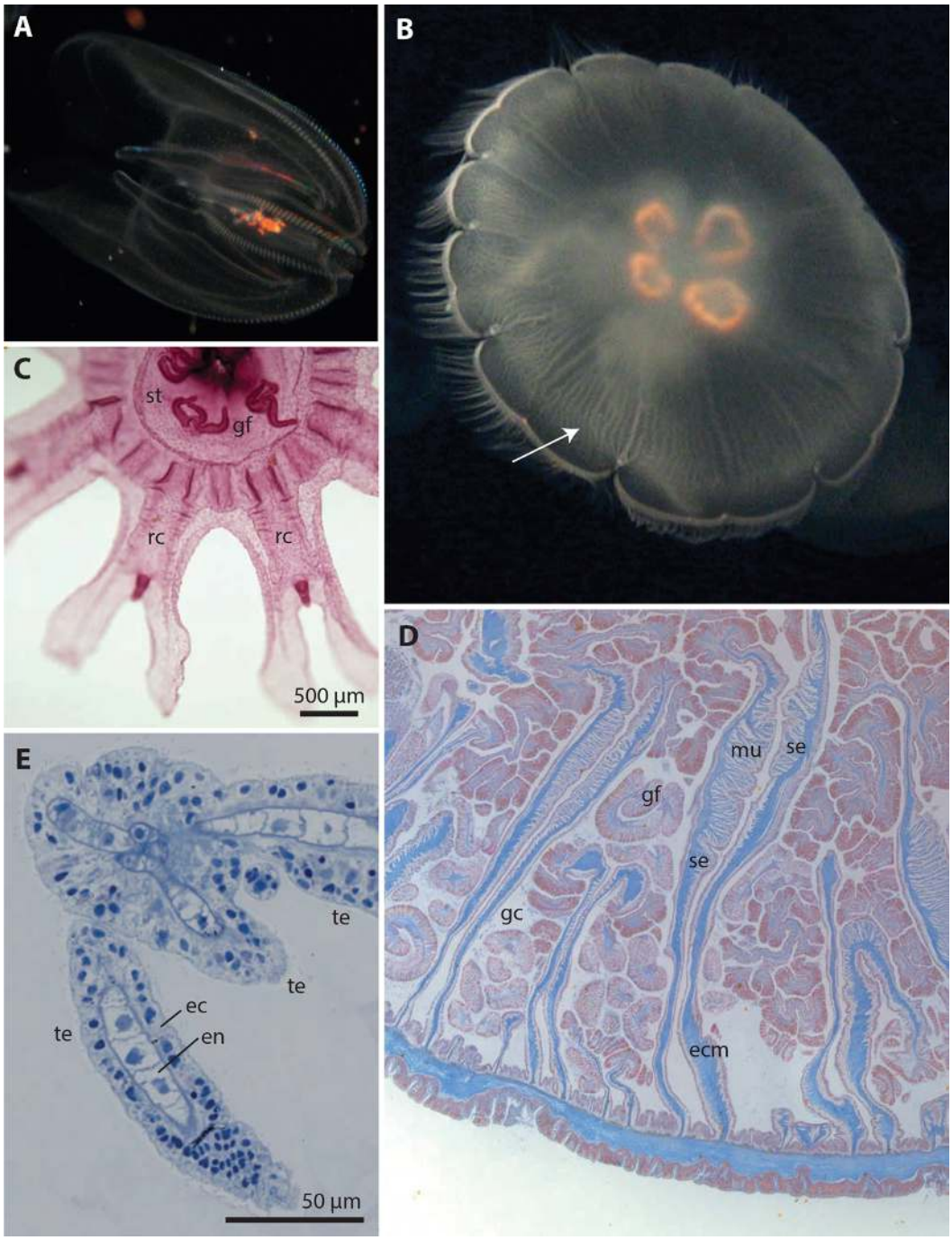


FIGURE 4.3 Ctenophores and Cnidaria. (A) The ctenophore *Mnemiopsis leidyi*. (B) A medusa of the scypohozoan *Aurelia aurita*. Note fine peripheral branching of gastric canals (arrow). (C) Ephyra (young medusa) of *A. aurita* showing branching of central stomach (st) into radial canals (rc) and presence of gastric filaments (gf) in the stomach. Red color is from staining. (D) Histological section with Azan staining through a polyp of the plumose sea anemone (*Metridium senile*) (Anthozoa)

showing septae (se) and their composition of central extracellular matrix (ecm; = mesoglea) and gastrodermis (red tissue lining the ecm), with local extension of the muscular part (mu). Gastric filaments (gf) originate on the inner side of the septae. As the polyp is strongly contracted, gastric filaments occupy much of the section, restricting the gastric cavity (gc) to small spaces. (E) Semi-thin section (staining with toluidine blue) through a modified medusa of *Halammohydra vermiformis* showing the origin of tentacles (te) from the central body. In the tentacles, ectodermal cells (ec) surround a central rod of vacuolated endodermal cells (en). ⏎

In medusae, the mouth opening is on the underside of the umbrella, at the tip of a tubular extension, the manubrium. The gastrovascular cavity extends in various forms throughout the umbrella and can also extend into the tentacles. Hydromedusae often have a system of four radial canals that extend from the central "stomach" and are connected by a peripheral ring canal (Thomas & Edwards 1991). Scyphomedusae have more complex systems with either strongly branching canals (**Figure 4.3B**) or a broadly extending stomach that is divided to various extent by dorsoventral septae into peripheral chambers (Lesh-Laurie & Suchy 1991). Gland cells secreting digestive enzymes occur in the gastrodermis and concentrated on so-called gastric filaments (**Figure 4.3C**). The gastrodermis consists of different types of secretory and digestive cells (Heeger & Möller 1987, Lesh-Laurie & Suchy 1991, Thomas & Edwards 1991). Ingested prey is broken and pre-digested extracellularly in the gastrovascular cavity, and final digestion of macromolecules takes place intracellularly.

In rhizostomes the manubrium does not have one central mouth opening, but many small pores or slits (e.g., Nagata et al. 2016). It has been shown that there is a directed water current through the rhizostome body including a separation of ingestion and egestion currents in the manubrium (Avian et al. 2022).

Cnidaria is the sister group of Bilateria in most analyses (e.g., Hejnol et al. 2009).

4.4 XENOTURBELLIDA

Seven species of *Xenoturbella* have been described so far; they occur endobenthic in muddy sediments or epibenthic in the deep sea (Rouse et al. 2016). They are more or less "simply" organized bilaterians. Their nervous system is a plexus without significant condensation into ganglia or a brain, their musculature is a grid of longitudinal and circular musculature (Raikova et al. 2000). Excretory organs, body cavity, or circulatory systems are lacking. The ventral mouth opening leads into a sac-shaped intestine that largely follows the oval shape of the animal and is not further structured. The mouth opening does not show further elaborations. The body wall musculature continues in the mouth region into longitudinal and circular musculature surrounding the intestine, but no significant strengthening of musculature is seen around the mouth (Raikova et al. 2000). The mouth can be opened and part of the intestinal cells can be pushed forward, so that they can receive contact to food through the mouth opening (Ehlers & Sopott-Ehlers 1997). *Xenoturbella* feeds on different prey, but mainly on bivalves (Bourlat et al. 2008). How exactly food is taken up has not been described. The intestinal cells have no cilia, but have microvilli for surface extension. Westblad (1949) describes the intestinal tissue to be syncytially organized, but no fine structural data on the intestine are present. Intestinal cells rest on a basal extracellular matrix.

Gonads are not present in *Xenoturbella*, but gametes originate and mature in the parenchyma surrounding the intestine. Oocytes have been observed among intestinal cells, and this has been interpreted as a migration of oocytes between intestinal cells into the intestinal cavity, from where they

are given off to the sea water through the mouth (Westblad 1949). This is contrasted by the observation that gametes are released by rupture of the epidermis (Nakano et al. 2023). A male genital pore was recently discovered by Andrikou et al. (2025), this becomes important in the discussion on the origin of the hindgut, see below under Acoelomorpha.

Xenoturbella has a very basal position within Bilateria, but there are several possibilities for the exact position. Probably they are the sister group of Acoelomorpha, together named Xenacoelomorpha, but a basal position within Deuterostomia, a sister group position to all other Bilateria or even a position within Acoelomorpha are discussed (see Cannon et al. 2016, Hejnol & Pang 2016, Redmond 2024, Rouse et al. 2016 for further reading and references).

4.5 ACOELOMORPHA

Acoelomorpha is a group of about 400 species of small marine worms. Originally being integrated into flatworms (Platyhelminthes), it was shown that they are an independent clade (e.g., Ruiz-Trillo et al. 1999) with relationship to Xenoturbellida (see above) and a basal position within Bilateria, probably as the sister group of all remaining Bilateria (= Nephrozoa).

Acoelomorpha consist of the sister taxa Acoela (about 400 species) and Nemertodermatida (18 species; Meyer-Wachsmuth & Jondelius 2016, Meyer-Wachsmuth et al. 2014). Species of Nemertodermatida possess an epithelialized, sac-shaped intestine and, if at all, a very weak pharynx. There are very few observations on feeding. Gut contents are diatoms, copepods or nematodes (see Gavilán et al. 2019), which are probably taken up with some kind of lip formation, but further details are unknown. The digestive tract is lined by strongly interdigitating cells of two kinds:

glandular and absorptive cells (Tyler & Rieger 1977), suggesting extracellular as well as intracellular digestion.

Within Acoela, phylogenetic analyses agree in the presence of two basally branching taxa, Diopisthoporidae and Paratomellidae and a large taxon Bursalia including all other acoels (Achatz et al. 2013, Jondelius et al. 2011). Species of Bursalia have a peculiar condition of the digestive system, which is modified into a central digestive syncytium and peripheral "wrapping" cells. Gavilán et al. (2019) describe different conditions and review functional interpretations. The central syncytium may dissolve during digestion (probably to set free digestive enzymes), but is permanent in most species. In some species it forms a central lumen, but usually is a spongy central mass. The peripheral, "wrapping" cells participate in phagocytosis and may regenerate the central syncytium.

The two basal branches are not very well investigated, but include species with a central digestive syncytium (*Diopisthoporus* cf. *longitubus*, see Gavilán et al. 2019) as well as *Paratomella rubra*, a species with cellular organization of the digestive system (Ehlers 1992). Here, more or less two layers of cells are present. All cells show signs of phagocytosis, but gland cells appear to be absent (Ehlers 1992). It is tempting to see an evolutionary trend from the epithelial gut in Nemertodermatida over the non-epithelial, but cellular gut as in *P. rubra* to the central syncytium as in most acoels (Ehlers 1992, Smith & Tyler 1985), but more recent phylogenies (Achatz et al. 2013, Jondelius et al. 2011) show that the picture is more complex and syncytial digestive systems may have evolved in more than one acoel lineage (Gavilán et al. 2019).

Recently, Andrikou et al. (2025) have shown that the transcription factors Caudal and Brachyury, which are expressed in the hindgut of most bilaterians, are expressed in the region of the male genital pore. They

therefore suggest that the hindgut may have evolved by a connection of the male genital system with the intestine.

4.6 PLATYHELMINTHES (THE FLATWORMS)

Platyhelminthes (flatworms) is a diverse taxon with either free-living or parasitic lifestyle. Most free-living forms are very small and occur in marine or freshwater sediments; specimens in the taxa Tricladida and Polycladida can grow in size to the centimeter-scale. Most triclads live in freshwater, but some are marine and an entire subtaxon, Geoplanidae, is terrestrial. Polyclads are entirely marine, they live in or on the sediment, and several are even capable of active swimming. Parasites are often larger in size, at least in the adult stage. Tapeworms (Cestoda) have lost their intestine completely and are taking up nutrients entirely through the integument.

Most flatworms are predators and feed on other animals (Jennings 1974). Some feed on plant material or are parasitic. Those parasitic taxa that have a digestive tract feed on blood, mucus or host tissue. Prey is consumed as whole and digested internally or is surrounded by the pharynx and exposed to digestive fluids right there. The pharynx may also be used to pierce and suck out prey (Rieger et al. 1991).

The digestive system of flatworms is sac-shaped, but is very diversely organized. In general, it is composed of the mouth opening, an ectodermal pharynx, and the endodermal intestine. Sometimes further structures are used in prey capture, which do not belong to the digestive tract. This is most evident in Kalyptorhynchia, which have a muscular proboscis that is used in prey capture (see Smith et al. 2015 for review and further references; **Figure 4.4A**). The proboscis can have various shapes, from a muscular bulb to paired muscular flexible lips. Hard elements derived from

extracellular matrix or intracellular deposits in epidermal cells are present in several species in the form of hooks. Glands with toxic content are assumed to be present at least in some kalyptorhynch species (Smith et al. 2015). Two further and quite derived cases of structures used in prey capture are *Prorhynchus* (Lecithoepitheliata), which uses the stylet from the male genital for prey capture (**Figure 4.4C**; Reisinger 1968, Steinböck 1927, Tyler et al. 2018) and *Antromacrostomum* (Macrostomida), where pockets with modified, needle-shaped rhabdites are present in the pharynx (Faubel 1974) and are assumed to be used in prey capture.

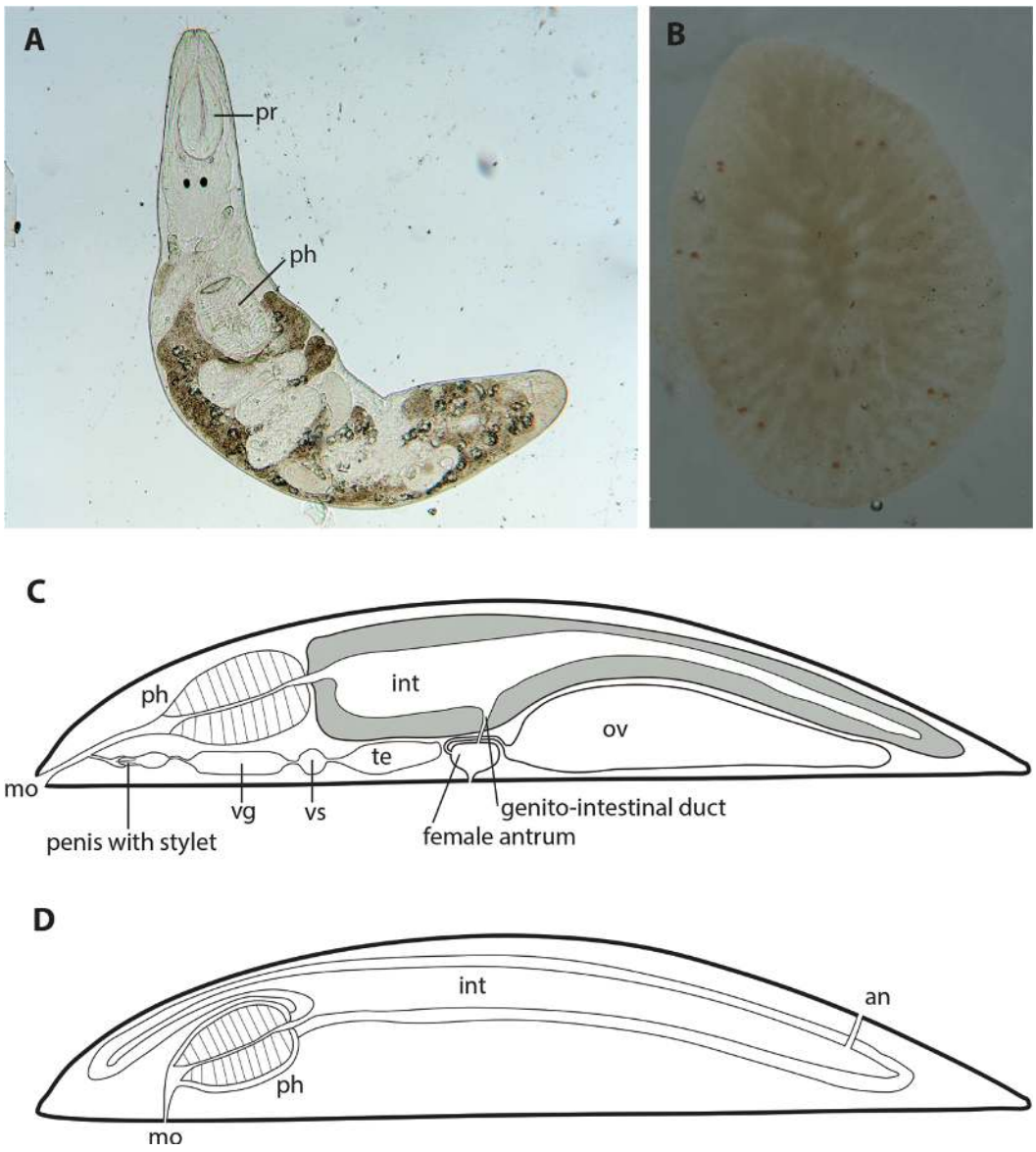


FIGURE 4.4 Platyhelminthes. (A) Unidentified specimen of Kalyptorhyncha showing the spatial division between proboscis (pr) and pharynx (ph). (B) Unidentified specimen of Polycladida showing how the intestine (darker color) forms multiple branches starting from a central gastric cavity. (C) Schematic representation of Prorhynchidae showing a connection between the reproductive system and the intestine (genito-intestinal duct) and the connection of the penis to the mouth region. int = intestine, mo = mouth, ph = pharynx, ov = ovary, te = testes, vg = vesicula granulorum, vs = vesicula seminalis. The drawing is based on figures of *Geocentrophora sphyrocephala* in Karling (1974; Figure 4) and *Prorhynchus stagnalis* in Bresslau (1928–1933; Figure 84). (D) Schematic

representation of the anal opening (an) in Polycladida. The drawing is motivated from a figure of *Leptoteredra maculate* in Bresslau (1928–1933; Figure 77). ⏎

The mouth opening can be in various positions on the ventral side, either in the anterior part of the body or in the central region of the animals. At the mouth opening the epidermis and underlying musculature more or less turn inward and continue into the pharynx tissue (Rieger et al. 1991). Often there is a ring of glands and sensory cells around the mouth opening and two sphincters are present to open and close the mouth.

The region behind the mouth opening is muscular and is called the pharynx. Several different types of pharynx are distinguished in flatworms, and they are diagnostic for certain subgroups. There are three main types and several subtypes of these (Ehlers 1985, Rieger et al. 1991). The pharynx simplex is a muscular tube behind the mouth, while the two other types of pharynges, the pharynx plicatus and the pharynx bulbosus, are within a pharyngeal cavity, which gives them more freedom of movement. Especially the pharynx plicatus can be very long and is protruded completely through the mouth opening as in triclads ("planarians"). In the pharynx bulbosus, the pharyngeal musculature is set off from the neighboring parenchymal tissue by a septum, which makes the action of pharyngeal musculature more autonomous, flexible, and effective. The pharyngeal epithelium consists of epithelial and gland cells. The musculature originally is a continuation of the subepithelial body wall musculature, but the layering of muscles (longitudinal versus circular musculature) can vary, and additional radial muscle fibers may be present (Rieger et al. 1991). In some triclad species, the number of pharynges and of mouth pores can be multiplied, e.g., in *Planaria montenigrina* (Mrázek 1904) or *Digonopyla harmeri*, where more than 100 mouth pores can be present in large animals (Fischer 1926).

The transition between the pharynx and the intestine is sometimes called esophagus. This is the region where ectodermal (epidermis in free-living and neodermis in parasitic taxa) and endodermal tissues meet, but the region usually is not very elaborated.

The intestine may be a simple sac or a blindly ending tube, but may also become strongly branched. The extent of branches roughly depends on the size of the animals. Especially in large marine polyclads and large parasitic digeneans such as the giant liver fluke *Fasciola hepatica* multiple branches are present and reach into all parts of the flattened body (**Figure 4.4B**). The intestinal epithelium (also called gastrodermis) contains absorptive and secretive cells. Additionally, a nerve plexus and musculature are present. Intestinal cells are ciliated in some groups and without cilia in others (Ehlers 1985). In general, food is broken down by a combination of extracellular and intracellular digestion (Jennings 1974). In some taxa, there are differences in the distribution of secretory and absorptive cells in the intestinal epithelium (Jennings 1974).

Anus-like structures were described from some species, but there is never an ectodermal hindgut and at least in some cases the anus may function only temporarily. In most cases, the anal openings are dorsal. Species with an anus (or several anuses) are scattered among flatworms. Examples are *Haplopharynx rostratus* (Macrostomorpha) (Karling 1965), *Tabaota curiosa* (Seriata) (Marcus 1950) and some cotylean polyclads. These may have a single anal pore as in *Leptoteredra tentaculata* (Kato 1943a; **Figure 4.4D**) or multiple pores as, e.g., in *Cycloporus* species (Hyman 1951, Kato 1943b). Among parasitic flatworms, one or two anal pores occur in some fish trematodes (Hyman 1951, Stunkard 1931). Anal pores occur preferably in long or large species, in which the retour of indigestible particles to the

mouth opening may become anatomically difficult. All the cases of anal structures appear to be derived within flatworms.

Interestingly, there are connections between the genital system and the intestine in some species (**Figure 4.4C**). This refers to the male as well as to the female system and is thought to lead excess sperm or yolk into the intestine, so that it can be digested. One organ of the female reproductive system, the bursa, has histological resemblance to the intestine and was hypothesized to be derived from the posterior part of the intestine. The bursa acts as storage organ for allosperm or digests sperm. The presence of genito-intestinal ducts and the nature of the bursa have sparked a debate about the evolution of the hindgut (e.g., Reisinger 1929, Remane 1951). However, it must be kept in mind that genito-intestinal ducts occur, just as anal structures, scattered in derived taxa and are lacking in basally branching taxa, which makes it less likely that they are ancestral features for flatworms.

The old view that Gnathostomulida (and later the entire taxon Gnathifera) is the sister group of Platyhelminthes within a taxon Plathelminthomorpha (Ax 1985), which conveniently explains the presence of a sac-shaped gut in both groups, is not supported by more recent analyses. Both Gnathifera and Platyhelminthes belong to the taxon Spiralia, but they are not directly related and Gnathifera consistently branch off earlier than Platyhelminthes (e.g., Hejnol et al. 2009, Bleidorn 2019, Laumer et al. 2015, Struck et al. 2014, Marlétaz et al. 2019). This nested position within Spiralia makes the interpretation of the nature of the digestive tract a bit complex. When flatworms primarily lack a hindgut, then the through-gut must have evolved several times within Spiralia. Alternatively, flatworms may have lost their hindgut, which is in

correspondence with the absence of the genes *caudal* and *brachyury*, at least in the tricladid *Schmidtea polychroa* (Martín-Durán & Romero 2011).

4.7 GNATHOSTOMULIDA

Gnathostomulids are known with about 100 species from marine sediments (Sterrer & Sørensen 2015). They are microscopically small, flatworm-like animals, but belong to a taxon called Gnathifera (see below). Their digestive system starts with a ventral mouth opening. The oral cavity leads into a pocket that contains the pharynx with hard elements (jaw) and the narrow esophagus that leads into the intestine. The intestine is tube-like and follows the elongate shape of the animal. In the posterior end, a temporary anus may be present. Gnathostomulids scratch their food from surfaces, such as sand grains, and feed mainly on algae.

The mouth opening is ovoid or slit-like, without further elaboration. Oral cavity, esophagus, and intestine lack cilia, but have microvilli. Centrioles were reported from intestinal cells in some species (Herlyn & Ehlers 1997, Lammert 1991, Sterrer & Sørensen 2015).

The pharynx is the most conspicuous and name-giving feature of gnathostomulids. It is a muscular organ with hard jaw elements. The pharynx can move and expose the hard elements through the mouth opening, so that food can be scratched from surfaces. The hard elements consist of an unpaired basal plate and paired elements that act like a forceps (Sørensen et al. 2003, Sterrer & Sørensen 2015). In some species, an additional unpaired dorsal element called jugum can be present. The paired jaw elements are fused in the posterior region. Basal plate and jaws are equipped with tooth-like structures. Only in the genus *Agnathiella* jaws are absent (Sterrer 1971). The hard elements are composed of cuticular material that is secreted by the pharyngeal cells. The jaws have a characteristic fine

structure that suggests that cuticular material is secreted around microvilli and creates concentric patterns in sections. This fine structure corresponds to the jaws (mastax) in rotifers and argues for their phylogenetic relationship (Rieger & Tyler 1995). The pharyngeal hard elements are moved by a complicated set of cross-striated musculature (Herlyn & Ehlers 1997, Sterrer & Sørensen 2015).

The intestine is a blindly ending tube, intestinal cells have microvilli, but lack cilia (Lammert 1991). In two species of the genus *Haplognathia*, which belongs to the comparably large and long Filospermoidea, it was observed that intestinal and epidermal cells interdigitate in the posterior region of the intestine and that no extracellular matrix separates these two tissues in this location (Knauss 1979). This structure is interpreted as a temporary anus. It is not known whether such a structure is present in further species of gnathostomulids.

The above-mentioned fine structure of the jaw and its homology with the jaws in rotifers and micrognathozoans as well as gene sequence comparisons have supported the monophyly of a taxon Gnathifera, to which also the jawless and endoparasitic Acanthocephala belong (e.g., Vasilikopoulos et al. 2024). As explained under Platyhelminthes, Gnathifera are a basal taxon within Spiralia. The presence of the genes *caudal* and *brachyury* and, if present, their expression patterns, are unknown in gnathostomulids.

4.8 MICROGNATHOZOA

Two species are present in the genus *Limnognathia*, which belongs to Micrognathozoa. The first species, *Limnognathia maerski*, was described in 2000 (Kristensen & Funch 2000) and since then found in disjunct locations around the world (De Smet 2002, Giribet et al. 2023, Sato et al. 2025).

Limnognathia is very small (80–150 µm) and probably feeds on bacteria and diatoms. The digestive tract very much resembles the one in gnathostomulids (see above). It has a flexible mouth opening, followed by a pharynx, esophagus and midgut. The jaw is composed of different hard elements (De Smet 2002, Kristensen & Funch 2000, Sørensen 2003) which are movable by specialized musculature (Bekkouche et al. 2014). The fine structure is comparable to that of the jaws in gnathostomulids and rotifers (Kristensen & Funch 2000, Sørensen 2003). The midgut narrows toward the posterior end of the animal. While the intestinal cells have microvilli (but no cilia), the posterior cells lack them, and there is no extracellular matrix between gut and epidermal cells. This structure is interpreted as a temporarily functional anus. In addition, it was observed that larger, unwanted food items are spit out of the mouth opening (Kristensen & Funch 2000).

The jaw and its fine structure show that *L. maerski* belongs to Gnathifera. It shares with Gnathifera the cellular nature of the epidermis and with Syndermata the presence of an intracellular (intrasyncytial in the case of Syndermata) dense layer.

4.9 CONCLUSIONS

This little review may allow some conclusions:

- Sac-shaped digestive systems occur in a diversity of taxa, but are primarily restricted to small organisms or those composed of two germ layers. Sac-shaped digestive systems also evolved secondarily in a number of cases, but mostly in single species or small groups of species and rarely in larger taxa (such as in articulate brachiopods).

- Sac-shaped digestive systems occur primarily in animals with a basal position in the tree of life. Platyhelminthes and Gnathifera (with Gnathostomulida and Micrognathozoa) may be an exception due to their position within Spiralia and indicate that the through-gut may have evolved several times within Spiralia.
- Sac-shaped digestive systems evolved into different shapes and can become quite complex. The flow of gut contents is not known in all taxa, but in some cases (e.g., ctenophores), a well-defined flow of gut contents is present.
- Sac-shaped digestive systems may perform the function of other organ systems, in particular of body cavities or circulatory systems, to distribute nutrients throughout the animal.
- A number of organisms with a sac-shaped digestive system developed secondary openings for defecation, either temporary or permanent. None of these anal structures has a hindgut made out of ectodermal tissue. Anal structures seem to be additional to defecation through the mouth. Their formation may be due to functional constraints in comparably large organisms. Their homology to the anus in animals with a through-gut is questionable.

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5 Evolution of Immunity

The Gut as an Interface with the Environment

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5.1 INTRODUCTION

The evolution of the through-gut represents a pivotal development in the history of metazoans, which significantly improved digestive efficiency and nutrient absorption (**Figure 5.1**). Although the mechanisms driving its development in urbilaterians, the last common ancestor of all bilaterians, remain enigmatic, its development has had a profound impact (Kiela & Ghishan, 2016; Yaguchi et al., 2024). This gastrointestinal tract (or gut) is more than merely an organ designated for digestion and nutrient absorption, which are clearly fundamental processes for animal growth, survival, and evolution. It is a very complex microenvironment that operates at the interface of the body's internal compartments and the outside world, integrating both biotic and abiotic signals from the surrounding environment (**Figure 5.2**). In recent decades, the diverse environmental cues and complex microbial communities that occupy or pass through the digestive tract of animals have been recognized as exerting a significant

influence on host physiological processes. These include organ development and function (e.g., digestive and respiratory tracts), metabolic regulation, immune maturation, and diverse neurobehaviors (Sommer & Backhed, 2013). These observations have inspired the recognition of animals as metaorganisms; as complex symbiotic associations occur between the host and the sum of its stable microbial communities, a holobiont emerges (Simon et al., 2019).

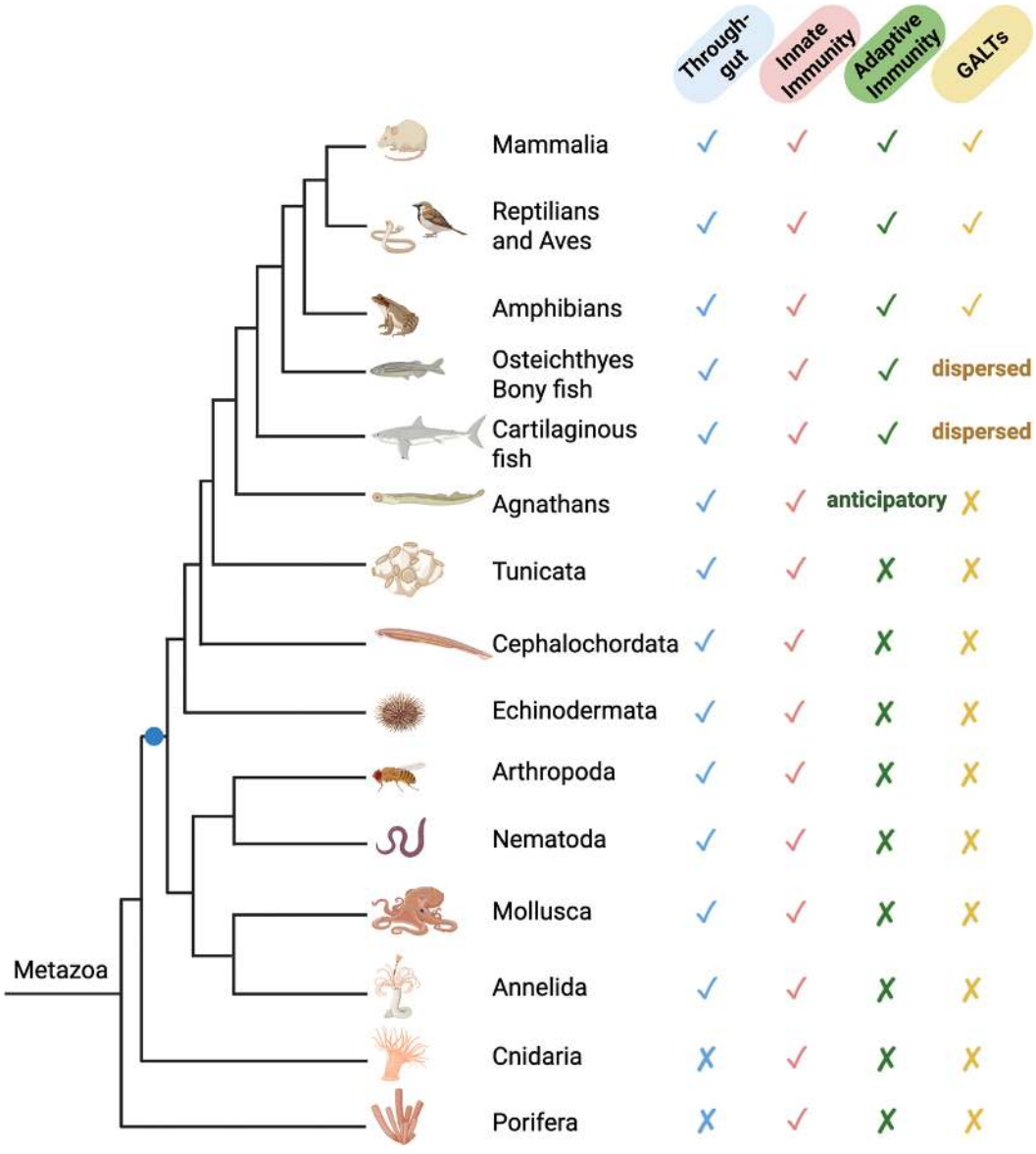


FIGURE 5.1 Evolutionary distribution of gut morphology and immune system features across metazoans. A phylogenetic tree of representative metazoan lineages is shown alongside the presence (✓), absence (✗), or variation of key traits, such as having a through-gut and diversification of immune functions (*i.e.*, innate immunity, adaptive immunity, and the presence of gut-associated lymphoid tissues (GALTs)). Major taxa are illustrated and labeled as commonly known, via class or phyletic designation. Emphasized is the appearance of the through-gut with the divergence of bilaterians, which includes a split between protostomes and deuterostome organisms (below and above the blue dot, respectively). Cnidarians possess a gastric cavity, while sponges lack a defined digestive organ. Innate immunity is foundational, and adaptive immunity evolved in vertebrates, at first with jawless vertebrates (Agnatha), though it is described as 'anticipatory' rather than classical and demonstrates more advanced structures in the lamina propria, like GALT. ⏎

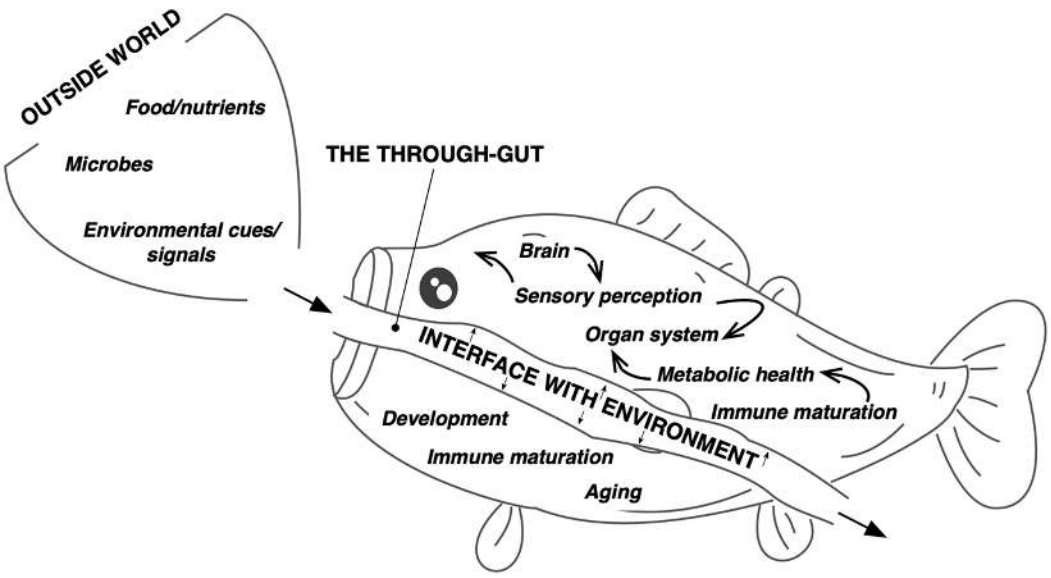


FIGURE 5.2 Gut as an interface with the external world. Artistic rendition of the through-gut, using a fish body plan for illustrative purposes. The through-gut originated in the earliest bilaterian animals and transformed the evolutionary history of animals by expanding their ability to explore, sense, respond to, and consume nutrients from new environments. With the external environment passing through the sterile, internal confines of their bodies, diverse exogenous signals are encountered that can mediate regionalized and systemic sensory responses, influencing the

development and maturation of even distant organ systems. These processes facilitated the co-evolution of animals with their environment (including microbes), promoting the development of the gut as a major sensory organ capable of establishing and maintaining complex, synergistic relationships with microbes. With an enteric nervous system of increasing complexity, the evolving gut becomes a primary interface and sensory site with the environment. ⏎

In the gut, microbes find a stable and mostly safe environment (away from the extremes of nature) with suitable nutrients. At the same time, the host gains advantages, including food fermentation and digestion, production of essential nutrients, protection against pathogens, and stimulation of immune maturation. The latter includes fine-tuning of immunological tone, resulting in an immunological state of readiness and responsiveness that requires careful regulation. Hence, within this ecosystem, host and microbe interact and collaborate to ensure proper digestive function, with broader implications for overall physiology and health. Additionally, given the significance of microbiomes to holobiont function, they often play crucial roles in evolutionary adaptation for many animals, particularly in response to environmental changes, by enhancing fitness. Indeed, microbiomes may be especially important in mediating acclimation and adaptation to environmental changes because microbes can more readily respond to environmental challenges. This can include alterations in microbiome composition, as well as through genetic and phenotypically plastic changes in individual microbial lineages. Animal hosts, on the other hand, may respond more slowly than their microbiomes to a changing environment due to their longer generation times and smaller population sizes (Petersen et al., 2023).

The gut is considered a holobiont ecosystem because it plays a vital role in establishing and maintaining homeostasis, highlighting how its functions are integrated. This dynamic process is crucial for the proper functioning of

organs and overall physiology. Important functional exchanges that shape systemic homeostasis rely on the host's ability to maintain physicochemical barriers, including the mucus layering of the epithelial surface, diverse immune defenses (humoral and cellular), and neural regulation via the enteric nervous system. Subsequently, the microbiota that occupy this space are comprised of a diverse array of prokaryotic and eukaryotic microorganisms, including bacteria, archaea, fungi, protozoans, and helminths (and their viruses), which interact in ways that include both synergistic and antagonistic behaviors in sharing and competing for resources. A significant challenge faced by host immune systems at mucosal interfaces is to distinguish between pathogens and the normal, beneficial intestinal microbiota. The host must remain vigilant to combat the former, while maintaining a more homeostatic relationship with the latter. In this chapter, we will describe and discuss components of mucosal barriers that protect the gut as a primary interface with the environment. We will also highlight the crucial role that host-microbiota exchanges serve in shaping host physiology and in the development and evolution of the animal immune systems. Indeed, a crucial aspect of the gut's functional development is the establishment of microbial communities and the subsequent maturation of regionalized immune tissues and systemic responses. This process enables the body to tolerate beneficial microbes while remaining vigilant toward potentially harmful ones, ultimately achieving the homeostasis that is a defining feature of health and well-being.

5.2 STRUCTURAL AND IMMUNE ELEMENTS OF THE HOST MUCOSAL BARRIER IN THE GASTROINTESTINAL TRACT

As highlighted in the introduction, in the digestive tract exists an intricate interplay between the intestinal microbiota, the intestinal epithelial layer, and the local mucosal immune system (**Figure 5.3**). While these interactions are primarily localized within the context of the gut and its complex biophysics, signals originating from this region can also influence systemic immunity and the general physiology of the host. To establish an infection in the digestive tract, pathogens must overcome these defensive layers. In this section, we will focus on the host-defensive components, specifically the epithelial barrier and the mucosal immune system, which are dedicated to interfacing with the outside world, outlining their main features and functions.

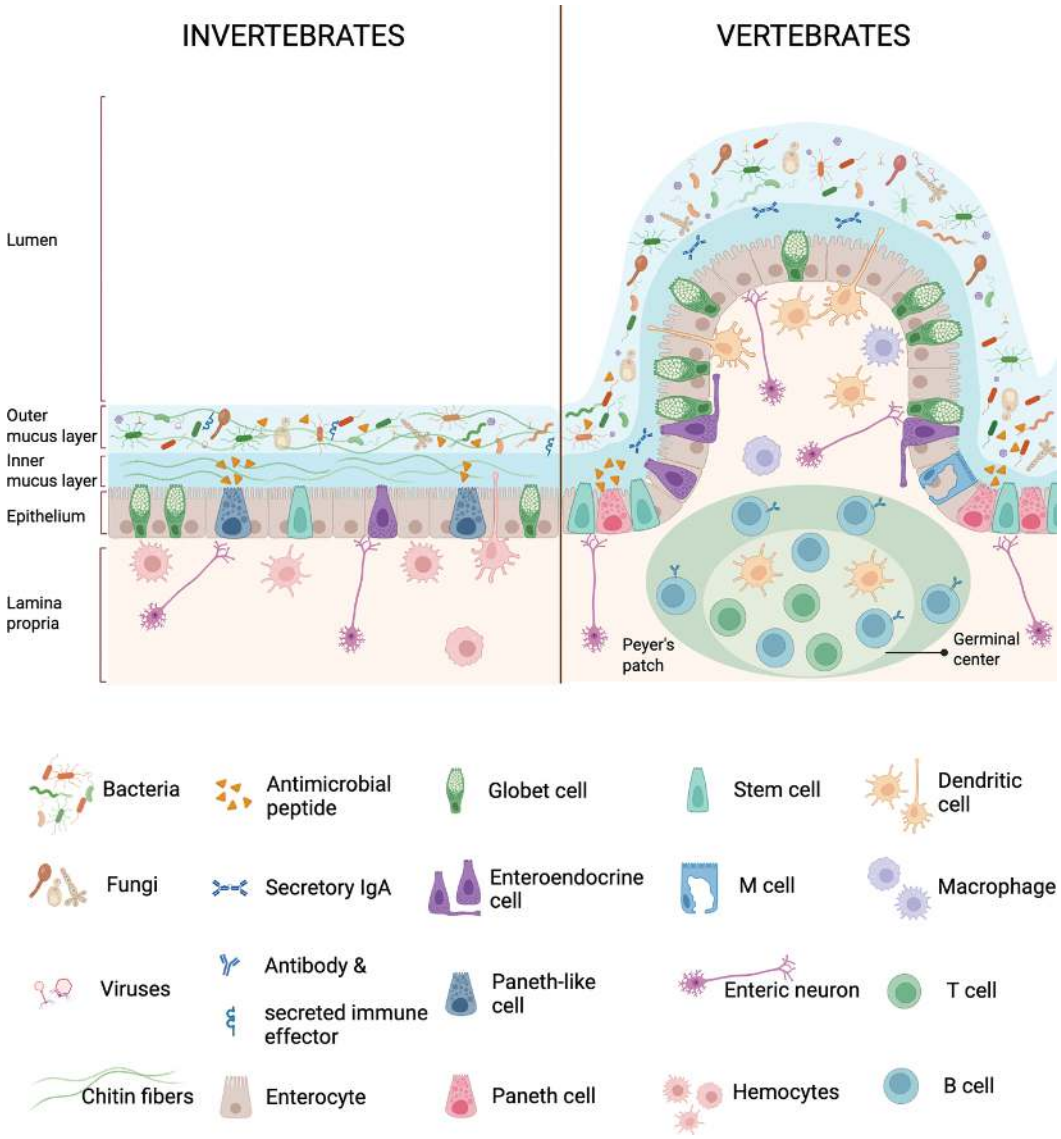


FIGURE 5.3 Simplified comparative illustration of gut epithelial barriers in invertebrates and vertebrates. The gut is a multifunctional interface integrating microbial, immune, and sensory signals to maintain homeostasis. Polarized epithelia and mucus-associated immune effectors restrict most commensal activity to outer mucus layers; in many invertebrates and aquatic animals, chitin further reinforces this barrier while providing a microbial substrate. Conserved epithelial cell functions support secretion, antimicrobial defense, neuroendocrine signaling, and tissue renewal. Vertebrate guts exhibit greater structural and cellular organization, including epithelial folding, a complex enteric nervous system, and diverse immunocytes organized into gut-associated lymphoid tissues

(GALT). Together, these features illustrate the evolutionary integration of barrier, microbial, immune, and sensory functions at the gut–environment interface. ⏎

5.2.1 Mucus and the Epithelium as a Physicochemical Barrier

In all metazoans that evolved a gastric cavity or a through-gut, the epithelial barrier plays a crucial role in protecting the body's internal environment from external threats, including potential pathogens. This physical barrier separates the commensal bacteria in the gut from the underlying tissues, such as immune cells localized in the lamina propria, via a single layer of different cell types that are continuously replaced by new cells (**Figure 5.3**). The epithelium of the intestines can include folding patterns, and when it does, the base of those folds is known as the crypt region. The crypts house stem cells that are essential to renewal and repair; invertebrates, however, have a more heterogeneous localization of these cells within the gastrointestinal tract (Liebing et al., 2025; Takashima et al., 2013). The epithelial cells are connected by tight junctions that firmly link the cells together and regulate the permeability and polarity of the epithelium (Liebing et al., 2025). The assembly of tight-junction complexes is a dynamic process that can be disrupted by certain bacteria or fungi through the release of toxins (Kwak et al., 2012; Libertucci & Young, 2019) or mycotoxins (Akbari et al., 2017; Gao et al., 2020), respectively. The epithelium includes diverse specialized cell types that can be categorized into secretory epithelial cells, such as Paneth cells (or cells with a Paneth-like function in invertebrates), goblet cells, enteroendocrine cells, and absorptive cells, such as enterocytes (**Figure 5.3**) (Liebing et al., 2025). In the context of gut immunological homeostasis, the goblet cell and Paneth cell are particularly relevant. Indeed, goblet cells produce mucus that coats

and reinforces the physical barrier of the epithelial cell layer, whereas Paneth cells have a role in innate immune defense among both vertebrates and invertebrates. The mucus that coats the epithelium is a viscous, aqueous fluid containing gel-forming mucin (MUC) glycoproteins (as both membrane-associated and secreted molecules). The gel can also be rich in lipids, DNA, inorganic salts, and diverse effectors, including antimicrobial peptides (AMPs) like defensins, produced by Paneth cells, as well as immunoglobulins (Ig) like IgA (transported by another cell type, the microfold cell or M cell) and other secreted immune factors that serve to enhance the physical barriers established by the epithelium (Dishaw, Litman, & Liberti, 2024; Okumura & Takeda, 2017). The composition and physicochemical properties of mucus vary depending on its anatomical location. In the large intestine of vertebrates, the intestinal mucus is layered into two zones: an inner, firm layer and an outer, loose one, both composed primarily of the MUC2 glycoproteins, which are heavily glycosylated via N-acetylgalactosamine (GalNAc) O-linkages (**Figure 5.3**). The inner layer, firmly anchored to the intestinal epithelium, is dense and free of bacteria, acting as a barrier. The inner mucus layer is converted into the loosely associated outer layer via breakdown of MUC2 by glycosylases secreted by the host during digestion or by microbes that feed off its polysaccharide chains. Microbial activity in the outer mucus layer correlates with their occupation of diverse niches throughout the gut lumen, where they often form stable communities and adherent biofilms. The stability of the mucus barriers is often impacted even by diet; for example, when dietary fiber is scarce, mucin-degrading bacteria increase in number, leading to degradation of the inner mucus layers and increasing the risk of microbial contact with the epithelium. Phylogenetic analyses have revealed that secreted or gel-forming mucins were present in the earliest metazoans and are well

developed in ctenophores and cnidarians. In contrast, membrane-anchored mucins diversified later in the chordates (Lang et al., 2007, 2016).

Some mucins possess glycosylation patterns that include polymerized N-acetylglucosamine (GlcNAc), resulting in the formation of interwoven chitin fibers (**Figure 5.3**). In arthropods, the peritrophic matrix (PM) in the midgut acts as a glycocalyx barrier composed of chitin fibers bound to peritrophins, specialized glycoproteins with chitin-binding domains (CBDs) (Lehane, 1997; Syed et al., 2008), and sometimes MUC domains (Dias et al., 2018). Peritrophins may have evolved from MUC genes by acquiring CBDs. The PM complements mucus gels in providing protection from damage, pathogens, and toxins, while also aiding digestion by localizing enzymes. Although chitin-rich mucus fibers were first identified in arthropods, similar versions have also been found in invertebrate chordates such as *Ciona robusta,* and in other aquatic vertebrates, including zebrafish, salmon, and axolotls (Dishaw et al., 2016; Nakashima et al., 2018; Tang et al., 2015). This indicates that chitin-rich mucus is more widespread than previously thought. These specialized mucus gels reduce mucin solubility in water and can enhance the barrier against infection. In nature, crystallized chitin provides structural support while non-crystallized forms can be found interwoven with and stabilizing mucin gels, particularly in aquatic environments; their presence in insect midguts is likely a remnant of their aquatic ancestry (Dishaw et al., 2024).

In summary, the mucus lining of the epithelial barrier serves as a primary defense against microbial invasion by preventing direct contact with epithelial cells, thus limiting tissue inflammation and microbial translocation. It also functions as a reservoir for antimicrobial effector molecules, such as secretory IgA and defensins, which also contribute to host protection. Mucus production and degradation are regulated by

complex host-microbe interactions, influenced by microbe-associated molecular patterns (MAMPs) and bacterial metabolites, and are sensitive to microbiota composition and microbial behaviors. There is a reciprocal relationship between mucus and microbes on mucosal tissue surfaces, where changes in microbiota or inflammatory profiles can alter mucus structure, potentially increasing susceptibility to infection.

5.2.2 The Immune System as a Master Regulator of Microbial Interactions

The mucosal immune system comprises innate immune components, which are conserved across all invertebrate and vertebrate taxa, as well as adaptive immune elements that evolved specifically in vertebrates. To briefly recall the main differences between these two branches of the immune repertoire, the innate immune system serves as a first line of protection, responds immediately and non-specifically to pathogens, and includes physical barriers (such as epithelia of various anatomical compartments), immune cells (e.g., macrophages and neutrophils), and inflammatory responses. In contrast, the adaptive immune system most often provides follow-up protection, is activated to supplement innate immunity, or responds when the innate system fails to restore homeostasis; however, innate immunity is essential in its ability to instruct the adaptive immune system about the nature of the pathogenic challenge. Adaptive immunity responds specifically to pathogens and possesses immunological memory, enabling it to recognize and mount more effective responses upon re-exposure to the same pathogen. Key players in this system include B cells, which are responsible for antibody production, and T cells, which either kill infected cells or assist other immune cells (Flajnik & Kasahara, 2010; Litman et al., 2005, 2010; Vivier & Malissen, 2005). Traditionally, the innate immune

system has been defined as a system lacking memory; however, recent findings have highlighted a phenomenon known as trained immunity, indicating a form of functional memory in the innate response, acting mainly via epigenetic mechanisms (Netea et al., 2020). This finding suggests that a limited amount of immunological memory may also exist among non-vertebrates.

Notably, it has been hypothesized that the adaptive immune system may have evolved, in part, not just to confer protection against invading pathogens, but also, owing to its specificity and regulatory pathways, to recognize and help manage the complex communities of beneficial microbes living on or within vertebrates (McFall-Ngai, 2007). Invertebrates, relying solely on their innate immune systems, are similarly exposed to microbial challenges and are nonetheless capable of maintaining physiological homeostasis. However, initial high-throughput sequencing and microbial community profiling revealed that a defining feature of vertebrates is their association with diverse, co-evolved bacterial communities. These microbial consortia contribute to host fitness by expanding metabolic capabilities through an extensive repertoire of microbial gene products. In contrast, invertebrates are generally believed to host less diverse, more limited microbial communities (McFall-Ngai, 2007). Currently, this topic is more widely debated due to the increasing number of studies on the microbial communities of invertebrates, which have highlighted a greater degree of complexity than previously thought. Hypothetically, the adaptive immune system may play a key role in managing the extensive microbial compartmentalization observed in larger digestive tracts, such as those of vertebrates, where microbes have more niches to occupy, potentially increasing the impact of microbial diversity.

An important feature of the immune system, particularly in the context of mucosal environments like the gastrointestinal tract, is immune tolerance. Here, the immune system avoids harmful responses to non-pathogenic stimuli, like self-antigens, commensal microbes, and environmental antigens (e.g., dietary or food antigens), thus preventing unnecessary inflammation and maintaining tissue integrity and homeostasis with beneficial microbes. The polarized nature of antigen receptor recognition responses in the intestinal epithelium, see Section 2.3, serves essential roles in mediating and maintaining tolerance. Maintaining tolerance is essential to preventing chronic inflammation, allergies, and autoimmunity, and it involves both the innate and adaptive immune components that act synergistically while remaining poised for pathogenic defense (Round & Mazmanian, 2009). In such a case, as well as after an injury, the immune system triggers an inflammatory response as a defense mechanism designed to eliminate harmful stimuli and initiate tissue repair. Inflammation is characterized by the release of pro-inflammatory cytokines (e.g., TNF-α, IL-1β, IL-6), activation of the complement system, recruitment of innate immune cells (like neutrophils and macrophages) via chemokines, and activation of innate signaling pathways that can amplify responses (e.g., NF-κB, MAPKs) (Belkaid & Hand, 2014). While acute inflammation is protective and resolves, chronic inflammation, particularly in the gut, can lead to tissue damage and diseases such as inflammatory bowel disease. To prevent overreacting to harmless antigens while still being able to manage real infections, a careful balance between inflammation and tolerance is necessary (Hooper et al., 2012; Round & Mazmanian, 2009). By presenting antigens in a non-inflammatory context, specialized dendritic cells (DCs) and intestinal epithelial cells in the gut promote tolerance by influencing immune responses that lead to inactivity or non-responsiveness. As a result,

the host can withstand dietary molecules and trillions of commensal microbes without experiencing inflammation and the collateral damage that can result (Belkaid & Hand, 2014; Mowat & Agace, 2014).

5.2.3 Immune System of the Gastrointestinal Tract: Components and Structural Organization

The delicate equilibrium between immune tolerance and inflammation involves different cell types, their molecular components (such as specific receptors), and the corresponding signaling pathways. In animals, the through-gut is, by volume, the largest surface area for antigenic exposure and interactions with symbiotic microbes. It is often assumed, then, that the bulk of the immune system is housed in this organ; however, at least in humans it is now recognized that the bulk of immune cells are found in bone marrow, lymph nodes and spleen (Sender et al., 2023), while about 3% of the total immune cells of the body and 5% of the lymphocytes are distributed in the gut. Conversely, because of the extensive amount of antigenic exposure occurring at these surfaces, about 70% of all antibody-producing B-cells or plasma cells are indeed found in mucosal tissues, with the gut housing the bulk of them (Belkaid & Hand, 2014; Campos Canesso et al., 2025; Pabst et al., 2008). Thus, mucosal surfaces are a major site of immune surveillance, responses, and tolerance, and a key site of antibody production.

In invertebrates, the innate immune system encompasses cell types broadly labeled as hemocytes or coelomocytes, performing surveillance functions by freely moving from hemolymph (or blood) into gut tissues, mediating phagocytosis, encapsulation, and immune signaling. These functions can be carried out by diverse subpopulations of hemocytes or coelomocytes, although their classification is not always straightforward

(Franchi & Ballarin, 2017; Xin & Zhang, 2023). On the other hand, in vertebrates, diverse well-characterized innate immune cell types include (i) phagocytic cells, including monocytes, DCs and macrophages, that have a role in engulfing and destroying pathogens, removing debris, and presenting antigens, thus activating the downstream effect of the adaptive immune system; (ii) granulocytes, characterized by cytoplasmic granules containing antimicrobial compounds with cytotoxic activity (Shao et al., 2023), and (iii) innate lymphoid cells (ILCs), that include classic cytotoxic natural killer cells, lymphoid tissue inducer and non-cytotoxic populations. These ILCs have important effector functions during the early stages of immune responses against microorganisms and also contribute to tissue repair, the anatomical containment of commensal microorganisms, and the maintenance of epithelial integrity at barrier surfaces (Sonnenberg & Artis, 2012; Spits et al., 2013). Interestingly, ILCs represent a primitive form of lymphocyte-like immunity that precedes the adaptive immune system and is found in early jawless vertebrates (Litman et al., 2010; Yoder & Litman, 2011).

Innate immune cells detect microbes via a limited number of germline-encoded receptors, known as pattern recognition receptors (PRRs). These identify and bind MAMPs, which are specific and highly conserved molecular structures shared by diverse microorganisms that can be both commensals or pathogens (e.g., diverse bacteria, fungi, or viruses). These include lipids, such as lipopolysaccharides (LPS), diverse cell-surface proteins, lipoteichoic acid, a lipid-anchored phosphate-rich glycan polymer, or nucleic acids, such as bacterial DNA. MAMPs are often essential for microbial survival and can include unique molecular or subcellular characteristics that are not found on or within host cells. The recognition of these molecular signatures enables the immune system to distinguish them

from self-antigens, thereby initiating innate immune responses and inflammation when necessary, and facilitating the activation of the adaptive immune response (Brubaker et al., 2015; Janeway, 1989; Janeway & Medzhitov, 2002; Li & Wu, 2021). The recognition of pathogens by PRRs is based not only on binding to MAMPs but also on other features, such as microbial (often virulent) behaviors and the secretion of toxins and other virulence factors.

PRRs can be divided into the following five types based on protein domain homology: Toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), and absent in melanoma-2 (AIM2)-like receptors (ALRs). These receptors can be grouped into membrane-bound receptors and soluble, unbound intracellular receptors. The former group consists of the TLRs and CLRs, which are found at the cell surface or on endocytic compartment membranes where they participate in surveillance of microbial ligands in the extracellular space and within endosomes. The NLRs, RLRs, and ALRs form the latter group and remain in the cytoplasm, where they survey the presence of intracellular pathogens. This distinct compartmentalization of receptors is crucial for preventing inappropriate or unnecessary immune responses to self-molecules (Brubaker et al., 2015).

Both membrane-bound and cytoplasmic PRRs are composed of ligand recognition domains, intermediate domains, and effector domains. Once recognizing their ligand, PRRs activate downstream signaling pathways, that can result in diverse effector functions, such as recruiting or releasing of AMPs, inflammatory cytokines (that can amplify or regulate responses), chemokines (that help recruit other immunocytes), hormones and growth factors that can result in opsonization of microbial cells, activation of

complement and coagulation cascades, phagocytosis (to neutralize and eliminate certain threats), and induction of apoptosis (inducing cell death of infected cells). These processes contribute to the activation of an acquired immune response via the induction of costimulatory molecules on lymphocytes, such as T and B-cells (see below), and by increasing the capacity of DCs to present antigen on their cell surface. These processes mediate an inflammatory microenvironment, with a primary focus on restoring homeostasis.

PRRs are also often broadly expressed on not just innate immune cells, such as hemocytes, DCs, and macrophages, but also on some cell types often considered non-immune, like intestinal epithelial cells (Li & Wu, 2021). Here, receptors like TLRs, NLRs, and CLRs recognize intestinal microbiota and their products. Importantly, PRRs, such as TLRs, are primarily expressed in a polarized manner on epithelial cells of the intestines, and repeated engagement of TLR ligands helps mediate tolerance to commensal microbes. In contrast, engagement of these receptors on the basolateral side of the gut epithelium drives strong responses (as this indicates a pathogenic breach) (Abreu, 2010; Marques & Boneca, 2011; Peterson & Artis, 2014). Their role in the gut, for example, is much better understood among vertebrate model systems, e.g., mammals, where their function and signaling pathways have been extensively elucidated, in particular during specific pathologies (Brubaker et al., 2015; Li & Wu, 2021). Comparative studies among invertebrate models, such as the cnidarian *Hydra spp.*, insects like *Drosophila melanogaster*, mollusks like the cephalopod *Euprymnia scolopes*, or urochordates like *C. robusta* have exemplified the crucial (and conserved) roles of PRRs in gut immunity. These immune-phylogenetic studies highlight the involvement of PRRs not only in defending the host against pathogenic microbes but, more

interestingly, in facilitating a wide range of interactions with the diverse microbial world, which includes promoting long-term colonization of microbiota by driving immune tolerance to commensal microbes (Chu & Mazmanian, 2013; Sasaki et al., 2009). Thus, tolerating symbiotic microbes has likely been one of the most vital aspects of immune evolution, alongside the preservation of 'self.'

Indeed, it is widely recognized that an ancient function of innate immune signaling is to achieve and maintain homeostasis by balancing the recognition of commensal bacteria and other members of the microbiota, maintaining barrier defenses, and promoting immune tolerance (Chu & Mazmanian, 2013; Dishaw et al., 2014; Sasaki, Ogasawara et al., 2009). Moreover, it is important to emphasize that the 'arms race' motivates both the host and pathobionts (microbes with pathogenic tendencies) to co-evolve adaptive strategies that increase their respective fitness. In insects, it has been demonstrated that the repertoire of PRR effectors can expand in response to the wide variety and constantly evolving range of pathogens. This flexibility raises the possibility that insect immune systems are more complex than previously thought (Wang et al., 2019). Insects may not be the only invertebrates to exhibit this kind of immune plasticity; other invertebrates and even vertebrates may have adopted it as a more general evolutionary strategy to deal with pathogenic variability across taxa since many PRR or immune effectors evolve as multi-gene families (Dishaw et al., 2012; Hibino et al., 2006; Nei & Rooney, 2005; Peng et al., 2024; Vinkler et al., 2023; Zhang et al., 2008, 2015).

By serving crucial roles in modulating microbial settlement and shaping the dynamics of symbiotic interactions in mucosal environments, AMPs are essential innate immune effectors of the gut. AMPs are usually small peptides of about 10–60 amino acid residues and a net positive charge,

making these diverse and bioactive macromolecules attracted to the generally negatively charged membranes of microbes such as bacteria, fungi, parasites, and viruses. Since the discovery of the first AMP, many others have been identified and isolated from diverse organisms across all kingdoms. The last update (January 2025) of the Antimicrobial Peptide Database reports 5099 peptides, including either natural (3306), synthetic (1299), and predicted (231) AMPs (https://aps.unmc.edu/home). Studies of these molecules have significantly increased in recent years, due to the emergence of antibiotic-resistant microorganisms and the fact that AMPs represent a promising and more specific alternative to antibiotic drugs (Huan –, 2020). Although AMPs have been classically assumed to have broad-spectrum activity and simple kinetics, recently, evidence suggests an unexpected degree of specificity and a high synergistic capacity among some of these molecules. These properties make AMPs attractive for translational applications. However, the clinical use of these molecules requires an understanding of their natural biology to minimize the risk of collateral damage and avoid the crisis of resistance now facing conventional antibiotics (Lazzaro –, 2020).

Classifying AMPs is challenging due to their diversity, but they can be grouped by their source or producing organism, structural features, amino acid-rich types, and their activity or mechanism of action (Huan et al., 2020). Examples of AMPs most thoroughly investigated and found in a broad range of organisms are the α and β- defensins, lysozymes, galectins, and regenerating proteins, which are produced by both immune and non-immune cell types, such as macrophages or intestinal epithelial cells, and the specialized secretory epithelial cells, such as the aforementioned Paneth cells, respectively (Gubatan et al., 2021). AMPs serve important roles in maintaining gut homeostasis and immune tolerance through different

mechanisms: (i) AMPs help regulate the gut microbiome by influencing the composition and distribution of gut microbiota, preventing harmful bacteria overgrowth and facilitating the growth of beneficial bacteria; (ii) AMPs protect the epithelial barrier by enhancing the physical barrier via increased production of tight junction proteins and mucins, reducing mucosal damage and permeability (Marin et al., 2019); (iii) AMPs directly fight pathogens via broad-spectrum antimicrobial activity, disrupting membranes, binding microbial components like LPS, and sequestering essential metals (like iron, zinc, and manganese) to inhibit microbial growth; (iv) AMPs influence the immune response by interacting with immune cells to reduce inflammation, recruit additional immune cells (like neutrophils and T cells), and promote tolerance to beneficial bacteria, and (v) AMPs respond to inflammation via increased synthesis, which helps to restore homeostasis and protect against further damage. Overall, AMPs are crucial for managing gut ecology, preventing infections, and helping the immune system tolerate commensal microbiota (Gubatan et al., 2021).

Among vertebrates, gut homeostasis and immune tolerance are further regulated by the involvement of the adaptive immune system, which emerged in jawless vertebrates, such as agnathans (lamprey and hagfish) (**Figure 5.1**). The jawless (or earliest) vertebrates have an adaptive system that does not encompass the prototypical immunoglobulin (Ig) domain-based effector molecules, such as the receptors of T-cells (TCR), B-cells (BCR), or secreted antibodies, but instead involves secreted effectors known as the variable lymphocyte receptors (VLRs) (Kasahara & Sutoh, 2014). Rather than possessing Igs, the VLRs possess recognition domains composed of leucine-rich repeating segments; remarkably, some of these segments have also been noted to use somatic recombination to generate variability, as originally observed in the Ig gene regions of TCR and BCR

(Kasahara & Sutoh, 2014). The VLRs (encompassing VLR-A/B/C) represent an adaptive or anticipatory-type immunity that facilitates developmental and morphological plasticity, in addition to the advantages conferred by increased polymorphism, which includes the recognition of a larger portion of the antigenic world (Guo et al., 2009). Moreover, in lamprey, differential responses of VLR gene products have been observed following exposure to gut-derived *Shewanella* strains, thus indicating a role of these intestinal bacteria in the adaptive immune responses of lamprey Li et al., 2021). Surprisingly, VLR specificity is sufficient for even the development of specific biotechnology tools, similar to traditional Ig antibodies from mammals (Bela-Ong et al., 2024).

The adaptive immune system of jawed vertebrates, based on TCR and BCR genes, emerged during the evolution of cartilaginous fishes. With the emergence of tetrapods, distinctive types of antibodies evolved that can be secreted into mucosal environments for immune protection, such as IgT of teleost fishes, IgX of amphibians, and IgA of birds and mammals. Many studies have focused on investigating the role of mammalian IgA in the gut environment, acting not just to protect against pathogens and toxins, but in shaping gut microbiota composition and modulating bacterial behaviors (Okai et al., 2017; Pabst & Slack, 2020). The IgT mucosal antibody molecules, which are also expressed in the gut and coat most intestinal bacteria in bony fish (or teleosts), are distantly related to IgA and have some functional similarities with IgM (Piazzon et al., 2016; Salinas, 2015). In fact, it has been discovered that depletion of IgT causes microbiome dysbiosis characterized by a marked overgrowth of pathobionts (Xu et al., 2020). IgX, which is expressed in the gut and is believed to be crucial for mucosal immunity, is a probable ortholog of IgA in amphibians (Mussmann, Du Pasquier, & Hsu, 1996). Removal of the thymus early in

development revealed a T-cell independent immune response when exposed to intestinal microbiota; expression of IgX is independent of T-cells and still serves significant roles influencing gut microbial colonization (Mashoof et al., 2013). Interestingly, although no direct evidence exists for their role in the digestive tracts of birds or reptiles, IgY is a principal systemic antibody (like IgG in mammals) and is primarily transferred from the circulatory system to the egg yolk, where it is stored and functions to protect the developing embryo (Li et al., 2016). In a mouse model of *Salmonella typhimurium* infection, IgY specific for *S. typhimurium* plays a protective and immunomodulatory role, alleviating intestinal inflammation and damage while maintaining a balanced immune response during bacterial infection, thus acting as a passive immune agent in the digestive tract (Li et al., 2016). In conclusion, from an evolutionary point of view, diverse classes of antibodies have an important role in regulating symbiosis with microorganisms, but debating homology of these molecules is unnecessary given that common selective pressures have driven diverse taxa to innovate and repurpose various extracellular secreted immune molecules possessing Ig domains for the management of the complex antigenic nature of the gut (Liberti et al., 2022).

While emerging prior to the jawless vertebrates, invertebrate chordates are an interesting example of how adaptive evolutionary selective pressures have favored the independent evolution of a secreted Ig-based effector system, the Variable Ig domain-containing Chitin Binding Protein (VCBP) found in lancelets and tunicates. These effector proteins are secreted into the gut directly from specialized intestinal epithelial cells, where they can simultaneously bind bacteria (via the N-terminus Ig domain) and fungi (via the C-terminus chitin binding domain, CBD), influencing microbial behavior, like e.g., bacterial settlement and biofilm formation (Dishaw et

al., 2011, 2016; Liberti et al., 2019), whereas the influence of VCBP binding to fungi is still under investigation. Besides protochordates, other invertebrates possess secreted immune effectors containing Ig-like domains, like the Fibrinogen-Related Proteins (FREPs) of the gastropod *Biomphalaria glabrata* (Adema, 2015) and the Down Syndrome Cellular Adhesion Molecules (DSCAMs) of the arthropod *D. melanogaster* (Dong et al., 2006; Watson et al., 2005). Although the products of FREP and Dscam genes are primarily recognized for their roles against parasitic and bacterial pathogens, respectively, recent findings do suggest they may also play broader roles in the regulation of gut microbiota. For instance, FREPs might contribute indirectly to microbiome management by controlling parasitic infections, as parasite load can significantly influence microbiome composition and structure (Fredensborg et al., 2020; Leung et al., 2018). Related to Dscam, the gene's taxon-specific and highly diverse transcriptional repertoire suggests not only its role in pathogen recognition but also a complex interaction with distinct microbial taxa, potentially influencing the assembly of microbial communities in mucosal environments such as the gut lumen. This functional versatility is consistent with the large, multi-domain nature of these effector molecules, which likely enables them to bind a variety of targets and mediate multiple regulatory signals. These findings, which include the origin of VCBPs, VLRs, vertebrate antibodies, and other secreted effector systems, suggest that related selective pressures have driven the conserved and sometimes independent origins and evolution of diverse secreted effectors that can help manage the complexity of the microbiomes associated with the lumen of mucosal tissues of animals.

The development of more complex digestive systems and rich symbiotic microbial communities necessitates the reciprocal need for a more

sophisticated and anatomically organized immune regulatory network. In vertebrates, the sub-mucosal surfaces of the *lamina propria* can be diffusely populated with diverse effector cells, whereas regionalized specialization results in the so-called mucosal-associated lymphoid tissue (MALTs), which, depending on their localization in the body, receive specific names, such as gut-associated lymphoid tissue (GALTs) for the digestive tract (Mowat & Agace, 2014).

These tissues, located in the mucosa and submucosa and adjacent to the epithelium, are essential for protecting the body from pathogens the host may encounter in the gastrointestinal tract, and also help shape the developing and maturing postnatal immune system. MALTs appear to have first evolved as a network of diffuse leucocytes that are disseminated along the mucosal surfaces of vertebrates such as cartilaginous and bony fishes (Salinas, 2015; Smith et al., 2019), whereas more organized lymphoid structures can be found within the mucosal epithelia of amphibians, reptiles, birds, and mammals (**Figure 5.3**) (Cebra et al. 1998; Lillehoj & Trout, 1996; Mowat & Agace, 2014; Zimmerman et al., 2010). In mammals, GALT achieves greater complexity, characterized by cell-rich niches and includes distinct tissues such as Peyer's patches, isolated lymphoid follicles, and mesenteric lymph nodes.

Localized in the small intestine of vertebrates, Peyer's patches are the best characterized among GALT regions. These structures are not encapsulated like lymph nodes but always contain germinal centers that play a crucial role in the development of class-switched antibodies (class-switch recombination from IgM to IgA) (**Figure 5.3**). They serve important roles in the formation of memory B-cells, indicating continued immune stimulation, presumably in response to luminal antigens or MAMPs (Mowat & Agace, 2014). The germinal center of Peyer's patches appears to

be a unique site for expanding and synchronizing gut IgA B-cell responses; these responses are dependent on repeated exposure to antigen and the effective distribution of antigen-activated B-cells into multiple Peyer's patches (Lycke & Bemark, 2012).

Peyer's patches are also populated by innate immune cells, like macrophages and DCs, that facilitate a strong coordination between innate and adaptive immune systems. Briefly, the overlying epithelium to the Peyer's patches contains specialized epithelial cells, known as microfold cells, or M cells, that continuously sample and transport particulate antigens (like MAMPs) from the lumen into the underlying DCs, which then present them to adaptive immune cells, like B- and T-cells, and subsequently tuning the immune response (Dillon & Lo, 2019). M cells are also utilized in the active transport of IgA that is produced in Peyer's patches and destined for secretion to the lumen; however, they are not the only source of active transport of mucosal antibodies.

Polymeric Ig receptors (pIgR) are expressed on the basolateral surface of most polarized gut epithelial cells, or enterocytes, where they bind polymeric antibodies, such as IgA. These bound antibodies are then transported via transcytosis onto the apical surface, where the ectodomain of the pIgR is cleaved but remains attached as a secretory component to help stabilize these effector molecules in the physiologically challenging environment of the gut lumen (Dishaw et al., 2024; Kaetzel, 2014). Hence, the maturation of IgA responses in the GALT and their active translocation to the gut lumen helps promote symbiosis and colonic homeostasis, although the precise mechanisms have yet to be elucidated (Nakajima et al., 2018). A proposed mechanism includes glycan–glycan interactions among IgA, bacteria, and mucus; these interactions can influence gene expression and the function of members of the gut microbial community (like

Bacteroides thetaiotaomicron, a prominent gut symbiont of the phylum Bacteroidetes). Interestingly, this type of interaction has been observed also in invertebrate chordate, like the ascidian *C. robusta*, where VCBP molecules, through a glycan-glycan interaction involving the CBD of the protein and chitin fibers present within the mucus layer, can become tethered to mucus and modulate microbial interactions via the Ig domain; the precise dynamics (and outcomes) of these interactions are yet to be deciphered (Dishaw et al., 2011, 2024; Liberti et al., 2021).

These stabilizing interactions promote symbiosis among commensal bacterial species required for colonic homeostasis (Nakajima et al., 2018). Tethering antibodies to the mucus can, in turn, stabilize microbial communities and disrupt settlement dynamics among pathogens (Dishaw et al., 2024). Additionally, by utilizing the antigen-binding Ig-variable region, secretory IgA specifically blocks certain bacterial epitopes, thereby interfering with the direct adhesion of commensal bacteria to the apical surface of intestinal epithelial cells. Secreted IgA can also limit microbial motility by non-specifically binding bacteria through glycans associated with the secretory component (the pIgR-derived polypeptide) and constant regions of IgA. In addition to neutralizing pathogens in the intestinal lumen, SIgA can intercept microbes and toxins inside intestinal epithelial cells. Notably, SIgA delivers these protective functions without activating inflammatory responses, such as complement, an innate effector system that is very efficient at lysing microbes and amplifying inflammatory responses. In this way, SIgA mediates its diverse effector functions and minimizes collateral damage to the epithelial barrier (Gutzeit et al., 2014; Strugnell & Wijburg, 2010).

The dedicated lymphoid structures that help recognize the MAMPs provide the anatomical, physiological, and immunological basis for the

maturation of antibody responses, where selection for high-affinity B-cell clones (among the entire pool of B-cells) takes place. In the gut, these activities reveal the GALT as a principal location for priming adaptive immune responses.

5.3 THE GUT MICROBIOTA AS SYMBIONTS AND METABOLIC PARTNERS INFLUENCING HOST PHYSIOLOGY

It is now clear that the gut immune system has evolved not only to defend against pathogenic breaches but also to help mediate symbiotic relationships. Tolerating these microbes and resisting unnecessary responses is essential given that the gut microbiome, due to its functional integration via metabolic and immunological crosstalk, is now increasingly recognized as a 'forgotten organ' (O'Hara & Shanahan, 2006). For example, the microbiota's significant integration into host physiology with its metabolic output (e.g., vitamins, short-chain fatty acids, bile acid metabolism), modulation of host immune status, and its important developmental influences on host tissues (e.g., GALT, epithelial differentiation/turnover) inspired the original proposal. However, it has gained significant traction since then and inspired translational research (Ma et al., 2024) into the metabolic and endocrine functions of the microbiome (Fan & Pedersen, 2021; Fujisaka et al., 2023), in defining its dramatic influences on host immune maturation (Brown, Bashir, & Zeng, 2025), its utility in microbiome-targeted therapies (Gilbert et al., 2025; Sorbara & Pamer, 2022), as well as inspiring the expansion of holobiome (and symbiosis) concepts (S. F. Gilbert, 2019; Simon et al., 2019).

By serving crucial roles as symbionts and metabolic partners, animal-associated microbiota enable hosts to adapt to ecological niches and dietary

challenges. The examples below illustrate how microbiota facilitate thriving in diverse environments through specialized metabolic partnerships, facilitating the exploitation of novel diets and the developmental maturation of critical tissues, such as immune tissues and systemic responses.

A representative standard of symbiosis is the bioluminescent relationship between the Hawaiian bobtail squid, *E. scolopes*, and the bacterium, *Vibrio fischeri*. The squid selectively recruits *V. fischeri* to its light organ, where the bacterium provides counterillumination camouflage that facilitates the squid's hunting behavior (McFall-Ngai et al., 2012; Rader & Nyholm, 2012). A specific two-component sensor kinase has been identified that regulates settlement and biofilm formation, and its transfer to other bacteria can mediate colonization of the light organ (Brooks & Mandel, 2016; Fung et al., 2024).

A large repertoire of metabolic exchanges is now recognized as essential to human health and can change during aging (Best et al., 2025) and in disease-specific cases (de Vos et al., 2022; Fan & Pedersen, 2021; Marcelino et al., 2023; Porcari et al., 2025). Specific population-based adaptations are also known, such as the presence of *Bacteroides plebeius* in the gut of Japanese individuals, which enables the metabolism of seaweed polysaccharides. The *Bacteroides* strain acquired the gene horizontally from the marine bacterium, *Zobellia galactanivorans* (Hehemann et al., 2010). Similarly, dietary restrictions can have a further influence on the structure and function of the gut microbiota, thereby affecting health outcomes (Fackelmann et al., 2025; Graf et al., 2015).

Herbivorous mammals, or ruminants, have evolved partnerships with *Fibrobacter* species to break down lignocellulose, a capability lacking in mammalian genomes (Neumann & Suen, 2018). Likewise, captive elephants and baboons show reduced *Collinsella* species, which are fiber-

associated, and increased *Lactobacillus*; these studies reveal how captivity and human influence on diet can also impact the microbiome (and dietary capacity) of wild animals (de Jonge et al., 2022; Moy et al., 2023).

Members of microbiomes interact in complex ways that can involve competition, synergy, or cross-feeding (Levy & Borenstein, 2013). Their metabolic output involves metabolites that are often important to the host, helping to define the functional landscape of the microbiome. Full metagenomic sequencing of gut microbiomes has enabled the predictive modeling of genetic pathways, facilitating a deeper understanding of the functional ecology of individualized or personalized microbiomes (Levy & Borenstein, 2014). Increasingly, this effort is integrated with other '-omics' data that includes full proteomes and metabolomes so that the physical metabolites and/or enzymatic potential of the system are fully recognized (Bauer & Thiele, 2018; Heinken et al., 2021). These efforts have led to a deeper understanding of the metabolic contributions of gut microbiomes that influence health, as a legitimate organ-like system. Furthermore, it identifies potential therapeutic targets in precision medicine (Best et al., 2025; Porcari et al., 2025; Rios Garza et al., 2023).

And more recently, microbial network models are being integrated with host immune data (including transcriptomic, proteomic, and single-cell resolution) to better understand how local immune status, developmental stage, or immune pathology shape, and are shaped by, gut microbial ecology. The key advances are occurring at the intersection of microbiome ecology, mucosal immunology, and systems biology, with direct implications for precision medicine.

5.4 RECIPROCAL INTERDEPENDENCIES: HALLMARK OF SYMBIOSIS

It has been well described that members of the gut microbiome are essential for immune maturation Arpaia & Rudensky, 2014; Arpaia et al. (2013; Atarashi et al., 2011; Furusawa et al., 2013; Lubin et al., 2023), which includes the recruitment and expansion of immune cells and tissues within the GALT, as well as at distal sites (Ahern et al., 2014). These immune-modulatory features often include keystone species that influence the expansion of regulatory T-cells (T-regs), cytokine production, the synthesis and secretion of AMPs, and the maturation of mucosal barriers. Investigating the reciprocal influence of microbiota networks and host immunity, such as co-analysis with host transcriptomes, has led to a better understanding of hallmark features of the onset and maturation of inflammatory bowel diseases (Lloyd-Price et al., 2019), for example. Further, spatial microbiome and immune profiling reveal interaction networks that influence localized anatomical sites, e.g., stem cell readiness in specialized niches (Anandakumar et al., 2024; Liu et al., 2022; Lotstedt et al., 2024; Sharma & Thaiss, 2020; Tropini et al., 2017).

During early human infancy (preterm and term), microbial assembly and functional networks have been linked to the training of innate immunity, desensitization of TLRs, and the establishment of cytokine production thresholds (Donald & Finlay, 2023; Ignacio et al., 2024; Kollmann et al., 2012; Sprockett et al., 2018). Improper timing and signals during this window can predispose infants to chronic immune-related issues, such as food allergies and inflammatory conditions (Gensollen & Blumberg, 2017; Hoskinson et al., 2023). This dependency on the type and timing of specific microbial cues for proper development is an increasingly recognized feature of the co-evolutionary patterning among animal holobiont units (Bosch & McFall-Ngai, 2021; McFall-Ngai & Bosch, 2021).

Under optimal conditions, synergy with the gut microbiota also helps reduce the potential of unnecessary inflammatory responses (**Figure 5.4**). For example, the commensal microbiota can have both direct and indirect influence on barrier defenses in at least four important ways. First, specific members of the microbiota, such as *B. thetaiotaomicron*, can help stimulate host epithelial gene expression to activate the expression of genes regulating mucus production (Hooper et al., 2001). In addition, butyrate, a short-chain fatty acid derived from the metabolism of dietary fiber by *Firmicutes* bacteria, can also stimulate mucus production, along with the enhancement of epithelial cell tight junctions (Antharam et al., 2013; Van den Abbeele et al., 2013). Second, commensal microbiota can form structured biofilms on the surface of and within the barrier mucus layers, which help prevent settlement and invasion by pathogens (Ren, Zhou, Xu, Jia, & Yang, 2024; Swidsinski, Weber, Loening-Baucke, Hale, & Lochs, 2005; Walsham et al., 2016). Notable about this form of indirect defense against pathogens is that host immune systems have evolved strategies involving immune effectors that promote such settlement of the surface, thus 'encouraging' the formation of 'beneficial' biofilms (Bollinger et al., 2003; Dishaw et al., 2014; Donaldson et al., 2018). Third, occupation of the mucosal surface by commensal flora enhances protection of the host surface through competitive exclusion of pathogens (Britton & Young, 2014). Fourth, commensal microbiota modulate host gene expression via distinct signals, e.g., polysaccharide A (PSA) from *B. fragilis*, can modulate host responses via IL-10-producing T-regs that promote and maintain an anti-inflammatory tone (Round & Mazmanian, 2010; Round, O'Connell, & Mazmanian, 2010). Other MAMPs, at sub-threshold levels, are known to maintain or promote innate immune readiness via PRRs, such as TLRs, without triggering inflammation (Round et al., 2011). Thus, homeostasis of

the gut includes coordinated responses mediated by both the host immune system and members of the microbiota.

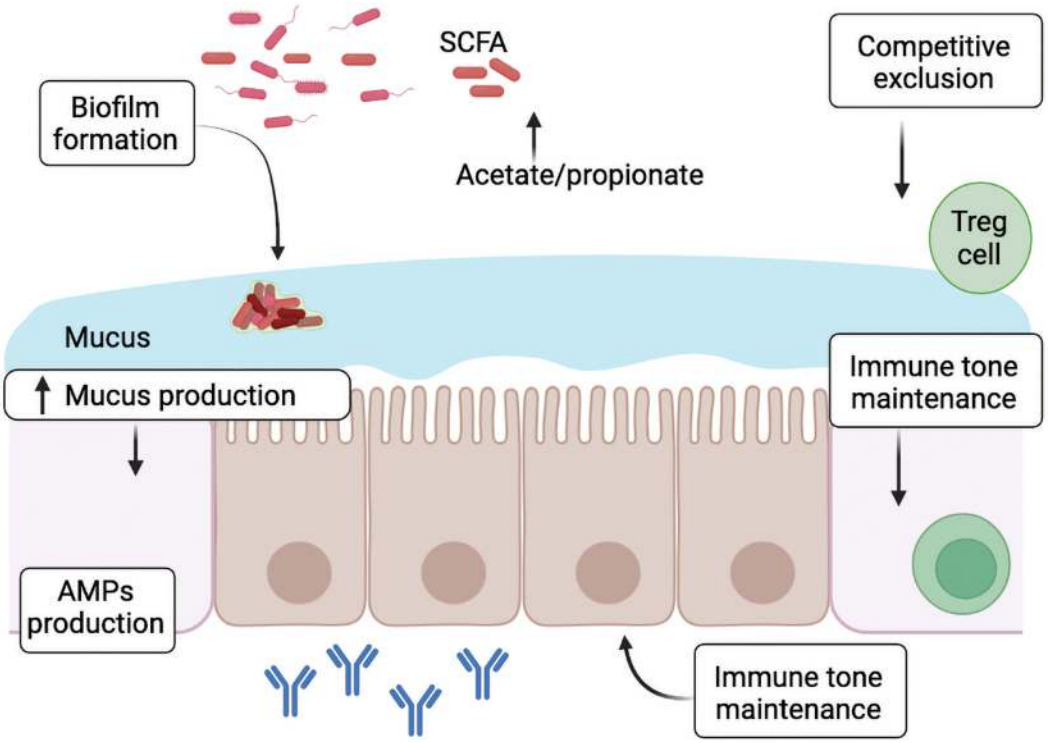


FIGURE 5.4 Simplified illustration of the interplay governing homeostasis at the gut epithelial surface. Interactions between host and gut microbiota involve bi-directional synergies that promote homeostasis and the maintenance of a steady-state immunological tone involving tolerance toward commensal microbiota. For example, mucin foraging preferences among commensal microbiota drive colonization behaviors (including settlement and biofilm formation) in some bacteria, and this promotes additional mucus production. Bacterial secondary metabolites, such as short-chain fatty acids (acetate, butyrate, or propionate), positively influence barrier integrity and promote a tolerogenic state. Constitutive production of host AMPs and other secreted effectors like antibodies influences settlement behaviors, kills or neutralizes pathobionts, influences microbe-microbe or host-microbe interactions, and helps mediate competitive exclusion. Enhanced barriers help reduce the likelihood of provoking unnecessary immune responses in the gut. ⏎

The evolution of reciprocal exchanges and interdependencies in the gut has led to developmental pathways becoming dependent on distinct environmental and microbial cues. These symbiotic exchanges between the host (immune system) and microbial ecosystems result in phylogenetic breadcrumbs that reflect dietary restrictions, environmental specialization, and other lifestyle traits with broad influence on the taxonomic evolution of metazoans.

5.5 EVOLUTIONARY EMERGENCE OF THE ENTERIC NERVOUS SYSTEM FOR THE INTEGRATION OF DIGESTIVE AND ENDOCRINE FUNCTIONS WITH HOST IMMUNITY

Phylogenetically, the forerunner of an enteric nervous system (ENS) was likely enteric neurons forming simple nerve nets, likely originating from neuroectoderm, during the transition from simple gastric cavities, such as those found in cnidarians, to the through-gut of bilaterians. Subsequently, the evolution of the neural crest in chordates facilitated a rapid expansion of more complex innervation patterns characteristic of vertebrates (Edens et al., 2025; Goldstein et al., 2013; Green, Uy, & Bronner, 2017). These innovations facilitated the regionalized specializations (e.g., foregut, midgut, hindgut), along with localized and temporally coordinated regulation of peristalsis, secretion, absorption, and immune surveillance that exists in a segmented gut (Spencer & Hu, 2020; Vicentini et al., 2022).

The emergence of the ENS further enabled the gut to act not just as a digestive system, but as a sensory and integrative hub that coordinates nutritional sensing, immune readiness, and neuroendocrine signaling (Kaelberer et al., 2018; Sharkey & Mawe, 2023), dramatically expanding the exploration and conquest of new environments among bilaterians. As a

major interface with the environment, the gut could now help coordinate sensing and responding to complex external signals. The ENS has been generally regarded as the 'second brain' not only because of its neuronal complexity, but also because of its semi-autonomic regulation as well as its ability to communicate bidirectionally with both the central nervous system (CNS) and the GALT (Rhee et al., 2009; Yoo & Mazmanian, 2017). However, due to its phylogenetic antiquity, with enteric neurons predating the emergence of complex brains and central nervous systems, the ENS has more recently (and more accurately) been referred to as the 'first brain' (Furness & Stebbing, 2018; Furness et al., 2013).

Four key features of the ENS are worth noting. First, sensory neurons detect stretch, microbial metabolites, and biophysical features of the gut, such as pH (redox) and osmolarity (Hamilton, Wall, Robinson, Guillemin, & Eisen, 2022; Sharkey & Mawe, 2023). Second, enteroendocrine cells (EECs) release hormones such as serotonin (5-HT), Peptide YY, or Glucagon-like peptide-1 that regulate important aspects of nutrient intake, such as digestion and appetite, but can also coordinate immune readiness (Batterham et al., 2002; Grondin & Khan, 2024; Kaelberer et al., 2018; Wachsmuth et al., 2022). Third, the ENS mediates neuro-immune crosstalk, largely mediated by neuropeptides and neurotransmitters, which helps modulate immune tone and readiness, AMP production, and epithelial renewal (Kabata & Artis, 2019; Yang, Almanzar, & Chiu, 2023). And fourth, microbial feedback loops regulate the development and function of ENS. For example, gut microbiota colonization promotes the differentiation of EECs and modulates the plasticity of neural circuits via microbial-derived metabolites such as butyrate and tryptamine (Arora, Vanslette et al., 2021; Damiani et al., 2023; Fung et al., 2017).

Thus, the neuronal and endocrine circuits of the gut, therefore, do more than support digestion; they sense and interpret environmental cues, and help the host mount (adaptive) responses to both nutritional and microbial challenges. In this way, the ENS helps define the gut as an environmental interface, linking external stimuli (via ingested content) to internal physiological outcomes.

5.6 CONCLUSIONS AND FUTURE DIRECTIONS

The evolution of the gastrointestinal tract in bilaterians represents one of the most profound innovations in animal biology, not only as a site of digestion and absorption, but as a multifunctional interface that integrates sensory, neuroendocrine, immune, and microbial inputs. This chapter has explored how the gut emerged not merely as a conduit for nutrient flow but as a site of continuous environmental negotiation, where the immune system plays a central role in managing the dynamic interplay between host and microbiota, as well as other exogenous signals.

We have shown that the gut mucosal barrier evolved structural and cellular complexity that enables both defense and, importantly, tolerance with mucus, epithelial cells, and immune effectors forming a layered and responsive barrier. Notably, this barrier does not function in isolation. It is shaped by, and in turn shapes, the commensal microbiota. The immune system, often regarded as solely a pathogen-fighting entity, has evolved into a master regulator of microbial symbioses, fostering mutualistic interactions while maintaining vigilance against invasive threats on mucosal surfaces.

Central to this symbiosis is the awareness that microbiota are not passive occupants, but active metabolic partners that expand the physiological capacity of the host. They influence nutrient (energy) harvesting, barrier integrity, epithelial turnover, and immune tone. This dynamic co-

dependence is reciprocal: host development and immune calibration are now understood to require microbial signals, and in many cases, the presence of microbes altogether. The gut, therefore, emerges as a chimeric organ, or the ‘forgotten organ,’ shaped by evolution not only through host genomic adaptations, but also through deep co-evolution with microbial partners.

This complexity is further amplified by the emergence of the ENS, which integrates local and systemic inputs to coordinate digestion, immunity, and behavior. The gut is thus a sensory organ in the most literal sense, detecting physical and chemical cues, translating microbial signals, and orchestrating hormonal and neuronal outputs that affect the host on a systemic level.

Looking forward, several frontiers emerge on the horizon, spanning from different biological fields, that will facilitate a better understanding of the interdependencies between host and microorganisms. Examples include: (i) Evolutionary ecology of symbiosis. Investigating how gut-microbe-immune relationships evolve in different ecological contexts (e.g., dietary shifts, environmental change, host range expansions) may reveal principles of adaptive symbioses that apply beyond the gut; (ii) Clinical translation. Viewing the microbiota as a functional ‘organ’ opens new windows into precision medicine, where microbial networks, immune tone, and gut–brain signaling can be leveraged as diagnostic biomarkers or therapeutic targets in diseases ranging from inflammatory bowel disease to neurodevelopmental disorders; (iii) Developmental and comparative models. Increasing the use of nontraditional model systems (e.g., marine invertebrates) may help uncover conserved and derived strategies for gut immunity and symbiosis, illuminating evolutionary transitions in mucosal interface design and (iv) Systems-level integration. As multi-omics and spatial mapping tools evolve, the capacity to resolve gut microbe–immune–neuroendocrine

interactions at single-cell and spatial levels will improve. This will allow us to model the gut not as a linear tract, but as a dynamic ecosystem with regionalized specializations.

In conclusion, the gut is more than a mere digestive tract; it is a gateway to the 'self' and continuously engages with the outside world. Its evolution reflects a delicate equilibrium between openness and defense, between autonomy and interdependence. To understand the gut is to understand how organisms negotiate the boundaries of identity, survival, and symbioses.

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6 Comparative Study of Cell Types and Their Regeneration in Echinoderm Digestive Systems

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6.1 INTRODUCTION

In 1955, Libbie Hyman published a landmark book describing much of what was then known about the phylum Echinodermata. This "tour de force" work provided a detailed account of the anatomical features of all echinoderm classes, with particular emphasis on the histology of their organs. Within this volume, she compiled descriptions of cell types reported in earlier studies. At the time, histological and cellular characterization of

echinoderms—and most other organisms—relied primarily on light microscopy combined with various chemical stains, which sometimes offered a degree of specificity in identifying cell types. Cells were typically described based on morphology and location, as observed in tissue sections or fluid suspensions, with or without staining.

In subsequent decades, cellular characterization advanced significantly through the use of electron microscopy (TEM and SEM), and immunohistochemistry using antibodies or molecular markers that targeted specific cellular components. These innovations often revealed additional cell types that had not been previously distinguished. For instance, in mammals, the gene ***lgr5*** was identified as a marker of stem cells in the intestinal luminal epithelium (Tan and Barker 2014). These cells, located in crypts, serve as the origin for all other luminal phenotypes and maintain a persistent stem cell population throughout the organism's life. Thus, molecular markers, mainly the presence of specific proteins or mRNAs, served to identify particular cell populations.

More recently, single-cell (scRNA-seq) and single-nucleus RNA (snRNA-seq) sequencing technologies have revolutionized cell classification. These methods rely, not on morphological traits, but on transcriptomic profiles—using mRNA expression as a proxy for protein production. It is now possible to generate a comprehensive catalog of cell populations within an organism or organ based solely on their gene expression profiles.

Looking ahead, researchers face two major challenges. First, integrating molecular and morphological data to define true cell populations. For this, tools like hybridization chain reaction fluorescence in situ hybridization (HCR-FISH) enable visualization of the expression of specific genes—identified through scRNA-seq—at the cellular level. These genes are

typically probed in small numbers using gene-specific probes and visualized via fluorophores. At a broader scale, spatial RNA sequencing provides an integrated view of gene expression and cellular localization within tissues.

The second challenge is to compare cell types across species to identify cellular orthologues. This involves determining whether similar cells in different organisms share evolutionary and functional relationships. For example, Lgr5-expressing cells have been identified in the holothurian intestine (Mashanov et al. 2017). However, unlike their mammalian counterparts, these cells are not located in crypts, and their capacity to differentiate into other cell types remains uncertain. Are these cells true orthologues of mammalian Lgr5+ stem cells? Questions like this will become increasingly common in the coming years as comparative cell biology continues to evolve, now with the optics of gene expression profiling.

An additional challenge of scRNAseq and snRNAseq is how to clearly define a cell population. Cells can change their gene expression in response to environmental, developmental and/or homeostatic changes. Researchers must then determine whether a cell population is in a different “state” or whether the differences in gene expression account for a “different” cell population. Such distinctions are hugely important in developmental systems where cells might change progressively from one stage to another, thus becoming a “different” cell. Similar problems are found by researchers working with echinoderms where changes in cell phenotypes, might be the result of the cellular dedifferentiation and/or transdifferentiation that has been described during organ regeneration in several species (García-Arrarás et al. 2001; Kalacheva et al. 2017; Mashanov et al. 2005). In these cases,

phenotypes that belong to transient cell stages might shed some light into the genetic profiles that underlie the regenerative process.

In this chapter, we focus on the digestive tract of the sea cucumber *Holothuria glaberrima* and its regenerative process, using it as a case study to explore the future of echinoderm cell phenotype research, comparative analyses, and the integration of these findings with functional and developmental stages. To this end, we have compiled a catalog of the cell phenotypes identified by traditional methods in the holothurian digestive tract, along with their known functional roles. Additionally, we have incorporated the limited available data from scRNA-seq and/or snRNA-seq studies to offer researchers a preliminary reference point. This serves as a guide to what can be anticipated as these molecular techniques become more widely adopted in the near future.

6.2 THE DIGESTIVE TRACT: FOCUS ON THE INTESTINE

The intestine is the main structure of the holothurian digestive tract. At the anterior end, the intestine connects with the esophagus (or in some species to a short stomach) while in the posterior end it connects to the cloaca. The intestine itself is usually divided into three components; a descending segment, an ascending segment, and a second descending segment (Smiley 1994). The first two are considered "small intestine" while the second descending portion is considered "large intestine".

The histology of the holothurian digestive tract has been described at the light and electron microscope level in two extensive reviews, one by Jean-Pierre Feral and Claude Massin (1982) and a second one by Scott Smiley (1994). The basic layout of the digestive tract is that of two epithelial layers separated by a layer of connective tissue (**Figure 6.1**). One of these layers,

the mesothelium, faces the coelomic cavity while the other, the luminal or mucosal epithelium faces the lumen of the digestive tract. In echinoderms, the mesothelium includes the proper coelomic epithelial cells or peritenocytes and an underlying muscle layer. Both peritenocytes and muscle cells are attached to a basement membrane that separates the mesothelium from the connective tissue. Neurons have also been described within the mesothelial layer. Changes in the cell types or width of the layers serve to define the different regions of the digestive tract. For example, in the esophagus, the muscle layer is prominent with little connective tissue, while in the descending posterior or large intestine the connective tissue layer has an increased size. Likewise, there might be differences in the presence or size of villi in the luminal layer. For example, in *Isostichopus* species, the larger villi are present in the most anterior part of the intestine when compared to the posterior region (Vergara and Rodriguez 2015).

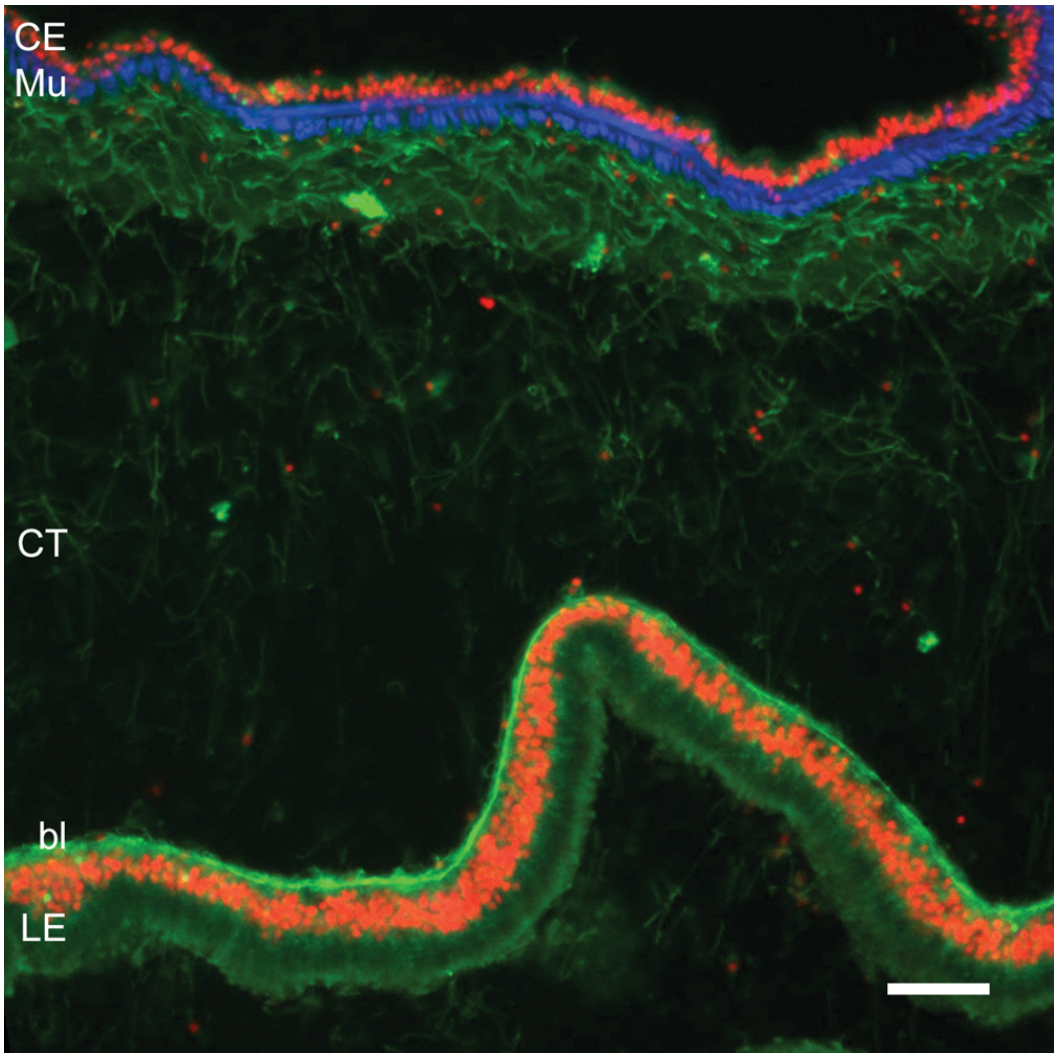


FIGURE 6.1 Histological section of the *Holothuria glaberrima* intestine. A longitudinal section of the second descending (large intestine) digestive tract component. The section has been labeled with an antibody against collagen (green), fluorescently labeled phalloidin that recognizes the muscle actin (blue) and a nuclear stain, DAPI (red). The three main tissue layers of the digestive tract are observed; the Mesothelium that is made up of the coelomic epithelium (CE) and muscle (Mu), the Connective Tissue layer (CT) and the Luminal Epithelium (LU). The basement lamina (bl) that separates the CT from the LE is clearly observed. Bar = 50μm. ⏎

The digestive tract is anchored to the body wall by a mesentery, which also runs from the anterior region of the animal to the posterior part, but

changes its attachment site to the body wall- from dorsal (in the descending "small" intestine) to medial (in the ascending "small" intestine) and then to ventral (in the second descending or "large" intestine). The mesentery layers (mesothelium and connective tissue) are continuous with those of the digestive tube at one end and with the coelomic epithelium and the connective tissue of the body wall at the other end. In recent years, the mesentery of vertebrates has been recognized as a novel organ, thus indicating that it has distinct functions from those of other organs (Coffey et al. 2020). In this respect it is important to highlight that the holothurian mesentery plays a pivotal role in the process of intestinal regeneration (García-Arrarás et al. 2019).

In the following sections we explore the cell types found within the various layers of the holothurian digestive tract. We focus on the large intestine of *H. glaberrima* and use this as a point of reference for comparisons with other regions of the digestive tract and/or with other species.

6.3 MESOTHELIUM

The echinoderm mesothelium, according to Smiley (1994), is a mesodermally derived epithelium that covers the coeloms and most internal organs and that may be simple, pseudostratified or even stratified according to its location (**Figure 6.2A,B**). In the holothurian gut, the mesothelium has been described as having four layers: the perivisceral mesothelium (corresponds to the coelomic epithelium or peritenocytes), a layer of nerve elements, and two layers of muscle, one longitudinal and one circular (Kawaguti 1964). However, the neurons and fibers present are interspersed with muscle cells and coelomic epithelia, thus difficult to define as a "layer" (García-Arrarás et al. 1999, 2001).

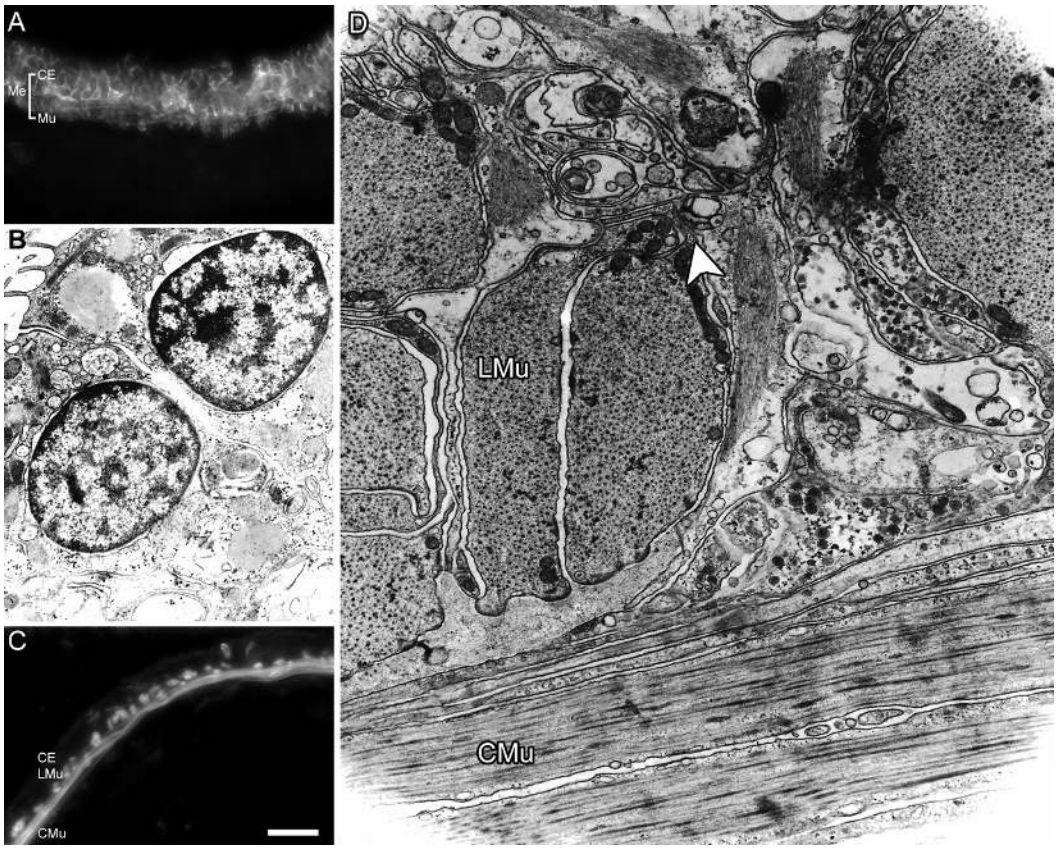


FIGURE 6.2 Mesothelial layer of *H. glaberrima* large intestine. The mesothelial layer is made up by an external layer of coelomic epithelial cells (also called peritoneocytes) (A, B) and two internal layers of muscles (circular and longitudinal) (C, D). (A) The photograph of the mesothelium was taken to emphasize the cells in the coelomic layer, as labeled with monoclonal antibody ERG1 (see Mashanov et al. 2010). (B) Coelomic epithelial cells are shown in an electron micrograph, with centralized nuclei, some vacuoles, and filaments. These filaments, some of which are observed in the area below the nuclei, form the peduncle that anchors the cells to the basal lamina found below the muscle layer. (C) Transverse histological section of the large intestine showing the two muscle layers, longitudinal (Lmu) and circular (Cmu) as labeled with fluorescently labeled phalloidin. The muscle layers lie below the other mesothelial component, the coelomic epithelial (CE) cells. (D) Micrograph of the enteric muscle layers and their interaction with a diverse plexus of fibers with different type of vesicles. Note the muscle extensions that are associated with the fiber plexus. Ce-Coelomic epithelium, Me-mesothelium, Mu-Muscle. Bar (A)= 75μm (B) = 5μm (C) = 25μm, (D) = 5μm. ⏎

There are three main cell types in the mesothelium: coelomic epithelial cells, neurons and muscle cells; each will be described as follows.

6.3.1 Coelomic Epithelium

In simple terms, the coelomic epithelium is a thin, delicate layer of cells that lines all the organs contained within the coelomic cavity, acting as a protective and dynamic barrier essential for organ function and regeneration. The cell layer is sometimes referred to as "perivisceral mesothelium" and the cells have also been termed as "adluminal mesothelial cells" (Smiley 1994) and more recently as "tenocytes" or "peritoneocytes" (Vandenspiegel et al. 2000; Guatelli et al. 2022; Medina-Feliciano et al. 2025). Although several functional roles have been attributed to the coelomic epithelium, especially in maintaining internal stability and supporting organ regeneration, its precise cellular composition and the extent to which it contributes to these processes in echinoderms remain only partially characterized.

In the digestive system, the coelomic epithelial cells can have different morphologies depending on the species and the digestive tract region. In *H. glaberrima,* they have been described as having an elongated squamous morphology, but in another sea cucumber species, *Cladolabes schmeltzii*, they are described as having a goblet shape through most of the intestine and a granular content that varied depending on the region of the intestine (Kamenev et al. 2013). They have a single cilium that projects into the coelomic cavity. Under the electron microscope, the cells have been shown to have a slender projection or peduncle that extends from the squamous cell body to the basement membrane (Mashanov et al. 2004). The cellular extension contains bundles of microfilaments or tonofilaments, whose molecular nature remains unknown (Mashanov et al. 2004; Smiley 1994).

The cells are attached to each other by zonula adherens (desmosomes) and bind to the basal lamina via hemidesmosomes (**Figure 6.2B**).

The coelomic epithelium of the intestine is continuous with that of the mesentery. To date, no major differences have been reported in the cell types comprising the coelomic epithelium of the mesentery and those of the digestive tract, suggesting that both structures share a similar cellular composition. Since the coelomic epithelium covers the internal wall of the body (coelom) cavity, it might be the case that its function varies according to the anatomical region.

Little is known of the coelomic epithelium function in the normal intestine, but in other organs it produces a non-adhesive surface to avoid organ friction, secrete apical glycosaminoglycan, and regulate the coelomic fluid dynamic (Winters and Bader 2013).

The coelomic epithelium stands out for its remarkable versatility and repair potential (Winters and Bader 2013). This functional plasticity is especially evident in echinoderms. The echinoderm coelomic epithelium has been proposed as a source of both stem/multipotent cells and direct source of circulating cells, including immune cells (Guatelli et al. 2022). In the starfish *Asterias rubens*, for example, coelomic epithelial cells collected from the aboral surface of the body wall display intensified activity in protein synthesis, membrane trafficking, and secretion of signaling molecules, including growth and regeneration factors when compared to coelomocytes, which primarily serve immune functions (Lyukmanova et al. 2024). A similar regenerative activation has been documented in *Marthasterias glacialis*, where the perivisceral coelomic epithelium near the wound site becomes notably thicker within 24 hours post-amputation. Peritoneocytes in this region exhibit increased apocrine secretion and accumulation of phagosomes, while the underlying muscle layers undergo

disorganization and remodeling (Guatelli et al. 2022). These rapid ultrastructural changes suggest an early and active role of the coelomic epithelium in tissue repair.

In the sea cucumber *Holothuria forskali*, it has been shown that the coelomic epithelium plays a critical role in the regeneration of Cuverian tubules (Vandenspiegel et al. 2000). These tubules, which function as a defense mechanism and share numerous embryological and structural similarities with the intestine, are naturally regenerated after expulsion. The coelomic epithelium has been identified as the source of many of the cell types involved in this regenerative process.

The role of the coelomic epithelium in forming muscle cells has best been studied in the somatic musculature. Like the visceral muscle system, the somatic muscle is also surrounded by a layer of coelomic epithelial cells which has been shown to play an active role in muscle regeneration (Dolmatov et al. 1996; García-Arrarás and Dolmatov 2010). Following muscle injury, the new muscle bundles are formed due to dedifferentiation, proliferation, migration, and immersion of the coelomic epithelial cells into the connective tissue. (Dolmatov et al. 1996). This process is marked ultrastructurally by the loss of cilia, reorganization of centrioles, and the appearance of nascent actin and myosin filaments within the cytoplasm, features indicative of early muscle differentiation (Dolmatov et al. 1995).

Comparable regenerative activity has also been documented in another sea cucumber species, *C. schmeltzii*, which regenerates its posterior intestine following asexual fission. In this species, coelomic epithelial cells remain largely epithelial in nature but contribute directly to tissue formation by differentiating into myocytes and releasing lipoprotein granules into the coelomic cavity—possibly to support the energetic demands of regeneration (Kamenev and Dolmatov 2015). This contrast in strategy suggests a

conserved role of the coelomic epithelium across species, with variation in the degree of epithelial-to-mesenchymal transition depending on the regenerative context.

In *H. glaberrima*, coelomic epithelial cells undergo a transition and morphological changes during intestinal regeneration that will be described together with the changes in muscle cells below.

6.3.2 MUSCLE

As in vertebrates, the muscular system of holothurians is divided into visceral and somatic muscles (Romer 1972; García-Arrarás and Dolmatov 2010). However, in terms of microscopic structure, both muscle types more closely resemble vertebrate smooth muscle, characterized by non-striated, spindle-shaped fibers and the absence of organized sarcomeres. Within the digestive tract, the visceral musculature is composed of myoepithelial cells that have been most thoroughly described through electron microscopy (Filimonova and Tokin 1980; Mashanov et al. 2004; Kamenev et al. 2013;). These cells are confined to the basal region of the coelomic epithelium, where they are anchored to the basal lamina via hemidesmosomes and interconnected with one another through desmosomes, creating a cohesive contractile network. Histologically, myoepithelial cells are oriented either perpendicular to the long axis of the gut, forming the inner circular musculature, or parallel to the long axis constituting the outer longitudinal musculature. In cross sections, the longitudinal muscle layer is positioned adjacent to the coelomic epithelium, while the circular muscle layer lies next to the internal connective tissue (**Figure 6.2C,D**). Morphologically, these cells are elongated and mono-nucleated. Their cytoplasm is composed of densely packed myofilaments that form the contractile apparatus composed of actin and myosin filaments, the main proteins involved in

muscle contraction. In the remaining cytoplasmic region, the cells contain mitochondria, rough endoplasmic reticulum cisternae, and spherical electron-dense granules (Gabella 1997).

There are no obvious differences among the various muscles of holothurians, namely body wall muscle, visceral muscle and muscle from the mesentery. They all appear to consist of a uniform population of the same muscle cell phenotype, where differences lie on the thickness or orientation of the muscle layers. Despite the similar appearance of muscle cells, the question remains of whether all smooth muscle cells share uniformity in their functional roles, metabolic pathways, biochemical characteristics, and developmental origins and trajectories.

6.4 THE MESOTHELIUM DURING REGENERATION

Following evisceration, the mesentery is the epicenter of the formation of the new intestine, and the mesothelium plays its commanding role (García-Arrarás et al. 2019). In *H. glaberrima*, this involves extensive cellular reorganization of the mesentery mesothelial layers. Both the coelomic and myoepithelial cells in the mesothelium undergo significant morphological, molecular, and functional changes that underlie the formation of the new intestine.

The process begins shortly after evisceration when the coelomic epithelial cells and myoepithelial cells of the mesothelium undergo dedifferentiation. Dedifferentiation of the coelomic epithelium has been documented by EM in the sea cucumber *E. fraudatrix* (Mashanov et al. 2005) but it is in the dedifferentiation of myoepithelial cells where it has been closely studied with the use of electron microscopy and with markers such as fluorescent phalloidin (García-Arrarás and Dolmatov 2010; García-Arrarás et al. 2011; Candelaria et al. 2006). In this case, mature muscle cells

dismantle their contractile machinery, which is expelled as membrane-bound “spindle-like structures” (SLSs) and revert to a less differentiated state.

The dedifferentiated muscle and coelomic epithelial cells form the coelomic epithelium of the anlage, a transient blastema-like structure that will give rise to the new intestine (Quispe-Parra et al. 2021). The cells of this new coelomic epithelium appear homogeneous and, at present, it remains unknown if the dedifferentiated cells can be distinguished by their gene profile or if they form a homogeneous population. What is clear is that this new coelomic epithelium plays an important role in the formation of the new intestine. Dedifferentiated cells proliferate and differentiate into various cell types. The cells of the anlage celomic epithelium can also migrate into the underlying connective tissue, contributing to the formation of the mesenchymal cells of the intestinal rudiment. Moreover, the gene expression profile of the anlage coelomic epithelium greatly differs from the mesothelium of the normal mesentery, from where it originated (Medina-Feliciano et al. 2025).

Recent single-cell transcriptomic analyses have confirmed the pluripotency of the anlage coelomic epithelium. Coelomic epithelial cells within the regenerating anlage give rise to a diverse array of cell types, including mesenchymal, muscle, and neuronal cells, positioning the epithelium as a progenitor source central to visceral regeneration (Medina-Feliciano et al. 2025). These findings reinforce earlier histological observations and expand our understanding of coelomic epithelial plasticity. We have identified a population of enteric muscle precursors expressing muscle-specific genes (e.g., *titin, myl1, myh7, actg,* and *cnn3*) during regeneration Medina-Feliciano et al. (2025). Notably, these muscle progenitors exhibited transcriptional similarities with cells previously

classified as coelomic epithelial-derived, suggesting that at least a subset of muscle cells may originate from the coelomic epithelium anlage.

Further supporting this link, hybridization chain reaction-FISH (HCR-FISH) analysis localized *myh7* expression not only to differentiated muscle layers in normal tissue but also to cells at the basal side of the coelomic epithelium during regeneration—regions previously shown to harbor myogenic activity. While these findings highlight the coelomic epithelium as a likely source of muscle precursors, they are limited to a single regenerative stage (9 days post-evisceration). Ongoing research using single-nucleus RNA sequencing (snRNAseq) aims to capture the dynamic evolution of these cellular states across multiple time points.

6.5 CONNECTIVE TISSUE

The intestinal connective tissue has been described as composed of sparse, isolated cells within an electrolucent ground substance and collagen fibers (Byrne 2001; Quiñones et al. 2002; Mashanov et al. 2004). This ground substance is rich in proteoglycans (Vazquez-Velez et al. 2016). The connective tissue layer is subdivided according to their connective tissue fiber density; the region near the mesothelium has a high density while the region closer to the luminal epithelium has a low density (see **Figure 6.1**) (Feral and Massin 1982; García-Arrarás et al. 1998). The connective tissue layer has been assumed to be similar in fiber and cell content all along the digestive tract with only changes in its width or relative area. For example in *H. glaberrima* the connective tissue layer of the posterior descending (large) intestine corresponds to about three-fourths of the organ area, while in the anterior descending (small) intestine the same layer corresponds to about one-third of the organ area (García-Arrarás et al. 1998).

A layer of “outer connective tissue” supposedly underlying the coelomic epithelium was originally described in various holothurians. including *H. glaberrima* (Hyman 1955; García-Arrarás et al. 1998), but has since been considered to be an artifact of staining with chemical dyes. Thus, the only connective tissue layer is the one that is bound by the luminal epithelium on one side and the mesothelium on the other.

Cells in the connective tissue of the intestine are the least characterized of those present in the organ. This is probably due to the fact that most of them lack salient features that can be observed in light microscopy. In general terms, three types of cells have been described in the connective tissue. These are spherulocytes, a neuron-like cell and mesenchymal cells (**Figure 6.3**).

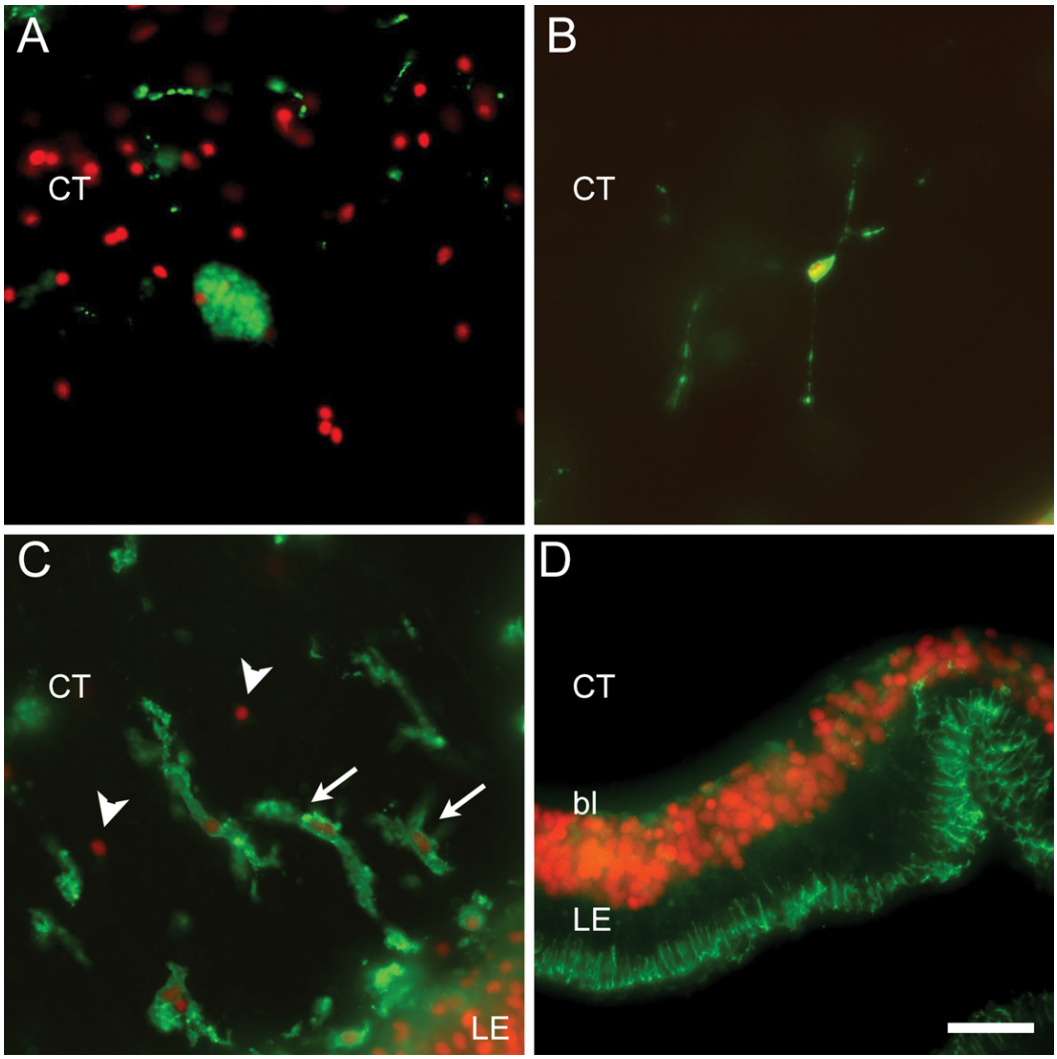


FIGURE 6.3 Cellular components of the large intestine connective tissue (CT) layer. Histological section of *Holothuria glaberrima* large intestine showing (A) immune system cells, sometimes named as "morulas" or "spherulocytes" due to their large content of vesicles and the peripheral nucleus and (B) neuron-like cells labeled with antibody RN1 (green) (see Díaz-Balzac et al. 2007). The latter cell phenotype extends fibers that form a connective tissue neuronal network. (C) Most cells in the CT layer have a mesenchymal phenotype (arrows) as labeled here with antibody KL4 (green) (see Medina-Feliciano et al. 2025) while DAPI (red) shows the cells nuclei. These cells correspond to about 75% of the cells in the connective tissue. Note the nuclei of a few non-labeled cells (arrowheads). (D) Luminal epithelium (LE) layer of *Holothuria glaberrima* large intestine. The longitudinal section shows the polarized morphology of the enterocytes. The cells form a pseudo-

stratified layer where the nuclei (DAPI-red) are found basally, in the lower third of the cells, near the basal lamina (bl) that separates the cells from the connective tissue (CT) layer. The apical region, here labeled with a novel monoclonal antibody, faces the lumen. LE- luminal epithelium layer. Bar = 25 µm. ⏎

6.5.1 Spherulocytes

Spherulocytes or morula cells are probably immune cells that are found throughout the animal; they are concentrated in the coelomic fluid and sparsely present in all of its organs. The presence of multiple vacuoles or spherules give them their distinctive name as well as their tendency to stain with histochemical dyes (**Figure 6.3A**). Our lab has described these cells as morula cells in an initial study using Milligan trichrome dye in *H. glaberrima* (García-Arrarás et al. 1998). These cells accounted for a small percentage (~14%) of the cells in the intestinal connective tissue and will be discussed in a separate section along with other coelomocytes.

6.5.2 Neuron-Like Cells

The *neuron-like cell* of the connective tissue was identified thanks to a monoclonal antibody (RN1) that labeled a neuronal component (**Figure 6.3B**) (Díaz-Balzac et al. 2007). (Prior to this, they were probably grouped under the mesenchymal cell category). The antigen that it recognized was initially unknown but has since been characterized as a START-domain containing protein (Rosado-Olivieri et al. 2017). These cells show small and somewhat elongated cell bodies and extensive bipolar or multipolar projections that ramify and form a network of fibers within the connective tissue. These projections, characterized as long, thin, varicose fibers, appear to extend to other intestinal layers, including the muscle and luminal epithelium. In addition, these cells and fibers are found within the

connective tissue of the mesentery, the body wall and of other organs. These cells probably correspond to the juxtaligamental cells that have been described in other echinoderm classes which have been shown to have multiple large electron-dense vesicles in their cytoplasm and that have been proposed to modulate connective tissue properties (Wilkie 1979; Welsch et al. 1995).

6.5.3 Mesenchymal Cells

The mesenchymal cells, referred to with different names ("fibrocytes", "fibroblasts", "lymphocytes" or "amoebocytes") and may correspond to several populations or functional stages (**Figure 6.3C**). They constitute about 80% of the connective tissue cells. In their chapter on digestive systems of the Holothuroidea, Feral and Massin (1982) refer only to two types of cells within the connective tissue (fibrocytes and coelomocytes). The term "fibrocyte" refers to most connective tissue cells while "coelomocyte" refers to the "morula" cells. In the study of echinoderm tissues, Smiley (1994) treats the connective tissue compartments in all holothurian organs as one. The cells are classified as a variety of mesenchymal cells that include fibroblasts (~10um diameter cells with a prominent nucleus with condensed heterochromatin, that secrete the connective tissue), scleroblasts (produce ossicles), and coelomocytes—these can also be found in the coelomic cavity or coelomic fluid.

In our early histological studies with Milligan trichrome dye in *H. glaberrima*, we categorized the mesenchymal connective tissue cells into lymphocytes and amoebocytes; roughly divided into 38% lymphocytes and 48% amoebocytes (García-Arrarás et al. 1998). These two mesenchymal cell types probably account for the "fibrocytes" described by Feral and Massin (1982). Similarly, in a study of the digestive tube of the sea

cucumber *E. fraudatrix*, Mashanov et al. (2004) described the cells within the connective tissue compartment as being morula cells, amoebocytes and fibroblasts. Once again, the mesenchymal cells in the connective tissue are separated into two populations.

Therefore, what is consistent in all experiments using morphological observations of holothurian intestinal connective tissue is the presence of a spherule-containing coelomocyte cells (sometimes referred to as "morula") and two mesenchymal cell types that varied in their description in terms of size and morphology.

Although most of these mesenchymal cells within the connective tissue of the intestine look similar there are hints to differences among them. For example, during intestinal regeneration, coelomic epithelial cells undergo epithelial-mesenchymal transitions (EMT) and ingress into the underlying connective tissue (García-Arrarás et al. 2011). Some of these cells migrate toward the forming lumen and establish themselves in the connective tissue that lies across the basal lamina from the luminal epithelium. These cells, because of their morphology and spatial location, are similar to myofibroblasts described in vertebrates, though no marker has been found to link them at the molecular level.

Similarly, other cell types appear during intestinal regeneration, such as cells named hemocytes (García-Arrarás et al. 1998). Other cells that have been highlighted during regenerations include phagocytic amebocytes (Candelaria et al. 2006) and spherulocytes (García-Arrarás et al. 2006). The latter are also probably coelomocytes, although a different type of coelomocyte (see coelomocyte section). Some of these cells will probably correspond to components of the immune system, (as discussed later in this chapter) while others might be developmental stages of a cellular

population. Future studies will be needed to clearly distinguish among these alternatives.

In this respect, our scRNA seq data from regenerating intestinal tissues provide some interesting insights (Medina-Feliciano et al. 2025). We identified two putative connective tissue cell populations that possibly correspond to different states of a cell type closely associated with ECM production and maintenance. These cells were characterized by the expression of *erg* (transcriptional regulator ETS- related gene), an oncogene that has been associated with mesenchymal cells in other echinoderms (Meyer et al. 2023; Tominaga et al. 2023). Other highly expressed genes in these cell populations were those involved in cellular/substrate adhesion. Thus, although the present data support the presence of some cell types in the connective tissue of regenerating intestines, it still remains unclear whether other subpopulations are present in normal intestinal connective tissue or how these populations, described by their transcriptional profile relate to those described traditionally by morphological and histochemical methods.

6.6 LUMINAL EPITHELIUM

The structure of the luminal epithelium is shared by all echinoderm species, with some variations (Jangoux 1982). Both Feral and Massin (1982) and Smiley (1994) in their comprehensive reviews of the holothurian anatomy describe two main types of luminal epithelia in their digestive tract. The first is ectodermal derived and is present in the most anterior (pharynx, esophagus and stomach) and most posterior (rectum and cloaca) digestive tract regions. The main type of cell present is T-shaped cells that are covered by a cuticle and share some morphological similarities to epidermal cells. Mucous-producing cells are interspersed with these cells. A later

study on *E. fraudatrix* confirms some of these findings, although it questions the embryological origin of the luminal epithelium, stating that its origin is endodermal and not ectodermal (Mashanov and Dolmatov 2004)

The second type of luminal epithelium, and the focus of our discussion, is that found in the intestinal divisions. This epithelium, whose main cell components are the intestinal enterocytes, is arguably the most important cell type of the digestive tract, being responsible for the absorption of nutrients from the ingested food. In addition to the enterocytes, the luminal epithelium also has mucous-producing cells, enteroendocrine cells, and immune system cells. These will all be discussed in the next sections. Although our work is centered around *H. glaberrima*, similar results have been documented in other species such as *Apostichopus japonicus* and *E. fraudatrix*. For instance, in *E. fraudatrix*, enterocytes with basal nuclei and mucocytes with granular content have been identified by electron microscopy (Mashanov et al. 2004). Similarly, in *C. schmeltzii,* studies have documented the presence of different types of enterocytes and granule-containing cells (Kamenev et al. 2013).

6.6.1 Enterocytes

Enterocytes constitute the main cell of the intestinal epithelium (Feral and Massin 1982). These are tall columnar cells that form a densely packed pseudostratified epithelium specialized in nutrient absorption (**Figure 6.3D**). The cells show a polarized morphology, with an apical surface characterized by numerous mitochondria and vacuoles, and the presence of microvilli, significantly increasing the absorption area (**Figure 6.4**). The basal region contains the cell nucleus and lies close to the basement membrane and to numerous neuronal fibers. Enterocytes attach to each other via zonula adherens forming an important barrier to the invasion of

pathogenic organisms from the lumen. Thus, these cells are crucial for the maintenance and restoration of normal digestive function in homeostatic and regenerative regeneration.

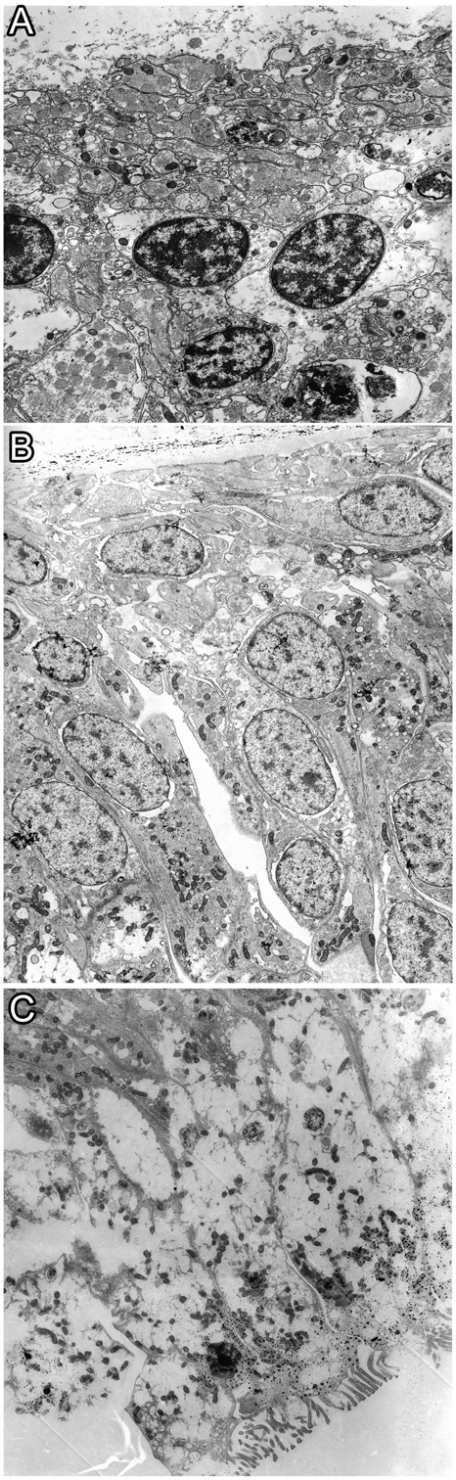


FIGURE 6.4 Electron micrograph showing the luminal epithelium of *Holothuria glaberrima* large intestine. Three different regions are shown in this cross section: (A) the basal lamina that separates the luminal epithelium from the connective tissue. A plexus of nerve fibers with different types of vesicles is found at the base of the enterocytes. This plexus is part of the luminal epithelium and does not cross into the connective tissue area. (B) the enterocyte basal region shows the accumulation of nuclei and the presence of some granules and mitochondria in the cell cytoplasm. (C) the apical region of the luminal epithelium is devoid of nuclei. The cells microvilli projecting into the luminal cavity can be observed. Large vesicles and abundant mitochondria are present in their cytoplasm. Bar = 5 μm. ⏎

Three enterocyte subtypes have been described in holothurians: vesicular enterocytes, granular enterocytes, and so-called "typical" enterocytes (Feral and Massin, 1982; Smiley 1994). However, the distinctions among these subtypes remain unclear, and they may represent different stages of maturation rather than truly distinct cell types. The terminology adds to the confusion, since Feral and Massin (1982) reported vesicular enterocytes as the most common cell type in the holothuroid gut—arguably making them the "typical" enterocytes. Moreover, the morphological descriptions of the vesicular and the "typical" subtypes show no major differences, suggesting that they likely represent the same cell type and exemplify the recurrent problem in echinoderm scientific literature of multiple names being applied by different authors to similar cells. Nonetheless, the description of vesicular and granular enterocyte subtypes is found in the description of many echinoderm species and even in juveniles soon after metamorphosis (Heinzeller and Welsch 1994; Dolmatov et al. 2016).

The main difference between the vesicular and granular enterocytes lies in the type of vesicles or granules found in their apical region. The vesicular enterocytes vesicles are thought to harbor mucous material, while the

granules in the granular enterocytes are thought to contain digestive enzymes (Smiley 1994).

A more comprehensive description of the holothurian luminal cells was made by Mashanov and Dolmatov (2004) in the species *E. fraudatrix*. They compared the luminal cells from different regions of the digestive tract. In the first descending region of the intestine, they described two different vesicular enterocytes, mainly by their staining to methylene blue and toluidine blue. One of the subtypes is only found in the anterior portion of the bulb. The main cells are tall columnar, with oblong or spindle-shaped nuclei located in the basal third of the epithelium and electron-lucent vesicles in their apical region. They have few microvilli and their mitochondria are distributed evenly throughout the cytoplasm. The cells of the ascending region of the small intestine are similar, but the supranuclear cytoplasm contains numerous large phagosomes, and mitochondria are found mainly in the basal part of the cell. Finally, cells in the second descending region (large intestine) also have the vesicular phenotype, contain microvilli (though less than those in the previous region) and the region above the nuclei is filled with large secretory vacuoles. Mitochondria are evenly distributed throughout the cytoplasm. Thus, what stands from Mashanov and Dolmatov's (2004) study is that the vesicular cell type is the one found in this species and that different enterocyte subtypes might be present in the different digestive tract compartments. Similar to what Mashanov and Dolmatov described in *E. fraudatrix*, the luminal epithelium of *H. glaberrima* appears to contain mainly the vesicular phenotype (**Figure 6.4**). In fact, we would like to suggest the possibility that the vesicular enterocyte is the main component of holothurian luminal epithelia and that what has been called the granular enterocyte might correspond to the

mucocytes (see next section) that have been described as a different cell type. Future studies will be able to define these cell populations correctly.

Some studies have shown that enterocytes can be characterized by specific molecular markers. In particular, a study using orthologs of mammalian intestinal stem cell markers found that *bmi1* and *lgr5* are abundantly expressed in the adult intestine of the sea cucumber *H. glaberrima* (Mashanov et al. 2017). Notably, the expression levels of these markers vary significantly across different regions of the digestive tract.

Unlike the mammalian intestine, where stem cells are localized within well-defined crypts, the luminal epithelium of the sea cucumber lacks such crypt structures. Instead, proliferating cells are dispersed throughout the tissue, with Bmi1+ and Lgr4/5/6+ cells interspersed among marker-negative cells (Mashanov et al. 2017).

In addition, the expression of the four Yamanaka factors has been examined in both normal and regenerating digestive tracts of *H. glaberrima*. Homologs of *myc, soxB1, oct1/2/11,* and *klf1/2/4* show differential expression along the length of the digestive tube (Mashanov et al. 2014). For instance, in the luminal epithelium of the intestine, homologs of *myc* and *soxB1* exhibit high expression, whereas *klf1/2/4* is expressed at lower levels. In contrast, the *oct1/2/11* homolog is strongly expressed only in certain luminal cells of the first descending region (anterior small intestine) but not in the ascending region (posterior small intestine).

These molecular findings highlight the heterogeneity of the luminal epithelial cells in the sea cucumber digestive tract—heterogeneity that cannot be discerned by morphology alone. This underscores the potential of using molecular markers and advanced technologies to further characterize distinct cell populations within and across different regions of the digestive system.

6.6.2 Mucocytes

Mucocytes are secretory cells that contain mucous in their expanded apical portion (**Figure 6.5A**). They have been studied mainly in se urchins (Holland and Ghiselin 1970). Some investigators have described these cells as similar to Goblet cells found in other species (Feral and Massin 1982). This mucous serves a protective function against pathogens and facilitates the passage of the intestinal content. In particular, the mucocytes are thought to play an important role in the secretion of mucus to form the fecal pellets.

6.6.3 Enteroendocrine Cells

Enteroendocrine cells are specialized in the secretion of hormones that regulate various intestinal functions, such as motility and enzyme secretion. These cells can be identified using immuno and cytochemical techniques, mainly those aimed at their neurotransmitter, neuropeptide or hormonal contents.

Two types of cells have been described to correspond to enteroendocrine cells (Díaz-Balzac et al. 2016). The first are elongated bipolar cells whose oval body transverses from the luminal cavity to the basal membrane (**Figure 6.5B**). The cell's nuclei are usually found in the middle to lower half of the cell, while immunocytochemical detection of the active substances is found within their cytoplasm. The second type of cell shows a round body, located close to or near the basal lamina and is usually unipolar. These cells have been shown to express the neurotransmitter GABA, and their processes are possibly the ones that form the nerve plexus that underlies the luminal epithelium.

Similar to other species, enteroendocrine cells have been found to express a large number of neuropeptides. A partial table of some of these

can be found in a previous publication (García-Arrarás et al. 2019). In recent years, more have been added to this list (see Tinoco et al. 2023; Cai et al. 2023; Huang et al. 2025). Most of the neuropeptide characterization and mapping have been the work of the Martinez lab and the Elphick lab, working on the sea star *A. rubens*. However, while the work is solid in showing the presence of the neuropeptide in the enteroendocrine cells of the luminal epithelium, it remains unclear if two or more neuropeptides are expressed in the same cell or if each neuropeptide is expressed by a specific cell population. Therefore, a full description of the different enteroendocrine populations characterized by their neuropeptide expression profile remains to be done.

6.7 LUMEN EPITHELIAL CELLS UNDERGO CHANGES DURING INTESTINAL REGENERATION

The intestinal lumen develops bidirectionally, from both the anterior and posterior ends, eventually merging to form a thin intestinal tube that will continue growing with time. Regardless of whether regeneration follows anterior or posterior evisceration, the luminal epithelium of the posterior anlage originates from the endoderm-derived epithelial lining of the cloaca (García-Arrarás et al. 1998; Mashanov et al. 2017). In the anterior anlage, however, two distinct modes of formation have been described. In posteriorly eviscerating holothurians, such as *H. glaberrima* and *A. japonicus*, the esophagus remains within the coelomic cavity after evisceration (García-Arrarás et al. 1998; Shukalyuk and Dolmatov 2001; Mashanov et al. 2014). In these species, the new intestinal lumen forms by the invasion of tubular remnants of the esophagus into the thickened connective tissue (Mashanov and García-Arrarás 2011). Conversely, in anteriorly eviscerating species, including *Eupentacta quinquesemita* and *E.*

fraudatrix, nearly the entire gut and the oral complex are expelled, leaving only the cloaca intact (Mashanov et al. 2005; Okada and Kondo 2019). In this case, the anterior mesenteric margin thickens to form the anlage, which becomes filled with mesenchymal cells. The luminal epithelium is subsequently derived from the proliferation and differentiation of cells from the esophageal mesothelium. In *E. fraudatrix* (Mashanov et al. 2005) the luminal epithelium arises from dedifferentiated mesothelial cells that migrate into the underlying connective tissue (Mashanov et al. 2005). A slightly different mechanism has been reported in a closely related species, *E. quinquesemita* (Okada and Kondo 2019). In this case, researchers did not observe mesothelial invaginations. Instead, they proposed that lumen formation occurs through a cavitation-like process, in which cells within the connective tissue of the intestinal rudiment aggregate and undergo a mesenchymal-to-epithelial transition (MET), eventually forming epithelial structures with internal cavities. These cavities later fuse to establish the main lumen of the anterior intestine (Okada and Kondo 2019).

What is particularly noteworthy is that in both *E. fraudatrix* and *E. quinquesemita*, the luminal epithelium—normally an endoderm-derived tissue—is regenerated from cells of mesodermal origin, whether mesothelial or connective tissue cells. Crinoids have also been documented to regenerate their digestive tract via a transdifferentiation mechanism. However, in this case the precursor cells are the juxtaligamental cells found in the connective tissue. These cells were observed to transdifferentiate by MET to form the luminal epithelium (Kalacheva et al. 2017).

Molecular changes accompanying shifts in cell populations during regeneration of the luminal epithelium have also been observed (Medina-Feliciano and García-Arrarás 202; Su et al. 2022.). A study investigating the role of homologs of the four Yamanaka factors during digestive tract

regeneration in *H. glaberrima* revealed differential expression patterns. While the expression of these factors is not synchronized at bulk mRNA levels of the whole regenerating structure, in the luminal epithelium, all four factors show strong expression (by in situ hybridization), coinciding with elevated cell proliferation (Mashanov et al. 2015).

Following the formation of the luminal epithelium, neuroendocrine cells have been found to appear soon after lumen formation (Tossas et al. 2014). These findings indicate that intestinal enteroendocrine cells not only regenerate but also emerge at very early stages during the formation of the luminal epithelium. However, the results suggest that the different subpopulations of neuroendocrine cells do not appear simultaneously but rather arise at distinct phases of the regenerative process: the first to appear are those identified with the anti-calbindin antibody, followed by cells expressing GFSKLYamide, and the last subpopulation to emerge corresponds to cells labeled with the anti-Pax6 marker.

The cellular origins of the luminal epithelium warrant further investigation. While enterocytes are generally believed to arise from proliferating progenitor cells within the epithelium, the lineage and origin of enteroendocrine cells remain poorly understood. Even less is known about the emergence and differentiation of mucocytes during intestinal regeneration. These unresolved questions are likely to be clarified through future single-cell RNA sequencing (scRNA-seq) studies.

In conclusion, the digestive epithelium of holothurians during regeneration consists of a variety of cell types that play complementary and essential roles in restoring intestinal function. The regeneration of this epithelium represents an excellent model for studying cellular plasticity, epithelial differentiation, and the functional organization of complex tissues. Furthermore, comparisons between different *Holothuroidea* species

reveal common patterns in intestinal regeneration, suggesting evolutionarily conserved mechanisms within this phylum of echinoderms.

6.8 ENTERIC NERVOUS SYSTEM

The anatomical and molecular description of the enteric nervous system of echinoderms has been extensively studied, in part due to our research on *H. glaberrima*. Our laboratory has performed neurohistochemical analyses demonstrating that the holothurian enteric nervous system can be divided into four components, initially named: the mesothelial plexus, the basiepithelial plexus, the connective tissue plexus, and the neuroendocrine component (García-Arrarás et al. 1999, 2001; Díaz-Balzac et al. 2007, 2010, 2014, 2016; Tossas et al. 2014, Nieves-Rios et al. 2020). Initial descriptions were based on the anatomical location, distribution, and morphology of neuronal fibers using classical immunohistochemical markers. Over time, the application of new molecular markers refined these observations, confirming the existence of distinct neuronal populations and revealing greater cellular and functional complexity within each plexus. Two of these cell populations have already been described: the neuronal-like cells in the intestinal connective tissue and the enteroendocrine cells associated with the luminal epithelium. This section serves to characterize the remaining two components, in terms of the morphology and molecular expression of their cells, highlighting how our knowledge has evolved over decades of research.

6.8.1 MESOTHELIAL PLEXUS

This plexus, has been referred to by many names, including the "hyponeural" (Jangoux 1982), "subcoelomic" plexus (Martínez et al. 1993), "intestinal" plexus (García-Arrarás et al. 1991), "coelothelial" or "outer"

plexus (Heinzeller and Welsch 1994), and “muscular”, “visceral” or “serosal” plexus (García-Arrarás et al. 1999; Byrne 2001). Morphologically, this plexus is distinguished by a dense network of thick compact fibers in the mesothelium that are associated with both the coelomic epithelium and the muscular layers of the viscera (**Figure 6.5C,D**, see also **Figure 6.2**). It contains isolated neuronal somata that typically exhibit a rounded to slightly ovoid shape. The fiber bundles are notably thick and compact, often interwoven with muscle fibers, which implies a significant role in motor control and the integration of visceral functions. These neurons not only innervate the adjacent and underlying muscle cells but probably some of their fibers include those observed within the connective tissue and luminal epithelium. Some researchers have speculated that the neurons in this plexus originate from the coelomic epithelium overlying the plexus (Vandenspiegel et al. 2000).

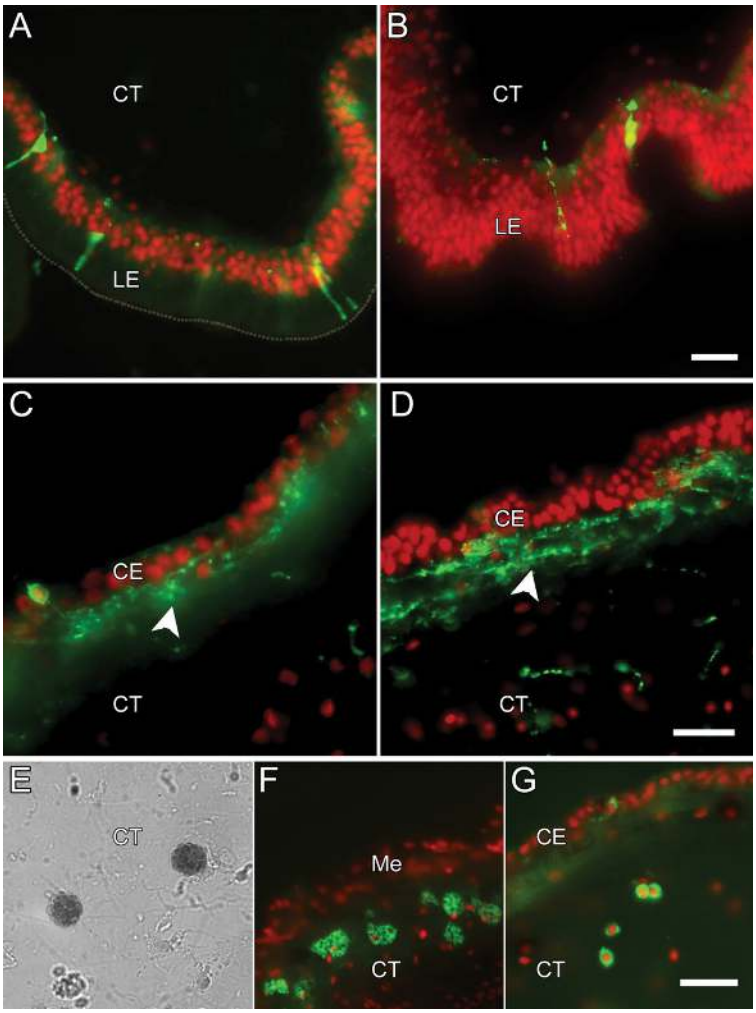


FIGURE 6.5 Mucocytes and enteroendocrine cells in the luminal epithelium layer. A cross section of *Holothuria glaberrima* large intestine labeled with (A) a novel monoclonal antibody that recognizes mucocytes and (B) an antibody against the neuropeptide GFSKLYFa (see Díaz-Miranda et al. 1995). The cells' nuclei are labeled with DAPI (red). Notice how the extensions of the mucocytes extend to the apical region of the luminal epithelium (dashed lines) and face the luminal space (L). Extensions of the enteroendocrine cells are found in the basal region of the luminal epithelium where they form part of the plexus shown in (A). (C, D) Neuronal plexus associated with the mesothelial layer. Histological section of the large intestine showing (C) a neuropeptide-expressing neuron (arrow) and (D) the fiber plexus (arrowhead) that innervate the mesothelial muscles. Neurons and fibers are labeled with an antibody against the neuropeptide GFSKLYFa (see Díaz-Miranda et al. 1995), while the cell nuclei are labeled with DAPI (red). (E, G) Coelomocytes

(immune cells) within the intestinal connective tissue. Free-roaming cells that constitute the holothurian immune system are found within the large intestine of *Holothuria glaberrima*. Different types of coelomocytes have been described. These include spherule-containing cells that can be identified by (E) histochemical dyes in light microscopy (in this case by toluidine blue) or (F) by monoclonal antibodies (green) (see García-Arrarás et al. 2006). Other cell types, such as (G) small round cells, named as lymphocytes are labeled by the presence of fibrinogen mRNA (see Medina-Feliciano et al. 2025). Me-Mesothelial layer, CT-Connective tissue layer. DAPI- red. Bar = 25 µm in all, except E-F = 40µm. ⏎

Early characterization of this plexus identified immunoreactivity to several neuropeptides, including GFSKLYFamide, galanin, and cholecystokinin (García-Arrarás et al. 1991, Díaz-Miranda et al. 1995, 1996). While many of these peptides have not been fully characterized as to their gene or peptide sequence, studies by Maurice Elphick and his colleagues provide important context for understanding neuropeptide diversity among echinoderms. For instance, SALMFamide peptides, first identified in *A. rubens* (Elphick et al. 1991), were later found to be localized in cells and fibers within the digestive tract, including the basiepithelial plexus (Moore and Thorndyke 1993). Similarly, GFSKLYFamide belongs to the same neuropeptide family and has been identified in holothurians in both serosal and connective tissue layers (Díaz-Miranda et al. 1995). It induces relaxation of the intestine and the longitudinal muscle in the body wall of the sea cucumber. In 1999, Inoue and colleagues described *A. japonicus* NGIWYamide-positive cells in the intestinal mucosa and mesentery, which were initially misidentified as part of the basiepithelial plexus (Inoue et al. 1999). Additional neuroactive substances include fibers or cells labeled with anti-tyrosine hydroxylase (TH), anti-GABA, and anti-phosphate histone H3 (PH3), indicating the presence of excitatory, inhibitory and dividing cells and highlighting the

molecular diversity of this plexus. The large number of neurotransmitters/neuropeptides described in this plexus needs to be analyzed in terms of cells expressing one or more of these neuroactive substances in order to describe the neuronal populations that are present.

In 2016, a more refined molecular characterization was achieved. RN1 (the antibody that recognizes the STARD molecule) and an antibody against β-tubulin were found to extensively label the large nerve bundles within the mesothelial plexus (Díaz-Balzac et al. 2016). Additional markers including pax6 and nurr1, which are associated with neuronal progenitors and dopaminergic lineages, were used to identify previously undescribed motor neuron-like cells near the muscle layer. These cells were circular to slightly elongated and were morphologically distinct from the earlier neuropeptide-positive populations. Calbindin-immunoreactive cells exhibited intermediate labeling, and some co-expressed RN1, further confirming neuronal identity. These findings suggest that this plexus includes multiple distinct neuronal populations, some of which may be functionally analogous to motor neurons in the vertebrate myenteric plexus.

6.8.2 Basiepithelial Plexus

While the mesothelial plexus has been best described in holothurians, the best-described nervous component of the echinoderm enteric nervous system is likely the basiepithelial plexus. Morphologically, the basiepithelial plexus is formed by abundant longitudinal fibers and a few scattered neuronal cell bodies. This plexus is prominent in asteroids and echinoids, forming an extensive nervous layer adjacent to the luminal epithelium (Hyman 1955). In most echinoderm groups, the basiepithelial plexus is situated directly at the base of the luminal epithelium, as originally described by Chia and Koss (1994). In contrast, in holothurians like *H.*

glaberrima and *Holothuria mexicana* this plexus is found deeper, within the connective tissue, beneath the basement membrane of the muscle layer (Díaz-Balzac et al. 2010). This distinct localization reflects significant histological differences among echinoderm groups and underscores the importance of species-specific anatomical characterization.

Histochemical and immunohistochemical studies identified catecholaminergic cells and fibers in this plexus, particularly in the esophagus of *Heliocidaris erythrogramma* (Cobb and Raymond 1979) and in the stomach of *A. rubens* (Cottrell and Pentreath 1970). The anterior digestive tract has also been described as having GABA-immunoreactive fibers and unipolar neurons (Newman and Thorndyke 1994). Additionally, GFSKLYFamide-positive fibers and cells were described in the basiepithelial plexus of *H. glaberrima* (Díaz-Miranda et al. 1995).

Work on other echinoderms provided further insights into neuropeptide diversity in this plexus. SALMFamide peptides, including S1, were shown to be expressed in basiepithelial neurons of *A. rubens* (Moore and Thorndyke 1993). In *M. glacialis*, adrenomedullin-like immunoreactivity was localized to this plexus in the cardiac and pyloric stomachs and the pyloric caeca but was absent in the intestine and rectal caec Martínez et al. (1989). Other neuropeptides, including pancreatic polypeptide, PYY, α-MSH, and the enzyme nitric oxide synthase, were also found in fibers of this plexus (Martínez et al. 1996), indicating functional neurochemical complexity. Later, we confirmed in *H. glaberrima* the anterior localization of this plexus and its continuity with the central nerve ring using RN1 and calbindin.

An interesting characteristic of the basiepithelial plexus was the restricted localization found in *H. glaberrima* (Díaz-Balzac et al. 2010). This plexus was only found in the anterior regions of the digestive tract, and was not

present in the second descending region of the intestine, highlighting the importance of regionalization of the digestive system and the fact that histological characteristics cannot be extrapolated from one region to another consistent with patterns described in *M. glacialis* (Martínez et al. 1989).

Recent single-cell transcriptomic studies in the sea urchin *Strongylocentrotus purpuratus* larvae have expanded our understanding of gut-associated neurogenesis in echinoderms (Paganos et al. 2021). Neural progenitor cells expressing the transcription factor soxC, which regulate neuronal differentiation and survival, were detected in the ciliary band and gut regions during late gastrula and early larvae before metamorphosis. Whereas *brn1/2/4* expression was detected in post-mitotic neuronal precursors that followed *soxC* expression, appearing after progenitor commitment at late gastrula. Additionally, mature neurons expressing *elav*, an RNA-binding protein essential for neuronal mRNA regulation, were found within the esophagus co-expressing neuropeptides such as SALMF and NGFFF (Paganos et al. 2021). This evidence shows the presence of distinct neurogenic domains within the intestinal plexus of echinoderms and highlights its evolutionary conservation that remains to be further characterized in holothurians.

The enteric nervous system has also been studied during intestinal regeneration (García-Arrarás et al. 1999, Tossas et al. 2014. Intestinal re-innervation has been described, showing the appearance of the new cells and fibers. However, no new cell phenotype that is distinct to the regenerating intestine was found.

In summary, the enteric nervous system components of holothurians have been mainly characterized in terms of the localization of the neurons and on their neurotransmitter or neuropeptide content. In many ways, this is similar

to what has taken place in other animal groups. However, little is known about whether neurons within specific layers or plexuses share distinct genomic signatures or express common protein profiles. Neither is it known if their roles are motor versus sensory. Furthermore, the existence and characterization of enteric glial cells in echinoderms remain entirely unexplored. Therefore, an in depth understanding of the enteric nervous system is warranted.

6.9 COELOMOCYTES

Coelomocytes are a morphologically diverse group of "free-roaming" cells found in the coelomic fluid of all echinoderms (**Figure 6.5E–G**). These cells form the cellular component of the echinoderm immune system. Although traditionally associated with the coelomic fluid, coelomocytes are also abundant in various tissues (Hyman 1955; Smith et al. 2018) and have been implicated in functions beyond immunity, such as wound healing and regeneration (Vazzana et al. 2018).

Due to the relative ease of isolation, coelomocytes are among the best-characterized cell types in echinoderms. However, their classification has been historically inconsistent and traditionally done via morphological analysis. Researchers have used different names for the same cell types across species or echinoderm classes, while in other cases, distinct cell types have been referred to by the same name. These discrepancies, along with methodological variations, such as differences in cell isolation, fixation protocols, staining dyes, and even environmental factors like temperature, seasonality, or the physiological state of the specimens, combined with a lack of consensus and comprehension regarding the role of each cell type, have made comparative studies particularly challenging (Queiroz et al. 2022).

Efforts to improve coelomocyte classification have been undertaken in various echinoderm groups (e.g., sea urchins and others). Recently, Queiroz and Custódio (2024) proposed a refined classification of holothurian coelomocytes based on a multidisciplinary approach that includes morphological, cytochemical, and functional criteria. Their comprehensive review offers a framework that may provide a deeper understanding of coelomocyte diversity and function.

In Holothuroidea, eleven primary coelomocyte types have been described: phagocytes, spherule cells, fusiform cells, crystal cells, giant cells, vibratile cells, lymphocytes, hemocytes, brown bodies, progenitor cells, and minute corpuscles. With the exception of the last three types, all have been reported in *H. glaberrima*. As in other holothurians, coelomocytes in *H. glaberrima* have been documented in multiple tissues, including the body wall, tentacles, respiratory tree, hemal system, hydrovascular system, digestive tract, and gonads. A consistent feature across tissues is their close association with connective tissue layers.

Determining whether cells described within an organ originate from the freely circulating coelomic fluid remains challenging. In our study, such cells could have infiltrated the digestive tract tissues, where they may be engaged in immune-related or other physiological functions. Notably, four of the eight coelomocyte types reported in *H. glaberrima* have been specifically associated with the digestive system. For clarity, we will follow the classification provided by Queiroz and Custódio (2024) in describing them.

6.9.1 Spherulocytes

These cells have received various names, including: amoebocytes with spherules, morula cells, and granulocytes (**Figure 6.5E,F**). They are

typically large, ranging from 8 to 20 μm in diameter and distinguished by a cytoplasm filled with many vacuoles of various sizes. Their nucleus is often not visible in live preparations and tends to be eccentrically located (Queiroz and Custódio 2024). Histological staining has been critical for their identification, with dyes such as methylene blue and toluidine blue. However, different staining techniques may label distinct subpopulations or developmental stages of these cells, making their classification inconsistent. For instance, in the intestinal tissue of *H. glaberrima*, some spherule-containing cells react with specific dyes while others do not (García-Arrarás et al. 2006). Thus, multiple subpopulations have been described in various species, sometimes labeled as types I, II, or III spherule cells based on morphology and staining behavior. Spherule cells have been implicated in a wide array of functions, including cytotoxic activities, participation in brown-body formation, wound healing, graft rejection, and potentially collagen biosynthesis. Their numbers increase markedly following immune challenges or during regenerative processes, highlighting their importance in holothuroid physiology.

Spherulocytes are found within the intestinal tissue of holothurians. They are usually associated with the connective tissue and correspond to about 15% of the cells in the tissue, as characterized using histological dyes (García-Arrarás et al. 1998). Spherulocytes have also been labelled with monoclonal antibodies, and have been observed migrating into the regenerating tissues and possibly playing important roles in ECM deposition (Cabrera-Serrano and García-Arrarás 2004; García-Arrarás et al. 2006). As stated above, different populations of cells have been described using various dyes, however, it remains unclear whether these populations correspond to different cell types or different cell stages. Similarly, an electron microscope study performed in *H. glaberrima* described three

different spherule-containing cell types in intestinal tissues that differ in their types of vesicles (Candelaria-Suria 2005). Interestingly, two of them were found in normal and regenerating intestines while the third one was only present in the intestines of regenerating animals.

6.9.2 Phagocytes

Phagocytes are active amoeboid cells. In the scientific literature, they are called various names: Phagocytic amoebocytes, cells with long pseudopods, hyaline amoeboid corpuscules, and bladder amoebocytes (Hyman 1955). They have a large round and central nucleus, and their size ranges from 8 to 50 μm in diameter. They have a varying appearance depending on their cytoplasm that divides phagocytes into filopodial or petaloid subtypes; however, these subtypes are considered as different stages of the same cell. The known functions of phagocytes are elimination of dead cells and foreign particles, clotting, coelomocyte aggregation, and wound repair. In the regenerating intestinal tissues of holothuroids, phagocytes have been observed in the process of phagocytizing and/or degrading ingested cellular and ECM debris, such as the spindle-like structures of dedifferentiating muscle and the extracellular collagen matrix (García-Arrarás et al. 2019; Candelaria et al. 2006). However, it remains to be clearly shown that these phagocytic cells within the regenerating intestinal tissues, originate from coelomic fluid coelomocytes or other immune-associated cell types.

6.9.3 Lymphocytes

Also called progenitor cells, lymphocytes are small, round, cells, typically measuring 3–8 μm in diameter, with a large nucleus-to-cytoplasm ratio. Their name is a misnomer since they are probably not related to the vertebrate immune cells. In addition, there is little evidence that they are

progenitor cells. Moreover, their lack of salient features, other than their small size and little cytoplasm, makes it so that probably many different cell types are included within this cell category. Nonetheless, our recent characterization of a cell population expressing immune-related genes, specifically a fibrinogen C domain-containing protein, provides some evidence of a lymphocyte cell within the intestinal tissues (**Figure 6.5G**) (Medina-Feliciano et al. 2025). In this case, the cells expressing this mRNA are found in the normal and regenerating intestine, in most other organs, as well as in the coelomic fluid, suggesting that at least a population of coelomocytes with lymphocyte-like features is found within the intestinal tissues.

6.9.4 Hemocytes

Cells described as hemocytes in *H. glaberrima* are the only ones that do not conform to the classification of Queiroz and Custódio (2024). For these authors, hemocytes show variable features including different sizes and shapes. However, a salient feature of these cells is that they are colored due to the presence of hemoglobin. In *H. glaberrima* we named as hemocytes a cell type only found in the regenerating intestine (García-Arrarás et al. 1998). These were circular or oval cells of ~9 um in diameter. When stained with Milligan trichrome dye, they showed a central red-stained nucleus and clear lightly stained cytoplasm. Whether these cells are a distinct cell population or are developmental or physiological stages of other populations remains to be determined.

6.10 CONCLUSIONS

Previous studies of holothurian digestive tracts have identified roughly a dozen distinct cell populations (**Figure 6.6**). These cell types were

characterized primarily through classical histological methods, including light and electron microscopy, histochemical staining, immunocytochemistry, and, in some cases, in situ RNA hybridization. Based on these approaches, the cells can be broadly grouped into seven major categories: coelomic epithelium, muscle cells, neurons, mesenchymal cells, enteroendocrine cells, coelomocytes, and enterocytes. Many of these cell types undergo marked phenotypic changes during regeneration. In addition, novel or transient cell populations may emerge, reflecting the dynamic shifts in cellular identity and function that accompany the regenerative process.

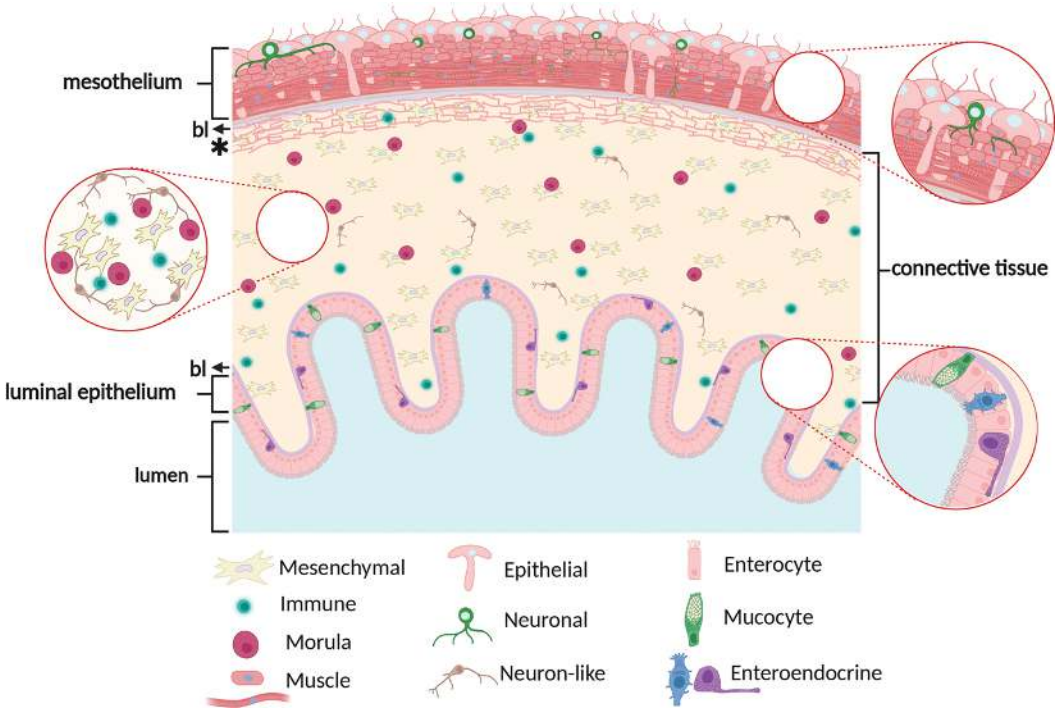


FIGURE 6.6 Cellular phenotypes of *Holothuria glaberrima* large intestine. Diagram showing the various cell populations that can be presently identified in the mesothelium, connective tissue, and luminal epithelium layers of the sea cucumber. Note the presence of basal lamina separating both epithelia layers from the connective tissue layers. *asterisk serves to highlight the large component of extracellular matrix, including collagen that is found in the connective tissue area close to the

mesothelium. At present, close to a dozen cell types can be identified in the large intestine. This number is likely to increase as neurons and enteroendocrine cells are classified according to their neurotransmitter or hormone content. Likewise, future studies will probably find divisions among the connective tissue mesenchymal cells and immune cells. ⏎

Equally important is the relationship of these adult cell types to those in the embryo, where far more information is available. In echinoderms, the sea urchin has long served as a model for reconstructing gene regulatory networks governing embryonic development and cell differentiation (Annunziata et al. 2019). Comparing embryonic and adult cell populations raises compelling questions: Are the same cell types found in both regenerating and embryonic stages? Where are the insulin-like peptide–expressing cells identified in the larval gut (Perillo et al. 2018) located in the adult? Do specialized endocrine-like populations exist within specific regions of the adult intestine?

With the advent of single-cell RNA sequencing (scRNA-seq) and single-nucleus RNA sequencing (snRNA-seq), the holothurian digestive system is entering a new era of cellular and molecular resolution. These technologies are expected to reveal previously unrecognized cell types, uncover transcriptional differences linked to distinct regions of the digestive tract or spatial tissue organization, and—most intriguingly—clarify lineage relationships between resident, dedifferentiated, and regenerating cells. Together, these insights will deepen our understanding of the cellular complexity and plasticity that underlie intestinal regeneration in holothurians.

Nonetheless, the biggest impact of these new technologies will be the characterization of cellular orthologs. Identifying orthologs is a cornerstone of comparative biology. As new technologies advance, their greatest impact will be in enabling deeper and more precise comparative studies. By

determining cellular orthologs across species, we can uncover conserved cellular functions, trace genetic pathways preserved through evolution, and gain critical insight into the history and divergence of life. This approach not only bridges biological findings across species but also enhances our understanding of human biology through model organisms, ultimately revealing the shared molecular toolkit that underpins all life.

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Index

Absorptive cells, 48, 52, 104, 107, 121

Acinar cells, 47, 50, 52, 55, 57–58, 60, 75

Acoela, 103–104

Acoelomorpha, 1, 21, 103

Adaptive immunity, 117, 122

Alimentary canal, 4, 8, 19–20, 87–88

Ambulacraria, 2, 8, 49, 81

Amphioxus, 7–8, 12, 14, 79

Apostichopus japonicus, 158

Archenteron, 1–2, 8–10, 16–19, 50

Asterias rubens, 80, 152

Asteroidea, 172

Bacteria, 41, 43, 82, 109, 118–124, 126–128, 130–131, 133

Basal lamina, 151–153, 156–157, 160, 163, 171

Blastopore, 1–2, 4, 10, 17–18, 21–22, 49

Bmp, 4–7, 17, 46

Brachyury, 9, 17, 20, 23, 104, 107–108

Brain–gut axis, 15, 71, 88

Caenorhabditis elegans, 23, 85

Cdx, 4–5, 7–10, 12, 17, 20

Cell differentiation, 55, 74, 88, 170

Cell fate, 6, 58, 81

Cephalochordates, 2, 53, 58, 78

Choanocytes, 41, 58

Chordata, 49, 52–53, 56

Ciona intestinalis, 7, 80

Cnidaria, 1–2, 16, 18, 21, 43–44, 56, 58, 60, 77, 86, 89, 97, 99–102, 117, 121, 125, 134

Coelomic epithelium, 149–155, 165, 170

Coelomocytes, 85, 124, 155, 157, 162, 168–170, 172

Collagen, 149, 155, 169, 171

Colon, 14, 72, 126, 128, 130–131, 133, 135

Commensal bacteria, 130

Connective tissue, 12, 40, 149–150, 153–158, 160, 162–165, 168–169, 171

Ctenophora, 43–45, 56, 58, 77, 97

Deuterostomes, 1–2, 17, 21, 49, 58, 78, 88–89

Digestive enzymes, 38, 40, 43–48, 52–54, 60, 74, 78, 83, 101, 104, 159

Duodenum, 5, 55, 72–74

Echinodermata, 49, 56, 172

Echinoidea, 172

Endocrine cells, 2, 8, 15–16, 47, 56, 72–84, 86–89, 120–121, 135, 158, 161–165, 170

Endoderm, 2, 4–10, 16–17, 19, 21, 23, 40, 45–49, 52, 54–56, 60, 72, 74, 81, 85, 87, 89, 97, 101–102, 105–106, 158, 163–164

Enteric nervous system, 15, 118, 120, 134, 165–167

Enterocytes, 47, 72–73, 83, 89, 121, 130, 156, 158–161, 164, 170

Enteroendocrine cells, 2, 15–16, 47, 72–74, 120–121, 135, 161–165, 170

Epithelial cells, 9, 37, 40, 43–44, 47–49, 52, 55–56, 58, 72, 84, 99–100, 119–123, 125, 127–130, 149–154, 157, 161, 163

Epithelial-mesenchymal transition, 157

Esophagus, 1, 4, 6–7, 9–10, 12, 14–15, 50, 52–53, 80, 84, 98–99, 108–109, 149–150, 158, 163, 166–167

Eupentacta quinquesemita, 163, 172

Evisceration, 154–155, 163, 172

Exocrine cells, 37–43, 45, 47–49, 51–59, 77

Extracellular digestion, 2, 37, 40, 43–45, 49, 58–59, 97

Extracellular matrix, 99–103, 106, 108–109, 171

Fgf, 4–6, 46, 55, 74

Foregut, 4, 6, 9, 14, 19–21, 45, 47, 50, 54–55, 74, 83, 97, 134

FoxA, 5, 9–10, 17, 19, 21, 23, 81, 89

Gastrodermis, 99–102, 106

Gastrointestinal tract, 119, 123–124, 129, 135

Gastrulation, 1, 4, 6–7, 9–10, 17, 23, 40, 44–45, 50

Gata4 19, 21, 48, 52–53, 55

Gata6 5, 50, 81

Gene regulatory network, 1, 40, 88, 170

Germ layers, 40, 97, 109

Gland cells, 43–44, 83, 86, 97, 101, 104, 106

Gut microbiome, 127, 131–132

Gut patterning, 1–3, 5–13, 15, 17, 19, 21, 23

Hedgehog, 5, 46, 55, 74

Hepatic caecum, 8, 52–53

Hepatopancreas, 7, 46, 48, 50, 53, 84–85

Hindgut, 4, 7–10, 12, 14, 16, 19–21, 45, 47, 50, 52–53, 79, 82–83, 103–104, 107, 109, 134

Hnf1 4, 44, 50, 52–53, 55–56

Hnf4 4, 10, 19–20, 44–46, 48, 50, 52–53, 55–57

Holothuria glaberrima, 148–149, 156, 162, 171–172

Holothuroidea, 157, 164, 168

Homeostasis, 47, 54–55, 71–72, 75–76, 82, 118–119, 122–123, 125–127, 130, 133–134

Hox genes, 5, 7–10, 16–17, 20

Hydra, 84, 86, 89, 102, 125

Hydrozoa, 99–101

Immune cells, 119, 122–125, 127, 129–130, 132, 152, 155, 162, 170–171

Immune system, 117–119, 122–127, 129, 131, 134–135, 156, 158, 162, 168

Innate immunity, 117, 122, 132

Insulin, 5, 8, 53–54, 60, 74–76, 78–86, 88–89, 171

Insulin-like peptide, 8, 53, 79, 81, 83–86, 88, 171

Intestinal regeneration, 150, 153, 157–158, 163–164, 171–172

Intestine, 1, 4–7, 9–10, 12, 14–15, 17, 44, 47, 50, 52–53, 72–74, 79–81, 84–85, 103–108, 119, 125, 129, 148–162, 164, 166–167, 169–172

Jak/Stat, 5

Liver, 1, 4–6, 8, 37, 47, 52–55, 75–76, 80, 82, 106

Lumen, 12–13, 15, 38, 46, 48–49, 52–53, 72–73, 82–83, 101, 104, 121, 129–130, 149, 156–157, 159, 163–164

Lymphocytes, 124–125, 157, 162, 168, 170

Macrophages, 122–125, 127, 129

Marthasterias glacialis, 80, 152

Mesenchymal cells, 15, 154–155, 157–158, 163, 170–171

Mesenchyme, 4

Mesentery, 150, 152–154, 157, 166, 172

Mesoderm, 1–2, 4–5, 9, 16–19, 40, 55, 74, 79, 83, 150, 164

Metamorphosis, 3, 7, 10, 49, 53, 81, 159, 167

Microbiome, 116, 118, 127–129, 131–132

Microbiota, 118–119, 121–122, 125–128, 131–136

Midgut, 4, 7–10, 12, 14, 19–21, 45, 47–48, 50–53, 76, 79, 82–83, 88–89, 109, 121–122, 134

Mist1 4

Mnx, *4*, 7, 81, 88

Mucocytes, 158, 161–162

Mucosa, 4, 12, 15, 56, 72, 84, 118–119, 122–124, 126–130, 132, 134–136, 149, 166

Mucus, 38, 44, 54, 56, 64, 98, 104, 118–122, 130, 133–135, 161

Muscle cells, 77, 150, 152–154, 165, 170

Myoepithelial cells, 9, 99, 153–154

Nematostella, 2, 86, 89

Nematostella vectensis, 86

Neurons, 15–16, 19, 77, 81, 84, 86–89, 134–135, 150, 162, 165–167, 170–171

Neuropeptides, 16, 72, 78, 87–89, 135, 163, 165–167

Nkx2.1 4

Nkx2.2 4, 81, 89

Nodal, 4–6

Notch, 5, 7, 41, 50, 55, 74, 89, 94

Ophiuroidea, 172

Organoids, 6

Pancreas, 4–8, 37, 46, 48, 50, 52–55, 58, 60, 72, 74–77, 79, 81, 84–85, 88–89

Patiria miniata, 51

Pax6 4, 17, 88, 164, 166

Pdx1 4–5, 7–10, 12, 17, 20, 55, 60, 74, 79, 81–82, 88

Phagocytes, 168–169

Phagocytosis, 41, 77, 104, 124–125

Pharynx, 6–8, 10, 14, 44–45, 50, 52–53, 86, 98–99, 101, 103–109, 158

Placozoa, 1, 40, 43, 58, 86

Porifera, 40–41, 43, 56, 58, 89

Protostomes, 1, 17–21, 46, 50, 82

Ptf1a, 4, 7, 48, 50, 52–53, 55, 57–58, 60, 74

Ptychodera flava, 10, 81

Pyloric caeca, 50, 80, 167

Pyloric sphincter, 5, 9–10, 13–14, 16–17, 81

Rbpj, 4, 41, 50, 55, 57–58

Regeneration, 86, 148–155, 157–159, 161, 163–165, 167–172

Regionalization, 1, 9, 40, 48–49, 58, 97, 167

Retinoic acid, 5, 125

Sea cucumber, 8, 49, 148, 152–154, 157, 161, 166, 171–172

Sea star, 8, 49–51, 80, 88, 163

Sea urchin, 8–12, 16–17, 23, 49–52, 60, 80–81, 88, 167–168, 170

Secretory cells, 44, 48, 86–87, 121, 161

Single-cell RNA sequencing, 164, 171

Single-nucleus RNA sequencing, 155, 171

Sox2, 4, 7–8

Spherulocytes, 155–156, 158, 169

Sphincter, 5, 7–10, 12–17, 81, 106

Sponges, 1–2, 40–41, 43, 58, 117

Stem cells, 6, 22–23, 47, 71–72, 119–123, 148, 150, 156, 158, 161

Stichopus japonicus, 158

Stomach, 1, 4–7, 9–10, 12, 14, 17, 44–45, 50–51, 53, 56, 72, 74–75, 80–81, 84, 98–99, 101–102, 158, 166–167

Strongylocentrotus purpuratus, 11, 167

Symbiosis, 128, 130–132, 136

Tgfβ, 7

Tgif2, 4

Through-gut, 14, 40, 44–45, 58, 97, 99, 101, 103, 105, 107, 109, 116–119, 124, 134

Transcriptomics, 6, 22, 38, 41, 44, 50, 81, 86

Transdifferentiation, 148, 164

Tunicates, 2, 6–8, 12, 53, 128

Vertebrates, 1–2, 4, 6–10, 12, 14–17, 49, 54–59, 72, 76–79, 82, 84, 88, 117, 119–124, 126–129, 134, 136, 150, 153, 157, 172

Villi, 47, 72–73, 78, 82, 99, 103, 108–109, 120, 150, 159–160

Wnt, 4–7, 9, 17, 46, 55, 74

Wnt10, 9

Wound healing, 168–169

Xbp1, 4, 47–48, 50, 53, 55, 57

Xenacoelomorpha, 1, 21, 103

Zebrafish, 53, 89, 121