Evolutionary Origin and Developmental Relationships of Germline, Gonads, and Embryonic Mesodermal Precursors

Petra Kováčiková

Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
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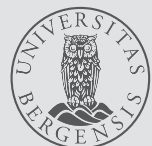

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Scientific environment

The work presented in this thesis was conducted in the laboratory of Prof. Dr. Hejnol, at Sars International Centre for Marine Molecular Biology, University of Bergen, Norway and Friedrich Schiller University of Jena, Germany. The thesis is part of the PhD program of the Department of Biological Sciences of the University of Bergen.

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We say in science that in all our research we stand on the shoulders of giants (great researchers of the past). Here are some giants of today, on whose shoulders this thesis was built.

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Abstract in English

Germ cells, oocytes and sperm, are essential cell types for species propagation in all sexually reproducing animals. Their differentiation can proceed by two different mechanisms: inheritance and induction. In this thesis, I investigate the germ cell origin and specification mechanism in a neglected group of Xenacoelomorpha — Nemertodermatida. Studying nemertodermatid worm *Meara stichopi*, I detected a previously undescribed population of neoblast-like cells which enable this animal a limited regenerative capacity and which I propose to serve as a cellular source for germ cell induction. Focusing on the regeneration part, I probed *M. stichopi*'s wound healing process in post-oviposition and post-amputation. This revealed wound-induced cell proliferation and expression of evolutionarily conserved transcription factors *egr* and *runt*. In the second part, I use the collected single-cell transcriptomic data from hatchling, juvenile, and adult stage to show molecular differentiation of neoblasts into major cell types and into germ cells.

Abstract in Norwegian

Kjønnscellene, oocytter og sædceller, er essensielle celletyper for artsforplantning hos alle seksuelt reproduserende dyr. Differensiering av kjønnsceller kan skje via to ulike mekanismer: arv og induksjon. I denne oppgaven undersøker jeg hvordan kjønnsceller blir spesifisert og oppstår i Nemertodermatida, en neglisert gruppe av Xenacoelomorpha. Ved å studere nemertodermatid-ormen *Meara stichopi*, oppdaget jeg en tidligere ubeskrevet populasjon av neoblastlignende celler som gir dette dyret noe regenerativ kapasitet. Min hypotese er at disse neoblastlignende cellene ogå gir opphav til kjønnscellene i disse dyrene. Med søkelys på regenereringsdelen undersøkte jeg M. stishopis sårhelingsprosess etter oviposisjon (egglegging) og etter amputasjon. Disse forsøkene viste sårindusert celleproliferasjon og uttrykking av konserverte transkripsjonsfaktorer som egr og runt. I den andre delen gjør jeg transkriptomiske dataanalyser av enkeltceller (scRNA-Seq) fra klekkings-, ungdoms- og voksenstadiet for å vise molekylær differensiering av neoblaster til somatiske celletyper og til kjønnsceller.

List of Publications

Manuscript I: Kovacikova, P., Stracke K., Vellutini B.C., Hejnol, A. (2023): The stem cell system of nemertodermatid *Meara stichopi* (preprint)

Author Contributions

I hereby declare that I have written this thesis myself, with the help and contributions of other people in form of comments and formal corrections.

I also declare that I have written Manuscript I, having received comments and corrections from my co-authors and from Francesca Pinton.

These are the detailed contributions of authors for the Manuscript I:

Petra Kováčiková (me), Dr. Katharina Stracke, Dr. Bruno C. Vellutini and Prof. Dr. Andreas Hejnol are the authors of this paper. I performed the comparative analysis of RNA-binding domains in nemertodermatid and Xenoturbella species based on the initial search strategy from Dr. Vellutini. I performed the cell proliferation assay, and combined fluorescent *in situ* hybridizations of *piwi1*, *vasa*, *egr*, and *runt* in intact and amputated animals. I performed colorimetric *in situ* hybridizations of regeneration GRN genes. I also performed immunostainings of intact and regenerating animals. Dr. Katharina Stracke performed initial amputation experiments and colorimetric *in situ* hybridizations of piwi1, piwi2, nanos and vasa in intact and amputated animals presented in Suppl. Fig. 4 a. Dr. Bruno C. Vellutini performed colorimetric *in situ* hybridizations of RNA-binding proteins presented in Fig.1c. Prof. Dr. Andreas Hejnol was involved in the design and supervision of the project.

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CHAPTER 1: Introduction

1.1 The Germ cell – what it is and what it is not

All sexually reproducing organisms start their life with an interaction. An interaction of two cells, the gametes – the oocyte and the sperm. Gametes are morphologically differentiated products of cells collectively known as germ cells. Germ cells arise in embryonic development from the primordial germ cells (PGCs) – the cell lineage committed to the germ line. Germ cells differ from the rest of the cells forming the body – the somatic cells – in their number of chromosomes. Being haploid, carrying half of the somatic chromosome count, the product of their interaction becomes a diploid one-cell embryo called a zygote. In the following development, a zygote "germinates" through successive cell divisions, giving rise to the whole animal. When exposed to the right interaction, a differentiated germ cell can generate a plethora of cell types and thus can be seen as a universal cell.

Historically, the unique position of the gamete at the onset of an individual's life has brought it to the spotlight of early works trying to explain the concepts of reproduction and development itself (Bowler, 1971). As Bowler summarizes, at the beginning of endeavors in embryology, observing the eggs, sperm, or early development left many authors such as William Croone, Anthony van Leeuwenhoek and Jan Swammerdam tempted to report that the body is formed from what is already present in one of the gametes alone; be it either sperm or an oocyte; thus assigning the important role to one and reducing the role of the other. These original works of the 17th century, often never really making such claims openly nor providing the evidence for them, formed the grounds for the preformation theories of development due to inaccurate translations and misinterpretations of their rather ambiguous language. The preformation saw the organism as the "miniature" already present at the time of fertilization and only expanding afterward. These hypotheses were rooted in the inability to explain the "self-organizing" property of cells' constituent material, composing the embryo in an orderly, reproducible fashion. Despite the careful examination of the observations of that time and long-lasting disputes, preformation continued to gain popularity. This was mainly brought about by a conceptual switch from gametes containing a

predetermined “miniature” to the gametes that carry a predetermined “design” of a physical nature guiding the following development.

The idea of unequal gametes was finally rebuked by direct evidence that both - the sperm and the oocyte - are essential for the successful creation of a zygote in the process of fertilization (Spallanzani, 1789). Observing the gradual formation of the embryo in the process of development laid the basis for a competing theory of development coined by William Harvey called the epigenesis (1847, Chapter On Animal Generation). Although epigenesis also recognizes the presence of the material inside the gametes as essential, this material is considered “unformed” since the complexity of form increases and emerges over time (Nicoglou & Wolfe, 2018).

These seemingly opposing lines of thinking in embryology were somehow unified by the discovery of the DNA as the molecular hereditary agent (Hershey & Chase, 1952). Shaping our understanding of germ cells as the ultimate vessels for the genetic material that carries the molecular instructions for the next generation. The offspring, self-instructed by the received genetic material (predetermination) and exposed to the contextual information from the environment (epigenesis), undergoes development until it reaches the reproductive stage and produces gametes of its own, restarting the life cycle again. This fact alone makes germ cells indispensable for life to carry on. Furthermore, being the only point of molecular exchange between the two generations, they become the subject of evolution to act upon.

Right next to the energy expense for individual’s survival, it is the reproduction, which is fundamental for evolutionary fitness, i.e., increasing one’s genetic code in a population. To successfully make progeny, the individual must invest energy to protect their most prized possession - the material for the next generation, the germ cells - until they are put into action in the act of reproduction. And from a cell-centric view, the privilege of being chosen for this purpose ensures a certain molecular immortality. The specific combination of gene alleles and mutations present in the particular germ cell will be the combination that the individual passes on and will form the genetic background of the future zygote. As a result, germ cells are maintained by the present

individual to ensure future progeny. Unlike their somatic neighbors that function to maintain the individual’s body - the soma - in its lifetime but will cease to exist with its decay. Therefore, it is essential for the individual to accurately select which cells are to be used for this purpose – a problem that arises only for organisms composed of multiple cells.

Indeed, in unicellular organisms, all life-mediating functions are performed by a single cell. Although there is not much room for a selection at the cellular level, the same cannot be said about the genome. As illustrated by the lifestyle of ciliates, unicellular organisms that form two separate nuclei (Karrer, 2012), selecting which genes will be passed on to the progeny is still possible in the genome maintained by a single cell. Outside their predominant asexual reproduction by cell fission, the ciliates are able to reproduce sexually, increasing the plasticity of the population through the genetic recombination (Zhang et al., 2022). When this occurs, the two individuals exchange their haploid micronuclei - a “germline” sub-portion of the genome that is mostly transcriptionally inactive. The recombinant diploid micronucleus then drives the formation of the new macronucleus - a “somatic” sub-portion of the genome. The genome rearrangement of the micronucleus is epigenetically templated and involves vast sequence eliminations, fragmentations, and amplification, finally producing the somatic chromosomes of the macronucleus (Lindblad et al., 2017; Nowacki et al., 2008). This complex molecular mechanism demonstrates a way of protecting the germline genome from accumulating mutations by repressing its expression. The functionally separated germline genome is devoid of active transcription outside the time that prepares the cell for meiotic recombination and conjugation. This strategy is remarkably efficient for a unicellular lifestyle but would be quite unattainable in a body composed of more than one cell.

On the way to multicellular life, the early metazoans must have found a way to separate these tasks of the body upkeep and of investment into the progeny. In the same way that each somatic cell is specialized to perform its function, the germ cells, too, are specialized through differentiation.

1.2 Germline Differentiation Modes

How do the germ cells become different from the rest without sacrificing their cell potency? And simultaneously, how to resist the waves of somatic cell differentiation as the early embryo is being patterned into germ layers? When does an individual clearly set the germ line apart from the somatic cells? These dilemmas and their solutions highly depend on the organism's life cycle.

The studies on several model organisms selected for their fast reproduction time have revealed two extreme modes of germline differentiation. These are called the inheritance mode and the inductive mode, or their alternative names: preformation and epigenesis (Extavour & Akam, 2003). Although any connection with the theories bearing the same name described above is purely semantic, they too represent two seemingly opposite poles of the spectrum. However, the inheritance and inductive modes describe the mechanisms of germline differentiation, not the embryonic development itself. To avoid any confusion between the two phenomena, germline specification modes will not be referred to by their alternative names here.

In the inheritance mode, the germline specification relies on the accurate placement of molecular determinants in the cytoplasm upon cell division. Germline fate is thus determined cell-autonomously by the RNAs and proteins important for germ cell fate that reside in a specific portion of the mother cell's cytoplasm – the germ plasm – a term coined by August Weismann (1893).

According to Weismann's theory, the germ plasm, linked by the continuous chain of cell divisions throughout the generations, is immortal. It is important to note, however, that he used this term for cell's nuclear content rather than the cytoplasmic inclusions conferring germ cell fate that we understand it to be today (and the way it is described in the paragraph above). Based on his description of its structure (Weismann, 1893), the "germ plasm" – a nuclear content – carries determinants and controls the cell's cytoplasm, morphology, and eventually, its fate. Throughout the organism's ontogeny, the germ plasm gradually disintegrates, leaving only a few specific determinants to

enforce a cell to adopt its germ cell identity in the body. His ideas, progressive as they were for the pre-molecular era, outlined mechanistically the principles of central dogma in molecular biology and laid the foundations for the understanding of heredity.

The molecular determinants in today's understanding of the germ plasm can be sequestered early on in development, polarizing the oocyte (Figure 1.1 a.)). Such strict maternal control of the germline specification can be found in development of *Drosophila melanogaster* (Mahowald, 1962), *Caenorhabditis elegans* (Strome & Wood, 1983), *Danio rerio* (Yoon et al., 1997), and *Xaenopus laevis* (Ikenishi et al., 1986). For the parent cell to effectively ensure that its cytoplasmic content is neatly organized, there must be certain molecular mechanisms at play.

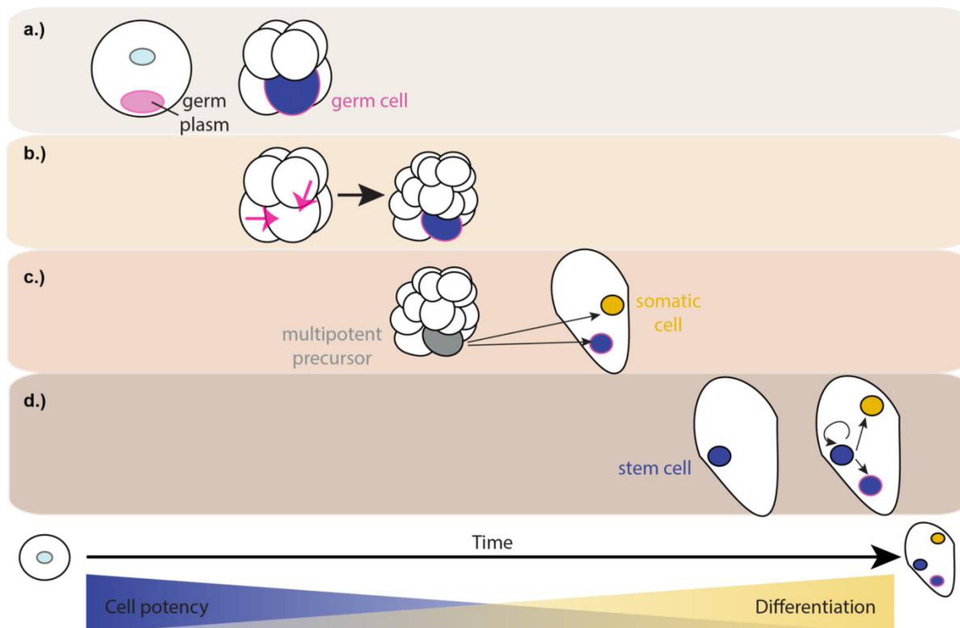


Figure 1.1 Germline differentiation modes in animal development. Germline can be specified at various time points during development from oocyte to adult organism. Along this developmental trajectory, the potency of somatic cells decreases as their differentiation and specialization increase. The only exceptions to these processes are stem cells and germ cells, which withstand the waves of somatic differentiation and retain high cell potency until

adulthood. **a.)** Early sequestration of determinants in germ plasm (pink cloud) in the oocyte's cytoplasm leads to asymmetric distribution of cell fate in subsequent cell division and represents a strict inheritance mode of germline (blue cell outlined in pink) specification. **b.)** In the early inductive mode, the germline lineage is specified from early blastomeres by the inductive cell signaling (pink arrows) from neighboring cells. **c.)** Multipotent precursor cells represent a less stringent inheritance mode, where germline determinants might be sequestred later on in development. These precursor cells are of mixed germ and soma potential and give rise to germline cell lineage and somatic cells (yellow cell). **d.)** Generation of germ cells can occur in adults continuously in animals with robust stem cell systems and regenerative capacities. Germ cells are specified *de novo* from stem cells (blue cells outlined in black), which can also differentiate into somatic cells.

Such specific mechanisms, orchestrating the distribution of maternal determinants, have been observed in the oocytes of the frog *Xenopus laevis* (Czołowska, 1972; Heasman et al., 1984). In the gonad of the mother organism, where the female germ cell maturation occurs in the process of oogenesis, the oocytes become highly polarized by the sequential events of active RNA and protein localization (King et al., 2005). When the maturation is complete and the oocyte is fertilized by the sperm, the resulting zygote, inheriting the oocyte's cytoplasm, is polarized too. The entire cell content is organized along this first developmental axis, the animal-vegetal (AV) axis, important for future sets of blastomere cleavages. All the maternal determinants in the form of RNA and proteins are sequestered in the electron-dense inclusions called the germinal granules (Kloc et al., 2001, 2002). Harbored in the vegetal pole of the zygote, these granules await to be inherited by the vegetal blastomeres of the early embryo – the early precursors of the PGCs (Ikenishi et al., 1986).

The inheritance mode can also act in a somewhat less time-stringent manner, when the germ plasm is not inherited from the mother germ cell but rather from the parent blastomere. In this case, determinants, which might be broadly expressed initially, get sequestered through subsequent cleavage divisions, leading to a formation of cell lineage with a dual soma and germ potential in some species. In ascidian development,

despite the early asymmetric distribution of some maternal germline determinants, such as *vasa* transcripts (Fujimura & Takamura, 2000; Goto et al., 2022; Paix et al., 2009) and protein (Takamura et al., 2002), the posterior blastomeres loaded with these determinants do not differentiate directly into PGCs but act as the multipotent somatic and germline precursors (Figure 1.1 c.)). It is only at the 64-cell stage when the definitive germ-soma dichotomy is formed, as the precursor blastomere divides asymmetrically into a somatic sister blastomere — which forms the gut wall — and a germline sister blastomere, containing maternal determinants, that migrates into a juvenile gonad (Shirae-Kurabayashi, 2006). Similar use of multipotent precursors formed by asymmetric cell division can be found in sea urchins. The vasa-positive micromeres of 16-cell stage give rise to micromeres which later form larval somatic skeletogenic cells and small micromeres that further accumulate germline markers and are thought to be the source of the PGCs in the adult (Juliano et al., 2006; Yajima & Wessel, 2011a, 2012). Although not completely understood on a mechanistic basis, germline determination through multipotent precursors could represent a more plastic solution to autonomous specification. Since the early primed precursors do not fully segregate from somatic tissue, the germline can be compensated for and regenerated after the removal of its source tissue as shown by the microsurgical experiments in ascidians and sea urchins (Takamura et al., 2002; Yajima & Wessel, 2011a).

An even more plastic solution to a germline specification is seen in the inductive mode, where germ cells are not generated cell-autonomously — by their cell lineage history — but are conditionally selected (Figure 1.1 b)). The most studied example of inductive germline formation occurs in the mouse embryo. The synergy of the Bone Morphogenetic Protein 4 (Bmp4) secreted from the extraembryonic ectoderm and the int/Wingless family protein 3 (Wnt3) secreted by epiblast cells trigger signaling in the competent, neighboring proximal epiblast cells (Lawson et al., 1999; Ohinata et al., 2009). The signal transmitted through the canonical Wnt cascade by the activation of Brachyury/T transcription factor (TF), a known mesodermal TF, initiates a transcriptional program of the PGCs (Aramaki et al., 2013). This transcriptional network has been shown to rely on the three key TFs (Magnúsdóttir et al., 2013):

B-lymphocyte induced maturation protein 1 (Blimp1; Ohinata et al., 2005; Vincent et al., 2005), PR (PRDI-BF1 and RIZ) domain-containing transcriptional regulator 14 (Prdm14; Yamaji et al., 2008), and transcription factor AP2 gamma (AP2γ; Weber et al., 2010); their activity helps the pluripotent epiblast cells escape the somatic differentiation and adopt a germ cell fate instead. Thus, though still segregated in embryogenesis, the mouse germline is not "hard-wired" in the developmental program, but rather arises from competent, pluripotent cells, as a consequence of the morphogenetic interplay of cellular signaling in a specific niche of the embryo.

Similarly, the BMP signaling is involved in conditional germline specification in axolotls where PGCs arise from non-patterned undifferentiated mesodermal precursors (Chatfield et al., 2014; Johnson et al., 2003). In axolotls, however, in contrast to the murine PGCs, the BMP and Brachyury activity do not induce the *Blimp1* expression, and their activity alone is not sufficient to establish the PGC fate directly during early embryogenesis. Instead, additional Fibroblast growth factor (FGF) signaling ensures the undifferentiated state of the intermediate mesoderm throughout the gastrulation by the MAPK activity, as both seem to be required for the onset of germline-specific gene expression in explants and *in vivo*. Chatfield and colleagues (2014) thus proposed the axolotl germline to be specified only after all the somatic lineages are patterned in gastrulation which is in stark contrast to most Metazoa.

Orthologues of vertebrate BMP2/4 were also identified as a source of the inducing signal for the PGC formation in crickets (Donoughe et al., 2014), suggesting that the same signaling pathway is used by invertebrates in the inductive PGC specification. Through functional studies, the authors have also shown the role of BMP downstream effector Blimp-1 in this insect (Nakamura & Extavour, 2016). Blimp-1 expression is activated by the BMP signaling in the presumptive abdominal mesoderm, where the PGCs originate from in the cricket embryo, and its levels affect the number of induced PGCs. Nakamura and Extavour (2016) have remarked that a role of BMP/Blimp-1 signaling module in PGCs specification was most likely present in the bilaterian ancestor, but was lost in the lineages relying on the inheritance and was replaced in the

axolotls. Their hypothesis is supported by the expression data of BMP2/4 and Blimp-1 from rabbit and chicken (Hopf et al., 2011; Wan et al., 2014).

In the species mentioned so far, the germline – soma segregation is executed once and for all, either very early (strict inheritance mode in *C. elegans* and *X. laevis*) or relatively late embryogenesis (multipotent precursors in sea urchin and ascidians, induction in mouse and acoels) or in postembryonic development (induction in axolotls). On the extreme side of the specification timeframe are animals with extensive regenerative capacities where the germline-soma boundary seems arbitrary as the germ cells can arise *de novo*, continuously throughout adulthood (Figure 1.1 d)). In notoriously regenerative planarians it is their robust stem cell system called neoblasts which provides the source for postembryonic germ cell generation in adult worms (Handberg-Thorsager & Saló, 2007; Newmark et al., 2008; Sato et al., 2006). A similar mechanism occurs in non-bilaterian metazoans: in sponges (Fierro-Constaín et al., 2017; Funayama et al., 2010; Gaino et al., 1984; Pilato, 2000) and hydrozoan cnidarians (Bosch & David, 1987; DuBuc et al., 2020; Leclère et al., 2012; Nishimiya-Fujisawa & Kobayashi, 2012; Varley et al., 2022), the germ cells arise from totipotent archaeocytes and/or choanocytes, and multipotent or pluripotent somatic interstitial stem cells (I-cells), respectively. Recent studies of anthozoan cnidarians report conditional and likely stem cell-based germline specification (Chen et al., 2020; Miramon-Puertolas & Steinmetz, 2023). These findings together with previously reported maternal input in the form of transcript localization and refinement in development (Extavour et al., 2005) can suggest some level of stem cell lineage dedication. Common progenitor stem cell lineage for somatic and germline would explain the fact that somatic mutations do not seem to propagate into the gametes (Barfield et al., 2016), yet, the parental mutations can be found in the offspring (López-Nandam et al., 2023; Vasquez Kuntz et al., 2022). Another evidence of a certain degree of stem cell lineage restriction — and hence germ-soma cell fate restriction — comes from the regeneration studies in ctenophores (Edgar et al., 2021), which seem to possess stem cells of unresolved potency. Interestingly, the origin of germline in these animals is still unknown. Morphological observations point to the endodermal larval

cells, and thus later specification as suggested by Extavour & Akam (2003). Whether the induction mode, despite being suggested by the molecular data (Alié et al., 2011; Reitzel et al., 2016), acts on the stem cells for germ cell formation is still unclear.

1.3 Evolution of germline differentiation

Historically, the scientific community considered the inheritance mode as a predominant way through which animals specialize their germline (e.g. Mahowald 1962). This is due to the limited number of taxa that were traditionally studied in developmental biology. The early landmark studies of *C. elegans*, *Drosophila*, zebrafish, and *Xenopus* embryos have described in detail the essential molecular mechanisms carrying out the process of the inheritance mode and named the key protein interactions leading to the germ plasm formation and its components (Voronina et al., 2011). While offering many valuable insights into the process, the selected species poorly represented the vast diversity of Metazoa. From such a skewed view, the inheritance mode was deemed the most parsimonious answer as to how the ancestor of multicellular animals segregated its germline.

However, with a closer look at various animal phyla including bilaterian outgroups, the inductive mode started to emerge as the ancestral way toward the germline (Extavour & Akam, 2003). This hypothesis is strongly supported by the comparative analyses of germ plasm components and nucleators (*C. elegans* and other nematodes - Bezares-Calderón et al 2010, *Drosophila* & other insects – Lynch et al 2011, zebrafish & other vertebrates – Skugor et al 2016,). As reviewed by Kulkarni and Extavour (2017), the germ plasm nucleators are often species or lineage-specific and display rapid sequence evolution (Whittle & Extavour, 2019). Clearly lacking sequence homology, a molecular sign of common descent, these proteins with germ plasm-organizing properties could not be inherited from a common ancestor. Not being orthologous, but rather performing the analogous functions of binding molecular determinants, the germ plasm nucleators illustrate that the solution to organize the maternal cytoplasm evolved in animals repeatedly and independently. The germ plasm nucleators thus illustrate how non-orthologous proteins — i.e., the proteins of non-common evolutionary descent — can evolve repeatedly and independently to perform analogous functions

(binding the molecular determinants) with the same goal, organizing maternal cytoplasm.

Similarly, highlighting the key role of a comparative approach, when one extends the scope of the study, the convergent evolution of the inheritance mode becomes even more evident. Many clades, for which the inheritance mode was considered a shared trait, group animals that specify their germline using different mechanisms.

A great example of germline formation diversity can be found in insects: holometabolous fruitfly *Drosophila* (Mahowald, 1977) and wasp *Nasonia* (Lynch & Desplan, 2010) use inheritance mode, whereas holometabolous honeybee *Apis* (Dearden, 2006) and hemimetabolous cricket *Gryllus* (Ewen-Campen, Donoughe, et al., 2013; Nakamura & Extavour, 2016) and milkweed bug *Oncopeltus* (Ewen-Campen, Jones, et al., 2013) use the inductive mode. Similarly, in amphibians, the anuran frog *Xenopus* (Ikenishi et al., 1986) uses the inheritance but urodeles — the axolotls — use the induction mode (Chatfield et al., 2014; Johnson et al., 2003). In echinoderms: euechinoderms (sea urchins and sea dollars) use a plastic form of inheritance with multipotent precursors while the expression data from their sister groups (asteroids and ophiuroids) point towards the use of induction (Fresques et al., 2016; Juliano & Wessel, 2009). Thus, it is only the complex and mosaic, yet more complete, picture of PGC specification, obtained by studying many diverse species within a phylogenetic context that can shed more light into the germline evolution.

Despite differences in specification modes, the outstanding diversity in their minute, detailed parts, such as the timepoint of segregation inherently linked to the developmental origin of the precursor cells, the molecular components of germline specification in Metazoa relies on a deeply conserved set of genes (Fierro-Constaín et al., 2017), traditionally thought of as germ cell markers (Ewen-Campen et al., 2010; Juliano & Wessel, 2010). These genes are used in key germline functions that heavily revolve around RNA processing: inhibition of somatic differentiation by translational repression (nanos) (Tsuda et al., 2003), genome protection by transposon repression (piwi) (Cox et al., 1998), and transcriptional regulation (RNA helicase vasa) (Yajima

& Wessel, 2011b). Their expression has been widely used in germline identification in new non-model species, however, many recent works have pointed out their role in the maintenance of the stemness of somatic stem cells in several taxa (ctenophores - (Alié et al., 2011; Reitzel et al., 2016), sponges - (Fierro-Constaín et al., 2017; Funayama, 2013; Funayama et al., 2010), cnidarians - (Leclère et al., 2012; Lim et al., 2014; Miramon-Puertolas & Steinmetz, 2023; Mochizuki et al., 2001; Rebscher et al., 2008; Seipel et al., 2003), planarians - (Shibata et al., 1999), annelids - (Dill & Seaver, 2008; Gazave et al., 2013; Giani et al., 2011)). This apparent connection between germline and stem cells has led to the proposal of an ancestral genetic "pluripotency module" (Ewen-Campen et al., 2010) or a germline multipotency program (GMP) (Juliano et al., 2010) utilized by primordial stem cells (PriSCs) (Solana, 2013), able to give rise to both, somatic stem cell-like cells, and to the germline. How could this program evolve and what does it mean for the relatedness of these two cell types?

1.4 Stem cells – a plastic connection

Outside the germ cells, somatic stem cells are the only other cell type in the adult organism retaining the ability to differentiate into non-self – a wide morphogenetic cell potency, while enjoying the prominence of "not specializing, but socializing". The stem cells are multi- or pluripotent precursor cells that are known to self-renew by clonal expansion and can replenish other somatic cell types (Rinkevich et al., 2022; Weissman et al., 2001). PGCs and stem cells are not only similar morphologically (high nuclear:cytoplasmic ratio, conspicuous nucleolus, roundish shape, electron-dense cytoplasmic granules) (Morita et al., 1969), but also on a molecular level as revealed by many transcriptomic and proteomic studies (Alié et al., 2015; Fierro-Constaín et al., 2017; Grudniewska et al., 2016; Kurosaki et al., 2006). The evolutionary and ontogenetic relatedness of these cell types has been suggested by many authors (Isaeva, 2011; Juliano et al., 2011; Solana, 2013; Srouji & Extavour, 2010) and is supported by the expression data from bilaterian outgroups (Alié et al., 2011; Bosch & David, 1987; Fierro-Constaín et al., 2017; Funayama et al., 2010; Leclère et al., 2012; Miramon-Puertolas & Steinmetz, 2023; Mochizuki et al., 2001; Reitzel et al., 2016; Varley et al.,

2022) and data from bilaterian animals (Davies et al., 2017; De Mulder, Kuales, et al., 2009; De Mulder, Pfister, et al., 2009; Grudniewska et al., 2016; Kimura et al., 2022; Pfister et al., 2008; Rebscher et al., 2007; Shibata et al., 1999).

One of the intensively studied somatic stem cell systems in invertebrates is the already mentioned pluripotent stem cell population of planarian flatworms called neoblasts. The neoblasts are located in the parenchyma of adult worms and constitute the only dividing cells in the animal (Baguña & Romero, 1981; Newmark & Sánchez Alvarado, 2000). Embryonic neoblasts are responsible for differentiating into all the cell types in development (Davies et al., 2017), including the germ cells (Handberg-Thorsager & Saló, 2007; Sato et al., 2006), which the adult neoblasts can restore in regeneration (Newmark et al., 2008). Outside of the homeostatic cell replacement and tissue turnover, the neoblasts also provide planarian flatworms with extraordinary regenerative capabilities (Morgan, 1898). Their whole body can be regenerated from small tissue fragments and regenerative ability can be restored in animals devoid of neoblasts, eliminated by X-ray irradiation, when a single neoblast cell is transplanted into an irradiated host (Wagner et al., 2011).

What is it that gives stem cells such a prominent character reminiscent of the immortal germ cells? Just like the germ cells, the stem cells too, must become different without really differentiating — adopting a somatic fate — withstanding the identity assignment of somatic cell types in embryogenesis. To achieve this and to fully facilitate their respective functions, both cell types use similar mechanisms that can be summarized in three main components: protection, selection, and restoration (Raz & Yamashita, 2021). Their genome needs to be protected from accumulating mutations, a collateral damage of repeated rounds of cell division, and from deleterious transposon activity that imposes a threat to the genome stability. Overall, this is largely enabled by repressing the levels of transcription and translation and by piwi-piRNA-mediated RNA silencing (eg. somatic stem cells in *Hydra* - Juliano et al., 2014; Teefy et al., 2020; neoblasts - D. Li et al., 2021) for post-transcriptional regulation in neoblasts see Krishna et al., 2019).

While not a universal rule, eg. a sea star - (Fresques & Wessel, 2018), transcriptional quiescence is required in the specification step of PGC precursor cells in several species: *C. elegans* and *Drosophila* (Seydoux & Dunn, 1997), ascidians – (Miyaoku et al., 2018; Tomioka et al., 2002), sea urchin (Swartz et al., 2014), *Xenopus* (Venkatarama et al., 2010). The transcriptional halt comes much later in stem cells, where it is a basis for their maintenance (Cheung & Rando, 2013; Freter et al., 2010). Not only do the global or targeted transcriptional and translational repression hinder the cell cycle progression and thus lower the risk of faulty replication upon cell division, but they also inhibit the expression of somatic transcription factors which would otherwise drive the cells to differentiate had they been active (Harris et al., 2011; Kadyrova et al., 2007; Lai et al., 2012; Miyaoku et al., 2018; Shirae-Kurabayashi et al., 2011). In germ cells, the initial transcriptional control is brought about by maternally inherited cytoplasmic determinants: eg. PIE-1 in C. elegans (Ghosh & Seydoux, 2008), germ-cell-less (gcl) and polar granule component (Pgc) in *Drosophila* (Hanyu-Nakamura et al., 2008; Leatherman et al., 2002; Martinho et al., 2004), nanos in *Xenopus* (Lai et al., 2012), or the BMP-induced transcriptional repressor Blimp-1 in mouse (Ohinata et al., 2005; Vincent et al., 2005), whereas in stem cells it depends on the presence of perinuclear nuage-like structure called chromatoid bodies (Hori, 1982). Chromatoid bodies of neoblasts resemble the germ granules as they consist of ribonucleoprotein particles (RNPs) with perinuclear distribution which and are enriched in GMP genes that exert RNA processing control (bruno - Guo et al., 2006; piwi - Palakodeti et al., 2008; Reddien et al., 2005; vasa - Shibata et al., 1999; tudor - Solana et al., 2009). The initial quiescence is further enhanced by the chromatin condensation, making most parts of the genome inaccessible for transcriptional machinery and only allowing key transcriptional activators to maintain the germ/stem cell signature expression (Lebedeva et al., 2018; Robert et al., 2015; Strome & Updike, 2015).

Since no protection mechanism is bulletproof, the faulty germ cells and stem cells, eg. damaged by genotoxic stress, need to be eliminated before having a chance to create an organismal or cellular progeny. Several DNA damage checkpoints along the germline differentiation route ensure that affected gametes in both male and female

gonads undergo cell death (Chakravarti et al., 2022; Lu & Yamashita, 2017). The same outcome awaits neoblasts exposed to the genotoxic stress (Stevens et al., 2018) .

Lastly, because of their integral role in fitness and homeostasis, an organism needs a mechanism to fall back on in case the initial pool of germ cell precursors or stem cells are lost or severely depleted upon environmental impact such as injury or nutrient depletion. In those situations, the restoration of somatic cell types depends on the animal's regenerative capacity and the rescue of its fertility and reproduction depends on the germline specification mode and maintenance.

Indeed, in species with determinative germline specification, the germline is inevitably lost upon surgical removal of its embryonic precursors (Sulston et al., 1983; Warn, 1975). However, if ablation is performed once the ontogenetically older precursor cells are formed, the germline can be restored even in some inheritance-using species if they possess so-called germline stem cells (GSCs) (Angelo & Van Gilst, 2009; Lin & Spradling, 1993). The GSCs, differentiated from PGCs, have a stem-like character in self-renewal, they are set aside cells responsible for continual gamete production throughout the lifetime (Lehmann, 2012; Lin, 1997, 1998; Spradling et al., 2011).

Restoration of somatic cells is governed by the regenerative capacityof an animal. If an injury leaves the initial stem cell pool too small to cater to the needs of the organism, selected progenitor cells can dedifferentiate into a stem cell-like phenotype and replenish the needed somatic cell type (eg. limb regeneration in salamander) (Sandoval-Guzmán et al., 2014). Dedifferentiation is particularly important for vertebrates with lineage-restricted uni/multipotent stem cells, but it also occurs in the invertebrates (Ferrario et al., 2020) and non-bilaterians (Edgar et al., 2021; Funayama, 2013).

In planarians, the amputation-induced stem cell depletion prompts neoblast cell division (Newmark & Sánchez Alvarado, 2000); if combined with the genotoxic stress, the remaining stem cells can replenish the initial pool by hyperproliferation (Stevens et al., 2018). Together with the highly regenerative animals apparently lacking the germ-soma barrier (mentioned above in section 1.2), planarians too can regenerate

germline from a somatic source (Newmark et al., 2008). However, the ability to restore germ cells is not exclusive to the animals without segregated germline as there is mounting evidence from several other species (annelids – (Dannenberg & Seaver, 2018; Özpolat et al., 2016), echinoderms – sea star (Inoue 1992,), sea urchin (Ransick et al 1996), ascidian (Takamura et al., 2002)).

Thus, many studies of homeostasis-compromising situations such as injury revealed the the role for GMP genes in mobilized stem cells, confirming their pleiotropic role outside of germline (Juliano et al., 2011; Juliano & Wessel, 2010) and supporting the idea that germline might have evolved from stem cells in the metazoan ancestor (Extavour 2008). To reconcile these similarities with the strongly opposing hypothesis of Weismann's barrier (Weismann et al., 1889), which holds the immortal germline to be separated from the soma, Solana (2013) proposed that the primordial stem cells (PriSCs) should be included in the germline circle. He loosely defines the PriSCs as multipotent cell types which express the GMP genes, contain germ plasm, and have a mixed germ and soma potential. According to Solana, such PriSCs exist in extant animals in different modes, based on their cell potency, corresponding to different cell types. The potency of PriSCs forms a broad gradient starting from unlimited potency (adult somatic stem cells able to generate germ cells: I-cells, archeocytes, neoblasts), through restricted potency (multipotent precursors that eventually give rise to PGCs by induction), to rudimentary potency (early blastomeres that carry maternal determinants to give rise to PGCs by inheritance but still exhibit very limited somatic potential) (Solana, 2013).

1.5 *Xenacoelomorpha* – a case for ancient bilaterian pluripotency in germline generation?

An ideal system to study the presence of such primordial stem cells are xenacoelomorphs for their irreplaceable role in uncovering the evolution of characters in Bilateria (Hejnol & Pang, 2016; Jondelius et al., 2019). Xenacoelomorpha comprises

three different groups: Acoela, Nemertodermatida, and *Xenoturbella*. They all have a blind gut, and lack true coelom and excretory systems (Haszprunar, 2015). Due to the worm-like morphology with often dorso-ventrally flattened body shapes, xenacoelomorphs used to be grouped together with platyhelminth flatworms forming "Turbellaria" (Karling, n.d.), a taxon no longer supported by modern sequence-based phylogenies (Baguñà & Riutort, 2004; Jondelius et al., 2002; Philippe et al., 2007; Ruiz-Trillo et al., 2002). The sister group relationships within the clade have been retrieved by phylogenomic analyses (Hejnol et al., 2009) but overall, their phylogenetic position within Metazoa has been largely disputed (Cannon et al., 2016; Kapli & Telford, 2020; Philippe et al., 2011). Some studies suggest Xenacoelomorpha as a sister group to all bilaterians with excretory system (Nephrozoa) (Cannon et al., 2016; Hejnol et al., 2009; Ruiz-Trillo et al., 2002), making xenacoelomorphs the closest proxy to early bilaterian evolution. Alternatively, this attraction to the base of the bilaterian tree is an artifact (Kapli & Telford, 2020; Philippe et al., 2019) and xenacoelomorphs represent a sister group to echinoderms and hemichordates, forming the clade Xenambulacraria (Philippe et al., 2011). Under this hypothesis, their bodies were secondarily reduced after the split within deuterostomes. Regardless of their exact phylogenetic position, studying these animals, their germline formation and potential stem cells closely related to the regenerative capacity may elucidate the relationship between germline and stem cells.

Early studies of the sister group Acoela revealed the presence of mitotic cells in adult specimens (Drobysheva, 1986), which, together with observed spontaneous regeneration (Costello & Costello, 1939), suggested that acoels might possess the adult pluripotent stem cell system similar to the one known from planarians. Indeed, studies on several acoel species have uncovered a spectrum of regenerative capacities in this group, ranging from posterior regeneration (De Mulder, Kuales, et al., 2009; Perea-Atienza et al., 2013), or head and tail regeneration (Bailly et al., 2014; Sprecher et al., 2015) to whole-body regeneration (Srivastava et al., 2014), and regeneration processes connected with the asexual reproduction (Gschwentner et al., 2001; Sikes, 2009; Sikes

& Bely, 2010). The neoblast-like stem cell system, located in the mesenchyme, surrounds the gonads in bilateral bands (De Mulder, Kuales, et al., 2009) or can be homogenously scattered throughout the body (Gschwentner et al., 2001; Srivastava et al., 2014). Cell proliferation assays and evidence of *piwi* expression in two acoel species identified the pluripotent neoblast-like stem cells which serve as a cellular source for the PGCs (De Mulder, Kuales, et al., 2009; Gschwentner et al., 2001).

Acoel neoblast-like stem cells are, together with the gonadal tissue, derived from endomesodermal precursors in embryogenesis (Chiodin et al., 2013). Recent blastomere labeling in acoel *Hoftenia miamia* traced the embryonic origin of neoblasts to a single micromere pair 3a/3b of the 16-cell stage (Kimura et al., 2022); a gastrulation stage in *Hofstenia* (Kimura et al., 2021). Generally, a gastrulation event in acoels is marked by the single internalization of the endomesodermal precursors – a macromere pair 3A/3B – (Henry et al., 2000) and typically starts earlier (at the 14-cell stage) in other acoel species investigated so far (Hejnol, 2015). However, in *Hofstenia*, there is a second internalization event at dimple stage (unique to this species), during which the 3a/3b progeny delaminate (Kimura et al., 2022). Furthermore, neither the 3A/3B blastomeres, nor their parent blastomeres produce any progeny with mesodermal fate. (Kimura et al., 2022). The lack of endomesodermal precursor in *Hofstenia* worm might be explained by modifications of acoel duet cleavage pattern seen as early as the 4-cell stage. Its first micromere duet divides before the macromeres in an unequal division (Kimura et al., 2021) as opposed to the convolutids (Henry et al., 2000) where the first cells to divide are vegetally placed macromeres. There is also a temporal shift, with the third blastomere duets (3a/3b and 3A/3B) produced by a totipotent parent macromere pair 2A/2B at the transition to 12-cell stage in convolutids (Henry et al., 2000) and isometridae (Cetrangolo, 2020), while in *Hofstenia* they are not produced until the 16-cell stage (Kimura et al., 2021) by the parent macromeres that already show restricted fates with no mesodermal derivatives (Kimura et al., 2022). Thus, it is unlikely that the fate of 3a/3b blastomeres, producing neoblast-like cells in *Hofstenia*, is conserved in other acoel species. As is the case in the nervous system or the reproductive organs, acoels display a huge variety of character states rather than their conservation (Achatz et al., 2013). Indeed, this micromere pair in *Neochildia*

fusca (Henry et al., 2000) and *Isodiametra pulchra* (Cetrangolo, 2020), both species showing more derived characters (Jondelius et al., 2011), produce ectodermal progeny. It is possible that the different fate distribution in early blastomere precursors of acoel embryos represents a developmental constraint imposed on different species. This would restrict the potency of adult pluripotent stem cells and in turn, affect a range of regenerative abilities found in adults.

The data describing regenerative abilities of *Xenoturbella* species are scarce. In fact, there is only a single report. Nakano (2015) claims to have witnessed "some degree of tissue regeneration" in pieces of animals fissioned by the collection-induced wounds of different planes. He also documents a regenerated piece that survived the wound-induced fission and emphasizes the role of the statocyst for its viability; as the other non-statocyst piece degenerated after a couple of days (Nakano, 2015).

Thus, whether the neoblast-like cells represent a symplesiomorphy of Xenacoelomorpha remains to be answered by investigating the presence of such potential cells in yet many more representatives of the group. Particular attention should be turned to the most neglected group of Xenacoelomorpha: meiofaunal nemertodermatids, members of which have been briefly inspected for the presence of putative aPSCs previously (Smith III et al., 2009).

Meara stichopi (Westblad, 1949) is a member of Nemertodermatida, the least studied group of Xenacoelomorpha comprising only about 20 species (Meyer-Wachsmuth & Jondelius, 2016). Despite not being numerous, this mostly meiofaunal group holds the promise of expansion thanks to cryptic species capacity (Meyer-Wachsmuth et al., 2014). Thus, its environmental importance and biodiversity are yet to be discovered and fully explored and appreciated in years to come by the improved taxonomic sampling. *M. stichopi* lives as an endosymbiont in the foregut of holothurian sea cucumbers *Parastichopus tremulus* in the North Sea (Bush & Baldrige, 1981; Westblad, 1949). Interestingly, there are reports of the same genus, *Meara sp.*, collected in the Bahamas (Lundin, 1998; Smith III et al., 1994, p. 97; Sterrer, 1998),

from the foregut of a different holothurian host *Holothuria (Vaneyothuria) lentiginosa enodis* with a brief ultramorphological description of epidermis and no further description of their ecology (Lundin, 1998). This species, however, was not revisited by researchers since the mentioned morphological datasets were compiled, and was not included in the most recent phylogenetic hypothesis of Nemertodermatida (Meyer-Wachsmuth & Jondelius, 2016). Thus, its relatedness to *Meara stichopi* remains to be tested by molecular approaches.

Based on the specimen collection data in the North Sea, *M. stichopi* has been proposed to have an annual life cycle (Børve & Hejnol, 2014). Its development from egg to hatchling was observed under laboratory conditions during winter months (Børve & Hejnol, 2014) and can take up to ten weeks (Figure 1.2 A). In their natural environment, probably the muddy sea sediment, the hatchlings or juveniles likely get ingested by the sea cucumber in the spring to further grow until they reach sexual maturity and reproduce in the digestive tract of their host. The spawned oocytes likely leave the host's digestive tract. Embryonic development proceeds through the holoblastic stereotypic cleavage (Børve & Hejnol, 2014), which from 4-cell stage onwards differs from the acoel duet cleavage pattern (Boyer, 1971; Henry et al., 2000) and from the cleavage pattern of *Nemertoderma* – the only other nemertodermatid species with described development so far (Jondelius et al., 2004). The oval-shaped embryo undergoes cleavage and gastrulation (24- to 64-cell stage) within a transparent eggshell (Figure 1.2 A). After about 5 weeks (Børve & Hejnol, 2014), the compacted and ciliated embryo starts to rotate within the eggshell, a stage that is found in other xenacoelomorphs whose development has been studied so far (Bush, 1975; Cetrangolo, 2020; Jondelius et al., 2004; Kimura et al., 2021; Nakano et al., 2013; Shannon & Achatz, 2007). At this point, the formed musculature and basiepidermal nerve fibers can be detected by immunostainings (Børve & Hejnol, 2014). Developed hatchlings (Figure 1.2 B) escape from the eggshell at around week nine or ten with formed muscles of all groups covered by an established nervous system composed of a subepidermal nerve net, anterior commissures, and two dorsal bilateral bands axon tracks condensations that cross posteriorly. The nervous system already shows some subfunctionalization based on FMRFamide and serotonin reactivity in the neurite

bundles and ventrally located subepidermal cells of unknown function. The gonads, the mouth opening, and the gut are lacking (Børve & Hejnol, 2014). The hatchlings are immediately able to glide and move around thanks to the muscles and the ciliated epidermis with serotonin-positive sensory cells (Børve & Hejnol, 2014), while using the anteriorly located double statocyst as a gravitational sensory organ (Børve & Hejnol, 2014; Westblad, 1949). The sexually immature juveniles (Figure 1.2 C) are thought to enter the host together with the ingested food, to grow and reproduce within its foregut (Børve & Hejnol, 2014). It is not currently known when the animals reach sexual maturity, what determines it, or whether it is host dependent.

The adults of *M. stichopi* display a fully ciliated drop-like body (Figure 1.2 D,E) with an apparently pseudostratified epidermis: the outer cuboid interdigitating multiciliated cells (Lundin & Hendelberg, 1995), and the underlying cylindrical cells (Børve & Hejnol, 2014). Epidermal cells are withdrawn from the epidermal layer as "degenerating bodies" (Lundin & Hendelberg, 1996) and can be found well below the muscle sheet (Westblad, 1949), probably thanks to the thin layer of extracellular matrix and the lack of basal lamina (Lundin & Hendelberg, 1995). They are intercalated with rhabdoid cells (Lundin & Hendelberg, 1995) producing polyphenol substances (Smith III et al., 1994), and sensory cells (Lundin & Sterrer, 2001) formed between the thick cylindrical epithelial cells and the underlying neurons in development and showing a positive serotonin immunostaining (Børve & Hejnol, 2014). In between the cilia, the extraepidermal symbiotic bacteria can be found (Lundin, 1998). The only body opening is a ventrally located mouth leading into the blind epithelial gut with a lumen that is surrounded by the gonads (Westblad, 1949). The musculature consists of three layers: circular, longitudinal, and diagonal forming U-shape fibers around the mouth opening (Meyer-Wachsmuth et al., 2013). The nervous system is entirely basiepidermal and forms dorsolateral neurite bundles along the body with anterior commissures (Raikova et al., 2000) and the submuscular nerve fibers around the anteriorly positioned double statocyst (Børve & Hejnol, 2014).

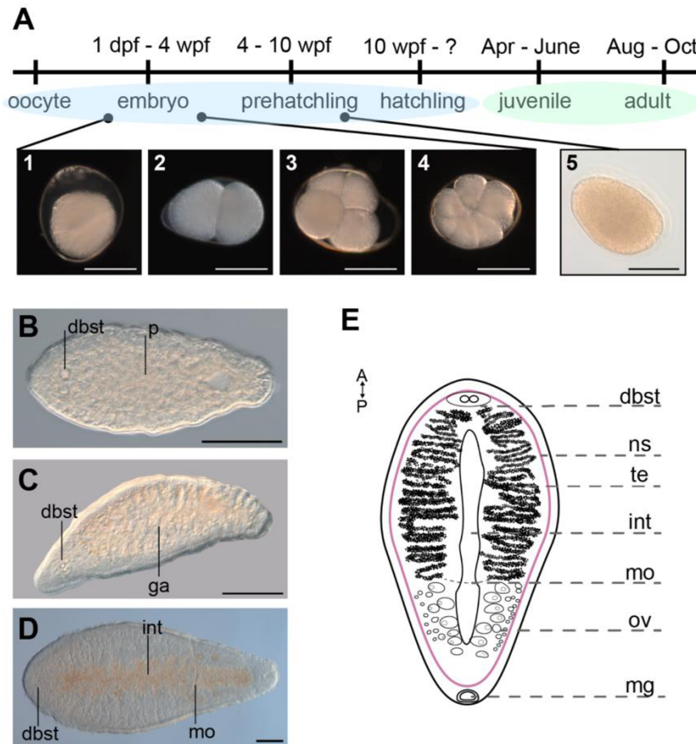


Figure 1.2 Development and anatomy of nemertoderamtid *Meara stichopi*. A. Timeline of M. stichopi's development. Brightfield images of zygote (1), 2-cell stage (2), 4-cell stage (3) and 12-cell stage (4) embryonic stages and prehatchling stage (5) are shown. The staging of stages in blue oval is according to the lab cultures. The staging of stages in green oval is according to the sampling data. **B.** Lab-cultured hatchling stage with pronounced double statocyst (dbst) and undifferentiated central parenchyma (p). **C.** Wild-caught juvenile stage with medially positioned gonad anlage (ga). **D.** Adult stage showing well-developed morphology. **E.** Schematic of the adult worm showing all major morphological characters. Anterior is facing left in B-D and up in E. Image of adult *M. stichopi* is reproduced from Børve & Hejnol, 2014. dbst – double statocyst; dpf – days post fertilization; ga – gonad anlage; int – intestine; mo – mouth; mg – male gonopore; ns – nervous system; ov – ovaries; p – parenchyma; te – testes; wpf – weeks post fertilization. Scale bars 100 µm

The adult animals are hermaphroditic with follicular gonads, having multiple anteriorly (pre-orally) located testes and multiple posteriorly (post-orally) located ovaries (Westblad, 1949). The mature gonads are located in the parenchyma between the body wall and the intestine (Figure 1.2 E). The gametes in the female part mature lateromedially, from young oocytes found dorsally close to the body wall and mature oocytes found more ventrally, proximal to the medially located intestine (Westblad, 1949). The mature eggs are deposited via mouth (Sterrer, 1998; and own observations). Male germ cells develop into uniflagellate and filiform spermatozoons (Lundin & Hendelberg, 1998) with a small acrosomal vesicle on top (Buckland-Nicks et al., 2019). The sperm which can be found in the epithelia, parenchyma, and the posterior (ovaries-containing) part of the animal (Sterrer, 1998; Westblad, 1949) was considered to be allosperm (Lundin & Hendelberg, 1998). Fertilization has not been directly observed but is thought to occur externally based on the presence of the sperm around the spawned oocytes (Børve & Hejnol, 2014) in contrast to the proposed internal fertilization in *Nemertoderma* species (Jondelius et al., 2004). The mature sperm in *M. stichopi* are stored in the sperm vesicle of the male apparatus connected to the male gonopore opening located dorso-posteriorly (Westblad, 1949). The sperm bundles are released from the gonopore and the mouth (Børve & Hejnol, 2014; and own observations).

CHAPTER 2: Aims of the study

The overall aims of this thesis were focused on exploring the developmental and evolutionary relationship between germline and stem cells. As introduced in the above sections, these two cell lineages share many similarities on a molecular level. In the past, these similarities have led to the hypothesis proposing their common evolutionary descent from primordial stem cells (PriSCs) (Solana, 2013). Such primordial stem cells contribute to gamete precursors in sexually reproducing animals where their activity and morphogenetic potential are temporally restricted by the germline specification timepoint. On the other hand, the PriSCs in asexually reproducing animals or animals with extensive regenerative capacities can generate germ cells and somatic cells, and in some species are maintained until adulthood and can self-renew. Testing this hypothesis, in my thesis I investigated the potential presence of such cell population in the hermaphrodite marine worm *Meara stichopi* (Nemertodermatida). This species was selected for several reasons:

a. Because of their phylogenetic position, the question of how this species specifies the germline becomes very informative for our understanding of the evolution of germline in Metazoa.
b. Nemertodermatida belongs to the group Xenacoelomorpha (Cannon et al., 2016; Philippe et al., 2011), which shows a highly disputed phylogenetic position in the metazoan tree of life. Studying nemertodermatids, whose features are considered less derived than those of acoels or *Xenoturbella* (Achatz et al., 2013; Sterrer, 1998) should improve our interpretations of data coming from this enigmatic group.
c. The adult stem cell population has been documented in their sister group Acoela (De Mulder, Kuales, et al., 2009; Gschwentner et al., 2001; Ramachandra et al., 2002; Srivastava et al., 2014), thus *M. stichopi* represents a perfect candidate to explore the evolution of this trait in Acoelomorpha.

The goals of the thesis can be divided into the following subsections.

2.1 Evolutionary and developmental origins of germline with a focus on Nemertodermatida

It has been proposed that the inductive mode — where cell differentiation relies on signaling — and not the inheritance mode — where cells inherit the fate determinants — is the ancestral mode of the germline specification (Extavour & Akam, 2003). Therefore, studying germline specification in nemertodermatids brings valuable data into these discussions whether we consider nemertodermatids as a part of Xenacoelomorpha - a sister group of all other bilaterians with excretory system (Cannon et al., 2016), or as a part of Xenambulacraria (Philippe et al., 2011) and thus reduced deuterostomes. In this aim, I set to identify the specification timepoint of the germline in *Meara stichopi*. To achieve this, taking into account the symbiotic lifestyle and hypothesized annual life cycle (Børve & Hejnol, 2014), several developmental stages of *M. stichopi* were collected throughout 4 years of sampling seasons with the help of the Hejnol lab members. Investigation of the gene expression of a conserved set of genes previously identified as germline markers — *vasa, piwi, nanos* — were carried out to approximate the occurrence of germ-soma separation during this animal's development. Likewise, because of its phylogenetic relationship with all other bilaterians, the relationship between germline and mesoderm is especially of interest. To this account, the collected single cell and bulk transcriptomic data allows the inference of single cell RNA sequencing (scRNASeq)-informed developmental trajectories, improving our understanding of nemertodermatid cell types and their molecular signature throughout development. With this design, the presented thesis brings long-awaited pieces of information from nemertodermatid species into the field of Xenacoelomorpha and germline research.

2.2 Connection of primordial germ cells and stem cells

I intended to determine the presence of a potential stem cell population in *M. stichopi*. The dynamics of cell proliferation were inferred by means of direct visualization in homeostasis, throughout the three coarse stages of the lifecycle: hatchling, juvenile, and adult. This overview offers a basis for the estimation of the size of potential stem cell pool size throughout development. As a factor predetermining regenerative abilities, this too varies greatly amongst different acoel species: from the embryonic “stem neoblasts” (Ramachandra et al., 2002) or stem cells facilitating asexual reproduction (Gschwentner et al., 2001) in *Convolutidae*, to neoblast-like stem cells maintaining the somatic cell types and enabling posterior regeneration in *Isodiametra* (De Mulder, Kuales, et al., 2009; Perea-Atienza et al., 2013) or whole body regeneration in *Hofstenia* (Srivastava et al., 2014). To determine the cell potency of putative neoblast-like cells in *M. stichopi*, I evaluated their expression levels of the germline multipotency genes (Juliano et al., 2010) in the collected transcriptomic data and *in situ* - comparing the homeostatic state with the injury-induced state. The wound healing process in injured animals is compared to the aforementioned regenerative capabilities in the acoels in its time progression and molecular similarities, mainly focusing on the previously described regeneration gene regulatory network (Gehrke et al., 2019). Further examining the molecular aspects of both processes — germline and stem cell differentiation and maintenance — I analyzed the transcriptomes of available *Xenoturbella* and nemertodermatid species, comparing the broad magnitude of expressed classes of RNA-binding proteins. The findings are discussed in the phylogenetic context of life history-subjected changes.

CHAPTER 3: Materials and Methods

All the material and methods used for the here presented work are are described in respective Materials and Methods sections of respective manuscripts, but the following section will provide more detailed protocols for some of the used techniques.

3.1 Animal collection and maintenance

The sea cucumbers (*Parastichopus tremulus* Gunnerus, 1767) were collected using the Schander-sledge at locations described in Børve and Hejnol (2014). Nemertodermatid worms of species *Meara stichopi* were removed from the dissected sea cucumber foregut. The data presented in this study comes from specimens collected in the years 2018-2022. After the dissection, the worms were transferred to glass culture bowls with filtered deep-sea water sampled at the collection spot and kept at 8 °C. The water was changed regularly, twice a week. During the spawning season (late August to mid-October), the gravid adults of sizes 1-2 mm were left to spawn naturally. The fertilized eggs were collected, transferred to small Petri dishes, and kept in the deep-sea water supplemented with the antibiotic mix (100 mg pentamycin G, 25 mg streptomycin, 25 mg gentamycin; dissolved in 10 ml of natural seawater and used 1:100). The embryos were kept until they hatched out the eggshell (ca. 2 months). These lab-cultured hatchlings of sizes up to ~ 400 µm were directly fixed or kept for the cell proliferation experiments. Consistently with the presumed life cycle of *M. stichopi* (Børve & Hejnol, 2014), juveniles (a stage devoid of gonads) of sizes 500-800 µm were collected in April or May and adults in late August to October.

The adult brachipods of species *Terebratalia transversa* (Sowerby 1846) were collected near San Juan island, Washington, USA during the spawning season (January). The spawned eggs were reared as described previously (Freeman, 1993) until the late larva stage. Several developmental stages were fixed for ISH.

3.2 Animal fixation

Adults and various developmental stages of *M. stichopi* and *T. trasversa* were fixed at room temperature with 4% paraformaldehyde (PFA) solution in seawater for 1 h. Subsequently, the samples for ISH were washed in PTw (1x PBS with 0.1 % Tween-

20) and samples for immunostaining were washed in 1xPTX (1xPBS with 0.1 % Triton X-100). After a quick was in Milli-Q® H_2O (ISH) or 1xPBS (immunostaining), the samples were transferred into 100% MeOH or 1xPBS, and stored at -20 °C or 4 °C, respectively.

3.3 Transcriptome assembly and annotation for *M. stichopi*

To obtain a reference transcriptome, total RNA was extracted from hatchlings, juveniles, and gravid adults. Each sample tube contained 80, 30 or 8 animals of a respective stage. Samples were always processed in duplicate. RNA extractions from hatchlings and juveniles were done with the Nucleospin® RNA XS kit (Macherey Nagel), while the extraction from adults with Direct-zol RNA Miniprep (Zymoresearch). RNA quality and concentration were checked using the Agilent BioAnalyzer and the samples were stored at -80 ˚C. Total RNA from hatchlings and juveniles was used to prepare stranded libraries (TruSeq RNA library kit, Illumina) that were sequenced on the Illumina platform in the NorSeq Sequencing core (Oslo, Norway), yielding 111 M paired-end reads for hatchlings and 140 M paired-end reads for juveniles. Stranded libraries generated from adult worms total RNA were sequenced on the Illumina platform in the Genomics Core Facility (EMBL Heidelberg, Germany), yielding 139 M paired-end reads. The reads were quality trimmed using trimmomatic (Bolger et al., 2014) bundled within the Trinity package (Grabherr et al., 2011), and normalized using the insilico_read_normalization.pl script from Trinity package using the --*KMER_SIZE 21* and --*max_cov 150* options. 52 486 803 reads (15.65 %) selected in the normalization process were assembled into the reference transcriptome using the Trinity v 2.12 with the following parameters: --*include_supertranscripts* --*SS_lib_type* RF. As a quality check, the normalized reads were mapped back to the assembly with bowtie2 (Langmead & Salzberg, 2012). The mapback rate of the assembly was 78%. The redundancy was removed with cd-hit v 4.8.1 (W. Li & Godzik, 2006), using default settings. The resulting transcriptome had 236 923 transcripts and BUSCO v5.2.2 (Manni et al., 2021) completeness of 92.3%. The assembled transcriptome was used as an input to find coding regions with Transdecoder (Haas,

BJ. https://github.com/TransDecoder/TransDecoder), homology predictions with blastx, blastp (v 2.7.1) using the Uniprot database (release 2020), and Pfam (release P21) were used to improve the final proteome prediction. The best hits from homology predictions were used for annotation.

3.4 Single cell dissociation of *M. stichopi*

Hatchlings

The cells at this stage are more fragile, therefore in order to preserve their cells, a layered dissociation approach was chosen. The samples were processed in triplicate. Around 200 animals were pre-incubated in Calcium Magnesium free artificial sea water (CMFSW: 31 g NaCl, 0.8 g KCl, 0.29 g $NaHCO_3$, 1.6 g Na2SO_4, 1 l distilled water) for 20 min. In the meantime, the Protease XIV (Sigma-Aldrich) dissolved in Low Ca^{2+} no Mg^{2+} artificial sea water (ASW) (26.88 g NaCl, 0.74 g KCl, 0.16 $CaCl_2$, 2.38 ml HEPES, 1 l distilled water, pH7.6) at the final concentration of 10 mg/ml was heat activated for 20 min at 39 ˚C. After the pre-incubation, the enzyme was added to the animals at the final concentration 4 mg/ml. The samples were triturated gently for 1 min, left on a shaker (room temperature, 550 rpm) for 10 min, and then centrifuged briefly (600 g). The supernatant (already containing the cells) was removed, diluted with Low Ca^{2+} no Mg^{2+} ASW, and kept on ice until the next centrifugation step (from now on: supernatant tube). The pellet was resuspended in the dissociation solution with a final enzyme concentration of 8 mg/ml. The samples were triturated gently for 2 min and then left on a shaker (room temperature, 550 rpm) for 15 min and triturated a few more times. Dissociation was stopped by adding CMFSW up to 1 ml of total tube volume and the samples were centrifuged together with the supernatant (4.5 min, 700 g, 4 ˚C). The pellet from the "supernatant" tube was resuspended in a small volume of Low Ca^{2+} no Mg^{2+} ASW and added to the resuspended pellet of the corresponding samples. The samples were filtered through 40µm cell strainer (Falcon), washed with 1 ml of Low Ca^{2+} no Mg^{2+} ASW and centrifuged (4.5 min, 700 g, 4 ˚C). The pellet was resuspended in 80 µl No Ca^{2+} no Mg^{2+} ASW and 20 µl of 1x PBS. Cell viability was

assessed by FDA/PI staining (PI – 1 mg/ml concentration 1:500 dilution, FDA – 11 uM concentration 1:100 dilution, both Sigma-Aldrich) to be between 70-80 % for all the replicates. Cell concentration was meassured with a hemocytometer. The three replicates had cell concentrations of: 880 cells/µl; 3250 cells/µl; 3150 cells/µl.

Juveniles

Animals were pre-incubated in CMFSW for 15 min. In the meantime, Protease XIV dissolved in Low Ca^{2+} no Mg^{2+} ASW at the final concentration of 10 mg/ml was heat activated for 20 min at 39 ˚C. After the pre-incubation, the enzyme was added to the animals in the final concentration of 8 mg/ml. The samples were triturated gently for 1 min, left on a shaker (room temperature, 550 rpm) for 20 min, and then centrifuged briefly (800 g). The supernatant (already containing the cells) was taken out, diluted with Low Ca^{2+} no Mg^{2+} ASW, and kept on ice until the next centrifugation step. The pellet was resuspended in the dissociation solution with the final enzyme concentration of 10 mg/ml. The samples were triturated gently for 2 min and then left on a shaker (room temperature, 550 rpm) for 10 min and triturated a few more times. Dissociation was stopped by adding CMFSW up to 1 ml of total tube volume and the samples were centrifuged together with the supernatant (4.5 min, 800 g, 4 ˚C). The pellet in a "supernatant" tube was resuspended in a small volume of Low Ca^{2+} no Mg^{2+} ASW and added to the resuspended pellet of the corresponding samples (200 µl of Low Ca^{2+} no Mg^{2+} ASW). The samples were filtered through the 40 µm cell strainer, washed with 1 ml of Low Ca^{2+} no Mg^{2+} ASW, and centrifuged (4.5 min, 800 g, 4 ˚C). The pellet was resuspended in 80 µl CMFSW and 20 µl of 1x PBS. The cell suspension was washed again, resuspended in the same volume, and filtered through the 40 µm cell strainer (Falcon). The cell viability was assessed by FDA/PI staining (PI – 1 mg/ml concentration 1:500 dilution, FDA – 11 uM concentration 1:100 dilution) to be between 70-80 % for all the replicates. The cell concentration in the suspension was: 20000 cells/µl; 1300 cells/µl; 1550 cells/µl.

Adults

Three tubes with 6-10 gravid animals each were incubated in CMFSW for up to 20 min. In the meantime, the Protease XIV dissolved in Low Ca^{2+} no Mg^{2+} at the final concentration 10 mg/ml was heat activated for 20 min at 39 ˚C. After the pre-incubation step, the animals were transferred to a low-binding tube with 100 µl of protease XIV dissociation solution and incubated in the thermomixer at 24 ˚C, 550 rpm. After 30 min, the tube was flicked to see the progress of dissociation and after 1 h, the solution was gently triturated every 10 min. Dissociation was stopped after 1.5 h by adding 300 µl of Low Ca^{2+} no Mg^{2+} ASW. The tube was centrifuged (4 min, 800g), the pellet was resuspended in 300 µl of Low Ca^{2+} no Mg^{2+} ASW, washed and centrifuged again (4 min, 800g), and resuspended in 80 µl of CMFSW and 20 µl of 1x PBS. Resuspended cells were filtered once through the 40 µm cell strainer (Falcon) and twice through the 20 µm cell strainer. The cell viability was assessed by FDA/PI staining (PI – 1 mg/ml concentration 1:500 dilution, FDA – 11 uM concentration 1:100 dilution) to be between 70-80 % for all the replicates. The cell concentration in the suspension was: 8800 cells/µl; 12500 cells/µl; 17000 cells/µl.

3.5 Single-cell RNA sequencing of *M. stichopi*

Chip loading and library preparation

Hatchlings & Juveniles: Fresh cell suspension volumes containing ca. 40 000 cells were loaded into the 10x Chromium 3' gene expression (double index) chips v3.1 (10x Genomics, USA). The CMFSW was used as a cell dilution buffer in all cases to reach the cell suspension volume as advised by 10x Genomics guide.

Adults: The fresh stock cell suspension volumes containing ca. 40 000 cells were loaded into the 10x Chromium 3' gene expression (single index) chips v2 and v3.1 (10x Genomics). The CMFSW was used as a cell dilution buffer in all cases to reach the cell suspension volume advised by 10x. To account for different loading volumes in v2 (90 µl) and v3.1 (70 µl), and hence different salt concentrations, a portion of nuclease-free water was replaced by 1xPBS for the v3.1 chip.

After the Chromium Controller runs, we followed the manufacturer's instructions to obtain the cDNA, quantified on Qubit (Invitrogen) and Bioanalyzer (Agilent), and prepared libraries. The resulting libraries were tested for size distribution and quantified on Bioanalyzer (Agilent) and then stored at -20 ˚C. The adult libraries were sequenced on Illumina platform in the Genomics Core Facility (EMBL Heidelberg, Germany), the hatchlings and juvenile libraries were sequenced on Illumina platform in the NorSeq Sequencing core (Oslo, Norway).

Data analysis and clustering

The reads obtained from single-cell RNA sequencing were mapped to the reference transcriptome of *M. stichopi* using the Cell Ranger (v 6.0.2, 10x Genomics). A custom script kindly provided by Dr. Daniel Leite from Telford lab was used to make a gtf file for the reference transcriptome. Reads from one of the hatchling replicates were excluded from the following analysis due to contamination. Output of the cellranger count — the gene expression matrices — were analyzed in Seurat v4 (Hao et al., 2021). The raw gene expression matrices were used to create Seurat objects while keeping the genes expressed in at least 10 cells and keeping the cells expressing at least 150 features (parameters *min. cells* = 10, *min. features* = 150). For each dataset, only the genes with at least 3 counts were kept. The matrices of replicate libraries were merged into a single Seurat object per stage. Each dataset was further quality filtered using the following criteria. The hatchling dataset, to have the UMI counts of at least 300, a number of features between 150 and 2500, a log10genesPerUMI metric greater than 0.8, a percentage of mitochondrial genes less than 5 %, and a percentage of rRNA genes less than 10 %. The juvenile dataset, to have the UMI counts of at least 500, a number of features between 250 and 2500, a log10genesPerUMI metric greater than 0.8, a percentage of mitochondrial genes less than 5 %, and a percentage of rRNA genes less than 5 %. The adult dataset, to have the UMI counts of at least 580, a number of features between 280 and 2500, a log10genesPerUMI metric greater than 0.8, a percentage of mitochondrial genes less than 5 %, and a percentage of rRNA genes less than 5 %. This yielded a total of 4359, 3568, and 35328 cells for hatchling, juvenile, and adult stage, respectivelly (Suppl. Table 1). Each sample in datasets was log normalized, and

variable features were selected. Replicate samples per stage were subsequently batch-corrected via the Seurat CCA method (Stuart et al., 2019). All three resulting stages were integrated using the standard rPCA workflow with 2738 anchors. The integrated dataset contained 43255 cells and 67546 genes, the median UMI count was 938, and the median genes expressed per cell was 413. The integrated dataset was scaled with nCount, mitochondrial and rRNA percentages regressed out. The PCA was calculated using 30 dimensions. The subsequent nonlinear dimensionality reduction was performed using UMAP method *"umap learn"* with *metric = correlation* and parameters *dims = 1:30*, *min.dist = 0.001*, *spread = 0.8*, *n.neighbors = 5*. Nearest neighbors were found using 30 dimensions and default parameters. The Leiden clustering with the igraph method was performed with a resolution 0.3 which yielded 16 clusters. The cluster markers were obtained with FindAllMarkers using the Wilcoxon test, including genes expressed in at least 25 % of cells, and returning markers that have a p-value less than 1e-5. To annotate the clusters, top 100 markers were selected based on the average log2 fold change (avglog2FC) and used as a query in the blastx search with UniProt human database. Human homologs were subsequently used for the GO search. If the blastx hit was missing the homology predictions from the transcriptome annotation were used. To calculate cluster correlation, Pearson correlation was calculated from the average cluster expression obtained with AverageExpression. For cluster-specific URD analysis, each cluster was subsetted as a separate Seurat object, renormalized, scaled, and reintegrated.

3.6 Single cell trajectory inference

To evaluate a pseudotime ordering of detected cell clusters a semi-supervised inference using Slingshot was performed (Street et al., 2018). The cluster 1 or cluster 5 was used as a starting cluster. The resulting cluster-based minimum spanning tree (MST) of global lineages in the dataset was used to infer three categories of cell clusters with respect to differentiation: initial, intermediate, and terminal. To ensure the best stage representation possible, the integrated dataset object was split by stage and downsampled to include a maximum of 90 cells per cluster identity class. The downsampled objects were merged again and subset based on the MST-inferred

differentiation categories. Their gene expression matrices and metadata were used as the input to create the URD object (Farrell et al., 2018) with *min.cells* and *min.counts* parameters set to 3. To calculate diffusion maps, an auto-detected sigma was used. The cells from cluster 1 were used as the root cells, cells belonging to the "terminal" category were used as the tips of the tree. For the logistic to bias the transition probabilities, parameters *optimal.cells.forward =20*, *max.cells.back =40* were used. The URD tree was built using all the tips, "preference" divergence method, 25 *cells.per.pseudotime*, 8 *bins.per.pseudotime.window* and *p.thresh=0.001*. To extract markers and differentially expressed genes along the URD tree segments, the aucprTestAlongTree was used with parameters *log.effect.size =0.4*, *auc.factor=1.25*, *max.auc.threshold=0.85*, frac.must.express=0.1, frac.min.diff=0, *must.beat.sibs=0.1.*

3.7 Gene cloning and orthology assessment

Putative orthologs of genes of interest were found in the transcriptome of *M. stichopi* and *T. transversa* (SRX1307070) using the tBLASTx search. Gene orthology of genes of interest identified by tBLASTx was tested by reciprocal BLASTx against NCBI Genbank database and followed by phylogenetic analyses. Protein sequences for the phylogenetic analysis were obtained from the GenBank, UniProt, or OrthoDB databases and aligned with MUSCLE (R. C. Edgar, 2004). Alignments were trimmed with TrimAl (Capella-Gutiérrez et al., 2009) using the following parameters: *-gt 0.8 -st 0.001 -cons 60*. Trimmed alignments were used to construct maximum likelihood phylogenetic trees with IQ-TREE (Minh et al., 2020), using ModelFinder (Kalyaanamoorthy et al., 2017) and Ultrafast Bootstrap (Hoang et al., 2018) options. The genes of interest were amplified from cDNA of *M. Stichopi*, or *T. transversa* using gene-specific primers (Sigma-Aldrich). PCR products were purified and cloned into the pGEM-T Easy vector (Promega), the inserts were sequenced and used as templates for the probe synthesis.

3.8 Probe synthesis and the *in situ* hybridizations

The labeled antisense riboprobes for colorimetric whole mount *in situ* hybridization (WMISH) or fluorescence-based detection in the whole mount *in situ* hybridization (FISH) were prepared as follows. The genes of interest were amplified from the cDNA

prepared from pooled *M. stichopi* or *T. transversa* stages using the gene specific primers. Amplified sequences were ligated into the pGEM® T Easy Vector (Promega), which was transformed into the competent *E. coli* using the heat shock method. The transformed bacteria were plated on the LB plates with Ampicilin (1 µl/ml) and freshly added X-Gal (Sigma-Aldrich), allowing for the blue-white selection, and incubated at 37 °C overnight. The next day, the positive colonies were picked, screened by PCR, and grown on another tracking LB plate. The positive clones, carrying the inserts of a correct size, were inoculated into the liquid LB medium with Ampicilin (1 µl/ml), grown overnight at 37 °C in a shaker (250 rpm), to be used for the miniprep isolation with NucleoSpin® Plasmid kit (Macherey-Nagel) the next day. The isolated plasmids were linearized in the PCR reaction, sequenced in the sequencing facility of the University of Bergen (Norway), reamplified, and used as the templates for the antisense riboprobes. For colorimetric WMISH, the probes, obtained by the *in vitro* transcription reaction using MEGAscript™ kit (Invitrogen, Thermo Fisher) were labeled by the Digoxigenin-11-UTP base analog (Roche), were diluted in the Hybe buffer (50% Formamide; 5x SSC (pH4.5); 50 µg/ml Heparin; 0.1% Tween-20; 1% SDS; 100 µg/ml Salmon Sperm DNA; prepared in DEPC-treated H_2O) and used at 1ng/µl. The WMISH was conducted as described in (Hejnol & Martindale, 2008) with the following modification: proteinase K treatment was performed for 15 min for *M. stichopi* and 10 min for *Terebratalia transversa*. Hybridization steps were carried out at 64 °C for approximately 72h. The Dig-11-UTP labeled riboprobes were detected with the alkaline phosphatase (AP)-conjugated anti-Dig antibody diluted to 1:5000 in the Boehringer-Mannheim Blocking buffer. The signal was visualized by the AP-catalyzed colorimetric reaction from the NBT/BCIP substrate. The samples were imaged using the Axiocam HRc camera mounted on Axioscope Ax10 (Zeiss, Germany). Images were edited in Fiji and Adobe Photoshop CS6. Pairwise stitching (Preibisch et al., 2009) was used for images that were too large.

For the FISH, the riboprobes were labeled post-transcription with DIG and DNP, using the *Label*-IT kit (Mirus BIO), following manufacturer's instructions. DIG-/DNP-labeled riboprobes were used at 1ng/µl and detected with the anti-DIG or anti-DNP

POD antibodies (Sigma Aldrich) used at 1:250. The signal was amplified using the TSA Plus Cy3/Cy5 Kit (Perkin Elmer, USA) for 1 h. The samples were imaged using the confocal laser scanning microscope (CLSM) Leica SP5 (Leica, Germany). Images were edited in Fiji (Schindelin et al., 2012) and Adobe Photoshop CS6.

The probes for the hybridization chain reaction (HCR) were designed by Molecular Instruments, Inc. (USA). The HCR staining protocol was based on (Ibarra-García-Padilla et al., 2021a; S Bruce et al., 2021) with the following modifications: the pre-hybridization step and hybridization step were extended to overnight incubations.

To simultaneously visualize proliferating cells, DIG-labeled FISH probes, and HCR probes in *M. stichopi*, following modifications were introduced. Samples were pretreated as described in the standard WMISH protocol (above) with the proteinase K treatment of 15 min. After the hybridization step for DIG-labeled probes (left out in the control samples that were only stained with HCR probes), the HCR pre-amplification at 37 °C was performed overnight. The samples were washed according to the standard protocol, prehybed for 1h with warm HCR hybe buffer at 37 °C, and subsequently hybridized at the same temperature overnight. The next day, after the last wash in 5xSSCT, the EdU was developed following manufacturer's instructions, and samples were blocked for 1h in Boehringer-Mannheim Blocking buffer and incubated with the anti-DIG antibody (1:5000) at 4 °C overnight on a rocker. Next, samples were washed, and the signal was amplified using the TSA Plus Cy3/Cy5 Kit (Perkin Elmer, USA). Finally, DAPI (1:1000) was used to counterstain the nuclei.

CHAPTER 4: Summary of the findings

The findings of my PhD research which are presented in detail in the enclosed publication are summarized below.

1. *Meara stichopi* has neoblast-like cells located in the parenchyma of the animal, in the proximity of the muscle fibres and nervous plexus, and are retained until the adulthood. These cells express a known set of germline multipotency program (GMP) genes (*piwi, vasa, nanos*); however, as their number decreases throughout development, it is likely that their main function is to contribute to developing structures, mainly the gonads.
2. The progression of gonad formation was traced by comparing *piwi1* and *nanos* expression in gonad-lacking juveniles and sexually mature adults. Gene expression studies revealed the *piwi*- and *nanos*-expressing cells of gonad primordia of juvenile animals. These genes were also expressed in other structures of the juveniles, confirming their pleiotropic roles.
3. Testing the differentiation potential of the adult *bona fide* stem cell-like cells by exposing the animals to injuries did not lead to a whole-body regeneration within the timeframe of six days. The amputation experiments revealed that the mechanisms of wound healing and blastema formation were considerably slower than in the acoel species whose regenerative capacities have been studied thus far. This process was accompanied by increased cell proliferation in the proximity of the wound. Several genes are involved in the regeneration process of *M. stichopi* based on their expression in the blastema-proximal region. Namely, the GMP genes (*piwi1*, *vasa*, *nanos*) and early growth response (*egr*) and *runt* transcription factors.
4. I explored the activity of a regeneration gene regulatory network (GRN) found to control the wound-induced gene expression in the acoel *Hofstenia miamia* and planarians. To this aim, I identified the orthologous genes in the transcriptome of *M. stichopi* and documented their expression in homeostasic and regenerating animals. Under homeostatic conditions, the expression of *egr*, *runt*, *deaf-1*, *neuregulin 2* (*nrg2*), *follistatin*, and *nemo-like kinase* (*nlk*) — the components of

regeneration GRN — was observed to be biased to the posterior, ovaries-bearing end, in sexually mature animals. On the other hand, their expression was always absent in the smaller, sexually mature individuals with less ripe gonads bearing early stages of germ cells, and in sexually immature juvenile stages of *M. stichopi* that are devoid of gonads. Homeostatic regeneration GRN gene expression in the ovaries-proximal region can be explained by the oviposition mechanism in *Meara* which occurs through the mouth and likely involves the disruption of the intestine as the eggs are being released from the follicles. This suggests that the genetic components of the regeneration GRN are present in *M.stichopi*'s transcriptome, and function in oviposition-induduced injury response. However, their ability to orchestrate a full whole-body regeneration upon amputation was either lost, temporally restricted to younger stages, or modified due to developmental constraints adapted to *M.stichopi'*s life history.

4.1 Single-cell RNA Sequencing Results

I collected single-cell transcriptomes from three developmental stages: hatchlings, juveniles, and adults. Each stage was sampled with a biological replicate: two libraries from the hatchling stage, three from the juvenile stage, and four from the adult stage. Figure 4.1 outlines the experiment design starting from sample collection throughout the years to the data analysis.

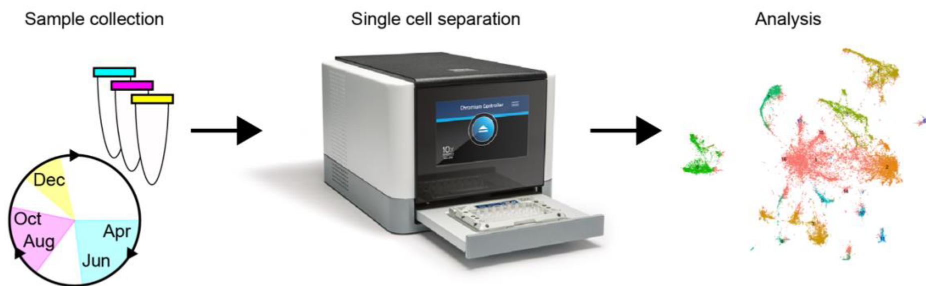


Figure 4.1 **Experiment design for single-cell RNA sequencing of *M. stichopi*.** Hatchling (yellow), juvenile (cyan), and adult stages (magenta) were collected in a designated timeframe, dissociated into single cells and loaded onto 10x chip. The 10x-processed cells

were used to prepare the sequencing libraries. Sequenced samples were analyzed as described in the text. The collection timepoints for *M. stichopi* stages are according to its life proposed life cycle (Børve & Hejnol, 2014). The image of 10x Controller is from the 10x website.

The raw gene expression matrices were quality filtered and batch-corrected per stage using Seurat CCA method (Stuart et al., 2019). The resulting three Seurat objects were integrated together using the rPCA integration workflow (see Material and Methods). The integrated dataset contained 43 255 cells consisting of 4 359 cells from the hatchling stage, 3 568 cells from the juvenile stage, and 35 328 cells from the adult stage (Suppl. Table 1). Clustering with a Leiden algorithm and a resolution of 0.3 yielded 16 cell clusters (Figure 4.2 B).

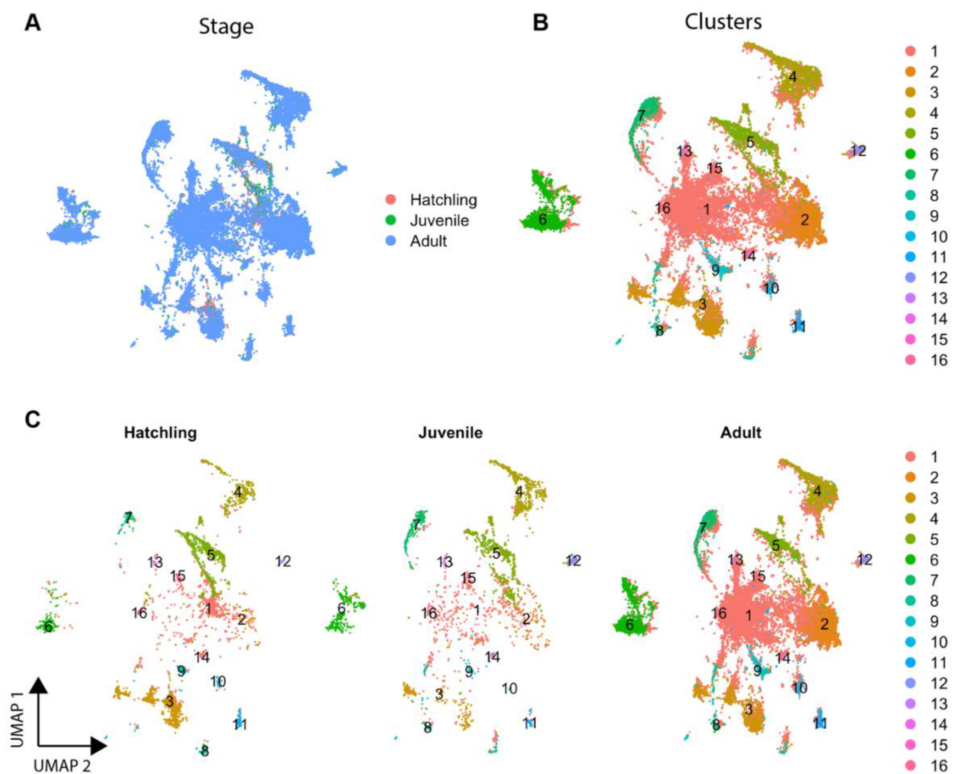


Figure 4.2 The UMAPs of integrated hatchling, juvenile, and adult scRNA-Seq datasets. A. UMAP colored by developmental stage showing a good integration of stages in each cluster. B. UMAp colored by cluster identity. C. The same UMAP split by developmental stage showing how cluster cell densities vary based on a developmental timepoint.

Generally, all clusters showed a good intermixing of all three developmental stages (Figure 4.2 A) except for the clusters 10, 14, and 15 which contained less than 10 cells from the hatchling or juvenile stages. Surprisingly, however, there were no stage-specific clusters. With only varying factors throughout the developmental timepoint being the cell densities (Figure 4.2 C).

<table>
<tr><th>Sample</th><th>Cells</th><th>10x Kit Version</th><th>Cells Batch-corrected</th><th>Median UMI</th><th>Median genes</th><th>No of Genes</th></tr>
<tr><td>Ad1</td><td>7365</td><td>v2</td><td rowspan="4">35328</td><td rowspan="9">938</td><td rowspan="9">413</td><td rowspan="9">67546</td></tr>
<tr><td>Ad2</td><td>20021</td><td>v2</td></tr>
<tr><td>Ad3</td><td>6971</td><td>v2</td></tr>
<tr><td>Ad4</td><td>971</td><td>v3.1</td></tr>
<tr><td>H1</td><td>1964</td><td>v3.1</td><td rowspan="2">4359</td></tr>
<tr><td>H2</td><td>2395</td><td>v3.1</td></tr>
<tr><td>J1</td><td>501</td><td>v3.1</td><td rowspan="3">3568</td></tr>
<tr><td>J2</td><td>223</td><td>v3.1</td></tr>
<tr><td>J3</td><td>2844</td><td>v3.1</td></tr>
</table>

Supplementary Table 1: Single-cell RNA Sequencing experiment technical parameters

4.1.1 Cluster cell identities description

Cluster 1 contains a high proportion of cells expressing zing metalloproteinases, protocadherins FAT2 and FAT4, and protein crumbs homolog 1 (CRUM1) (Figure 4.3). The GO terms associated with this cluster include secretory granules, both extracellular and intracellular secretory vesicles and endomembrane system. This cluster might represent epithelial cell identity.

Cluster 2 does not have any GO term associated with it. Its cells express high levels of microtubule nucleation factor (SSNA1), tubulin alpha chain (TBA), tubulin polymerization promoting protein family member 3 (TPPP3), and patched domain-containing protein 3 (PTHD3) which was found to be male germ cell-specific in mouse (Fan et al., 2007). High levels of transcripts involved in tubulin polymerization and maintenance, together with a prerequisite for active hedgehog signaling (Figure 4.3) make this cluster a good candidate for some stage of male germ cell identity.

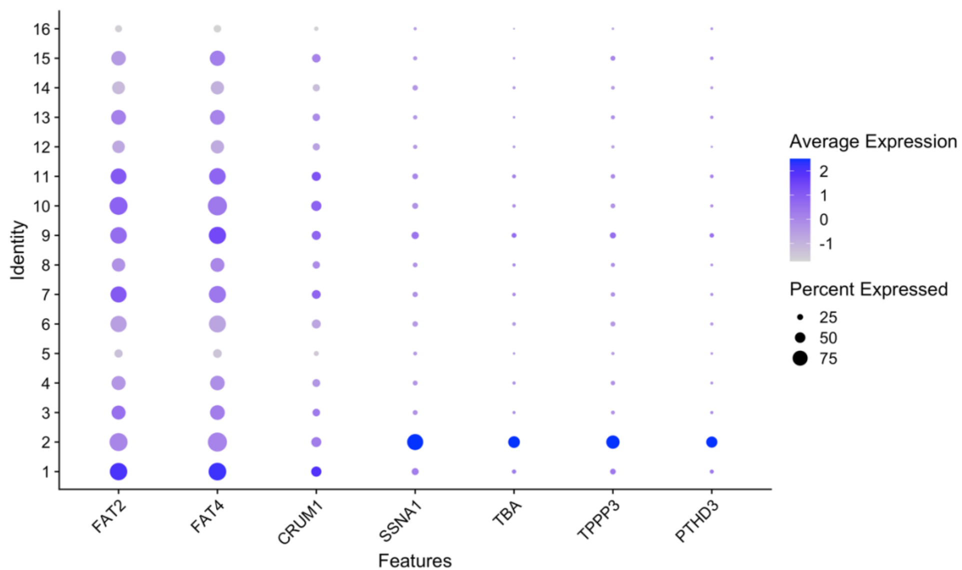


Figure 4.3 Dotplot showing marker expression for cluster 1 and cluster 2.

Clusters 3 is associated with GO terms secretory vesicle, exocytic and synaptic vesicle membranes and vesicles. The cells in this cluster show enrichment in genes involved in neuronal fate and neurosecretion (Figure 4.4 A) such as neuroendocrine protein 7B2 (7B2), secretagogin (SEGN), genes involved in voltage gated Calcium signaling such as calmodulin (CALM) and polycystin (PKD2). This together with high expression of neural proliferation differentiation and control protein (NPDC1), synaptotagmin (SY65), and Unc5 Netrin receptor (UNC5), which is required for the axon guidance in

Drosophila (Keleman & Dickson, 2001) suggest that these cells share a neuronal phenotype. A subset of cells in this cluster expresses high levels of pro-neural markers ELAV1, Sox2 and neuroD (Figure 4.4 B).

Cluster 8 shares neuroendocrine and neuronal markers 7B2, SEGN, NPDC1, with cluster 3. It also shows high expression of Gamma aminobutyric acid receptor subunits rho 2 and 3 (GBRR2, GBRR3) and Netrin receptor UNC5B (Figure 4.4 A). Thus, these cells likely represent another group of neurons. Interestingly, some of the top markers such as axonemal dynein, tubulin polymerization promoting protein, and intraflagellar transport protein suggest a subset of these cells might possess a sensory cilium, which is also supported by the GO terms for this cluster. Such sensory cells with cilia protruding into the epithelial layer have been observed in *M. stichopi* before (Børve & Hejnol, 2014) .

Cluster 11 shares neuroendocrine markers 7B2 and SEGN with clusters 3 and 8 and is also enriched in the expression of GABA receptor subunit alpha 5 (GBRA5) (Figure 4.4 A).

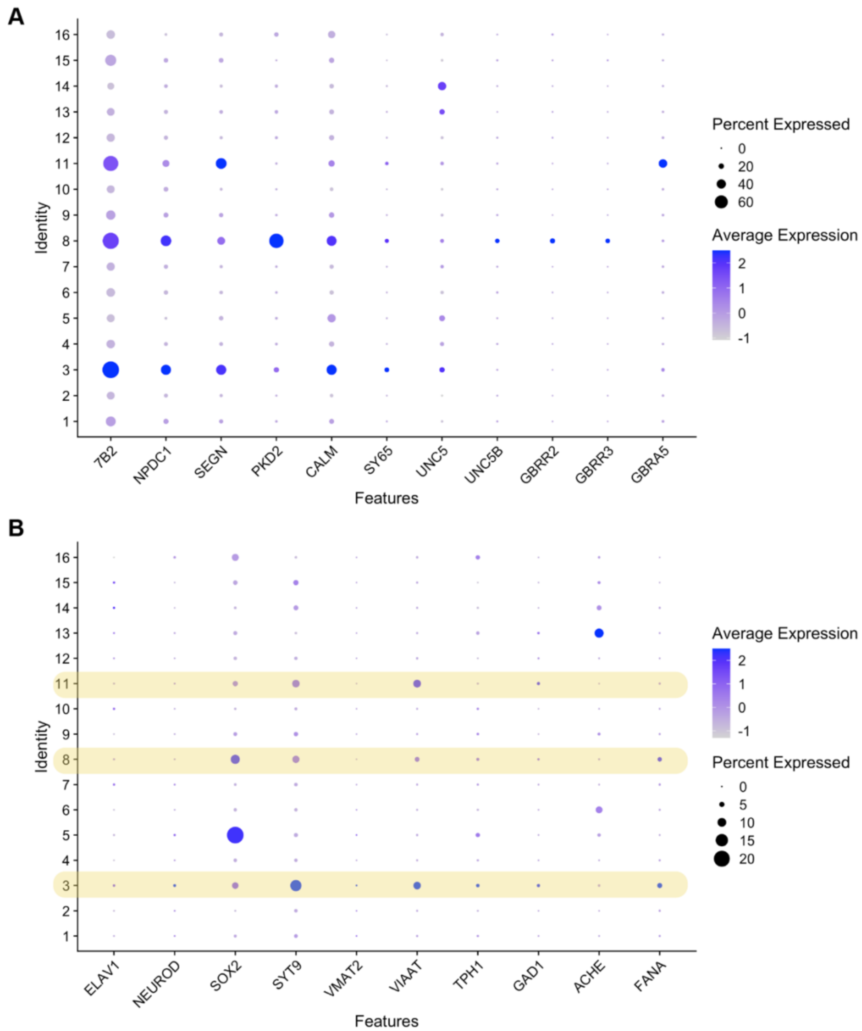
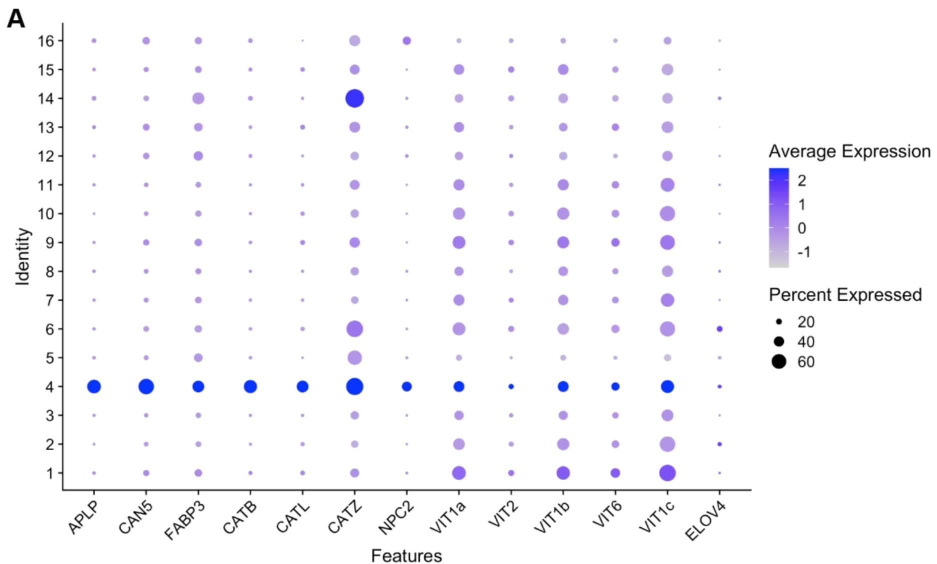


Figure 4.4 Neuron and neuroendocrine-like cell expression profile. A. Dotplot showing marker expression for neuron/ neuroendocrine-like clusters 3,8, and 11. **B.** Dotplot showing expression of pro-neural marker genes in these clusters (highlighted in yellow).

Cluster 4 is associated with GO terms lytic vacuole, lysosome, secretory vesicle, and pigment granules. Its cells show high expression of genes involved in lipid metabolism (Figure 4.5 A), such as apolipophorin (APLP), fatty acid binding protein type 3 (FABP3), and NPC intracellular cholesterol transporter 2 (NPC2), and genes involved in protein degradation such as calpain (CAN5) and cathepsins (CATB, CATL, CATZ). This expression profile is consistent with cells of digestive character. This cluster is also marked by high expression of vitelogenins and a specific expression of endodermal markers GATA456 and hepatocyte nuclear factor 4-alpha HNF4A (Figure 4.5 B).



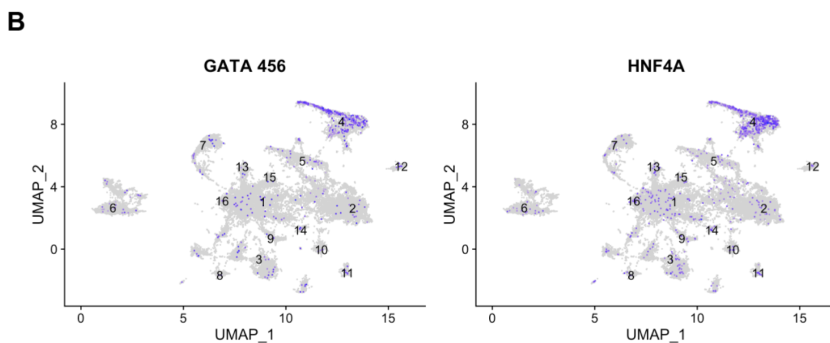


Figure 4.5 Digestive cells expression profile. A. Dotplot showing expression of top markers for digestive cells. **B.** Feature plots showing enriched expression of endodermal markers GATA456 and HNF4A in cluster 4 consisting of digestive cells.

Cluster 5 shows high expression of several histone variants, including late histone 2 variants and linker histone H1. More importantly, it also shows a high level of proliferating cell nuclear antigen (PCNA) and mitotic apparatus protein p62 (MP62), showing that these are actively proliferating cells. This cluster is also marked by a specific expression of transcription factor Prospero 1 (PROX1) found to regulate stem cell self-renewal and differentiation in Drosophila (Choksi et al., 2006), and Hes family transcription factors HES1A and HES1B which negatively regulate stem cell differentiation (Hu & Zou, 2022). The GO terms associated with this cluster include replisome, telomerase holoenzyme complex, ribosomal subunits, and ribonucleoprotein complex. Altogether, this cluster is a good candidate for putative neoblast cell identity.

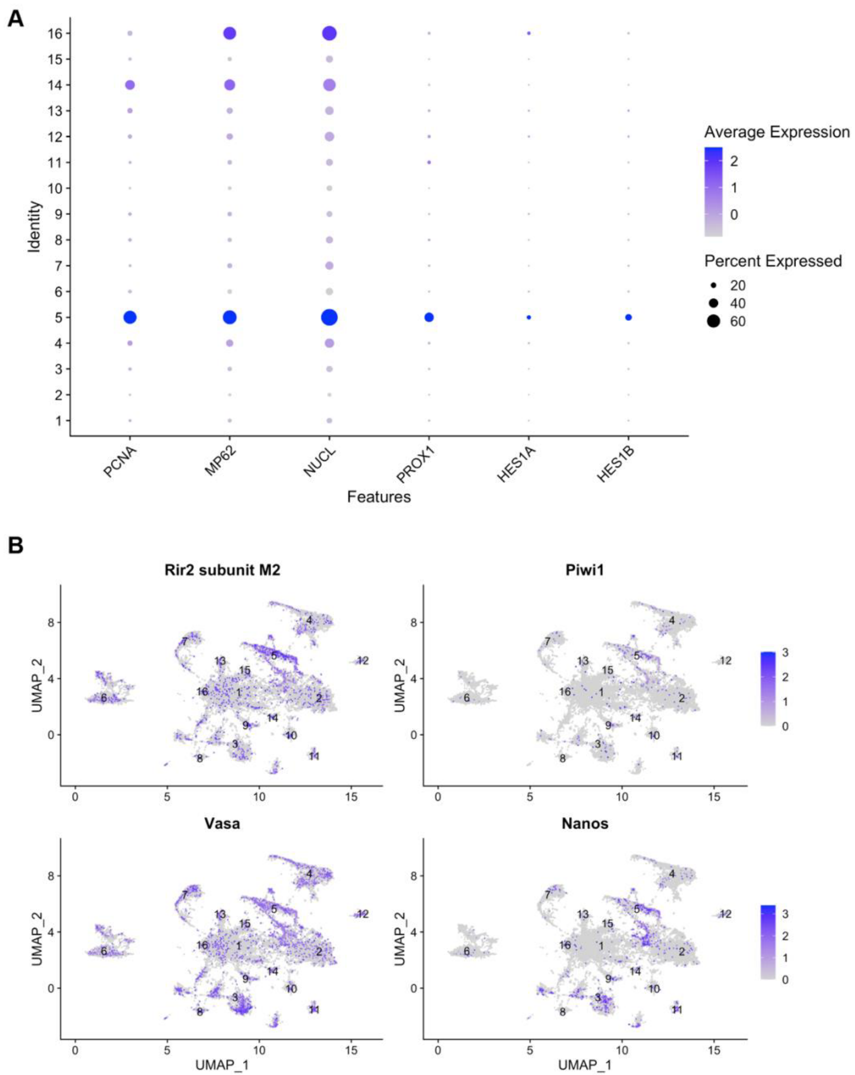


Figure 4.6 Proliferating cells expression profile. A. Dotplot showing expression of top marker genes for proliferating cells cluster. B. Feature plots showing expression of known neoblast marker *Rir2* and germline multipotency program genes *Piwi1*, *Vasa*, and *Nanos*.

Cluster 6 shows specific enrichment in tubulin alpha and beta chains (TBA,TBB), flagellar dynein heavy and light chain (DYH2, DYL1), Sperm axonemal maintenance protein Cfap97d1 (CF97D) and Four and half Lim domains protein 2 (FHL2). There is also a high expression of genes encoding Calcium binding proteins calmodulin and calretinin, which have both been shown to play a role in sperm motility and capacitation (Dressen et al., 2018; Leclerc et al., 2020). All the above suggests that a high proportion of this cluster represents male germ cells at various stages of spermatogenesis, which is corroborated by high expression of spermatogenesis-associated protein 17 (SPT17), sperm associated antigen 8 (SPAG8) and meiosis-specific nuclear structural protein (MNS1) (Figure 4.7 A, B). Surprisingly this cluster also contains cells from hatchling and juvenile — stages thought to be devoid of gonads. It is possible that these genes in hatchling and juvenile stages function in the formation of primary cilia and not the sperm axoneme as is presumably the case in the adult. Alternatively, there might be a pool of PGCs primed for meiosis present already in the hatchling and juvenile without the gonads being morphologically developed.

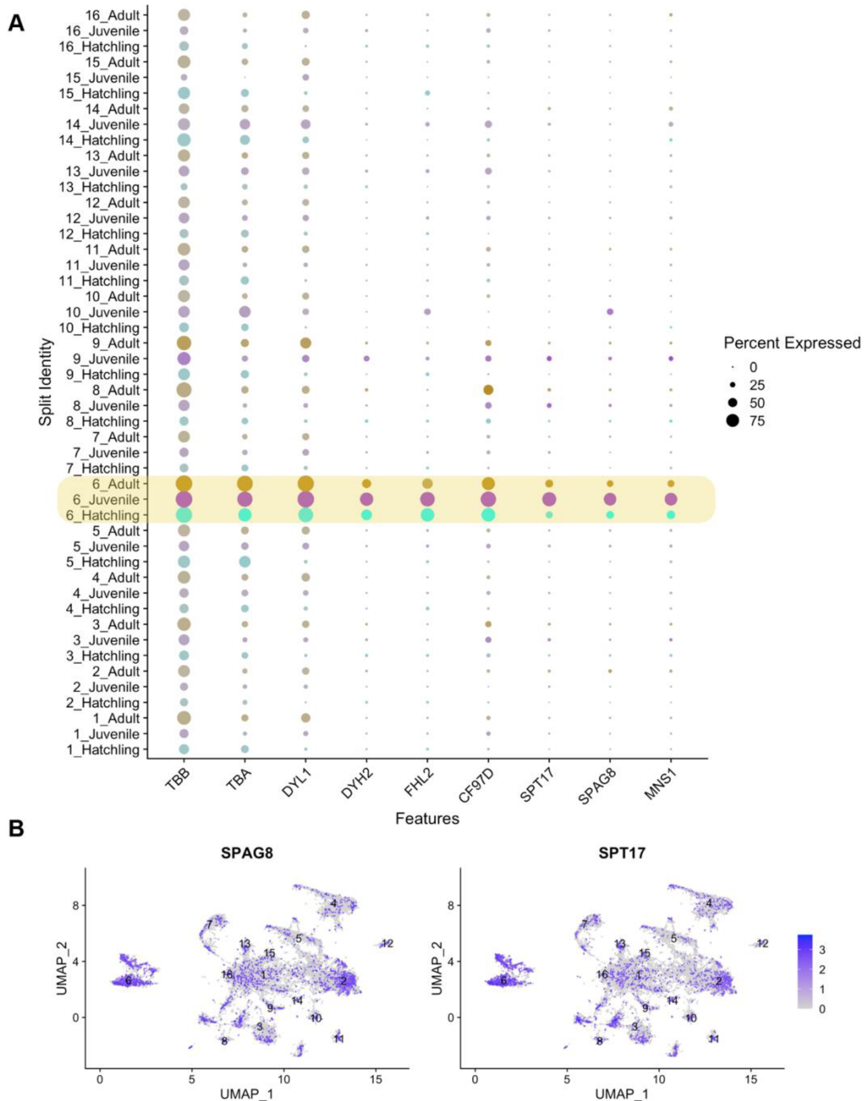


Figure 4.7 Male germ cells cluster expression profile. A. Dotplot showing expression of top markers for cluster 6 in each developmental stage. Hatchling expression in cyan, juvenile expression in magenta, and adult expression in yellow. Cluster 6 expression is highlighted in a yellow box. B. Feature plots showing expression of spermatogenesis ans sperm markers SPT17 and SPAG8.

Cluster 7 shows high expression of spondins (SSPO1 and SSPO2), fibrillin (FBN1), and sortilin-related receptor (SORL), suggesting that this cluster corresponds to the secretory cells (Figure 4.8). Other genes amongst top markers are coatomer subunits, and proteins involved in orchestrating vesicular transport and motility such as MAGE-like protein 2 (MAGL2), and kinectin (KTN1) highlighting the role of vesicular transport for this cell identity. GO terms for this cluster: translocon complex, nuclear outer membrane-endoplasmic reticulum membrane network; support the existing secretory pathway.

Cluster10 is marked by high expression of spondins (SSPO3, SSPO4) and adhesive plaque matrix protein 2 homolog (FP2) which suggests it represents another secretory cell identity (Figure 4.8). It also shows high expression of genes involved in vesicular trafficking, as is also supported by the associated GO terms. A subset of these cells expresses 5-hydroxytryptamine (serotonin) receptor 1 B (5HT1B) and islet cell autoantigen (ICA69), which is involved in neurotransmitter secretion (Pilon et al., 2000).

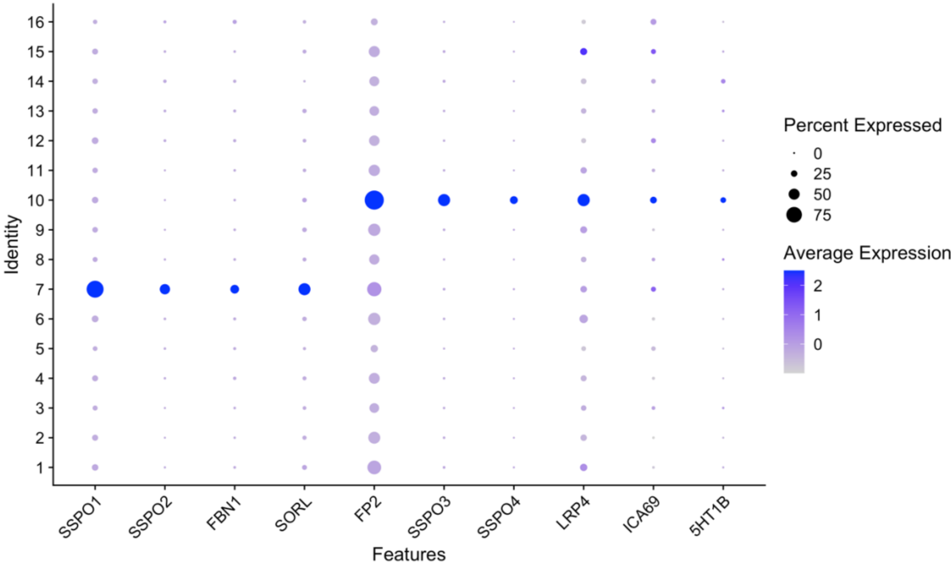


Figure 4.8 Secretory cell expression profile. Dotplot showing expression of top markers for clusters 7 and 10.

Cluster 9 is enriched in tubulin alpha and beta chain, and ciliary dynein, together with axonemal radial spoke head 1 homolog (RSPH1). This cluster does not show any other specific signature. Based on the cilia presence, it might represent a stage of spermatogenesis, alternatively, it might represent restitution cells — degenerating ciliated epithelial cells which lie in the integument and parenchyma (Westblad, 1949).

Cluster 13 shows high expression of genes involved in muscle activity: tropomyosin (TPM), myosin regulatory light chain (ML12B), myosin heavy chain from striated muscle (MYS), actin (ACT), myophilin (MYPH), Four and a half LIM domain protein 1 (FHL1), and titin. This cluster thus likely corresponds to the muscles.

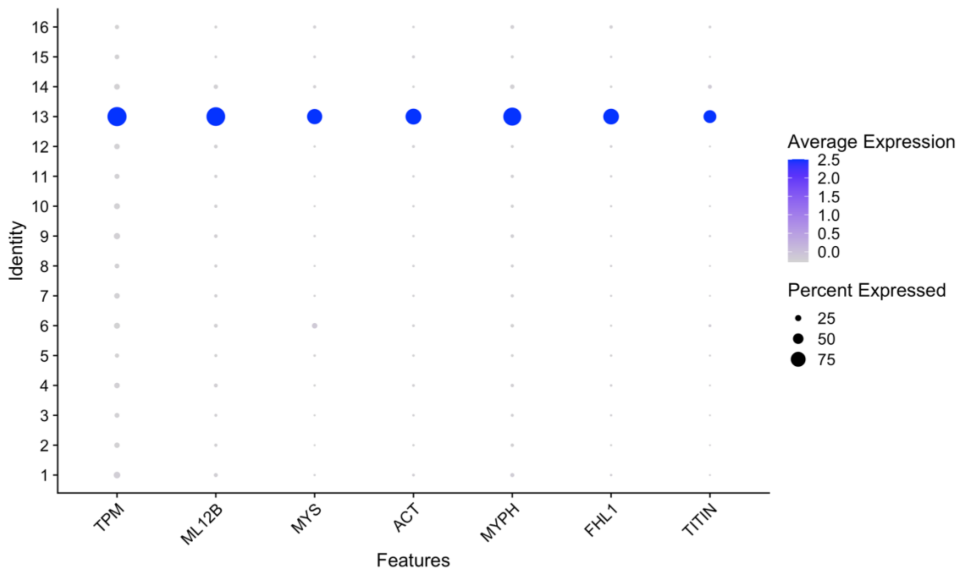


Figure 4.9 Muscle cell expression profile. Dotplot showing expression of top markers for clusters 13.

Cluster 12 is marked by a high expression of homologs of IgGFc-binding protein (FCGBP), Chromodomain helicase DNA binding protein 5 (CHD5), Zonadhesin

(ZAN), and several lectin homologs (LADD, LECM2, FUCL6). These secretory lectin-producing cells might have a function in the lumen of the gut.

Cluster 14 is marked by a high expression of DNA binding protein inhibitor (ID2) homolog involved in cellular growth, senescence, differentiation, and apoptosis (Ruzinova & Benezra, 2003), and apoptosis inducing factor 1 (AIFM1). The rest of markers for this cluster includes genes involved in cytoskeleton organization and actin dynamics. This cluster might represent differentiating cells or alternatively senescence undergoing cells.

Cluster 15 shows high expression of enzymes involved in lipid metabolism, amino acid metabolism, transmembrane transport and it is also enriched in GABA receptor subunit alpha 5 (GBRA5) and serotonin recpetor (5HT2B). A subset of these GABA-ergic and serotonergic cells also likely exhibit neurotransmitter-producing activity since they express Multiple C2 and transmembrane domain-containing protein 1 homolog (MCTP1) and Vesicle-associated membrane protein/ synaptobrevin-binding protein homolog (VP33).

Cluster 16 is enriched in genes with proteolytic activity such as transmembrane protease serine 3, carboxypeptidase E (CBPE), plasminogen (PLMN), chymotrypsinogen A (CTRA), chymotrypsin-like protease and low choriolytic enzyme (LCE). It is also enriched in the expression of vitelline membrane outer layer protein 1 homolog (VMO1), acidic mammalian chitinase (CHIA) and pancreas transcription factor 1a (PTF1A). Given the catabolic nature of expression profile of this cluster and the expression of *ptf1a*, it might represent a specialized subset of digestive cells. Alternatively, due to the high expression of VMO1, it might constitute the cells of the ovaries.

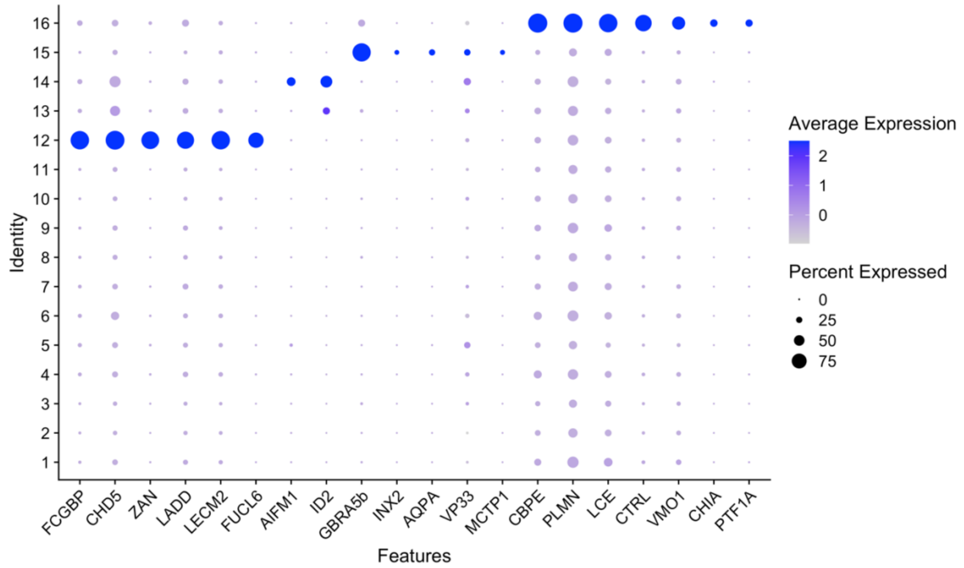


Figure 4.10 Miscellaneous clusters expression profile. Dotplot showing expression of top markers for clusters 12, 14, 15, and 16.

To examine the similarity of cluster expression profiles, an average cluster expression was calculated for each cluster and used for Pearson correlation (Figure 4.11). The cluster 12 and 16 were the most distinct, not correlated with the expression of any other cluster. Interestingly, the average expression of clusters 8, 9, 10, and 11 was highly correlated.

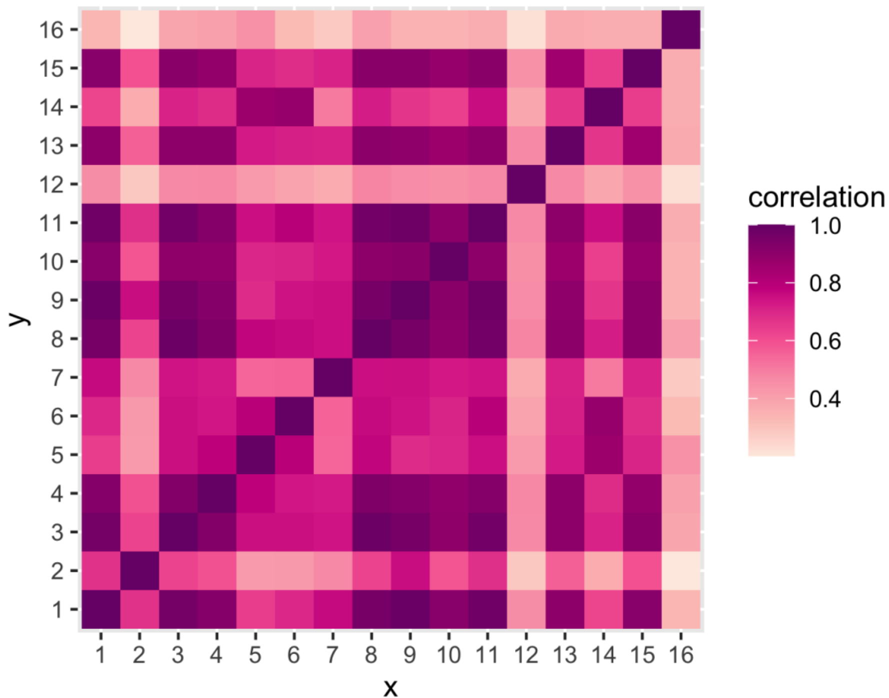


Figure 4.11 Heatmap of Pearson correlation of cluster average expression. Color intensity represents the value of Pearson correlation between the expression of average cells from clusters.

4.1.2 Putative neoblasts as a cellular source in differentiation

Gene expression of cluster 5 showed enrichment in homologs of neoblast markers *PCNA* (Orii et al., 2005) and *rir2* (Eisenhoffer et al., 2008). This prompted me to explore the expression of GMP genes in these cells throughout three studied developmental stages more closely. Genes *piwi1*, *vasa*, *nanos*, *argonaute a* (*ago a*), *argonaute c* (*ago c*), *tudor 1* (*tdrd1*) and *pumilio 2* (*pum2*) are all expressed in the subset of cells from cluster 5 at relatively high levels (Figure 4.12), although their expression is also detected in other clusters.

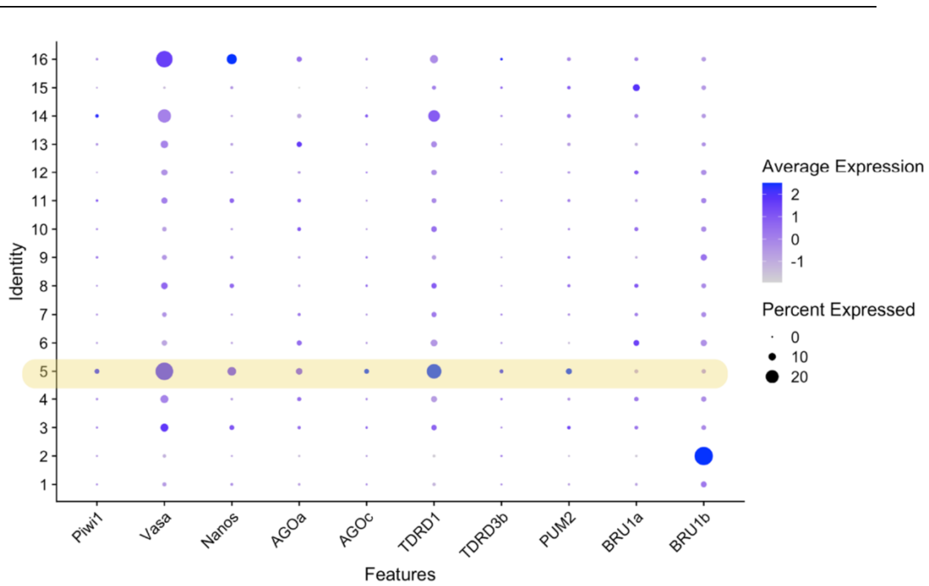


Figure 4.12 Expression of germline multipotency program (GMP) genes in the integrated dataset. Dotplot shows the expression of selected GMP genes in each cluster. Cluster 5 (highlighted in yellow) shows enrichment in in most of them, except for *bruno 1b*, which is enriched in cluster 2.

Confirming their pleiotropic role in development, *piwi1*, *vasa*, and *nanos* are expressed in a subset of multiple hatchling cell identities (Figure 4.13). Their levels, however, seem to decrease as development proceeds (Figure 4.13 and 4.14), perhaps reflecting the increasing differentiation of cell types. The decrease in expression levels is particularly noticeable for the levels of piwi1. Unlike the expression of *rir2*, and *vasa* which both persist in the cluster 5 and other clusters in juvenile and adult stages, *piwi1* levels seem to be gradually reduced (Figure 4.14). This is unexpected as it shows strong expression detected by in situ hybridization in adults (Manuscript I in this thesis). The opposite trend is seen in the expression of *bruno 1b* which is strongly expressed mostly by adult cells from cluster 2 (Figure 4.13).

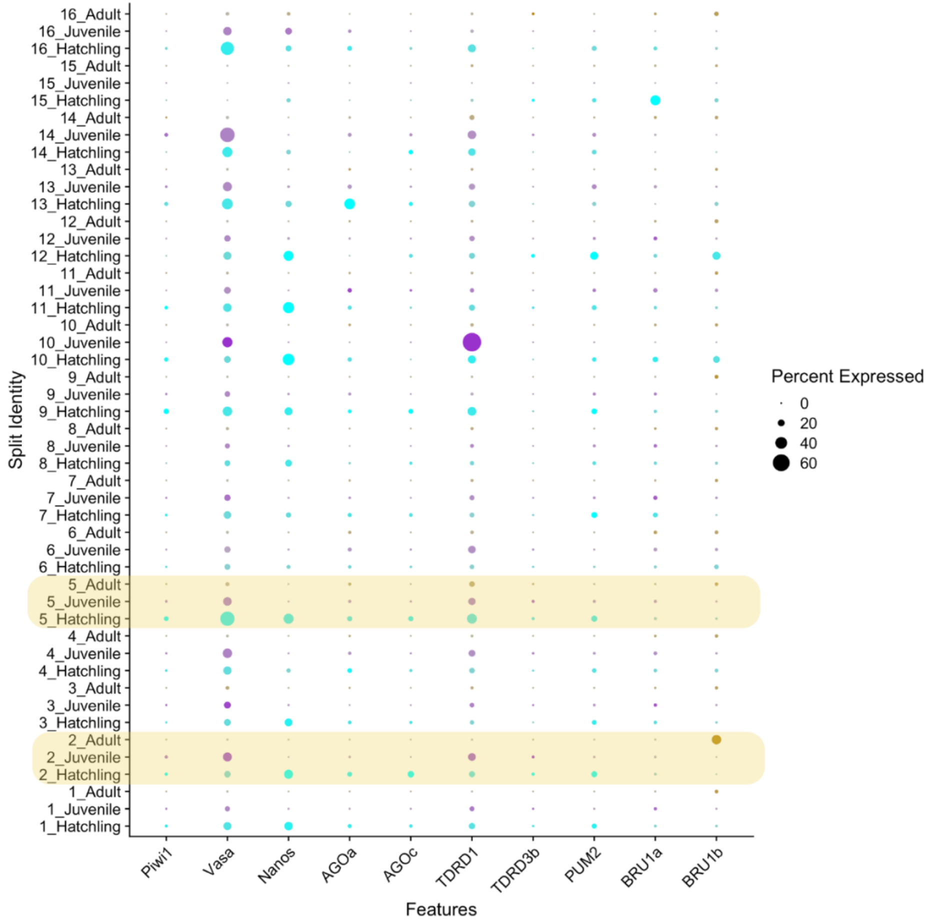


Figure 4.13 Expression of germline multipotency program (GMP) genes in each developmental stage. Dotplot shows the expression of selected GMP genes in each cluster. Cluster 5 and cluster 2 which show highest level of coexpression are highlighted in yellow.

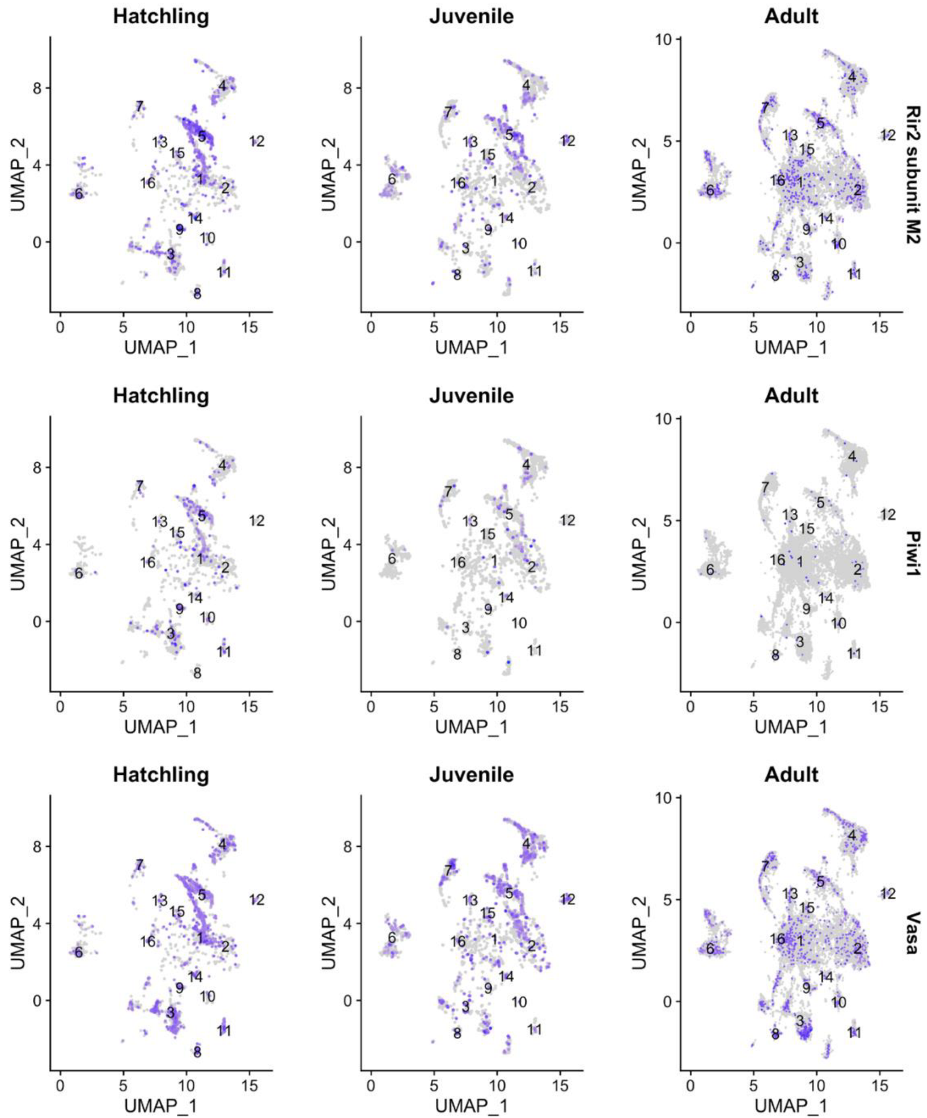


Figure 4.14 Expression of neoblast marker *rir2* and *piwi1* and *vasa* in each developmental stage. Feature plots show cells expressing the genes. Note the strong expression in cluster 5 at the hatchling stage.

Next, I wanted to get an understanding of cell differentiation trajectories throughout development. To order cells in pseudotime, I first generated a minimum spanning tree (MST) with slingshot. The resulting tree showed a branched topology with cluster 1 positioned at the center of the tree (Figure 4.15). Taking this MST as a pseudotime proxy, I split the clusters into three categories based on the level of supposed differentiation: initial, intermediate, and terminal. The intermediate clusters were connecting the terminal branches of the MST with the initital cluster, and terminal clusters were always at the end of the MST lines. To select the initial cell cluster, I opted for two different strategies: (i) MST-inferred cluster 1 and (ii) biology-informed cluster 5.

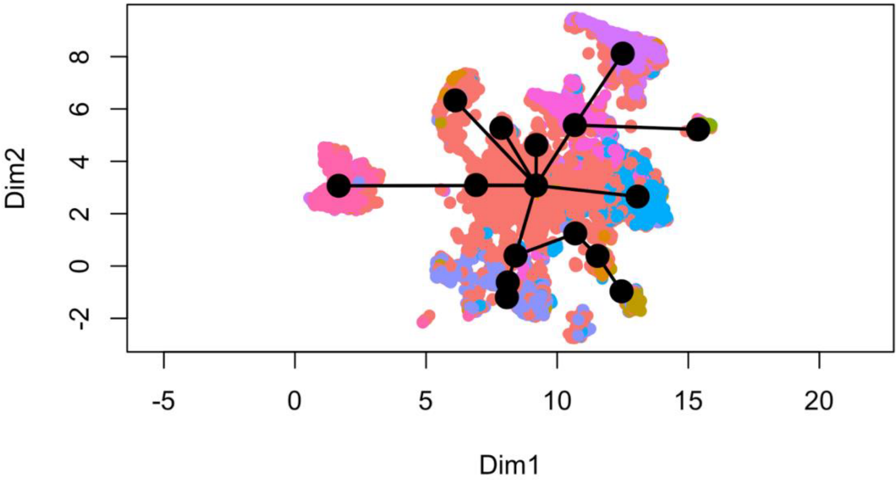


Figure 4.15 Minimum spanning tree of integrated dataset produced by slingshot. Cluster nodes (dots) are connected by lines that reflect transitions. Note the central position of node cluster 1.

Both hypotheses yielded differentiation trajectories visualized as molecular trajectory trees with terminal cell clusters as the tips of the tree. Cluster 1 as putative neoblasts hypothesis produced a differentiation tree with branched topology (Figure 4.16 A). In this tree, the muscle cluster 13, the male germ cell cluster 6, the neuronal-/neuroendocrine-like clusters 3 and 11, and the secretory cell cluster 7 shared a common progenitor cell population which was separated from the cells producing the rest of the cluster tips 2, 8, 4, 12, and 15. This tree topology assumes separate progenitors for the neuronal-/neuroendocrine-like clusters 3,8, and 11 and thus seems unlikely. Cluster 5 as putative neoblasts hypothesis produced a differentiation tree where each terminal tip cluster had its own progenitor, thus producing a topology with a polytomy (Figure 4.16 B). According to this trajectory hypothesis, the putative neoblast cluster 5 gives rise to lineage-primed progenitors which gradually differentiate into all terminal cell identities. Recently, Hulett. et al. (2023) described neoblast subtype dynamics during postembryonic development in acoel *Hofstenia*. The authors identified several regulators of cellular differentiation stemming from neoblasts. The muscle regulators *foxF* and *six1*, digestive/endodermal regulators *foxA* and *ikzf-1,* and neural regulators *vax* and *nk2-1* (Hulett et al., 2023). I recovered a subset of these genes in branching points of the inferred trajectory trees in *M. stichopi* dataset (Figure 4.16 A,B). In contrast to *Hofstenia*, the *ikzf-1* was clearly defining neuronal-/neuroendocrine-like lineages.

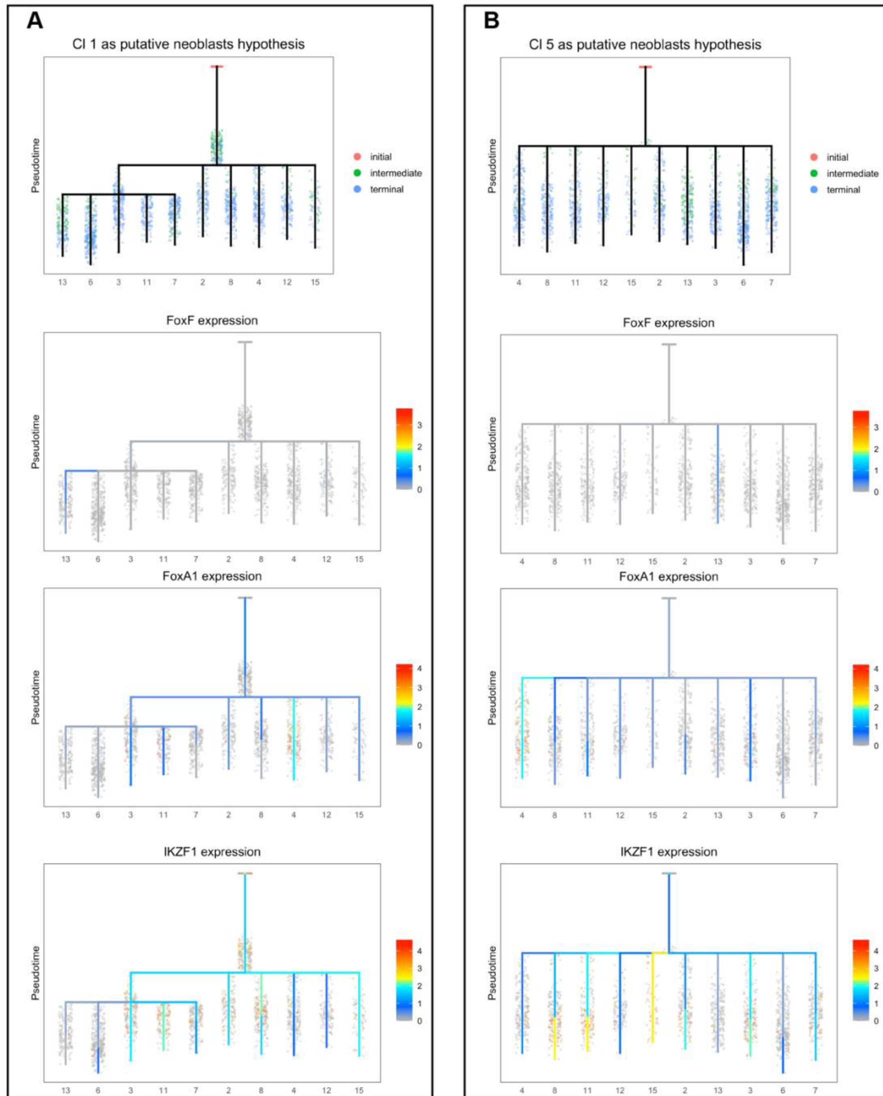


Figure 4.16 Molecular trajectory trees depicting potential neoblast hypotheses with differentiation markers. Hypothesis in panel A takes the cluster 1 as the putative neoblast cell population, while hypothesis in panel B takes cluster 5 as the putative neoblast cell population. Differentiation markers are shown for muscles (*foxF*), endodermal cells (*foxA1*) and neuronal /neuroendocrine-like clusters 3,8, and 11 (*ikzf-1*).

4.1.3 Putative neoblasts as a cellular source for germ cells

To see whether such molecular signatures of lineage-primed differentiation would be present in the cluster 5, I subsetted this cluster and looked at differentially expressed genes. Subclustering revealed 6 cell identities (Figure 4.17 A), out of which two (cluster 4, and cluster 6) are adult-specific (Figure 4.17 C). The pan-neoblast marker *PCNA* is abundantly expressed in all but cluster 4 (Figure 4.17 B). Same as in the whole dataset, *piwi1* expression is not broad but rather found in a subset of cells in clusters 1, 2, and 3 (Figure 4.17 D). Importantly, these clusters also contain cells positive for the *sox2* expression, suggesting they might indeed represent pluripotent cells. Transcription factor *hes1B* is detected in clusters 1, 2, 4, and 5. However, there is no significant correlation in coexpression of *piwi1* and *hes1B* or *piwi1* and *PCNA*. Such correlation is detected for *vasa* and *PCNA* coexpression (data not shown). Cluster 3 is enriched in the *ikzf-1*, and achaete-scute homolog 1 (*ascl1)* expression, reflecting some degree of differentiation. Surprisingly, the subclustering also revealed a population of cells in cluster 6 with high levels of two meiosis markers: synaptonemal complex protein 3 (*sycp3*) (Figure 4.17 D) and meiotic recombination protein *DMC1* (data not shown). This suggested an existing connection of proliferating cells — putative neoblasts — and germ cells in *Meara stichopi*.

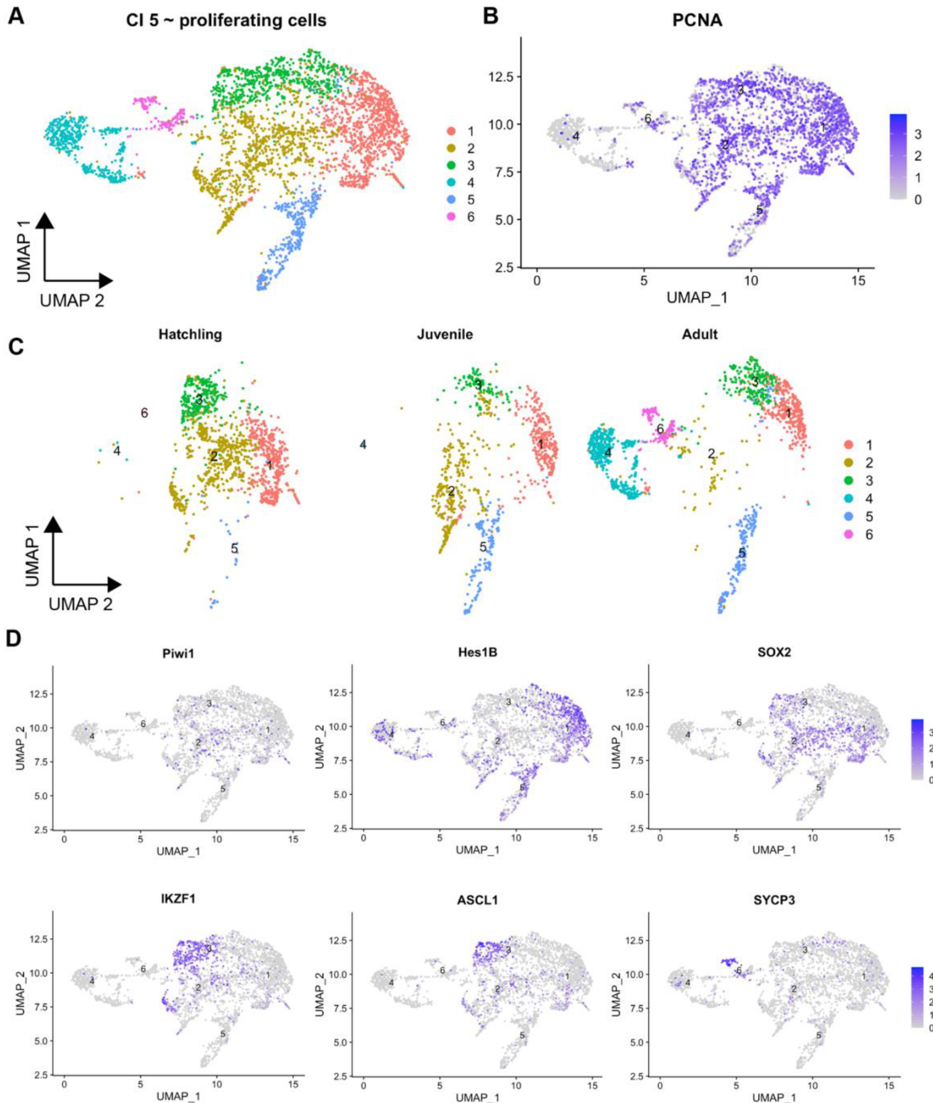


Figure 4.17 Putative neoblast dynamics throughout developmental stages and molecular signs of their differentiation. A. UMAP of a subclustered clsuter 5 — putative neoblasts. B. feature plot showing PCNA expression in these cells. C. UMAPs of subclustered putative neoblasts for each stage. D. Feature plots showing the expression of several marker genes.

To seek molecular evidence that would support such a connection, I used the pseudotime ordering again. This time, however, focusing only on the putative neoblasts (cluster 5) and germ cells (cluster 6). Nonlinear dimensionality reduction of these cells (Figure 4.18 A) revealed a close association of hatchling and juvenile cells, but adult cells seemed more distinct, connected with juvenile cells with very few transitions. Pseudotime ordering of selected cell populations reflected this phenomenon by the visible gap between juvenile cells from cluster 6 and adult cells from cluster 5 (Figure 4.18 B). Nonetheless, the proposed trajectory tree copies the true ordering of developmental stages with the hatchling putative neoblasts at the root and adult cells at the tips of the tree. When queried with a set of marker genes (Figure 4.18 C), the obtained molecular trajectory conveys a clear differentiation path for germ cells and putative adult neoblasts. Indeed, the cells at the root and the stem constitute the entire *sox2* expression, with the adult cells completely negative for *sox2*, while the *PCNA* and mitotic marker *MP62* span almost the entire stem of the tree and the left branch leading toward the putative adult neoblasts. Interestingly, there is a clear gap in both mitotic markers, where the juvenile cells from cluster 6 reside, corresponding to when the meiosis is initiated. This part of the tree is marked by the meiosis-specific nuclear structural protein 1 (*mns1*). Subsequently, other two meiosis markers, *sycp3* and *dmc1* are detcted at the branch point in the subset of adult cells from cluster 5. Complementing this expression pattern, the *hes1b* is expressed in hatchling, juvenile and adult cells from cluster 5, and in the putative adult neoblasts in the left branch of the tree, while the spermatogenesis marker *SPT17* spans the meiotic cells and right branch of the tree.

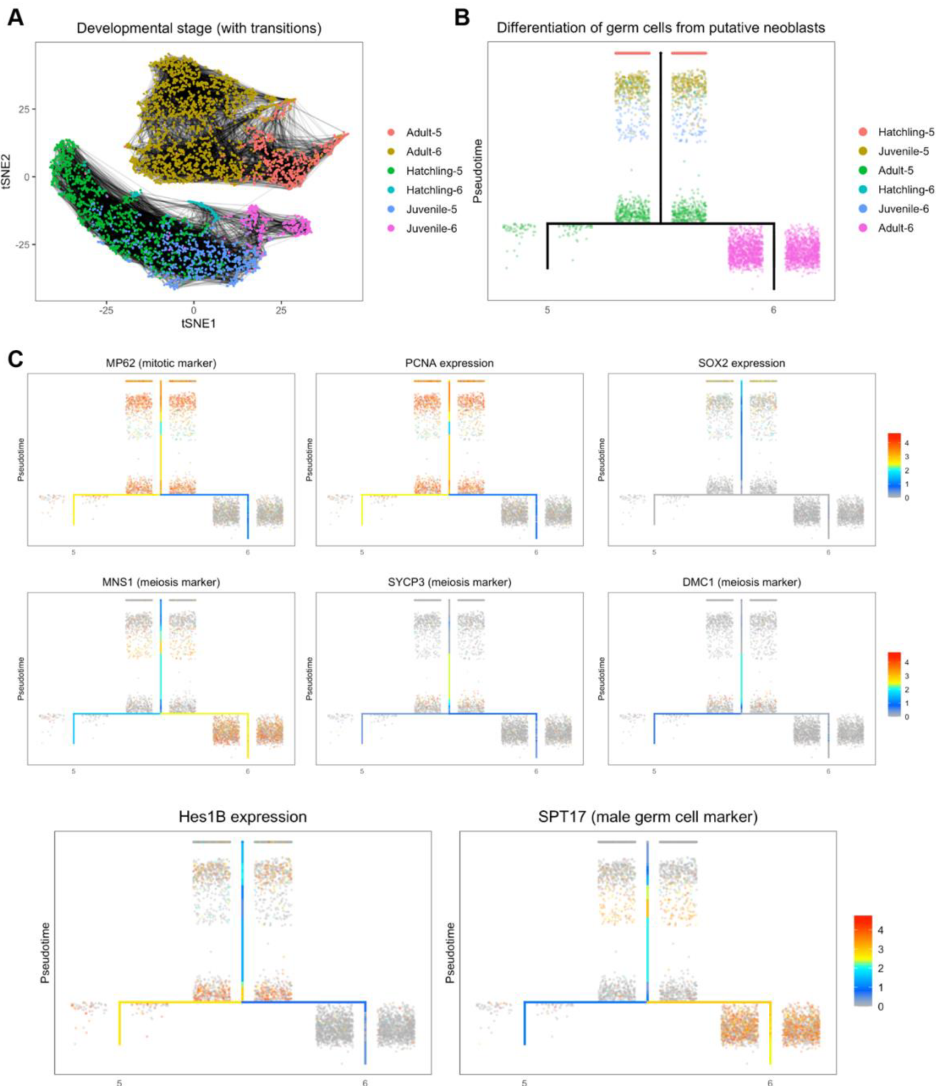


Figure 4.18 Mode of germ cell differentiation from putative neoblasts. A. Nonlinear dimensionality reduction plot of cluster 5 and cluster 6 cells colored by developmental stage and cluster membership, lines depict the connections from the pseudotime diffusion map of the data. B. Pseudotime inferred molecular trajectory for the same cells. C. Expression of genes from the marker set mapped onto pseudotime trajectory.

4.2 Additional Results

Additionaly, I performed a transcriptomic survey in the transcriptome of a brachiopod *T. transversa*, looking for the germline mulitpotency program (GMP) genes. To get a better insight about their activity in germline specification in brachiopods, I performed a whole mount in situ hybridization (WMISH) with selected GMP genes (Figure 4.19). RNA-binding proteins *bruno*, *pl10*, *pumilio* and *piwiA* are all expressed at radial gastrula stage, but only *bruno* shows asymmetric distribution in the ectoderm, while *piwiA, pl10, and pumilio* show rather broad expression in all germ layers. *boule* shows no expression at this stage. At the asymmetric gastrula stage, *pl10* expression disappears and reappears again at the bilateral gastrula stage, which seems to be the onset of the *boule* expression. Indeed, it is expressed specifically in two small bilaterally symmetrical domains at the anterior border of the mesoderm (Andrikou & Hejnol, 2021; Passamaneck et al., 2015). At the bilobe stage, mesodermal expression of *piwiA, pl10 and pumilio* becomes more pronounced, although all three are also weakly detected in the ectoderm. Strong ectodermal expression of *bruno* is evident, particularly from the lateral view (Figure 4.19 insets). In larval stages, *boule* expression continues to label specific domains of mesoderm in all three lobes — apical, mantle, and pedicle — lobes. Subportion of *boule*-expressing pedicle mesoderm cells likely represent multipotent precursors that give rise to germline. This domain also co-expresses *bruno, piwiA, pl10, and pumilio*.

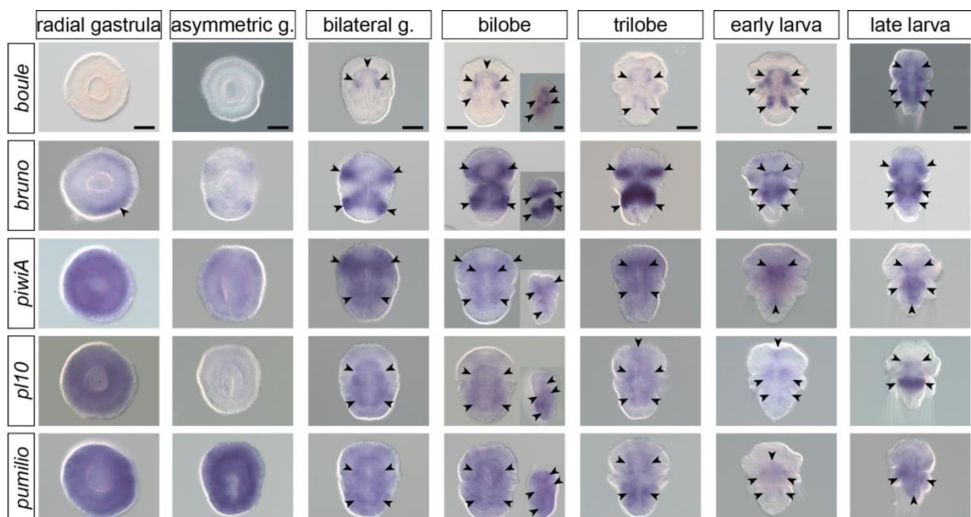


Figure 4.19 Expression of selected germline multipotency program (GMP) genes in the development of *T. transversa*. All stages are imaged from the vegetal or ventral view except for the insets in the bilobe stages which show a lateral view. Expression domains are labeled by notched arrowheads. *boule* starts to be expressed at the bilateral (late) gastrula stage, while *bruno, piwiA, pl10*, and *pumilio* all start to be expressed at the radial (early) gastrula stage. All genes, except *bruno*, are detected in mesodermal derivatives throughout development. Scale bars 50 µm.

CHAPTER 5: Discussion and perspectives

5.1 Gonad development and RNA binding proteins

The aims of this thesis were to investigate the evolutionary and developmental relationships of germline, gonads and mesodermal precursors. To research these phenomena, I studied nemertodermatid worm *Meara stichopi* from clade Xenacoelomoprha. As xenacolomorphs lack the excretory system, their only mesodermal derivatives are the muscles and gonads. Our current knowledge of *M. stichopi*'s biology does not allow us to discern their gonad formation in great detail because of its symbiotic life cycle. The free-living juveniles ingested by the sea cucumbers are thought to grow inside their host foregut and sexually mature for several months, with the gravid animals spotted in their host again from mid August to mid October. The gonad formation thus occurs sometime during this intense growth period and likely involves the parenchyma proliferation as suggested by the species description work by Westblad (1949). Supporting his proposed mechanism, such prolific activity of central parenchyma cells is detectable in the juvenile stage (Manuscript I, Figure 2). In comparison to the hatchling stage where most proliferating cells are localized in the periphery, the juvenile central parenchyma shows morphologically distinguishable outgrowths containing proliferating cells, which I propose represent the early gonad anlage that later develops into the follicular testes and ovaries.

The process of parenchyma differentiation is accompanied by the expression of genes encoding the set of evolutionarily well-conserved RNA-binding proteins (RBPs). Indeed, the expression of *bruno a* (*bru a*), *mago nashi* (*mago*), *maelstrom* (*mael*), *staufen* (*stau*), *tudor* (*tdr*), *argonaute a* (*ago a*), *gustavus a* (*gus a*) and *boule* (*boll*) is detected in adult testes and/or ovaries. In juveniles, these genes are found in different regions of parenchyma, often lining the gonad primordium (Manuscript I, Figure 1c). The same genes have been implicated in the maturation of germ cells and stem cells in planarians (Guo et al., 2006; Handberg-Thorsager & Saló, 2007; Kuales et al., 2011; Pfister et al., 2008; Salvetti et al., 2005; Wang et al., 2007), and acoels (De Mulder, Kuales, et al., 2009; Hulett et al., 2023; Srivastava et al., 2014). This demonstrates that

they function similarly in *M. stichopi*, taking part in gametogenesis and possibly also in the stem cell maintenance. However, several works have described the plethora of other roles for the RBPs in model organisms such as *Drosophila* and mouse (Lasko, 2000; McKee et al., 2005; Gamberi et al., 2006; Kerner et al., 2011). Likewise in *M. stichopi*, the expression of *ago a*, *bru a*, *gus a*, and *orb2* in adults but mainly in juveniles also indicates these genes play a role in the nervous system too (Manuscript I, Figure 1c). I probed the overall capacity to encode conserved RNA-binding domains (RBDs) contained in the RBPs in the predicted proteomes of nemertodermatid species: *Ascoparia*, and *Nemertoderma westbladi*, and *Xenoturbella* species: *X. bocki* and *X. profunda*. The results are comparable with those reported by Kerner et al. (2011) for the sponge, *Drosophila*, and mouse. The RRM motif containing RBPs are consistently the most numerous group recovered in all studied proteomes, followed by the DEAD-box RBPs, KH-1 and KH-2 motif RBPs, and dsRBM-containing RBPs being the least numerous group (Manuscript I, Suppl. Fig. 1).

5.2 Putative neoblast-like cells in *M. stichopi* and limited regenerative capacity

The parenchyma cells — the cells found between the body wall and the gut that do not form the gonads are thought to be lacking in nemertodermatids. Indeed, many authors claimed that nemertodermatids and *Xenoturbella* are completely devoid of parenchymal cells (Rieger, 1981; Smith & Tyler, 1985). It is noteworthy that these claims are based on the ultrastructure of species *Nemertoderma* and *Flagellophora*, while the morphological description of *M. stichopi* in Westblad's original description (1949) and in a later comprehensive description by Sterrer (1998) does mention the parenchyme. Interestingly, the cell proliferation assay presented in Manuscript I. has revealed a cell population lying in a position where parenchyma cells are usually found. Occasionally, there were also proliferating cells present in the epithelial layer (Manuscript I, Suppl. Fig. 2 D-E), a feature that is absent in planarians and acoels (Egger, Steinke, et al., 2009). The proliferating parenchyma cells reside below the musculature and do not form a part of the gut or the gonads, they rather give an individual appearance (Manuscript I, Suppl. Fig. 2). Their nuclear morphology,

position, and the fact that they are mitotically active, all support the notion that these cells might represent putative neoblasts. Indeed, the labeled cells concentrate along the body walls, forming bilateral bands that start posteriorly to the double statocyst and stretch all the way to the posterior end (Manuscript I, Figure 2). Furthermore, this pattern is consistently detected throughout the worm's development starting from the hatchling stage (Manuscript I, Figure 2A), through the juvenile stage (Manuscript I, Figure 2B), to adulthood (Manuscript I, Figure 2C). A similar pattern is typical for the neoblasts of planarians (De Mulder, Pfister, et al., 2009; Egger, Steinke, et al., 2009; Newmark & Sánchez Alvarado, 2000) and neoblast-like cells of some acoels (De Mulder, Kuales, et al., 2009; Srivastava et al., 2014). Importantly, in my analyses, these cells were often in close proximity to the nerve bundles (Manuscript I, Figure 2 A''-A''' and B''-B'''). This association and potential interaction of neurons and putative neoblast-like cells which might have a functional aspect is also known from *Macrostomum* (Nimeth et al., 2004) but not from acoels where the neoblast-like stem cell system is more associated with the gonads and peripheral parenchyme (De Mulder, Kuales, et al., 2009; Gschwentner et al., 2001; Srivastava et al., 2014). I also detected proliferating cells associated with the gonads in *M. stichopi*. In addition to spermatogonia, forming follicles consisting of several cells (Manuscript I, Figure 2 E,G), the labeled cells were also found dorsolaterally at the rim of testes (Manuscript I, Figure 2F) and ovaries (Manuscript I, Figure 2H-J). It is possible these cells are presumptive germline stem cells, a separate cell lineage capable of self-renewal, which also reside at the periphery of planarian gonads (Sato et al., 2006). If there is indeed such cell population present in *M. stichopi*, it might originate from the putative neoblast-like cells or directly derive from the PGCs.

To investigate the function of putative neoblast-like cells, I examined the cell proliferation following the injury. Exposing the animals to two different homeostasis-compromising situations: traumatic oviposition and amputation caused a visible increase in proliferation in the vicinity of the blastema region (Manuscript I, Figure 4 B'i, Suppl. Fig. 3 A-D, Figure 5). The blastema itself contained proliferating cells (Manuscript I, Figure 4 B'i, Suppl. Fig. 3 and Figure 5) and was dorsally tapered (Manuscript I, Figure 4 C' and Suppl. Fig. 3 D). This represents a unique feature among

the so far studied flatworms, which all displace the blastema ventrally (Chiodin et al., 2011; Egger, Gschwentner, et al., 2009; Grosbusch et al., 2022; Srivastava et al., 2014). Displacing the wound to the dorsal side is probably facilitated by strongly developed dorsal muscle bands and the fact that animals slide with their ventral, mouth-containing, side up; dorsal side being the one that is in contact with the substrate. The presence of dividing cells in the blastema has been observed in *Macrostomum* (Egger, Gschwentner, et al., 2009) but it is in contrast to the regeneration blastema of acoel *Hofstenia* (Srivastava et al., 2014) and planarians (Morita & Best, 1974) where the blastema consists of post-mitotic progeny of neoblast-like cells. It is possible that due to the long EdU pulse, lasting for 24 h, some of the labeled cells that are present in the blastema represent a post-mitotic progeny. To distinguish between these scenarios, a shorter EdU pulse or different cell labeling technique is necessary.

Interestingly, both, the anterior and posterior regenerating pieces showed some degree of regenerative capacity, although in the case of post-oviposition injury only anterior, double-statocyst pieces were observed. The actin stainings showed disorganized muscle fibers which were in the wound-affected area (Manuscript I, Figure 4 B', C'), the blastema was also innervated by serotonergic neurons (Manuscript I, Figure 4 B, C). Proliferating cells were in the proximity of both structures (Manuscript I, Figure 4 B'i, C'i), perhaps reflecting their interaction in orchestrating the wound healing process and providing positional cues — a phenomenon previously demonstrated in planarians (Witchley et al., 2013) and acoels (Raz et al., 2017).

It is perplexing, however, that despite the intensive proliferation response seen in *M. stichopi*, I never observed the full whole-body regeneration. Neither was it observed in worms regenerating for the period of 4 weeks (Manuscript I, Suppl. Fig. 4), corroborating my observations. This suggests that *M. stichopi*'s regenerative capacity is rather limited and perhaps only covers the initial wound healing response and restricted regrowth of the tissue. There are multiple explanations for this from a comparative perspective (Figure 5.1). (i) An extensive range of regenerative capacity seen in acoels (Keil, 1929; Steinböck, 1954, 1963; Hanson, 1967; Gschwentner et al., 2001; De Mulder, Kuales, et al., 2009; Chiodin et al., 2011; Srivastava et al., 2014)

might have been acquired as an apomorphy on the acoelomorph lineage leading towards acoels (Figure 5.1A). (ii) The regenerative capacity was present in the acoelomorph (Figure 5.1B'), and/or xenacoelomorph ancestor (Figure 5.1B) but was lost or modified in the lineage leading towards nemertodermatids. This scenario is supported by observations of limited regeneration in *Xenoturbella* (Israelsson, 2006; Nakano, 2015) and unpublished reports of neoblasts present in nemertodermatids (Rieger & Ladurner, 2003; Smith III et al., 2009). The data to support it, however, are still scarce. (iii) The regenerative capacity was reduced and/or lost in *M. stichopi* as the result of its adaptation to the symbiotic life cycle (Figure 5.1C).

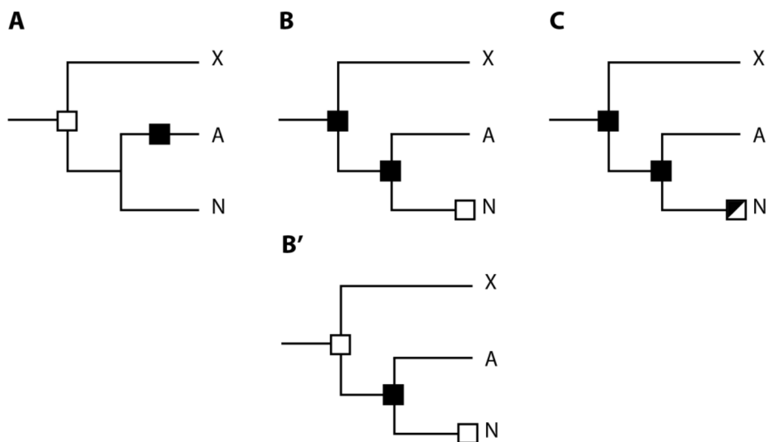


Figure 5.1 Different scenarios of evolution of regeneration in *Xenacoelomorpha*. A. Regeneration was absent in the xenacoelomorph common ancestor and was only acquired in the lineage leading towards Acoela. **B.** Regeneration was present in the xenacoelomorph common ancestor and acelomorph common ancestor but lost or modified in nemertodermatids. B'. Regeneration was absent in the xenacoelomorph common ancestor but present in acelomorph common ancestor and lost or modified in nemertodermatids. **C.** Similar to **B.**, but regeneration might be present in some nemertodermatids, while lost in others such as M. stichopi. X – *Xenoturbella*; A – Acoela; N – Nemertodermatida.

5.3 Conservation of regeneration GRN and its functionality

Recently, epigenetic profiling of regeneration-induced chromatin response in acoel *Hofstenia* recovered several early wound-induced genes and associated GRN with a master regulator *egr* (Gehrke et al., 2019). Orthologs that constitute this regeneration GRN: *egr*, *deaf1*, *fstl*, *mtss*, *nlk*, *nrg2*, *runt* are present in the transcriptome of *M. stichopi* (Manuscript I, Suppl. Fig. 6). Their expression in intact adults is rather weak, mostly found in the posterior end, whereas the expression in juveniles is nearly absent or completely absent (Manuscript I, Suppl. Fig. 5). I hypothesize that the expression seen in the adult in homeostasis is connected to the traumatic oviposition and probably caused by the release of the oocyte from the follicle into the lumen of the gut. This would explain the absence of expression in the posterior of sexually immature stage. The oviposition by means of the body wall rupture and the following regeneration are also described in acoel *Polychoerus* (Costello & Costello, 1939). Upon the amputation, there was a strong induction of *egr* and *runt* expression in anterior and posterior regenerates (Manuscript I, Figure 5 A-D). This is consistent with wound response in *Hofstenia* (Gehrke et al., 2019) but differs from regeneration in *Convolutriloba*, where only the anterior regenerates show *runt* induction since posterior regeneration proceeds by morphallactic processes (Sarfati et al., 2023). In *M. stichopi*, both genes showed accumulation in the blastema region and a broad expression domain at 24 hpa in muscles and neurons (Manuscript I, Figure 5 A-D and E-H). Furthermore, both, *egr* and *runt* were coexpressed with *piwi1*$^{+}$ cells, a subset of which were also proliferating (Manuscript I, Figure 5 A''-D''). During initiation of wound response in acoels (Gehrke et al., 2019; Hulett et al., 2023; Sarfati et al., 2023) and planarians (Sandmann et al., 2011; Wenemoser et al., 2012), both *egr* and *runt* start to be expressed several hours post-injury. The expression data in Manuscript I present a single timepoint, 24hpa, so it is not possible to distinguish whether the wound response in *M. stichopi* has a delayed onset or whether the levels of transcripts persist until then. Likewise, without the chromatin-level information or functional validation, it remains uncertain whether the whole GRN is wired the same in nemertodermatids.

5.4 GMP genes and their role in putative neoblasts and beyond

One of the main points of focus of the thesis was to investigate the expression dynamics of known GMP genes. To this end, I performed fluorescent in situ hybridizations for *piwi1* and *vasa* (Manuscript I, Figure 3). Both genes were strongly expressed in the follicles of gonads, staining multiple, if not all, stages of spermatogenesis and oogenesis (Manuscript I, Figure 3 B-C and H-I). Outside of gonads, the signal was also present in the somatic cells located in the parenchyma (Manuscript I, Figure 3 D-F and J). Indeed, *piwi1* expression pattern highly resembled the bilateral bands of proliferating cells, forming a continuous signal from anterior to the posterior end (Manuscript I, Figure 3 A). According to these results, both *piwi1* and *vasa* are expressed in putative neoblast-like cells in homeostasis (Manuscript I, Figure 3) and post-amputation (Manuscript I, Figure 5 A'-D' and Suppl.Fig. 4 b and c). The blastema in anterior and posterior regenerates contained *piwi1* and *vasa*-expressing cells, suggesting that both are involved in regeneration process. In planarians, orthologs *smedwi-2* (Reddien et al., 2005), *smedwi-3* (Palakodeti et al., 2008), and *vasa* (Shibata et al., 1999) are contained within the ribonucleoprotein particles in neoblasts and control their role in regeneration. Similarly in *Hofstenia*, *piwi-1* is iindispensable for regenearation (Srivastava et al., 2014). In contrast, RNAi of *piwi1* in *Isodiametra* reduces the *vasa* expression but does not lead to a reduction in cell proliferation nor impedes the posterior regeneration (De Mulder, Kuales, et al., 2009). Thus, it remains to be seen whether *piwi1* and/or *vasa* are essential for the regeneration in *M. stichopi* and how their knockdown affects the cell proliferation dynamics.

Given the strong expression of *piwi1* detected by in situ hybridization, we would expect to see its enrichment in high proportion of cells in the single-cell transcriptomic data. On the contrary, its transcript levels are quite low in the integrated dataset, showing a clear decrease throughout animal's development (Figure 4.12 – 4.14). The same is not true for the *vasa* transcripts which are detected at hatchling, juvenile, and adult stages. This might be a technical artifact explained by the possible exclusion of majority of *piwi1*$^{+}$ cells in the filtering step of the analysis, or by different 10x kit versions used (v2 for the adult samples and v3 for the hatchling and juvenile samples). Such differences in cell and gene dropout events between v2 and v3 have been demonstrated before (Yamawaki et al., 2021) with v3 outperforming the v2. Despite

this issue, the *piwi1* transcripts were detected in a subset of several cell identities with the strongest expression in cluster 5 — putative neoblast-like cells and cluster 14 (Figure 4.12). Interestingly, these two clusters showed high correlation of average expression (Figure 4.11), which might convey that cluster 14 represents differentiating neoblast-like cells.

Demonstrating the pleiotropic and essential role of GMP genes in development, *vasa*, and *nanos* and to an extent also *piwi1* were detected in multiple cell identities of a hatchling (Figure 4.13). Indeed, while high proportions of almost all hatchling cell identities express these three genes, *piwi1,* and *nanos* but not *vasa* are expressed in fewer cells of juveniles, and adults. This might reflect the high potency of cells at the hatchling stage. Indeed, the putative neoblast-like cells constitute the most numerous cluster at this stage, while in the adults it is the cluster 1 and 2 (Figure 4.2 C). Consistent with the role og GMP genes and RBPs in neoblast biology (Shibata et al., 1999; Reddien et al., 2005; Salvetti et al., 2005; Sato et al., 2006; Handberg-Thorsager & Saló, 2007; Palakodeti et al., 2008; Solana et al., 2009; Y.-Q. Li et al., 2011), the *piwi1*, *vasa*, *nanos*, *ago a*, *ago c*, *tdrd1* and *pum2* are all detected in the subset of cells from cluster 5 at relatively high levels (Figure 4.12).

5.5 Nemertodermatid cell type diversity

Using the scRNA-Seq, this thesis brings new insights into the cell type diversity of nemertodermatids and may serve as a resource for their future studies. Sampling three developmental stages: hatchling, juvenile and adult revealed 16 putative cell identities (Figure 4.2). The clustering with higher resolution parameter typically yielded finer separation (eg. putative neoblast cluster separated into three clusters based on the cell cycle phase; data not shown) but it also split major cell types into subsets that are not supported by animal's biology. Surprisingly, there were no stage-specific clusters in the integrated dataset, if we do not count those represented by less than 10 cells from hatchling or juvenile stages. A limited cell type diversity is also known from *Xenoturbella* species (Robertson et al., 2022). My analysis recovered all major cell

identities: epithelial cells (cluster 1, Figure 4.3), gut/digestive cells (cluster 4, Figure 4.5), muscles (cluster 13, Figure 4.9), neuron/neroendocrine-like cells (clusters 3, 8, and 11, Figure 4.4), and secretory cells (clusters 7 and 10, Figure 4.8). Transcriptional signatures which define these cell identities are largely shared among corresponding cell types from available scRNA-Seq xenacolomorph datasets (Duruz et al., 2021; Hulett et al., 2023; Robertson et al., 2022). However, there were also species-specific genes among the top markers present in almost all cell identities. Without the genomic information, it is difficult to ascertain their function.

Importantly, the scRNA-Seq analysis revealed a neoblast-like cell identity (cluster 5, Figure 4.6) defined by the expression of known neoblast markers: *PCNA*, *rir2*, and *piwi1* (Orii et al., 2005; Reddien et al., 2005; Eisenhoffer et al., 2008). Interestingly, a subset of cells in this cluster also expresses transcription factor *prospero1* (Figure 4.6 A), which is expressed in planarian gamma neoblasts – the intestine progenitors (Fincher et al., 2018). Given that neoblasts are the major source of all adult cell types in planarians (Davies et al., 2017) and acoel *Hofstenia* (Hulett et al., 2023; Kimura et al., 2022), I probed their capacity to differentiate into terminal cell types in *M. stichopi* by using a pseudotime lineage trajectory (Figure 4.16 B). With putative neoblasts as root cells, the resulting molecular trajectory displays a topology where each terminal cell identity has its own progenitor. Among the genes that were differntially expressed along the branches of this tree, several markers were retrieved. The transcription factor *foxF* was specific for the muscle lineage, transcription factor *foxA1* was specific for the endodermal lineage, and transcription factor *ikzf-1* was specific for the neural/neuroendocrine lineage (Figure 4.16 B). The same regulators of neoblast-derived cellular differentiation are known from the postembryonic development of *Hofstenia* (Hulett et al., 2023). Interestingly, the only difference is the expression of transcription factor *ikzf-1* which in *Hofstenia* labels the endodermal-like progenitors (Hulett et al., 2023). This difference might be caused by different wiring of developmental program mirrored in distinct blastomere division patterns in early cleavage of both animals (Børve & Hejnol, 2014; Kimura et al., 2021). As is also the case for adult neural structures. While in *M. stichopi* they are entirely basiepidermal with the exception of statocyst-associated neurons (Børve & Hejnol, 2014), in

Hofstenia, they form two layers with the internalization below the muscle sheath (Hulett et al., 2020).

Further supporting the hypothesis that putative neoblasts are a cellular source in development and homeostatic tissue turnover in *M. stichopi*, I also detected expression of *ikzf-1* and *ascl1* — pro neural markers — in a subset of subclustered neoblast cells (Figure 4.17 D). This shows that *M. stichopi*'s neoblast-like cells might function as a source for cell progenitors in a similar way to planarian and acoel neoblasts.

5.6 Origin of germ cells in nemertodermatids

What differentiation mode do nemertodermatid germ cells utilize? And what serves as their cellular source? Finding a support in the presented transcriptomic data, I propose that *M. stichopi*'s germ cells arise by differentiation from neoblast-like cells by induction. The fact that the meiotic marker *sycp3* is expressed in a subset of putative neoblasts (cluster 5, Figure 4.17 D), which are adult-specific, suggests that this induction is not restricted to the early stages of development but rather occurs throughout animal's life. Surprisingly, the onset of expression of meiosis-associated genes occurs much earlier in development than previously thought. Indeed, it is already in the hatchling and juvenile cells belonging to cluster 6 when the gene *mns1* starts to be expressed on differentiation trajectory from neoblasts to differentiatied germ cells of adult cluster 6 (Figure 4.18 C). This implies that there are germ cell-fated cells already present in the hatchling, well before the gonad anlage is formed. Similarly, expression data from *Isodiametra* suggest there are *piwi1*$^{+}$ germ cells already present at the hatchling stage (De Mulder, Kuales, et al., 2009). On the other hand, in *Hofstenia* it is only from early adult when the germ cell signature starts to be detectable in the transcriptomic data (Hulett et al., 2023). One possible reason for this might be that in the study of Hulett et al, the authors did not look at the meiosis genes but rather genes that define a phenotype of differentiated germ cells. Alternatively, the production of germ cells in *M. stichopi* might be accelerated as the adaptation to its symbiotic life cycle. It is important to note however, that despite its symbiotic life cycle, *M. stichopi*

was retrieved as more basal than species *Flagellophora*, *Nemertinoides* or *Sterreria* in modern phylogenies (Meyer-Wachsmuth & Jondelius, 2016). Arguably still, future studies that include more nemertodermatid species are necessary to see how representative the data from *M. stichopi* really are.

CHAPTER 6: Bibliography

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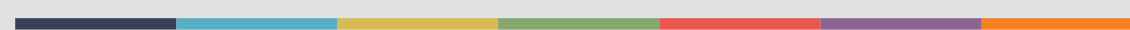

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