

Identifying embryonic mechanisms that induce a germ cell fate in sea stars

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A dissertation submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in the Division of Biology
and Medicine at Brown University

Providence, RI

May, 2017

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2. Wessel GM, Brayboy L, **Fresques T**, Gustafson EA, Oulhen N, Ramos I, Reich A, Swartz SZ, Yajima M, and Zazueta V. (2014). The biology of the germ line in echinoderms. *Mol. Reprod. Dev.* 81(8):679-711.
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7. **Fresques T**, Swartz Z, Juliano C, Morino Y, Kikuchi M, Akasaka K, Wada H, Yajima M, Wessel GM (2016). The Diversity of Nanos Expression in Echinoderm Embryos Supports Different Mechanisms in Germ Cell Specification. *Evolution & Development* Jul;18(4):267-78.
8. Oulhen N, Heyland A, Carrier TJ, Zazueta-Novoa V, **Fresques T**, Laird J, Onorato TM, Janies D, Wessel G (2016). Regeneration in bipinnaria larvae of the bat star *Patiria miniata* induces rapid and broad new gene expression. *Mech. Dev.* Nov; 142:10-21.

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Acknowledgements

I would like to thank the Wessel laboratory for the wonderful time spent together. Gary Wessel, in particular, was a wonderful mentor throughout my graduate career. Thank you for challenging me and teaching me how to think like an independent scientist. I cannot imagine what graduate school would have been like without Nathalie Oulhen, I am extremely thankful for your help and kindness throughout the years. I would also like to thank all other members of the Wessel laboratory that have contributed to my development as a scientist: Lynae Brayboy, Zak Swartz, Adrian Reich, Mamiko Yajima, Vanesa Zazueta, Dons Xu, Isabela Ramos, Henry Vargas, and Peter Baek. Thank you for contributing to a fun and studious laboratory atmosphere.

Thank-you to my committee for all of your support and helpful feedback throughout the years: Robbert Creton, Casey Dunn, Cassandra Extavour, Rich Freiman, and Kristi Wharton.

I am appreciative of Geoff Williams for training me how to use the various microscopes in the Leduc Bioimaging Center. I also would like to thank him for teaching me and helping me troubleshoot sample preparation techniques.

The MCB program has helped me in so many ways throughout the years. Thank you to Elaine Butler for always being willing to help me. Thank you to all of the graduate program directors: Mark Johnson, Judith Bender, Art Salomon, and Tricia Serio.

I would not have been able to devote as much time to my research if it wasn't for the wonderful administrative staff. Thank you to Tammy Glass for handling all of my purchasing needs. Thank you to the stock room: Kenny, Jerry, and Gill for delivery of all of my scientific reagents. Thank you to Jennie for handling all of our mailroom needs.

I would like to thank my fellow graduate student friends that have also supported me throughout the years. Abbie Frederick-Maguire and Christine Scaduto, thank you for your friendship, advice, and support throughout the years. You definitely made my graduate school experience enjoyable.

I would like to thank my family for their support throughout the years. To my mom, for being the strongest and kindest role model I could ever have. Thank-you to my sisters for always reminding me the importance of balance in life. And thank you to my dad for always believing in me.

I would also like to thank my wonderful fiancé Matthew Hirakawa, for his support throughout the years. You are the only person I would have wanted to share this journey with. Thank you for loving me, supporting me, cooking for me, and being my best friend.

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Chapter 1: Introduction

The fusion of an egg and sperm jump-starts the complex process of animal development. This single cell undergoes many rounds of cell division to produce an embryo composed of multipotent cells. Simultaneously, subsets of these multipotent cells begin to differentiate into specific cell fates. It is extremely important that during this process the cell lineage that will become eggs and sperm, the **germ cell lineage**, also begins to differentiate. This process, termed **germ cell specification**, is required for the new animal to be able to reproduce when it gets older. Accordingly, germ cell specification is essential for the survival of most animal species (Figure 1).

How is the germ cell fate specified during animal development? Simply put, different animals use different mechanisms. In fruit flies, for example, the germ cell fate is specified by the localization of maternal molecules in the posterior pole of the egg. Whatever embryonic cells happen to inherit these maternal molecules during early cleavage stages become the germ cell lineage and they will eventually give rise to eggs or sperm (Figure 2) [1]. This mechanism is generally referred to as **inherited** germ cell specification (also referred to as preformation) [2]. This mechanism is likely the predominant mode of germ cell specification in roundworms, zebrafish, frogs, and many more animals [3-5]. However in other animals, such as the mouse, the germ cell fate is specified by an entirely different mechanism. In the mouse most of the early cells remain multipotent and any cell has the ability to become a germ cell. It is not until embryonic day 6.75 that the germ cell lineage is first specified. By this time, extraembryonic cell types have begun to differentiate and produce signals that pattern the multipotent cells contributing to the embryo proper (epiblast cells). The special combination of signals that are received in approximately 40 cells in the posterior part of

the epiblast tissue cause these cells to become the germ cell lineage (Figure 3) [6]. This mechanism is generally referred to as **inductive** germ cell specification (also referred to as epigenesis) [2]. This mechanism is likely the predominant mode of germ cell specification in mammals, axolotl, crickets, and many other animals [7-9].

In an effort to determine when each germ cell specification mechanism arose during animal evolution, previous researchers examined germ cell specification literature in animals sampled from different major animal phylogenetic groups (Figure 4). The authors find that the inductive mechanism is the most prevalent mechanism and that it is the only mechanism found in outgroups to the bilaterian lineage. Therefore, they conclude that the inductive mechanism is likely the ancestral mechanism for germ cell specification. An interesting finding in this review is that the inherited mechanism evolved many different times during animal evolution (Figure 4) [2]. It is still unclear how an inherited mechanism can evolve from an inductive mechanism or what evolutionary factors contribute to this change, although many hypotheses have been put forth.

One hypothesis that explains the frequent evolution of inherited germ cell specification is that germ cells are protected from potentially deleterious mutations when they are specified early and become quiescent (Figure 5). Mutations can be introduced via cell replication errors [10, 11], transcriptional errors [12], or through the mobility of transposable elements [13]. However, the acquisition of a germ cell fate protects germ cells from such mutational influences. In general, primordial germ cells are transcriptionally quiescent [14], they have reduced mitotic activity [15-18], and they employ their own piRNA pathway to inhibit the mobility of transposable elements [13]. If protection from mutation is a significant benefit for inherited germ cell specification, then

we would expect increased levels of general mutation rates in animals that use an inductive mechanism for germ cell specification. In order to test this hypothesis, future studies should compare general mutation rates in closely related animals that use different modes of germ cell specification. General mutation rates can be estimated by a few different methods, including: direct sequencing of reporter constructs during many generations, calculating the frequency of de novo mutations that contribute to monogenic disorders, and by calculating SNP rates between many animals within a single species [19-21]. With the availability of transcriptomic and genomic sequences from a variety of animal species, a comparison of SNP rates is the most efficient method to estimate general mutation rates in many different animals. If an inherited germ cell specification mechanism protects the germ cell lineage from mutagenesis then we would expect decreased levels of general mutation rates in animals that use an inherited germ cell specification mechanism relative to closely related animals that use inductive mechanisms.

Another hypothesis that explains the frequent evolution of an inherited germ cell mechanism is that this process frees embryonic molecules usually involved in inductive germ cell specification so that these molecules can change and be used for novel beneficial functions (Figure 6). Authors that support this hypothesis further explain that novel components can also be added to gene regulatory networks governing early developmental processes and this allows rapid evolution of protein coding sequences, morphological innovations, and increased speciation rates (Figure 7) [22]. In support of this hypothesis four vertebrate lineages (teleost fish, birds, rodents, and frogs) that have inherited germ cell specification mechanisms have increased numbers of species

compared to closely related lineages that use inductive mechanisms for germ cell specification (actinopterygian fish, turtles, non-rodents, and salamanders) [22]. In addition, when phylogenetic trees are generated with random subsets of protein coding sequences from animals that use differing mechanisms for germ cell specification a large percent of the trees are inconsistent with the species tree and incorrectly predict that animals that use inductive mechanisms are more closely related. Evans et al' interpretation is that protein coding sequences in animals that use the inductive mode are more similar to each other while the animals that use the inherited mode have attained more changes in their sequences [23]. In addition, relative-rate tests show that sequences in fish and amphibians that use inherited mechanisms for germ cell specification have an increased number of genes that evolve at a faster rate [23]. This study is the first attempt to test the consequences of the evolution of an inherited germ cell specification, based on their research Evans et al. conclude that germ plasm enhances vertebrate evolution.

However, a more recent study concludes that the inherited mechanism of germ cell specification does not correlate with increased protein evolutionary rates in diverse bilaterian animals (Figure 7) [24]. Whittle et al. note that the species trees generated in the above study are based on incomplete sequence data sets (transcriptomes that bias a particular tissue or do not include the majority of protein coding sequences in the genome). In addition, Whittle et al. note that Evans et al. did not use the third position of the codon for protein translation in their analyses. This is problematic because changes observed in sequences could be due to increased overall mutation rates that are not dependent on a release from a selective pressure. In other words, if the third coding

position was taken into account then you could compare non-synonymous changes (mutations that change the amino acid) to synonymous changes (neutral mutations that don't change the amino acid) to determine if protein coding changes aren't simply due to an increased overall mutation rate in the animal that does not depend on PGC specification mode. Whittle et al. then computed the ratio of non-synonymous to synonymous changes (dN/dS ratios) in 7 closely related animal model pairs that use differing methods of germ cell specification. They selected these pairs based upon completeness of genome sequences available and based upon how closely related these animals were, to ensure the changes in the 3rd nucleotide (non-synonymous) were not saturated. Overall their results show that within seven animal-model pairs there is no relationship between increased dN/dS ratios and germ cell specification mode [24]. Although rapid evolution of many protein coding sequences could still occur in anuran amphibians and teleost fish in response to an inherited germ cell specification mechanism (this remains to be tested with more complete genome sequences), overall this hypothesis does not hold true in all vertebrates or in any non-vertebrates tested. Nevertheless, this hypothesis may still hold true among sequences directly involved in germ cell specification. For example, genes directly involved in inducing germ cells may have elevated rates of change in animal lineages that use inherited mechanisms for germ cell specification.

Another possible explanation for the prevalence of the inherited germ cell specification is that its evolution is dependent on other developmental processes (Figure 8) [24]. For example, a faster time from egg to larva (or the adult body form) may be beneficial for an organism as they could feed, reproduce, and/or move away

from predators more easily. Earlier or maternal specification of the body plan could cause a faster time from egg to feeding larva. If germ cell specification is dependent on any of the molecules that are required for specification of the body plan then germ cell specification would also become earlier/under maternal control. In order to test if an inherited germ cell specification mechanism depends on other developmental processes, future studies should calculate how many cell divisions it takes for many developmental milestones to occur in closely related animals that use different mechanisms of germ cell specification. Such milestones could include: establishment of early axes (ie: animal/vegetal, anterior/posterior, dorsal/ventral, and left/right), larval feeding, larval mobility, metamorphosis, and reproduction. Such studies will illuminate if the timing of these developmental processes correlate with an inherited or inductive germ cell specification mechanism. For example, if a faster time from egg to larva causes the evolution of an inherited germ cell specification mechanism, then we would expect less cell divisions to occur from the egg to larval feeding stage in an animal that uses an inherited germ cell specification mechanism relative to a closely related animal that uses an inductive mechanism.

A likely possibility is that every distinct time an inherited germ cell specification mechanism evolved a different combination of these hypotheses were contributing factors. Indeed, the widespread presence of both germ cell specification mechanisms suggests that the evolution of an inherited germ cell specification mechanism is context-specific. The presence of the inductive mechanism in many animals argues that the inherited mechanism is not universally more beneficial than the inductive mechanism, otherwise all animals would have evolved an inherited germ cell specification

mechanism. This suggests that a unique combination of evolutionary factors contribute to every distinct time an inherited germ cell specification mechanism evolves. In order to rigorously test if these hypotheses contribute to the evolution of an inherited germ cell specification mechanism and to come up with new ones, four things need to be accomplished. 1) We need to find multiple closely related animal model pairs in which one animal uses an inherited mechanism for germ cell specification while the other uses an inductive mechanism. These animal model pairs should also be from diverse phylogenetic backgrounds. 2) We need to study how germ cells are specified by inherited and inductive mechanisms in these animal pairs. 3) We need to compare these mechanisms within animal model pairs to infer how inherited germ cell mechanisms evolved from an ancestral animal that used an inductive germ cell specification mechanism. 4) We need to compare these evolutionary theories between animal model pairs to determine if there are any recurring themes.

Germ cell specification mechanistic research is available for three such animal model pairs. Within amphibians, axolotls use inductive mechanisms for germ cell specification while frogs use inherited mechanisms. Within insects, crickets use inductive mechanisms for germ cell specification while fruit flies use inherited mechanisms. Lastly, within echinoderms, I have learned that sea stars use inductive mechanisms for germ cell specification while sea urchins use primarily inherited mechanisms. The latter group will be the focus of this review. Briefly: I will outline what is known about germ cell specification in all echinoderms, I will speculate what evolutionary changes might have occurred to cause a shift in germ cell specification

mechanism, and I will compare these evolutionary changes with other animal model pairs.

Germ cell specification in the sea urchin

Primordial germ cells are specified in the sea urchin relatively early in development (Figure 9). During the 4th and 5th cleavage divisions, the primordial germ cells arise as the product of two unequal cell divisions and are also known as small micromeres. As soon as these cells are made they selectively accumulate conserved germ cell factors including: Nanos mRNA and Nanos and Vasa protein. In addition, removal of the small micromere cells results in a perfectly normal sea urchin with gonads that contain somatic tissue but do not contain any germ cells (Appendix D) [25]. Previous research suggests that sea urchins use primarily inherited mechanisms for germ cell specification.

Primordial germ cells are specified relatively early in development (during cleavage stages) when developmental decisions rely on more maternal factors and less embryonic gene products. Previous research in sea urchins shows that embryos develop relatively normally when they are not able to transcribe embryonic gene products [26, 27]. Therefore, developmental decisions that specify the germ cell lineage at the 32-cell stage are most likely dependent on maternally stored molecules and less likely dependent on products of embryonic transcription. Furthermore, primordial germ cells become quiescent soon after they are specified, which makes them unlikely to depend on embryonic signals for any subsequent specification or development. They accumulate epigenetic markers of low transcriptional activity [28], they do not undergo cell divisions [29, 30], and they also have low translational activity [31]. In addition,

when these cells are transferred to a dish they still express germ cell factors cell-autonomously [32]. Because of these reasons we conclude that the small micromeres are fully specified by the 32-cell stage and they do not require embryonic gene products for their specification. We propose this germ cell quiescence could be a mechanism to shield the early forming primordial germ cells from embryonic signals that would otherwise lead them to a somatic fate.

The selective retention of maternal messages in primordial germ cells is required for germ cell specification. Previous work in our laboratory shows that the depletion of an mRNA deadenylase (CNOT6) in the small micromeres is required for the selective retention of maternal mRNAs. Furthermore, this mechanism is required for the retention of conserved germ cell factors: Vasa protein and Piwi RNA [28]. This study suggests that retention of maternally loaded germ cell factors are required for the small micromeres to retain a germ cell fate.

Even though sea urchins use primarily inherited mechanisms for germ cell specification, some research suggests that inductive mechanisms also contribute to germ cell specification. Mainly, Nanos is embryonically transcribed at the 32-cell stage [16]. I propose that the transcription of Nanos is dependent on vegetally localized maternal molecules and therefore, is ultimately dependent on inherited mechanisms. In addition, sea urchins have a brief ability to re-specify their germ line by entirely inductive mechanisms at the 16-cell stage [33]. I propose this mechanism is likely a remnant of the ancestral inductive mechanism. Furthermore, germ cells are only induced in an experimental situation, when the germ cell precursor cells are removed. This suggests that although sea urchins have remnants of inductive germ cell specification

mechanisms, the inherited germ cell specification is the predominant mode of germ cell specification.

Germ cell specification in the sea star

Primordial germ cells are specified relatively late in development in the sea star (Figure 10). The conserved germ cell factor Vasa does not accumulate selectively in a set of cells until the early gastrula stage [34]. Shortly after, Vasa accumulates in a similar vegetal ring domain as Nanos. However, this field of Nanos and Vasa cells is further restricted during embryogenesis until the larva stage when germ cell factors all accumulate in a pouch that buds off of the endoderm (also referred to as the posterior enterocoel) (Chapter 4). Germ cell pouch (**GCP**) removal experiments result in juvenile larva with significantly fewer morphologically distinct primordial germ cells [35]. The remaining primordial germ cells are likely either leftovers that were not removed at the beginning of the experiment or are a result of germ cell regeneration. Nevertheless, removal experiments show that cells in the germ cell pouch contribute to the germ cell lineage. In summary, the GCP is likely the embryonic source of primordial germ cells in the sea star.

Since primordial germ cells are specified relatively late in development, the decisions that govern the accumulation of germ cell factors likely rely more on embryonic gene products and less upon maternally stored material. The germ cell factor Nanos, for example, is transcribed at the early gastrula stage and requires embryonic transcription for its presence [36]. The germ cell factors Vasa and Piwi accumulate broadly in early development and are subsequently restricted to the GCP (Chapter 2) [37]. These restriction events are likely dependent on embryonically produced signals

assuming the sea star follows a similar maternal to embryonic transition as the sea urchin. That is, maternally produced molecules are degraded in bulk at the blastula stage [26]. Our data suggests the accumulation of germ cell factors are mostly dependent on inductive mechanisms in sea stars.

The cells that accumulate conserved germ cell factors in early development are able to respond to signals, in fact they require the response to embryonic signals for further specification of the germ cell lineage. My work shows that the restriction of the germ cell factors Vasa and Nanos requires the embryonic production of Nodal signal (Chapter 4, in preparation). In addition, cells that express Nanos and Vasa are not quiescent. Preliminary results suggest they do not accumulate epigenetic markers that indicate low transcriptional activity, they have normal transcriptional activity, and they also have normal translational activity (Appendix A, Oulhen unpublished). This means that germ cell precursors are not only capable of responding to embryonically produced materials, but they are actually dependent upon them for their specification.

Germ cell specification in other echinoderm species

To determine when inductive or inherited mechanisms of germ cell specification arose during echinoderm development it is necessary to analyze how germ cells are specified in other echinoderm species (Figure 11). Based upon Vasa and Nanos mRNA localizations it becomes apparent that the majority of echinoderm clades are more like the sea star and use more inductive mechanisms for germ cell specification. Vasa mRNA and protein does not accumulate in a restricted embryonic domain until the larva stage in embryos that belong to asteroid, holothuroid, and cidaroid clades [34]. In addition, Nanos mRNA does not accumulate in a specific embryonic domain until the

blastula stage in embryos that belong to the ophiuroid clade and the mid-gastrula stage in the asteroid, holothuroid, and cidaroid clades (Chapter 3) [36]. In other words, outside of the echinoid clade (the clade containing sea urchins) Nanos and Vasa does not accumulate in a restricted set of cells during the early cleavage stages. Unfortunately, access to embryos from the crinoid clade, the sister group to other echinoderms, is very difficult. From the existing research I propose that the ancestral echinoderm mechanism for germ cell specification is inductive and that the echinoid clade represents a shift in evolution to an inherited mechanism of germ cell specification.

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Figures and Tables

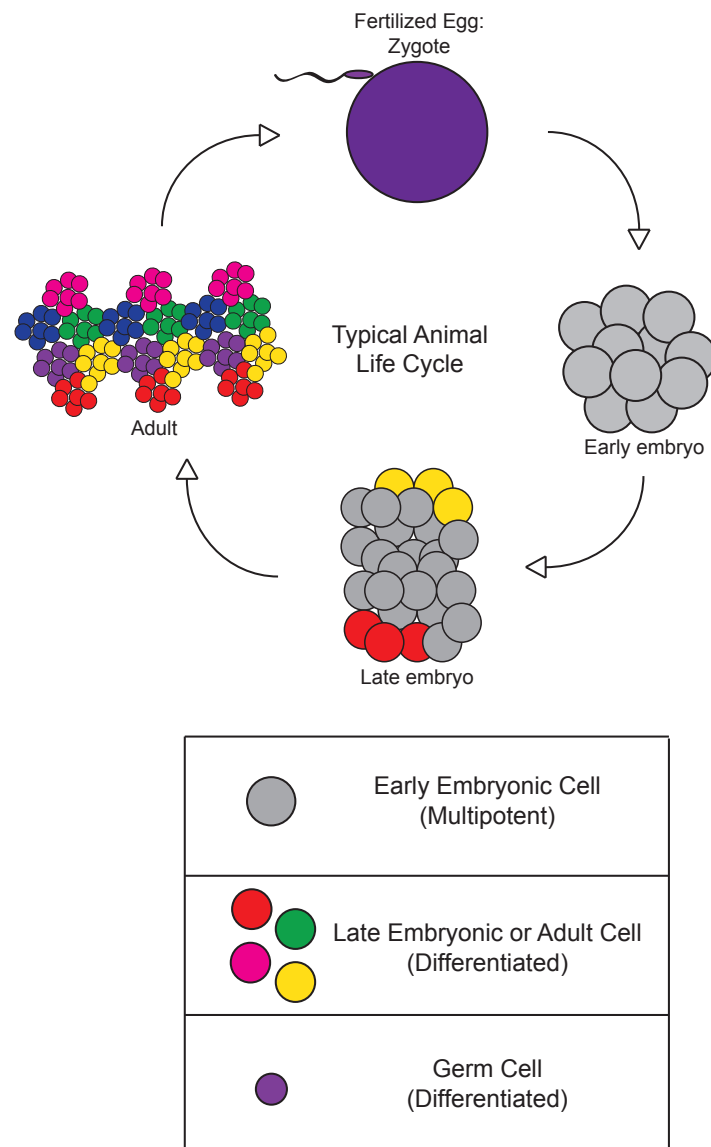


Figure 1- Germ cell specification during the typical animal life cycle

After fertilization, early embryonic cells (gray) retain the potential to become many cell types. As development proceeds, different cell types begin to differentiate (red, green, pink, yellow). The specification of the germ cell lineage (purple) is essential for the adult animal to be able to reproduce.

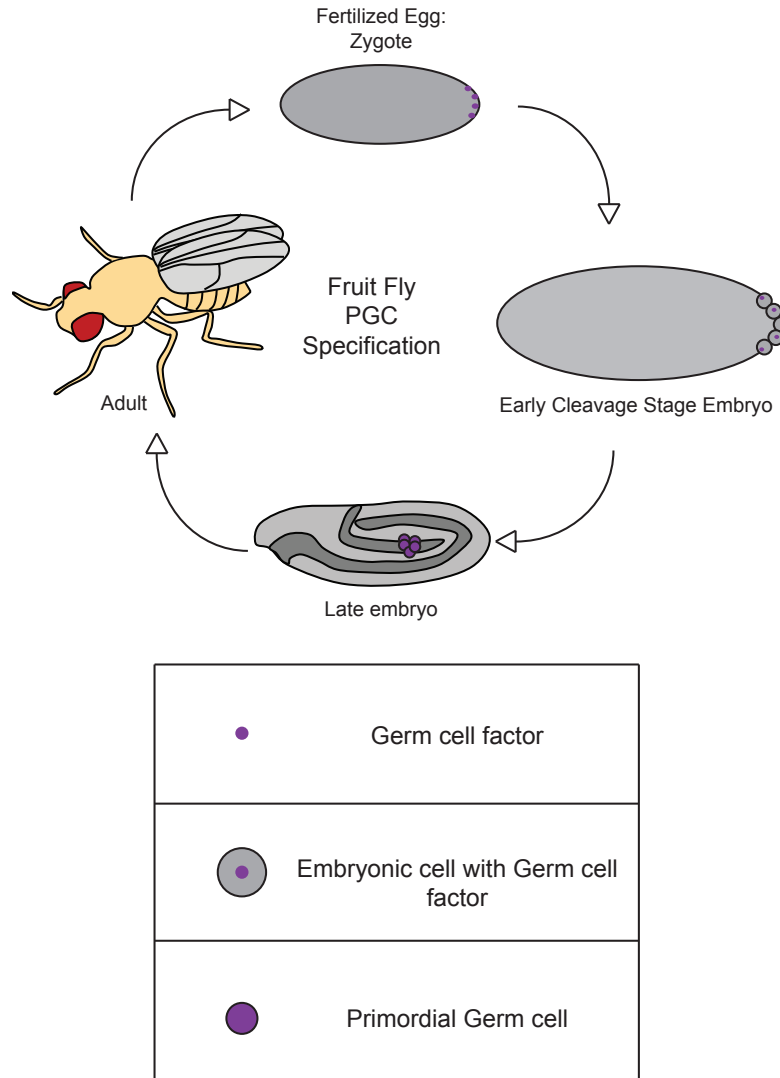


Figure 2- Germ cell specification in the fruit fly

During early cleavage stages whichever cells inherit maternally localized germ cell factors (purple) become the germ cell lineage [38].

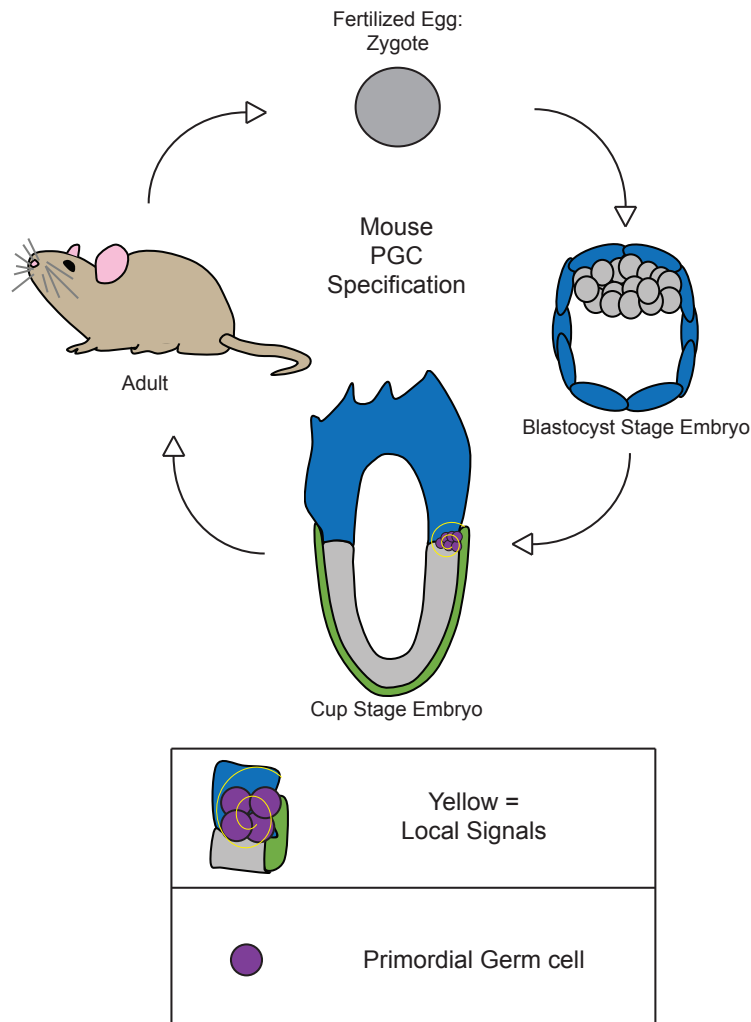
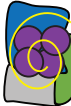


Figure 3- Germ cell specification in the mouse

During the cup stage of development, signals that emanate from surrounding tissues (blue, green) instruct a subset of cells in the epiblast (gray) to become the germ cell lineage (purple) [39].

	Egg	Embryo	Examples:
Inherited			Fly Worm Frog Zebrafish
Inductive			Mouse Axolotl Cricket

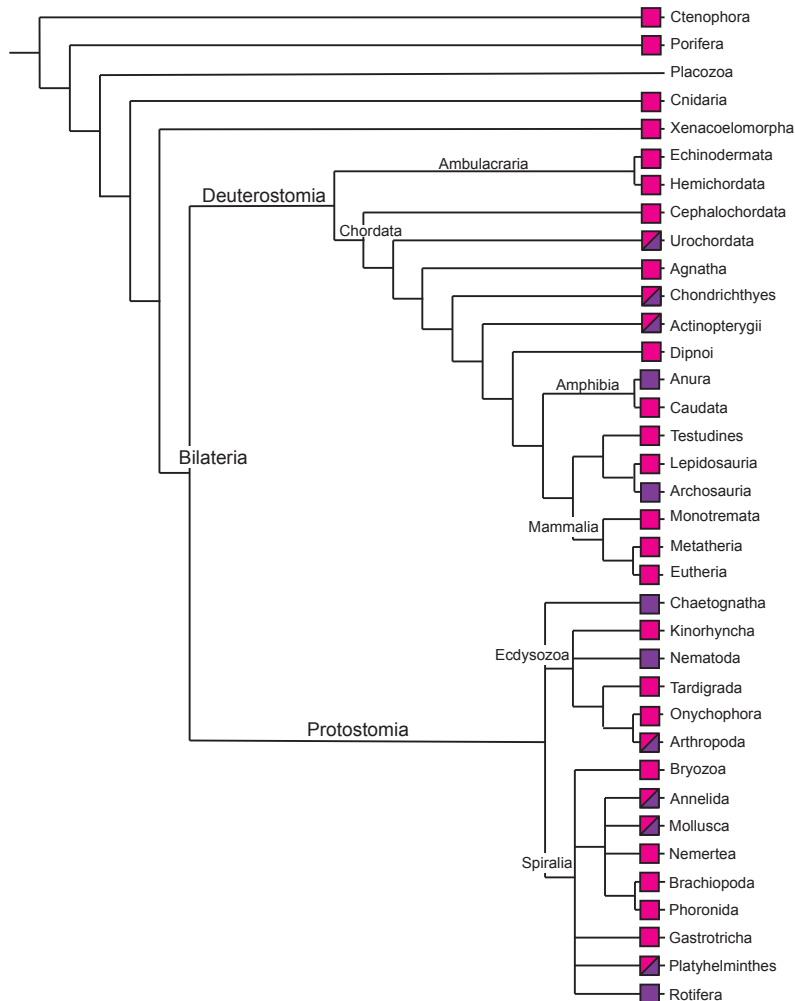


Figure 4- Evolution of germ cell specification in animals

A summary of Extavour and Akam's analysis of the literature on germ cell specification in diverse animal species [2]. Bilaterian outgroup and protostome topologies are updated based on Dunn et al.'s recent study [40]. Pink squares represent a clade that uses inductive mechanisms for germ cell specification. Purple squares represent a clade that uses inherited mechanisms for germ cell specification.

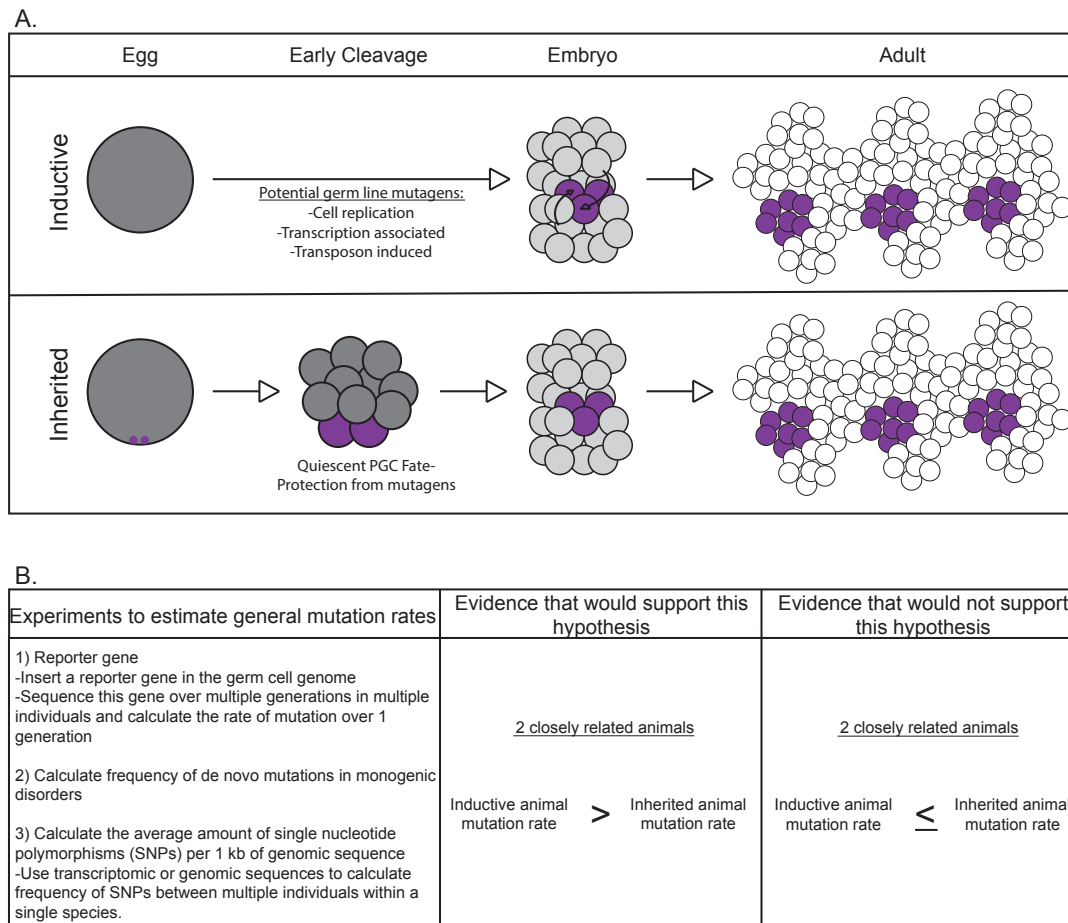


Figure 5- Protection from mutation hypothesis

A. When primordial germ cells are specified late in embryogenesis (inductive) the cells that become the germ cell lineage have the potential to accumulate mutations from cell replication, transcription, and transposons. When primordial germ cells are specified early in embryogenesis (inherited), they become quiescent and have less likelihood of mutation.

B. In order to test if an inherited germ cell specification protects germ cells from mutagenesis we need to calculate general mutation rates. If the mutation rate is generally higher in an animal that uses an inductive germ cell specification mechanism when compared to a closely related animal that uses an inherited mechanism this would support the hypothesis that an inherited germ cell specification mechanism generally protects germ cells from mutagenesis.

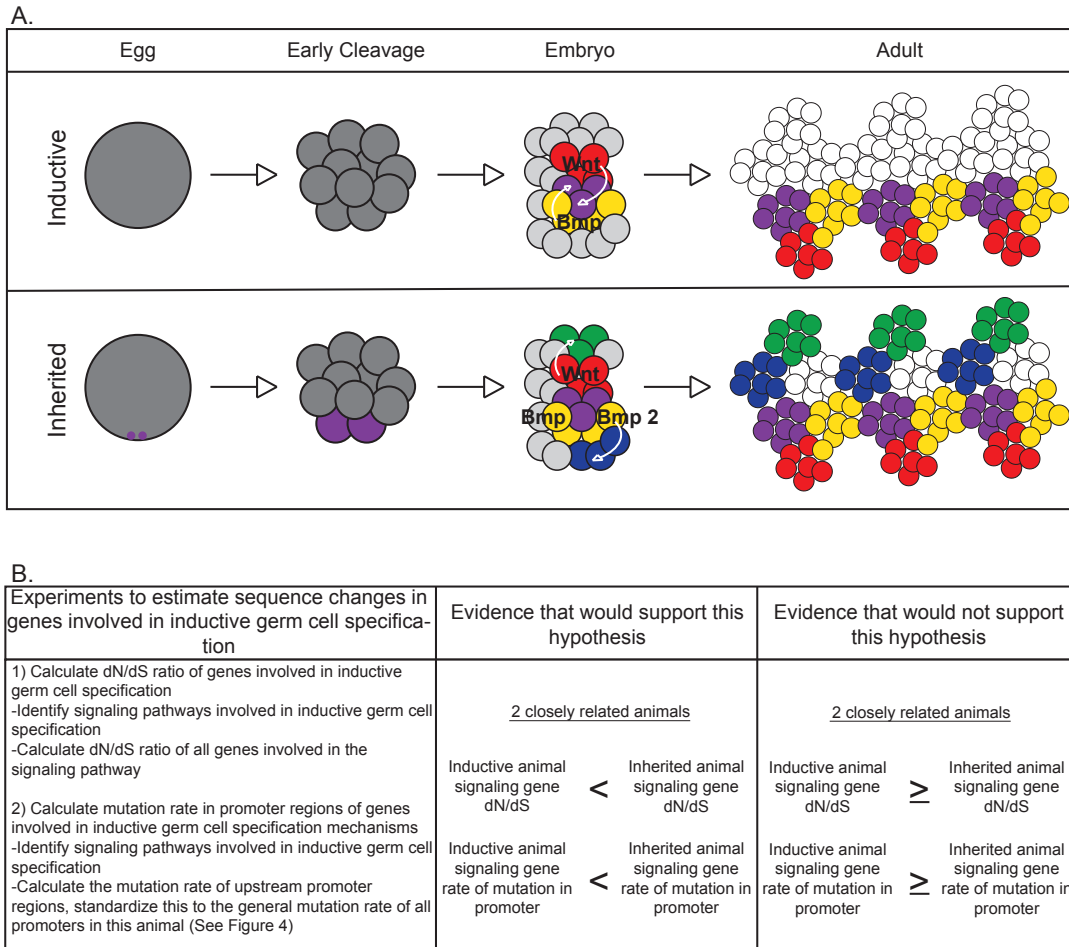


Figure 6- Evolution of novelty hypothesis

A. When primordial germ cells are specified late in embryogenesis by signaling molecules, the signaling molecules themselves are under evolutionary constraint. Most changes to the signaling molecules would be deleterious to the germ line and would inhibit the resulting animal from being able to reproduce. In contrast, when an inherited germ cell specification evolves, the signaling molecules that were under constraint can then change. This may cause new cell types to evolve or cause a change in the body plan. Previous authors hypothesize that increased variation in adult morphology may cause increased levels of speciation [22, 41].

B. In order to test if an inherited germ cell specification mechanism causes molecules that are normally involved in inductive germ cell specification to undergo increased sequence evolution we need to 1) estimate dN/dS ratios of genes involved in inductive germ cell specification and 2) calculate the mutation rate of upstream promoter regions of genes involved in inductive germ cell specification. If the dN/dS ratio or the rate of mutation in promoters of genes involved in inductive germ cell specification are lower in an animal that uses an inductive germ cell specification mechanism relative to a closely related animal that uses an inherited germ cell specification mechanism then this would

support the evolution of novelty hypothesis for genes involved in inductive germ cell specification.

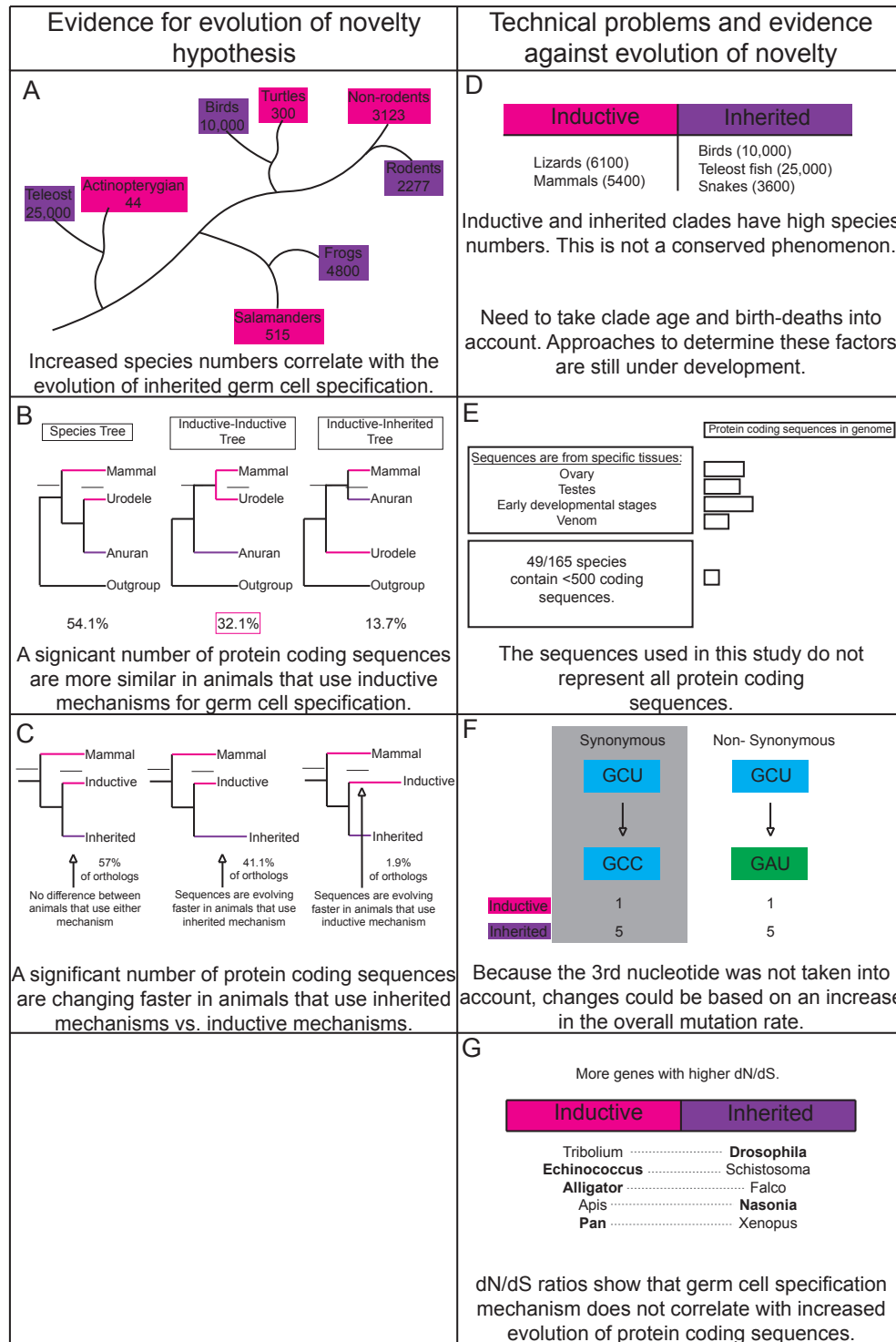
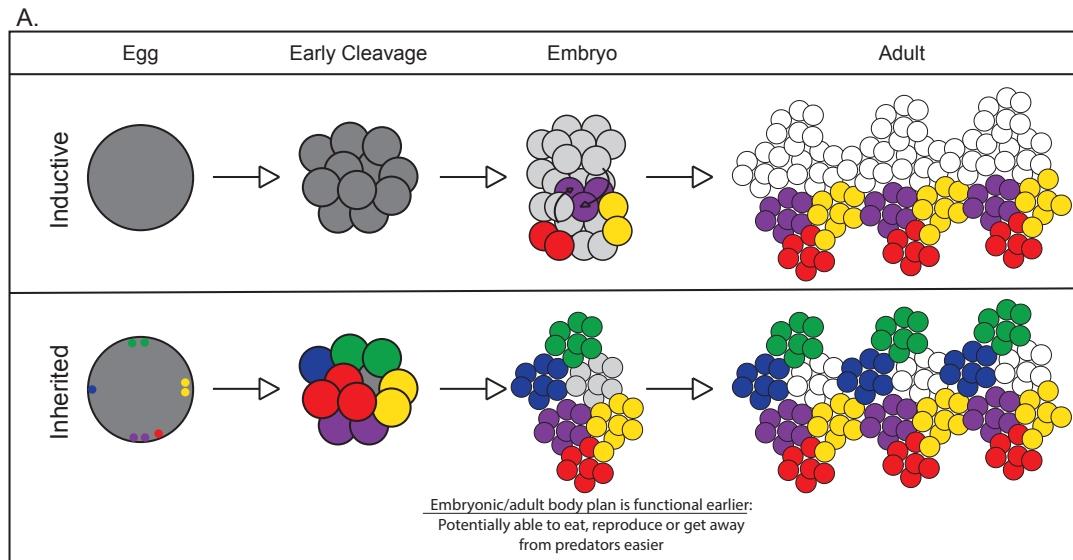


Figure 7- Evidence for the evolution of novelty hypothesis, technical problems, and evidence against evolution of novelty

- A. Within vertebrates, lineages that use inherited mechanisms of germ cell specification (purple) tend to have increased numbers of species when compared to closely related lineages that use inductive mechanisms of germ cell specification (pink) [22].
- B. When phylogenetic trees based on orthologous proteins do not follow the species tree they usually support a phylogeny where animals that use inherited germ cell specification mechanisms are more closely related [23].
- C. Relative rate tests show that 41.1% of orthologous sequences change faster in animals that use an inherited mechanism for germ cell specification [23].
- D. Increased species numbers correlate with animals that use both inherited and inductive mechanisms of germ cell specification. In addition, approaches to calculate rates of speciation are still under development as clade age and birth-deaths need to be taken into account [24].
- E. In the previous study, the protein coding sequences used to identify orthologous proteins between species do not accurately represent all protein coding sequences in the genome. Some sequences are from highly differentiated tissues. In addition, 49/165 species datasets contain less than 500 coding sequences in total [24].
- F. In the previous study, synonymous mutation rates are not taken into account (gray). Therefore, increased changes in protein coding sequences in animals that use inherited mechanisms could simply be doing so because they have increased overall rates of mutation [24].
- G. When dN/dS ratios are calculated there is no correlation between an increase in dN/dS and germ cell specification mechanism [24].



B.

Experiments to estimate how germ cell specification mechanism correlates with the timing of developmental phenomena	Evidence that would support this hypothesis	Evidence that would not support this hypothesis
1) Calculate the amount of total cell divisions required for specific developmental milestones -Milestones can include: axes formations, feeding, mobility, metamorphosis, reproductive competency	<u>2 closely related animals</u> Inductive animal No. of cell divisions for a particular developmental phenomenon $>$ Inherited animal No. of cell divisions for the same developmental phenomenon	<u>2 closely related animals</u> Inductive animal No. of cell divisions for a particular developmental phenomenon $\leq$ Inherited animal No. of cell divisions for the same developmental phenomenon

Figure 8- Other developmental processes hypothesis

A. Mechanisms that allow earlier differentiation of cell types or decreased time from egg to a feeding embryo/adult form could be beneficial. If these mechanisms are selected, earlier specification of the germ cell lineage could be a secondary effect.

B. In order to test if the evolution of an inherited germ cell specification mechanism is a result of earlier specification of other developmental processes we need to calculate the amount of cell divisions it takes for many developmental milestones to occur. If it takes more cell divisions for a particular developmental phenomenon to occur in an animal that uses an inductive germ cell specification mechanism relative to a closely related animal that uses an inherited germ cell specification mechanism then this would support the hypothesis that the evolution of an inherited germ cell specification mechanism is dependent on the evolution of earlier specification of other developmental processes.

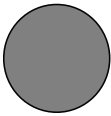
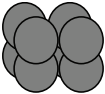
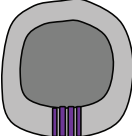
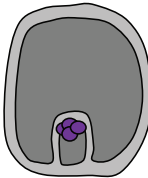
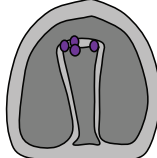
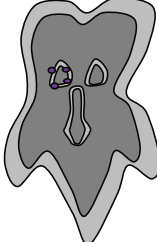
	Egg	8 Cell	16 Cell	32 Cell	Blastula	Mid- Gastrula	Late- Gastrula	Larva
								
Vasa RNA							X	
Vasa Protein				X				
Nanos RNA				X				
Nanos Protein				X				
Piwi RNA							X	
Piwi Protein								X
H3K9me3				X				
Low RNA Pol2					X			
Low bxn					X			
Low tin					X			
Slow cell cycle				X				

Figure 9- Germ cell specification in the sea urchin

Two unequal cell divisions at the 4th and 5th cleavage stages result in 4 small cells called small micromeres (purple). Small micromeres are the primordial germ cells in the sea urchin. They accumulate many primordial germ cell markers as soon as they are formed. After they are formed they travel up the developing gut and enrich in the left coelomic pouch (Appendix D) [25].

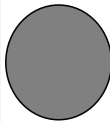
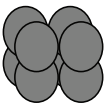
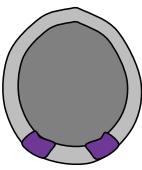
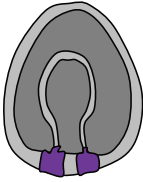
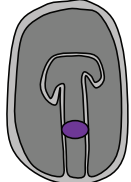
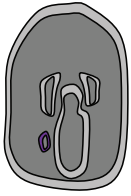
	Egg	8 Cell	16 Cell	32 Cell	Blastula	Mid- Gastrula	Late- Gastrula	Larva
								
Vasa RNA						x		
Vasa Protein								x
Nanos RNA						x		
Nanos Protein								x
Piwi RNA								x
Piwi Protein								
H3K9me3								
Low RNA Pol2								
Low tbn								
Low tln								
Slow cell cycle								

Figure 10- Germ cell specification in the sea star

A few germ cell factors accumulate in a ring of cells in the vegetal hemisphere at the early gastrula stage. This field of cells is restricted to a dorsal patch in the developing gut by the late gastrula stage. And this field of cells is further restricted to the left side of the developing gut by the larva stage. During the larva stage cells that accumulate germ cell factors invaginate out of the gut and form a germ cell pouch, GCP. Although, conserved germ cell factors accumulate in these cells the germ cell lineage does not show any evidence of quiescence during early embryogenesis (Chapter 2-4, Appendix A and E) [36, 37, 42]. A gray box indicates that this phenomenon has not yet been experimentally tested.

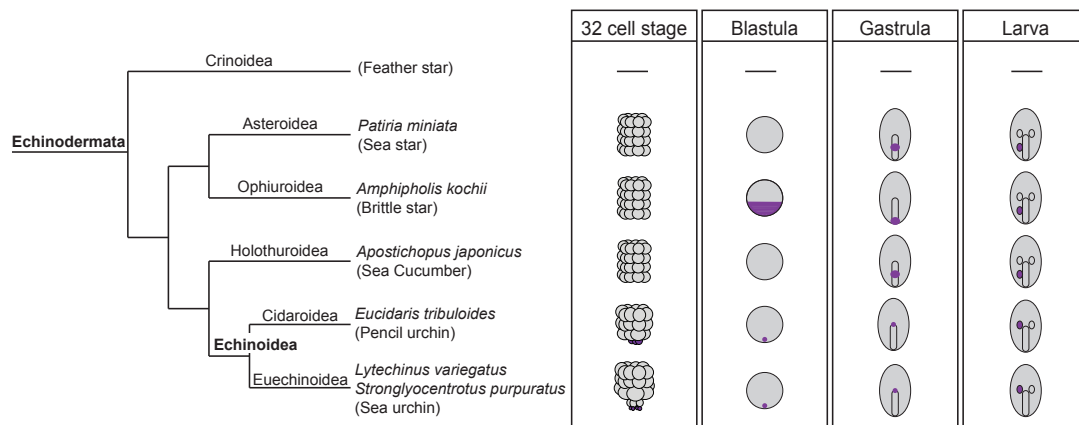


Figure 11- Germ cell specification in echinoderms

Purple indicates the accumulation of the conserved germ cell factor, Nanos. Current research supports the hypothesis that germ cells are specified early, likely by inherited mechanisms, in the euechinoid clade. In contrast, evidence suggests that germ cells are specified later, likely by primarily inductive mechanisms, in asterooid, ophiuroid, and holothuroid clades (Chapter 3) [34, 36].

Table of Definitions

Germ cell lineage	Any cell that retains the potential to become an egg or sperm under normal developmental conditions.
Primordial germ cell (PGC)	A lineage restricted cell that will become an egg or sperm.
Germ cell specification	The processes in development that contribute to the production of a primordial germ cell fate.
Inductive germ cell specification	When embryonic produced gene products are primarily used to specify the germ cell lineage. Generally, embryonically produced signals instruct which cells will become the germ cell lineage. Typically occurs after cleavage stages.
Inherited germ cell specification	When maternal molecules are primarily used to specify the germ lineage. Typically occurs during early cleavage stages.
Small micromere lineage (Smm)	The primordial germ cells in sea urchins. They are 4 cells that arise as a product of 2 unequal cell divisions at the 32-cell stage [25].
Vasa (Germ Cell Factor)	A translational regulator involved in specification and maintenance of the germ cell fate. The Vasa protein contains an ATP-helicase domain. Generally, Vasa binds to target mRNAs and can either activate or inhibit their translation through an unidentified mechanism. Recently, Vasa has been shown to interact with precursor piRNAs and is required for nucleo-cytoplasm transport [43].
Nanos (Germ Cell Factor)	A translational inhibitor involved in specification and maintenance of the germ cell fate. The Nanos protein contains 2 CCHC-Zinc finger domains. Generally, the Puf domain protein, Pumilio, binds to the TGTNNNT consensus site in the 3'UTR of target RNAs. Pumilio recruits Nanos, which further recruits machinery that will degrade the target mRNA or inhibit it from being translated into a protein [13].
Piwi (Germ Cell Factor)	A translational and transcriptional inhibitor involved in specification and maintenance of the germ cell fate. The Piwi protein is part of a larger family of Argonaute proteins. Generally, small piRNAs (22nt) guide Piwi to target RNAs for degradation. Subsequently, the genetic loci producing these target RNAs are also transcriptionally shut off. The majority of the target RNAs are transposons, mutagenic sequences that threaten the genetic integrity of the germ line [13].

Germ Line Index (GLI)	A recent term used to standardize when primordial germ cells are specified during development. It is calculated $(\# \text{ of total cells})/(\# \text{ of primordial germ cells})$ [44]. Mouse- 87 Nematode- 50 Fly- 20 Sea urchin- 8
Germ Cell Pouch	The site where putative germ cells localize during the early larva stage of the sea star. Germ cells localize in a patch in the left-dorsal gut and invaginate (bud off) to form a coelomic pouch. This is also referred to as the posterior enterocoel in previous literature [42]. I rename this pouch in this dissertation based upon current literature that shows the cells in this pouch accumulate germ cell factors and contribute to morphologically distinct primordial germ cells. I will generally refer to this structure as the germ cell pouch for the purposes of this dissertation. However, I use technical terminology (posterior coelom) when I discuss the presence of this structure in different echinoderm species.

Box 1- The evolution of the germ cell lineage itself in relationship to multicellularity

Previous authors propose that the evolution of the germ cell lineage was an extremely important event in the evolution of multicellularity. During the evolution of multicellularity it is generally thought that individual cells were in competition with each other to be able to contribute to the next generation of cells. Therefore, when a particular set of cells were tasked to contribute to the next generation, while the rest of the cells lost this ability (the evolution of the germ cell lineage), then this eliminated competition within cells of the same organism. This effectively causes evolution to act on all cells in an organism as one unit. In other words, factors that benefit the whole organism are no longer in competition with factors that benefit individual cells within one organism. As a result, the evolution of a germ cell lineage is a necessary event in the evolution of multicellularity [45].

Chapter 2: Selective accumulation of germ-line associated gene products in early development of the sea star and distinct differences from germ-line development in the sea urchin

Fresques T*, Zazueta-Novoa V*, Reich A, and Wessel G. (2014). Selective accumulation of germ-line associated gene products in early development of the sea star and distinct differences from germ-line development in the sea urchin. *Dev. Dyn.* 243(4):568-587.

I performed half of the experiments in this study and wrote approximately 1/3 of the manuscript.

Abstract

Background: Echinodermata is a diverse Phylum, a sister group to chordates, and contains diverse organisms that may be useful to understand varied mechanisms of germ-line specification. **Results:** We tested 23 genes in development of the sea star *Patiria miniata* that fall into five categories: 1) Conserved germ-line factors; 2) Genes involved in the inductive mechanism of germ-line specification; 3) Germ-line associated genes; 4) Molecules involved in left-right asymmetry; and 5) Genes involved in regulation and maintenance of the genome during early embryogenesis. Overall, our results support the contention that the posterior enterocoel is a source of the germ line in the sea star *P. miniata*. **Conclusion:** The germ line in this organism appears to be specified late in embryogenesis, and in a pattern more consistent with inductive interactions amongst cells. This is distinct from the mechanism seen in sea urchins, a close relative of the sea star clad. We propose that *P. miniata* may serve as a valuable model to study inductive mechanisms of germ-cell specification and when compared to germ-line formation in the sea urchin *S. purpuratus* may reveal developmental transitions that occur in the evolution of inherited and inductive mechanisms of germ-line specification.

Introduction

Evolutionary changes have resulted in a diverse series of mechanisms to accomplish the task of germ-line specification. Three extremes in these mechanisms are widely recognized in the animal kingdom and include 1) germ-cell derivation from adult multipotent stem cells (e.g. neoblasts in planaria, I-cells in hydra), 2) inheritance of maternal factors in early embryogenesis (e.g. pole plasm in *Drosophila melanogaster*, germ plasm in *Xenopus laevis*), and 3) cell-cell communication resulting in induction of a germ-line lineage [e.g. mouse and axolotl; 1, 2]. Because these different mechanisms of germ-line specification are polyphyletic, transitions between these germ-line specification mechanisms appear to have occurred multiple times within animal evolution [1].

When an embryo exhibits a germ-line determination mechanism that exceeds one biological threshold or another, the investigator usually classifies that mechanism as either inductive or inherited. While this is important for ease and clarity in communication it does not reflect the biological mechanism(s) effectively. Since germ-line determination is likely a result of multiple parallel pathways and activities leading to determination, the transition from one state to another in an embryo may result from a continuum of changes instead of a series of binary switches. Part of the problem with our current definitions of germ-line specification results from the fact that most of what we know comes from a small set of animals; mostly developing by an inherited mechanism. These animals include *D. melanogaster*, *Caenorhabditis elegans*, *X. laevis*, and *Danio rerio*, each with rich contributions of genetic, biochemical, and embryological analysis [e.g. 3, 4-6]. Unfortunately, we do not have as rich a data set for understanding the inductive mechanisms of germ-line determination. The mouse is the best understood model of inductive germ line determination and in this embryo, the epigenetic changes essential to

prime PGC formation occurs as a result of signaling between cells [e.g. 7]. This epigenetic reconfiguration then diverts the fate of the cells from a somatic direction, and instead leads to a germ line fate. Whether these features seen in mice are conserved in other organisms using inductive mechanisms is not yet clear and having a strong data set of inductive germ-line features from multiple different animals will be important in order to establish a baseline from which comparisons can be made between inherited mechanisms. Reaching this goal may then reveal the features of germ cell specification shared by inductive mechanisms, those that are unique to inherited and inductive mechanisms, and how evolutionary transitions between each mechanism may occur.

Preliminary experiments in sea stars suggest that this animal uses inductive mechanisms to specify their germ cells. The conserved germ-line factor Vasa becomes restricted to the posterior enterocoel (PE; see Fig. 1) only by the early larval stage [8]. In addition, PE removal experiments show that the cells from this structure contribute to the future germ cell lineage [9]. This is very different than what is known in the closely related taxon of sea urchins. Sea urchins specify their germ cells as early as the 32-cell stage when 4 small micromere cells (sMics) arise as the product of two unequal cell divisions (see Fig. 1). Multiple germ cell marker RNAs and proteins accumulate in the sea urchin sMic lineage [10-12], they express these markers cell autonomously [13], and removal of these cells results in loss of the germ lineage in adults [14]. In comparison to sea urchins, sea stars and all other groups within echinoderms do not have a sMic lineage. This leads us to believe the sMic lineage and the associated mode of early germ-line specification arose independently in the echinoid (sea urchin) lineage from a common ancestor that used inductive processes for germ-line determination.

We hypothesize that sea stars use an inductive mechanism of germ cell specification that may represent the ancestral mode of germ cell specification in echinoderms [1]. To begin to test this hypothesis we undertook the current study with the goal of analyzing the expression of germ cell factors in sea stars based on candidate genes that are important for both inductive and inherited modes of germ cell specification in other organisms. In this way we can compare the expression patterns of germ-line associated markers between sea stars, sea urchins, and other model organisms – especially mice - to test if sea stars specify their germ line in an inductive mode and if so, test if this mode has mechanisms conserved over long periods of evolutionary time.

The genes studied here fall into five categories: 1) Conserved germ-line factors; 2) Genes involved in the inductive mechanism of germ-line specification; 3) Germ-line associated genes; 4) Molecules involved in left-right asymmetry; and 5) Genes involved in regulation and maintenance of the genome during early embryogenesis. Overall, our results support the contention that the PE is a source of the germ line in the sea star *P. miniata* and that there is no specific accumulation of germ cell markers in any cells prior to PE formation. Our results lead us to conclude that the endomesoderm retains the expression of many pluripotency-associated genes which later give rise to the PE. In addition, we found that PE formation and *P. miniata* PGC specification is likely determined by inductive interactions amongst cells which simultaneously cause both the accumulation of germ cell determinants and the loss of somatic cell markers in the presumptive PE. We propose the sea star may serve as a valuable model for future study of the inductive mechanisms of germ-line determination, and when compared to the data sets in sea urchins already available, may serve as a useful comparative model for

understanding the developmental transitions between an inductive germ-line determination mechanism and an inherited mechanism.

Results and Discussion

We selected the genes used in this study by first identifying genes that are associated with PGC specification in a variety of animals that exhibit diverse mechanisms of germ-line determination. Genes involved in both inherited and inductive mechanisms were chosen as well as those involved in left-right asymmetry. This latter group was selected because the hypothesis being tested is that the PE contributes to the primordial germ cells – the PE structure is on the left side of the midline in the larva. We obtained sequences of *D. melanogaster* and mouse proteins of all the genes tested here from NCBI. Orthologous protein sequences from sea urchins were found by BLAST analysis against the published sea urchin database (Spbase.org). *P. miniata* orthologous protein sequences were found by BLAST analysis against a nascent *P. miniata* ovary transcriptome database. The top *P. miniata* hit was used for reciprocal-BLAST analysis to the non-redundant NCBI database to test orthology. Alignments using these orthologous sequences from *Mus musculus*, *D. melanogaster*, *S. purpuratus* and *P. miniata* were performed to further test authenticity (Tables 1-5 and Supplementary Fig. 1). The lists of primers used for PCR amplification of each gene in sea star and the sizes of predicted and acquired PCR products are shown (Tables 1-5).

Conserved germ-line determinants – Select expression in the Posterior Enterocoel

The genes in the first set we explored are those most highly conserved amongst animals as being part of the germ-line determination mechanism (Table 1). Vasa, Nanos,

and Piwi are a classical cluster of germ-line factors, found in all animals at some point in the construction or maintenance of a new germ line in both inductive and inherited germ-line formation mechanisms. Vasa is a dead-box helicase involved in regulating the translation of RNAs in the germ-line. We found in the sea star that Vasa gene expression (mRNA accumulation) is ubiquitous in eggs and early embryos and first becomes restricted to the vegetal pole of the blastula. During gastrulation, Vasa mRNA becomes enriched in the middle region of the archenteron and by early larval stages is restricted to the left side of the mid-archenteron where the PE buds. Vasa remains selectively expressed in the PE throughout the development of the larva (Fig. 2). We also noted less detectable amounts of Vasa transcripts are present in the perimeter of the left and right coelomic pouches in larval stages (Fig. 2; late larva; asterisks). Overall these results support the contention that the PE is at least relevant for consideration of the origin of germ-line determination in this organism. The result also speaks more generally to Vasa function – the transcript is broadly expressed in the egg and early embryo – clearly it is not strictly a germ-line factor. Previous results in sea stars show that Vasa protein accumulates ubiquitously early in development as well. Not until the PE forms does the Vasa protein become restricted [8], much like the expression of its mRNA. This is a particularly important distinction to be made since in sea urchins the protein does not accumulate coincident with the mRNA. Protein translation is widespread in the sea urchin embryo but degradation is selective to the somatic cells. Therefore, the sMics, the PGC lineage in sea urchins, accumulate the Vasa protein selectively even with broad mRNA presence. This selection (degradation of Vasa protein outside of the presumptive PGCs) appears to be a function of at least one E3-ubiquitin ligase activity, Gustavus [15].

Piwi is a conserved germ-line factor involved in small RNA-mediated degradation of transposons, and its accumulation is similar to Vasa in *P. miniata* development. That is, broad early expression with selective accumulation in the PE by the early larval stage (Fig. 2). Although the Piwi transcript does not follow as tight an expression domain as Vasa, it is clear that Piwi transcripts accumulate in the archenteron as soon as it is formed and later in gastrulation the Piwi mRNA is enriched in the region where the PE forms - within the mid-region (future mid-gut) of the archenteron (Fig. 2, late gastrula, early larva). By the late larval stage Piwi transcripts disappear from the gut, but are retained in the PE (Fig. 2, larval stages). In the late larval stage we also note Piwi transcripts in the posterior portions of the left and right anterior coelomic pouches.

In the sea urchin, Piwi expression is much like Vasa, that is, broad early expression with protein accumulation selective to the sMics, and only subsequently during gastrulation does the mRNA become restricted to the sMics [10, 16]. In that respect it is similar in the sea star where the vegetal plate and endodermal tissues are the last to down regulate the Piwi mRNA while being retained in the future germ line, either the sMics in sea urchins or the PE in sea stars (Fig. 2, late larva, asterisk). Clearly both Vasa and Piwi gene expression patterns support the contention that the PE contributes to the germ line, and that their expression is not uniquely germ line.

Nanos is another conserved germ-line factor found in all animals studied. Nanos interacts with Pumilio to bind the 3'UTRs of select mRNAs bearing a Pumilio Response Element (PRE, also referred to as Nanos Response Element, NRE). Binding of this complex to the mRNA reduces translation of the encoded protein either through the translational machinery [17, 18] or by degrading the mRNA by recruiting deadenylase

activity [18-20]. Pumilio is usually expressed more broadly than Nanos and can interact with a variety of regulators of the bound mRNA. Nanos on the other hand is often restricted to the germ line and its expression is associated with decreasing the cell cycle of the PGC through binding of cyclin mRNAs. Mis-expression of Nanos in non-germ line cells will often lead to developmental abnormalities or death in the somatic cells [6, 21]. In sea urchins, Nanos is tightly regulated by a variety of means to be specific to the germ line cells [e.g. 22]. In the sea star, Pumilio, but not Nanos, is broadly expressed throughout much of the embryo early in development. Following gastrulation Pumilio became enriched to the gut but was largely excluded from the coelomic pouches and the PE (Fig. 2). Nanos however first appears significantly detectable in the PE when it forms (Fig. 2, late larva, asterisk). In this regard, it is like in sea urchins, where Nanos appears only once the sMics form. This is unlike other germ-line marker transcripts that accumulate in larger expression domains prior to restriction (see Vasa and Piwi, Fig. 2).

Boule/Dazl expression overlaps that of Pumilio but with distinct characters. Boule gene expression generally is present uniformly in early development with slight enrichment in the endomesoderm. In early larvae, the message is enriched in the esophagus significantly over other regions of the embryo or gut. In later larvae, expression is enriched in the esophagus in addition to a small number of cells (Fig. 2, asterisks), which may be the precursors to the dorsal ganglia in late larva [23].

Overall this gene set supports the hypothesis that the PE is a special derivation. In concert, the Piwi, Nanos and Vasa selective mRNA expression, and Vasa protein expression [8] argues that the PE contributes to the germ line. The endomesoderm however, seems to retain many pluripotency-related genes not otherwise present in the

ectoderm or future mesodermal cells. Perhaps the endoderm retains broad developmental potency utilizing these various germ cell markers that only later in development become restricted to the PE.

Gene regulatory molecules involved in inductive specification of germ cells in the mouse are conserved in echinoderms

The mechanism of inductive germ-line specification is thought to be the most ancient mechanism used by animals [1]. Many signaling molecules involved in inductive germ-line specification are not unique to the germ line during animal development and are instead used in a variety of developmental processes. The list of genes involved in the inductive mechanisms of germ-line determination is small – most work thus far has been accomplished only by genetic approaches and only in the mouse. This set of genes is important to test in order to determine if the signaling pathways and downstream effector molecules that are required for inductive germ-line specification in the mouse are conserved in other animals.

Blimp1 (B lymphocyte-induced maturation protein) also known as Prdm1 (Positive regulatory domain I element of the β -IFN gene promoter, containing Zn-fingers) is a transcriptional regulator. Blimp1 is believed to generally repress its target genes, although some evidence indicates that Blimp1 can also serve as an activator of gene expression in certain contexts [24, 25]. Blimp1 was first discovered in the immune system, but it is expressed and functional in many tissues during development [26], and in many animals studied [e.g. 27]. In mice, Blimp1 is a key factor for germ-line determination [28-31]. Blimp1 appears to repress genes within the presumptive germ cells of mice (including Hox genes, esp. Hoxb1) whereas these same genes remain active in neighboring cells as they begin somatic differentiation. Therefore, Blimp1 in the mouse germ line appears

to function in retaining a pluripotent fate by repressing genes important for somatic fates commitments. In contrast, the sea urchin *Blimp1* is involved in endoderm gene specification; *Blimp1* appears to activate *Wnt8* but repress *Notch* and *HesC* expression. Knocking down *Blimp1* results in a lack of endoderm morphogenesis and perhaps conversion to non-endodermal fates [32-34]. In the sea star oocyte, *Blimp1* transcripts are in low abundance, but in the blastula they accumulate significantly in a torus of cells surrounding the vegetal pole [Fig. 3, asterisks, and 35, 36]. In mid-gastrulae, expression of *Blimp1* extends into the posterior archenteron. In late gastrula and early larval stages, expression is restricted to the midgut and hindgut regions of the archenteron, with a slight clearing in the gut region where the PE will form, followed by a marked absence in the PE. *Blimp1* is enriched in the stomach, intestine and anus of the late larva, and enrichment persists later in development to the esophagus. Were *Blimp1* to have a conserved function in the sea star as in the mouse, it may be in retaining some potentiality of cell fate in the endoderm. Removing its function may, as in mice, enable cells to differentiate into mesodermal lineages and thereby lose their potential for endodermal and germ-line fates.

Prdm14 is closely related to *Blimp1/Prdm1* and is a key regulator of mammalian germ cell development [37]. It plays a critical role in cell fate pluripotency by suppressing the expression of differentiation marker genes. In mice, *Prdm14* is expressed early in germ line determination, by 6.5dpc, and its up-regulation is likely in response to *Bmp4* signaling. Recently it was shown that *Prdm14* functions to ensure pluripotency through two pathways: 1) it antagonizes activation of the fibroblast growth factor receptor (*Fgfr*) signaling by the core pluripotency transcriptional circuitry, and 2) it represses expression

of *de novo* DNA methyltransferases that would otherwise modify the epigenome to a primed (somatic) epiblast-like state. Prdm14 exerts these effects by recruiting polycomb repressive complex 2 (Prc2) specifically to key genomic sites and repressing nearby gene activity [38]. Prdm14 in the sea star is present widespread and has a very similar expression pattern to Bmp2/4 (below) in embryos and larvae. That is, Prdm14 transcripts accumulate in the archenteron of the gastrula and larvae showed strong Prdm14 staining in the mouth opening, stomach, intestine, anus, and the preoral and postoral ciliary bands, but not in the anterior coeloms nor in the PE (Fig. 3, asterisks). In the oocyte, Prdm14 transcripts are ubiquitous (Fig. 3). Unlike in mice, Prdm14 in sea stars or sea urchins does not correlate with the accumulation of other PGC markers. Instead, Prdm14 localizes to ciliary bands in urchins and to the embryonic gut and ciliary bands of sea star larva. This leads us to hypothesize that Blimp1 and Prdm14 may be involved in retaining gut pluripotency early in sea star development, which may be an essential role, but this role does not appear to be sequestered to the germ line.

Bmp2/4 is a critical signaling component for germ-line specification in the early mouse embryo [29, 39]. It is expressed by cells in the extra-embryonic tissues and is required for cells in the posterior epiblast to become PGCs. Zhou, Meng [40] also found Bmp4 enhances germ cell derivation *in vitro* from ES cells, although currently the link is not clear, if any, between the Bmp4 signaling and Blimp1 expression [41]. The sea star, as in sea urchin, appears to have a less diverse family of Bmp signaling molecules and the closest ortholog to the mouse Bmp4 in the sea star is a Bmp2/4 gene [42, see Supplementary Fig. 1]. Bmp2/4 in sea star is present broadly in development (Fig. 3). Remarkably, the Bmp2/4 transcripts are enriched in the nuclei of young oocytes, but not

in nuclei of full-grown oocytes or embryonic cells. Some gene expression is regulated by transcript retention in the egg nucleus of the sea urchin [43, 44] though this is the first example of selective transcript retention in the germinal vesicle (GV) of a sea star. In gastrula and early larval stages, Bmp2/4 transcripts accumulate very similarly to Prdm14, and show a clear accumulation in the gut of the embryo, but not in the anterior coeloms nor in the PE. In late larvae, Bmp2/4 is expressed largely around the opening of the mouth, anus and the preoral and postoral ciliary bands (Fig. 3; asterisks). Recently, another report on Bmp2/4 in *P. miniata* showed enriched Bmp2/4 expression in the future site of mouth formation in blastula stage embryos [23]. We did not see this profile here although it may have been a time point missed in our analysis – we focused on PE formation here and terminated the in situ hybridization analysis to best reveal signal information in later embryos and larvae.

The Wnt signaling pathway is thought to regulate several aspects of germ-line function. These include germ-line stem cell functions [e.g. 45], germ-cell migration [e.g. 46], and germ cell-soma interactions in the gonad [e.g. 47]. Most importantly, and in regards to mouse inductive PGC specification, Wnt3 signaling is required to prime the cells in the posterior epiblast so they are competent to respond to Bmp4 PGC specification signals [48]. In addition, Wnt8 is a somatic marker in axolotls that is expressed during gastrulation in areas of germ cell precursor formation [49, 50]. We chose to determine the expression patterns of Wnt3 and Wnt8 in the sea star to determine if their expression correlates with PE formation. Moreover, in sea urchins as in many embryos, the Wnt signaling pathway is involved in broad scale axial patterning [e.g. 51, 52]. In sea urchins, the nuclearization of β -catenin (a mark of active canonical Wnt

signaling) begins in the micromeres at the 16-cell stage, the precursors to the sMics. β -catenin and Blimp1 further trigger Wnt8 expression [53, 54]. These expression profiles are common in all indirect-developing sea urchins examined so far [55, 56]. Furthermore, in sea urchins Wnt8 is the primary component of the early endomesoderm gene regulatory network, and its role is to activate the β -catenin/Tcf signal transduction system [33, 53]. In sea stars, Wnt8 and Wnt3 expression patterns show a segmented, circumferential pattern with an overlapping border in the posterior third of the ectoderm (Fig. 3). As in sea urchins, Wnt8 transcripts are not maternally expressed in the sea star. By the late blastula stage, however, Wnt8 transcript enrichment is seen as a ring around the vegetal half of the embryo (Fig. 3; asterisks). This expression pattern continued until mid-gastrula, but its enrichment decreased during early and late larval stages. The Wnt3 expression pattern in *P. miniata* follows a similar expression pattern as Wnt8, but the circumferential pattern is closer to the vegetal pole of the embryo (Fig. 3, asterisks). This pattern continued during the gastrula and larval stages and both genes appeared to have continuous expression domains. Neither of the Wnts is detectable in the invaginated endoderm (where the greatest enrichment occurs for Blimp1, Prdm14, and Bmp2/4 transcripts) nor in the PE [see also 23]. We conclude from this small sampling that at least Wnt 3 and 8 are not directly linked to PE formation, though indirect instruction remains possible especially if they are conferring competency to respond to later specification signals (e.g. Bmp2/4).

Lin28 plays multiple roles in regulating cellular homeostasis at least in part by binding and regulating miRNAs [57]. A well-known target of Lin28 negative regulation is let-7, a miRNA critically involved in developmental regulation in *C. elegans* [58]. Lin28

expression is linked to pluripotency in a variety of animals. For example, *Lin28* is essential for proper PGC development in mice [57]. *Let-7* usually represses *Blimp1*, therefore, the presence of *Lin28* in the germ line suppresses *let-7* and enables *Blimp1* to accumulate and specify germ-line cells. *In sea star* oocytes, *Lin28* transcripts accumulate ubiquitously (Fig. 3) and then become restricted to the vegetal pole of the blastula and then to the archenteron in the early gastrula stage. During late gastrulation, *Lin28* transcripts are restricted to the archenteron within two enriched domains. The first one forms a ring in the upper part of the archenteron and the second domain forms a ring closer to the vegetal pole (Fig. 3, asterisks). *Lin28* transcripts accumulate in the lower part of the forming esophagus of the early larva, and in the stomach, intestine, anus and PE in the late larva, but not in the coelomic pouches. Importantly, a conserved *let-7* miRNA was found in sea urchins [59, 60], although its site of accumulation is not known, nor whether it is present in sea stars.

Assuming *Lin28* functions similarly in sea stars as it does in other organisms the marked enrichment in the endoderm (along with *Blimp1/Prdm1* and *Prdm14*) supports the hypothesis that it may be involved in retaining pluripotency in this tissue early in sea star development. Since *Lin28* transcripts are not present in the PE, either they do not function in germ-line determination, or they have already accomplished their germ-line role (in the endoderm) prior to this time. We hypothesize that the endoderm (invaginated epithelium) may have significant pluripotentiality that is lost in other tissues. We do not see any direct evidence that *Bmp2/4*, *Wnt3*, or *Wnt8* expression patterns correlate with PE formation. However, it is still possible that these signaling pathways may act on precursor PE cells at an earlier stage to confer the competency to respond to later PGC inducing signals. It

is also possible that Bmp signals may act at a distance during PGC specification in this embryo as well.

Germ-line associated genes

We examined the expression patterns of germ-line associated genes to test correlations with PE formation in sea stars. This cohort of genes is associated with sex determination and, potentially, regulation of germ-line factors (Tables 3-5).

CNOT6 is important for PGC development in sea urchins (Swartz *et al.*, submitted). CNOT6 has deadenylase activity and is recruited to mRNAs for widespread mRNA degradation. CNOT6 is down regulated in the PGCs, and thus these cells retain mRNAs for prolonged times. This is especially important considering the reduced transcriptional activity of the PGCs when compared to their neighboring somatic cells. Indeed, in sea urchins, the sMics retain their maternal (as well as microinjected, exogenously generated) mRNAs for many days whereas their neighboring cells turnover the same transcripts within about one day [61, 62]. CNOT6 is present ubiquitously in the sea urchin embryo except for a marked depletion in the sMics, and this selective depletion of CNOT6 mRNA appears to result from nanos expression selectively in the sMics (Swartz *et al.*, submitted). The CNOT6 mRNA has two nanos/pumilio response elements in its 3'UTR that causes degradation of CNOT6 selectively in the sMics in a nanos-dependent fashion. The CNOT6 present in all other cells of the embryo contributes to the egg – embryo transition e.g. a clearing of the general, maternal, and pluripotent egg mRNAs and “freeing” the somatic cells to differentiate. In contrast, the depletion of CNOT6 in the sMics contributes to the retention of these same mRNAs and their pluripotency (Swartz *et al.*, submitted). It is not clear yet if the sea star has cells that effectively retain maternal

mRNAs, so here we determined the expression of CNOT6 to test if such a mechanism may exist. CNOT6 mRNA is present uniformly in the sea star oocytes. By the blastula stage, we detected a depletion of CNOT6 transcripts around the blastopore (Fig. 4, arrows). CNOT6 transcripts are enriched in the ectoderm and archenteron of mid-gastrula stage embryos but a clear region remains within the vegetal pole (Fig. 4, arrow). In late gastrulae and early larval stages, CNOT6 is still expressed in the ectoderm and in the gut but is depleted in the PE and in the coelomic pouches. In the late larval stage, transcripts are enriched in the gut and both the preoral and postoral ciliary bands (Fig. 4, asterisks). We predict that general mRNA retention is greater in the endodermal cells through early larval stages that, once again, emphasizes the endoderm as a tissue retaining its pluripotency.

Gustavus is an E3 ubiquitin ligase that binds Vasa and leads to its degradation. Originally found in *Drosophila* [63], it is now also known to bind and regulate Vasa protein accumulation in the sea urchin. This function is particularly important in sea urchins since Vasa protein is translated in all cells from ubiquitous maternal mRNA but becomes uniquely retained in sMics as a result of Gustavus-mediated Vasa protein turnover in all somatic cells. Since Vasa protein first appears to be ubiquitous in the sea star embryo, and then clears to become enriched in the PE in late larval stages [8], we tested the location of Gustavus mRNA in the sea star. In sea star oocytes, Gustavus transcripts are present ubiquitously (Fig. 4). Gustavus transcripts accumulate in the vegetal pole of the blastula and around the blastopore in early gastrulae (Fig. 4). In the late gastrula, Gustavus transcripts are enriched in two rings; one ring forms at the base of the forming anus (blastopore) and another ring forms at the foregut, leaving a less-dense region in

the midgut, the site where the PE will form (Fig. 4). During early larval stages, Gustavus is expressed in the gut, but not in the coelomic pouches nor in the PE, the sites of greatest Vasa protein accumulation [8]. In late larvae, transcripts remain in the stomach, intestine, anus and ciliary bands (Fig. 4). We hypothesize that in the sea star larval stages, Gustavus activity in the midgut may degrade Vasa protein while the lack of Gustavus activity in the PE and anterior coelomic pouches allows Vasa protein to accumulate selectively in these structures.

Echinoderm SoxE is a transcription factor of the HMG family and is the ortholog of the vertebrate member Sox 9 [64]. Sox9 is involved in sex determination in all vertebrates examined; it is regulated positively by SRY in (male) mammals and is enriched in the somatic cells of the presumptive male gonad due to a positive autoregulatory feedback loop [65, 66]. In other vertebrates, including fish, reptiles, and amphibians, Sox9 may be activated by differing mechanisms, including temperature and other environmental factors and is important for both male and female gene expression leading to sexual dimorphism [31, 67-71]. In sea urchins SoxE is expressed in the left coelomic pouch approximately 50 percent of the time while it has a broader distribution the remaining time [10, 72]. The SoxE transcript in sea stars is present at low levels in oocytes and is difficult to detect at blastula and mid-gastrula stages. A strong enrichment is then seen in the left anterior coelom and a mild enrichment appears at the midgut in late gastrulae (Fig. 4). During early larval development, SoxE transcripts are retained in the most posterior tip of the left anterior coelom (Fig. 4, asterisk), then in the same site of both anterior pouches, and in the PE. The expression of SoxE in sea stars is similar to sea urchins in larval stages since

it accumulates similarly in the left coelomic pouch and shows a bi-modal expression pattern [10].

Ovo is a Zn-finger transcription factor that is important for oogenesis in *Drosophila* and spermatogenesis in mice [73, 74]. In sea urchins, Ovo is expressed ubiquitously in the blastula stage and is enriched in the vegetal pole at the mesenchyme blastula stage, an expression domain similar to Vasa. However, unlike Vasa, Ovo expression is not detected in gastrula and larval stages [10]. In the sea star, Ovo transcripts are not detectable in the oocyte but are detectable ubiquitously at low levels in the blastula stage (Fig. 4). During gastrulation Ovo transcripts accumulate throughout the developing gut. In early larvae Ovo transcripts are enriched throughout the gut and esophagus, and in late larva Ovo transcripts are enriched in the gut and pre-oral and post-oral ciliary bands. Although we did not find any obvious correlation between the expression of Ovo and the formation of the PE, its consistent link to the invaginating epithelium may indicate its involvement in the retention of developmental potentiality leading to PE formation.

Changes in cadherin expression are intimately linked to morphogenetic processes that involve the loss of epithelial character and the delamination of cells from an epithelial sheet [75-77], a character often seen in PGCs [78]. In sea urchin embryos, the cellular movements associated with the ingression of primary mesenchyme cells (PMCs) and convergent-extension of the archenteron involve the dynamic regulation of intercellular adhesion, including the loss of cell adhesion molecules [79, 80]. The sMics of the sea urchin appear to retain their cadherin through development until they reach the tip of the archenteron during gastrulation. Premature depletion of the G-cadherin protein appears to disrupt sMic patterning and expression of several cell-specific markers [13] and

cadherin orthologs appear important for germ cell function in many animals [81, 82]. Therefore we were interested to test the profile of the G-cadherin orthology in this sea star.

The sequence of the sea star ortholog of G-cadherin shows significant sequence identity to all classic cadherins, particularly in the cytoplasmic domain, the region predicted to be involved in catenin binding (Supplemental Fig. 1). G-cadherin mRNA accumulates ubiquitously in oocytes, but is not detectable in blastula and early gastrula stages (Fig. 4). In the late gastrula, G-cadherin transcripts are enriched in two domains; one in the foregut and another in the hindgut (Fig. 4, asterisks). This creates a midgut region with less G-cadherin transcripts, the same location in which new morphogenetic movements lead to PE formation. This profile also overlaps the *Gustavus* mRNA profile and perhaps this region supports the evagination of the epithelium leading to PE formation by decreased cell-cell adhesion. G-cadherin transcripts then decreased overall in the larval stages and became restricted to the mouth opening, stomach, intestine, and anus.

Overall, our analysis of the expression of germ-line associated genes in sea star embryos reveals how mechanisms of PGC biology vary between animals that use different modes of PGC specification. Yet, overall similarities are seen; the RNA degradation machinery such as the deadenylase, CNOT6, may be conserved in its down-regulation in PGCs once they are formed to preserve their inherited transcripts and retain greater developmental plasticity. The ubiquitin pathway and specific E3 ligases (such as *Gustavus*) may be conservatively involved in down-regulating germ cell determinant protein levels outside of the germ cells once they are specified. Transcription factors that

are conserved in somatic sex determination seem to have bi-modal expression patterns and be involved in sex determination regardless of the mode of PGC specification. Finally, changes in adhesion, such as through G-cadherin seem to be linked to germ cell function in many animals.

Left/Right asymmetry molecules

The PE forms only on the left side of the *P. miniata* gut during development and we hypothesize that conserved left/right signaling pathways may be involved in PE formation (and therefore PGC specification). Consequently, we identified and tested the gene expression patterns of signaling molecules involved in left/right asymmetry. Major mechanisms for establishment of the left/right axis in sea urchin and other organisms include complex epigenetic and genetic cascades [83; Table 4]. The initial symmetry-breaking event is not clearly understood, and is probably different between species [84], yet this axial organization still requires a specific, and likely conserved, set of gene activities. For example, the Tgf-beta family member Nodal is expressed on the left side of chordate embryos and is widely used to specify structures on the left side differently than on the right. Nodal function activates additional genes involved in development of the left/right asymmetry, including the additional Tgf-beta factor Lefty, and the homeobox transcription factor Pitx2 [85, 86]. In the sea urchin however, this pathway is reversed and is instead expressed on the right side [72]. Overexpression of Nodal throughout the sea urchin embryo results in developmental repression of the adult rudiment on the left side, and removal of Nodal in the sea urchin results in duplicated rudiments on both the right and left sides suggesting that a main function of Nodal is repression of developmental derivatives of the right coelomic pouch [72, 87-89]. In the sea star, Nodal is not expressed

in oocytes, but accumulates strongly in early blastula stages in the ectoderm (Fig. 5). Nodal mRNA remains transiently in the ectoderm until the late gastrula stage and is absent there in larval stages. A second domain of Nodal expression, however, occurs in mid gastrula embryos when Nodal message accumulates in the midgut and on the right side of the invaginated epithelium. We also see Nodal mRNA transiently within the posterior region of the right coelomic pouch, but to a much lesser extent.

Lefty follows a similar expression pattern as Nodal in the ectoderm. However, Lefty does not accumulate in the archenteron until later in gastrulation when it accumulates in the right side of the archenteron and in the right coelomic pouch (Fig. 5, late gastrula, asterisk). Pitx2 transcripts, although ubiquitous in *P. miniata* oocytes, do not accumulate significantly in early development until the late gastrula stage. Pitx2 transcripts follow a similar expression domains as Nodal and Lefty, albeit much delayed (Fig. 5, late gastrula, asterisk) and this is similar in the closely related sea star *Asterina pectinifera* [90]. Pitx2 is expressed in the posterior portion of the right coelomic pouch which persists from the late gastrula stage until late larval stages, although its accumulation in the right pouch of *A. pectinifera* is much broader than in *P. miniata*. We noticed that in the sea star *P. miniata*, the initial break in left/right asymmetry within the archenteron (as seen with the right-sided expression of Nodal) occurs at the same time that Vasa expression is restricted to the left side of the archenteron.

Therefore, we hypothesize that left/right signaling and Nodal are required for the initial break in left/right asymmetry of Vasa expression and therefore of PE specification. Overall the sea star left/right asymmetry program appears to closely mirror the program in the sea urchin, even with the significant expression in the right side of the archenteron.

Thus, the reversal of the left/right program in echinoderms likely occurred prior to the sea urchin-sea star split; phylogenetic analyses suggests Nodal on the right may be ancestral and that reversal may have occurred in the chordate lineage [72, 87-89]. This also may be related to the reversal of the dorsal/ventral program in chordates relative to non-chordates [91].

Genomic maintenance during morphogenesis and early embryogenesis

The last group of genes we studied encodes molecules related to genomic and epigenomic regulation during morphogenesis to test if these genes are associated selectively with sea star PE cells (Table 5, Fig. 6). Baf250 is an E3 ubiquitin ligase that functions in selective histone (H2B) turnover. Since Baf250 associates with the mammalian SWI/SNF complex it is thought that this histone-turnover machinery regulates epigenetic modifications that lead to selective gene activity [92]. Such modifications are of particular interest in mammalian embryos with the identification of selective epigenetic reprogramming during germ cell formation [7]. In the sea star *P. miniata*, Baf250 transcripts accumulate ubiquitously in oocytes. In blastula stage embryos, Baf250 transcripts become enriched in the blastopore (Fig. 6, asterisk), and in gastrula embryos transcripts accumulate in the archenteron. During larval stages, Baf250 accumulates in the esophagus, stomach, intestine, anus, and in the PE but not in the coelomic pouches.

Recent results in zebrafish suggest that the DNA repair elements Brca1 and Brca2 are involved in germ line development [93]. We find that Brca1 and 2 in the sea star have overlapping expression profiles: Brca1 transcripts are expressed ubiquitously throughout early development until the late gastrula stage when they become enriched in the midgut (Fig. 6). Brca2 transcripts are also present in oocytes and they accumulate in the gut of

late gastrula stage embryos (Fig. 6, asterisk). The accumulation of both Brca transcripts in oocytes suggests there might be a role for the maternal message in provisioning early blastomeres with Brca proteins that could affect DNA repair during the rapid cleavage divisions that occur.

Traffic jam is an atypical basic leucine zipper transcription factor that regulates somatic-germ cell interactions and its loss results in male and female infertility in *Drosophila* [94]. Here we found that transcripts of the ortholog of Traffic Jam, called Maf, are ubiquitously distributed in oocytes but are not enriched in blastula and mid-gastrula stage embryos. Maf transcripts are enriched slightly in the ciliary bands of the larva (Fig. 6, asterisks but not in the PE).

Conclusions

Our results support the contention that the PE is a source of germ line cells in the sea star *P. miniata*. Several gene expression patterns reflect a broad initial expression of germ line markers in the embryo followed by a restriction to the PE. These include Vasa, Nanos, and Piwi (see Fig. 7). While these results on their own do not prove that the PE contains the germ line, they are complementary to other studies that suggest that these cells give rise to sea star germ cells. The PE is most likely the site of germ cell formation in this animal based on four criteria: 1) PE removal experiments result in larvae with significantly less germ cells [9], 2) Vasa immunolabeling experiments show the PE is the first restricted site of Vasa protein localization [8], 3) conserved germ line factors accumulate selectively in the PE *e.g.* nanos and piwi in addition to vasa, and 4) the PE exhibits rapid depletion of mRNAs encoding factors involved in somatic cell fates *e.g.* Blimp1. These observations and the fact that the selective expression in the PE is

relatively late in development – following gastrulation - leads us to reason that the germ line in this organism is determined by inductive interactions amongst cells.

Blimp1 is a somatic factor in echinoderms (as it is necessary for endomesoderm gene regulatory networks, e.g. Spbase.org) and the selective loss of somatic markers in the PGCs represents a broad theme in the inductive mode of germ cell specification (see also Fig. 7). In the mouse inductive mode of germ cell specification, markers of mesoderm, such as Hoxb1 and Hoxa1, are lost from PGCs as soon as they receive PGC inducing signals [39]. The loss of Blimp1 transcripts in the PE of sea stars contributes to the hypothesis that when an animal uses the inductive mode for germ cell specification it is conservatively induced from a pluripotential mesodermal lineage.

A consensus revealed in this study was that the gut appears to harbor a gene set making it retain pluripotency and germ line potential. Many factors, including Vasa, Piwi, Blimp1, and Prdm14, are enriched in the endomesoderm of the gut and suggest the gut retains developmental potential whereas the ectoderm is devoid of many of these same factors. This result may simply mean that the endomesoderm forms later in its differentiation program when compared to the ectoderm, or that the cells within this tissue give rise to many more cell types later in development. The additional possibility is that the endomesoderm is the site of germ line formation and retains developmental potency. The gene expression profile on its own is not convincing and will certainly require functional analysis with metrics of germ line success to understand functionality of the genes tested here. However, when the precursor cells to the PGCs are disrupted in sea urchins Vasa is up-regulated throughout the endoderm of the remaining embryo [11]. In this experimental case within the sea urchin, Vasa mimics the same broad endodermal

expression profile that we see with many pluripotency-related genes in sea stars. Thus, these two echinodermal representatives may have diverged in their germ line determination by transposing the germ line program earlier in sea urchins, and to the sMics. Sea urchins represent a unique model in that this animal retains the ability to use multiple mechanisms for PGC specification upon its disruption [compensatory Vasa upregulation and recovery of germ line cells; 11, 14]. We favor the conclusion that the mechanism in sea urchins is derived and perhaps tending towards an inherited mechanism of germ line determination, especially when compared to the sea star.

Two extreme mechanisms of germ-line determination appear in animal development - inherited vs inductive [1, 31, 95, 96]. Embryos using inherited mechanisms usually establish their germ line early in development by acquisition of a specific region of egg cytoplasm - maternally deposited in the oocyte. This is the best known mechanism and is used by many model organisms e.g. fly, worm, frog, and zebrafish. Mice are the best studied organism that uses inductive mechanisms of germ-line determination. In this mechanism, cell interactions are responsible, usually later in development, to establish a germ line lineage. Results presented here support the contention that the sea star PE is a site of germ line formation and the evidence suggests this structure may fit better with the criteria of an inductive mechanism. This means functional studies that determine signaling networks required for PGC specification in this organism may complement the genetic and tissue culture approaches used in mice to reveal inductive germ-line determination mechanisms. Furthermore, comparisons between the differing mechanisms of PGC specification between sea star and sea urchins will be useful to understand transitions that occur in the evolution from one mode to another.

Materials and Methods

Animals and embryo culture

Patiria miniata were collected from several sites in southern California [www.scbiomarine.com; phalmay@earthlink.net] and embryos were grown basically as described [97]. Briefly, sperm were collected from a gonad biopsy and placed into a microfuge tube on ice. Oocytes were collected from a gonad biopsy and matured in vitro with 2 μ M 1-Methyl-Adenine. Resultant eggs were fertilized with a dilute sperm suspension and embryos were cultured as previously described [98]. Samples from different developmental stages (oocytes; hatched blastula, 18.5 hours post-fertilization (hpf); mid-gastrula, 27.5 hpf; late gastrula, 47 hpf; early larva, 3 days post-fertilization (dpf); late larva, 4-7 dpf) were collected, fixed and stored in 70% ethanol at -20 as described [99].

RNA analysis

Whole mount *in situ* RNA hybridizations were performed using digoxigenin-labeled RNA probes as previously described [99]. cDNAs from oocytes and early development stages were used as templates for PCR reactions. Primers designed to amplify each gene of interest included a T7 RNA polymerase sequence in the 5' end of reverse primers. The resultant PCR products were used as templates for transcription by T7 RNA polymerase to yield an antisense RNA probe with the DIG RNA Labeling Kit (SP6/T7) (Roche Applied Science, IN, USA). Oocytes and embryos were fixed, hybridized, and the signals were detected essentially as described [99]. Negative controls for these experiments included the use of a non-relevant transcript probe (Neomycin). Oocytes and embryos were visualized on a Zeiss Axioplan microscope, and the specimens are oriented with their left side to the left i.e. we position the larva ventral side down, left side of the larva to the left

of the image. This is the opposite of the classic human orientation scheme but we think it makes the structures easier to interpret.

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Figures and Tables

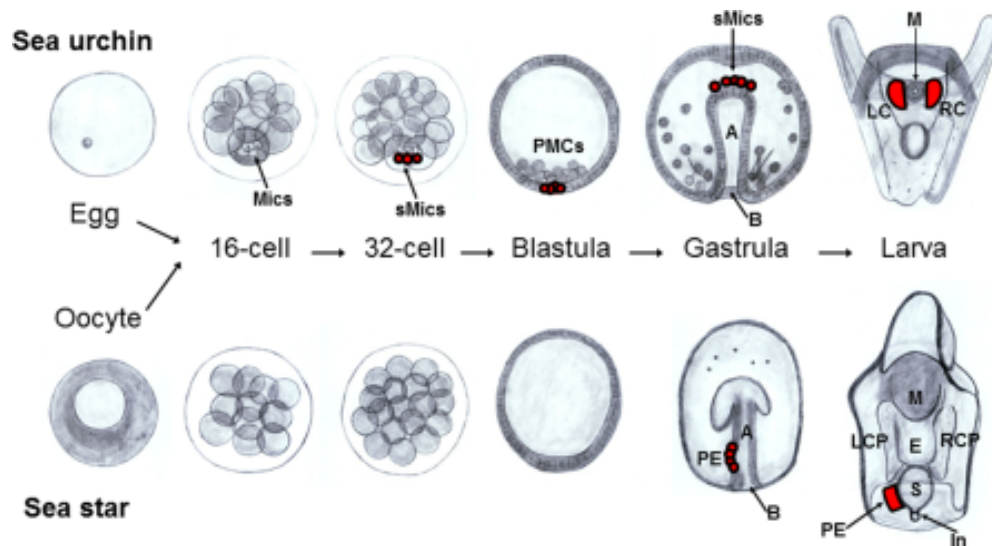


Figure 1- Schematic representation of the developmental stages in sea star and sea urchin

Sea urchins exhibit two asymmetric cleavage events within the cells at the vegetal pole (bottom). These asymmetric divisions result in 4 micromeres forming at the 16-cell stage, followed by 4 large and 4 small micromeres (sMics, in red) forming at the 32-cell stage. The PMC (descendants of the large micromeres) ingress to form the skeleton, whereas descendants of the sMics remain relatively quiescent through embryogenesis, divide only once before gastrulation, and then integrate into the larval coelomic pouches where the adult rudiment forms. Unlike in sea urchins, the sea star has symmetrical cell divisions and does not segregate its germ-line cells during early development. Instead, a PE (in red) projects from the dorsal wall of the archenteron into the blastocoel and then moves to the left side in late gastrula-early larval stages. Left and right anterior (top) coelomic pouches are present on both sides of the esophagus in larvae, and they subsequently extend posteriorly. Later in development, the left coelomic pouch integrates cells of the PE as it extends posteriorly. The blastopore is located at the lower opening of each embryo in the gastrula stage. Mics=Micromeres, sMics=Small micromeres, PMCs=Primary mesenchyme cells, LCP=Left coelomic pouch, RCP=Right coelomic pouch, PE=Posterior enterocoel, LC=Left coelom, RC=Right coelom, M=Mouth, E=Esophagus, S=Stomach, In=Intestine, B=Blastopore, A=Archenteron.

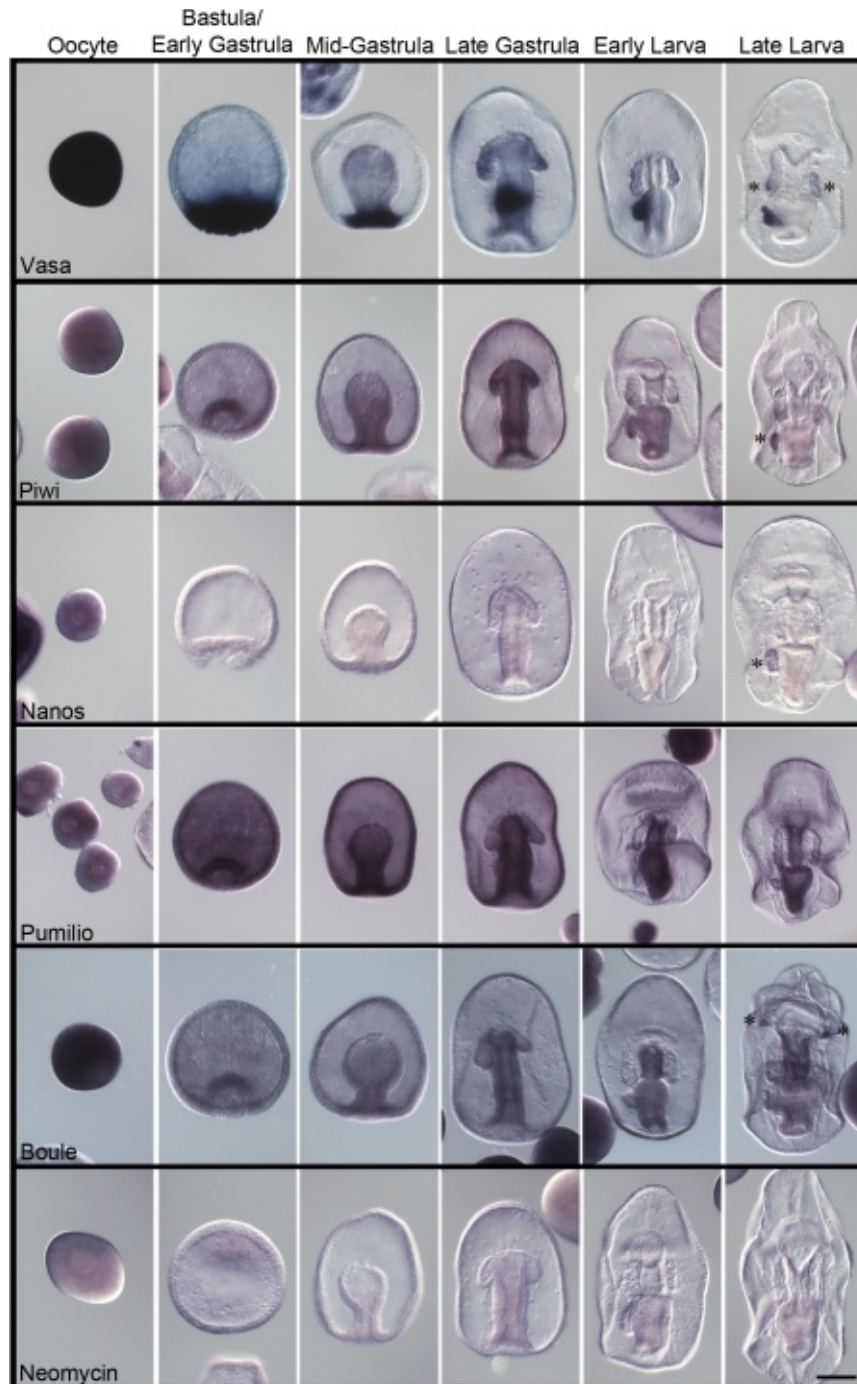


Figure 2- Expression of conserved germ-line determinants during *P. miniata* embryonic development

Line 1) Vasa transcripts are widespread in oocytes and become restricted to the vegetal pole in blastula stage embryos. During gastrulation Vasa is then restricted to the center of the archenteron and later to the left side of the archenteron. As soon as the PE is formed Vasa persists in the PE. **Line 2) Piwi** transcripts are widespread in oocytes, become restricted to the archenteron during gastrulation, then to the center of the

archenteron during late gastrulation, and persist in the PE of larva as soon as it is formed. **Line 3) Nanos** expression is only detected broadly in immature oocytes and in the PE of older larva. **Line 4) Pumilio** transcripts accumulate broadly throughout the embryo during early development and become enriched in the esophagus and stomach during larval stages. **Line 5) Boule** transcripts are widespread in oocytes but are not present during development until larval stages. Boule transcripts accumulate in the oral ectoderm/ciliary band and in the esophagus. A sequence for the **Neomycin (Neo) resistance gene (Line 6)** was used as a negative control for the hybridization procedure. Asterisks (*) represent areas of emphasis for mRNA accumulation. Dorsal views of the larva. Scale bar represents 100 μm .

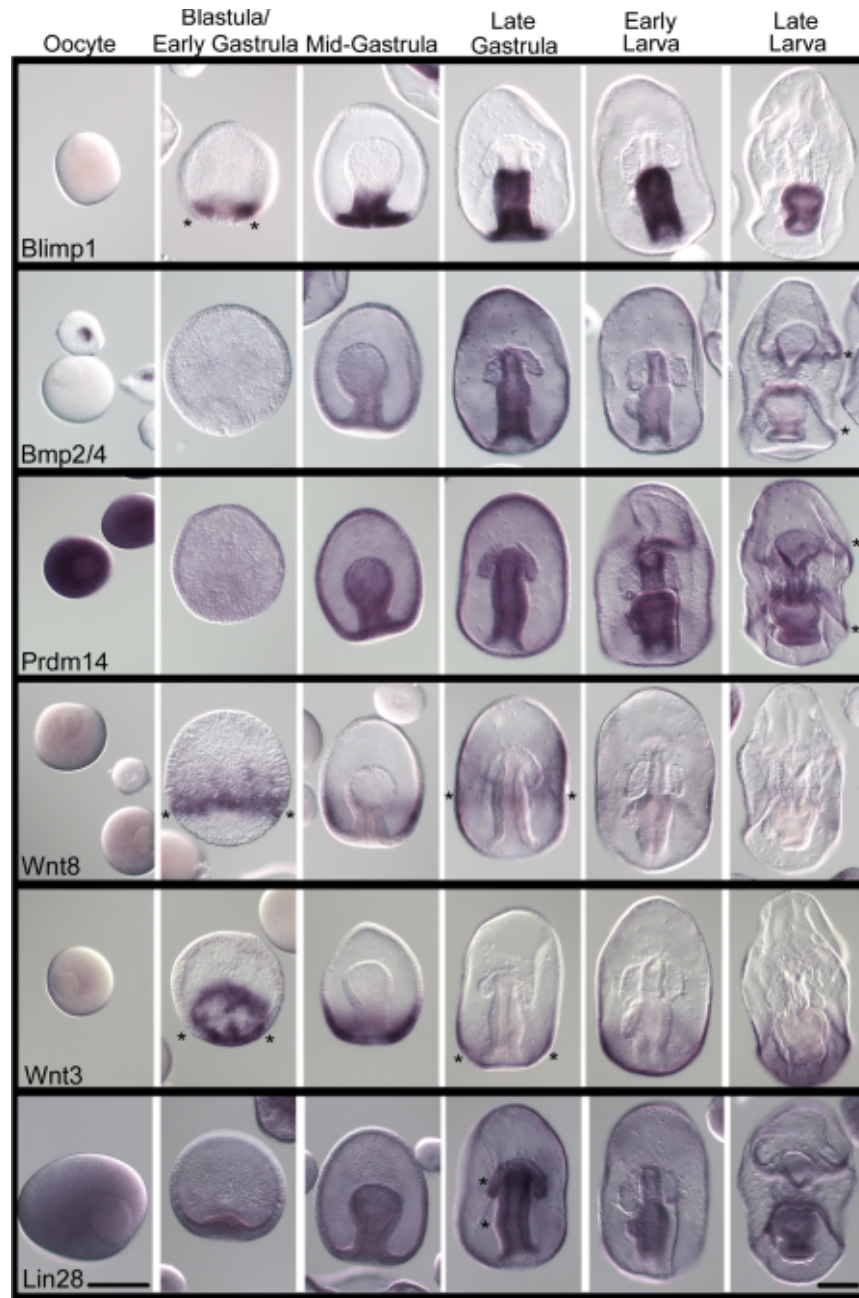


Figure 3- Expression of genes involved in inductive germ-line specification during *P. miniata* embryonic development

In situ hybridization showing **Line 1) Blimp1** transcripts, which first accumulate in the vegetal pole of the blastula, and remain largely endodermal through development. Of note is the rapid loss of Blimp1 in the newly formed PE (see also Figure 7). **Line 2) Bmp2/4** transcripts are seen in young oocyte germinal vesicles (oocyte nucleus). During development Bmp2/4 is expressed throughout the embryo except for in the coelomic pouches and PE. **Line 3) Prdm14** is present throughout development and largely throughout the embryo with the exception of the coelomic pouches. **Lines 4 and 5) Wnt 8 and Wnt 3** (respectively) form a complementary pattern with Wnt3 being more vegetal

and Wnt 8 more equatorial in the embryo and larva. No selective accumulation is seen in the coelomic pouches or PE in larval stages. **Line 6) Lin28** is expressed broadly during early development until the late gastrula stage when it becomes enriched in the foregut. In larval stages, Lin28 is enriched in the gut and PE and it is distinctly absent from the coelomic pouches. Asterisks (*) show areas of emphasis for transcript detection. Dorsal views of the larva. Scale bar represents 100µm.

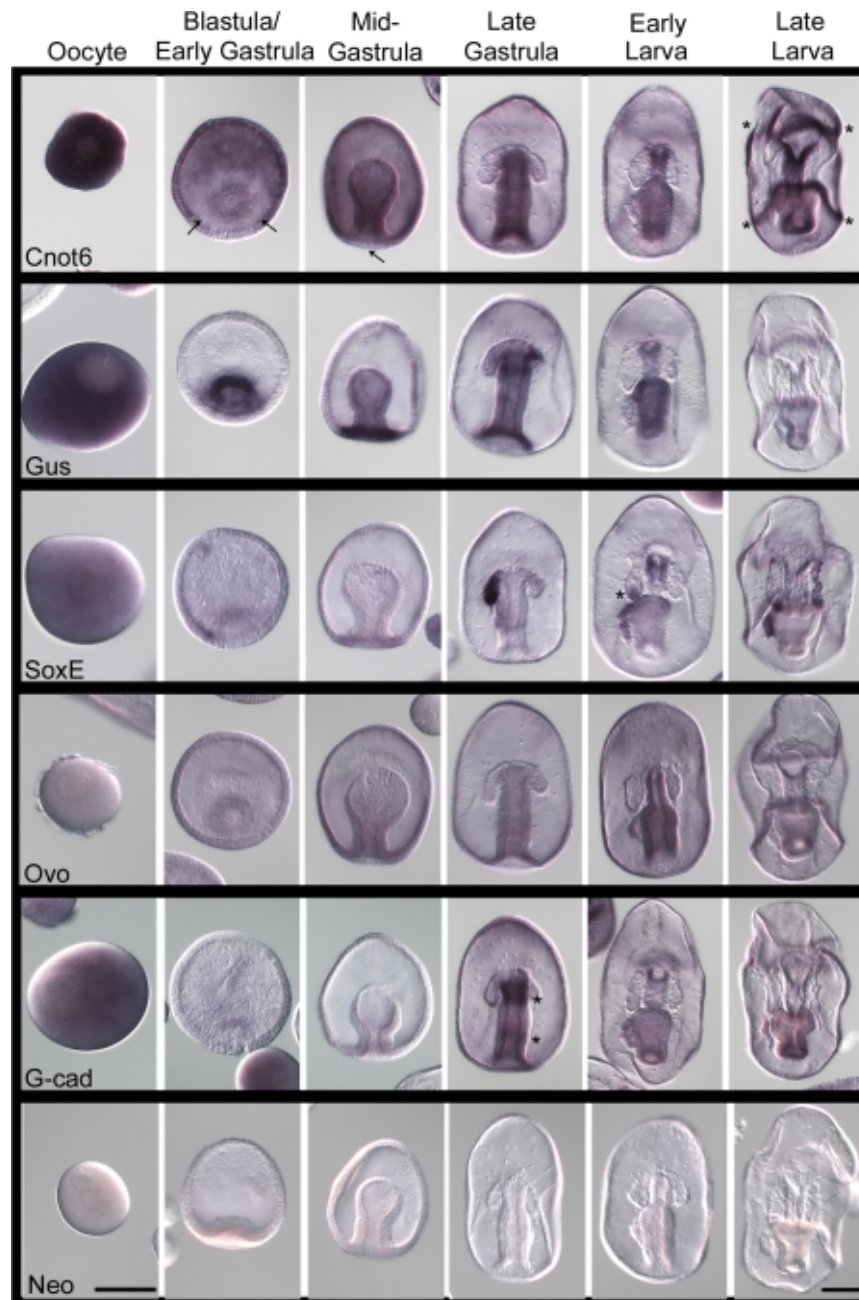


Figure 4- Expression of germ-line associated genes during *P. miniata* embryonic development

Line 1) Cnot6 is nearly uniform throughout development and is absent from the vegetal-most region of the embryo, the coelomic pouches, and the PE as well as the ciliary band in larvae. **Line 2) Gustavus (Gus)** is enriched in the developing mesoderm in early development. Gus transcripts become enriched in the mouth and stomach in larval stages. **Line 3) SoxE** (the Sox 9/10 ortholog) is largely absent from the early embryo but accumulates significantly in the left coelomic pouch following gastrulation. SoxE

transcripts then accumulate in the PE and the tips of both coelomic pouches during larval stages. **Line 4) Ovo** is present in the gut of the embryo and larva, with no significant enrichment in the pouches or the PE. **Line 5) G-cadherin** accumulates in the gut during the late gastrula stage in distinct bands in the foregut and midgut regions. In larval stages G-cadherin becomes enriched in the mouth and stomach. A sequence for the **Neomycin (Neo) resistance gene (Line 6)** was used as a negative control for the hybridization procedure. Asterisks (*) show areas show areas of emphasis for transcript detection. Arrows emphasize notable areas of transcript depletion. Dorsal views of the larva. Scale bar represents 100 μm .

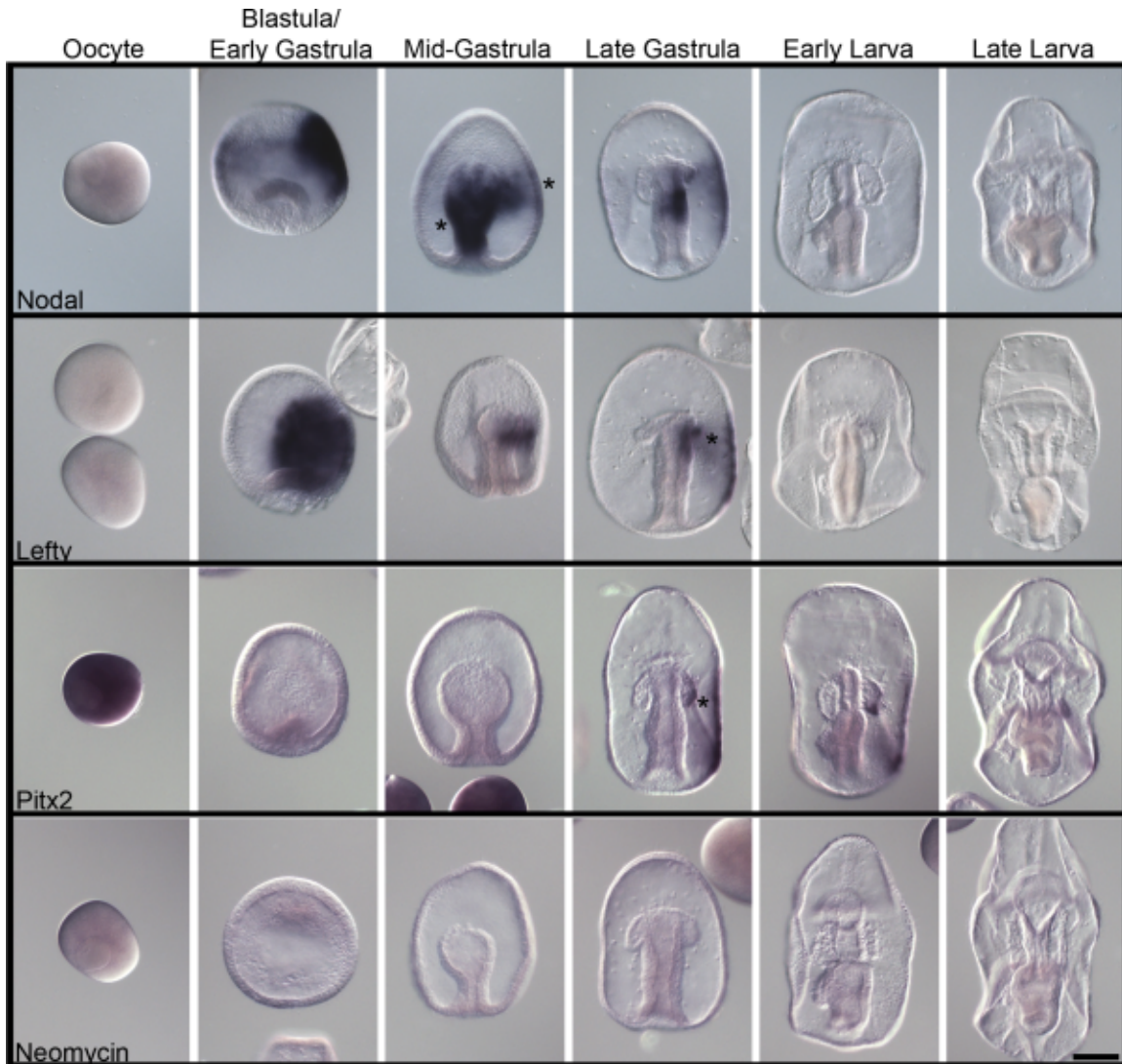


Figure 5- Expression of left/right asymmetry markers during *P. miniata* embryonic development

Line 1) Nodal is first expressed in the ectoderm at the blastula stage. A second expression domain of Nodal appears in mid-gastrula stage embryos when it is expressed symmetrically in the developing archenteron. Nodal expression becomes restricted to the right side of the archenteron in late-gastrula stage embryos. **Line 2) Lefty** is first expressed in the ectoderm at the blastula stage. A second expression domain of Lefty appears in late-gastrula stage embryos when it is expressed asymmetrically in the right side of the archenteron. **Line 3) Pitx2** is first expressed ubiquitously in oocytes. Pitx2 expression localizes to the right coelomic pouch and right ectoderm in late gastrula staged embryos stage. Pitx2 expression in the right coelomic pouch and right ectoderm persists through late larval stages. A sequence for the **Neomycin (Neo) resistance gene (Line 4)** was used as a negative control for the hybridization procedure. Asterisks (*) show areas of emphasis for transcript detection. Dorsal views of the larva. Scale bar represents 100 μ m.

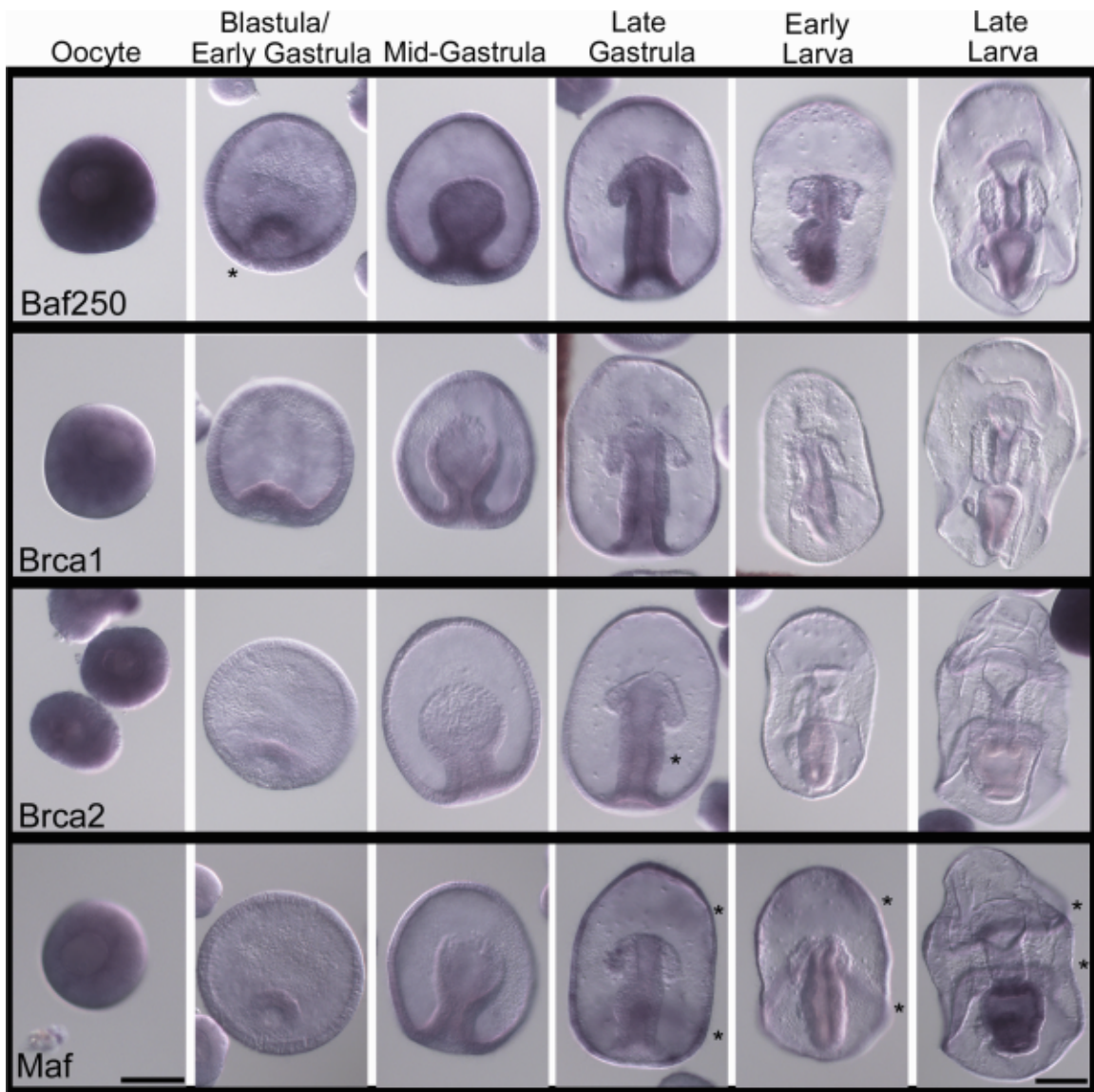


Figure 6- Molecules involved in genomic regulation and maintenance during *P. miniata* embryonic development

Line 1) Baf250 is expressed ubiquitously in the embryo throughout development with some enrichment in the gut and depletion in the coelomic pouches. **Lines 2 and 3) Brca1 and 2** are enriched in the gut of late gastrula embryos and we note no significant accumulation in the PE. **Line 4) Maf** is most apparent in the stomach of the larva and no specific enrichment in the PE. Asterisks (*) show areas of emphasis for transcript detection. Dorsal views of the larva. Scale bar represents 100 μ m.

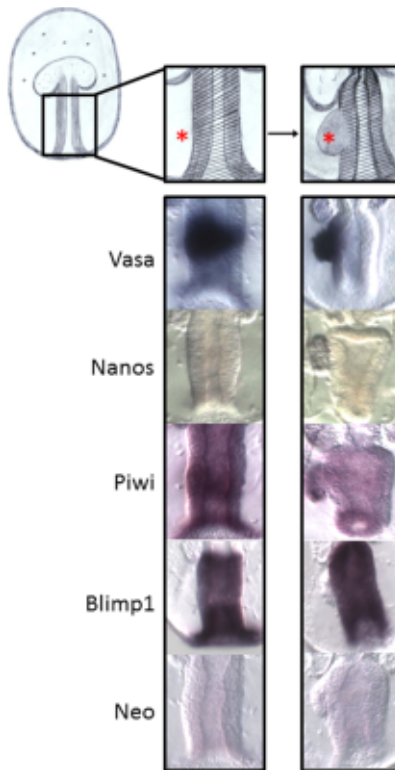


Figure 7- Transcript dynamics during posterior enterocoel formation

The conserved germ-line determinant, Nanos, accumulates specifically in the PE (red asterisk) after formation. During gastrulation, the conserved germ-line determinants Vasa and Piwi, become enriched in the midgut (shadowed area). As soon as the PE is formed Vasa and Piwi transcripts start to become restricted to the PE and to clear from the nearby stomach. This is in stark contrast to the somatic cell marker, Blimp1. During gastrulation, Blimp1 transcripts similarly become enriched in the midgut, however, as soon as the PE is formed, Blimp1 transcripts are restricted to the stomach and clear from the nearby PE.

Genes	Domains and Function	Referen ce	Orthologs (organism)	%Identities, % Similarities	NCBI or Spbase Reference number	Pm transcript number	Primers sequences	Size of amplify product
Piwi	PAZ (piwi, argonaute, zwiile) domain, required for negative regulation of transposable elements in germ cells	Juliano <i>et al.</i> , 2006	Mm-Piwi1L Dm-Argonaute 3, isoform G Sp-Seawi	51%, 74% 40%, 59% 50%, 71%	NP_067286.1 NP_001163498.1 AAG42534.1	Pm_33095	F:CGACGGCAGCCAGATC ACCTA R:taatacgactcactatagggCCA GGCAGCAGTACTTCTTGA	728 bp
Nanos	CCHC Zn- finger domain, With partner, Pumilio, serves as a negative regulator of translation in germ cells	Juliano <i>et al.</i> , 2006 Lai <i>et al.</i> , 2013.	Mm-Nanos2 Dm-Nanos Sp-Nanos2	54%, 65% 62%, 73% 39%, 50%	NP_918953.2 NP_476658.1 NP_001073023.1	Pm_4079	F:GGAGATTGAGAGCGAA GAT R:taatacgactcactatagggTGT TGAATTCATGAGGCAAA	971 bp
Vasa	CCHC Zn-finger and DEAD-box domains, ATP-dependent RNA helicase involved in germ-line specification	Juliano <i>et al.</i> , 2006	Mm-Vasa Dm-Vasa Sp-Vasa	61%, 76% 53%, 69% 69%, 82%	NP_034159.1 NP_723899.1 NP_0011396 65.1	Pm_1519	F:CGGTCCAGAAGTACGG GATA R:taatacgactcactatagggGTA GAAGCTGGTTGCCTTGC	992 bp
Pumilio	Puf domain, With partner Nanos, RNA-binding protein that negatively regulates translation of target RNA in germ cells	Lai <i>et al.</i> , 2013.	Mm-Pum2 Dm-PumF Sp-Pum	55%, 65% 79%, 88% 65%, 71%	NP_109648.2 NP_001247002.1 SPU_006847	Pm_2787	F:GGTAGTAACATGGGGG ACCAG R:taatacgactcactatagggGG CCTTGTTGTTGACCTTGC T	805 bp
Boule/ Dazl	RNA binding protein involved in germ-line determination in mammals	Xu <i>et al.</i> , 2001. Shah <i>et al.</i> , 2010.	Mm-Boule Dm-Boule Sp-Boule	59%, 71% 51%, 62% 37%, 46%	NP_083543.2 NP_729457.1 SPU_008194.1	Pm_22341	F:TCGGTTCATAACTGCCA TCA R:taatacgactcactatagggTTA TGGCACCTGGTGAGAG	925 bp

Table 1- Conserved germ-line determinants

Genes	Domains and Function	Reference	Orthologs (organism)	%Identities, % Similarities	NCBI or Spbase Reference number	Pm transcript number	Primers sequence	Size of amplify product
Blimp1/ Prdm1	Transcription factor involved in germ line determination in mice	Saitou <i>et al.</i> , 2010. Kurimoto <i>et al.</i> , 2008.	Mm-Prdm1 Dm-Blimp1 Sp-Blimp1/Krox1b	78%, 87% 67%, 79% 43%, 55%	AAI29802.1 NP_647982.1 NP_001073021.1	Pm_43022 (AAP35029.1)	F:CCATTCTCCGTACTCGTG GT R:taatacgactcactatagggCGG AAGTCTGTGCATGAGAA	912 bp
Prdm1 4	PR domain Zn-finger, transcriptional regulator involved in germ line determination in mice	Saitou <i>et al.</i> , 2010. Yamaji <i>et al.</i> , 2008	Mm-Prdm14 Dm-Prdm14 Sp-Prdm14	87%, 94% 37%, 55%** 94%, 97%	NP_001074678.2 XP_794184.3	Pm_65577	F:AACCGCTCTTCCGATCTG T R:taatacgactcactatagggGTGT GACGCGAAGGCTTTT	402 bp
BMP2/4	TGF-beta ligand signaling molecule. Critical for induction of PGCs in mice	Saitou <i>et al.</i> , 2010. Saitou, 2009.	Mm-BMP2 Dm-Decapentaplegi c, isoformA Sp-BMP 2/4	45%, 61% 40%, 55% 50%, 65%	NP_031579.2 NP_477311.1 SPU_021497	Pm_4348	F:CGTGCCACAGTACATGC TGGA R:taatacgactcactatagggGCTC GCTGACAGACCGAGCTA	590 bp
Lin28	Cold shock and a cluster of two CCHC Zn-finger domains, RNA binding protein that is required for PGC development	Bussing <i>et al.</i> 2008. West <i>et al.</i> 2009.	Mm-Lin28B Dm-Lin28 Sp-Lin28	53%, 61% 55%, 75% 64%, 80%	NP_001026942.1 NP_647983.1 SPU_027195	Pm_90489	F:GGCCGACGAGGGCAAG CTGTG R:taatacgactcactatagggGGC CAGTCACCGACTCCGCCT	215 bp
Wnt3	Wnt ligand signaling molecule. Involved in germ line competency in mice.	Saitou <i>et al.</i> , 2010. Ohinata <i>et al.</i> , 2009.	Mm-Wnt3 Dm-Wnt2 Sp-Wnt3	53%, 69% 43%, 57% 61%, 73%	NP_033547.1 NP_476810.1 XP_790595.2	Combo of: HP125189.1 and cloning	F:TAAATTCATCAGCCCCAA GG R:taatacgactcactatagggATGG CTTCGTTCTTGAATGC	975 bp
Wnt8	Wnt ligand signaling molecule. Correlates with localization of PGC's in axolotl.	Bachvarova <i>et al.</i> , 2001. Johnson <i>et al.</i> , 2003.	Mm-Wnt8b Dm-Wingless, isoform B Sp-Wnt8	59%, 73% 40%, 58% 48%, 67%	NP_035850.2 NP_723268.1 NP_999832.1	Pm_82262	F:GCAGCGACAACATCAAA TTCCG R:taatacgactcactatagggGCTC TTCCGATCTGACGGCTG	365 bp

Table 2- Genes involved in the inductive mechanisms of germ-line specification

Genes	Domains and Function	Reference	Orthologs (organism)	%Identities, % Similarities	NCBI or Spbase Reference number	Pm transcript number	Primers sequence	Size of amplify product
SoxE	HMG box, transcription factor involved in sex determination.	Juliano <i>et al.</i> , 2006	Mm-Sox9 Dm-Sox100B Sp-SoxE	77%, 90% 61%, 79% 82%, 89%	NP_035578.3 NP_651839.1 SPU_016881	Pm_60849	F:CGTCTATTTCGCGACGCC GTGT R:taatacgactcactatagggGCTT CTTCCTTGGGTGTGGTC	496 bp
Ovo	C2H2 Zn-finger domain, transcription factor involved in Drosophila oogenesis and mouse spermatogenesis	Dai <i>et al.</i> , 1998	Mm-Ovo2L, isoform A Dm-Ovo, isoform A Sp-Ovo	59%, 71% 69%, 72% 53%, 66%	NP_081200.2 NP_525077.2 SPU_012448	Pm_17822	F:CCACAGACGACACACAT CTCA R:taatacgactcactatagggGTGA AGGCCTTGCTGCAATAC	503 bp
Gustavus	SPRY and SOCS box domains, E3 ubiquitin ligase specific receptor involved in the regulatory balance of Vasa ubiquitylation.	Styhler <i>et al.</i> , 2002 Gustafson <i>et al.</i> , 2011	Mm-Gustavus Dm-Gustavus isoform G Sp-Gustavus	62%, 78% 65%, 79% 74%, 90%	NP_083311.1 NP_001246140.1 SPU_004717	Pm_19970	F:GGAGGATCTTCGGAGCG GTAG TGCC R:taatacgactcactatagggGGC AGTGGTAGCTGGTAGATGT CT T	804 bp
Cnot6	EEP domain, deadenylase involved in mRNA decay	Swartz <i>et al.</i> , 2013. Wahle <i>et al.</i> , 2013.	Mm-Cnot6 Dm-TwinB Sp-Cnot6	60%, 77% 59%, 76% 66%, 78%	NP_997649.1 NP_732967.1 XP_779942.3	Pm_19426	F:CTGGACCTATCGGCGAA TAA R:taatacgactcactatagggTGAT GGTCTGGATCAGCTTG	877 bp
G-Cadherin	Laminin G, Ca ²⁺ -binding EGF-like, and Cadherin tandem repeat domains, cell-cell adhesion molecule	Yajima and Wessel, 2012.	Mm-Fat tumor suppressor 1 Dm-NCad isoform G Sp-GCad	26%, 43% 34%, 51% 39%, 54%	NP_001074755.2 AAN10997.1 SPU_010840	Pm_6651	F:CGACAAGTTCAGGCTAG ACTC R:taatacgactcactatagggGTGA CGACAATGTGCATGGTG	690 bp

Table 3- Germ-line associated genes

Genes	Domains and Function	Reference	Orthologs (organism)	%Identities, % Similarities	NCBI or Spbase Reference number	Pm transcript number	Primers sequence	Size of amplify product
Nodal	Tgf-beta ligand Involved in left-right asymmetry	Grande <i>et al.</i> , 2009.	Mm-Nodal Dm-Dpp Sp-Nodal	51%, 74% 42%, 62% 53%, 69%	NP_038639 NP_477311.1 ABK33664.1	gil30705207 3 gb HP126 404.1 isotig21602. Pminagast	F:CGGTGGATCGTCTACCC TAA R:taatacgactcactatagggCCC GATCAAATTGTAAAAATGC	966 bp
Lefty	Tgf-beta signaling molecule, involved in left-right asymmetry	Grande <i>et al.</i> , 2009.	Mm-Lefty Dm-Dpp Sp-Lefty	24%, 40% 21%, 36% 28%, 46%	NP_034224.1 NP_477311.1 NP_001123281.1	lcl scaffold5 11856 75.8	F:ATGGAGTCTCGCGTAGC TGT R:taatacgactcactatagggCATG TTTGTTGACGGGTCTG	537 bp
Pitx2	Homeobox domain, transcription factor involved in left-right asymmetry	Grande <i>et al.</i> , 2009.	Mm-Pitx2 Dm-Ptx1 Sp-Pitx2	74%, 82% 60%, 65% 73%, 82%	NP_001035969.1 NP_733410.2 SPU_004599	Pm_22862	F:GCGTCAGGGTGTGGTTT AAG R:taatacgactcactatagggGTTC AAGTTCTGGTGGCTCA	329

Table 4- Left-Right asymmetry molecules

Genes	Domains and Function	Reference	Orthologs (organism)	%Identities, % Similarities	NCBI or Spbase Reference number	Pm transcript number	Primers sequence	Size of amplify product
Brca1	N-terminus RING finger domain, two nuclear localization signals and an acidic C-terminus. It is involved in DNA repair, transcriptional regulation, chromatin remodeling, cellular growth control and genome stability	Hoshino <i>et al.</i> , 2007	Mm-Brca1 Sp-Brca1	34%, 55% 62%, 75%	NP_033894.3 SPU_011027.3a	Pm_3175	F:GGATCTTCCCAGAGTAC GACT R:taatacgactcactatagggGGA GCCAAGAGTTGTCAGAGT	822 bp
Brca2	N-terminus acidic transcriptional activation domain and a C-terminus DNA binding domain. It is involved in maintenance of genomic stability in response to DNA damaging agents	Rodríguez-Marí <i>et al.</i> , 2011	Mm-Brca2 Sp-Brca2	36%, 57% 42%, 62%	NP_001074470.1 SPU_013435	Pm_34269	F:GGAGAAGCACAGCGAGG GAGG R:taatacgactcactatagggGGC TGGAACCCAGCCTGAAGA	729 bp
Baf250	It has a DNA binding domain called ARID. It is a component of catalytic cores that regulate the expression of Homeobox genes early in development	Li <i>et al.</i> , 2010	Mm-Arid1B Dm-OsaA Sp-Baf250	43%, 69% 38%, 52% 75%, 88%	NP_001078824.1 NP_732263.1 SPU_023530	Pm_47085	F:CCAGTGTGCATGCCCTC AGTA R:taatacgactcactatagggCCTC TCTCCTTAAGTGCATGG	506 bp
Maf	Traffic Jam/Maf is a transcription factor that controls gonad morphogenesis. TJ protein activates piwi expression and tj gene is a piRNA cluster which define the Piwi targets for silencing, in Drosophila	Saito <i>et al.</i> , 2009	Mm-MafB Dm-Tjam Sp-Maf	51%, 72% 57%, 72% 61%, 80%	NP_034788.1 NP_609969.2 SPU_025888	Pm_71778	F:CCAAGCCTTGATGAGCT CTAT R:taatacgactcactatagggGGA CTACTCGGCAAACTAACG	629 bp

Table 5- Regulation and genomic maintenance during morphogenesis and early embryogenesis

Chapter 3: The Diversity of Nanos Expression in Echinoderm Embryos Supports Different Mechanisms in Germ Cell Specification

Fresques T, Swartz Z, Juliano C, Morino Y, Kikuchi M, Akasaka K, Wada H, Yajima M, Wessel GM (2016). The Diversity of Nanos Expression in Echinoderm Embryos Supports Different Mechanisms in Germ Cell Specification. *Evolution & Development* Jul;18(4):267-78.

I performed the experiments that contributed to Figures 1, 2, 3, and 6. I also wrote the manuscript with guidance from G. Wessel.

Abstract

Specification of the germ cell lineage is required for sexual reproduction in all animals. However, the timing and mechanisms of germ cell specification is remarkably diverse in animal development. Echinoderms, such as sea urchins and sea stars, are excellent model systems to study the molecular and cellular mechanisms that contribute to germ cell specification. In several echinoderm embryos tested, the germ cell factor Vasa accumulates broadly during early development and is restricted after gastrulation to cells that contribute to the germ cell lineage. In the sea urchin, however, the germ cell factor Vasa is restricted to a specific lineage by the 32-cell stage. We therefore hypothesized that the germ cell specification program in the sea urchin/Euechinoid lineage has evolved to an earlier developmental time point. To test this hypothesis we determined the expression pattern of a second germ cell factor, Nanos, in four out of five extant echinoderm clades. Here we find that Nanos mRNA does not accumulate until the blastula stage or later during the development of all other echinoderm embryos except those that belong to the Echinoid lineage. Instead, Nanos is expressed in a restricted domain at the 32-128 cell stage in Echinoid embryos. Our results support the model that the germ cell specification program underwent a heterochronic shift in the Echinoid lineage. A comparison of Echinoid and non-Echinoid germ cell specification mechanisms will contribute to our understanding of how these mechanisms have changed during animal evolution.

Introduction

Eggs and sperm are essential for the reproduction of most animals. Thus, the **germ cell lineage**, any cell that retains the potential to give rise to an egg or sperm, is essential for animal development. **Germ cell specification** is the process when the germ cell lineage set aside from the rest of the somatic cells. Even though the germ cell lineage is a conserved requirement for sexual reproduction, there is not one common germ cell specification mechanism. Instead, studies of animal development reveal multiple mechanisms for germ cell specification that can belong to two major groups: inherited and inductive (also referred to as preformation and epigenesis) [1].

An **inherited mechanism** of germ cell specification occurs relatively early in development. A defining characteristic of this mechanism is the early localization of maternally supplied germ cell determinant molecules (RNAs and proteins) in a restricted domain of the egg or early embryo. As a consequence of cellular division, whichever cells inherit these germ cell determinant molecules are instructed to take on a germ cell lineage fate. The other cells that did not receive these germ cell determinant molecules instead will become somatic lineages. Early development in animals such as fruit flies, nematode worms, frogs, and zebrafish is characteristic of the inherited mechanism of germ cell specification [2-7]. However, many other animals use a different cellular mechanism for germ cell specification.

An **inductive mechanism** of germ cell specification occurs relatively later in development. A defining characteristic of this mechanism is that maternally supplied germ cell determinant molecules (RNA's and proteins) either do not accumulate during early development or only accumulate in large embryonic domains in early development. As a consequence, embryonic transcription, cell signaling, and cell

interactions instruct which cells will become the germ cell lineage and which cells will become the somatic cell lineage. Early development in animals such as crickets, salamanders, and mice is more characteristic of the inductive mechanism of germ cell specification [8-10].

Despite the similarities that appear in germ cell specification mechanisms that allow categorization into two general mechanisms, it is clear that one if not both of these mechanisms have independently evolved many times throughout animal phylogeny. For example, within Chordates both mammals and salamanders use an inductive mechanism of germ cell specification whereas zebrafish and frogs use the inherited mechanism of germ cell specification (Supplement Figure 1). Similarly, within the protostomes both worms and fruit flies use the inherited mechanism of germ cell specification whereas crickets use the inductive mechanism of germ cell specification (Supplement Figure 1). Recent studies have aimed to understand how these different germ cell specification mechanisms have evolved so frequently in closely related organisms [11]. However, in order to address this evolutionary question thoroughly, it is important to understand how germ cells are specified in diverse animal lineages.

Echinoderms are an informative group of animals to study the evolution of germ cell specification mechanisms for three reasons. **First**, echinoderm embryos are convenient models to study the evolution of early developmental processes. Embryos from each extant echinoderm clade are accessible and amenable for experimentation in early development. For example, they are large, optically clear, develop externally, and are mass-produced (on the order of hundreds of thousands). In addition, a variety of molecular tools are available for gene perturbation in the majority of echinoderm

embryos. **Second**, previous studies suggest that different species within the echinoderm clade use different mechanisms for germ cell specification. **Third**, echinoderms occupy an evolutionarily important position within animal phylogeny because echinoderms and Chordates are sister groups that belong to the Deuterostome lineage (Supplemental Figure 1). Therefore, comparisons between Chordates and echinoderms are essential to understand ancestral Deuterostome mechanisms of germ cell specification. For these reasons the echinoderm clade is a great model to study the evolution of germ cell specification mechanisms.

Previous studies of germ cell specification mechanisms in echinoderms have largely focused on the localization of the germ cell marker Vasa (Figure 1A) [12-14]. Vasa is an RNA helicase that is required to specify and/or maintain the germ cell lineage fate in many animals [15]. Vasa accumulates broadly during the early development of almost all echinoderm species tested (Figure 1A, light blue, dark blue, 32-cell stage) and is restricted to the coeloms by the larva stage (Figure 1A, light blue, dark blue, larva stage). These results have led previous authors to hypothesize that in most echinoderm species germ cell specification occurs later in development by inductive mechanisms [16]. By contrast, Vasa protein is restricted to a specific lineage early during sea urchin development, by the 32-cell stage. In fact, this specific lineage is an evolutionary derived characteristic that only occurs in the Euechinoid group of echinoderms. These data led to the hypothesis that during the evolution of the Euechinoid lineage there has been a shift for germ cell specification to occur earlier in development by inherited mechanisms (Figure 1A, dark blue, 32-cell stage, sea urchin) [16]. It is important to note that Vasa has other functions during the early development

of some animals that are independent of germ cell specification [17-20]. Therefore, in order to rigorously test these hypotheses, it is necessary to determine where other germ cell markers accumulate during the development of diverse echinoderm species.

Here, we test the localization of the germ cell marker Nanos during the embryonic development of several echinoderm species. Nanos is an RNA binding Zinc finger protein that is required for the specification and/or maintenance of the germ cell lineage in many animals [21-24]. In the sea urchin, Nanos is a more selective marker of the germ cell lineage as both the mRNA and protein is restricted to a small number of embryonic cells at the 32-cell stage (Figure 1A, purple, 32-cell stage, sea urchin) [25-27]. This is at a developmental time when Vasa mRNA accumulates in every cell of the sea urchin embryo (Figure 1A, light blue, 32-cell stage, sea urchin). Because Nanos is a more selective marker for the germ cell lineage, we use it here to further test how the germ cell lineage is specified in diverse echinoderm embryos.

Results

Diversity in Echinoderm Nanos sequences

Nanos protein sequences were found in one species from each extant echinoderm clade. An alignment of the echinoderm Nanos protein sequences reveal each protein has 2 conserved CCHC zinc fingers that are characteristic of the Nanos protein family and are necessary for function (Figure 2A, asterisks) [28, 29]. In addition, echinoderm Nanos proteins contain a conserved upstream domain that is found in non-protostome Nanos sequences and is also required for function (Figure 2A, underline) [30, 31]. This domain has been called the NIM domain in vertebrates and is required to

recruit the deadenylase protein CNOT1 to Nanos [32]. Sequence analyses reveal there is a Nanos ortholog in every echinoderm database that has been examined.

A phylogram of echinoderm Nanos protein sequences reveals that all Nanos sequences do not follow the species tree (Figure 1A; Figure 2B, grey). We note that the *Apostichopus japonicus* Nanos protein, a Holothuroid sequence, is not placed as a sister group to Echinoids (Figure 1A and Figure 2B, grey). This disparity could be explained by the relatively low amount of conserved amino acids between each species (Figure 2A, non-highlighted amino acids). Nanos appears to be a protein where many amino acid changes have occurred throughout echinoderm evolution with the exception of the zinc finger and the NIM domains, both regions essential for its function.

Nanos expression in Asterozoa

The sister groups Asteroid and Ophiuroid comprise the Asterozoa clade [33-35]. In the Asteroid, *Patiria miniata* (a sea star), Nanos is not detectable by in situ RNA hybridization until the mid-gastrula stage when it accumulates in the endomesoderm (Figure 3A). By the late-gastrula stage, Nanos is further restricted to the precursor cells that give rise to the left posterior coelom, a dorsal patch in the middle of the developing gut (Figure 3A, asterisk). In the larva stage, Nanos accumulates in the left posterior coelom (Figure 3A, arrow). This expression pattern is consistent with Vasa expression in sea stars and further supports the idea that the left posterior coelom is the site of germ cell formation (Figure 1A, light blue and dark blue, larva stage, sea star) [13]. Unlike the initial widespread early expression of Vasa, Nanos is first expressed at the mid-gastrula stage in a restricted number of cells. Because Nanos expression is based

upon embryonic events, Asteroids likely use an inductive mechanism for germ cell specification.

In the Ophiuroid, *Amphipholis kochii* (a brittle star), Nanos first accumulates at the blastula stage in a domain that contains roughly half of the cells in the embryo (Figure 3B, asterisk). We hypothesize this first expression domain gives rise to the endomesoderm by homology to the sea star. Later in development, Nanos is expressed in a second domain (Figure 3B, arrow). We suggest the second expression domain appears dorsally as Nanos is restricted dorsally in the sea star. However, further endomesoderm and dorsal markers will be necessary to test the identity of these early Nanos-positive expression domains. In the late gastrula stage the first Nanos expression domain is restricted to a few cells in half of the blastopore (Figure 3B, asterisk) and the second Nanos expression domain is restricted to a few cells in the blastocoel (Figure 3B, arrow). By the 3-day old larval stage the first Nanos expression domain becomes restricted further to a few cells in half of the stomach epithelium. This is the first report of germ cell marker expression in Ophiuroid embryos, to the best of our knowledge. Furthermore, using Vasa protein labeling we have identified a left posterior coelom in an Ophiuroid embryo for the first time (Supplemental Figure 2B, asterisk). It remains to be tested if the Nanos enriched cells at the 3-day larva stage will migrate to the left posterior coelom at a later time point in larval development. However, we hypothesize that this will occur because Vasa protein accumulates in the left posterior coelom at the 1-week old larval stage (Supplemental Figure 2B, asterisk). Together, these results suggest germ cell markers accumulate in the left posterior coelom of an Ophiuroid larva. In addition, the relatively later initial expression of Nanos (blastula

stage) and the dependence on embryonic events to restrict the Nanos positive domains suggests Ophiuroids also use inductive mechanisms for germ cell specification.

Nanos expression in Holothuroids

In the Holothuroid, *Apostichopus japonicus* (a sea cucumber), Nanos is first expressed in the mid-gastrula stage in a dorsal patch biased to the left side of the developing gut (Figure 4, asterisk). These Nanos positive cells likely develop into the left posterior coelom visible in the larva stage (Figure 4, arrow). This expression pattern is similar to Vasa expression in the sea cucumber because Vasa also accumulates in the left posterior coelom (Figure 1A, light blue and dark blue, larva stage, sea cucumber) (Supplemental Figure 2A, arrow) [14]. However, Nanos expression is more restricted than Vasa because Vasa accumulates in all of the other coeloms at the early-larva stage. These results demonstrate that germ cell markers accumulate in the left posterior coelom of a Holothuroid embryo. In addition, the initial expression of Nanos in Holothuroid embryos is relatively late in development (mid-gastrula stage) and suggests they use inductive mechanisms for germ cell specification.

Nanos expression in Echinoids

The sister groups Cidaroid and Euechinoid comprise the Echinoid clade. In the Cidaroid, *Eucidaris tribuloides* (a pencil urchin), Nanos is first detected at the early blastula stage in 2-4 vegetal cells (Figure 5A, asterisk). These cells are likely the micromeres, the smaller cells that arise from a variable number of unequal cell divisions at the 16-cell stage [36]. Subsequently, these Nanos positive cells accumulate in the left coelom (Figure 5A, arrows). Although Vasa is not restricted to the micromere lineage in this animal, it does end up accumulating in the left coelom (Figure 1A, light blue and

dark blue, 32-cell stage and larva stage, pencil urchin) [13]. Together, these results indicate that the left coelom accumulates germ cell markers in a Cidaroid embryo. Furthermore, Nanos is initially expressed early in development (during the first few cleavages), which is likely more dependent upon the localization of maternal molecules. This is more consistent with an inherited mechanism of germ cell specification.

In the Euechinoid, *Lytechinus variegatus* (a sea urchin), Nanos is first detected at the 128-cell stage in a restricted number of vegetal cells (Figure 5B, asterisk). These are likely the small micromeres, the smaller cells that arise from two series of unequal cell divisions at both the 16 and 32-cell stages. Subsequently, these cells migrate to the tip of the developing gut and end up in the left coelom (Figure 5B, arrows). This expression is very similar to both Nanos and Vasa protein expression in the related sea urchin, *Stronglyocentrotus purpuratus* (Figure 1A, dark blue, 32-cell stage and larva stage, sea urchin) [12, 26]. In summary, these results demonstrate that the left coelom accumulates germ cell markers and that the small micromeres have a conserved germ cell fate in the Euechinoid lineage. In addition, Euechinoids also likely use an inherited mechanism of germ cell specification because Nanos is initially expressed very early in development (at the 32-cell stage).

Discussion

Three major developmental shifts in Nanos expression

We notice **three** major developmental stages in which Nanos mRNA first begins to accumulate.

1) Nanos expression is associated with cells that arise as a result of unequal cell divisions in Echinoids. The study of Nanos expression in Echinoid embryos is

particularly important to understand evolutionary transitions in germ cell specification because the Echinoid clade has a derived feature in their early development related to germ cell specification. They are the only echinoderm embryos that undergo unequal cell divisions during early cleavage (Figure 6A, pink). It was already known that Nanos is first expressed in the small micromere lineage of sea urchins, cells that arise at the 32-cell stage as a result of two unequal cell divisions (Figure 1A, purple, 32-cell stage, sea urchin) [25, 26]. Here, we test if Nanos expression is associated with cells that arise from unequal cell divisions in a different Echinoid lineage, the pencil urchin. The pencil urchin undergoes a variable number of unequal cell divisions at the 16-cell stage to give rise to the micromere lineage (Figure 1A, 32-cell stage, pencil urchin). This study suggests Nanos is expressed in the micromere lineage of the pencil urchin (Figure 5A, asterisk). We hypothesize that unequal cell divisions are linked to an earlier restricted expression of Nanos. Furthermore, the sea urchin Nanos-positive small micromere cells have previously been shown to directly contribute to the germ cell lineage [37]. Because Nanos is expressed early in development in a restricted number of cells and these cells have been shown to contribute to the germ cell lineage (at least in the sea urchin) we hypothesize that Echinoids display inherited mechanisms of germ cell specification.

2) In contrast to the Echinoid lineage, both Asterozoa and Holothurozoa do not start expressing Nanos until later in development (Figure 3A; Figure 4, asterisk). Animals in both of these clades contain a left posterior coelom and Nanos is first expressed at the mid-gastrula stage in left posterior coelom precursor cells (Figure 3A; Figure 4, asterisk). In addition, removal studies in the sea star show that the left posterior coelom contributes to morphologically distinct germ cells [38]. Because Nanos

is expressed later in development and in cells that contribute to germ cells (in the sea star) we hypothesize that these animals use inductive mechanisms to specify the germ cell lineage.

3) Nanos expression in the Ophiuroid lineage is the most perplexing. It is expressed at the blastula stage in a large embryonic domain equal to roughly half of the cells of the embryo (Figure 3B, asterisk). Because Nanos expression is not restricted to a small number of cells early in development we postulate that Ophiuroids use inductive mechanisms for germ cell specification. Ultimately though, it will be important to examine more species from each echinoderm clade to better refine these models.

Taken together, the results are consistent with the hypothesis that echinoderms ancestrally specified their germ cell lineage following gastrulation via inductive mechanisms. This is because the majority of extant echinoderm clades (Asteroids, Ophiuroids, and Holothuroids) use inductive mechanisms for germ cell specification. In sea stars and Holothuroids (brittle stars are less clear at this point), germ cell markers accumulate in a restricted embryonic domain late in embryogenesis (during the late gastrula stage/early larva stage). Furthermore, our results are also consistent with the hypothesis that an evolutionary transition has occurred in the Echinoid lineage for the germ cell lineage to be specified earlier by inherited mechanisms. In these animals, germ cell markers accumulate in a restricted number of cells very early in development, during the first few cleavages. This is consistent with a previous comprehensive study in animals which proposes that the inductive germ cell specification mechanism is ancestral, while there have been several independent acquisitions of the inherited germ cell specification mechanism [1].

Cells within left coeloms contribute to the germ cell lineage in echinoderms

In most of the animals studied here, Nanos and Vasa accumulate in either the left coelom or the left posterior coelom (Figure 1A; Figure 6A, purple, larva stage). We notice that germ cell markers accumulate in the left posterior coelom in almost every echinoderm lineage (Figure 6A, asterisk). However, the Echinoid lineage seems to have lost this structure and germ cell markers accumulate instead in the left coelom (Figure 6A, arrows).

In addition to accumulating germ cell markers we argue that the posterior left coelom also contributes to the germ cell lineage in Asteroids, Ophiuroids, and Holothuroids. In support of this hypothesis, the left posterior coelom has been removed from sea star larva and results in juveniles with about half of the normal number of morphologically distinct germ cells [38]. We also argue that the left coelom contributes to the germ cell lineage in all Echinoids. The small micromere cells have been removed from sea urchins before they reach the left coelom and results in an adult that develops normally, but is lacking any gametes [37]. From these results, we propose that left-sided coeloms contribute to the germ cell lineage in all echinoderm species.

It is important to note that the left coelom is a mesodermal lineage (Figure 6B, red). In addition, the left posterior coelom also accumulates many mesodermal markers in sea stars and sea cucumbers [39] (unpublished results). This is significant because in all animals that have been studied that use an inductive mechanism for germ cell specification their germ cell lineage was induced from a mesodermal lineage [8, 9, 40]. These results further support the hypothesis that the most recent common ancestor of Bilaterian animals had a germ cell lineage induced from a mesodermal lineage.

Nanos expression correlates with the expression of larval skeletogenic genes

We notice that the germ cell lineage is linked to genes that are required for specification of the larval skeleton. Both the Echinoid and Ophiuroid lineages likely acquired larval skeletons independently because larval skeletons are absent from other clades (Figure 6A, green). In the sea urchin (Echinoid), it has already been shown that the cell lineage that gives rise to the germ cells at the 16-cell stage also gives rise to the larval skeleton [41-44]. In addition, the cell lineage that gives rise to the larval skeleton in the pencil urchin (Echinoid) also accumulates Nanos [45] (Figure 5A, asterisk). This shows that both the germ cell lineage and the larval skeletal cell lineage share a common precursor cell lineage in Echinoids. In addition, this study found that Nanos is expressed in half of the cells during the blastula stage of an Ophiuroid embryo (Figure 3B, asterisk). This expression pattern resembles the vegetal expression pattern domain where five larval skeletal genes accumulate in Ophiuroids, although the skeletal genes appear to localize in a more restricted domain [46-48]. We hypothesize that the gene regulatory networks that control the germ cell lineage and the larval skeleton lineage are co-dependent in both Echinoids and Ophiuroids and that this may be an evolutionary restriction placed on the evolution of both cell lineages. In other words, whenever the gene regulatory networks that control the skeletal cell lineage or germ cell lineage experience a heterochronic shift in development the other lineage may follow.

In support of this hypothesis, the expression of germ cell markers also correlates with the expression of genes associated with larval skeletal specification even in animals that do not have larval skeletons. Asteroids do not have larval skeletons, yet genes that are involved in specification of the larval skeleton in Euechinoids (e.g. Alx

and Ets) are also co-expressed with Nanos in the left posterior coelom of the Asteroid larva (Figure 3A, arrow) [39, 46]. Together, these results support the hypothesis that the gene regulatory network that specifies the echinoderm germ cell lineage is in some way intertwined with the gene regulatory network that specifies the Echinoid and Ophiuroid larval skeletons.

Mechanisms that may contribute to evolutionary transitions in germ cell specification

We notice three developmental changes that are involved in the evolution of an inherited germ cell specification mechanism in the Echinoid clade. **First**, we propose that the evolution of early unequal cell divisions within the Echinoid clade was important for the evolution of earlier accumulation of germ cell markers. Therefore, early unequal cell divisions may be a broader strategy for the evolution of an inherited mechanism of germ cell specification. To explore this model further it will be important to understand the cellular and molecular tools that are required for an early unequal cell division in the Echinoid lineage. In addition, it will be important to understand the upstream regulatory molecules that are required for the earlier accumulation of germ cell markers. **Second**, we propose that the earlier specification of the germ cell lineage in the Echinoid lineage is accompanied by an earlier specification of the larval skeletal lineage because these two cell fates overlap. This suggests there may be an upstream factor that is required for the specification of both cell lineages, such as a general determinant of the endo/mesoderm cell fate (ie. Wnt). This may be an evolutionary constraint placed upon the evolution of an earlier germ cell specification in Echinoids. To explore this model further it will be important to define the upstream regulators of both of these cell lineages. **Third**, there is a loss of the posterior left coelom in the Echinoid lineage. This

further emphasizes the importance of the posterior left coelom in echinoderms that use the ancestral inductive mechanism of germ cell specification, while this structure is not required for germ cell specification in Echinoids and has been lost. To explore how the posterior left coelom cell lineage has been lost in the Echinoid lineage it will be necessary to understand the molecules that are required to induce the left posterior coelom cell lineage in non-Echinoid embryos. The future study of these three developmental changes in diverse echinoderm embryos will significantly impact the broader field examining the evolution of germ cell mechanisms within all animals.

Materials and Methods

Embryo Culture-

Asteroid (sea star): The species, *Patiria miniata*, were collected from the Southern coast of California, USA from either Pete Halmay (PeterHalmay@gmail.com) or Josh Ross (info@scbiomarine.com) and cultured as described [49]. Briefly, oocytes and sperm were collected by dissection. Oocytes were isolated by passing diced ovary tissue through a cheesecloth and collecting the oocytes in a beaker. Oocytes were washed 2 times with filtered sea water and incubated 1 hour with 1-methyl adenine (3.0 μ M, Acros Organics) to induce maturation. Eggs were fertilized with diluted sperm and excess sperm was washed out with filtered sea water. Embryos were cultured with continuous stirring at 16° C until the larval stage.

Ophiuroid (brittle star): The species, *Amphipholis kochii*, were collected from Himi (Toyama Prefecture, Japan) and cultured as described [46]. Briefly, animals were induced to spawn by a 1-2 hour cold shock at 4° C. Eggs were fertilized with diluted sperm and cultured in artificial sea water at 23° C.

Holothuroid (sea cucumber):

Adults of the species *Apostichopus japonicus*, were collected near Misaki Marine Biological Station, the University of Tokyo in Kanagawa Prefecture, Japan and cultured as described [50]. Briefly, spawning was induced by injecting animals with 10 μ M cubifrin. Eggs were fertilized with sperm that had been obtained by mincing of the testis. Embryos and larvae were cultured at 20° C in filtered sea water containing 75 mg/L of penicillin and streptomycin.

Cidaroid (pencil urchin):

The species, *Eucidaris tribuloides*, were collected from Florida, USA by KP Aquatics LLC (www.sealifeinc.net). Animals were induced to spawn by injection with 0.5 M KCl. Eggs were fertilized with diluted sperm and excess sperm was washed off 1 time with filtered sea water. Cultures were kept in 10 cm Petri dishes at 15° C.

Euechinoid (sea urchin):

The species, *Lytechinus variegatus*, were collected from Florida, USA by Pelagic Corporation. Animals were induced to spawn by injection with 0.5 M KCl. Eggs were fertilized with diluted sperm and excess sperm was washed off 1 time with filtered sea water. Cultures were kept stirring at 25° C until the larval stage.

Fixing Embryos-

Ophiuroid (brittle star): Embryos were fixed overnight at 4° C in a solution containing 4% PFA, 0.1 M MOPS (pH 7.0), and 0.5 M NaCl. Embryos were first washed with 50% Ethanol in PBS, then washed in 70% Ethanol, and stored at -20° C in 70% Ethanol.

All other embryos were fixed essentially as described with a few exceptions [51]. Briefly, embryos were resuspended in MOPS buffered PFA (5% PFA, 162.5 mM NaCl, 32.5 mM MOPS pH 7.0, 32.5% filtered sea water) and incubated overnight at 4° C. Embryos were washed 5 times with MOPS Buffer (0.1 M MOPS pH 7.0, 0.5 M NaCl, 0.1% Tween 20) and stored at -20° C in 70% Ethanol.

Whole Mount RNA in situ Hybridization-

General: RNA in situ analyses were done essentially as described [51]. Briefly, embryos were hybridized with 0.1-0.5 ng/ul of DIG labeled RNA probe for 1 week at 50° C.

Embryos were incubated with anti-DIG Alkaline Phosphatase FAB fragments overnight at room temperature (1:1500, Roche Diagnostics). After washing, embryos were stained with NBT/BCIP for 6-24 hours. The reaction was stopped with 5 mM EDTA in MOPS Buffer. Embryos were stored up to 1 day at 4° C and imaged on a Zeiss Axiovert 200M Microscope with an AxioCam MRc5 color camera. In all cases embryos were also incubated with the same concentration of Neomycin probe as a negative control.

Asteroid (sea star): 0.2 ng/ul of probe was used for hybridization. Alkaline Phosphatase staining took approximately 6 hours.

Ophiuroid (brittle star): 0.2 ng/ul of probe was used for hybridization of eggs through the mid-gastrula stage embryos. 0.5 ng/ul of probe was used for hybridization of late-gastrula and larva stage embryos. Alkaline Phosphatase staining took approximately 13.5 hours and 10 hours, respectively.

Holothuroid (sea cucumber): 0.5 ng/ul of probe was used for hybridization. Alkaline Phosphatase staining took approximately 7 hours.

Cidaroid (pencil urchin): 0.5 ng/ul of probe was used for hybridization. Alkaline Phosphatase staining took approximately 6 hours.

Euechinoid (sea urchin): 0.1 ng/ul of probe was used for hybridization. Alkaline Phosphatase staining took approximately 24 hours.

Sequence Analysis for Probe Design-

Asteroid (sea star) and Ophiuroid (brittle star): Sequences were obtained using de novo development transcriptome databases. The sea urchin, *Stronglyocentrotus purpuratus*, Nanos2 protein sequence (NP_001073023.1) was used as a query to find Nanos gene sequences by performing a tblastn analysis. A reciprocal blastn was then performed using the non-redundant nucleotide database on NCBI to test authenticity.

Holothuroid (sea cucumber) and Cidaroid (pencil urchin): Sequences were obtained using de novo ovary transcriptome databases [33] using the same method as described above for Asteroids.

Euechinoid (sea urchin): Sequences were obtained by degenerate PCR followed by 5' and 3' RACE. Degenerate PCR primers are as follows: Forward- TTYTGYAARMAYAAAYGGIGAR, Reverse- RTTIAVIGGRCARTAYTTIA. 5' RACE primers are as follows: Outer- ACTCTAGTCATTTGCACACC, Inner- GGGCTATTGTCCAAGTCAA. 3' RACE primers are as follows: Outer- CAATTCTAAGGGCGTACACC, Inner- GCTCTGTGGGACGAATGG.

Probe Design and Synthesis-

General: Nanos sequences were amplified by PCR and ligated into PGEM-T Easy Vector (Promega) and transformed into competent XL-1 Blue Bacteria. Primers used for PCR amplification are listed in Supplement Table 1. Following sequence verification,

plasmids were digested and used for antisense transcription using the DIG RNA Labeling Kit (Roche).

Holothuroid (sea cucumber) and Cidaroid (pencil urchin): Nanos sequences were amplified by PCR. However, the reverse primer contained a T7 RNA polymerase recruitment sequence at its 5' end. Following sequence verification, PCR products were used for antisense transcription as described above.

Phylogenomic Analyses-

Sequences used for alignments were found as described above for Asteroid, Ophiuroid, Holothuroid, Cidaroid, and Euechinoid Nanos. Crinoid Nanos sequences were obtained using the species *Oxycomanthus japonicus* Ovary transcriptome using the same method described above for Asteroids, accession number: GAZO01037160 [33].

The Nanos protein alignment was made using BioEdit software that used a ClustalW analysis (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>). Nanos protein phylogram was made using phylogeny.fr [52, 53].

Vasa Immunofluorescence-

Embryos were fixed with 90% ethanol for 1 hour. After a PBS wash Vasa primary antibody [12] was added to embryos at a 1:200 dilution for 3 hours at 25° C or overnight at 4° C Embryos were washed with PBS, stained with a fluorescent secondary antibody, and counterstained with Hoechst.

Acknowledgements

Funding for this research was provided by the NIH: (2RO1HD028152 to GMW)

(NIGMS 1F31GM112479-01, Award No. 004102 to TMF) (Training grant T32GM007601 to Brown University) and by the NSF: (EPSCoR-RI Graduate student fellowship, 1004057 to TMF). Also, thank you to the members of PRIMO for a rich working environment.

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Figures and Tables

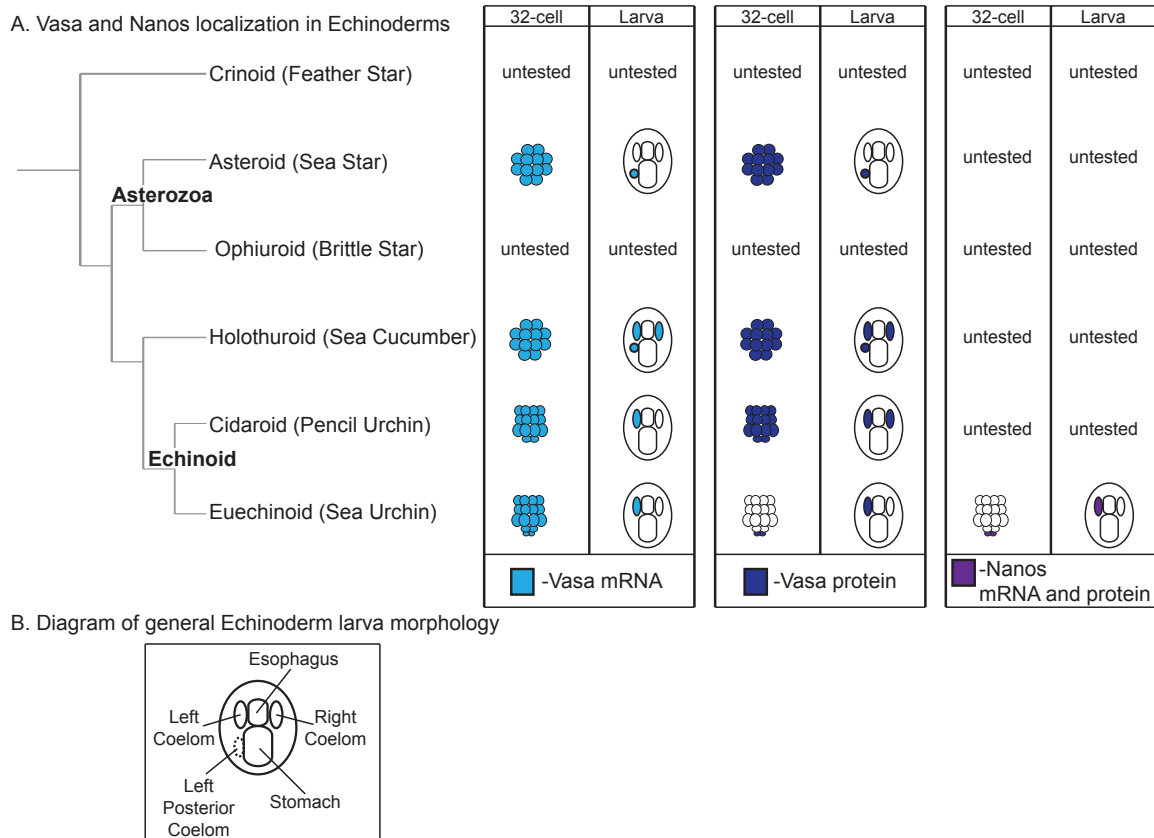


Figure 1- Vasa and Nanos localization in Echinoderms

A. A summary of known Vasa and Nanos mRNA and protein localization in Echinoderm embryos before this study. Light blue= Vasa mRNA, Dark blue= Vasa protein, Purple= Nanos mRNA and protein. In Asteroids, Holothuroids and Cidaroids Vasa mRNA and protein localizes broadly at the 32-cell stage and both are restricted to the coeloms by the larva stage. In Euechinoids, Vasa mRNA localizes broadly at the 32-cell stage and accumulates in the left coelom at the larva stage. However, Vasa protein in Euechinoids is restricted to a subset of embryonic blastomeres at the 32-cell stage and accumulates in the left coelom at the larva stage. Nanos mRNA and protein expression has only been reported in Euechinoids and is restricted to a subset of embryonic blastomeres at the 32-cell stage. By the larva stage Nanos mRNA and protein accumulates in the left coelom. **B.** A diagram of general echinoderm larva morphology. The dotted left posterior coelom is not present in all Echinoderm clades. All larval views are dorsal images with the anterior side up as a standard for this paper.

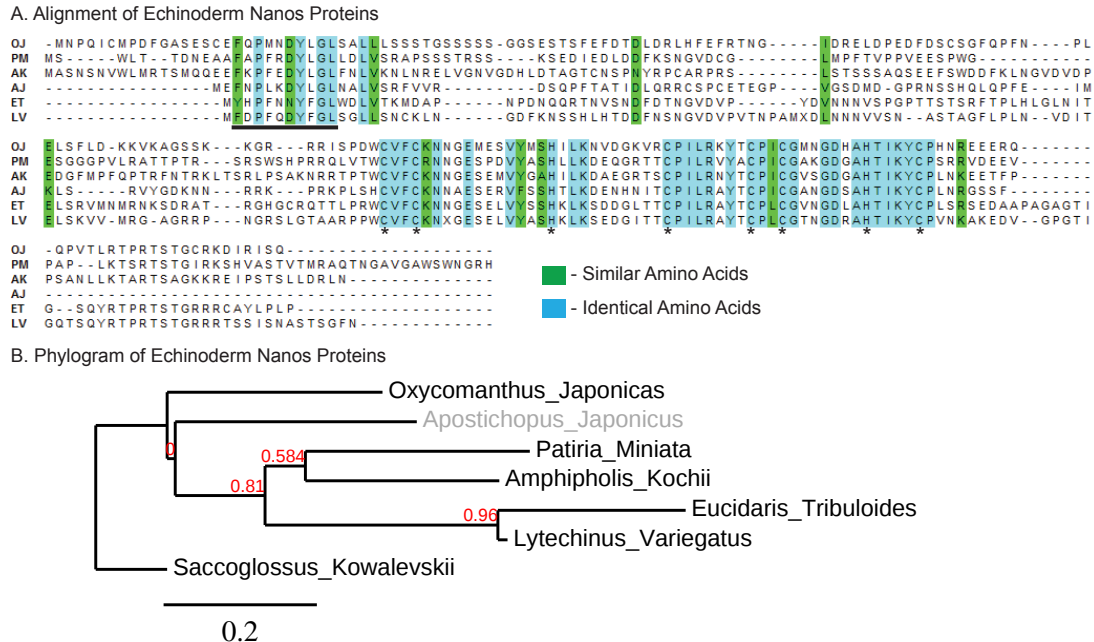
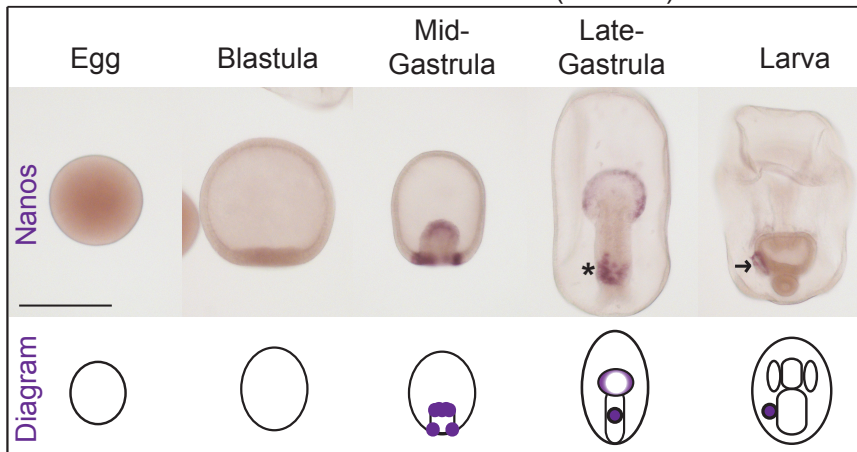


Figure 2- Nanos sequence analysis in Echinoderms

A. An alignment of Nanos proteins found in Echinoderms from each major clade using ClustalW2 and BioEdit. All similar amino acids are highlighted in green and all identical amino acids are highlighted in blue. Asterisks identify the conserved Nanos CCHC zinc fingers. Underlined amino acids identify the NIM domain, a non-protostome conserved protein interaction domain. OJ= *Oxycomanthus japonicus*, Crinoid; PM= *Patiria miniata*, Asteroid; AK = *Amphipholis kochii*, Ophiuroid; AJ= *Apostichopus japonicus*, Holothuroid; ET = *Eucidaris tribuloides*, Cidaroid; LV = *Lytechinus variegatus*, Euechinoid. **B.** A phylogram of Echinoderm Nanos protein sequences using phylogeny.fr. Clades in grey do not share the same relationships as the species phylogenetic tree.

A. Nanos mRNA localization in an Asteroid (sea star).



B. Nanos mRNA localization in an Ophiuroid (brittle star).

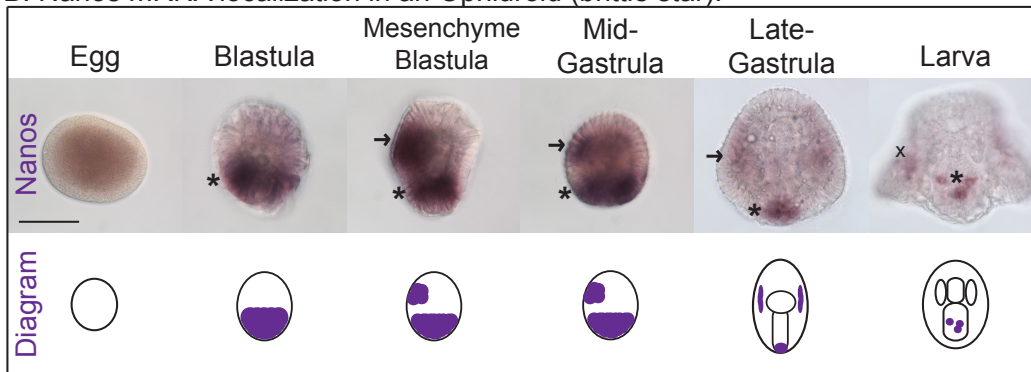


Figure 3- Nanos mRNA localization in an Asteroid and an Ophiuroid

A. Nanos mRNA localization in an Asteroid (sea star, *Patiria miniata*). Nanos mRNA first accumulates in mid-gastrula embryos in the endomesoderm. Subsequently, Nanos accumulates in the tip of the developing gut and in a dorsal patch in the middle of the developing gut, asterisk. By the larva stage Nanos is restricted to the left posterior coelom, arrow. Scale bar is 200 μ M. **B.** Nanos mRNA localization in an Ophiuroid (brittle star, *Amphipholis kochii*). Nanos mRNA first accumulates in blastula embryos in half of the cells of the embryo, asterisk. Subsequently, Nanos accumulates in a second expression domain at the mid-gastrula stage, arrow. At the late gastrula stage, Nanos is restricted to a few cells at the blastopore, asterisk, as well as to some mesenchymal cells within the blastocoel (arrow). By the larva stage, Nanos is further restricted to a few cells within the epithelium of the stomach, asterisk. Note: The larval stage is viewed dorsally and pigment cells are stained non-selectively throughout the blastocoel, X. Scale bar is 50 μ M

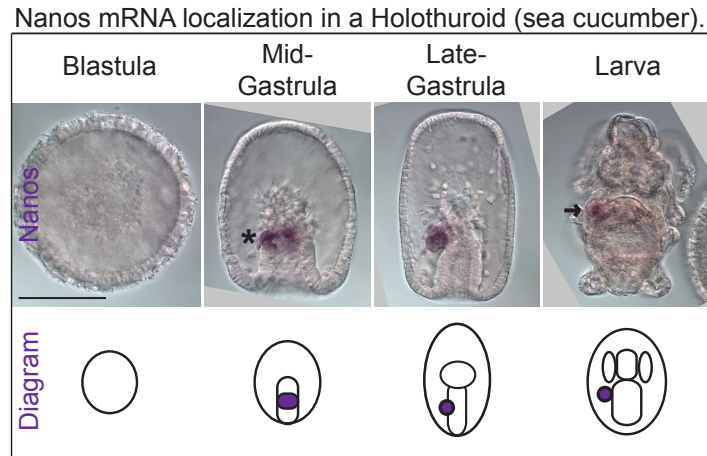
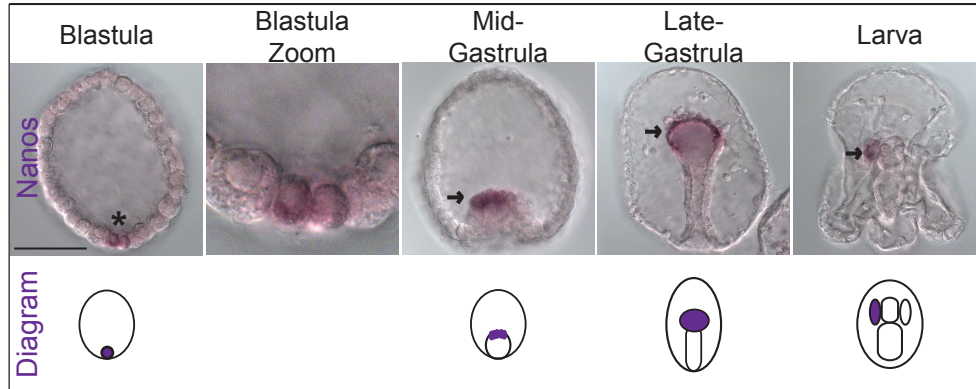


Figure 4- Nanos mRNA localization in a Holothuroid (sea cucumber)

In the sea cucumber, *Apostichopus japonicus*, Nanos mRNA first accumulates in mid-gastrula embryos in a dorsal domain in the middle of the developing gut, asterisk. Nanos is restricted to the left posterior coelom during the gastrula and larva stages (viewed dorsally), arrow. Scale bar is 100 μ M.

A. Nanos mRNA localization in an Cidaroid (pencil urchin).



B. Nanos mRNA localization in an Euechinoid (sea urchin).

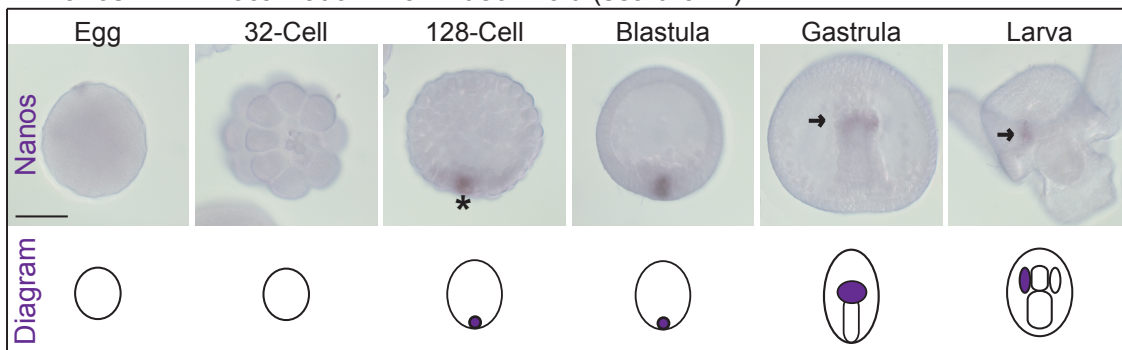
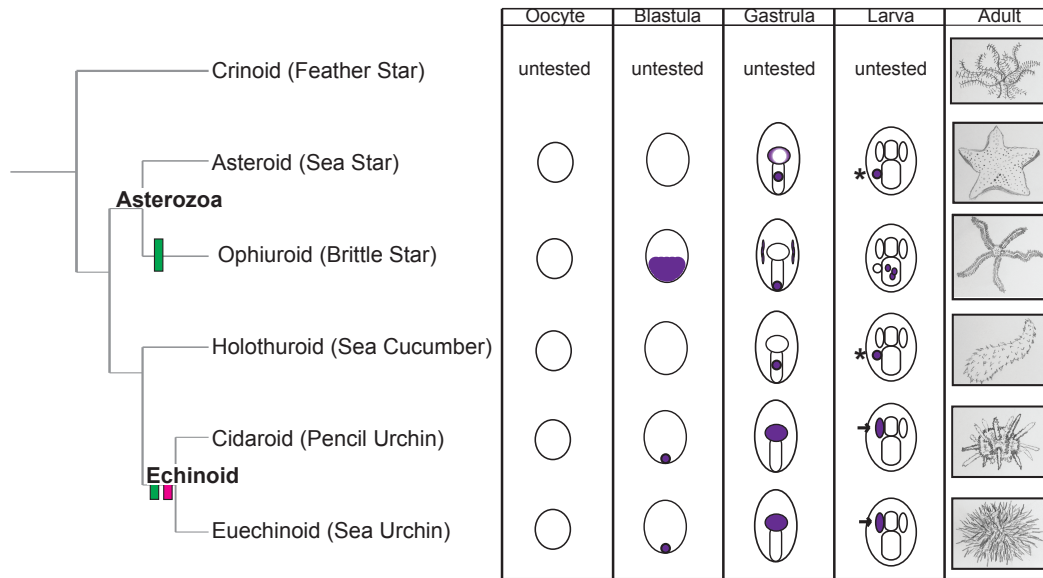


Figure 5- Nanos mRNA localization in a Cidaroid and an Euechinoid

A. Nanos mRNA localization in a Cidaroid (pencil urchin, *Eucidaris tribuloides*). Nanos mRNA first accumulates in blastula embryos in a restricted number of cells at the vegetal plate, asterisk. Subsequently, Nanos accumulates in the tip of the developing gut during the gastrula stage, arrow. By the larva stage (viewed dorsally) Nanos is restricted to the left coelom, arrow. Scale bar is 100 μ M. **B.** Nanos mRNA localization in an Euechinoid (sea urchin, *Lytechinus variegatus*). Nanos mRNA first accumulates in 128-cell embryos in a restricted population of cells at the vegetal plate, asterisk. Subsequently, Nanos accumulates at the tip of the developing gut during the gastrula stage, arrow. By the larva stage (viewed dorsally) Nanos is restricted to the left coelom, arrow. Scale bar is 60 μ M.

A. Nanos mRNA localization in Echinoderms



B. General Echinoderm development and germ layers

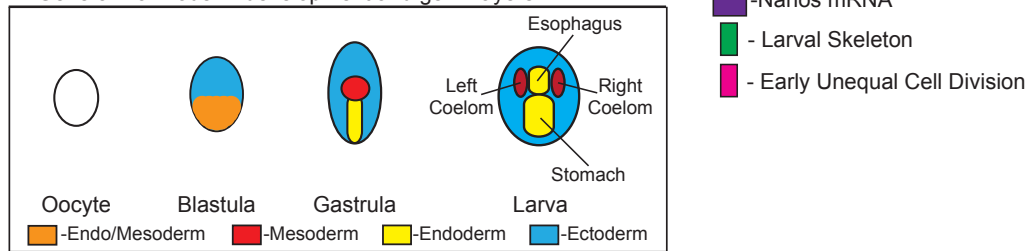
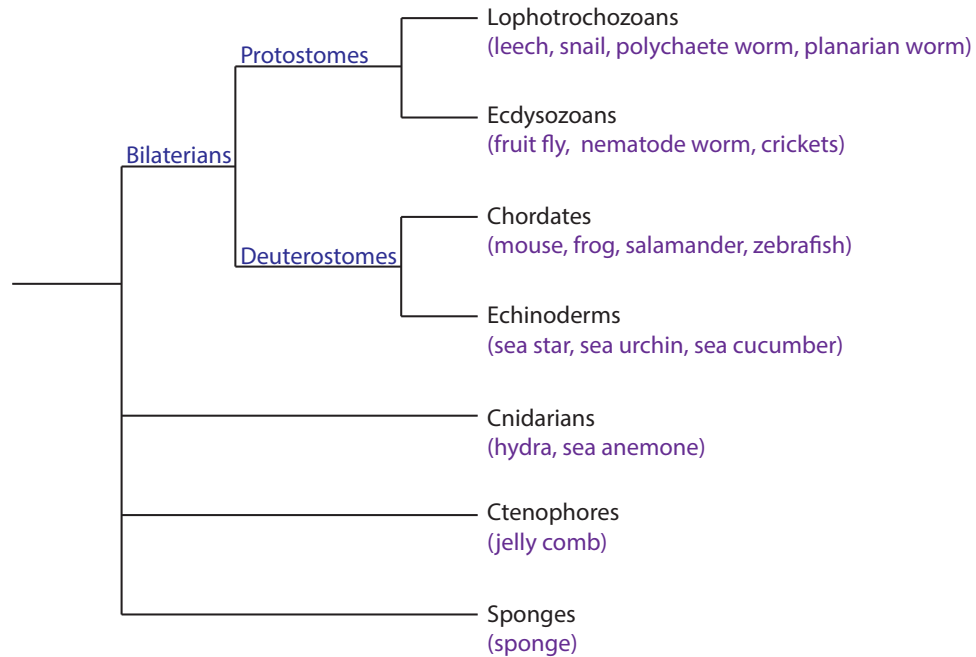


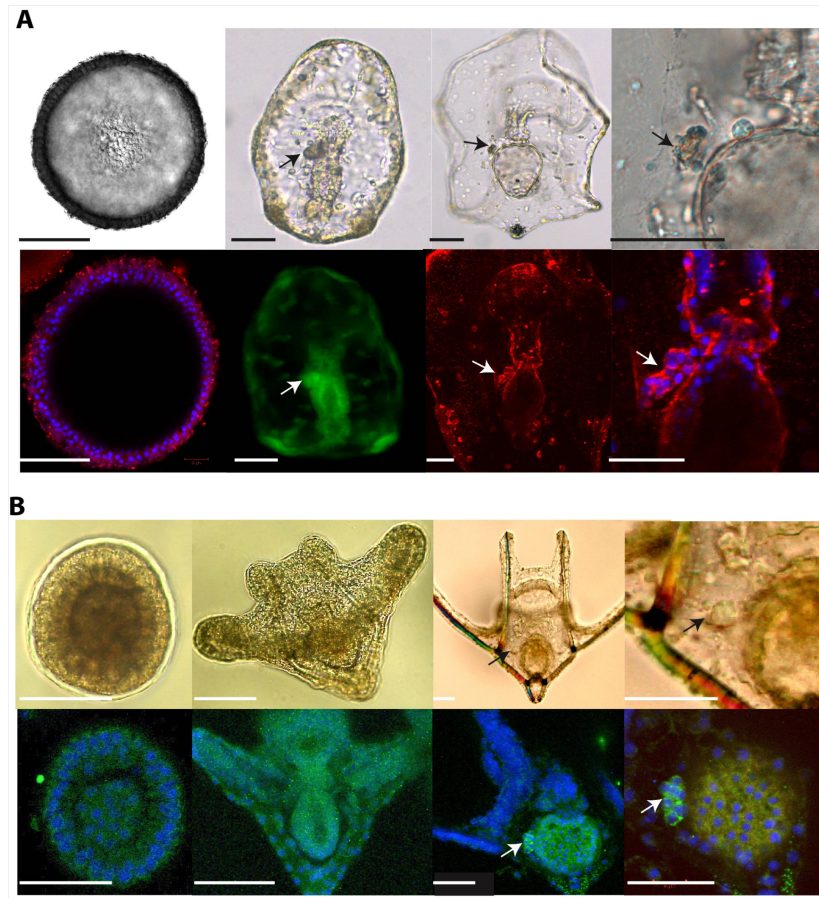
Figure 6- A Summary of Nanos mRNA localization in Echinoderms

A. In both Asteroids and Holothuroids, Nanos is enriched in the left posterior coelom by the larva stage, asterisks. In both Cidaroids and Euechinoids Nanos accumulates in the left coelom by the larva stage, arrows. In contrast, Nanos accumulates in the stomach at the larva stage in Ophiuroids. Purple= Nanos mRNA, Green= larval skeleton, Pink= early unequal cell divisions. **B.** A diagram of general Echinoderm development and the embryonic germ layers.



Supplemental Figure 1- Metazoan Phylogenetic Tree

A simplified version of the metazoan phylogenetic tree. Chordates and Echinoderms belong to the Deuterostome clade. Lophotrochozoans and Ecdysozoans belong to the Protostome clade. Together, Deuterostomes and Protostomes make up the Bilaterian clade. Ctenophores, Cnidarians and Sponges are outgroups to the Bilaterians. We did not specify the relationships between the outgroups as they are still controversial [54].



Supplemental Figure 2- Vasa protein localization in a Holothuroid and an Ophiuroid

A. Vasa protein localization in a Holothuroid (sea cucumber, *Apostichopus japonicus*). Vasa protein accumulates broadly during early developmental stages. By the larva stage Vasa protein accumulates in the left posterior coelom, arrow. **B.** Vasa protein localization in an Ophiuroid, (brittle star, *Amphipholis kochii*). Vasa protein accumulates broadly during early developmental stages. By the larva stage Vasa protein accumulates in the left posterior coelom, arrow. Scale bar is 50 μ M.

Species: (Accession Number)	Primers for Probe Design:	Nucleotide Sequence:
Asteroid "sea star" <i>Patiria miniata</i> (KU594505)	F: ATATGAGCTGGCTGACAACG R: TGATATTCAATGCTAGGCCTAATAGA	ATGAGCTGGCTGACAACG GACAACGAGGCCGCATTGCCCCCTTTAGGGACTATCTTGGCCTGTTG GATCTGGTGTCCCGGGCACCGAGTTCTAGCACAGATCGTCAAAGAGCGAAGACATTGAGGACCTC GATGACTTCAAATCAAATGGGGTCGACTGCGGCCTGATGCCATTTACCGTGCCCCCGGTGAGGAG AGCCCTTGGGGCGAGAGCGGCGGCGGTCTGTGCTGCGGGCCACAACCCCAACCCGAAGCCGGTC CTGGTCCCACCCCGACGCCAACTAGTTACTTGGTGCGTATTCTGTGCGCAACAATGGGGAGTCCCCA GATGTGTACGCCTCCCATCTCCTGAAGGACGAGCGGGCAGGACGACCTGCCCGATCCTCCGGGTC TACGCCTGCCCCATCTGTGGGGCGAAGGGCGACGGAGCCACACCATCAAATACTGTCCGTCCCGA AGAGTTGACGAGGAAGTGCCAGCCCCACTGAAGACCTCCAGGACGTCCACGGGCATCAGAAAGTCG CACGTGGCGTCGACGGTGACCATGCGGGCGCAGACAAACGGAGCTGTGGGTGCTTGGAGTTGGAA CGGACGGCATT GA
Ophiuroid "brittle star" <i>Amphipholus kochii</i> (KU641124)	F: GCAAGCAACAGCAACGTTTG R: TCTTTTCCCAGCAGAGGTCC	ATGGCAAGCAACAGCAACG TTTGTTAATGCGTACGAGTATGCAACAGGAAGAATTCAAACCTTCG AGGACTACCTTGGCCTCTTCAACCTGGTAAAGAACTTGAACAGAGAGCTAGTGGGGAATGTCGGCGA TCATCTAGATACTGCTGGTGAATTCCCCGAACATCGGCCCTGTGCTCCGACCTAGCTCGCTTT CAACGAGTTCTAGTGCACAAAGTGAAGAGTTCAGCTGGGATGACTTTAAGTTGAATGGAGTGATGT GGATCCAGAGGATGGCTTCATGCCCTTCCAACCGACTAGATTTAACACTAGGAAGCTTACTTCTAGAC TACCGTCAGCCAAGAACCGCCGGACTCCAACCTGGTGTGTGTTTTGCAAGAATAATGGCGAGAGTGA GATGGTTACGGAGCCCACATCCTGAAAGACGCCGAGGGTCGAACCTCTTGCCAATTCTGCGCAAC TACACCTGCCCCATTTGCGGGGTGTCTGGAGATGGAGCTCATACCATCAAGTACTGTCCGTTGAACA AGGAGGAACATTTCCACCTCCGCCAATTTGCTGAAGACCGCCAGGACCTCTGCTGGGAAAAAGAG AGAGATTCCGTCTACATCATTGTTAGACCGGCTTAATT AG
Holothuroid "sea cucumber" <i>Apostichopus japonicas</i> (GAVS01020807)	F: CATTGAGATTTTGCCGTCCT R: TTGATGGTATGCGCTGAATC	ATGGAATTCAATCCG TAAAAAGACTATCTTGGGCTGAATGCCTTGGTTTCAAGGTTTGTGTTAGAGA TTCTCAGCCGTTCACTGCTACCATCGATTTACAGCGACGTTGCAGTCCATGTGAGACGGAGGGTCCT GTCGGATCCGATATGGATGGCCCTCGGAATTCGAGCCATCAACTGCAGCCTTTTGAAATAATGAAGT TGAGTAGAGTGACGGCGACAAAAACAACAGGCGGAAGCCAAGGAAGCCACTGTCGCACTGTGTCT TCTGTAAGAACAACGCAGAGAGTGAAAGGGTTTTCTCATCACATACATCAAGACGCGAGTAATCACAAC ATCACATGCCCAATACTTCGGGCTTACACCTGCCCAATTTGTGGAGCCAACGGAGATT CAGCGCATA CCATCAAGT ATTGCCCTTTGAATAGGGGCTCAAGTTTT TAA
Cidaroid "pencil urchin" <i>Eucidaris tribuloides</i> (GAZP01035152)	F: CGGACTCTGGGATTGGTAA R: TTGCAAGGTCACAAGAAAC	ATGTATCATCCATT AATAACTACTT CGGACTCTGGGATTGGTAACTA AGATGGATGCGCCGAATCC GGACAACCAGCAGCGAACTAACGTTAGTAACGACTTCGACACTAACGGAGTCGATGTCCCTTACGAC GTCAACAACAATGTGAGCCCGGGCCCCACCACAGTACCAGCCGATTCACTCCACTGCATCTTGGAC TGAACATCACTGAATTGAGTCGTGTTATGAATATGAGAAACAAGTCGGACAGGGCGACGAGGGGACA CGGATGTCGACAGACGACGCTGCCGCGATGGTGTGTTTTCTGTAAGAATAACGGCGAGATCGGAGTT GGTGTATTCTTCCACAAACTGAAATCGGACGATGGACTAACAACTGCCCAATTCTGCGGGCATAAC ACCTGCCCCCTGTGTGGTGTGAATGGAGACCTCGCGCACACCATCAAGTACTGCCCTCTTAGCAGG AGTGAGGATGCTGCCCCAGCTGGAGCCGGTACCATCGGGTCGCAATACCGAACCCCCCGTACAAGT ACGGGTAGGAGGAGGTGCGCTTACCTGCCGCTGCCT TGA
Euechinoid "sea urchin" <i>Lytechinus variegatus</i> (KU594506)	F: GGGCTATTGTCCAACCTGCAA R: CCATTCGTCACAGAGC	ATGTT CGATCCATTCCAAGACTACTTTGGCCTGAGT GGGCTATTGTCCAACCTGCAA ACTAAATGGAGA TTTCAAAAACCTAGTCATTTGCACACCGATGATTTCAACTCTAATGGAGTCGATGTTCTGTCACTAA CCCGCAATGRGCATCTGAACAACAACGTAGTGTCAAATGCAAGCACAGCTGGGTTCTACCTCTG AACGTCGATATTACCGAACTTTGAAAGTCGTGATGAGAGGAGCGGGCCGACGCCCAATGGCAGA TCACTGGGTACGGCAGCCCGGCCACCATGGTGCCTGTTTTGTA AAAACAAYGGAGAAAGTGAGTTG GTTTACGCCAGCCACAAGTTGAAGTCCGAGGATGGCATCACTACCTGCCCAATTCTAAGGGCGTACA

		CCTGTCC <u>GCTCTGTGGGACGAATGGCGACCGCGCCCACACCATCAA</u> TA <u>CTGCCC</u> GGTCAATAAGG CCAAGGAGGATGTCGGACCAGGTACCATCGGACAGACGTCGCAATATCGTACGCCACGCACATCAA CCGGTCGAAGACGAACCTCCAGCATCAGTAATGCCAGCACCAGCGGCTTCAACT TAG
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Supplemental Table 1- Primers and sequences used for in situ analyses

A list of the primers used for probe synthesis and the coding sequence of each Nanos mRNA. Primers used for probe synthesis are underlined within the coding sequence. If the primer is not underlined within the coding sequence then the probe includes sequences within the untranslated region of the mRNA as well. **ATG**= start codon, **TGA**= stop codon.

Chapter 4: Nodal causes sequential restriction of germ cell factors during primordial germ cell specification

Fresques T and Wessel GM (2017). Nodal causes sequential restriction of germ cell factors during primordial germ cell specification. (In Preparation).

I performed all of the experiments in this study and wrote the manuscript with guidance from G. Wessel.

Abstract

Specification of the germ cell lineage is required for sexual reproduction in animals. The timing and mechanism of germ cell specification varies greatly among animals but roughly clusters into either inherited or inductive mechanisms. How inductive mechanisms of germ cell specification generate a germ cell fate selectively is poorly understood. Here we show that germ cell factors in the sea star initially are present broadly yet they subsequently become restricted both dorsally and then in the left side of the embryo. We find that the Nodal embryonic signal is required for the subsequent restriction of two germ cell factors, Nanos and Vasa, during the early development of this animal. We learned that Nodal inhibits germ cell factor accumulation in three ways including: inhibition of specific transcription, degradation of specific mRNA, and inhibition of tissue morphogenesis. These results document a signaling mechanism required for the sequential restriction of germ cell factors which may be a conserved aspect of the inductive mode of germ cell specification.

Introduction

Eggs and sperm are the only potentially immortal cells in sexually reproducing animals. They are able to participate in reproduction and become an entirely new animal while all remaining cells, somatic cells, are destined to die. As such, the proper development of the **germ cell lineage**, cells with the developmental potential to become an egg or sperm, is essential for the survival of sexually reproducing animals. An early step required for proper germ cell development is **germ cell specification**. This step is when a subset of cells obtain a unique set of molecules that cause them to become germ cells. On the other hand, every cell that does not obtain these molecules will become somatic cells.

There are two main mechanisms that a sexually reproducing animal can use for germ cell specification. The **inherited** (Figure 1A) mechanism is when a subset of germ cell factors are asymmetrically localized in the egg or early embryo and the cells that happen to inherit these molecules will become the germ cell lineage. Animals such as frogs, nematode worms, flies, and teleost fish primarily use inherited mechanisms for the specification of the germ cell lineage [1-6]. An alternative mechanism of germ cell specification is called **inductive** (Figure 1a). This is when germ cell factors are either localized broadly in the egg and early development or are not expressed at all. Embryonic signals are required to determine which cells obtain germ cell factors and acquire a primordial germ cell fate. Animals such as mammals, axolotls, and crickets use primarily inductive mechanisms for the specification of the germ cell lineage [7-9].

Although the inductive mechanism of germ cell specification appears to be the ancestral mechanism in animals and is widespread throughout the animal phylogenetic tree [10], the mechanisms that directly induce a germ cell fate are only beginning to be

discovered from a limited repertoire of animals: mouse, cricket, and axolotl. In order to discover the breadth of this mechanism of germ cell specification and to determine which aspects are conserved between species it is necessary to determine how germ cells are induced in diverse animals.

The sea star may be a useful model to examine inductive germ cell specification for several additional reasons. The sea star: 1) Holds a unique position in animal phylogeny as an invertebrate deuterostome. This means comparisons can be made with chordates to understand germ cell specification in the deuterostome ancestor. 2) Preliminary data shows inductive mechanisms contribute to germ cell specification in the sea star [11-13]. 3) Germ cell factors are present widely in early development and provide a useful assay to determine how signaling molecules contribute to germ cell specification.

Here we show that Nodal signaling is required for the sequential restriction of Nanos and Vasa mRNAs in early development. Although the function of Nanos and Vasa remains to be tested in sea stars we strongly suggest that they are required for germ cell specification because 1) these factors are usually found together in the germ cell lineage [14] 2) these factors are required for germ cell specification in many animals [14] and 3) these factors accumulate in the posterior coelomic pouch structure, a structure that has previously been shown to contribute to primordial germ cells [15]. We propose that the sequential restriction of germ cell factors is a significant mechanism involved in germ cell specification. That is, germ cell factors are present broadly in early development and embryonic signals reduce the field of cells that retain germ cell factors until the last remaining group becomes the germ lineage.

Results

Germ cell factors are sequentially restricted during early development

We noticed in previous studies that the germ cell factors Vasa, Nanos and Piwi are present broadly in early development and then become sequentially restricted in *P. miniata* [12, 16]. The restriction of Vasa and Nanos in particular show a similar restriction pattern during two stages of embryonic development. First, at the mid-gastrula stage Vasa and Nanos accumulate in a vegetally localized ring. Then, by the late-gastrula stage these 2 factors are eliminated from cells in the ventral part of the developing gut (Figure 1C, i-vi). Second, at the late-gastrula to early-larva transition these same germ cell factors are then eliminated from cells in the right side of the developing gut, and the remaining left-sided cells form a germ cell pouch (GCP), previously referred to as the posterior enterocoel (Figure 1C, ix-xiv). In order to quantify the changes in mRNAs of germ factors during this dynamic period, we performed qPCR. Our results show that during the dorsal and left phases of restriction Vasa and Nanos mRNA significantly decreases (Figure 1C, xvii-xviii). This suggests that Vasa and Nanos mRNA is lost from cells in the ventral and right part of the developing gut.

Nodal is required for the restriction of germ cell factors

We next sought to determine what embryonic signal(s) could be involved in the dorsal and left restriction of Vasa and Nanos. Previous research in a closely related animal, the sea urchin, shows that Nodal is required for the patterning of the Dorsal/Ventral and Left/Right axes [17, 18]. In order to test if Nodal is relevant for this restriction in the sea star we first determined where Nodal mRNA was localized in sea star development [12]. We found that Nodal is expressed in the ventral side of the

developing embryo during the blastula stage and also is expressed in the right side of the embryo during the late gastrula stage (Figure 1C, vii and xv). These data suggest that Nodal is expressed either in close proximity to cells that will lose germ cell factor mRNA's or in these cells themselves (Figure 1C; i, ii, ix, and x dotted oval).

In order to test if Nodal is required for the dorsal and left restriction of Vasa and Nanos we perturbed Nodal signaling in two ways. First, we inhibited Nodal signaling with the pharmacological inhibitor SB-431542, previously shown to inhibit Nodal signaling in the sea urchin [18]. Our results show that when Nodal signaling is downregulated Vasa shifts its localization such that there is less restriction compared to embryos treated with DMSO as a control (Supplemental Figure 1, A-D). Second, we inhibited Nodal signaling by injection of a translation blocking morpholino. As embryos were injected with increasing amounts of morpholino we saw a dose dependent phenotype (Supplemental Figure 1, E-G). With an intermediate amount of Nodal morpholino the majority of embryos were inhibited from restricting Vasa in the left side of the embryo. With a higher concentration of Nodal morpholino the majority of embryos were inhibited from restricting Vasa in the dorsal side of the embryo. Together these results suggest that Nodal is required for the dorsal and left restriction of the germ cell factor Vasa.

Nodal's effect on the dorsal restriction of Vasa could also be due to a delay in development. We tested this premise by injecting a concentrated amount of Nodal morpholino and fixing embryos at an earlier developmental time point (when the controls were morphologically indistinguishable from the Nodal morphants). We found that two germ cell factors, Nanos and Vasa, failed to restrict to the dorsal half of the

embryos in 97% and 94% of Nodal morphants, respectively, while they were properly restricted to the dorsal half in 98% and 95% of control morpholino injected embryos, respectively (Figure 2, C and D). These data strongly suggest that Nodal is required for the sequential restriction of Nanos and Vasa and that Nodal knock-down is not simply causing a delay in embryonic development.

To test if embryos injected with Nodal morpholino had decreased Nodal signaling we performed several control experiments. First, we saw that two dorsal-sided markers (Nodal and Tbx2/3) became radialized in the majority of Nodal morphants (Figure 2, E and F). In addition, we saw that a known transcriptional target of Nodal signaling in animals, Lefty, is downregulated in Nodal morphants (Figure 3, B and C). As Nodal signaling is required for establishing the Dorsal/Ventral and Left/Right axes in the sea urchin these data suggest that the Nodal morphants indeed have decreased levels of Nodal signaling and that Nodal likely acts in a conserved way to establish these axes in both the sea urchin and sea star.

Nodal decreases levels of germ cell mRNA via both inhibition of transcription and post-transcriptional degradation mechanisms

Nanos and Vasa mRNAs significantly decreased in abundance during the developmental time of Nodal dependent spatial restriction to the dorsal and left side of the embryo (Fig 1C). In order to test if Nodal inhibits the transcription of Nanos and Vasa we used a nascent RNA capture system. We incubated control embryos and Nodal morphant embryos with the nucleotide analog EU at the mid gastrula stage. Following isolation of nascent RNA that had incorporated EU we saw that Nodal morphant embryos had significantly higher levels of both Nanos and Vasa RNA compared to control embryos via qPCR (Figure 3C). The results show that Nodal

signaling decreases both Nanos and Vasa transcription. In addition, we found that “old” (EU-minus, produced before the EU pulse) Nanos and Vasa mRNA was also significantly increased in Nodal morphant embryos compared to control embryos (Figure 3B). This suggests that Nodal is also required to decrease the half-life of Nanos and Vasa mRNA produced prior to the mid-gastrula stage. As a control, we measured the levels of Lefty RNA (a known target of Nodal signaling). As expected, we saw decreased levels of Lefty RNA in Nodal morphants compared to control embryos, reflective of decreased Nodal function (Figure 3, B and C). Together, these results imply that areas not influenced by Nodal signaling are able to retain important germ cell factor mRNAs, whereas cells under the influence of Nodal reduced both transcription and retention of germ cell factors. The overall result causes the selective retention of a germ cell fate in time and space.

Apoptosis is not a mechanism that contributes to the restriction of Nanos and Vasa positive cells in the ventral and right side of the embryo

Another mechanism that could contribute to the decrease in Nanos and Vasa positive cells is programmed cell death. To test this hypothesis we used a DeadEnd Fluorometric TUNEL System to determine if any cells in the ventral or right side of the developing gut are undergoing apoptosis. Our results show that very few cells are undergoing apoptosis during development in the sea star (Supplemental Figure 2, A and B). In addition, the localization of cells undergoing apoptosis is stochastic and does not correlate with Nanos and Vasa positive cells. These results show that programmed cell death does not significantly contribute to the loss of Nanos and Vasa mRNA positive cells in the ventral or right side of the developing gut.

Nodal inhibits tissue morphogenetic events involved in germ cell pouch formation

In addition to the molecular restriction of germ cell mRNAs, an obvious visual metric is also associated with this fate decision. While germ cell factors are being restricted to cells on the left side of the gut, these same cells undergo a distinct out pocketing from the left side of the endoderm and form a morphologically distinct pouch. To examine the morphogenetic events involved in normal germ cell pouch formation with greater detail, we used Scanning Electron Microscopy (SEM) to study this process. In normal development we notice that cells in the mid-dorsal part of the archenteron initially form a bulge in the epithelium (Figure 4Ai, these are likely cells that contain Nanos and Vasa RNA during the dorsal restriction of germ cell RNA) as we have seen by light microscopy. Slightly later in development, we notice that an out pocketing buds off of the dorsal-left part of the archenteron (Figure 4Aii and iii, these are likely cells that contain Nanos and Vasa RNA during the left restriction of germ cell RNA) consistent with its identity as the germ cell pouch.

To determine how Nodal affects the morphogenetic events involved in germ cell pouch formation we analyzed pouch formation in Nodal morphant embryos. By SEM, we found 2 phenotypes 1) ectopic bulging in the ventral and right side of the developing gut (Figure 4Bi and ii) and 2) ectopic pouch formation in the ventral and right sides of the developing gut (Figure 4Biii). These results suggest that Nodal signaling in the ventral side of the embryo inhibits cell shape changes that cause a bulge off the developing gut epithelium. These results also suggest that Nodal signaling in the right side of the embryo inhibits cell shape changes that cause the germ cell pouch to bud off

of the developing gut. In summary, Nodal signaling is required to inhibit cellular morphogenetic events leading to germ cell pouch formation.

Discussion

We present evidence within inductive germ cell specification that an embryonic signal is required for the sequential restriction of germ cell RNA's. We propose sequential restriction is involved in inductive germ cell specification in many animals. That is, germ cell promoting factors are initially present in broad embryonic regions and subsequent rounds of signals that inhibit these factors increasingly narrow the field of cells in which these factors are retained. This inductive mechanism can subsequently restrict the amount of cells that retain a multipotent early embryonic cell fate (as germ cell promoting factors are also required for multipotency [14, 19]). However most importantly, these restrictive signals can define which cells ultimately retain these factors and become primordial germ cells.

In the sea star, the conserved germ cell mRNAs Nanos and Vasa accumulate in broad embryonic domains and rely on embryonic signals for the restriction of these germ cell factors. This mechanism is likely present in other animals such as *Nematostella* based on a similar accumulation of germ cell factors: that is, early broad expression followed by sequential restrictions into smaller and smaller embryonic domains [20]. Future studies should test if these traditional germ cell RNAs are required for germ cell specification in these animals and if an embryonic signal is required for their subsequent restriction.

In some animals we see evidence of sequential restriction as part of the germ cell specification mechanism. In the cricket embryo, for example, traditional germ cell

factors Piwi and Vasa are present ubiquitously at low levels during early cleavage stages. However by the late blastoderm stage, these germ cell factors only accumulate in the germ band. The multipotent germ band gives rise to the embryo proper (which includes germ cells) while the other tissues are considered extra embryonic [9].

Therefore, at this stage in development one might conclude that the restriction in potential to become the germ lineage is associated with the selective accumulation of germ cell factors in the cells that can become the germ lineage and a selective loss of germ cell factors in the cells that can no longer become the germ cell lineage. In order to test this hypothesis, it will be important to determine if the Piwi and Vasa mRNA in the germ band are retained from the early embryo and if their germ band accumulation is dependent on a restrictive signal.

In addition, from seeing the sequential restriction mechanism in the sea star, we propose that sequential restriction is also present in other animals even where conserved germ cell factors (such as Nanos, Vasa, and Piwi) are not present in early development. In the mouse, for example, germ cell promoting factors are either 1) genes that promote a multipotent cell fate in vertebrates or 2) are widely used signals that promote a primordial germ cell fate in mammals. First, in the case of vertebrate specific genes such as Oct4, Sox2, and Nanog these factors accumulate selectively in the inner cell mass which gives rise to the multipotential epiblast [21]. The epiblast cells therefore retain the potential to become the germ cell lineage while the extraembryonic tissues lose it. Second, in the case of widely used signals (ie BMP4 and Wnt3) that promote a germ cell fate in mammals, these factors act selectively in the posterior epiblast [22, 23]. The posterior epiblast cells that receive these germ cell promoting

signals become the primordial germ cells. However, it is just as important to note that inhibitory signals such as Cer1 and Lefty1 (negative inhibitors of BMP and Wnt signaling) from the anterior visceral endoderm restrict the anterior epiblast from becoming the germ cell lineage [24-26]. Importantly, Nodal is the signaling pathway in the mouse that sets up the epiblast anterior-posterior axis [27]. More functional research in diverse animals will determine if Nodal specifically, or other TGF β factors, function conservatively in the sequential restriction of germ cell potential during early development.

This research and our analysis of the literature predicts two types of molecules that may function in inductive germ cell specification via sequential restriction:

First, we predict that broad patterning molecules that inhibit a germ cell fate are likely involved in germ cell specification. This is not the first time molecules involved in patterning the entire embryo have been implicated in germ cell specification. In the mouse, for example, Wnt3 is doubly required for specification of the mesoderm and the primordial germ cell fate [23]. In addition in the axolotl, the molecules FGF and BMP4 are required for the specification of multipotent mesoderm precursors which give rise to PGC's and other somatic cell fates [8]. A long-held question in these animals is how the germ cell fate is specified uniquely. Growing evidence suggests the combination of broad patterning signals that both promote and restrict the primordial germ cell fate throughout multiple stages in development is required for the proper specification of the primordial germ cell fate. Most importantly this predicts that broadly expressed molecules that inhibit the germ cell fate are involved in germ cell specification in many animals.

Second, we predict that molecules involved in cellular morphogenetic changes are also likely involved in inductive germ cell specification. In the sea star, we see that cellular morphogenesis is required for the formation of a germ cell pouch (Figure 4). Furthermore, the inhibition of cell morphogenesis restricts where this germ cell pouch forms (Figure 4). The formation of a coelom is also associated with germ cell specification in crickets and axolotl. In general, a coelom is a fluid-filled sac that is coated with cells of mesodermal origin. In crickets, germ cells accumulate adjacent to coelomic pouches as they are formed and germ cells subsequently localize medial to these coelomic pouches. Decreasing levels of Twist by RNAi, an important regulator of mesodermal morphogenesis, causes the complete loss of germ cells or a significant decrease in number of germ cells [9]. This shows that the proper morphogenesis of mesodermal coeloms is required for germ cell specification in the cricket. Germ cells are also associated with a coelom during axolotl development. At stage 45, germ cells protrude into a coelom [28]. In addition, an underappreciated event in germ cell specification in the mouse, is how the cells in the posterior proximal epiblast become nestled in the extra embryonic region to receive PGC inducing signals [29]. We argue that mesodermal morphogenetic events during development of these animals are likely required for germ cell specification to occur properly.

We urge many more studies in diverse animals to test how broad embryonic patterning signals that inhibit a germ cell fate and molecules that promote cellular morphogenetic events contribute to inductive germ cell specification. When this is done then we can more accurately speculate conserved and ancestral mechanisms of inductive germ cell specification. Identifying conserved features of inductive germ cell

specification will be important to ultimately understand the complete biology of conserved immortal cells- the germ lineage.

Materials and methods

Embryo culture-

Adult *Patiria miniata* animals were collected by either Pete Halmay (PeterHalmay@gmail.com) or Josh Ross (info@scbiomarine.com) off the California coast. Embryos were cultured essentially as described before [16].

In situ hybridization-

DIG-labelled RNA probes were made with a Roche DIG probe synthesis kit as described before [16]. A list of primers used for probe synthesis can be found in Supplemental Table 1. Embryos were incubated with probe for 1 week and were developed essentially as described before [16]. For statistical analysis of the localization of in situ probes in control vs. morphant embryos, experiments were performed either in duplicate or triplicate and a student t-test was used to analyze significance.

Pharmacological inhibition-

Embryos were incubated with 15 μ M SB-431542 at the developmental time points indicated.

Morpholino injection-

Oocytes were injected with either 0.1 mM, 0.5 mM or 1.0 mM of morpholino (Gene-Tools) in injection solution: 10% glycerol and 1 mM Texas Red Dextran or 0.83 mM FITC (Molecular Probes). After incubation for 1 day at 16° Celsius, injected fluorescent oocytes were selected and matured with 1-methyl adenine (3.0 μ M Acros Organics).

Eggs were fertilized with diluted sperm, washed, and incubated in filtered sea water at 16° Celsius until they developed into the desired embryonic stage.

Nodal morpholino sequence- 5' TCAAGTTCTTGGTCATTCTCGAAAC 3'.

cDNA synthesis-

RNA was extracted from ~150 embryos in each desired stage by using the RNeasy Micro kit (Qiagen). A reverse transcription reaction was performed using M-MLV Reverse Transcriptase (Promega) or M-MuLV Reverse Transcriptase (Thermoscientific, Maxima first strand cDNA synthesis kit).

qPCR analysis-

Sybr green was used for qPCR analysis (Thermofisher). Experiments were done either in duplicate or in triplicate and ubiquitin or 18s was used to normalize RNA levels between samples. All data is represented as fold-change relative to a specific sample. A student t-test was done to calculate significance. Error bars reveal +/- 1 standard deviation. A list of primers used can be found in Supplemental Table 2.

Nascent RNA Collection-

244 oocytes were injected with control morpholino and 305 oocytes were injected with Nodal morpholino as described above. 1 mM EU-RNA was added to embryos 27 hours post fertilization, at the early to mid-gastrula stage. RNA was isolated from larva stage embryos (2 days later) as described above. Nascently transcribed RNA that incorporated EU-RNA was isolated using a Click-It nascent RNA kit (ThermoFisher). RNA that did not bind to the streptavidin magnetic beads, termed "Old RNA", was precipitated overnight and resuspended in the same volume as nascently transcribed and captured RNA. cDNA synthesis and qPCR was performed as described above.

Scanning Electron Microscopy-

Embryos were fixed 1 hour at 25° C and overnight at 4° C in primary fix (1% glutaraldehyde, 80% sea water, pH 8.0-8.2). Embryos were washed 2 times in 1.25% Sodium Bicarbonate, pH 7.2-7.4 and then incubated in secondary fix for 1 hour at 25°C (2% Osmium tetroxide in 1.25% Sodium Bicarbonate). Embryos were rinsed 2 times in distilled water and then stained in Uranyl Acetate for 1 hour at 25°C. After 2 washes in distilled water, embryos were dehydrated in a series of ethanol dilutions. Embryos were incubated in the following ethanol dilutions 2 times for 5 minutes: 30%, 50%, 70%, 90%, 95%, and 100%. After dehydration embryos were placed in baskets and dried in CO₂ with a Ladd Research Industries critical point dryer. Dried embryos were poured on top of sample stubs with conductive carbon tabs. Samples were coated with gold using an Emitech K550 sputter coater. Samples were visualized on a Hitachi 2700 Scanning Electron Microscope.

Tunel Assay-

Double stranded breaks were labelled with fluorescein via a DeadEnd Fluorometric TUNEL System (Promega). Briefly, embryos were grown to the desired stage, fixed in 4% PFA in PBS, and permeabilized with 0.2% TritonX-100. Then Terminal Deoxynucleotidyl Transferase was added to the embryos in combination with fluorescein-12-dUTP. After 3 washes in PBS, embryos were imaged on a Zeiss LSM 800 confocal microscope.

Acknowledgements

We thank Geoff Williams for valuable training in sample preparation and advanced microscopy techniques.

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Figures and Tables

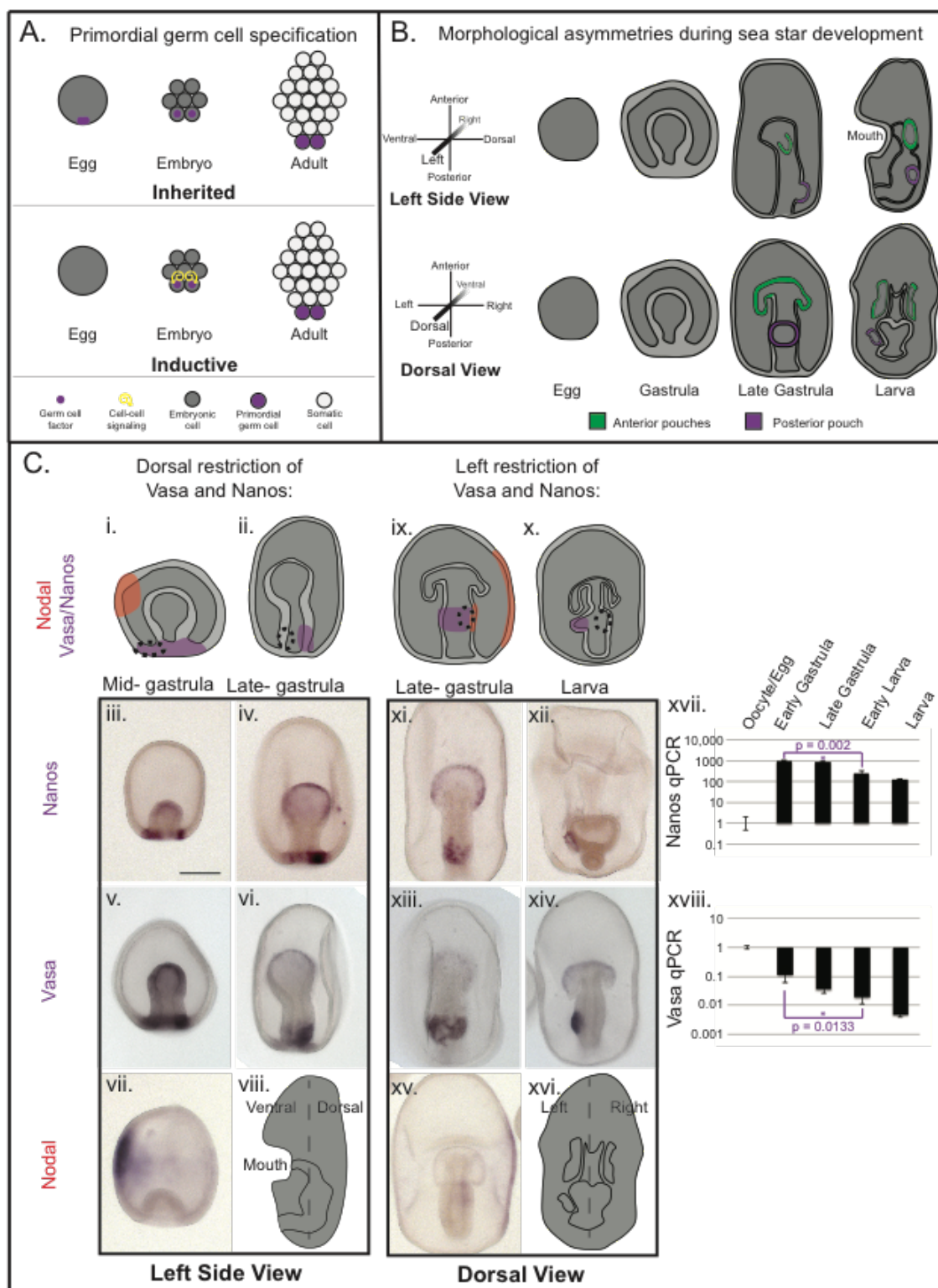


Figure 1

A) Primordial germ cells can be specified by either inherited mechanisms or inductive mechanisms. The inherited mechanism requires asymmetric localization of germ cell factors in the egg and whichever embryonic cells acquire these factors will become the germ cell lineage. In the inductive mechanism, germ cell factors are either not present or are present ubiquitously in the egg and embryonic signals instruct which cells will accumulate germ cell factors and will eventually become the germ cell lineage.

B) Dorsal/Ventral asymmetry in sea star development can be seen by two morphological events best visualized in the left side view. The mouth forms on the ventral side of the outer cell layer while a posterior pouch begins to form on the dorsal side of the developing gut (purple). Left/Right asymmetry in sea star development can be seen by one morphological event best visualized in the dorsal side view. The posterior pouch is only present in the left side of the larva (purple).

Ci-viii) Germ cell factors Vasa and Nanos are present in a vegetal ring at the mid-gastrula stage (purple). By the late gastrula stage Vasa and Nanos only accumulate in the dorsal side of the developing gut (purple). During the dorsal restriction of germ cell factors Nodal is present in the ventral side of the embryo (red). Areas outlined with a dotted circle lose the presence of germ cell factors. Scale bar is 100 microns.

Cix-xvi) Germ cell factors Vasa and Nanos are present in a dorsal patch at the late gastrula stage (purple). By the early larva stage Vasa and Nanos only accumulate in the left side of the developing gut (purple). During the left restriction of germ cell factors Nodal is present in the right side of the embryo (red). Areas outlined with a dotted circle lose the presence of germ cell factors.

Cxvii-xviii) Nanos and Vasa RNA sequentially decrease during early embryogenesis via qPCR analysis.

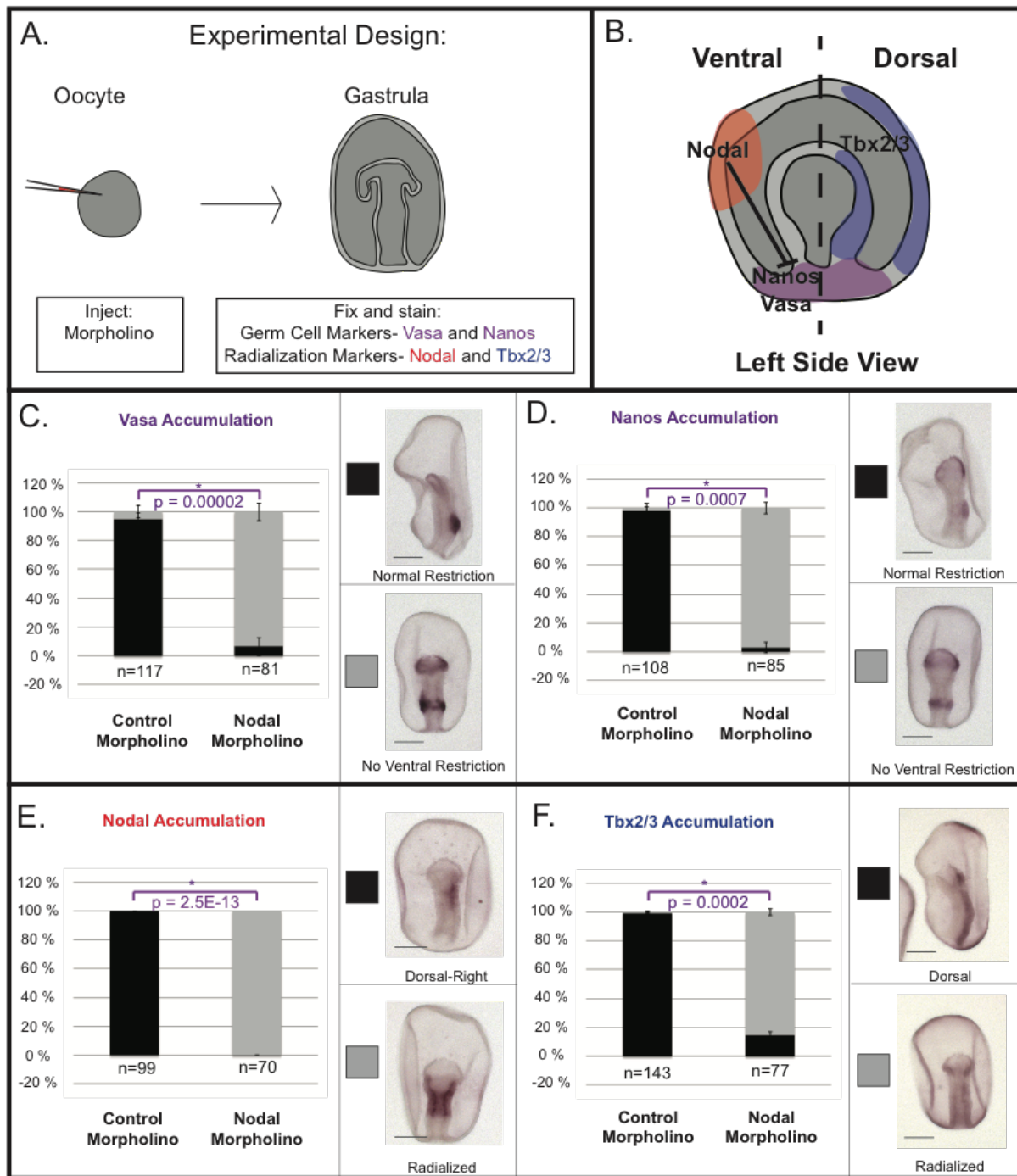


Figure 2

A) Experimental design: Oocytes were injected with Nodal morpholino or control morpholino and the resulting embryos were fixed at the gastrula stage. Germ cell markers (purple) and radialization markers (red and blue) were visualized by an RNA in situ hybridization. Scale bars are 100 microns.

B) Our results support the model that Nodal expression (red) in the ventral side of the embryo is required for the loss of germ cell factors (purple) in the ventral side of the embryo. Nodal expression in the ventral side of the embryo is also required for the later restriction of itself and Tbx2/3 to the dorsal side of the embryo.

C-D) Vasa and Nanos accumulate in a dorsal patch on the gut in 95% and 98% of embryos injected with control morpholino. In contrast, Vasa and Nanos are not restricted and instead accumulate in a ring around the gut in 94% and 97% of embryos injected with a Nodal morpholino.

E-F) Nodal and Tbx2/3 accumulate in the dorsal side of the embryo in 100% and 99% of embryos injected with control morpholino. In contrast, Nodal and Tbx2/3 are not restricted and instead become radialized in 100% and 86% of embryos injected with a Nodal morpholino.

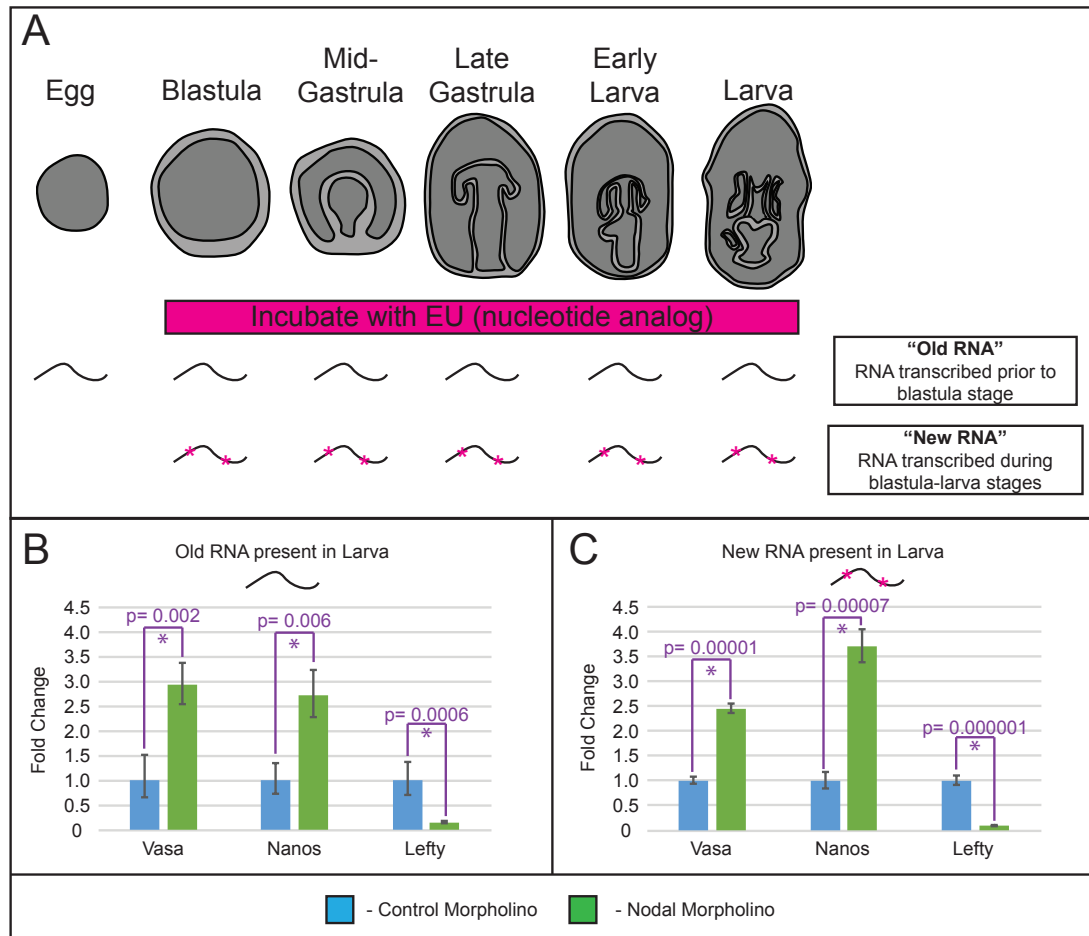


Figure 3

A) Experimental design: EU RNA nucleotide analog was added to embryos at the blastula stage. RNA was extracted from larva stage embryos and “New RNA” (EU+) was separated from “Old RNA” (EU-) by covalently attaching biotin to EU and using magnetic biotin beads.

B) qPCR analysis shows that germ cell factor RNA's (Vasa and Nanos) produced prior to EU labelling are significantly increased in embryos injected with Nodal morpholino (green) compared to embryos injected with control morpholino (blue). As a control for inhibition of the Nodal signaling pathway, Lefty RNA levels are significantly decreased in embryos injected with Nodal morpholino (green) compared to embryos injected with control morpholino (blue).

C) qPCR analysis shows that germ cell factor RNA's (Vasa and Nanos) produced during EU labelling are significantly increased in embryos injected with Nodal morpholino (green) compared to embryos injected with control morpholino (blue). As a control for inhibition of the Nodal signaling pathway, Lefty RNA levels are significantly decreased in embryos injected with Nodal morpholino (green) compared to embryos injected with control morpholino (blue).

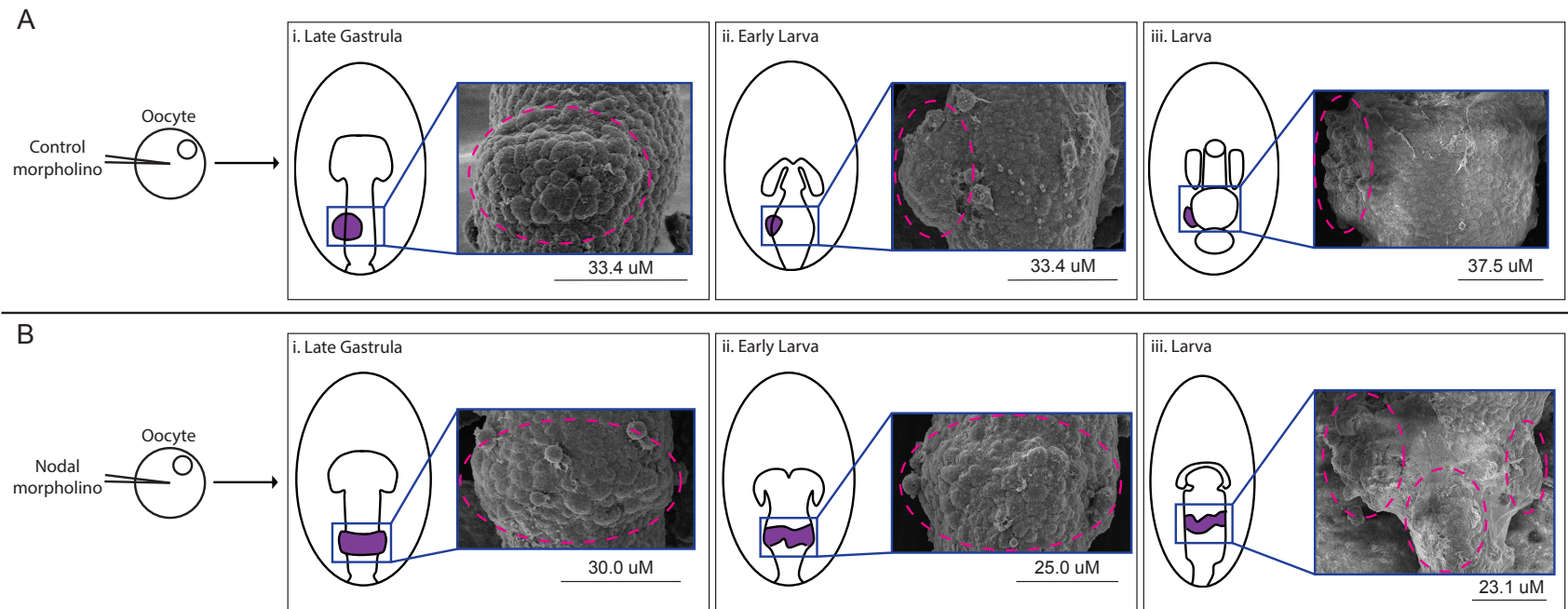
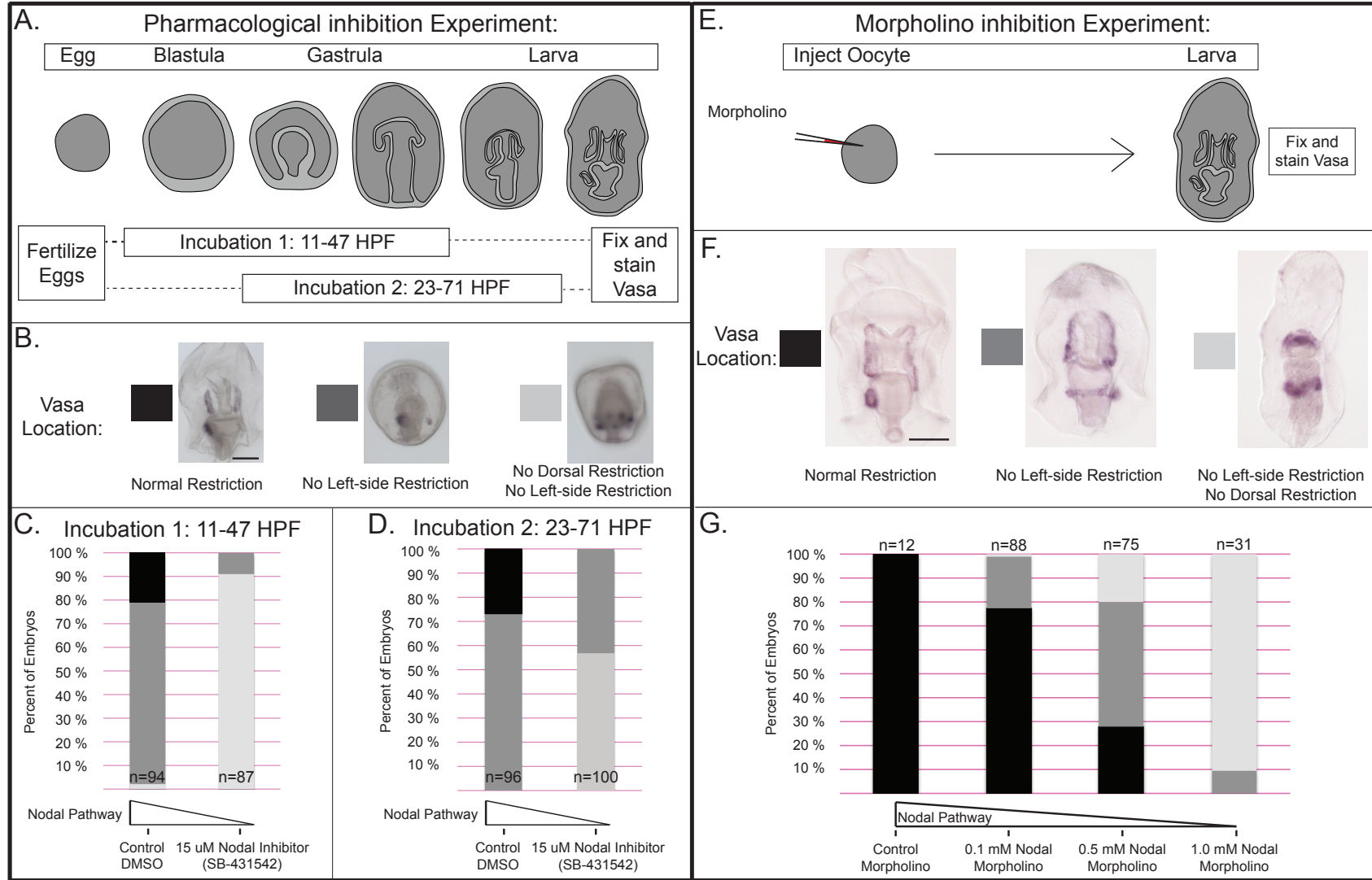


Figure 4

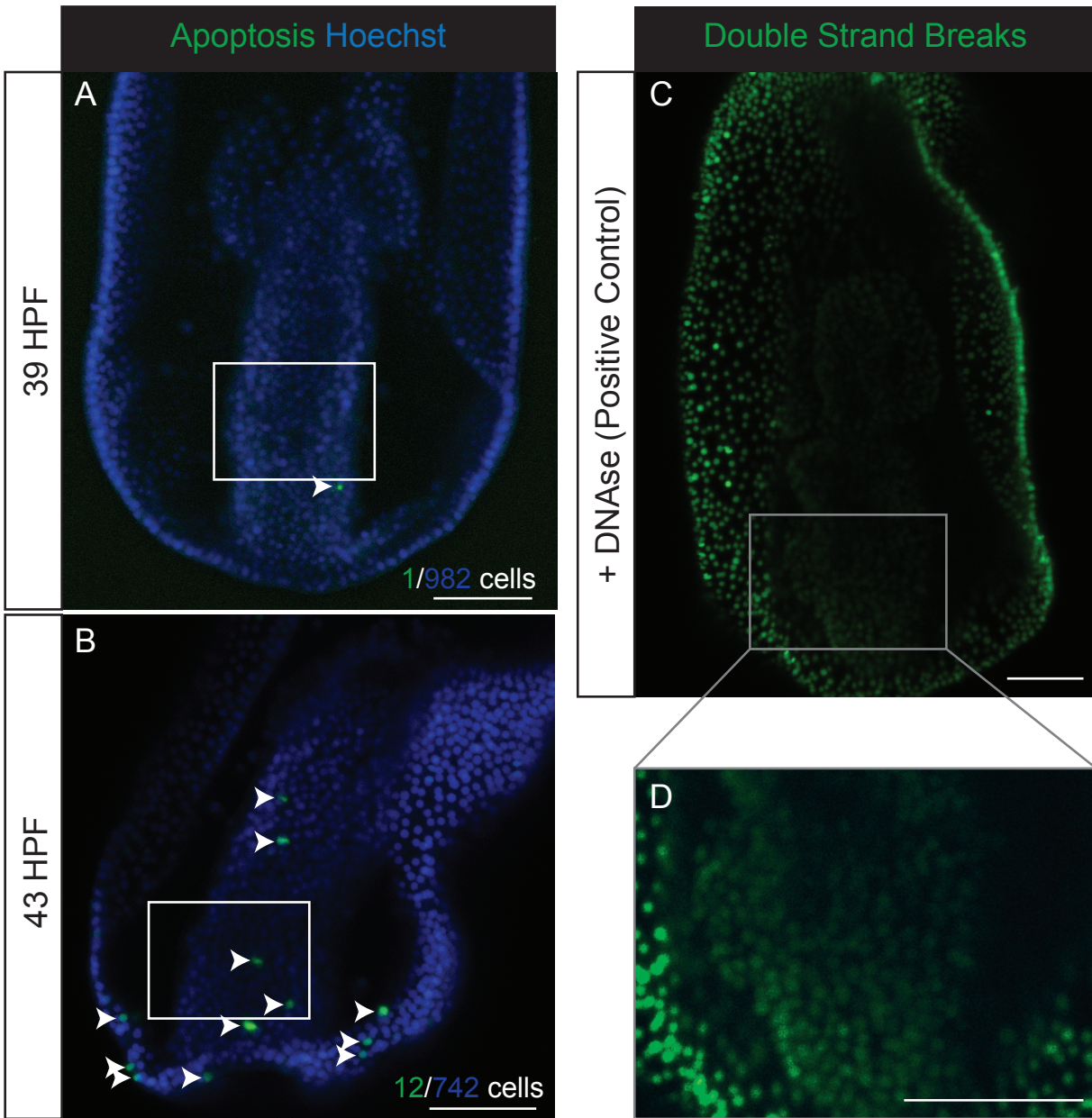
A) When oocytes are injected with control morpholino a patch of cells bulge out of the dorsal side of the gut during the late gastrula stage. By the early larva and larva stage, cells only bulge out from the left side of the gut and only 1 pouch begins to form.

B) When oocytes are injected with Nodal morpholino cells bulge out in a ring around the gut during the late gastrula stage and early larva stage. By the larva stage multiple discrete pouches begin to form radially all around the gut.



Supplementary Figure 1

- A)** Pharmacological experimental design: Embryos were incubated with 15 μ M Nodal inhibitor (SB-431542) from either 11-47 hours post-fertilization or from 23-71 hours post fertilization. Embryos were then fixed at the larva stage and Vasa mRNA was localized via an in situ hybridization.
- B)** When embryos were treated with inhibitor Vasa was localized in 3 different patterns. If there was normal restriction then Vasa was localized in the left side of the gut (black). If there was no left-side restriction then Vasa was localized in the dorsal side of the gut (dark gray). If there was no dorsal or left side restriction then Vasa was localized in a ring around the gut (light gray). Scale bar is 100 microns.
- C)** When embryos were treated with inhibitor at an earlier time point then there was a shift in Vasa localization such that there is less restriction in embryos treated with inhibitor relative to embryos incubated with DMSO as a control.
- D)** When embryos were treated with inhibitor at a later time point then there was a shift in Vasa localization such that there is less restriction in embryos treated with inhibitor relative to embryos incubated with DMSO as a control.
- E)** Morpholino experimental design: Oocytes were injected with Nodal morpholino or control morpholino at varying concentrations. The resulting embryos were fixed at the larva stage and Vasa was localized via an RNA in situ hybridization.
- F)** When embryos were injected with Nodal morpholino at different concentrations Vasa was localized in 3 different patterns. If there was normal restriction then Vasa was localized in the left side of the gut (black). If there was no left-side restriction then Vasa was localized in the dorsal side of the gut (dark gray). If there was no dorsal or left side restriction then Vasa was localized in a ring around the gut (light gray). Scale bar is 100 microns.
- G)** There is a dose dependent effect of Nodal morpholino upon Vasa restriction. Oocytes injected with morpholino at 0.1 mM result in 22% of embryos with a defect in left side restriction. Oocytes injected with 0.5 mM morpholino result in 52% of embryos with a defect in left side restriction and 20% of embryos with a defect in both left and dorsal side restriction. Oocytes injected with 1.0 mM Nodal morpholino result in 10% of embryos with a defect in left side restriction and 90% of embryos with a defect in both left and dorsal side restriction.



Supplementary Figure 2

A) During the ventral clearance of Nanos and Vasa RNA (~39HPF) only a few cells are fluorescently labelled with a marker of apoptosis (arrowhead) and they are not concentrated in a specific embryonic domain.

B) During the right clearance of Nanos and Vasa RNA (~43HPF) only a few cells are fluorescently labelled with a marker of apoptosis (arrowheads) and they are not concentrated in a specific embryonic domain.

C) Positive control, nuclei of embryos treated with DNase.

D) Inset of C. All scale bars are 50 microns.

Gene name	Primers	Accession
Lefty	F- ATGGAGTCTCGCGTAGCTGT R- CATGTTTGTGACGGGTCTG	N/A
Nanos	F- ATATGAGCTGGCTGACAACG R- TGATATTCAATGCTAGGCCTAATAGA	KU594505
Nodal	F- CGGTGGATCGTCTACCCTAA R- CCCGATCAAATTGTAAAAATGC	KC669538
Tbx2/3	F- GGCCAACGACATTTTGAAGT R- GCTTAACGCTGAAGGGTCTG	N/A
Vasa	F- CGGTCCAGAAGTACGGGATA R- GTAGAAGCTGGTTGCCTTGC	FJ605737

Supplemental Table 1- Primers used for in situ probe synthesis

Gene name	Primers
Lefty	F- GACCTGACCTCAATCCCTGC R- CTGATGCTGATGGGTGGTGT
Nanos	F- CCGAAGAGTTGACGAGGAAG R- CAACTCCAAGCACCCACAG
Ubiquitin	F- TTCGGTGAAAGCCAAGATTC R- CCCACCTCTCATGGCTAGAA
Vasa	F- TGGCTGATGCTCAACAAGAC R- AAAGTTTCCGCCTCCGTAAT
18S	F- CGCGAGATTGAGCAATAACA R- GTACAAAGGGCAGGGACGTA

Supplemental Table 2- Primers used for quantitative PCR

Chapter 5: Conclusions, Speculations, and Future Directions

Changes involved in the evolution of inherited germ cell specification in echinoids

Evidence suggests that the most recent common ancestor of echinoderms (at least asteroids, ophiuroids, holothuroids, and echinoids) used primarily inductive mechanisms for germ cell specification. However, an inherited germ cell specification mechanism likely evolved in the echinoid lineage (Chapter 3) [1, 2]. The nested position that echinoids hold within the echinoderm clade suggests they differ because they have derived traits. To better understand the origin of these unique derived traits I characterize development in another echinoderm species that does not have these derived traits, the sea star, *Patiria miniata*. Based upon mechanistic research in sea urchins (an echinoid) and sea stars (an asteroid) I find four main developmental phenomena that have changed in relation to the evolution of an inherited mechanism in the echinoid lineage. An inherited germ cell specification mechanism correlates with 1) early unequal cell divisions, 2) the loss of a posterior coelomic pouch, 3) changes in the Nodal signaling pathway, normally involved in germ cell restriction in the sea star, and 4) possible changes in the Wnt and BMP signaling pathways, likely involved in promoting a germ cell fate in the sea star (Figure 1).

1) Evolution of early unequal cell divisions that correspond to early localization of germ cell factors

Out of the 5 extant echinoderm clades, only the echinoid clade (which includes the sea urchin) has early unequal cell divisions. At the 5th cleavage the vegetal most cells, termed micromeres, divide into two vastly different cell fates, the large micromeres and the small micromeres (Figure 4). The large micromeres are bigger in size and take on a differentiated skeletal cell fate, while the small micromeres are smaller and take on

a germ cell fate (Appendix D) [3]. For the germ cell lineage to have evolved in this manner two things must have occurred: 1) early vegetal localization of molecules that contribute to unequal cell divisions and 2) early vegetal localization of molecules that contribute to the germ cell fate. In order to determine if these events act independently or together to confer a germ cell fate more work needs to be done to determine which vegetal molecules are involved in unequal cell division and which are involved in conferring a germ cell fate. I speculate on how to do so here.

To identify candidate molecules involved in unequal cell divisions I have examined two well-studied examples in the literature. The first example is the first cell division in the round worm, which is asymmetric in size and in daughter cell fates (Figure 2). (Intriguingly the germ cell fate is associated with the posterior smaller cell, analogous to sea urchins.) PAR proteins are asymmetrically distributed along the anterior/posterior axis such that Par1 and 2 localize in the posterior and Par 3 and 6 localize in the anterior. Once the Par proteins become asymmetrically distributed, the posteriorly localized heterotrimeric G protein subunit α links the cell membrane to the spindle microtubules with the help of adaptor proteins and dynein. The association of the spindle with the posterior membrane generates a force that causes the spindle to align parallel to the Anterior/Posterior axis favoring the posterior side. The second example of asymmetric cell division is in neuroblast cells in *D. melanogaster*, this cell division gives rise to one neuroblast cell and one ganglion mother cell (Figure 3). Proteins (such as Prospero and Numb) involved in specifying the ganglion cell fate are asymmetrically patterned prior to cell division. Cell polarity is established by the asymmetric accumulation of Par proteins as well. Par3 and 6 once again localize at one

pole, in this case on the apical side. The heterotrimeric G protein subunit α interacts with Par3 and 6 and associated proteins and this causes an asymmetric force on the spindle such that it rotates to be aligned with the apical-basal axis. Subsequently, the spindle arms become longer on the apical side, which generates the unequal cell size [4]. Overall, *D. melanogaster* and *C. elegans* use similar molecular machinery for the orientation of their mitotic spindles during asymmetric cell divisions.

Recently, our laboratory has tested if sea urchins use similar molecules for their early unequal cell divisions. Our laboratory found that the heterotrimeric G protein subunit α and the dynein binding protein NuMA are also required for the early asymmetric cell division in sea urchins (Poon et al., in preparation) (Figure 4). This shows that sea urchins likely use conserved molecules and mechanisms for the asymmetric placement of the spindle. In regards to asymmetric fate determinants, Vasa protein is the only known germ cell factor that asymmetrically accumulates in the vegetal pole. Our laboratory's preliminary results suggest Vasa protein accumulates asymmetrically on the vegetal-most spindle during early unequal cell divisions via localized translation (Poon et al., in preparation). Remaining questions include: How is Vasa protein preferentially translated on the spindle in the vegetal region? How do molecules involved in asymmetric positioning of the spindle accumulate in different regions of the vegetal cells? And do they both rely on similar machinery? The only known molecules that localize asymmetrically in the vegetal pole of sea urchins while asymmetric cells divisions take place are the Wnt signaling molecules Disheveled and nuclear β -catenin [5, 6]. It remains to be discovered if molecules involved in spindle placement or specification of the germ cell fate interact with these asymmetrically

localized Wnt signaling molecules. Once all of these phenomena are understood we can attempt to force unequal cell divisions and the translation of Vasa in the vegetal pole in non-echinoid echinoderms. For example, we can synthesize a sea urchin mRNA that codes for a protein that links Vasa translation to the vegetal pole and inject this construct into the oocytes of sea stars. In this way we can test which combination of molecules are sufficient for the evolution of an earlier (more inductive) mode of germ cell specification (Figure 4).

2) Loss of posterior coelomic pouches

Out of the 5 extant echinoderm clades, the echinoid clade is also the only member in which a coelomic pouch does not invaginate out of the mid-gut. Interestingly, this is the site that germ cell factors accumulate and the proposed site of germ cell induction in all other echinoderm clades [2]. I hypothesize that the posterior coelomic pouch was only able to be lost in echinoids after inherited germ cell specification evolved. This prompted me to ask: How is an entire larval structure lost? In order to answer this question, I must 1) determine what molecules are involved in the formation of a posterior coelomic pouch and 2) determine how the regulation of these molecules changed during echinoid evolution such that they are no longer used for pouch formation.

In the sea star, I found that the posterior coelomic pouch likely forms by cell shape changes in the left side of the developing gut that promote a territory of epithelial cells to bud off or invaginate (Fresques and Wessel, in preparation) (Figure 7). In order to identify molecules that might be involved in this morphogenesis I will analyze two examples in the literature in which epithelial layers undergo invagination. The first

example is neural tube formation in the frog embryo (Figure 5). Briefly, N-cadherin accumulates in neural cell precursors, while the remaining epidermal cells only have E-cadherin. This likely causes neural cell precursors to have a differential affinity for each other. N-cadherin also allows filamentous-actin and activated-myosin (actomyosin) to organize in the apical region of the neural cells which causes them to constrict and enter the body cavity [7]. The second example is mesoderm formation in the fruit fly (Figure 6). Briefly, the transcription factors Twist and Snail ensure the upregulation of N-cadherin and the downregulation of E-cadherin. Then, apical accumulation of a Rho activating protein causes actomyosin constriction. These contractions force the cytoplasm to the basal side of the cell, which results in furrow formation [8-10]. Together this shows that cadherins, filamentous actin, and genes involved in activating myosin are required for epithelial invagination in both frogs and flies.

We need to determine if similar molecules (cadherins, f-actin, and actomyosin) are involved in invagination in the sea star. Although we still need to determine where all cadherins localize during sea star development I have noticed that G-cadherin mRNA may accumulate in germ cell pouch precursor cells [11]. This may play a role in the adhesion of germ cell pouch precursor cells while they bud out of the epithelium. In the future we also need to determine where cytoskeletal molecules are localized to determine the mechanism that controls cell shape changes, in particular filamentous actin (f-actin), activated non-muscle myosin, and Rho GTPases. The RNA transcripts can be visualized with in situ RNA hybridizations, f-actin can be visualized by injecting Life-Act-GFP mRNA into the oocytes prior to fertilization, non-muscle myosin can be visualized with an antibody, and Rho GTPases can also be visualized with antibodies.

Lastly, we need to determine if any transcription factors or signals that promote epithelial invagination are expressed in germ cell pouch precursor cells, such as Snail, Twist, and BMP [12, 13].

Preliminary data suggests G-cadherin may be involved in germ cell biology in sea urchins and sea stars. In sea urchins G-cadherin has been shown to be required for germ cell maintenance. Germ cells in which G-cadherin was downregulated lose Vasa protein and improperly migrate into a group of skeletogenic cells [14]. I speculate that G-cadherin may be conservatively required for specification of the germ cell fate in both the sea urchin and sea star. However, the sea star likely uses it for pouch formation during the larva stage while the sea urchin uses it for asymmetric cell division during the 32-cell stage. It will be interesting to determine if G-cadherin underwent an heterochronic shift in the echinoid lineage as part of the germ cell specification pathway. If the cells that give rise to the posterior pouch in the sea star underwent a heterochronic shift in the sea urchin lineage then they would no longer localize as a patch in the endoderm during the larval stage, therefore no cells in the endoderm would have the program to initiate invagination. Together, this work will reveal the mechanisms that contribute to the loss of posterior pouch in the echinoid lineage.

3) Change in the germ cell restrictive signaling pathway, Nodal, during the establishment of Left-Right asymmetry

Previous research in an asteroid, the sea star, shows Nodal is required for the dorsal and left restriction of the germ cell fate (Chapter 4). In addition, germ cell factors are also restricted to the dorsal and/or left side of the developing gut in two other echinoderm clades, ophiuroids and holothuroids (Chapter 3) [2]. Because of this I conclude that Nodal signaling is probably required for germ cell restriction in these

animals as well. However, since the germ cell fate is specified much earlier in the sea urchin they no longer use Nodal signal for the restriction of their germ cell lineage. I therefore hypothesize that there are changes in the Nodal pathway in the sea urchin lineage that accompany this loss of function. Preliminarily, I see a difference in the initial expression of Nodal during the establishment of Left-Right asymmetry. The Nodal ligand is initially expressed broadly in the sea star endoderm and it subsequently accumulates in the right side of the endoderm and mesoderm (Chapter 2 and Figure 8). Although it is not known how Nodal mRNA becomes restricted to the right side of the endoderm I propose a conserved reaction diffusion mechanism is likely involved [15]. Specifically, Nodal is required for the expression of itself and its own inhibitor, Lefty. The increased diffusion rate of Lefty relative to Nodal causes Nodal to become restricted in the right side of the endoderm by being inhibited in the left side of the endoderm. In support of this hypothesis, the inhibition of Nodal signaling in sea stars causes a significant decrease in Lefty mRNA and a significant increase in Nodal mRNA which is coincident with the radialization of Nodal RNA (Chapter 4). In contrast, the Nodal ligand is initially expressed in a few cells in the right side of the endoderm in the sea urchin (Figure 8) [16]. This means that the widespread expression of Nodal has been lost in the endoderm of the sea urchin and that asymmetric Nodal expression does not depend on a reaction diffusion mechanism. Consistent with this, inhibition of Nodal does not cause a significant increase in Nodal mRNA or radialization of its expression domain [16]. These results show that the initial endodermal expression of Nodal mRNA and the presence of a reaction diffusion mechanism to restrict Nodal mRNA accumulation is different between asteroids and echinoids.

In order to determine if the evolution of an inherited germ cell specification mechanism caused a change in Nodal expression in echinoids many experiments need to be done. It will be necessary to determine how Nodal is expressed during Left/Right asymmetry in diverse echinoderm species. We need to test if Nodal is broadly expressed in the endoderm of other non-echinoid embryos by performing Nodal RNA in situ hybridizations. And we need to test if endodermal Nodal is restricted via a reaction-diffusion mechanism in other non-echinoid embryos by treating them with a Nodal inhibitor (SB-431542) and testing if this causes radialization of Nodal mRNA. If widespread endodermal expression is only lost in the sea urchin lineage, we need to determine what factors led to this change in expression. To do this we will have to determine how Nodal is regulated in the sea urchin and sea star. For example, we should compare the promoter region of the Nodal gene between sea urchin and sea star and look for any differences in sequence. Perhaps the loss of widespread endodermal Nodal in the echinoid lineage is a result of the loss of the left posterior enterocoel. The Nodal reaction diffusion mechanism is no longer used because Left-Right asymmetry is lost in the endoderm. The results of these experiments will reveal if Nodal expression in the endoderm is only changed in the echinoid lineage and the mechanisms that contribute to a change in Nodal expression.

Although Nodal does not have an effect on germ cell specification in sea urchins, it still mediates germ cell survival. Nodal is required to downregulate Nanos in the small micromeres that end up in the right coelomic pouch which subsequently causes them to undergo apoptosis. Meanwhile, the small micromeres that end up in the left coelomic pouch do not receive Nodal signal and survive [17]. It is interesting that Nodal has a

similar function in inhibiting a germ cell fate in both sea urchins and sea stars. However, Nodal is required during germ cell specification in sea stars while it is required much later than that in sea urchins. Germ cell death in the right coelomic pouch could occur because the left coelomic pouch is the primary embryonic tissue that metamorphoses into the adult sea urchin [18]. Although direct lineage analysis has not been completely done in other echinoderm clades it is clear that many different larval tissues that originate from anterior and posterior coelomic pouches undergo metamorphosis to contribute to the adult echinoderm animal [19]. I speculate that the loss of widespread Nodal expression in the endoderm and the loss of the posterior coelomic pouch in the sea urchin lineage allowed for a more localized embryonic territory to become the adult animal.

4) Change in positive signals that promote the germ cell fate in sea stars

I hypothesize that Wnt and BMP signaling members are involved in the promotion of a germ cell fate in sea stars. I propose that Wnt promotes a germ cell fate during the mid-gastrula stage when germ cell factors accumulate in a ring in the vegetal territory (Figure 9). Previous researchers have shown that a gradient of Wnt signaling in the vegetal hemisphere is required to pattern and specify the different vegetal cell territories [20]. In addition, differing Wnt ligand orthologs are expressed in concentric rings in the vegetal hemisphere [21]. Therefore, the precise amount of Wnt signaling conferred by a specific Wnt ligand present in the vegetal ring where germ cell factors accumulate could promote the germ cell fate. Based upon expression patterns of these Wnt ligands, Wnt 3, Wnt A/4 and Wnt16 are good candidates that could be expressed in the same ring as Nanos and Vasa. In support of the hypothesis that Wnt promotes a

germ cell fate, we incubated embryos with an inhibitor of GSK-3 (a negative regulator of Wnt signaling) in an effort to increase Wnt signaling levels. We then visualized Vasa mRNA by an in situ hybridization. We found that the Vasa expression domain increased in embryos that were treated with GSK-3 inhibitor relative to untreated control embryos (Appendix B). Although more experiments need to be done, the current data supports the hypothesis that Wnt signaling is required to promote a germ cell fate in sea stars.

Preliminary data in sea urchins also suggests Wnt signaling is involved in germ cell specification, albeit at an earlier developmental stage. When embryos are treated with a Wnt inhibitor Nanos mRNA levels significantly decrease in the germ cell lineage (Swartz et al., in preparation). The Wnt signaling that promotes specification of the germ cell lineage in the sea urchin is likely from maternally deposited disheveled and β -catenin asymmetrically localized at the vegetal pole [5, 6]. Whereas, the Wnt signaling that promotes specification of the germ cell lineage in the sea star is likely from an embryonically produced Wnt ligand. In order to determine the changes that occur in Wnt signaling that cause an earlier specification of the germ cell lineage in the sea urchin lineage we need to determine the precise Wnt ligand that promotes a germ cell fate in sea stars. We need to determine if this ligand similarly promotes a germ cell fate in other echinoderm groups. This can be achieved by inhibiting Wnt ligands either with translation blocking morpholinos or through Cas9 mediated gene disruption. We also need to determine how this ligand may be regulated differently during the early development of the sea urchin. At the same time we need to analyze the early Wnt signaling mechanism required for the transcription of Nanos in the small micromeres of

the sea urchin. How did this mechanism become functional so early in the evolution of sea urchin development?

I hypothesize that BMP also promotes a germ cell fate in the sea star, during the dorsal and left restriction of germ cell factors (Figure 10). In animal development BMP and Nodal are often opposing signals [17]. For example, during the patterning of the Dorsal/Ventral axis in sea urchins Nodal is required for the specification of Ventral cell fates while BMP is required for the specification of Dorsal cell fates [22]. Similarly, during the patterning of the Left and Right coelomic pouches, Nodal is required to confer a right coelomic pouch cell fate while BMP is required to confer a left coelomic pouch cell fate [17]. Because Nodal is expressed in the ventral and right part of the embryo in the sea star (Chapter 4), I hypothesize that BMP is active in the opposite domains, the dorsal and left parts of the embryo. Indeed, phosphorylated Smad 1/5/8 antibody (a marker of active BMP signaling) localizes in the dorsal part of the sea star embryo [23]. Given that Nanos and Vasa mRNA selectively accumulates in the dorsal and left parts of the embryo BMP is an excellent signaling candidate that could promote a germ cell fate during the gastrula and larval stages. In sea urchins, BMP signaling is active in the dorsal and left parts of the embryo but it does not have any direct effect on the germ cell fate [17, 22]. From the available data I hypothesize that BMP has lost its germ cell specification function in the sea urchin lineage.

Recent research in the sea urchin suggests that Delta/Notch signaling is required for the transcription of Nanos in non-small micromere somatic cells. This requires a mechanism that is dependent on the FoxY transcription factor (Swartz et al., in preparation). In order to test if a similar cascade is present in sea stars I have identified

three different FoxY orthologs in sea stars. Remarkably, they are localized in similar expression domains as Nanos mRNA (Appendix C). This suggests that FoxY is required for Nanos transcription in both sea stars and sea urchins. In the future it will be important to determine if a similar Delta/Notch→FoxY→Nanos cascade exists in the sea star and other echinoderm species. In addition, we need to determine if this cascade is required for the expression of Nanos in germ cells and/or somatic cells. These experiments will elucidate the evolutionary origin of the Delta/Notch→FoxY→Nanos cascade in echinoderms and if any changes may have occurred in the echinoid lineage. I hypothesize that FoxY induction of Nanos is important for specification of the germ cell fate in sea stars. This cascade may be a novel feature in the somatic cells of the sea urchin and the function in these somatic cells still remains to be determined.

Changes repeatedly involved in the evolution of inherited germ cell specification

Next, I will speculate on which phenomena are repeatedly involved in the evolution of an inherited germ cell specification mechanism. As previously mentioned, the inherited germ cell mechanism is thought to have evolved independently many times in animal evolution. To test if specific phenomena are repeatedly involved in the evolutionary transition to an inherited germ cell specification mechanism it is necessary to determine what changes are involved in other closely related model animal pairs that use differing mechanisms of germ cell specification. Besides sea urchins and sea stars, two other closely related animal model pairs use different mechanisms for germ cell specification. Within amphibians, axolotls use the inductive mechanism while frogs use the inherited mechanism. And within insects, crickets use the inductive mechanism while fruit flies use the inherited mechanism. I selected these animal model pairs based

on the amount of germ cell specification mechanistic research available. Below, I describe three phenomena that correlate with the evolution of inherited germ cell specification in these animal-model pairs.

1) Early presence of molecules that recruit/condense germ cell factors

In most cases, inherited germ cell specification is associated with the presence of germ plasm. Germ plasm refers to electron dense aggregates of mRNAs and proteins that are required for the specification of the germ cell fate.

The use of germ plasm to specify the germ cell fate via inheritance is seen in both flies and frogs. In the fruit fly, these granules are made up of a group of RNA's and proteins that include the conserved germ cell factors Vasa (protein) and Nanos (protein) [24]. Importantly, transformation of this germ plasm to ectopic parts of the embryo is sufficient to induce ectopic germ cells [25]. The scaffolding protein that is required for the assembly of this germ plasm is the protein Oskar [26]. Oskar itself is anchored to the posterior pole of the embryo with the presence of filamentous actin and the motor protein kinesin 1 that transports cargo along polarized microtubules [27-29]. In frogs, germ plasm is made of a group of RNA's and proteins that include the conserved germ cell factors Nanos (RNA) and Dazl (protein) [30-33]. The protein required for germ plasm assembly in the vegetal pole is Xvelo, a conserved vertebrate protein [34]. Ectopic germ plasm placed in the animal pole is able to induce a germ cell fate in frogs as well [35]. In addition, a kinesin-like protein is also required for assembly of the germ plasm in frogs as well [36]. Together, these examples show that early germ plasm assembly is dependent on a germ cell factor scaffolding protein that uses microtubules and kinesin motor proteins for asymmetric placement.

Germ plasm scaffolding proteins are not conserved and usually evolve from an existing protein that has no function in germ cells. Oskar is only present in insects and it is believed to have an ancestral function in the nervous system. Oskar has been co-opted in the fruit fly for germ plasm assembly [37, 38]. In addition, although an Xvelo homolog is required for germ plasm assembly in both frogs and zebrafish this protein is also present in other vertebrates that do not use inherited mechanisms for germ cell specification [39]. This means Xvelo likely was co-opted for germ plasm assembly separately in frogs and zebrafish. To the best of my knowledge, no convincing evidence is available for condensed germ plasm in sea urchin eggs, or in early development. However, the early localization of germ cell factors in the small micromere cells are required for the germ cell fate (Appendix D) [3]. I suspect the proteins in the vegetal cortex that help localize germ cell factors in the vegetal-most cells during small micromere formation are functionally equivalent to Oskar and Xvelo in that they recruit germ cell factors in a high enough concentration making them sufficient to stimulate a germ cell fate. I also suspect a kinesin dependent microtubule mechanism is required for the recruitment of germ cell factors to the vegetal-most cells during germ cell specification in the sea urchin. In summary, I propose that the evolution of inherited germ cell specification is dependent on the evolution of a molecule that uses kinesin/polarized microtubules to recruit/condense germ cell factors in a high concentration. Once germ cell factors accumulate at a certain threshold they are able to produce a germ cell fate.

2) Changes in signals/molecules that restrict the germ cell fate

Molecules that restrict the germ cell fate are an important part of the inductive germ cell specification mechanism in sea stars (Chapter 4). I propose that restrictive molecules are also likely required for specification of the germ cell fate in all animals that use induction. Previously, investigators have suggested that once an inherited germ cell mechanism evolves, mechanistic changes are able to occur in the signaling pathways that were once used for germ cell fate specification [40]. To test this hypothesis, I will analyze how inductive germ cell restrictive signals have changed in animal model pairs that use different mechanisms for germ cell specification.

In the sea star the Nodal signaling pathway is required for restriction of the germ cell fate (Chapter 4). I have already discussed the differences in the Nodal signaling pathway between the sea urchin (inherited) and sea star (inductive) animal model pair. Mainly, the widespread endodermal expression of Nodal is not present in the sea urchin, which coincides with the loss of the posterior coelomic pouch (the germ cell structure that relies on endodermal Nodal for asymmetric positioning in the sea star) (Chapter 2) [11, 41]. In regards to the axolotl and frog model pair there is also a change in a restrictive signal. In the axolotl (an animal that uses induction for PGC specification), high levels of Nodal signaling inhibit germ cell specification and instead directs cells to take on a blood fate [42]. Therefore, I consider Nodal to also have a restrictive effect on specification of the germ lineage in the axolotl. Previous researchers have noted differences in Nodal signaling in the closely related frog (an animal that uses inheritance for PGC specification). Mainly, the Nodal family has expanded to 25 ligands [40, 43]. In regards to the cricket (inductive), it still remains to be determined if restrictive signals contribute to specification of the germ cell fate. My preliminary BlastP analysis

suggests no Nodal sequence is present in the cricket transcriptome. However, I do note that PGC's arise dorsally in crickets [44]. Therefore, they likely use dorsal/ventral patterning mechanisms to restrict where germ cells arise as well. In the closely related fruit fly (inherited), Nodal is also not present and the Dorsal/Ventral axis is instead formed by the translocation of the Dorsal transcription factor into the nuclei of ventral cells [45]. In the future it will be important to determine if the pathway required to specify ventral cell fates inhibits the germ cell fate in crickets. If this is true, then it will be important to test if any changes have occurred in the upstream regulatory and coding sequences of members of this pathway. Overall, the acquisition of an inherited germ cell specification mechanism allows signals that restrict the germ cell fate in the inductive animal model to change in the inherited animal model. In sea urchins, the Nodal signaling pathway is restricted, possibly instructing metamorphosis to occur in a specific and localized region. While in the frog the Nodal signal pathway is expanded, possibly allowing for the evolution of novel mesodermal cell fates and a deviation from the ancestral tetrapod body-plan [40].

3) Changes in signals/molecules that promote the germ cell fate

A hypothesis about the molecules involved in inductive germ cell specification states that signals usually involved in promoting a germ cell fate are likely under stabilizing selection as any changes would likely be deleterious to reproduction [40]. However, when an inherited germ cell mechanism evolves these signals are no longer under such stabilizing selection because any changes no longer affect germ cell formation [40]. To probe this hypothesis further I will analyze how inductive germ cell

promoting signals have changed in animal mode pairs that use different mechanisms for germ cell specification.

Within the fruit fly (inherited) and cricket (inductive) model pair, BMP is required for promoting the germ cell fate in both animals. In crickets, GBB (the human ortholog of human BMP 5/7/8) and to a lesser extent DPP (the ortholog of human BMP 2/4) are required for the specification of germ cells [46]. Decreasing levels of these BMP ligands or the common downstream transcription factor pMAD by RNAi causes a significant decrease in germ cell numbers. Importantly, injection of the fly DPP protein (the ortholog of human BMP 2/4) in cricket embryos is sufficient to cause ectopic germ cells to form [46]. If BMP is the ligand required for specification of the germ lineage in the ancestral insect, then the fly BMP ligand still retains this function as it is still able to promote a germ cell fate in the cricket. In the fruit fly, nuclear pMAD (a marker of active BMP signaling) is also present in PGC's. A recent study in the fruit fly shows that intermediary levels of BMP signaling is required to maintain the PGC cell fate [47]. This is because a decrease in BMP signaling causes loss of Vasa protein and defects in formation of a germ-stem cell organelle (the spectrosome). An increase in BMP signaling also causes deleterious effects in the PGC's; specifically there is a decrease in the number of germ cells in the gonads [47]. It still remains to be determined if the function of BMP signaling in flies (maintenance of the germ cell lineage) is conserved in insects or if this is a novelty that arose in the dipteran lineage. If maintenance of the PGC fate is a novel function then perhaps it was able to evolve because BMP was no longer necessary for PGC specification. If maintenance of the PGC fate is ancestral then perhaps this part of its function is still required in fly PGC development. I favor the

hypothesis that BMP also acted ancestrally to maintain a germ cell fate and therefore posit that upstream promoter and coding sequences of BMP signaling members are under stabilizing selection. Any changes could be potentially deleterious to the germ line. This also could explain why the fly DPP is able to induce a germ lineage in the cricket: it likely has not diverged significantly from the cricket DPP sequence. More work needs to be done to determine the function and sequence divergence of BMP signaling members in many insects that use both inherited and inductive germ cell specification mechanisms. I speculate that a signal that induces a germ cell fate in an inductive animal model (crickets) retains a germ cell maintenance function in a closely related inherited animal model (flies).

In regards to the axolotl (inductive) and frog (inherited) model pair, BMP4 and FGF are required for germ cell specification in the inductive animal, axolotls. However, no direct comparisons have been conducted to determine if any changes have occurred to these signals in the frog lineage. Another set of genes that may promote the germ cell fate in axolotls are the conserved vertebrate pluripotency factors: Oct4, Sox2, and Nanog. These factors are required for ground state pluripotency in vertebrates including axolotls [48-50]. However, many changes occurred in these genes in the frog lineage. First, the frog has no orthologs of Nanog or Oct4 in the genome [51, 52]. Second, the Oct4 related *pou2* family of transcription factors are expanded [53, 54]. Third, the ability of this pluripotency network to generate a germ cell fate is lost in the frog lineage. Previous research with animal caps (the region with the highest expression of pluripotency factors) shows that animal caps from axolotls can be induced to become germ cells while animal caps from frogs cannot [55]. Previous authors suggest that

when an inherited germ cell specification evolved in the frog lineage the pluripotent state was able to change because the germ cell lineage no longer depended on the pluripotent state for their production [40].

I speculate that changes also occurred in signals that promote the germ cell fate in the sea urchin (inherited) and sea star (inductive) model pair. As stated above I propose that BMP and Wnt are signals required for specification of the germ cell lineage in the inductive animal, sea stars. The only change that I notice in the Wnt signaling pathway between sea urchins and sea stars is the differing expression of the Wnt ligand 3. In sea stars, Wnt3 is localized in a ring in the vegetal hemisphere of the early gastrula [21], possibly expressed in the same cells as Nanos and Vasa. However, Wnt3 is not detected during the early development of sea urchins [56]. It is tempting to speculate that expression of this ligand was lost during the evolution of the sea urchin lineage after inherited germ cell specification evolved because it had no more function in development. In order to probe this hypothesis it will be very important to study Wnt3's involvement in germ cell specification in diverse echinoderm species. For reasons I state in previous sections, BMP also likely promotes a germ cell fate during the gastrula stage of sea star development. I propose BMP is active in the dorsal ectoderm of the blastula stage embryo which helps promote the retention of germ cell RNA's in the dorsal side of the embryo. I also propose BMP is active in the left side of the mid-gut which helps promote the retention of germ cell RNA's in the left side of the developing gut. In the sea urchin, BMP is required to specify dorsal cell fates during the blastula stage, a function that is likely conserved in echinoderms, which means BMP signaling during the blastula stage is likely under stabilizing selection [22]. However, BMP is likely

no longer necessary in the endoderm of the gastrula stage of the sea urchin because the germ cell fate is specified much earlier. Although we still need to test if BMP is expressed and functional in the endoderm of sea stars, if it is, then it is reasonable to hypothesize that during the evolution of the sea urchin lineage, BMP expression in the endoderm was lost.

In summary, I speculate that sequences of signaling molecules that induce the germ cell fate in crickets have undergone few changes in insects because they may also have an ancestral germ cell maintenance function in insects. Previous researchers showed that some molecules required for the germ cell fate in axolotls are lost and some have diversified during the evolution of the frog lineage. And I speculate that signals that promote the germ cell fate in sea stars may have been lost or at least expression during specific developmental stages is lost during the development of the sea urchin lineage.

A summary of inductive germ cell specification mechanisms

From the previous discussion, I propose a 3 step germ cell specification process in sea stars. During the blastula stage a Wnt ligand likely promotes the germ cell fate in a ring of cells in a vegetal territory. In the mid-gastrula stage opposing Nodal and BMP signals promote retention of the germ cell fate in a dorsal patch of cells in the developing gut. And by the early larva stage opposing Nodal and BMP signals promote the formation of a germ cell pouch in a group of cells that bud out from the left side of the developing gut. This 3-step germ cell specification mechanism in sea stars highlights that induction of the germ cell fate is a multi-step process that requires several signaling events at different embryonic times.

I propose that a multi-step process is likely a part of the germ cell specification program in other animals that use induction for germ cell specification. Even though previous work in other models that use inductive mechanisms for germ cell specification (mice, crickets, and axolotl) emphasize one embryonic time point I find that earlier events in the development of these animals also contribute to germ cell specification. In mice, the potential to become the germ cell lineage is restricted at the blastocyst stage. In particular, inner cell mass cells expressing Oct4, Sox2, and Nanog retain the potential to become the germ cell lineage while cells in the trophectoderm are restricted to become extraembryonic tissue [57-62]. In crickets, I see a reduction in the potential to become the germ cell lineage during the blastoderm stage. Cells that accumulate Piwi and Vasa in the germ band retain the potential to become the germ cell lineage while cells that do not retain Piwi and Vasa are restricted to become extraembryonic tissue [44]. In axolotl development previous researchers have already shown that two separate embryonic stages are required for germ cell specification. During the gastrula stage BMP4 and FGF are required for germ cell specification. However, these cells are not lineage restricted to become PGC's. Further signaling from MAPK is required after gastrulation for specification of the germ cell fate [42]. In addition, an earlier step has also been proposed to be required for germ cell specification in the axolotl. Early in development when Oct, Sox, and Nanog are active they likely also have a germ cell fate retention effect whereby cells that have these factors can become the germ lineage while cells that do not have these factors cannot become a germ cell [40]. Although this first step still remains to be fully tested in axolotls, I find that inductive germ cell specification is a multi-step process in many animals.

This discussion is in agreement with most general themes involved in inductive germ cell specification. First, BMP ligands are required for germ cell specification in many animals that use induction for germ cell specification [42, 46, 63]. And second, germ cell specification generally occurs in cells of mesodermal origin [42, 44, 64, 65]. I also notice a few other underappreciated themes. As mentioned above, germ cell specification is a multi-step process that requires a special combination of germ cell promoting and restricting factors at several stages of development. Previous researchers have put forth the 'last cell standing' hypothesis which states that the germ cell lineage is one of the last cell lineages to become lineage restricted [40]. I wish to highlight a specific part of this hypothesis, which is that in order for germ cell precursors to remain germ cell precursors they must retain zygotic multipotency. The fertilized egg has the potential to become any cell type and the germ cell lineage must retain this potential to contribute to the next generation. As development proceeds sequential restrictions decrease the number of cells that retain this potency and can become the germ lineage. Potency can be conferred through the acquisition of factors such as Oct, Sox, and Nanog or with the retention or expression of germ cell factors such as Vasa, Nanos, Piwi, or Dazl. I argue the widespread expression of these multipotency factors is likely required in the early embryo for cells to remain multipotent prior to differentiation. However, as most cells begin to differentiate they lose these multipotency factors, become somatic, and lose their ability to contribute to the next generation. In contrast, the last cells to differentiate (or the 'last cells standing') retain these multipotency factors with the help of key signals throughout development and therefore retain the ability to contribute to the next generation.

Final thoughts

Inherited germ cell specification has evolved many times in animal evolution. The evidence I present here supports the hypothesis that the inductive germ cell specification mechanism is ancestral in echinoderms and that an inherited germ cell specification has evolved in the echinoid lineage. Future work determining and comparing germ cell specification mechanisms in sea stars, sea urchins, and other echinoderms will contribute significantly to our understanding of how an inherited germ cell specification mechanism evolves. Current research in diverse animals that use diverse germ cell specification mechanisms suggest that the evolution of an inherited germ cell specification mechanism requires the recruitment of germ cell factors in a high concentration. I propose that once a certain threshold is hit, the germ cell factors are able to specify a germ cell fate. Comparisons of molecules involved in germ cell specification in animal model pairs that use different mechanisms for germ cell specification support the idea that germ cell signals/molecules required for inductive germ cell specification change when an inherited germ cell specification evolves. My analysis shows that sometimes germ cell specification molecules are lost and sometimes they diverge when an inherited germ cell specification evolves. Furthermore, these changes in specification molecules coincide with the evolution of novel developmental features in some animals. For example, the evolution of a more restricted embryonic area that contributes to the adult animal in echinoids, the evolution of larval skeleton in echinoids, and the evolution of a novel body plan in frogs. My work also reveals that the inductive germ cell specification likely is the result of multiple germ cell promoting and restricting signals that occur at many developmental times. In order to rigorously test the hypotheses presented in this dissertation we need to determine

how specific molecules directly contribute to germ cell specification in diverse species.

When this happens we will have a better understanding of the specification and

evolution of the most potential cell lineage in animals, the germ cell lineage.

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Figures and Tables

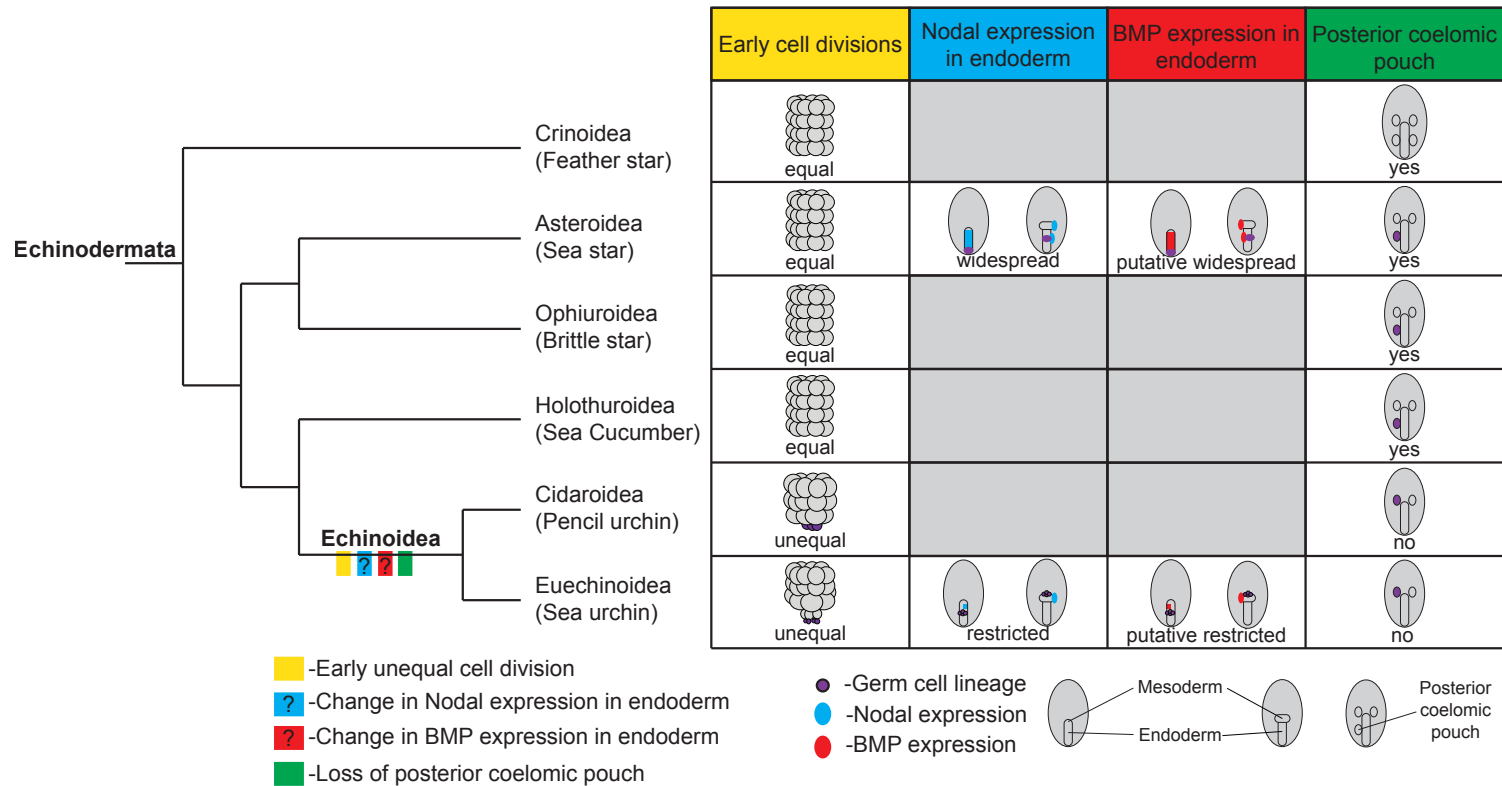


Figure 1- Changes involved in the evolution of an inherited germ cell mechanism in echinoderms

Four developmental phenomenon coincide with the evolution of an inherited germ cell specification mechanism in the echinoid lineage. 1) Early cell divisions are only unequal in the echinoid lineage (yellow). 2) Nodal expression in the endoderm is only restricted in the echinoid lineage (blue). 3) BMP expression is likely also only restricted in the echinoid lineage (red). 4) The posterior coelomic pouch is not present in the echinoid lineage (green). Gray boxes indicate a lack of experimental data.

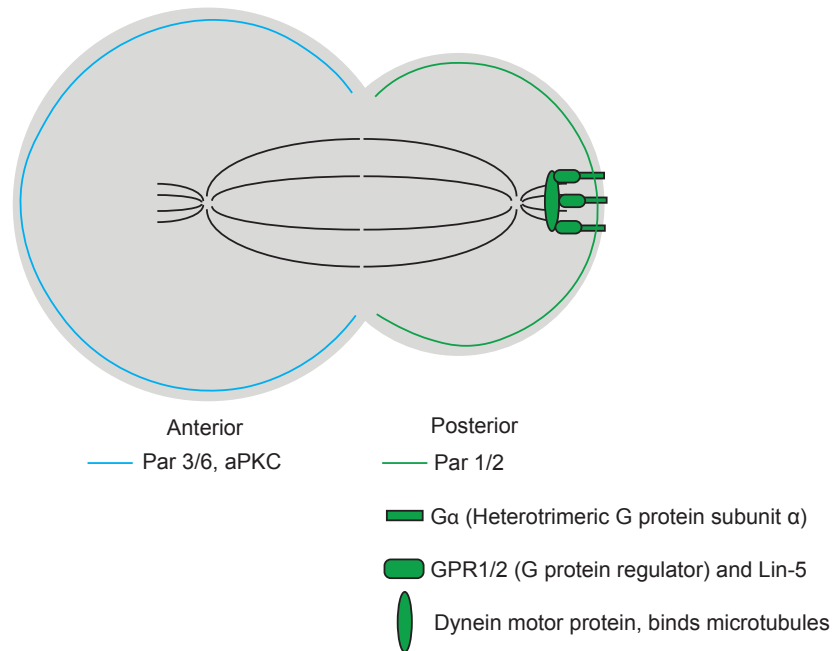


Figure 2- Asymmetric cell division in the round worm

The first cell division in *C. elegans* is asymmetric. The larger cell will become the future anterior part of the embryo while the smaller cell will become the future posterior part of the embryo. For this asymmetric cell division to occur Par proteins become asymmetrically located on the cell cortex in opposite domains. In the posterior pole, the heterotrimeric G protein subunit α links a complex of proteins including the dynein motor protein to the spindle microtubules. This effectively pulls the mitotic apparatus to favor the posterior side.

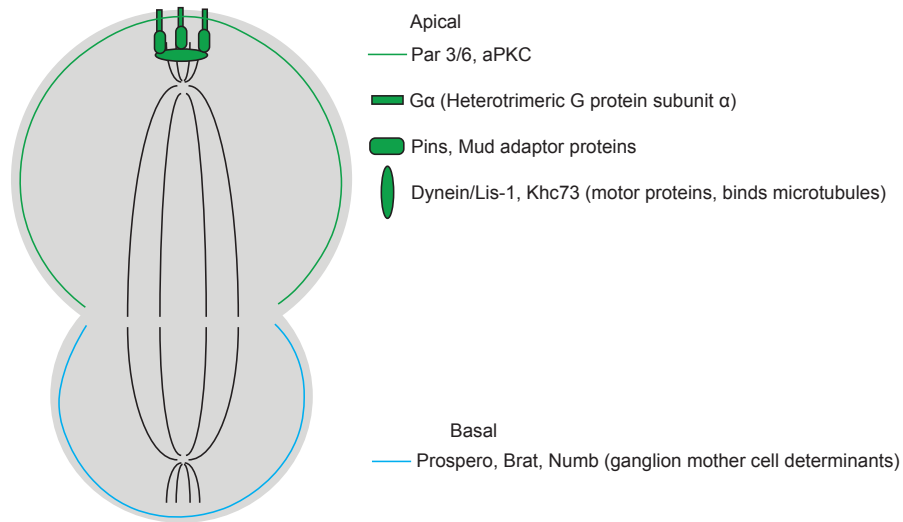


Figure 3- Asymmetric cell division in the fruit fly

During neuroblast differentiation one neuroblast stem cell divides asymmetrically to produce a large neuroblast cell and a small ganglion mother cell. For asymmetric cell division to occur Par proteins become asymmetrically localized in the apical cortex while ganglion mother cell determinants become localized in the basal cortex. For spindle re-orientation to occur the heterotrimeric G protein subunit α complexes with adaptor proteins and dynein to ultimately pull the mitotic apparatus parallel to the apical/basal axis. Subsequently, the microtubules become longer on the apical side to generate the unequal cell size.

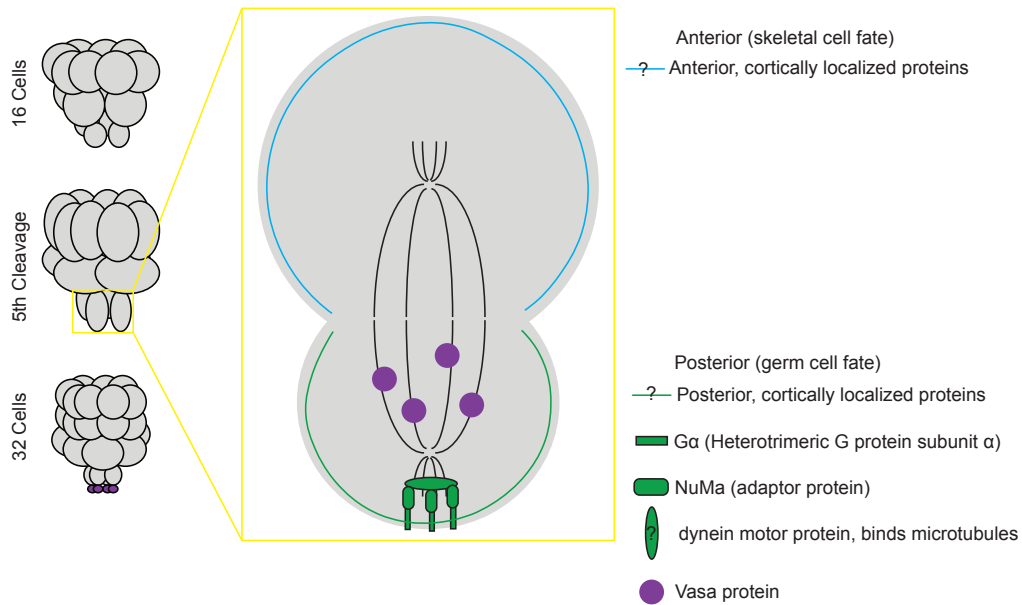


Figure 4- Asymmetric cell division in the sea urchin

During the 5th cell division the cells in the posterior pole undergo asymmetric cell divisions. The larger anterior cell will contribute to the larval skeleton while the smaller posterior cell will become a primordial germ cell. The initial asymmetric cortical proteins that help identify each pole remain to be identified. However, some conserved regulators of asymmetric cell division are required for this asymmetric cell division, including: the heterotrimeric G protein subunit α and its associated linker protein NuMa. Preliminary studies suggest that Vasa is translated on the spindle in the posterior cell, a mechanism that contributes to the accumulation of germ cell factors in the future primordial germ cells.

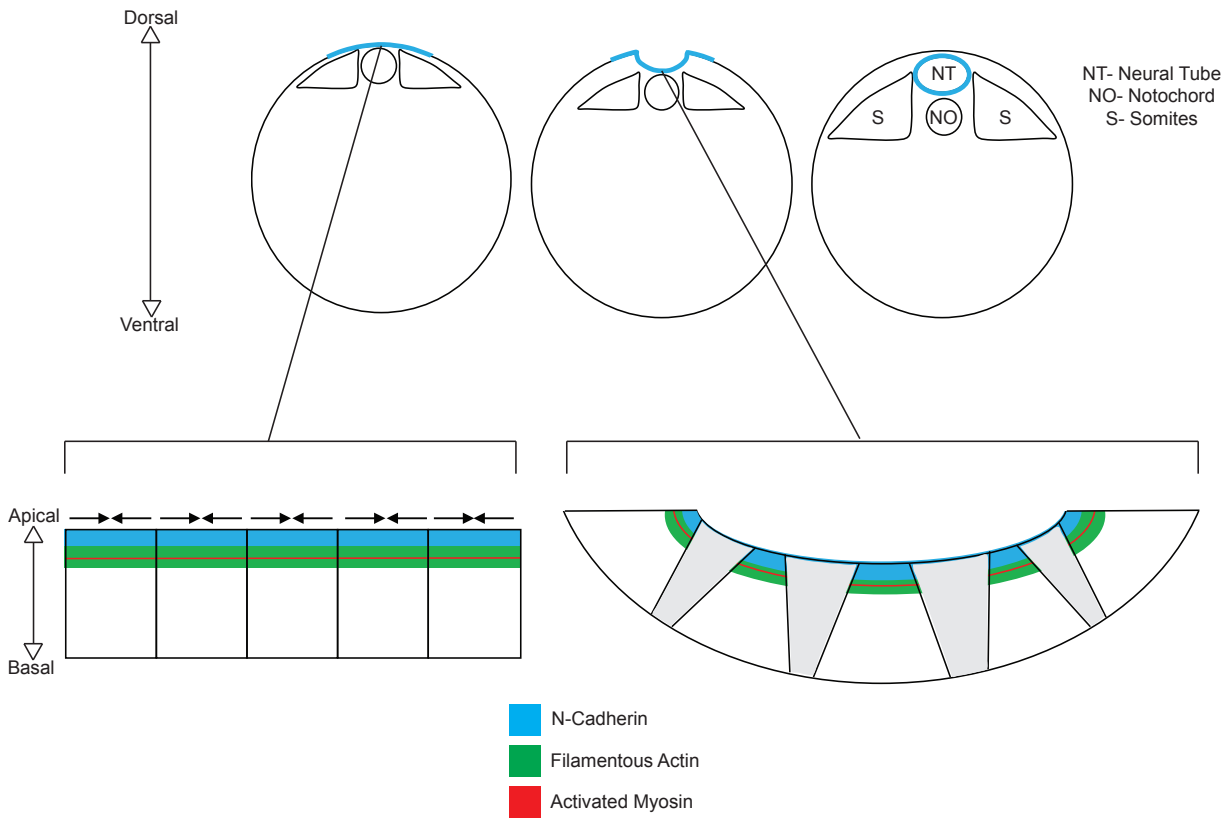


Figure 5- Invagination in the frog

Neural tube formation requires the invagination of dorsal cells. Briefly, N-cadherin localizes at the apical side of neural cells. Then apically localized actomyosin becomes active and contracts the cell membrane causing the neural cells to constrict and move into the body cavity.

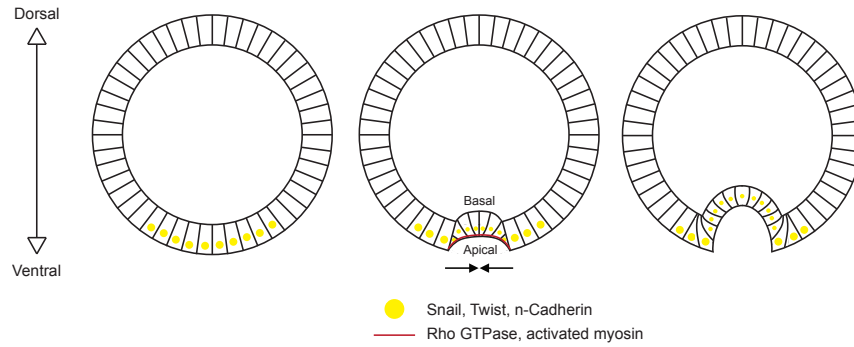


Figure 6- Invagination in the fruit fly

Mesoderm formation requires invagination of ventral cells. Briefly, the mesodermal transcription factors Snail and Twist upregulate N-cadherin expression in mesodermal cells. Then apically localized Rho GTPase activates myosin through a signaling cascade. Activated actomyosin contracts the apical side of the cells causing them to move into the body cavity.

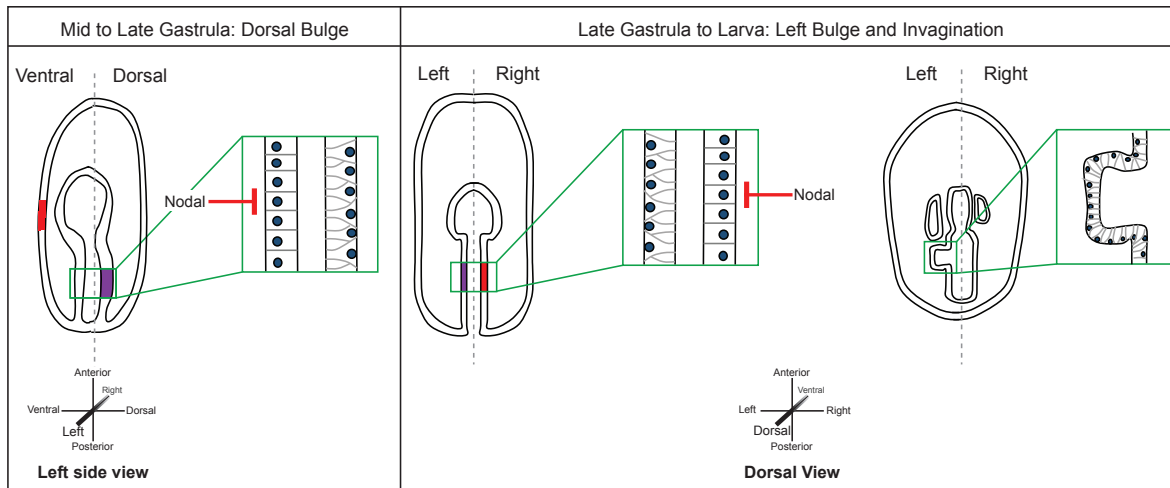


Figure 7- Invagination in the sea star

During the mid to late gastrula stage, germ cells accumulate in a dorsal patch in the developing gut (purple). At this point the dorsal patch of cells starts to bulge out of the developing gut. During the late-gastrula to early larva stage germ cells accumulate in a patch in the left side of the developing gut (purple). This left patch of cells then bulges out of the developing gut, invaginates, and subsequently forms its own coelomic pouch. The molecular mechanisms that govern this invagination are still unknown. However, Nodal (red) is required to inhibit bulging and pouch formation in the ventral and right sides of the developing gut.

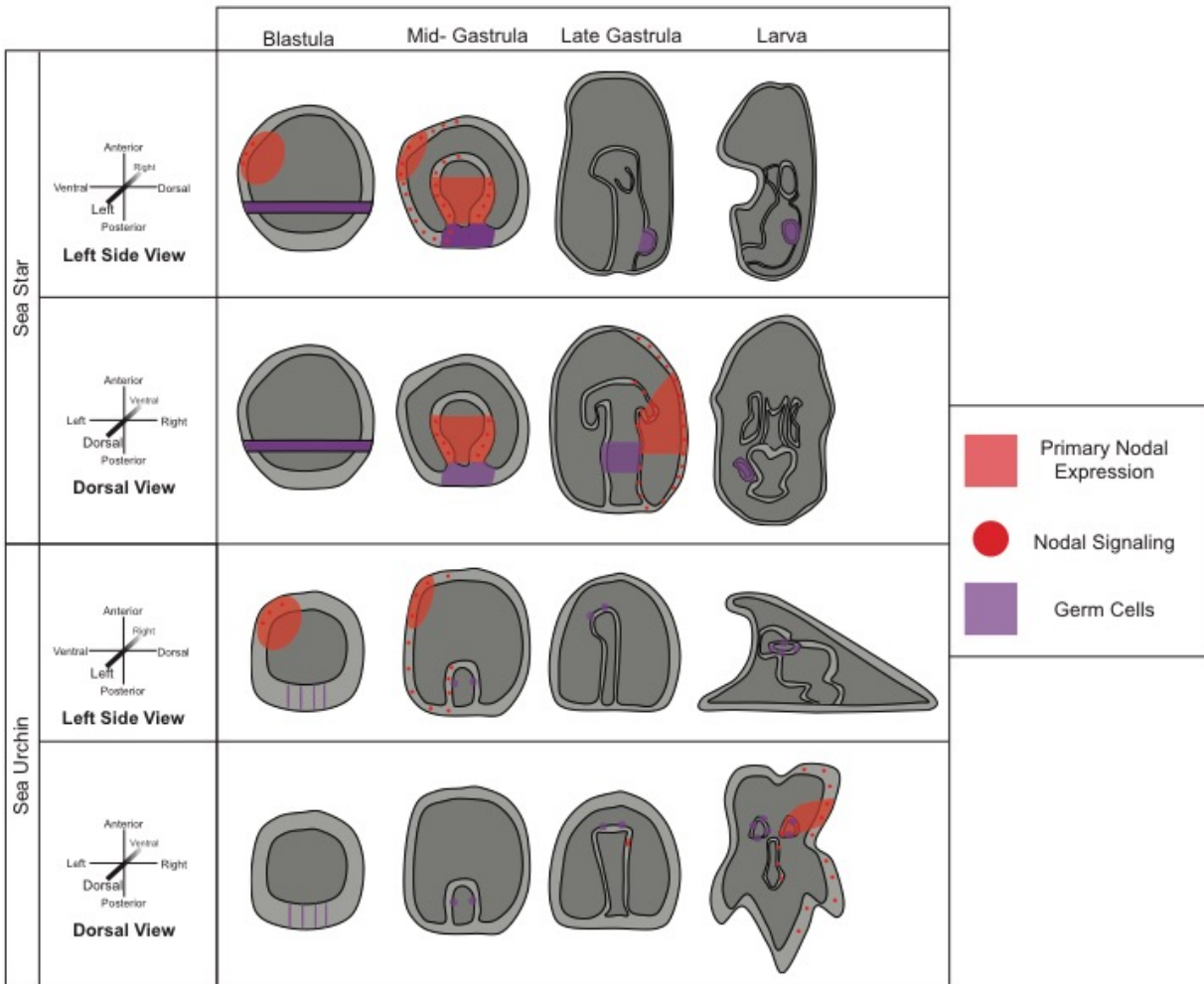


Figure 8- Nodal signaling during germ cell specification in the sea star and in the sea urchin

In the sea star blastula and mid-gastrula stage, Nodal is expressed in a patch of cells in the ventral ectoderm. Nodal expression is required for the loss of germ cell factors in the ventral side of the embryo. During the mid-gastrula stage Nodal is expressed ubiquitously in the developing endoderm. By the late gastrula stage Nodal is expressed in the right side of the endoderm, mesoderm, and ectoderm. Nodal expression is also required for the loss of germ cell factors in the right side of the embryo.

In the sea urchin blastula and mid-gastrula stage embryo, Nodal is expressed in a similar domain as the sea star. Nodal is expressed in a patch of cells in the ventral ectoderm. However, endodermal expression of Nodal is not similar to the sea star. Nodal is not expressed ubiquitously, instead it first appears asymmetrically in a few cells in the right side of the archenteron. Nodal expression persists in the right coelomic pouch and the right ectoderm in the larva stage. Germ cells are only affected by Nodal signaling in the larva stage when germ cells that enter the right coelomic pouch undergo apoptosis.

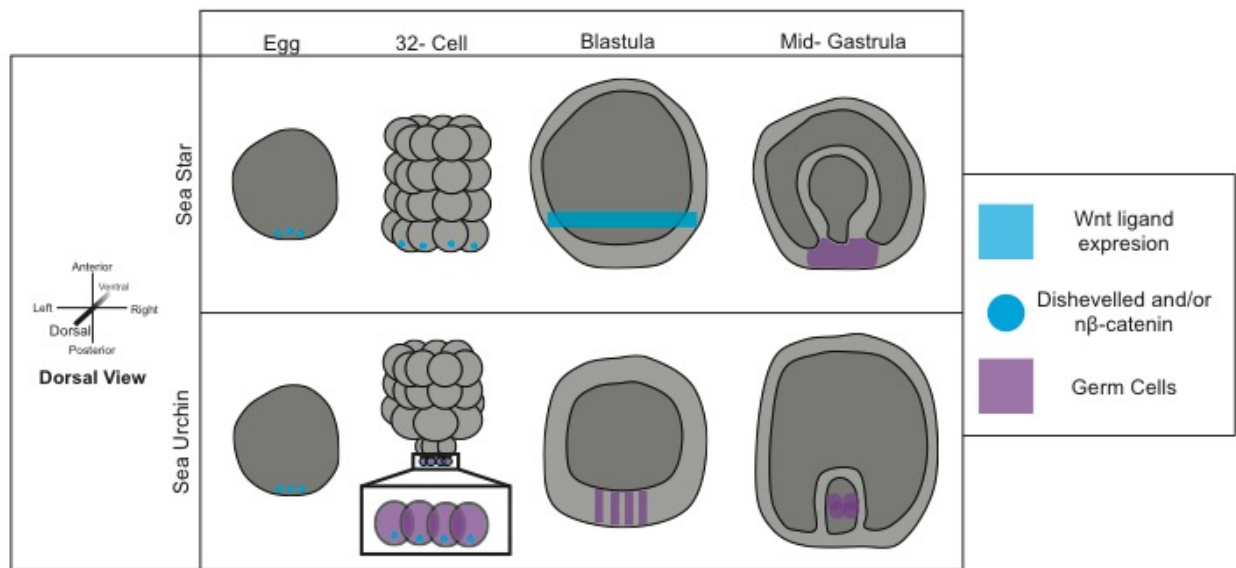


Figure 9- Wnt signaling during germ cell specification in the sea star and sea urchin

In the sea star β -catenin accumulates in the vegetal pole of the egg and in the nucleus of vegetal cells during early cleavage. Different Wnt ligands accumulate in concentric rings during the blastula stage. I hypothesize that one of these Wnt ligand domains promotes the germ cell fate which causes subsequent accumulation of Nanos and Vasa mRNA in a ring at the vegetal pole in the mid-gastrula stage.

In the sea urchin β -catenin and disheveled accumulates in the vegetal pole of the egg and in the nucleus of vegetal cells during early cleavage. I hypothesize that these Wnt signaling proteins promote the germ cell fate in the vegetal cells which causes them to become primordial germ cells.

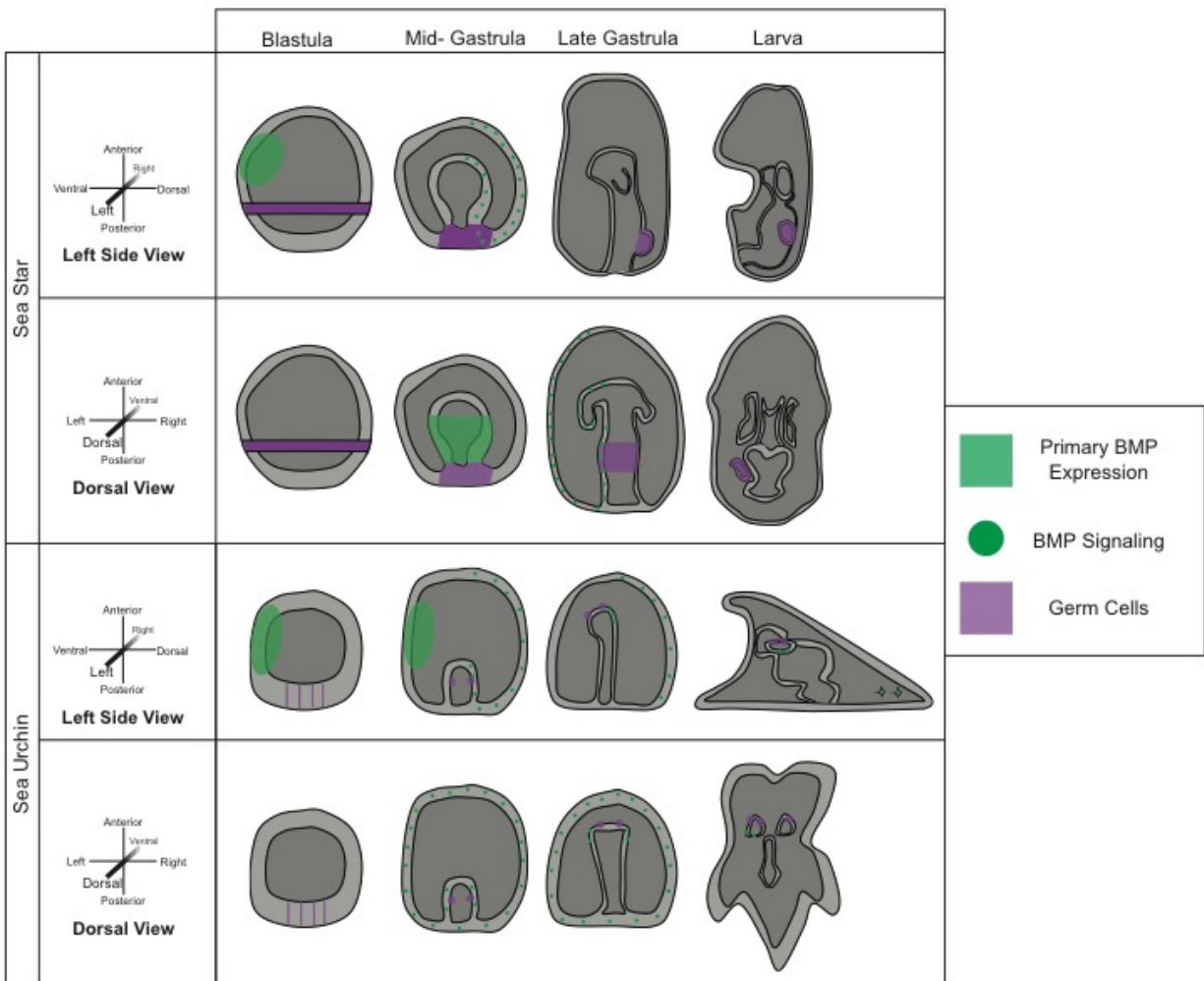


Figure 10- BMP signaling during germ cell specification in the sea star and sea urchin

In the sea star BMP is expressed in a ventral patch of the ectoderm in the blastula stage. However, a marker of BMP signaling (Phosphorylated Smad1/5/8) accumulates in cells in the dorsal half of the embryo. I hypothesize that BMP signaling in the dorsal side of the embryo promotes a germ cell fate. Although, BMP expression and markers for active signaling has not been tested in the gastrula stage I hypothesize that BMP accumulates broadly in the endoderm during the mid-gastrula stage and that BMP signaling subsequently occurs in the left side of the embryo. In this way BMP could also promote the germ cell fate in the left side of the embryo during pouch formation.

In the sea urchin BMP is also expressed in a ventral patch of the ectoderm in the blastula stage. Phosphorylated Smad 1/5/8 (a marker of active BMP signaling) accumulates in the dorsal half of the embryo, as in the sea star. Phospho-Smad 1/5/8 then accumulates in the left coelomic pouch in the larva stage.

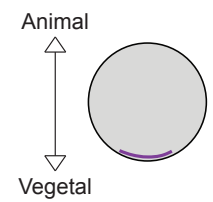
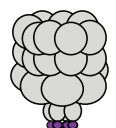
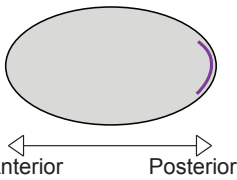
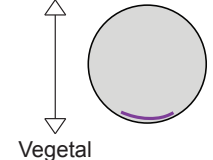
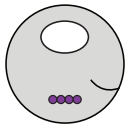
	Egg	PGC Specification
Sea Urchin		 Cleavage (32 Cells)
Fly		 Syncytial Blastula (512 cells)
Frog		 Early gastrula (~ 32,700 cells)

Figure 11- Germ Cell Factor Condensation

In the sea urchin, germ cell factors start to selectively accumulate in the vegetal cells at the 32 cell stage. It is at this stage that the small micromeres cells are formed, obtain germ cell factors in a high concentration, and become committed as primordial germ cells. The vegetal cortex likely has maternally localized molecules required for the selective accumulation of germ cell factors in the vegetal cells.

In the fly, Oskar localizes to the posterior pole of the egg. Oskar scaffolds the formation of germ plasm, therefore, germ cell factors are localized in a high concentration in the vegetal region of the egg. At the syncytial blastula stage, as nuclei migrate towards the posterior pole, cellularization occurs. It is at this stage that the cells that obtain this high concentration of germ cell factors become primordial germ cells.

In the frog, Velo localizes to the vegetal pole of the egg. Velo scaffolds the formation of germ plasm. During early development, the cells that inherit the vegetal cortex associated germ plasm continue to divide asymmetrically, producing both germ cells and somatic cells. It is not until the early gastrula stage that the germ plasm surrounds the nucleus and ensures both daughter cells inherit germ plasm components. At this point, a stable population of primordial germ cells are formed. Presumably, the germ plasm accumulates at some threshold concentration to produce a primordial germ cell fate at the early gastrula stage.

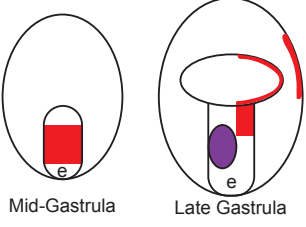
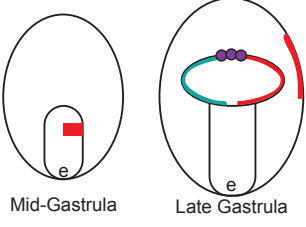
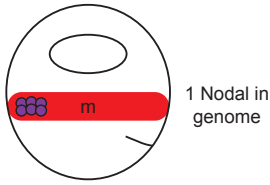
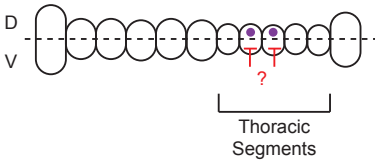
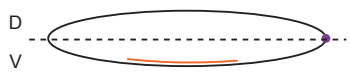
Inductive	Inherited	Changes associated with inherited germ cell specification
Sea Star  Mid-Gastrula Late Gastrula	Sea Urchin  Mid-Gastrula Late Gastrula	In the sea star endoderm (e), Nodal (red) is first expressed widespread. Nodal is required in the endoderm to pattern where the germ cell pouch will form (purple). In the sea urchin endoderm (e), Nodal is first expressed in a restricted domain. Nodal does not function in the endoderm and instead patterns where metamorphosis will occur (teal).
Axolotl  Early Gastrula 1 Nodal in genome	Frog  Early Gastrula 25 Nodals in genome	In the axolotl, Nodal (red) is first required to specify the mesoderm (m). Too much Nodal signaling causes germ cells (purple) to take on a blood cell fate. In the frog, Nodal (red) is also required to specify the mesoderm (m). Too much Nodal signaling has no effect on the specification of germ cells (purple). This correlates with an increase in Nodal coding sequences in the genome.
Cricket  Egg stage 5-8 Thoracic Segments	Drosophila  Blastula-Gastrula	In the cricket, primordial germ cells (purple) are first specified in an asymmetric pattern. They appear in the dorsal side of the 3-5th thoracic segments. In the fly, dorsal/ventral patterning is established by the nuclearization of the Dorsal protein in ventral cells (orange). In the future, it will be important to determine if a similar dorsal/ventral patterning mechanism is used during cricket development and PGC specification.

Figure 12- Changes in signals that restrict the germ cell fate

During the development of the sea star Nodal is required to restrict the germ cell fate in the endoderm. However, in the sea urchin germ cells are specified earlier in development. Nodal expression is different in the development of the sea urchin. Perhaps Nodal expression changed in the echinoid lineage because Nodal is no longer required in the endoderm for germ cell specification.

During the development of the axolotl high levels of Nodal signaling cause germ cells to become blood cells. However, in the frog Nodal signaling has no effect on germ cells because they are specified much earlier in development. The Nodal gene family has expanded to 25 members in the frog lineage. Perhaps this occurred in the frog lineage because changes in Nodal signaling would no longer effect the germ cell lineage.

During development of the cricket, germ cells first appear in the dorsal side of thoracic segments 3-5. In the future it will be important to identify what molecules are involved in the restriction of the germ cells to the dorsal side of the embryo and if these molecules have changed during the evolution of the fly lineage.

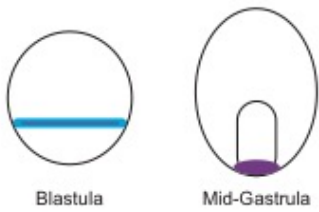
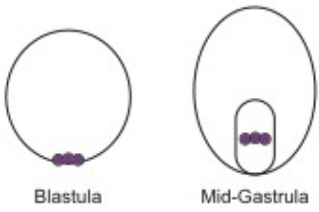
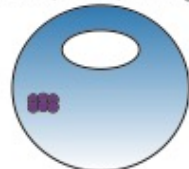
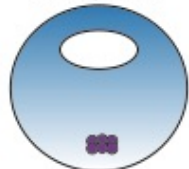
Inductive	Inherited	Changes associated with inherited germ cell specification
Sea Star  Blastula Mid-Gastrula	Sea Urchin  Blastula Mid-Gastrula	In the sea star, Wnt 3 is expressed in a ring in the vegetal territory (blue). I hypothesize this ligand may be required to promote the germ cell fate (purple) at the blastula stage. In the sea urchin, Wnt 3 is not expressed during early development. In addition, germ cells are already specified prior to the blastula stage.
Axolotl Oct4, Sox2, Nanog  Mid- Blastula Transition	Frog Pou2, Sox2  Mid- Blastula Transition	In the axolotl, a conserved vertebrate multipotency network (Oct, Sox, Nanog, blue) causes cells in the animal cap to be multipotent. Previous researchers suggest germ cell specification occurs in a subset of these multipotent cells. In the frog, the conserved multipotency network (blue) has changed. For example, Nanog and Oct4 are lost and the Pou family is expanded. PGC specification does not rely on this multipotency program.
Cricket  Egg stage 5-8	Drosophila  Blastula-Gastrula	In the cricket, BMP signaling (green) is required to specify the germ cell fate (purple). In the fly, BMP signaling (green) is required for maintenance of the germ cell fate (purple).

Figure 13- Changes in signals that promote a germ cell fate

During the development of the sea star I propose that Wnt ligand 3 is required for promoting the germ cell fate. This ligand is no longer expressed in the early development of the sea urchin. Perhaps this occurred in the echinoid lineage when germ cells were specified much earlier in development.

During the development of the axolotl previous authors have proposed that a pluripotency network is required to promote the germ cell fate in germ cell precursor cells. This pluripotency network has changed significantly in the frog lineage. Perhaps these changes occurred in the frog lineage when an inherited germ cell specification mechanism evolved because any changes to the pluripotency network would not be deleterious to reproduction.

During the development of the cricket BMP is required to promote the germ cell fate. BMP signaling is required for germ cell maintenance in the fruit fly. Perhaps, BMP is required for maintaining a germ cell fate in the most recent common ancestor. If this

is true then early BMP signaling in the PGC's was likely part of the evolution of an inherited germ cell mechanism in flies.

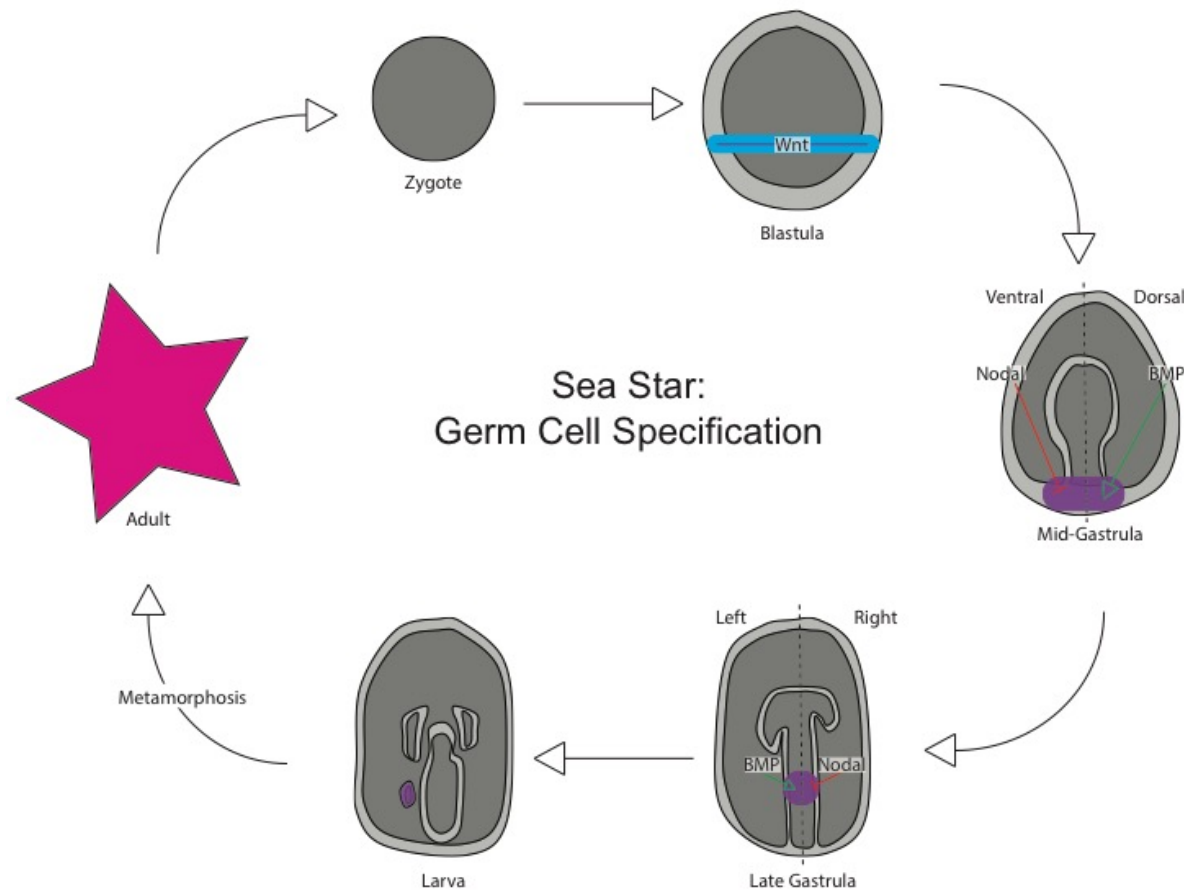


Figure 14- A model for germ cell specification in the sea star

I propose that Wnt signaling in the blastula stage promotes the germ cell fate in a ring of cells in the vegetal hemisphere. My research shows that Nodal is required to inhibit a germ cell fate in the ventral side of the mid-gastrula. I propose that BMP promotes a germ cell fate in the dorsal side of the mid-gastrula. My research also shows that Nodal is required to inhibit a germ cell fate in the right side of the late gastrula. I also propose that BMP promotes a germ cell fate in the left side of the late gastrula. Together, this multi-step program causes a germ cell pouch to form in the left side of the larva, which has previously been shown to contribute to primordial germ cells.

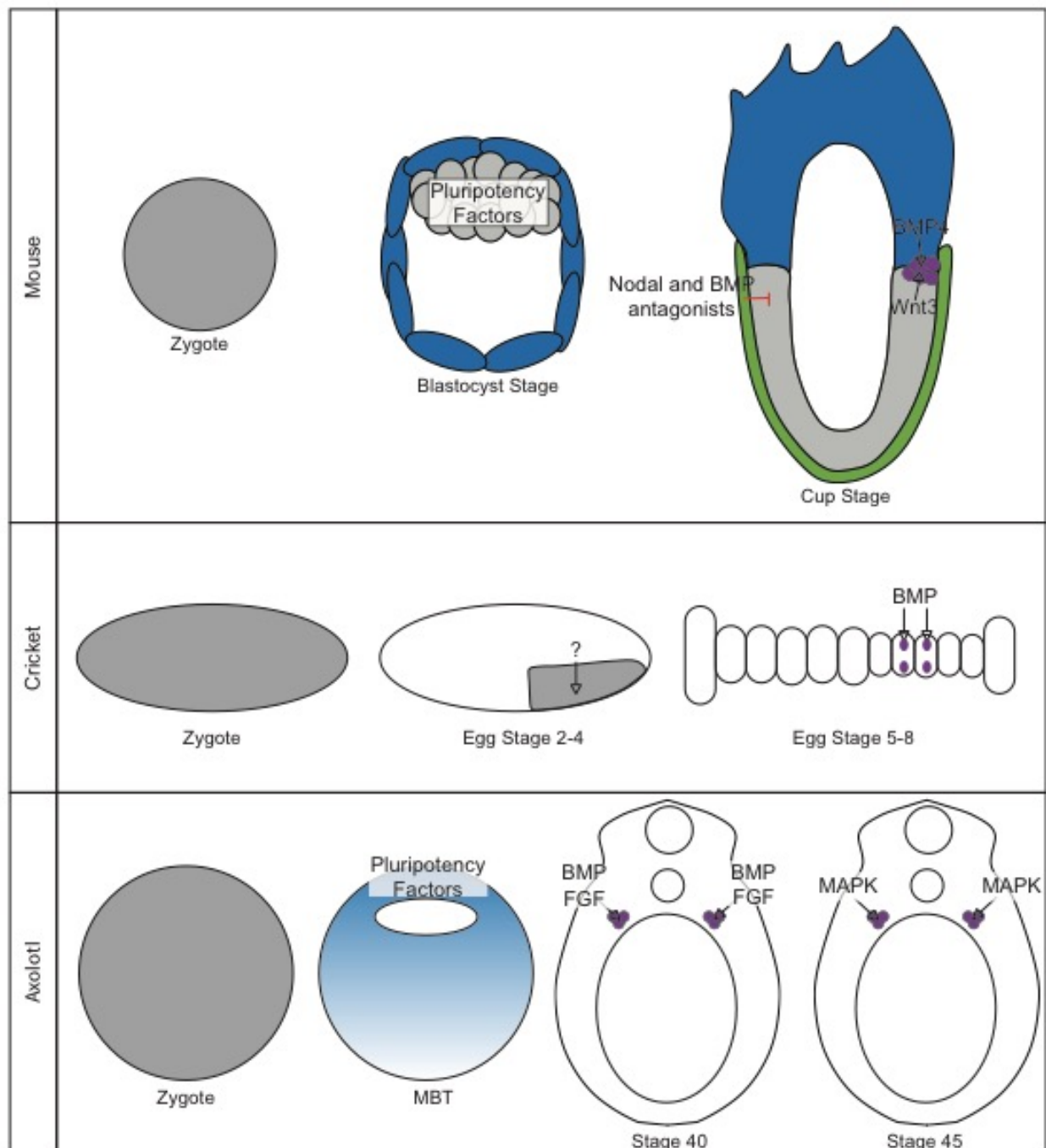


Figure 15- Inductive germ cell specification is a multi-step process

In the mouse the potential to become a germ cell is first restricted during the blastocyst stage when pluripotency factors cause the inner cell mass cells (gray) to retain the potential to become the germ cell lineage. Then during the cup stage a combination of multiple signals cause a specific set of multipotent cells to become

primordial germ cells. Nodal and BMP antagonists secreted from the visceral endoderm (green) inhibit the germ cell fate from forming in the anterior. The combination of BMP4 and Wnt3 signals in the posterior induce a set of cells in the posterior to become primordial germ cells.

In the cricket the potential to become a germ cell is first restricted at Egg Stage 2-4 when Vasa and Piwi accumulate in the germ band (gray). The germ band retains the potential to become the germ cell lineage while cells outside of this area become extraembryonic. Then during Egg Stage 5-8 BMP signals are required to promote the primordial germ cell fate.

In the axolotl, during the mid-blastula transition (MBT), pluripotency factors likely restrict which cells retain the potential to become the germ cell lineage. Then at stage 45 the combination of BMP and FGF signals causes promotion of the germ cell fate. Germ cells do not become lineage restricted until Stage 45 with the help of MAPK signaling.

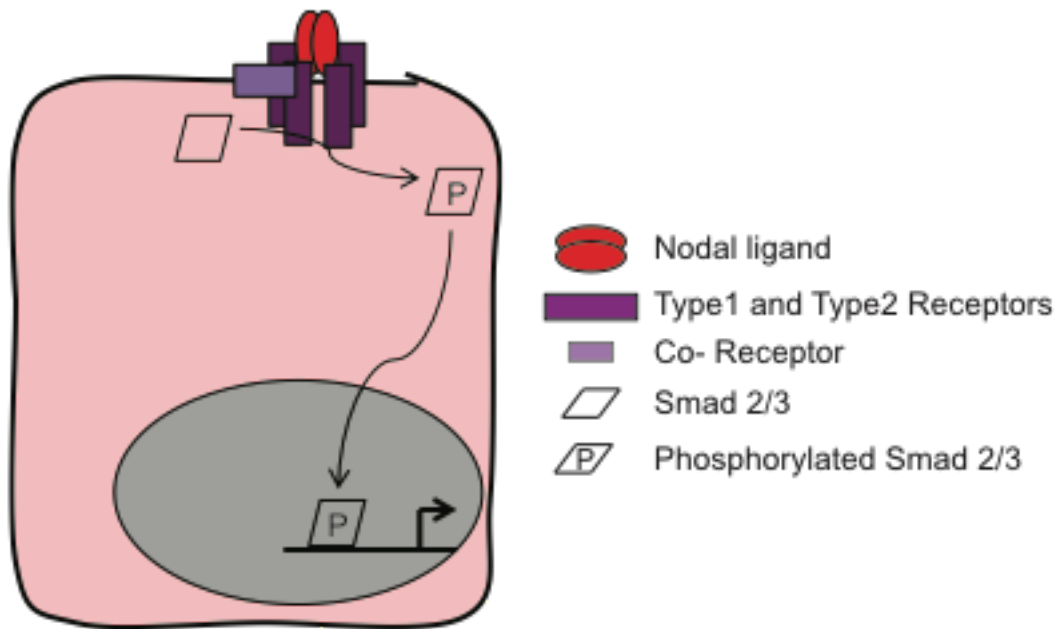
Table 1- Definitions, Asymmetric Cell Divisions

Par proteins	Proteins found in a <i>C. elegans</i> genetic screen that regulate the partitioning of cytoplasm in the early embryo [66].
Par 1	A Serine/Threonine kinase protein that localizes in the posterior pole during the first division in <i>C. elegans</i> . It is required for asymmetric cell division in <i>C. elegans</i> [67].
Par 2	A protein that contains a zinc finger domain and an ATP binding domain. Par2 localizes in the posterior pole during the first cell division in <i>C. elegans</i> . It is required for asymmetric cell division in <i>C. elegans</i> [68].
Par 3	Protein localized in the anterior pole during the first division in <i>C. elegans</i> . It is required for asymmetric cell divisions [69]. The <i>Drosophila</i> ortholog is termed Bazooka.
Par 6	PDZ-domain containing protein localized in the anterior pole during the first division in <i>C. elegans</i> . It is required for asymmetric division [70].
aPKC (Atypical protein kinase C)	This kinase was first found to co-localize with a mammalian Par3 homolog (ASIP) at the epithelial tight junction [71]. Subsequent studies in <i>C. elegans</i> and mammalian cell culture showed that aPKC complexes with Par3 and Par6 and is required for cell polarity [72-74].
Gα (Heterotrimeric G protein, subunit α)	When the heterotrimeric G protein complex subunit α is released from subunits β and γ , the alpha subunit remains free and able to interact with proteins that regulate asymmetric cell divisions [75]. Since G α is inserted in the membrane, G α tethers associated proteins to the plasma membrane. Generally, G α binds to scaffolding proteins such as Pins or GPR which further associate with microtubule motor proteins. Since G α is asymmetrically localized this allows the mitotic apparatus to be pulled in a specific direction.
Pins, GPR 1/2, AGS	G protein regulator proteins involved in asymmetric cell divisions. Pins is the <i>Drosophila</i> ortholog, GPR 1/2 are <i>C. elegans</i> orthologs, and AGS is the mammalian ortholog. These adaptor proteins bind G α and also linker proteins to aid in asymmetric placement of the mitotic spindle [75-78].
NuMA, Lin5, Mud	Linker protein involved in asymmetric cell divisions. This protein binds Pins (in a complex with membrane associated G α) and also binds dynein proteins. NuMA is the mammalian ortholog, Lin5 is in <i>C. elegans</i> , and Mud is the <i>Drosophila</i> ortholog [79-82].
Dynein/Lis-1	Dynein and its subunit Lis-1 are motor proteins that travel towards the minus end of microtubules. When motor proteins become physically associated with one pole they generate a force able to pull the mitotic spindle towards themselves [83].
Prospero	Transcription factor involved in specification of the ganglion mother cell fate during asymmetric cell division in <i>Drosophila</i> neuroblasts [84, 85].
Brat	Translational inhibitor involved in specification of the ganglion mother cell fate during asymmetric cell division in <i>Drosophila</i> neuroblasts [86-89].
Numb	Endocytic protein involved in specification of the ganglion mother cell fate during asymmetric cell division in <i>Drosophila</i> neuroblasts [90, 91].

Table 2- Definitions, Invagination

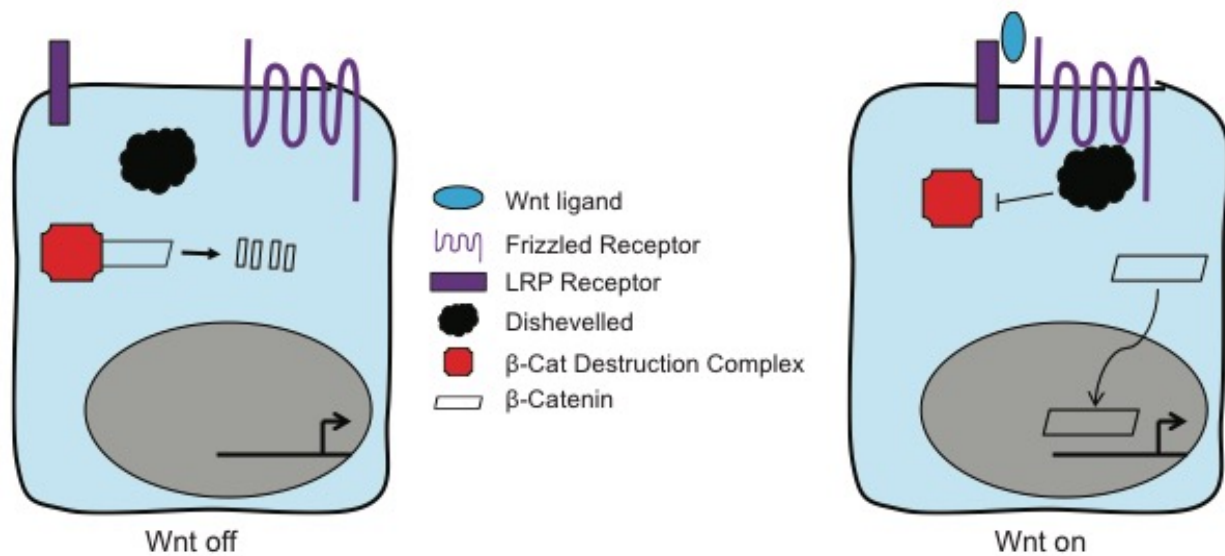
Cadherins	Family of transmembrane proteins that are homophilic (they bind to members of the same class). Generally, cadherins are expressed at the cell surface and enhance intercellular adhesion. The homophilic nature of cadherins is particularly important to regulate morphogenetic events [92].
N-cadherin	This protein was first found to be associated with neural cells and is generally required for cells to remain associated during morphogenetic events [93].
E-cadherin	This protein is generally associated with epithelial cells and is required for cells to remain attached together in a sheet [94].
Filamentous actin (F-actin)	One of the major cytoskeletal components of a cell. Actin can be found in globular forms (monomers) or can assemble into long filaments (polymers). During invagination, activated myosin motors “walk” along loose f-actin networks to contract the cell membrane or move a biological structure in a specific location [10].
Non-muscle myosin	Motor proteins that hydrolyze ATP to move along actin filaments [10].
Twist	Transcription factor involved in the upregulation of N-cadherin during mesoderm invagination in <i>Drosophila</i> [13].
Snail	Transcription factor involved in the upregulation of N-cadherin during mesoderm invagination in <i>Drosophila</i> [13].
Rho activating proteins	Family of proteins that regulate myosin's activity. Generally, when myosin is phosphorylated at certain sites it becomes activated [10, 95].
G-cadherin	This protein is required for germ cell specification in sea urchins [14].

Table 3- Definitions, Nodal Signaling Pathway



Nodal	An extracellular signaling ligand, a member of the Tgf- β superfamily [96]. Nodal is required for many developmental processes including: mesoderm formation, endoderm formation, neural patterning, Left/Right patterning, Dorsal/Ventral axis formation, and Anterior/Posterior axis formation [15].
Alk4, ActRII	Alk4, a type 1 serine-threonine kinase receptor that heterodimerizes with ActRII, a type 2 receptor. Nodal binds this receptor complex to initiate signaling [97-99].
Cripto/EGF-CFC	A co-receptor Nodal is dependent on for signaling [97, 99].
Smad 2/3	Intracellular Nodal signaling transducer. Activated Nodal receptor phosphorylates Smad 2/3 proteins. Smad 2/3 then accumulate in the nucleus to initiate Nodal mediated transcription [100].
Lefty	An inhibitor of Nodal-mediated signaling. Lefty is secreted and binds to Cripto (inhibiting it from interacting with Alk4/ActRII receptors) or Lefty binds Nodal itself and inhibits it from binding to the receptor [101, 102].
Reaction diffusion mechanism	The Turing model that suggests if an activator and inhibitor are expressed in the same initial region this condition can generate a graded morphogen. Generally, the activator autoregulates itself, so that wherever it is expressed signaling occurs in high levels. The inhibitor becomes more significant farther away from the activator region (as it can travel farther than the activator) and it inhibits the activator from being functional which leads to lower levels of signaling. In this fashion, a graded morphogen of signaling is established [15, 103, 104].

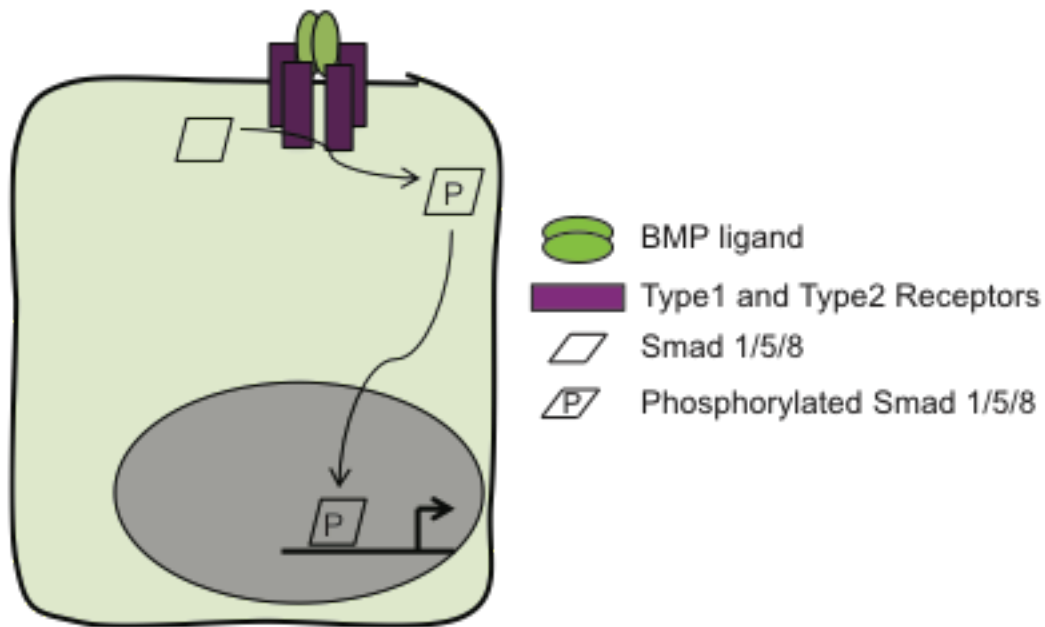
Table 4- Definitions, Wnt signaling pathway



Wnt	An extracellular signaling ligand and signaling pathway present in all animals. Wnt is involved in many processes, perhaps the most conserved function is its involvement in specification of the anterior/posterior axis [105, 106].
Frizzled (Fz) Low density lipoprotein receptor related protein (LRP)	Receptor complex that binds the Wnt ligand [107, 108].
Dishevelled	Intracellular protein that is activated upon Wnt binding to receptor. Activated dishevelled causes disruption of a β -catenin destruction complex [109, 110].
β-catenin destruction complex	Consists of APC (product of the adenomatous polyposis coli gene), glycogen synthase kinase 3b (Gsk-3B) and Axin. Together, these proteins cause phosphorylation of β -catenin at certain sites which target β -catenin to be degraded by the proteasome [110].
β-catenin	A multi-functional protein that includes transcriptional regulation. When it is not degraded it can enter the nucleus, interact with the TCF-Lef transcription factor and initiate transcription of target genes. It can also link cell adhesion molecules e.g. cadherins, to the actin cytoskeleton [110].
FoxY	Forkhead box transcription factor found in sea urchins. It is required for expression of germ cell factors in the germ cells. It is a putative target of

	β -catenin transcription in sea urchin germ cells [111] (Swartz et al, in preparation).
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Table 5- Definitions, BMP signaling pathway



BMP	Member of the Tgf- β superfamily of signaling ligands. BMP has many functions including: bone formation, embryonic development, and normal tissue homeostasis [112].
Alk2/3/6 (Type 1) BMPRII, ActRIIA, ActRIIB (Type 2)	Cell surface receptors required to transduce BMP signaling. Upon ligand binding, a conformation change causes Type II receptors to phosphorylate Type I receptors. Type I receptors are then able to phosphorylate Smad proteins [113].
Smad 1/5/8	Intracellular signaling transducers. Once these proteins are phosphorylated they can accumulate in the nucleus and stimulate specific gene expression [113].

Table 6- Definitions, General

Germ plasm	Electron dense aggregates composed of RNA, protein, and sometimes mitochondria. The RNAs and protein are typically germ cell factors such as Nanos and Vasa. These aggregates localize to a specific area in the egg cortex and are required for germ cell specification by inheritance. Embryonic cells that retain these maternal aggregates are often fated to become germ cells [24].
Oskar	The scaffolding protein in <i>Drosophila</i> that causes formation of germ plasm in the posterior pole [26]. The only determinant known to be sufficient for germ cell fate.
Xvelo	The scaffolding protein in <i>Xenopus</i> that causes formation of germ plasm in the vegetal pole. Xvelo is a disordered protein with a prion-like domain. This prion like-domain is required for the condensation of germ plasm into aggregates [34].
Dazl	RNA binding protein that belongs to the Daz (Deleted in Azoospermia) family of proteins. Daz family proteins are found almost exclusively in germ cells of diverse animal species. Daz family proteins are generally required for germ cell specification or for germ stem cell function in animals and Dazl is Daz-like [114].
Dorsal (transcription factor)	A NF- κ B related transcription factor required for dorsal/ventral patterning in the fly. Dorsal only enters the nucleus in ventral cells where it activates the transcription of ventral specific genes and inhibits the transcription of dorsal genes [45].
Spectrosome	A cytoplasmic structure required for cyst formation in the ovary of most insects. In most insects, incomplete cell divisions cause the formation of ovarian cysts. A cyst is comprised of 16 cells, one will become the egg and the other 15 will become nurse cells. The spectrosome is made up of remnants of the mitotic spindle that fuse together [115].
FGF	Fibroblast growth factor is a secreted ligand and cell signaling pathway with many functions including: cell growth, cell proliferation, angiogenesis, and development [116]. It has recently been shown to be required for germ cell specification in the axolotl [42].
MAPK	The MAPK cascade is an intracellular signaling relay system of three proteins that phosphorylate each other. The last kinase in the pathway is MAPK which goes on to enter the nucleus and active the transcription of downstream targets [117]. MAPK signaling is required for germ cell specification in the axolotl [42].
Oct4	Transcription factor that belongs to the Pou family. Oct4 is one of the core regulators of pluripotency in the inner cell mass cells during the mammalian blastocyst stage and in embryonic stem cells [58, 59].
Sox2	Transcription factor that belongs to the Sox family. Sox2 is one of the core regulators of pluripotency in the inner cell mass during the mammalian blastocyst stage and in embryonic stem cells [57].
Nanog	A divergent homeodomain transcription factor. Nanog is one of the core regulators of pluripotency in the inner cell mass during the mammalian blastocyst stage and in embryonic stem cells [60-62].

Appendix A: Germ cells are not quiescent during early development of the sea star

I contributed to all of the work in this Appendix with guidance from G. Wessel.

Introduction

In many animals, primordial germ cells (PGC's) become quiescent when they are first specified. In sea urchins, for example, PGC's become quiescent when they are formed at the 32 cell stage. Markers of quiescence in sea urchins include: decreased cell divisions [1], increased levels of H3K9me3 [2], decreased levels of actively transcribing RNA polymerase [2], and decreased levels of translation [3]. In order to determine if primordial germ cells become quiescent in the sea star I tested for quiescent traits during embryogenesis.

Materials and Methods

EdU Pulse

Embryos were pulsed with 10 uM EdU for 1 hour at a given time point during embryogenesis. EdU was visualized by using a Click-it Kit EdU Alexa 488 imaging kit (Thermofisher). DNA was labelled by incubating embryos with Hoescht (10 ug/ml) for at least 15 minutes. Embryos were visualized with a Zeiss 510 confocal microscope.

H3K9me3 labelling

Embryos were fixed overnight in MOPS buffered PFA. H3k9me3 was labelled by incubating embryos with rabbit anti-H3k9me3 primary antibody (Abcam 8898, 1:250) followed by incubating embryos with anti-rabbit Alexa 488 secondary antibody (Fisher, 1:500). DNA was labelled by incubating embryos with Hoescht (10 ug/ml) for at least 15 minutes. Embryos were visualized with a Zeiss 510 confocal microscope.

RNA Polymerase2 Phospho-Serine2 labelling

Embryos were fixed overnight in MOPS buffered PFA. RNA Pol2 P-Ser2 was labelled by incubating embryos with rabbit anti-RNAPol2PSer2 primary antibody (Abcam 5095, 1:250) followed by incubating embryos with anti-rabbit Alexa 488 secondary antibody (Fisher, 1:500). DNA was labelled by incubating embryos with Hoescht (10 ug/ml) for at least 15 minutes. Embryos were visualized with a Zeiss 510 confocal microscope.

Results

Pulsing embryos with EdU allows detection of DNA synthesis during cell cycling. In sea urchins, an EdU pulse during the first cell division labels the early embryo DNA with EdU. As development proceeds, somatic cells continue to divide and their EdU becomes diluted. Meanwhile, the germ cells do not undergo cell divisions and the nuclei retain high levels of EdU [1]. With the premise that the germ line does not form until gastrulation in the sea star, I pulsed embryos with EdU at 1DPF and at 4 DPF. Embryos were imaged 3 days later and the germ cells (cells in the posterior pouch) contained EdU labelling (Figure 1, A-D). This shows that germ cells are undergoing cell divisions at 1 DPF and 4 DPF because they have incorporated EdU. Furthermore, the levels of EdU in the germ cells were similar to levels in nearby somatic cells in the endoderm (Figure 1, A-D). One interpretation of this result is that EdU was diluted at a similar rate in germ cells and somatic cells. Taken together, this experiment is consistent with the hypothesis that germ cells are still undergoing cell divisions up to the larva stage and do not exhibit a detectable cell cycle quiescence as seen in the sea urchin.

H3k9me3 is an epigenetic marker of regions of the genome that are generally heterochromatic and not being transcribed. Primordial germ cells in the sea urchin have increased levels of H3k9me3 in their nuclei, consistent with a low level of nascent mRNA transcription [2]. To detect overall heterochromatic regions, I used an antibody to detect H3k9me3 in the nuclei of sea star larva. My results show that H3k9me3 is present in nuclei of the germ cell pouch and in nuclei of nearby somatic cells. Furthermore, I do not detect any qualitative differences between the levels of staining in germ cells vs. somatic cells. This experiment is consistent with the hypothesis that germ cells remain transcriptionally active in the larva stage.

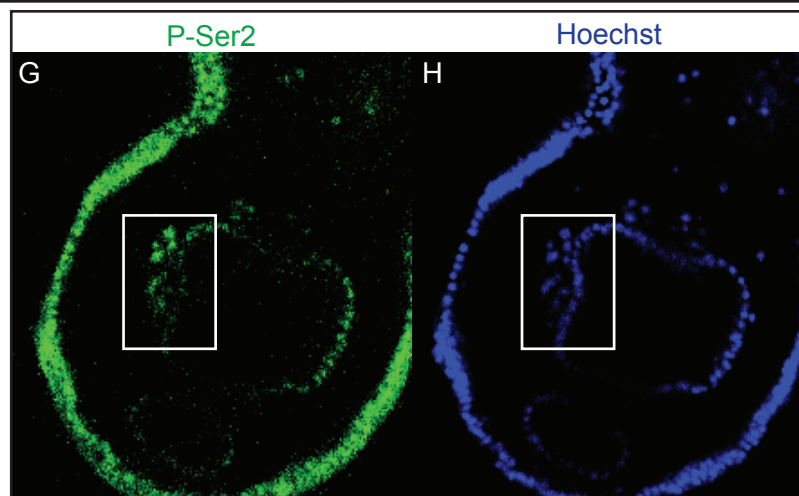
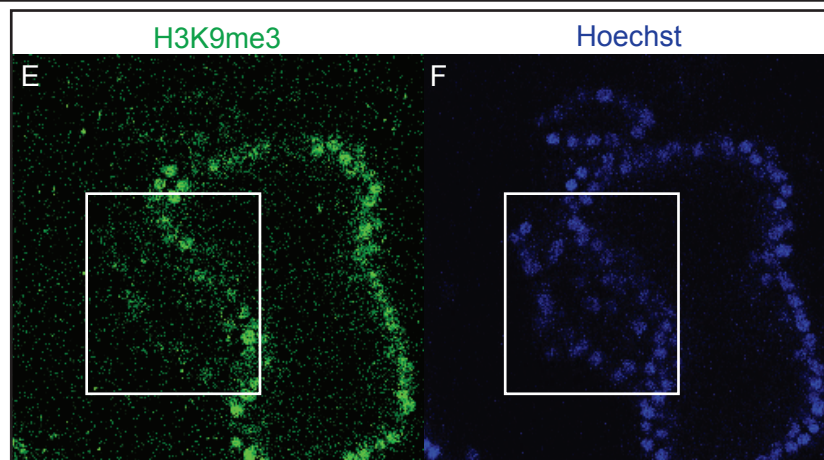
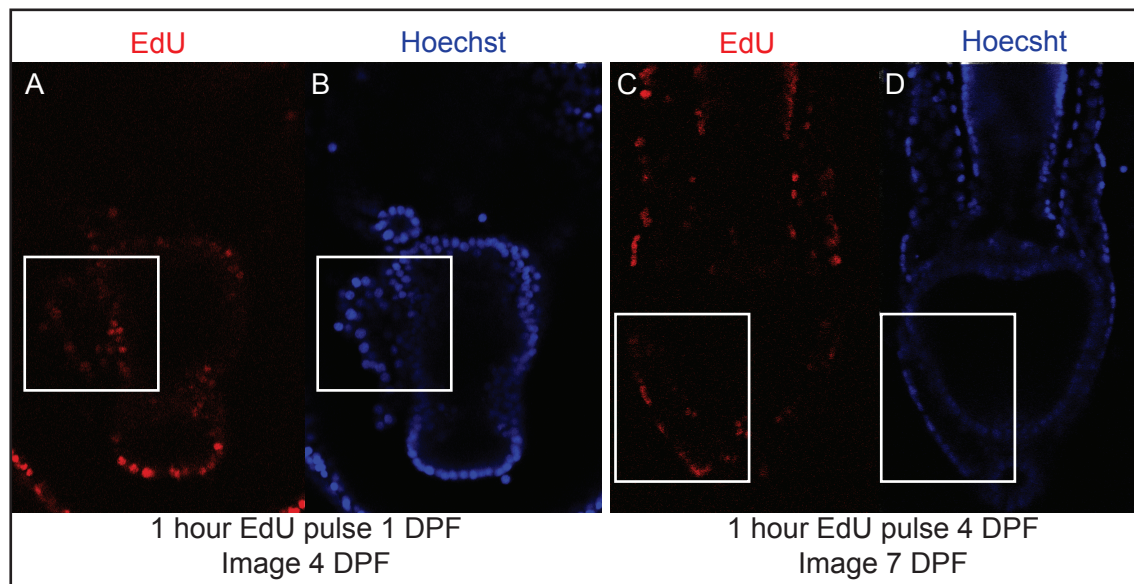
Phosphorylation of the C-terminal domain of RNA polymerase 2 at Serine 2 is a general marker of actively transcribing RNA pol 2. Primordial germ cells in the sea urchin, round worm, and fruit fly are depleted in this marker, showing they have low transcriptional activity [2, 4]. I used an antibody to detect active RNA pol 2 at the larva stage of the sea star and I found that active RNA pol2 is present in the germ cell pouch at similar levels as nearby somatic cells. This further supports the hypothesis that germ cells are transcriptionally active at the larva stage of the sea star.

Conclusions

I conclude that the germ cell lineage is not quiescent during embryogenesis of the sea star and is capable of responding to embryonic signals. In order for primordial germ cells to be specified by inductive mechanisms germ cell precursors need to be able to respond to extracellular signals. As such, primordial germ cells are transcriptionally active during their formation in animals that use inductive mechanisms for germ cell specification [5, 6]. Overall these results are consistent with the hypothesis that sea stars use inductive mechanisms for germ cell specification.

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Appendix A- The germ cell lineage is not quiescent during early embryogenesis in sea stars

In panels A-B embryos were pulsed with EdU for 1 hour at 1 Day Post Fertilization (DPF). Embryos were then fixed and imaged at 4 DPF. Cells in the PE (white box) do not selectively retain EdU, meaning they are still undergoing cell divisions. Panels C-D show similar results except embryos were pulsed with EdU at 4 DPF and imaged 7 DPF.

In panels E-F larva were stained with an anti H3k9me3 antibody. Cells in the PE (white box) do not have increased amounts of H3k9me3, meaning they do not contain epigenetic markers of heterochromatin.

In panels G-H larva were stained with RNA polymerase 2 phospho serine 2 antibody (a marker of active RNA polymerase 2). Cells in the PE (white box) do not have a lower amount of P-Ser2 staining, meaning they do not show low transcriptional activity.

Appendix B: Upregulation of Wnt signaling causes expansion of Nanos mRNA localization

I contributed to all of the work in this appendix with the help of an undergraduate trainee, Henry Vargas. G. Wessel also contributed valuable feedback.

Introduction

The Wnt signaling ligand is required for germ cell specification. In mice Wnt is required for upregulation of the transcription factor Brachyury (T) which is responsible for the transcription of Blimp1, one of the major transcriptional regulators involved in conferring a germ cell fate [1]. In addition, Wnt signaling has also been implicated in germ cell specification in sea urchins. In particular, Disheveled and nuclear- β -Catenin are localized in the vegetal pole of the egg and primordial germ cells inherit these molecules when they are formed at the 32 cell stage [2, 3]. In addition, when Wnt signaling is inhibited in sea urchins there is a decrease in Nanos mRNA levels (Swartz et al., in preparation). Here I test if Wnt signaling is involved in germ cell specification in the sea star by incubating embryos with a Gsk3 inhibitor.

Materials and Methods

Embryos were incubated with LiCl, a GSK3 inhibitor (30 μ M or 60 μ M), or water just after fertilization [4]. Embryos were fixed the next day in MOPS buffered PFA. A whole mount in situ hybridization was performed using a Nanos DIG labelled probe [5].

Results

Inhibition of GSK3, a part of the β -catenin destruction complex, causes upregulation of Wnt Signaling. As Wnt signaling is conservatively involved in patterning the vegetally localized endomesoderm I expect embryos treated with LiCl to have a vegetalized phenotype. In this experiment embryos treated with LiCl have decreased anterior/animal cells fates. This can be seen by measuring the distance from the top of the archenteron to the anterior ectoderm. The distance is greater in control embryos and non-treated embryos (A, D, E), when compared to embryos treated with LiCl (B-C). In the future it will be important to test if molecular markers of vegetal fates such as FoxA, Brachyury, and Ets are expanded in embryos treated with LiCl [6]. Nevertheless, this data shows that LiCl is working as predicted and is vegetalizing embryos.

Upregulation of Wnt signaling causes an expansion of Nanos mRNA. In embryos treated with vehicle (water) Nanos localizes in a narrow ring in the blastopore (A). In embryos treated with low levels of LiCl, Nanos localizes in a wide ring in the blastopore (B). In embryos treated with high levels of LiCl Nanos localizes in a dispersed ring in the ectoderm (C). In order to test if LiCl is causing a developmental delay I include images of Nanos localization in embryos at similar phenotypic stages. Nanos localizes in expanded domains in embryos treated with LiCl (B-C), compared to embryos at similar phenotypic stages (D-E). These results are consistent with the hypothesis that Wnt signaling promotes the germ cell fate in the sea star.

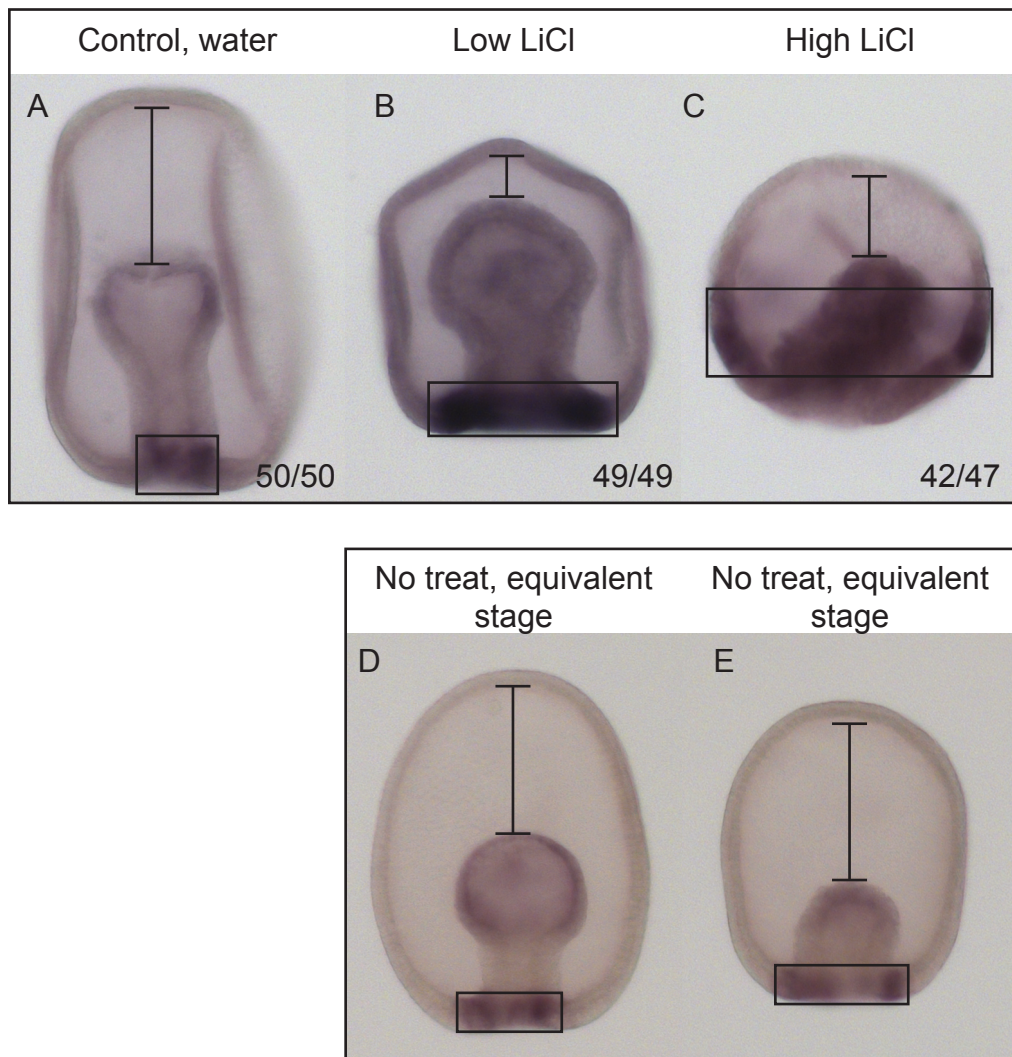
Conclusions

Wnt may be conservatively required for the promotion of a germ cell fate. Wnt signaling has been shown to be required for germ cell specification in the mouse. In addition, the inheritance of maternally localized intracellular Wnt signaling components is likely required for germ cell specification in the sea urchin. This experiment in sea stars shows that Wnt signaling also promotes a germ cell fate. It is interesting to note that Wnt is conservatively required for specification of the A/P axis and specification of the endomesoderm cell fate. This is significant because the posterior region of an egg

generally contains germ cell factors that contribute to the inheritance of the germ cell fate. In addition, the germ cell fate is also commonly induced from mesodermal cell precursors. This means that both mechanisms of germ cell specification correlate with Wnt signaling components. An observation that warrants future experiments to test if Wnt is ancestrally involved in germ cell specification.

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Appendix B- Upregulation of Wnt signaling causes expansion of Nanos mRNA localization

Embryos were treated with varying concentrations of GSK3 inhibitor or vehicle (water). As GSK3 causes the degradation of β -catenin, the GSK3 inhibitor, LiCl, should upregulate Wnt signaling. In embryos treated with water (A), Nanos localized in a narrow ring in the blastopore. In embryos treated with low concentration of LiCl (B), Nanos localized in a wide ring in the blastopore. While in embryos treated with high concentration of LiCl (C), Nanos localized in a dispersed ring in the ectoderm.

In order to test if the expansion of Nanos mRNA is due to a developmental delay, I included images of Nanos mRNA localization at similar phenotypic developmental stages. In embryos treated with low concentration of LiCl (B), Nanos accumulated in an expanded domain compared to embryos at an equivalent developmental stage (D). In embryos treated with high concentration of LiCl (C), Nanos accumulated in an expanded domain compared to embryos at an equivalent developmental stage (E).

Appendix C: Nanos and FoxY mRNA are expressed in similar domains during sea star embryogenesis

I contributed to all of the work in this appendix with guidance from G. Wessel.

Introduction

As germ cells derived from inherited mechanisms generally have low transcriptional activity, the majority of what we know about transcriptional control of germ cell specification is in mice (an animal that uses inductive mechanisms for germ cell specification). In mice, three transcriptional regulators are required for the specification of primordial germ cells: *Blimp1*, *Prdm14*, and *Ap2gamma* [1]. *Blimp1* also has been found to be required for germ cell specification in crickets [2]. In the sea urchin, a novel member of the forkhead transcription factor family, *FoxY*, has been implicated in the transcription of *Nanos* [3]. In order to test if *FoxY* is conservatively required for the transcription of *Nanos* in echinoderms I test if *FoxY* and *Nanos* are localized in similar expression domains in the sea star.

Materials and Methods

Whole mount in situ hybridizations were performed as described previously [4]. DIG labelled *Nanos* and *FoxY* probes were synthesized by transcription off of pGemT-EZ vectors within which regions of these genes were subcloned.

Results

Nanos and *FoxY* are expressed in similar domains. During the mid-gastrula stage *Nanos2* is transiently expressed in a ring surrounding the blastopore. *FoxY1* and *FoxY2* localize in a similar domain at the mid-gastrula stage. In addition, *Nanos3* and *FoxY3* accumulate in similar domains during the mid-gastrula and late gastrula stages. They both first accumulate in a ring in the vegetal territory and then accumulate in a dorsal patch of cells in the middle of the developing gut. These results support the hypothesis that *FoxY* is required for the transcription of *Nanos* in sea stars.

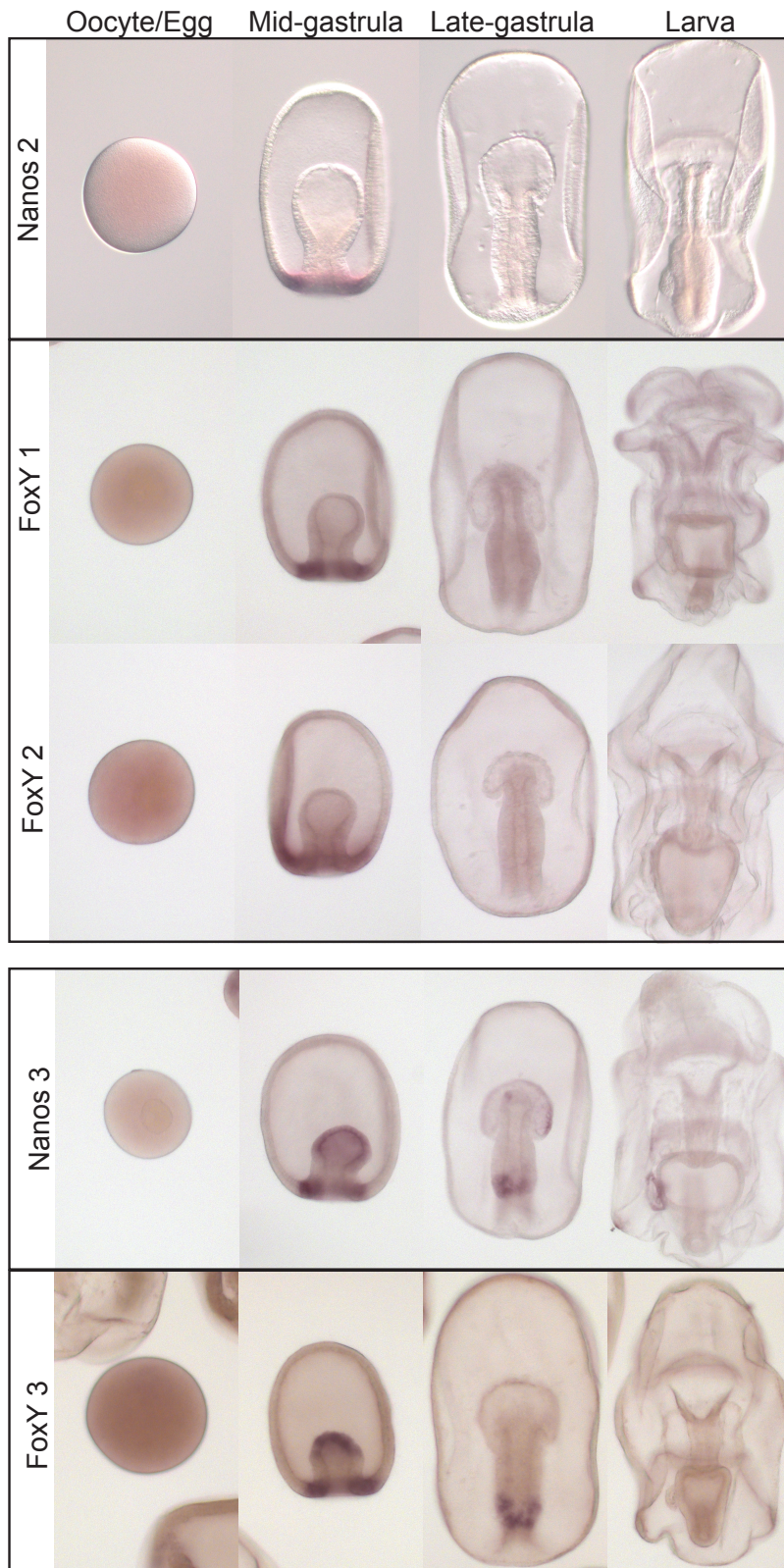
Conclusion

FoxY may be a conserved transcriptional regulator involved in germ cell specification in echinoderms. Previous research in the sea urchin shows that when *FoxY* is reduced, *Nanos* RNA and protein is depleted in the germ cell lineage [3]. The experiment presented here shows that *FoxY* and *Nanos* are expressed in similar embryonic territories in the sea star also. Future *FoxY* knockdown experiments in the sea star will be necessary to test if *FoxY* regulates *Nanos* mRNA during sea star development. If *FoxY* is conservatively found to be required for *Nanos* transcription in echinoderms then it will be important to test how *FoxY* is transcribed in germ cell precursors, if it is essential for germ cell fate, and if other *Fox* transcription factors are important for the transcription of germ cell factors in other animals.

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Appendix C- Nanos and FoxY mRNA are expressed in similar domains during sea star embryogenesis

Nanos 2 is transiently expressed in a vegetally localized ring at the mid-gastrula stage. FoxY1 and FoxY2 are expressed in a similar domain.

Nanos 3 is expressed in a ring at the vegetal pole at the mid-gastrula stage, a patch in the developing gut at the late gastrula stage, and in a left pouch at the larva stage. FoxY3 is expressed in a similar domain during the mid-gastrula and late-gastrula stage.

Appendix D: The biology of the germ line in echinoderms

Wessel GM, Brayboy L, **Fresques T**, Gustafson EA, Oulhen N, Ramos I, Reich A, Swartz SZ, Yajima M, and Zazueta V. (2014). The biology of the germ line in echinoderms. *Mol. Reprod. Dev.* 81(8):679-711.

I contributed to the research involved in Figure 17.

The Biology of the Germ line in Echinoderms

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SUMMARY

The formation of the germ line in an embryo marks a fresh round of reproductive potential. The developmental stage and location within the embryo where the primordial germ cells (PGCs) form, however, differs markedly among species. In many animals, the germ line is formed by an inherited mechanism, in which molecules made and selectively partitioned within the oocyte drive the early development of cells that acquire this material to a germ-line fate. In contrast, the germ line of other animals is fated by an inductive mechanism that involves signaling between cells that directs this specialized fate. In this review, we explore the mechanisms of germ-line determination in echinoderms, an early-branching sister group to the chordates. One member of the phylum, sea urchins, appears to use an inherited mechanism of germ-line formation, whereas their relatives, the sea stars, appear to use an inductive mechanism. We first integrate the experimental results currently available for germ-line determination in the sea urchin, for which considerable new information is available, and then broaden the investigation to the lesser-known mechanisms in sea stars and other echinoderms. Even with this limited insight, it appears that sea stars, and perhaps the majority of the echinoderm taxon, rely on inductive mechanisms for germ-line fate determination. This enables a strongly contrasted picture for germ-line determination in this phylum, but one for which transitions between different modes of germ-line determination might now be experimentally addressed.

[T]he Echinoidea lineage... may represent a transition from an inductive mechanism for germ-line specification to a more inherited-like mechanism.

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NSF; Grant sponsor: PEW Foundation;
Grant sponsor: CONACyT

Mol. Reprod. Dev. 81: 679–711, 2014. © 2014 Wiley Periodicals, Inc.

Published online 22 July 2014 in Wiley Online Library
(wileyonlinelibrary.com).
DOI 10.1002/mrd.22223

Received 27 April 2013; Accepted 23 July 2013

INTRODUCTION

Diversity in Development

As remarkable as the mechanism of fate acquisition is in early development, perhaps even more striking is the diversity by which this task is accomplished. Syncytial early development (e.g., insects), invariant cleavage (e.g., ascidians), variable cleavage (e.g., mammals), discoidal (partial) cleavage (e.g., birds), etcetera all contribute to the early embryogenesis—and it gets even more variable and complex after formation of the various cellular layers (germ layers) that give rise to the tissues outside (ectoderm), inside (endoderm), and in between (mesoderm). Not only is the final product diverse, but the mechanisms of

getting there are too. Certainly animals have many commonalities—the phylotypic stage, originally popularized by von Baer, is one good example (see e.g., Gilbert, 2013), yet diversity is more the rule than the exception.

Diversity of Germ Cell Development

Germ cells are not exempt from this developmental diversity—in fact they absolutely excel at this trait. The

Abbreviations: ABC transporter, ATP-binding cassette transporter; A–V, animal–vegetal; L/R, left–right [asymmetry]; LMics, large micromeres; PGC, primordial germ cell; sMic, small micromeres; UTR, untranslated region

germ line is the lineage of cells that leads to construction of eggs and sperm. While many tissues and cells are essential for this development, for example, the gonads (ovary and testis), the germ line is distinct from those because it is capable of passing hereditary information (genome) from one generation to the next, whereas all other cells (the so-called somatic cells, or soma) are programmed to terminate with the adult.

The first-popularized publication documenting the use of the term “germ” for the reproductive lineage is a comprehensive monograph on the origin of ‘germ cells’ in 38 species of marine hydroids, by August Weismann in (1883). In it, this giant in the field of developmental biology detailed the anatomy of various stages in the life cycle of these organisms, including the migratory precursors of *germ cells* (“Keimzellen”). The term *stem cells* (“Stammzellen”) was used in this monograph for the first time to point out putative migratory sperm progenitors. The terms *primordial germ cells* (“Urkeimzellen”) and *germ line* (“Keimbahn”) appear throughout the work, which charts the life cycle of the *Keimzellen*. The concept of *germ plasm* (“Keimplasma”) was discussed in a later publication, from an 1885 lecture given at the University of Freiburg, Weismann (1889), in reference to a distinct area of cytoplasm in the egg and early embryo that was always associated with the germ-line lineage. Here “germ” is used not to describe a pathogenic microbe, instead signifying the rudiment of a living organism, the initial stage in development, or the source for subsequent development (germ from the French *germe*, and the Latin *germen* meaning sprig, bud, or seed).

An important concept of the germ line is continuity. Following successful fertilization of functional gametes, the developing embryo will eventually make primordial germ cells (PGCs), a lineage that will commit largely if not exclusively to the germ cells. At some later point in development, the PGC population will expand through mitosis as germ-line stem cells and eventually begin gametogenesis (development of eggs and/or sperm). Successful fertilization of the next generation completes the cycle. This scheme is generally true for the germ line of most metazoans, although the details are markedly variable between even closely related species. For excellent perspectives on diversity in reproduction, consider the monographs by Birkhead (2002) and by Judson (2002).

Unique PGC Features

PGCs are precursors, formed in the embryo, that give rise to eggs or sperm. In many ways, they are the mother of all stem cells, and as such, their formation and development may have embellishments. Without a functional germ line, sexually reproducing organisms cannot reproduce, and the unique genetic composition of that individual is lost. While many cells of an organism are necessary for the germ line to develop and to be maintained, the germ-line lineage is distinct from the supporting cells (the soma) of the organism.

PGCs usually form in one portion of the embryo, sometimes even in association with the extra-embryonic tissues,

and in a space distinct from the somatic portion of the gonad. The PGCs almost universally migrate extensively from their birthplace to the developing gonadal tissues, and do so in a defined period of embryogenesis; if they fail to reach the developing gonad by the end of this time window, the PGCs usually die by apoptosis. It is feasible that this PGC migration is a ‘test’ that has been retained to select for PGCs bearing intact mechanisms for signal transduction, migration, homing, etcetera—characteristics essential for development of the next generation and reflective of an intact, and functional genome. Failure of the PGCs to apoptose may result in their conversion to teratomas (from the Greek *teras* for monster, or prodigy), which contain a variety of fetal-like tissues from all three germ layers. Although potentially dangerous for the individual harboring these growths, it does show the complete developmental potential retained by the PGCs.

Formation of PGCs in Animals

PGC fates may be acquired by a variety of contrasting mechanisms in animals, from a multipotent cell in adults, by *inherited* molecules, or predominantly by *inductive* cellular interactions. These developmental strategies are likely overlapping in mechanism with many shared intermediates.

The inherited mechanism, in which maternal instruction segregated within the oocyte drives development of PGCs in the cells that have acquired it. This mechanism has been widely studied in diverse organisms such as *Drosophila*, *Caenorhabditis elegans*, and *Danio rerio* (see e.g., Gao and Arkov, 2012; Lai and King, 2013; Voronina, 2013). Given their short generation time, the genetic screening approaches available, and their effective adaptation for laboratory research of these organisms, it is not coincidental that research on these organisms has led the field in elucidating mechanisms for germ-line specification. The PGCs in this group of organisms are segregated early during embryogenesis and contain electron-dense cytoplasmic organelles, called germ plasm (Geigy, 1933; Bounoure, 1934; Jazdowska-Zagrodzinska, 1966; Smith, 1966; Buehr and Blacklar, 1970; Illmensee and Mahowald, 1974; Okada et al., 1974; Eddy, 1975; Warn, 1975; Frohnofer et al., 1986; Ikenishi et al., 1986; Niki, 1986). The germ plasm in these animals was found to contain many unique RNAs and proteins that are conserved among diverse organisms, molecules such as Vasa (Seydoux and Braun, 2006; although see also Kassmer and Krause, 2013). These molecules are now often conveniently used as markers for researchers to identify PGCs in many organisms because of their conserved expression pattern in the germ line. Formation of these cells early in development means they are often insulated from normal developmental activities and are relatively quiescent for much of embryogenesis.

The inductive mechanism of PGC specification (fate determination by cell interactions) is less well understood, and is studied primarily in the mouse (Surani et al., 2008; Lesch and Page, 2012). This mechanism of germ-line

determination appears to rely on cell-to-cell signaling molecules, which impinge upon changes in the epigenetic landscape in cells that are otherwise destined to become somatic mesoderm. Inductive-derived PGCs generally form later in embryos than in the inherited strategy. Multiple epigenetic modifiers, including *Prdm* family members, appear necessary in the early mouse germ-line precursors to repress genes involved in a somatic mesoderm fate, including *Hox* genes. The frequency and distribution of animals utilizing inductive determination suggest that it is the basal or ancestral strategy of germ-line determination; animals using the inherited mechanism instead appear to have independently acquired this mechanism. This apparent transition in developmental mode is prevalent throughout phylogeny (e.g., Extavour and Akam, 2003), and may result in a continuum of mechanistic change between organisms (Juliano and Wessel, 2010; Seervai and Wessel, 2013).

The third general mechanism of germ-line determination, the adult-derived mechanism, is probably also reliant heavily on inductive interactions, if only because it is difficult to image a prolonged, segregated portion of maternal cytoplasm remaining in adults. Significant research is devoted to this topic in planaria and hydra (e.g., Chong et al., 2011; Rouhana et al., 2012) as it is associated with the vast potential for understanding totipotent adult stem cells.

Echinoderms have recently become popular for studying germ-line determination. The same properties that have made these animals amenable to understanding fertilization, calcium regulation, gene regulatory networks, sperm activation, chemotaxis (e.g., Ettensohn et al., 2004; Davidson, 2010; McClay, 2011; Peter and Davidson, 2011) make these embryos useful to understand germ-line determination. Large numbers of embryos ($>10^6$ per adult) can be obtained that are mostly transparent; develop predictably and synchronously; are facile to manipulation; and have a rich, classical literature describing them. Further, these animals represent a sister group to chordates in the deuterostome clade, and the research community has generated large amounts of genomic knowledge and resources (e.g., spbase.org; Weinstock et al., 2006).

Early Development of the Sea Urchin

Sea urchin embryos develop rapidly (15–60 min cell cycles depending on the species), synchronously, and predictably. Large cultures can be cultivated, treated, and dissociated, while individual embryos can be injected or manipulated to test important features of their developmental mechanisms. The first three cell divisions in the sea urchin are equal and yield a symmetrical eight-cell embryo (Fig. 1). The fourth cell division, however, is uneven—the four embryonic cells (blastomeres) that form on the animal

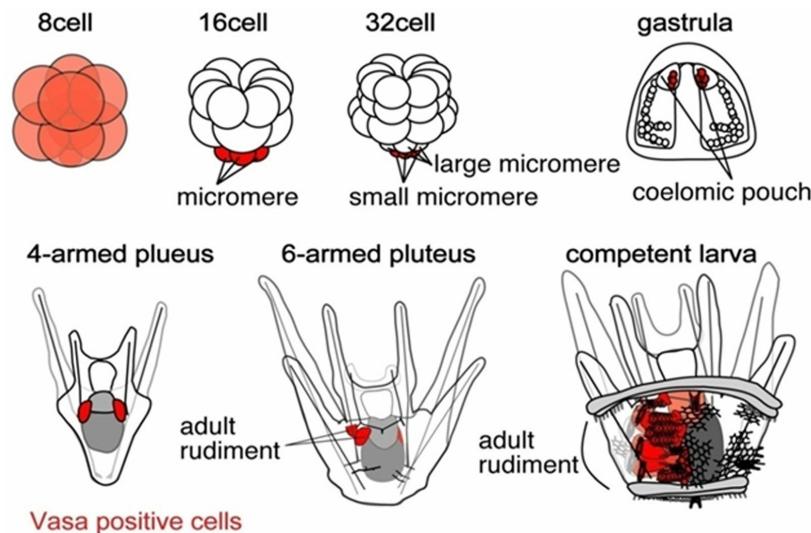


Figure 1. Diagram of the development of a sea urchin. Early development yields Vasa-positive cells (shown in red, beginning with a uniform Vasa positive egg and early embryo). At the 32-cell stage, the sMics are uniquely Vasa-positive. These cells move into the coelom during gastrulation, segregate into the left and right coelomic pouches, and expand to contribute to the germ cells of the adult, and likely also to some somatic cells of the rudiment.

hemisphere divide equally, yielding eight uniform cells of intermediate size (mesomeres), whereas cells of the vegetal-tiered hemisphere divide unequally, yielding the four largest cells (macromeres), and the four smallest cells at this stage (micromeres). The mesomeres and macromeres of the early embryo will form the majority of the ectoderm and endomesoderm, respectively, whereas the micromere lineage quickly diverges to extremes in cell fates and function.

The micromeres are an inductive signaling center of the early embryo. Classic experimental approaches by Horstadius found that micromeres were capable of inducing other embryonic cells to develop into endomesodermal fates (Horstadius, 1973). This property was most apparent when transplanted to the animal pole (opposite their normal site), resulting in a host embryo with a second, new endomesodermal axis (Ransick and Davidson, 1993). The mechanism behind this induction is not understood, but the micromeres do acquire nuclear beta-catenin shortly after their formation, and evidence suggests Notch-Delta, and Wnt8 signaling as candidates for this inductive process (e.g., McClay, 2011).

Further division of the micromeres yields the large micromeres (LMics) and the small micromeres (sMics)—two developmental extremes. LMics are fated to the skeletogenic lineage, and ingress into the blastocoel several hours later as primary mesenchyme cells. This ingression (individual cells detaching from an epithelium and moving into a new space) is associated with their loss of adhesion to other cells, secretion of enzymes associated with dissolution of the basal lamina, followed by an increase in adhesion for the extracellular matrix on the opposite side of the basal lamina (Fink and McClay, 1985; McClay, 2011). A fixed number of primary mesenchymal cells survive ingression, depending on the species, and this population will migrate throughout the vegetal hemisphere of the blastocoel. They eventually aggregate and begin depositing a calcite-based internal skeleton consisting of various rods, connections, and barbs; skeleton morphologies are species-specific (Ettensohn, 2009; McClay, 2011; Rafiq et al., 2012). While many treatments may inhibit developmental progression to actually forming skeletons, no reported experimental approaches, tests, or perturbations have diverted the fate of LMics from a skeletogenic one to a different cell/tissue type. Further, when cells are placed in culture, in isolation from all other cells of the embryo, they still replicate the primary mesenchymal cell phenotype and behavior, and even synthesize skeletal elements (e.g., Okazaki, 1975). The sMics, on the other hand, appear to contribute to the germ line (see below). Thus, within one cell division, the two progeny gain extremes of developmental fate: the LMics become the singular, somatic, skeletogenic lineage that will terminate with metamorphosis of the larvae, whereas the sMics contribute to the germ line and are thus generationally immortal.

Another distinction between the micromere descendants is their subsequent impact on the embryo. Micromeres are the major signaling center of the embryo (Horstadius, 1973; see also Ransick and Davidson, 1993). After cell

division, however, this inductive capability is retained by LMics but is essentially lost in the sMics. For example, the sMics in *Hemicentrotus pulcherrimus* have only 1/10th the inducing strength of one LMic (Kurihara and Amemiya, 2005). Horstadius (1973) documented the inducing capacity of micromeres by a series of transplants, either moving donor micromeres to ectopic sites in an entire host animal, or to various tiers of early embryos. When the animal-most tier (mesomere lineage) was opposed to the micromeres, a normal looking (though smaller) embryo formed with all the characteristics of a normal gastrula and larvae. This result showed both the strong inducing capacity of the micromeres, and the total plasticity of early embryonic cells to receive and respond to such signals by changing their developmental fates to become cell types, even different germ layer derivatives, that they would normally not acquire. Remarkably, a competent larva can form and metamorphose when derived from only mesomeres and micromeres; Minokawa and Amemiya (1999) showed that the larvae formed by these “animal cap inductions,” devoid of macromeres, were capable of metamorphosing to form a normal adult, and that the sMics would form from the micromeres and exhibit normal developmental programming. Development of these juveniles to reproductive age, however, was not reported.

The sMics Contribute to the Germ Line of Sea Urchins

Several lines of reasoning and experimentation suggest that the sMics contribute to the germ line. These include their cellular behavior, the molecules they harbor selectively, and results from perturbation studies—that is, in their absence, the embryo is not gravid (do not make gametes).

The behavior of the sMics first suggested their role in the germ line. These classically defined behaviors of PGCs include: (1) formation following (two) asymmetric divisions (Fig. 2); (2) a large nuclear-to-cytoplasmic volume ratio, with a cytoplasm depleted in many organelles, especially yolk (Pehrson and Cohen, 1986); (3) overly condensed chromatin relative to their siblings beginning at their formation (Tanaka and Dan, 1990); (4) a slow cell cycle relative to their neighbors, dividing only twice through development to the late gastrula stage (Fig. 3; Pehrson and Cohen, 1986; Tanaka and Dan, 1990); (5) transcriptional repression, as seen by chromatin modifications and RNA polymerase activity (Swartz et al., 2014); and (6) migratory pattern, reaching the tip of the gut during gastrulation and integrating into the coelomic pouches, the site of adult rudiment formation (see Cameron et al., 1987; Pearse and Cameron, 1991, for discussion of lineage tracing). Each of these sMic features is consistent with the hypothesis that they are, or at least contribute to, the germ line in the sea urchin. It should be noted that long-term labeling of the sMics has not been accomplished, so it is not possible to conclude what various fates the lineage may contribute to, but it appears that the sMics do not contribute to any functional structures of the embryo (Cameron et al., 1987; Cameron et al., 1991) and instead

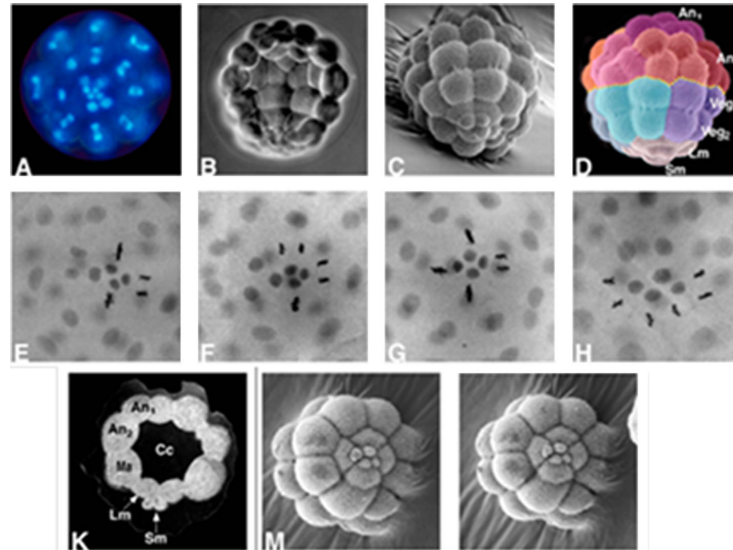


Figure 2. The morphology of the sMics. The early embryo of *Lytechinus variegatus* was imaged in a variety of ways to expose the various cells. **A:** DAPI-labeled 32-cell stage viewed from the vegetal pole. **B–D:** lateral view of 32-cell stage embryos viewed with differential interference contrast **B**, scanning electron microscopy (SEM) **C**, and pseudocoloring **D** to identify the various tiers of cells. **E–H:** DAPI-stained progressions, viewed from the vegetal pole, of the formation of the four sMics (centered) followed by cell division of the large micromeres adjacent to them. The cell cycle of the sMics is shown relative to their neighboring and sibling cells. **K:** Split 32-cell embryo fixed for SEM showing the various tiers of cells and the LMics and sMics at the bottom. **M:** A stereo view of a 32-cell stage embryo viewed from the vegetal pole. Stereo view glasses (B/W) or slightly crossed eyes are needed to view this pair. For more detailed information on the other cell types, see, for example, Horstadius (1973), McClay (2011), Gilbert (2013). Figure courtesy of John Morrill, personal communication.

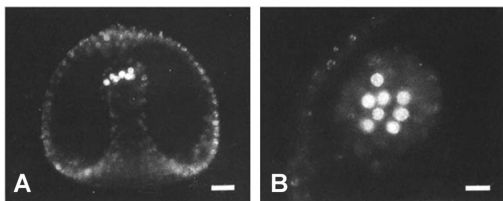


Figure 3. Sea urchin sMics exhibit slow cell-cycling, as revealed by bromo-deoxyuridine (BrdU) pulse-chase studies. The sMics divide much slower relative to the somatic cells. Late gastrula (**A:** side view; **B:** top view) showing sMics selectively labeled with bromo-deoxyuridine (BrdU). BrdU was pulsed in the embryo following fertilization, washed, and the embryos were incubated until various time points. The sMics retain the BrdU relative to other cells of the embryo because they divide much slower than other cells (soma) and thereby do not dilute the BrdU in their genome (Tanaka and Dan, 1990; similar results were first shown by Pehrson and Cohen, 1986). Scale bar in **A**, 10 μm ; in **B**, 20 μm .

is devoted to the germ line (Yajima and Wessel, 2011). Ideally the cells should be traced with a marker gene (fluorescent protein gene embedded in the genome) so that the fate can be traced longitudinally, throughout its development, particularly in the rudiment. This might be accomplished by identification of a sMic-unique promoter that can be used to drive GFP expression, or using a constitutive promoter driving GFP to mark transplanted sMics in a host embryo that is unlabeled or labeled with a different fluorescent protein. Certainly integrating genes into a select site of the genome now should be possible in this organism, especially with newly established zinc finger nuclease constructs or TALENs, as reported for a variety of other cell types and organisms (e.g., Xiao et al., 2013). One drawback for this approach becoming routine is the prolonged period needed to derive a subsequent, reproductively competent generation, which can take more than several months to multiple years, depending on the species, and limits long-term and repetitive analysis on lines of such genetically manipulated animals.

A screen for genes that, in other organisms, are involved in germ-line determination was undertaken to identify orthologous sequences involved in germ-line determination in the sea urchin, and its results also suggested that the sMics contribute to the germ line (Juliano et al., 2006). Fourteen selected genes were identified in the genome of *Strongylocentrotus purpuratus*, and were tested by in situ hybridization and qPCR. These included Boule (RNA binding protein), Vasa (DEAD-box containing RNA binding proteins), Seawi (piwi ortholog involved in piRNA function; see e.g., Mani and Juliano, 2013), germ cell-less (nuclear pore-associated protein; see e.g., Voronina, 2013), and Ovo (the zinc-finger containing transcription factor). The transcripts of almost all of the genes identified were found to accumulate uniformly in oocytes throughout oogenesis—no evidence was apparent to suggest that these oocytes compartmentalized or segregated the conserved mRNAs into a germ plasm. This does not eliminate the possibility that proteins derived from these transcripts are produced and aggregate distinctly in the cytoplasm, but at least the mRNAs inform us of the absence of transcript segregation and that the mechanism of germ-line acquisition may not be a strict inheritance mechanism in sea urchins. The transcripts encoding Pumilio (RNA binding protein), Tudor (scaffolding protein), MSY (RNA binding protein), and CPEB1 (translational regulator through polyadenylation) were also found to be uniformly distributed during embryonic development. In contrast, the transcripts for Vasa, Seawi, and Ovo became enriched in the vegetal plate of the mesenchyme blastula stage while Vasa, Nanos2, and Seawi localized in the descendants of the sMics at the tip of the archenteron during gastrulation, and were then enriched in cells of the left coelomic pouch in larvae (Juliano et al., 2006; Fujii et al., 2009). These results suggested that (1) the sea urchin does not use a germ-plasm mechanism of germ-cell determination and (2) the sMics are candidates that contribute to the germ line.

Ransick et al. (1996) indirectly tested if the sMics constitute a definitive PGC lineage during embryonic development. They removed the micromeres, the parent cells of the sMics formed at the fourth cleavage (Fig. 1), and cultured the remaining embryo. Remarkably, the animals developed rather normally, and even metamorphosed on time. Moreover, most of the adults made gametes. They concluded that the germ-cell lineage is not formed obligatorily by the fourth cell division, and instead, that it must segregate during postembryonic development, within the adult rudiment, or even following metamorphosis (Ransick et al., 1996). Support was garnered for this conclusion from the literature (McBride, 1903) in which cells with “germ-cell cytology” were described initially in the coelomic pouches of metamorphosing animals. This led to the conclusion that the germ line is not definitively established until the basic body plan of the adult is formed.

The adult body plan of sea urchins and other echinoderms is pentaradially symmetric starting with the adult rudiment, which forms within a bilaterally symmetric larva prior to metamorphosis. The sea urchin embryo is also a maximally indirect developer—that is, the adult has a

distinct body plan that forms only from the rudiment formed in the larvae (Peterson et al., 1997; Davidson and Erwin, 2006). Thus, were germ lines not formed until the adult body plan was established, then the germ line would not be specified until after rudiment formation (Dixon, 1994; Ransick et al., 1996). Other animals used for studies in germ-line determination, such as flies, nematodes, and frogs, are more “direct”, indirect developing organisms in terms of body axis formation. Each of these animals establishes an axis in early development that is basically the same as the adult axis, and they specify their germ lines early in development, whereas sea urchins, molluscs, and sea stars, among other organisms, do not form their adult body axis until late in larval development and their germ line is argued to be specified only following this period (Dixon, 1994; Ransick et al., 1996). A caveat to these conclusions for maximally indirect developers is that, with the exception of the sea urchin undergoing micromere removal (Ransick et al., 1996), assessment of the germ line has been made only cytologically, not functionally. It should be emphasized here also that no definitive germ plasm has been reported in sea urchins during embryonic development.

Removal of the micromeres at the 16-cell stage results in significant compensatory development and fate transitions within the embryo (Ransick and Davidson, 1993). Further, removal of the LMics (the primary mesenchymal cells) just prior to gastrulation results in trans-fating of pigment cells to primary mesenchymal cells, whereby other mesodermal cells change fate to become skeletogenic cells, a fate they would not normally display (Ettensohn and McClay, 1988). In the early embryo, micromeres have a capacity to induce new axial development when transplanted to ectopic positions, and their removal results in a delay of development in the mesendodermal tissues of the embryo even though the embryos form normal larvae (Horstadius, 1950; Ransick and Davidson, 1995). Removal of the micromeres also results in a significant up-regulation of Vasa, the RNA helicase, in all remaining cells of the embryo from *Strongylocentrotus purpuratus* (Voronina et al., 2008). This is significant in that Vasa is believed to be involved in germ-line specification and maintenance (Lasko and Ashburner, 1988; Lasko and Ashburner, 1990; Tomancak et al., 1998; Tanaka et al., 2000; Styhler et al., 2002; Salinas et al., 2007). Indeed, the micromeres and then the sMics are the sole cells that retain substantial Vasa in a normal embryo, and its up-regulation throughout the embryo following micromere removal may have a compensatory function in re-specifying the multipotent cell line or another germ-line lineage (Voronina et al., 2008).

Given the hypothesis that the sMics contribute to the germ line in adult sea urchins, this population was depleted at the 32-cell stage and the resultant embryos were raised to adulthood (Yajima and Wessel, 2011; Figs. 4 and 5). These embryos developed normally, on schedule with their control siblings and with all embryonic structures, and the larvae developed adult rudiments that metamorphosed. The adults, however, were sterile with gonads that lacked gametes, whereas adults from control and micromere-deleted embryos developed gonads containing gametes,

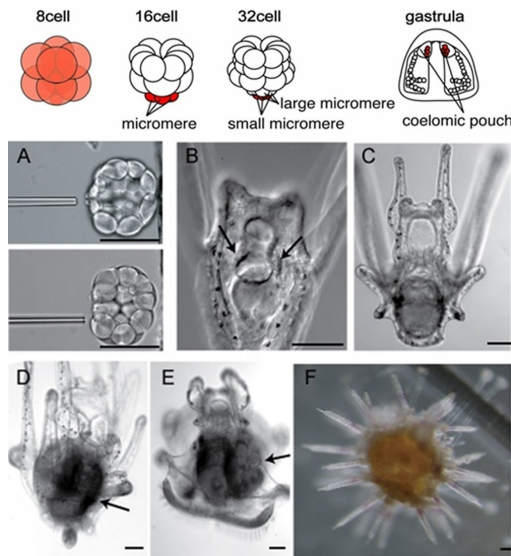


Figure 4. The sMics are dispensable for development. Diagram at top pointing out the sMic lineage (red). **A:** Method of sMic removal with a glass pipette. The resulting embryos develop into a larva (**B–E**), form an adult rudiment (**D, E**, arrow in **E**), and metamorphose into an adult (**F**) that does not produce gametes. Scale bars, μm .

suggesting that the sMics do indeed contribute to the germ line. (Recall that lineage analysis of the sMics has not been extended beyond gastrulation, so strictly referring to them as PGCs has not been resolved. It is possible that their lineage diverges in the adult rudiment to also contribute to somatic lineages that may enjoy compensatory development upon sMic removal earlier in development.) These results suggest that timing for an initial PGC-like specification might occur after the 16-cell stage, but that the PGC precursors have segregated by the 32-cell stage. The mechanism of specification of these cells is not understood, yet some interesting observations have been found. sMic-depleted embryos developed normally but did not express compensatory levels of *Vasa*, as did embryos from a micromere deletion, implying that the compensatory gene regulatory network was not activated in sMic-deleted embryos (Fig. 5). This is compatible with the finding that sMics have lost inductive or cell signaling capability, which was held by their precursors, the micromeres. Thus, the sMics may begin insulating their cellular interactions soon after their establishment, which is a feature similar to PGCs of other organisms (Seydoux and Braun, 2006). While direct lineage tracing experiments certainly need to be accomplished in the future, we can conclude from the results so far that germ-line specification in the sea urchin depends on the sMics, either directly as lineage products or

indirectly by a later signaling mechanism that directs neighboring cells to this fate.

Asymmetric Cell Divisions in Formation of sMics

The sea urchin embryo undergoes two sequential, asymmetric cell divisions of micromeres at the 4th and 5th cleavages to yield the LMics and the sMics. This cleavage pattern is invariant, is the first observable break in symmetry, and leads to differential cell fates. The mitotic spindles of these unequally dividing micromeres move toward the vegetal pole prior to cytokinesis and are peripherally located to the vegetal cortex; the aster of the micromere pole appears to be anchored to the vegetal cortex and presents a characteristic flattened shape. Quantitation of immunofluorescence signals of centrosomal materials demonstrated that micromere centrosomes are smaller than the macromere centrosomes (Holy and Schatten, 1991). These observations suggest that centrosomes of the micromere are different than other centrosomes, perhaps in content, density, or at least in distribution of components, thus providing a unique, microtubular environment at the vegetal pole. In addition to resultant cell-size differences, this asymmetric mitotic matrix possibly contributes to asymmetric distribution of germ-line components such as *Vasa* protein during the process of unequal cleavage at the 4th and 5th cell divisions (Figs. 6 and 7; Yajima and Wessel, 2011).

Although detailed mechanisms of these unequal cell divisions at the 16- and 32-cell stages is understudied in the sea urchin, several lines of functional evidence suggests a conserved mechanism of asymmetric cell division. This evidence includes pharmacological interference and dominant-negative protein expression, both indicating that the heterotrimeric G protein G_i and its interaction partner, activator of G-protein signaling (AGS), are necessary for the asymmetric cell divisions in many organisms such as *Drosophila*, *C. elegans*, and mammals (Morin and Bellaÿche, 2011). The AGS/Pins (activator of G-protein signaling/partner of inscuteable) family of proteins are conserved, multi-domain molecular scaffolds found in many organisms from yeast to humans, and are reported to be involved in asymmetric cell divisions so far in *Drosophila*, *C. elegans*, and sea urchins (Schaefer et al., 2000; Betschinger and Knoblich, 2004; Voronina and Wessel, 2006; Park and Rose, 2008; Krueger et al., 2010; Morin and Bellaÿche, 2011). In the sea urchin, inhibition of $G_{\alpha i}$ signaling by pertussis toxin interfered with micromere formation and led to defects in embryogenesis (Voronina and Wessel, 2006). Introduction of exogenous, dominant-negative AGS proteins, containing only the G-protein regulatory (GPR) domains, selectively prevented the asymmetric division during normal micromere formation (Voronina and Wessel, 2006). These results imply that cortical proteins located at the vegetal pole, where these proteins accumulate, regulate the asymmetric cell divisions in this embryo, and thus may be involved in cell-fate specification of the sMics to that of the germ-cell lineage.

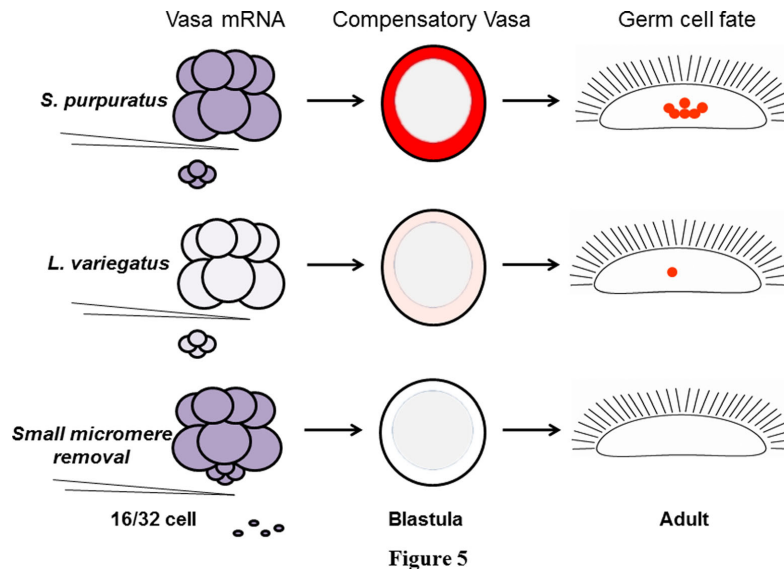


Figure 5. Summary of the Vasa-compensatory mechanism. Vasa mRNA is abundant in *S. purpuratus* 16-cell stage embryos, but not in *L. variegatus* embryos of the same stage. When micromeres are removed from embryos of each species, Vasa protein is upregulated in the blastulae in proportion to the amount of vasa mRNA present. Adults are obtained from each species at a frequency proportional to the amount of Vasa protein upregulated. When sMics are removed from either species at the 32-cell stage, no Vasa up-regulation is detectable and no germ cell production is seen.

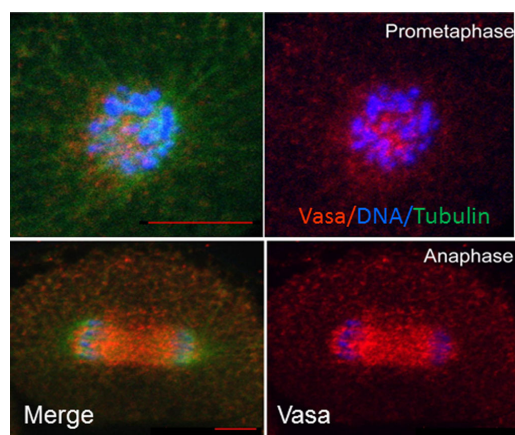


Figure 6. Vasa associates with the mitotic spindle in early cleavage divisions. Vasa is present throughout the cytoplasm in early embryos, but becomes enriched in the spindle during mitosis. Scale bars, μm . Reprinted with permission from Yajima and Wessel (2011).

Another pathway involving the Wnt-signaling mechanism may also be involved in these processes of asymmetric cell division and cell fate specification. Earlier studies of the testis in *Drosophila* found that germ-line stem cells (GSCs) normally divide asymmetrically, giving rise to one germ-line stem cell and one gonoblast (Yamashita et al., 2003). These germ-line stem cells use intracellular mechanisms involving centrosome function and a cortically localized Wnt-pathway member, Adenomatous Polyposis Coli (APC), to orient their mitotic spindles perpendicular to the stem-cell niche within the testis. This spindle directionality provides an asymmetric outcome in which one daughter cell remains in the niche and self-renews its stem cell identity, whereas the other cell becomes displaced from the niche and initiates differentiation into spermatocytes. In *C. elegans*, extrinsic Wnt signaling modulates spindle structures through APR-1 (the *C. elegans* APC-related gene)/APC during telophase of the asymmetric division of EMS cells at the four-cell stage, which in turn promotes asymmetrical nuclear localization of WRM-1/ beta-catenin and POP-1 (the *C. elegans* TCF/LEF transcription factor)/TCF (Sugioka et al., 2011). APR-1 is asymmetric along the cortex, and can cause an asymmetric distribution of astral microtubules. Perturbation of the Wnt

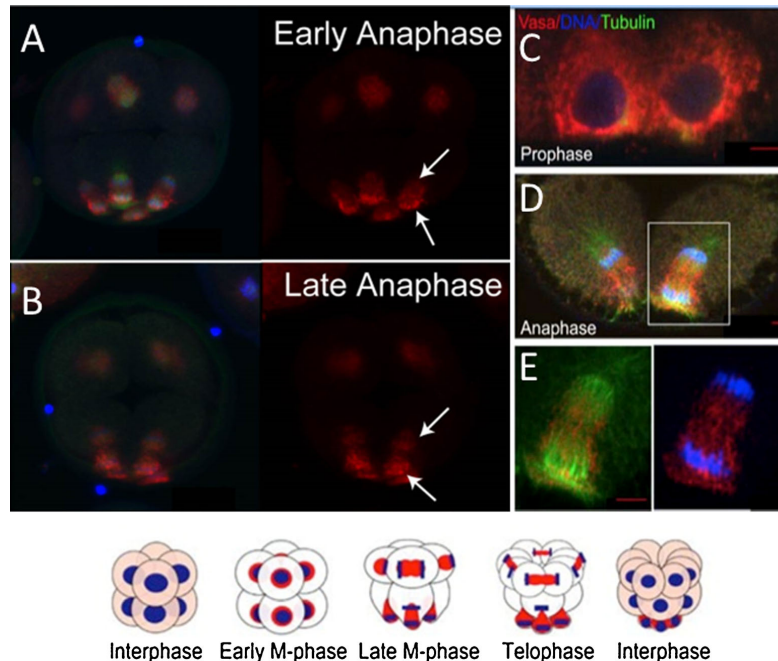


Figure 7. Vasa is asymmetrically partitioned into the micromeres. During anaphase of the 4th cell division in the vegetal tier of blastomeres, Vasa becomes enriched in the future micromere region of the spindle and concentrates in the vegetal-most region of the embryo. This phenomenon is repeated again to form the sMics, which retain the highest concentration of Vasa in the embryo. Whole embryos are shown in **A**; and **B**: are each ~90 microns in diameter, whereas the micromeres shown in **C**, **D**, and **E**: are each ~10 microns in diameter. Scale bars, μm . Reprinted with permission from Yajima and Wessel (2011).

signaling pathway altered this microtubule asymmetry and led to changes in nuclear WRM-1 (*C. elegans* beta-catenin) asymmetry, gene expression, and cell-fate determination, suggesting that the nuclear localization of proteins is regulated through the modulation of asymmetric microtubules and that this regulates cell fate (Sugioka et al., 2011).

The sea urchin embryo may have incorporated similar mechanisms for asymmetric cell division. Although still untested, these conserved Wnt pathway molecules, such as Wnt8 (Croce and McClay, 2006; Kumburegama and Wikramanayake, 2008), Dishevelled (Wikramanayake et al., 1998; Ettensohn, 2006), and nuclear beta-catenin, are localized at the vegetal pole and/or in the micromeres and sMics (Emily-Fenouil et al., 1998; Wikramanayake et al., 1998; Logan et al., 1999; Vonica et al., 2000). These Wnt-pathway molecules are thought to establish an initial animal–vegetal (A–V) polarity in this embryo, but may also participate in the asymmetric cell division of micromeres. Wnt and Delta, another micromere-enriched signaling molecule (Oliveri et al., 2002; Oliveri and Davidson, 2004), could be involved in signaling between adjacent cells to establish a differential signal between micromeres and macromeres at the 16-cell stage, leading to cell-fated

asymmetry in the micromeres that gives rise to the LMics and sMics at the next cell cycle. Importantly, a recent report demonstrated the involvement of Wnt3a in expansion of the early PGC population in the mouse embryo (Bialecka et al., 2012), implying that the Wnt pathway may be involved in PGC specification in many organisms. Testing direct contributions of Wnt pathway molecules to the asymmetric cell division for LMic and sMic formation and to the cell specification of PGCs in the sea urchin would be very useful, especially as it relates to the asymmetric distribution of germ-line determinants or factors. Directly linking a well-known signaling pathway to the distribution of a germ-line factor (Vasa) would provide important mechanistic insight for stem-cell determination in general, especially since germ-line factors are found broadly in stem cells and multipotent cells throughout phylogeny.

Calcium in Specification of sMics

Calcium dynamics in the micromeres. The ion calcium is a versatile intracellular messenger in eukaryotic cells. Calcium is the most ubiquitous of the intracellular

messengers, and most cell types contain conserved calcium signaling machinery (Carafoli et al., 2001). Its concentration within the cytoplasm is spatially and temporally controlled by ion channels, exchangers, and pumps, which coordinately are able to elevate calcium levels over 1,000-fold (to several micromolar amounts) in less than a second, and then promptly restore their low, resting levels (nanomolar concentrations). Calcium-transporting proteins are located in the plasma membrane and in the membranes of organelles such as the endoplasmic reticulum, the mitochondria, and the lysosomes, where they each play specific roles in the cellular homeostasis of calcium. In general, the transduction of the calcium signals is mediated by reversible binding to specific classes of proteins that act as calcium sensors.

Historically, fertilization calcium waves and local calcium signals, as well as their mechanisms of propagation and many of their fundamental components, were discovered and heavily studied using sea urchin eggs and sperm. It is known that calcium signaling at fertilization is accomplished by the coordinate action of various mechanisms, including different messengers (e.g., cADPR, NAADP, and IP₃), different intracellular stores (e.g., the endoplasmic reticulum and endolysosomes), and different calcium channels and receptors (e.g., plasma membrane voltage-gated ion channels), which have all been remarkably conserved through the course of evolution (Carafoli et al., 2001; Whitaker, 2006). Cell divisions utilize calcium during mitosis, so the question becomes: Do the distinct cells that are rapidly generated after the early cleavages in an embryo utilize calcium signals to accomplish coordinated tasks such as the asymmetrical mitotic divisions, axis patterning, and cell fate?

In sea urchin embryos, many large-scale cell fate decisions are made along the A–V axis. By the 60-cell stage, the A–V axis is further patterned to produce five distinct territories that are specified by a signal transduction cascade initiated by the micromeres at the 16-cell stage (McClay, 2011). The micromeres are characterized not only by their small size, but also by their distinct plasma membrane, cytoplasmic inclusions, and nucleus. The plas-

ma membrane of the micromere is mainly composed of a smooth, non-villous membrane (Yazaki, 2001). The smooth membrane is more adhesive than the villous membrane originally seen in other blastomeres (Kuraishi and Osanai, 1989), and lacks a specific egg surface protein (ES-1) (Yazaki, 1993). Intercellular ion communication remains between the micromeres and the macromeres during early development (Yazaki et al., 1999). The micromeres are also characterized by nuclear accumulation of beta-catenin, a cofactor of the TCF transcription factor that is required to specify the vegetal-cell fate in the sea urchin embryo (Logan et al., 1999).

The endoplasmic reticulum in the micromeres.

Staining the endoplasmic reticulum was accomplished by microinjecting the egg with a saturated solution of the fluorescent dye dicarbocyanine DiLC16 (according to the method of Terasaki and Jaffe (1991). The dye spreads from an oil drop into endoplasmic reticular membranes, but not into other organelles) and shows an apparent tubular membrane network in the cortex of the blastomeres without any obvious polarity until the two-cell stage. From the four-cell stage, however, the endoplasmic reticulum gradually accumulates in the vegetal half of the embryo, and finally becomes enriched in the vegetal-pole cortex of micromeres at the 16-cell stage (Fig. 8; Yazaki, 2001; Yazaki et al., 2004).

Plasma membrane currents and L-type voltage-gated calcium channels in the micromeres.

Calcium-dependent currents and steady state conductance in early sea urchin blastomeres were measured using whole-cell patch-clamp techniques, showing that L-type calcium channels are asymmetrically distributed along the A–V axis and very few are found in the micromeres (Dale et al., 1997). The calcium currents (all occurring during metaphase of the cell cycle) decreased from 8.5 $\mu\text{A}/\text{cm}^2$ at the four-cell stage to 5.4 pA/cm^2 at the eight-cell stage. In 16-cell stage embryos, calcium currents were 7.4 $\mu\text{A}/\text{cm}^2$ in the mesomeres, 2.3 pA/cm^2 in the macromeres, and

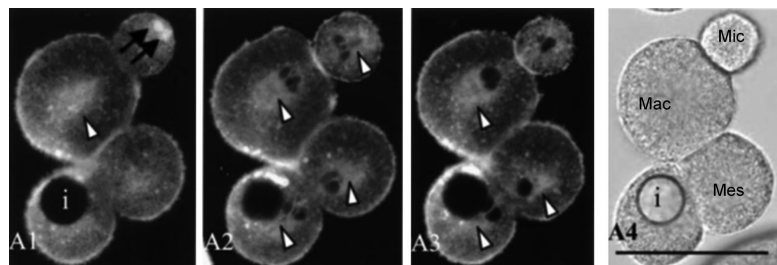


Figure 8. The endoplasmic reticulum is enriched at the vegetal pole in early development. Scale bar, μm . Reprinted with permission from Yazaki et al. (2004).

TABLE 1. Electrophysiological Properties of the 16-Cell Embryo (From Dale et al., 1997)

Cell	Cell diameter (microns)	Resting potential (mV)	Calcium current (microAmps/cm ²)
Mesomere	31	-25 ± 3	7.4 ± 1.7
Macromere	37	-27 ± 4	2.3 ± 1.2
Micromere	20	-9 ± 1	0

were not detectable in the micromeres (Table 1; Dale et al., 1997). Whether or not blocking any of these ion fluxes alters the asymmetric phenotype in the embryo is not known, but would be an important addition to the studies demonstrating that protein and organelle asymmetry contribute to this special cell division.

High resting calcium concentrations and characteristic global calcium oscillations in the micromeres. The intracellular concentration of calcium was measured directly in embryos at the 16-cell stage using the calcium indicator Fura-2/AM and a mixture of Dextran-conjugated Oregon green-BAPTA 488 and Rhodamine red injected originally into the egg (Yazaki, 2001). These injected embryos then were removed from their fertilization envelopes, and the blastomeres were separated at the four-cell stage. Following two more cell divisions, this procedure results in the formation of many "quarter embryos" consisting of two mesomeres, one macromere, and one micromere. Calcium fluxes in the cells were then assessed with the calcium indicators, which revealed that resting calcium concentrations are characteristically elevated in the micromeres during the 4th cleavage. Within a quarter embryo, the fluorescence-intensity ratio of micromere-to-macromere for two sea urchin species (*H. pulcherrimus* and *Pseudocentrotus depressus*) is significantly higher than 1.0, and is significantly different from the ratios in mesomere/macromere and mesomere/mesomere pairs ($P < 0.05$); all blastomeres other than the micromeres essentially have the same intracellular calcium concentration (Table 2) (Yazaki, 2001). Calcium levels subsequently oscillated for about 10 min in the micromeres. Repeated elevations of calcium were measured until the levels stabilized in later stages of the mitotic cycle (Fig. 9).

Oscillations do not occur in calcium-deficient seawater, and are evoked by the addition of calcium ions into the bathing medium, suggesting that calcium influx initiates the calcium transients. Since L-type voltage-gated calcium

channels seem to be absent from the micromere plasma membrane, other types of calcium channels must be involved in the initiation of these oscillations. Pharmacological studies, using a blocker of IP3-mediated calcium release (Xestospongin), a store-operated calcium entry inhibitor (2 aminoethoxydiphenyl borate (2-APB)), and an inhibitor of stretch-dependent ion channels (gadolinium), suggest that the high calcium concentrations and oscillations in the micromeres are triggered by calcium influx caused by the activation of stretch-dependent calcium channels, followed by the release of calcium ions from the endoplasmic reticulum (Yazaki, 2001; Yazaki et al., 2004). It is not clear if this calcium behavior is a common feature of asymmetric divisions, where the products are so disparate in size, yet a physiological explanation to the asymmetric cell division in this embryo seems important to consider for future studies. It is also important to consider how asymmetric signaling pathways (Wnts, G-proteins, as discussed above) may also impact physiological behaviors such as calcium flux in different cells.

Micromeres—Possible Role of Calcium As a Signaling Messenger

Embryos come in all shapes and sizes. In sea urchins, the specification of the micromeres and subsequent LMics and sMics is a direct consequence of the variation in the topography of cytokinesis during the very early divisions. The location of the mitotic spindle determines the point of the cleavage furrow constriction and membrane addition and, therefore, the resultant size of the daughter cells (Shuster and Burgess, 2002). In early sea urchins embryos, it is known that separation of sister chromatids is accompanied by a brief, sharp, small calcium transient, an order of magnitude smaller than those measured at fertilization (Groigno and Whitaker, 1998). The transient occurs 1–2 min before spindle elongation and always precedes it. Thus, it is reasonable to suppose that calcium is involved in controlling the formation of the furrow during cytokinesis. Whether or not calcium signals are directly involved in positioning the cleavage furrow to generate the embryos asymmetrical cells is less certain, however. The position of the furrow is determined by the disposition of the spindle's astral microtubules, and it appears that the spindle forms in the region of lowest microtubule density (Yoshigaki, 2001). It is known also that the contractile ring in the furrow is an actomyosin-based motility system (Kiehart et al., 1982). In smooth muscle, contraction is regulated by calcium via calmodulin activation of myosin light-chain kinase (MLCK). It has been shown in sea urchin that calmodulin is activated at the cleavage furrow, and that cleavage is inhibited by an anti-MLCK peptide (Torok et al., 1998). Thus, one can speculate that localized calcium signals and calmodulin activation may be involved not just in the control of the furrow but in determination of the position of the furrow (Wilding et al., 1995). It is also important to note that sea urchin embryos can divide and develop normally in seawater lacking calcium, so the inference is that release from internal stores is sufficient for proper signals, as happens at

TABLE 2. Relative Calcium Concentration in the 16-Cell Stage Blastomeres

Species	Micromere/macromere	Mesomere/macromere
<i>Hemicentrotus pulcherrimus</i>	1.14 (±0.018)	1.02 (±0.004)
<i>Pseudocentrotus depressus</i>	1.10 (±0.010)	1.00 (±0.004)

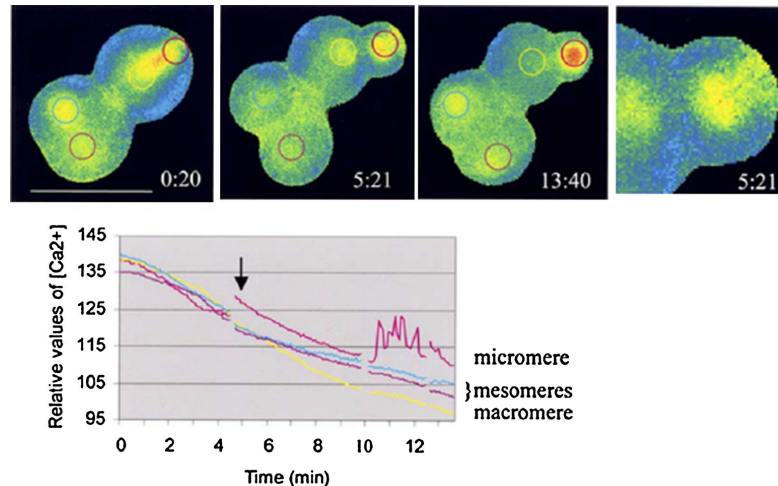


Figure 9. Calcium fluxes (red circle in photos, and red line in graph) are unique in the sMics. Scale bar, μm . Reprinted with permission from Yazaki (2001).

fertilization, but contributing influx from the external media is likely to occur in normal seawater.

Endoplasmic reticular accumulation, high resting calcium concentrations, and repetitive calcium transients markedly occur in the micromeres starting at the time that their fate is determined, right before the 4th division, together indirectly implicating a role for active calcium signaling during vegetal specification in sea urchin embryos (Yazaki, 2001; Yazaki et al., 2004). In sea urchins, it is known that Wnt proteins signal through multiple pathways that regulate developmental events, including cell polarity, cell fate, and body-axis specification (Logan and Nusse, 2004). The primary outcome is the stabilization of cytosolic beta-catenin; in fact, specification of the micromeres depends on the nuclear localization of beta-catenin by the late 16-cell stage (Logan et al., 1999; McClay, 2000), which will activate vegetal-specific genes by binding to a specific transcriptional regulator (TCF). Beta-catenin initially accumulates in the cytoplasm, and is then transferred to the nucleus only in the micromeres in the late phase of the 16-cell stage (20 min after the 4th cleavage) (Logan et al., 1999). Its nuclear localization is regulated by the activity of casein kinase I (CKI) and glycogen synthase kinase 3 β (GSK3 β) (Amit et al., 2002; Leonard and Ettensohn, 2007). Calcium gradients along the A–V axis begin before the 4th cleavage, and are likely initiated by stretch-dependent calcium influx, not by Wnt signals themselves. Since the increase in calcium apparently precedes nuclearization of beta-catenin, it is possible that calcium regulates protein kinase C (PKC) activity, thereby interfering with GSK3 β activity. This raises the suggestion that the local accumulation of endoplasmic reticulum in the vegetal half of four-cell

stage embryos is the first step in the process that gives rise to the micromeres.

The majority of data linking calcium signals and pattern formation comes from vertebrate embryos. It is known, for example, that in *Xenopus* and zebrafish, the canonical Wnt pathway does not involve calcium at all. In the alternative, planar cell polarity Wnt/calcium pathway, however, a second class of Wnts signals maintains ventral cell fate (Kuhl et al., 2000a), and these Wnts can activate PKC and calmodulin kinase II (CaMKII) and can interact with an exclusive subset of Frizzled receptors (a family of G-protein receptors) (Kuhl et al., 2000b). Wnt/calcium signaling occurs through Dishevelled (Dsh), a multivalent protein that interacts with the GSK3 β /beta-catenin pathway via a so-called DIX domain. Although Dsh was one of the first components described in Wnt pathways, its function still remains puzzling. It is known that a Dsh Δ DIX construct is able to activate both PKC and CaMKII (Sheldahl et al., 2003), and that a morpholino oligonucleotide directed against endogenous *Xenopus Dsh* blocks activation of PKC, as measured enzymatically (Kuhl et al., 2000b). Nevertheless, although sea urchin Dsh localizes to the vegetal cortex of early embryos, no links between Dsh and calcium effectors/signals have been reported so far (Leonard and Ettensohn, 2007).

Post-Translational Gene Regulation in the Determination of PGCs

Despite vast mechanistic variation, sexually reproducing metazoans employ a common group of molecules for PGC specification and development. One of the most

ubiquitous and extensively conserved factor is the DEAD-box RNA helicase Vasa. In all animals observed to date, Vasa protein is ultimately enriched in PGCs or multipotent PGC progenitor cells during embryo development (e.g., Raz, 2000; Gustafson and Wessel, 2010a; Lasko, 2011). Several animals accomplish this selectivity not by transcriptional mechanisms, but by post-translational activity regulating Vasa protein stability and turnover.

Protein Turnover Is a Conserved Mechanism for PGC Protein Enrichment

In *Drosophila*, Vasa protein localizes to the pole plasm in the developing embryo. In vitro protein interactions as well as genetic data suggest that Oskar may anchor Vasa to polar granules in the oocyte pole plasm, thereby contributing to its eventual incorporation into the pole cells of the developing embryo (Hay et al., 1990; Lasko and Ashburner, 1990; Breitwieser et al., 1996). *Drosophila* Vasa is ubiquitinated in the oocyte and Gustavus and Fsn are two paralogous E3 ubiquitin ligases required for Vasa ubiquitylation, thereby removing Vasa from regions outside the pole plasm (Styhler et al., 2002; Kugler et al., 2010). The deubiquitylating enzyme Fat facets is required for Vasa pole-plasm accumulation; thus, the persistence of Vasa is presumably in a war between ubiquitylating and deubiquitylating activities (Liu et al., 2003). Protein turnover also regulates the Vasa ortholog GLH-1 in *C. elegans*. Phosphorylation of GLH-1 by KGB1 (Jun N-terminal kinase) targets it for proteolysis, whereas association with the CSN-5 (COP9 signalosome subunit 5) enhances GLH-1 stability in the germ line (Orsborn et al., 2007). In addition, the oocyte-to-embryo transition in *C. elegans* relies heavily on selective maternal protein turnover (DeRenzo and Seydoux, 2004). In the embryonic germ line (P-lineage), PIE-1, MEX-1, and POS-1 proteins are stable, whereas they are selectively degraded in the developing somatic blastomeres (Reese et al., 2000; DeRenzo et al., 2003). Conversely, MEX-5 and MEX-6 proteins are selectively degraded in the P-lineage but are retained in the somatic blastomeres (Guo and Kemphues, 1995; Schubert et al., 2000). During zebrafish embryonic development, Vasa protein is uniformly distributed at the time of PGC specification (Yoon et al., 1997; Knaut et al., 2000). PGC-specific Vasa protein enrichment occurs, in part, through soma-specific Vasa proteolysis (Wolke et al., 2002). Thus, post-translational regulation of Vasa appears to be an important level of regulation that ultimately directs PGC specification.

Post-Translational Modifications Regulate Vasa Function in Germ Cells

Although it is currently unclear how protein turnover contributes to Vasa protein localization in mammals, both mouse (Mvh) and human (Ddx4) Vasa orthologs are subject to several post-translational modifications. These include serine, threonine, and tyrosine phosphorylation, as well as arginine methylation and lysine acetylation (Gustafson and Wessel, 2010b; Lasko, 2013; <http://www.phosphosite.org>).

Recent data in mice have identified Hat1 as the acetyltransferase responsible for Mvh acetylation at Lys405, and found that this post-translational modification is developmentally regulated and required for Mvh RNA-binding activity (Nagamori et al., 2011). It will be important to test how small-molecule inhibitors of the acetylation process, such as histone deacetylase (HDAC) inhibitors, influence the post-translational regulation, and function of Vasa and other germ cell-specific acetylated proteins. Vasa arginine methylation is also found in *Drosophila*, *Xenopus*, and mice. The arginine methyltransferase Capsuleen (dPRMT5/csul/dart5) is required for symmetric dimethyl-arginine modifications of Vasa in vivo (Kirino et al., 2010). Although the functional significance of these Vasa arginine methylations is currently unclear, they may facilitate its interaction with Tudor proteins and its localization to perinuclear nuage structures in germ cells, as occurs with several Piwi/Argonaut proteins (Liu et al., 2010; Chen et al., 2011; Mathioudakis et al., 2012).

Vasa Protein Enrichment in the sMics

In the sea urchin *S. purpuratus*, Vasa protein is present uniformly during early embryogenesis, but eventually becomes enriched in the 16-cell stage micromeres and subsequent sMics (Fig. 10) (Juliano et al., 2006; Voronina et al., 2008). Several pieces of evidence suggest that cell type-specific protein turnover is the major regulatory mechanism behind this sMic protein enrichment in the midst of uniform Vasa mRNA. First, although the Vasa untranslated regions (UTRs) are unable to direct sMic protein accumulation, the Vasa coding sequence alone is sufficient for sMic protein enrichment (Fig. 10c) (Gustafson et al., 2011). Second, Vasa protein accumulates throughout the embryo following proteasome inhibition (Fig. 10d) (Gustafson et al., 2011). As with *Drosophila*, sea urchin embryos express a Gustavus ortholog (and E3 ubiquitin ligase involved in target proteins for proteasomal degradation) that interacts with Vasa in vitro and likely in vivo. However, knockdown of endogenous Gustavus protein *reduces* endogenous Vasa protein and *reduces* the frequency of Vasa protein enrichment in the sMics, whereas overexpression of the Vasa-interacting domain of Gustavus results in ectopic Vasa protein accumulation throughout the embryo (Gustafson et al., 2011). Together, these data suggest that Gustavus may have two opposing functions: a degrading function outside of the future germ cells and a positive regulatory function that stabilizes Vasa protein in the sMics. Despite several protein domains indicative of an E3 ubiquitin ligase presumed to be involved in protein degradation, similar evidence was reported for a positive regulatory role of Gustavus in *Drosophila* (Kugler et al., 2010). To our knowledge, these are the only examples where a putative E3 ligase *promotes* the stability of its target ligand. We speculate, however, that this phenomenon of positive and negative modulation of protein stability may be more widespread, and may be a common developmental process underscoring the complex regulatory nature of protein ubiquitylation and turnover.

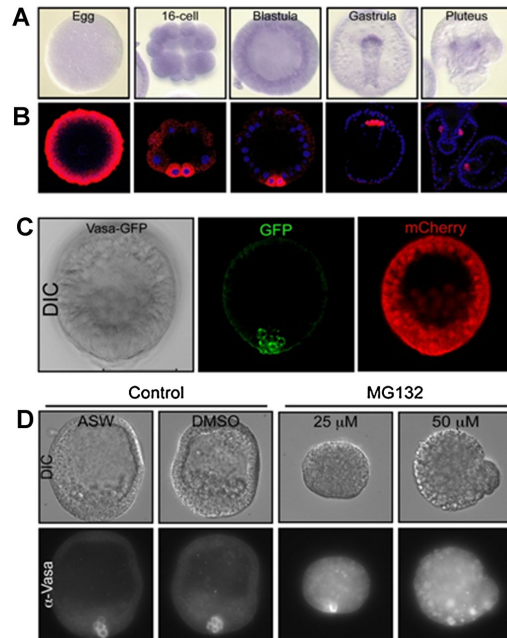


Figure 10. Post-translational Vasa regulation during sea urchin embryogenesis. **A:** Whole-mount in situ RNA hybridization of endogenous *vasa* transcript at the indicated developmental stages. **B:** Immunofluorescence localization of endogenous Vasa protein at the indicated embryonic stages. **C:** Two synthetic mRNAs were co-injected into fertilized eggs, then imaged at the blastula stage. One mRNA contained the *Vasa* open reading frame with a C-terminal GFP tag flanked by β -globin 5'- and 3'-UTRs. The control mRNA contained only the mCherry open reading frame flanked by β -globin 5'- and 3'-UTRs. **D:** Immunofluorescence localization of endogenous Vasa protein in embryos cultured in artificial seawater (ASW) alone, ASW containing 0.5% dimethyl sulfoxide (DMSO), or ASW containing 25 or 50 μ M MG132. Scale bars, 100 μ m.

This post-translational, regulatory conservation of *Gustavus* may also extend to vertebrate PGCs and germ cell development. Two *Gustavus* orthologs, SSB-1 and SSB-4, have been identified in the mouse and are expressed in several adult tissues, including the testis and ovary. In particular, their expression was found in somatic ovarian granulosa cells during folliculogenesis, but was not detected in the developing oocytes (Xing et al., 2006). While this work did not investigate the *Gustavus* orthologs during mouse embryonic development, these expression data may reflect contextual differences between their functions in embryo and adult gonads. Indeed, one of the zebrafish *Gustavus* orthologs is expressed in the PGCs during embryonic development, while both are enriched in the testis and ovary (Li et al., 2009).

Protein Turnover Is an Essential Mechanism in an Embryonic Gene Regulatory Network

While extensive data on transcriptional regulation comprises most of our current knowledge of gene regulation during sea urchin embryonic development, it is clear that post-translational regulatory mechanisms are essential for germ cell formation during embryogenesis. In addition to *Vasa*, other factors may be subject to similar post-translational gene regulation within the sMics. The transcript *seawi* (the sea urchin *piwi* ortholog) also is uniformly distributed throughout the early embryo, yet *Seawi* protein becomes enriched in the micromeres at the 16-cell stage just as seen with *Vasa* (Juliano et al., 2006; Rodriguez et al., 2005). Although *Vasa* and *Seawi* primarily function in a post-transcriptional regulatory context, the sea urchin embryo also utilizes selective protein turnover to adjust transcription factor abundance both within and outside the germ-cell lineage. For example, the transcription factor SoxB1 is degraded specifically in blastula vegetal cells comprising the endomesoderm (Angerer et al., 2005). This preferential SoxB1 protein turnover is dependent upon nuclear beta-catenin function, which is necessary for normal SoxB1 turnover and can cause ectopic degradation in animal blastomeres (Angerer et al., 2005). Spatial and temporal transcriptional regulation by beta-catenin is also dependent upon its differential protein stability and post-translational regulation within the developing embryo. In the 16-cell embryo, unphosphorylated beta-catenin translocates to the micromere nuclei, whereas beta-catenin in mesomeres and in macromeres is phosphorylated by GSK3 β and degraded. This intricate, post-translational regulation of beta-catenin is essential for proper A–V axis patterning in the early embryo (Emily-Fenouil et al., 1998; Wikramanayake et al., 1998; Logan et al., 1999; Sherwood and McClay, 1999; Angerer and Angerer, 2000) and documents the shared mechanistic utility of somatic and germ-line functions.

Transcriptional Regulation

Many in the sea urchin research community have sought to understand the transcriptional control of embryogenesis. Since the *S. purpuratus* genome sequence became available in 2006 (Weinstock et al., 2006), the work of many labs has identified a large set of transcription factors that are expressed lineage-specifically during development (e.g., Spbase.org). Despite these efforts, few genes are known to be selectively expressed in the sMics at any stage; further, it is not known how these few sMic enriched genes are transcriptionally regulated. The dearth of transcription factors therein may suggest that the sMics are a transcriptionally 'quiet' territory.

The chromatin state of the sMic indeed suggests broad transcriptional repression relative to the somatic cells of the embryo. Antibodies that distinguish between catalytic states of RNA Polymerase II (RNAPII) have proven useful for visualizing the transcriptional activity of cells. RNAPII is regulated by phosphorylation on its C-terminal domain (CTD), which contains numerous copies of the

heptapeptide repeat YPTSPS. Phosphorylation on Ser5 and Ser2 are associated with initiation and elongation, respectively (Nakamura and Seydoux, 2008). By immunofluorescence, sMic nuclei appear depleted for the Ser2 phosphoepitope by the blastula stage, which persists through gastrulation (Swartz et al., 2014). This observation suggests that the sMics are transcriptionally repressed during their migratory phase. A consequence of broad repression might be to insulate the sMics from differentiation as they translocate from the vegetal plate to the coelomic pouches, a time when they are exposed to numerous inductive signals as the surrounding endomesoderm differentiates (Swartz et al., 2014). The mechanism by which the sMics become depleted for RNAPII phospho-Ser2 (pSer2) remains unknown, although this depletion appears to be a hugely conserved feature of germ cells, shared by the PGCs of *Drosophila*, *C. elegans*, mice, and ascidians (Seydoux and Dunn, 1997; Seki et al., 2007; Shirae-Kurabayashi et al., 2011). Surprisingly, though, each of these organisms achieve RNAPII repression via distinct pathways involving genes specific to their species (Mello et al., 1996; Batchelder et al., 1999; Zhang et al., 2003; Ghosh and Seydoux, 2008; Hanyu-Nakamura et al., 2008). As these genes are not conserved in the sea urchin, it is likely that at least one other mechanism of repression evolved in the echinoderm clade. One possible player is the RNAPII phosphatase Ctdspl2/SCP2, whose transcript was found to be enriched in the sMics (Swartz et al., 2014). The timing of sMic pSer2 depletion differs from *Drosophila* and *C. elegans*, where it occurs before migration. In this regard, the sMics are more similar to mouse PGCs, where pSer2 is depleted during PGC migration to the genital ridge (Seki et al., 2007). It remains unknown, however, how mouse PGCs become depleted for pSer2.

Transcriptional activity is intimately linked with epigenetic modification to the DNA and surrounding nucleosomes. A faithful marker of repressive heterochromatin is histone 3 lysine 9 trimethylation (H3K9me3). Upon their creation at the 5th cleavage, the sMics are highly enriched for H3K9me3 compared to somatic cell nuclei, which persists to blastula stage. Thus, there appear to be at least two separate phases of sMic transcriptional repression: H3K9me3-positivity followed by pSer2 depletion as the sMics translocate. Histone modification has also been observed in the PGCs of *Drosophila* and *C. elegans*, which both display a loss of H3K4me, a marker of active euchromatin (Schaner et al., 2003). In *Drosophila*, this depletion is mediated by SU(VAR)3-3 (ortholog of the LSD-1 amine oxidase) (Rudolph et al., 2007), and occurs concomitantly with pSer2 depletion. Mice also display methylation transitions: during migration to the presumptive gonad, the PGCs lose H3K9me3 and acquire the repressive mark H3K27me (Seki et al., 2007). The transcriptional repressor Blimp1 is responsible for repressing the majority of inactivated genes in mouse PGCs (Ohinata et al., 2005), potentially by recruiting histone methyltransferase machinery (Ancelin et al., 2006). Blimp1 is conserved in the sea urchin, but it does not have a known role in the sMics; rather, it appears to function during specification of the endomeso-

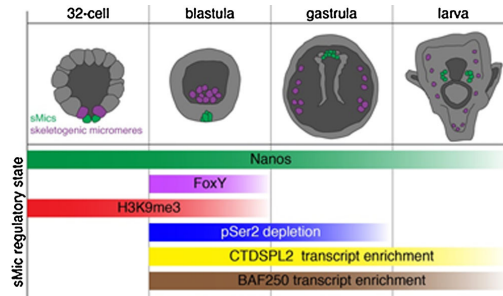


Figure 11. Temporal dynamics of sMic transcriptional activity. Sea urchin embryonic stages are schematized with the sMics indicated in green. Colored bars below indicate timing of events in the sMics.

derm, where it is required to activate Wnt8 (Minokawa et al., 2005; Livi and Davidson, 2006). In addition to specific histone modification, the nucleosome remodeling complex Swi/SNF has also been implicated in transcriptional repression; mutation of the ARID-containing factor Osa in *Drosophila* leads to derepression of the somatic transcript *zen* (Martinho et al., 2004). The SWI/SNF related factors BAF250, Brg1, and ISWI were each identified as sMic-enriched in sea urchins, but their involvement in regulating the transcriptome remains untested (Yajima and Wessel, 2012; Swartz et al., 2014).

Perhaps reflective of their transcriptional repression, only one transcription factor is known to be uniquely expressed in the sMics to date: the forkhead factor FoxY (Fig. 11) (Ransick et al., 2002). FoxY is only transiently activated in the sMics during blastula stage, and subsequently shifts into the non-skeletogenic mesoderm upon gastrulation. The early sMic phase of FoxY expression is dependent on Delta/Notch signaling from presumptive mesoderm, and *cis*-regulatory analysis of its promoter has revealed a functional Suppressor of Hairless site, the transcriptional effector of Delta/Notch signaling. All known FoxY targets are specific to the non-skeletogenic mesoderm cells, however; therefore, its function in the sMics is unclear. FoxY knockdown in its sMic expression phase has no effect on *nanos* transcript levels (Materna et al., 2013). *Nanos* is modestly reduced in larval stages with FoxY knockdown, although this is likely an indirect effect due to failure to form the coelomic pouches as a consequence of non-skeletogenic mesoderm defects (Song and Wessel, 2012). What happens to the sMics with FoxY knockdown has also not been determined. Still, germ cells in many species require a permissive niche environment, so failure to organize coelomic pouches could have profound effects on sMic gene expression.

RNAs Are Selectively Retained in the sMics

Several lines of evidence suggest that the sMics are transcriptionally repressed. How the sMics acquire germ-

line RNAs is, therefore, an important question. The sMics appear to initially inherit their RNAs rather than transcribe them *de novo*, consistent with the broad distribution of RNAs encoding Vasa and Seawi in early embryos that is later refined to the sMics during gastrulation (Juliano et al., 2006; Voronina et al., 2008). In other organisms such as *Drosophila*, mRNA localization is an important mechanism during early development (Becalska and Gavis, 2009) and localization of *nanos* RNA in the germ line depends on sites in its 3'-UTR (Gavis et al., 1996; Andrews et al., 2011).

One of the strengths of the sea urchin as a model organism is the ease and diversity of manipulations possible for studying developmental features. Investigators of this and many animals often inject mRNAs into eggs and early embryos to test the function or the regulation of proteins, and *Xenopus* β -globin UTRs are often used to direct constitutive translation throughout the embryos. This sequence of UTRs was shown to be innocuous to normal mRNA translational regulation in a variety of cases. Surprisingly though, injection of an mRNA coding for mCherry flanked by *Xenopus* β -globin UTRs showed a selective fluorescence signal in the sMics of gastrulae (Fig. 12). In situ hybridizations, using a mCherry probe, of the injected embryos indicate that this selective protein accumulation resulted from the prolonged retention of its RNA in the sMics (Fig. 1B) (Gustafson and Wessel, 2010c; Oulhen and Wessel, 2013). After microinjection at the one-cell stage, the RNA is strongly retained uniformly in embryos during early development until blastula. During gastrulation, however, the RNA becomes detectable only in the sMics (Oulhen and Wessel, 2013).

Retention of exogenous mRNA selectively in the sMics could result from either selective RNA decay in the non-sMic cells, and/or selective RNA protection in the sMics. mRNA decay can depend on *cis*-acting elements within

each RNA but also on the mRNA degradation machinery present at a specific time, in specific cells. RNA turnover often begins by deadenylation, followed by the action of exonucleases that degrade RNAs from 5'-to-3' or 3'-to-5' (Garneau et al., 2007). In eukaryotic cells, the 5'-to-3' degradation pathway depends on removal of the 5'-cap by the decapping proteins Dcp1 and Dcp2 (Scherer et al., 2006). The resulting uncapped RNA is then degraded by the 5' exoribonuclease, Xrn1 (Larimer et al., 1992; Hsu and Stevens, 1993). The 3'-to-5' degradation pathway is directed by a multi-subunit exonuclease complex, the RNA exosome (Garneau et al., 2007; Schmid and Jensen, 2008; Lykke-Andersen et al., 2011). mRNA can also be degraded by endonucleases, followed by degradation of decay products by exonucleases (Otsuka and Schoenberg, 2008). Finally, mRNAs can also be degraded by piRNAs and miRNAs (Selbach et al., 2008; Rouget et al., 2010). In zebrafish, miR-430 is the most abundant miRNA family expressed during early development, and accumulates during the maternal-to-zygotic transition. miR-430 facilitates the deadenylation and clearance of maternal mRNA during early embryogenesis (Giraldez et al., 2006). In *Drosophila*, a cluster of zygotically expressed miRNAs targets maternal mRNAs for turnover as part of the zygotic degradation pathway (Bushati et al., 2008). Remarkably in the sea urchins, neither the m⁷GTP cap nor the polyadenylation tail is required for sMic RNA retention of mCherry and GFP reporters containing *Xenopus* β -globin UTRs (Oulhen and Wessel, 2013). Interestingly, an RNA containing only the GFP open reading frame without a 3'-UTR is not selectively retained in the sMics (Fig. 13).

While the mechanisms of somatic-cell mRNA turnover have been explored in several organisms, how those RNAs remain protected in the PGCs is poorly understood. The best evidence comes from work in zebrafish, where it was

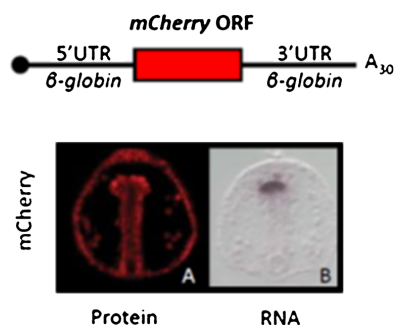


Figure 12. sMic retention of an exogenous RNA containing the mCherry open reading frame surrounded by the *Xenopus* β -globin UTRs. **A:** mCherry fluorescence at the gastrula stage after RNA injection. **B:** In situ RNA hybridization at the gastrula stage after injection of the RNA.

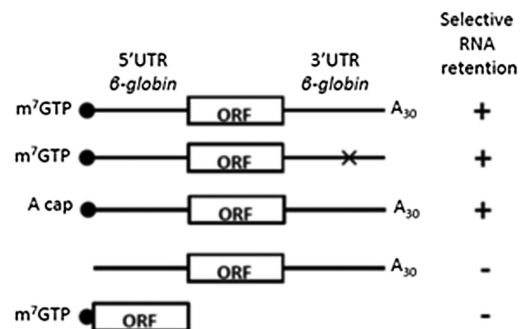


Figure 13. Summary of the RNA constructs tested for sMic RNA retention. The open reading frame is surrounded by *Xenopus* β -globin UTRs. The black circle represents the m⁷GTP cap, and the polyadenylation tail is symbolized by A₃₀. In the second construct, the "x" indicates the mutation of the polyadenylation signal in the 3'-UTR from β -globin.

first recognized that germ-line RNAs targeted by miR-430, including *nanos*, were differentially degraded in the soma versus the germ line (Koprunner et al., 2001; Mishima et al., 2006). That is, while miR-430 is present in the PGCs, its targets are protected within these cells. It was subsequently found that the germ-line RNA binding protein Dead end 1 (Dnd1) binds near and occludes miR-430 sites from Argonaute machinery (Kedde et al., 2007). Yet, Dnd1 is not conserved outside of vertebrates. Still, miRNAs are expressed in echinoderms during embryonic development (Kadri et al., 2011; Song et al., 2012), and could be involved in selective RNA retention, that is, degradation of mRNAs outside of the sMic but retention of mRNAs in the sMics due to protection of the target transcripts from the miRNAs.

In the sea urchin, the deadenylase CNOT6 is present in somatic cells but is specifically depleted in the sMics by Nanos, likely with its binding partner Pumilio (Fig. 3). CNOT6 is a core deadenylase of the conserved CCR4-NOT complex, a major cytoplasmic regulator of RNA stability (Wiederhold and Passmore, 2010). Reduced deadenylase activity provides the sMics with a generally stable environment for inherited RNA. Misexpression of CNOT6 in the sMics results in their failure to retain RNAs and accumulate Vasa protein, and possibly leads to their apoptosis. Conversely, global knockdown of CNOT6 expands the domain of RNA retention beyond the sMics and into the entire endomesoderm. These results indicate that differential CNOT6 regulation is central to the RNA retention phenomenon (Swartz et al., 2014, Fig. 15). This selective retention could also be explained by a differential RNA storage mechanism. Germ cells usually contain granules related to P bodies (processing bodies), called germ granules, which play a role in RNA accumulation and decay. They regulate maternal RNA levels required for germ-cell specification (Anderson and Kedersha, 2006). RNA injected at the one-cell stage might be subject to regulation as a maternal RNA by the embryo. Depending on the proteins present in the sMics, specific granules could protect this RNA from degradation.

In contrast to this selective RNA retention observed with non-specific *Xenopus* β -globin UTRs in gastrula, sMics also specifically retain reporter RNAs containing endogenous sequences. Injection of an RNA containing the GFP open reading frame surrounded by *nanos2* UTRs is exclusively retained in sMics of blastula whereas the RNA containing *Xenopus* β -globin UTRs is still detected in all the cells at this stage (Oulhen et al., 2013). *nanos2* UTRs accelerate the selective retention in the sMics, that is, these transcripts are more rapidly degraded outside of the sMics. Data obtained from two sea urchin species, *S. purpuratus* (*Sp*) and *H. pulcherrimus* (*Hp*), indicate that the *nanos2* 3'-UTR directs this specific and selective RNA retention in the sMics. A sequence composed of 357 nucleotides in *Hp* and 388 nucleotides in *Sp*, is required for this selective RNA retention. This sequence, present in *nanos2* 3'-UTR and defined as GNARLE for Global Nanos Associated RNA Liability Element, is required for the specific retention of the RNA in the sMics. GNARLE is sufficient for high protein enrichment in the sMics, suggesting a role for GNARLE in

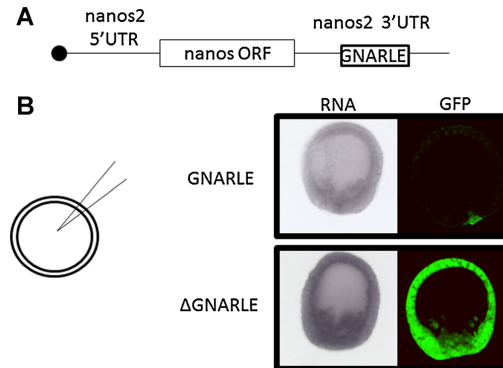


Figure 14. *Sp* GNARLE is necessary for selective RNA retention and protein accumulation in the sMics. **A:** Schematic representation of *nanos2* RNA, indicating the location of GNARLE in the 3'-UTR. **B:** RNAs containing the *Sp nanos2* 5'-UTR followed by a GFP open reading frame and by either GNARLE or Δ GNARLE 3'-UTR were injected in *S. purpuratus* fertilized eggs. At blastula stage, RNA retention was tested by in situ hybridization and protein accumulation was followed by GFP fluorescence.

inhibiting the translation in the non-sMics and/or stimulating translation in the sMics (Fig. 14). Therefore, GNARLE plays a role in both promoting selective RNA retention and translational activity of *nanos* in the sMics. *Hp* and *Sp* GNARLE are functionally conserved: injection of either *Sp* or *Hp* GNARLE RNA in *Sp* fertilized eggs give similar RNA retention and protein accumulation. *S. purpuratus* and *H. pulcherrimus* diverged between 7.2 and 14 million years ago (Lee, 2003). In contrast, no similar GNARLE was found in the sea urchin *Lytechinus variegatus*, which diverged from *S. purpuratus* between 30 and 50 million years ago (Smith et al., 2006), nor in the starfish *Patiria miniata*, which diverged from sea urchins approximately 500 million years ago (Hinman et al., 2003; Smith et al., 2006).

A combination of degradation in the non-sMics and protection in the sMics seems to be required for strong, selective RNA retention. How GNARLE regulates RNA stability is not well understood yet. Between 50 and 55 miRNAs have been identified in the sea urchin (Campo-Paysaa et al., 2011; Song et al., 2012). By sequence analysis, we found that GNARLE could potentially be targeted by three of these miRNAs in both *S. purpuratus* and *H. pulcherrimus*. Yet, perturbation of Dicer, an enzyme essential for miRNA biogenesis, does not affect the level of *nanos2* transcript significantly in *S. purpuratus* embryos, indicating that miRNAs are not or are not solely responsible for the decay of *nanos2* RNA outside of the sMics (Oulhen et al., 2013). In *Drosophila*, the *nanos* 3'-UTR contains a 547-nucleotide element mediating its RNA localization at the posterior pole of the syncytial embryo (Gavis et al., 1996). The protein rumpelstiltskin (rump), a heterogeneous

nuclear ribonucleoprotein (hnRNP) M homolog, binds *nanos* RNA directly to regulate its localization. More recently, the Argonaute family member Aubergine (Aub) was also found to be a *nanos* RNA localization factor, independent of its function in RNA silencing. Aub interacts with *nanos* mRNA in vivo and co-purifies with rump in an RNA-dependent manner (Becalska et al., 2011). The piRNA pathway was also shown to be required for *nanos* mRNA deadenylation and decay as well as translational repression in *Drosophila* embryos (Rouget et al., 2010). Aub is present in a complex with the RNA-binding protein Smaug, the deadenylase CCR4, *nanos* mRNA, and piRNAs that target the *nanos* 3'-UTR. In the sea urchin, preliminary data using a gel-shift assay, suggests that one or several proteins from mesenchyme blastula can bind to *Sp* GNARLE, and might be involved in RNA stability and or translational control (Oulhen and Wessel, unpublished data).

Left-Right Asymmetry in Germ Line Formation

The sea urchin develops as a bilaterally symmetrical embryo, but shows signs of left–right (L/R) asymmetry as

the larval form takes shape. The first morphological indication of symmetry break occurs following gastrulation, when the eight sMic descendants sort into the nascent coelomic pouches. The morphogenetic process of pouch formation requires Hedgehog signaling, based on inhibitor experiments, but perturbing this pathway does not affect coelomic pouch gene expression (Materna et al., 2013). Similarly, inhibiting BMP2/4 prevents bilateral sorting of the sMics—they instead remain centrally located at the foregut tip. It does not, however, alter their accumulation of *vasa*, *nanos*, or *seawi* RNAs (Luo and Su, 2012), possibly because those transcripts are inherited by the sMics rather than actively transcribed. The non-skeletogenic mesoderm constituents of the pouches, however, lose expression of numerous transcriptional regulators (e.g., SoxE, Pax6), indicating disruption to the sMic niche environment (Luo and Su, 2012).

Although some slight embryo-to-embryo variance is seen, the sMics usually split so that there are five in the left pouch, and three in the right (Pehrson and Cohen, 1986; Tanaka and Dan, 1990). The precise mechanism of this assortment is unresolved, but conserved L/R asymmetry genes are required. In late gastrula/early larval stages, *nodal* transcript is detectable asymmetrically on the right side ectoderm (Duboc et al., 2005). Pharmacological inhibition of Nodal during pouch formation causes symmetric assortment of sMics such that there are four on either side (Luo and Su, 2012). Multi-drug transporter (MDR) activity is also involved since treatment with MDR-inhibiting drugs randomizes sMic L/R sorting (Campanale and Hamdoun, 2012). This phenotype may point to a failure in chemoattraction—in *Drosophila*, the ABC transporter *mdr49* is required for secreting a lipid-modified peptide that attracts PGCs to the gonad (Ricardo and Lehmann, 2009; Fig. 16).

Nodal signaling also appears to impinge upon the survival of sMics in the right coelomic pouch, which have been proposed to apoptose due to loss of Nanos expression (Fujii et al., 2009; Juliano et al., 2010). By the pluteus stage, the right sMics normally become TUNEL-positive, indicative of apoptosis; inhibition of Nodal prevented this apoptosis whereas elevating Nodal signaling with exogenous Activin induced apoptosis in left side sMics. Consistent with these results, Nodal inhibition also causes the persistence of *vasa*, *nanos*, and *seawi* RNAs in the right coelomic pouch (Luo and Su, 2012). Nodal acts through the homeodomain repressor Pitx2 (Duboc et al., 2005), which is the likely repressor of FoxY in the right coelomic pouch cells (Materna et al., 2013). Therefore, one possibility is that *vasa*, *nanos*, and *seawi* transcripts are direct targets of this pathway. Alternatively, the loss of detectable germ-line transcripts in the right coelomic pouch could simply be due to Nodal-dependent apoptosis of the sMics.

The upstream-most signal for L/R asymmetric gene expression in the urchin embryo remains enigmatic. A potential organizing center has been recently discovered in the sea urchin even before coelomic pouch formation. In mid-to-late gastrula-stage embryos, a mesendodermal patch of *nodal*-positive cells appears on the right side of the archenteron. This *nodal* transcript accumulation requires

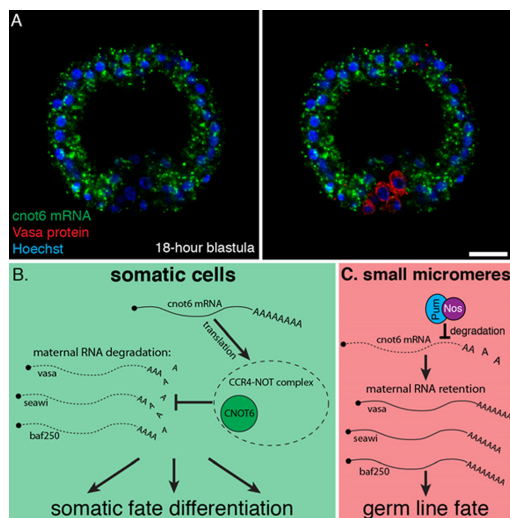


Figure 15. Differential accumulation of CNOT6 in somatic and germ-line blastomeres. **A:** *Cnot6* transcript (green) is detectable in all cells of an 18-hr blastula by fluorescence in situ hybridization, except in the sMics, which are labeled by Vasa immunofluorescence (red). **B,C:** Schematization of differential RNA retention in somatic cells versus sMics. **B:** *Cnot6* RNA is present in all somatic cells, and is translated and likely incorporated into a CCR4-NOT related complex, where it affects the degradation of germ-line RNAs. **C:** Nanos/Pumilio targets the *Cnot6* transcript for degradation, leading to a reduction in deadenylase activity in the sMics. This reduction creates a generally stable environment for both endogenous germ-line RNAs and micro-injected reporters.

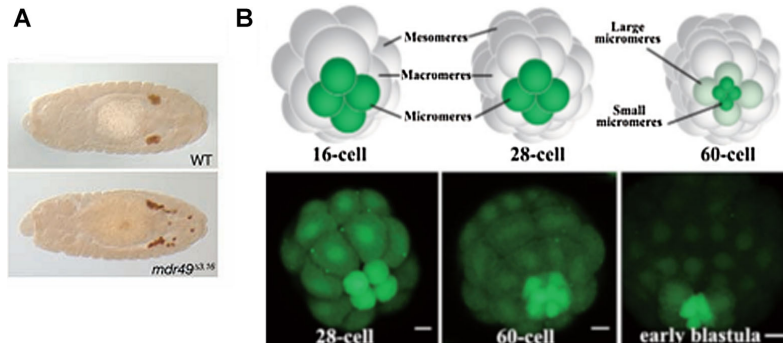


Figure 16. ABC transporters are involved in PGC success. **A:** In *Drosophila*, the ABC transporter *mdr49* is required for germ-cell migration to the mesoderm. An *mdr49*^{Δ3,16} embryo (bottom) shows lost germ cells. Reprinted with permission from Ricardo and Lehmann (2009). **B:** The fluorescent indicator calcein indicates the absence of fluorescence indicates active efflux, whereas the presence indicates a lack of ABC efflux activity. Remarkably, the sMics have low ABC-effluxing activity and, as a result, retain calcein in the cytoplasm. Scale bars, μm . Reprinted with permission from Campanale and Hamdoun (2012).

FGF and BMP signaling, and is repressed on the left side by Delta/Notch signaling. Perturbation of the mesendodermal organizer prevents subsequent accumulation of *nodal* transcript in the left-side ectoderm, which appears to involve mesendodermal/ectodermal crosstalk via Univin (TGF- β) signaling, and randomizes the placement of the adult rudiment (Bessodes et al., 2012). Since rudiment formation can still occur, it suggests that coelomic pouch development occurs largely normally. An interesting open question is whether or not the sMics properly home under these conditions, and if the resultant sea urchins are fertile. Further, although the L/R asymmetry is seen earlier in these embryos, the mechanism for this process is unknown.

Multi-Drug Resistant/ABC Transporters in Germ-Cell Development

ATP-binding cassette (ABC) transporters are transmembrane proteins found in nearly all cells that help control the flow of information between cells and their environment. These transporters provide energy-dependent shuttling of a variety of compounds across the plasma membrane to eliminate metabolites and secondarily remove drugs intended to work inside the cell (Higgins et al., 1990; Dean et al., 2001). Members of this transport family also provide protection in the export of toxins and xenobiotics (Deeley et al., 2006; Sarkadi et al., 2006; Dean et al., 2001). ABC transporters are highly conserved and present in all free-living organisms, from bacteria to humans. Their function is essential to the protection and normal development of the embryo. ABC efflux transporters are large membrane-bound proteins, with membrane-spanning domains and a cytoplasmic ATP-binding domain that is required to shuttle compounds in an ATP-dependent manner. In addition to

transportation and protection, ABC effluxers actively influence the fate of cells by modulation of cell differentiation, lysosomal and mitochondrial homeostasis, potassium channel regulation, pigmentation, and migration (Shipp and Hamdoun, 2012; Gökirmak et al., 2012). The ABC efflux transporter family is the parent family of multi-drug resistant transporters, which participate in chemotherapy resistance in cancer cells and antibiotic resistance in bacteria.

Remarkably, ABC transporters are implicated in the migration of PGCs. In *Drosophila*, *mdr49*, an ABCB efflux transporter, is essential for the appropriate migration of PGCs. Ricardo and Lehmann (2009) generated a loss-of-function allele for this transporter and demonstrated that mutant embryos showed germ cell-migration defects, which included migration through the posterior hindgut and lack of association with the somatic gonad. The PGCs formed, but during their migratory phase, moved to aberrant positions. In *C. elegans* and *Drosophila* embryos ABCC and ABCB members of the ATP-dependent transporters, respectively, efflux prenylated peptides from mesodermal cells to attract germ cells during their migration (Yabe et al., 2005; Ricardo and Lehmann, 2009). This finding highlights the functional diversity that multi-drug resistant transporters have during normal development, although exactly how these transporters are regulated in normal development is unknown. Data from the oncogene literature suggests that miRNAs are also involved in the regulation of ABC efflux transporters (Haenisch et al., 2008), specifically that ABCC2 is down-regulated in mature mouse testis by mir-379 (Haenisch et al., 2011). Yet, our understanding is limited in how all the diverse functions are intertwined to form a reproductively competent organism. Hamdoun et al. (2004) observed that the ABCC- and ABCB-type transporters were present in

eggs and cleavage-stage sea urchin embryos. The authors demonstrated that the fertilization response involves upregulation of multi-drug efflux transport activity as part of egg activation, and that this activity protects the embryo from exogenous toxins. Inhibition of the efflux activity further affects cell division, suggesting that the transporter activity also has some role in passage through mitosis; indeed, *S. purpuratus* embryos express ~20 ABC efflux transporters over the first three days of development (Shipp and Hamdoun, 2012; Gökirmak et al., 2012). *ABCB1a* mRNA is 10–100 times more abundant than mRNA of other ABC transporters in the developing embryo, and the *ABCB1a* protein localizes to the apical membrane of the embryo while *ABCC5a* is expressed on the basolateral membrane of polarized cells (Shipp and Hamdoun, 2012; Gökirmak et al., 2012).

It was recently found that the ABCB- and ABCC-type transport activity is downregulated in the sMics (Campanale and Hamdoun, 2012). This is unexpected given that ABC efflux transporters function to protect cells. In fact, the sMics retain significantly more calcein, a reporter of ABCB- and ABCC-type efflux from cells, than any other cell in the embryo (Campanale and Hamdoun, 2012). According to Campanale and Hamdoun (2012), this phenomenon is true in the sMics of other echinoid species as well, including the sea urchin *Lytechinus pictus* and the sand dollar *Dendraster excentricus*, indicating a conserved sMic activity from a common ancestor that diverged at least 250 million years ago (Smith et al., 2006). Logically, it seems rational that ABC efflux transporters function to protect stem cells from harmful toxins and differentiation (Zhou et al., 2001; Bunting, 2002), and hematopoietic stem cells express more efflux pumps than differentiated cells (Bunting et al., 2000). The loss of some ABC transporters in the sMics is contrary to what might be expected, so it will be important to learn if this observation simply represents a decrease in one family of ABC transporters or if overall activity actually decreases in these cells. Perhaps the absence of efflux normally ascribed to the ABCB- and ABCC-type transport activity contributes to the downregulation of metabolic activity detected in the sMics, which globally protects the cell. Another possibility is that sMics may transiently lose their ability to efflux in order to acquire other germ line-specific modifications.

Diversity of Germ-Line Development in Echinoderms

Echinoderms are a very diverse and ancient lineage, first appearing in the fossil record approximately 550 million years ago (Porter, 1977; Greenstein, 1993; Kroh, 2007; Kroh and Smith, 2010; Erwin et al., 2011; Smith et al., 2013). The ancient echinoderms diversified very rapidly, with four distinct body plans within 10–15 million years (Smith et al., 2013). Due to the rapid diversification of echinoderms over the subsequent 500 million years, it has been difficult to accurately determine the phylogenetic relationship between extant echinoderms. Part of the difficulty is that different results are obtained depending on the

methods for determining the phylogenetic relationships (rRNA sequences, mitochondrial sequences, morphology, larva, etcetera), which yields slightly different phylogenetic trees. Near unanimous agreement exists that the crinoids (feather stars and sea lilies) are the root of the echinoderm tree (Littlewood et al., 1997; Janies, 2001; Mallatt and Winchell, 2007; Janies et al., 2011; Mah and Foltz, 2011; Pisani et al., 2011), and there is little conflict over the close relationship between Holothuridea (sea cucumbers) and Echinoids (sea urchins and sand dollars). The majority of the conflicts arise from the uncertain position of Ophioids (brittle stars) within echinoderms (Okanishi et al., 2011; Perseke et al., 2012), but several studies suggest that Asterozoa (brittle stars and sea stars) are the sister group to Echinozoa (sea urchins, sand dollars, and sea cucumbers) (Fig. 17). A more comprehensive method for accurately determining the phylogenetic tree of echinoderms is needed.

However the phylogenetic relationships of echinoderms are resolved, though, it is clear that only the euechinoids (sea urchins and sand dollars) have sMics (Tanaka and Dan, 1990; Julianio and Wessel, 2009; Vellutini and Migotto, 2010). Therefore, when considering germ-line determination mechanisms and identifying which cells are responsible for this character in other echinoderms, the context will change dramatically. The cidaroids (pencil urchins) do have micromeres, or at the very least, asymmetrically dividing micromere-like cells at the 4th cell cleavage (Wray and McClay, 1988; Bennett et al., 2012). The micromeres and sMics are likely a derived trait unique to Echinoidea, as a single gain of micromeres, and subsequently sMics, in a single branch is more parsimonious than micromeres being the ancestral state and then lost independently at least three times. sMics are precursors of the PGCs of the sea urchin and therefore are protected in the embryo in a variety of ways discussed above. One hypothesis in their appearance in sea urchins might be their co-evolution with the hyaline layer. Cidaroids do not make sMics (at the 5th cell cleavage at the very least), do not have a hyaline layer, and instead depend on the fertilization envelope to constrain the blastomeres. Similarly, most echinoderms do not make hyalin, with the notable example of some brittle stars (Yamashita, 1985). Many developing embryos (e.g., sea stars) depend on the fertilization envelope to constrain the blastomeres, but the relatively small sMics may require an extra layer of constraint in the embryo to aid in their retention. The loss of a blastomere to even an eight-cell embryo is easily surmountable, but the loss of the PGCs at an early stage could lead to sterility (Yajima and Wessel, 2011).

It is important to note that echinoderms undergo both indirect and direct development, and different species from the Asterozoa, Ophiurozoa, Echinozoa, and Holothurians show this diversity. Direct-developing species do not form a feeding larval structure, which likely changes their PGC biogenesis. Some of the characteristics used to distinguish direct developers from indirect developers include egg, larval and post-larval size, rate of development, gonadal volume measurements, and numbers and volume of eggs. Although some of these characters are of questionable

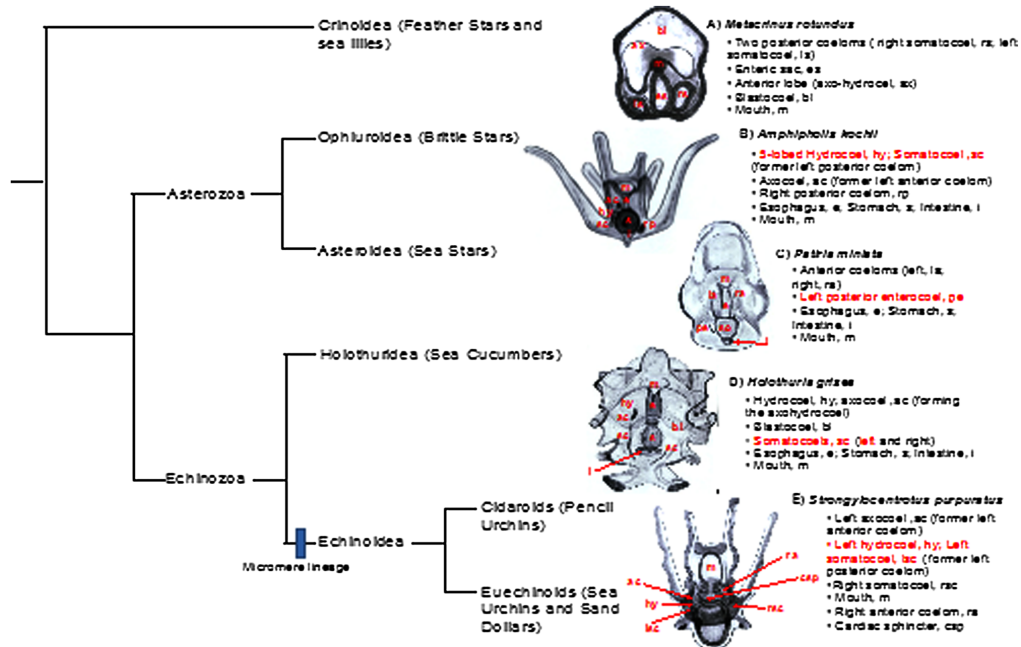


Figure 17. Members of the phylum Echinodermata. There are five extant classes of echinoderms: Crinoidea, Ophiuroidea, Asteroidea, Holothuroidea, and Echinozoa. Representatives of these classes: sea lily *Metacrinus rotundus*; brittle star *Amphipholis kochii*; sea star *P. miniata*; sea cucumber *Holothuria grisea*; sea urchin *S. purpuratus*. Schematic drawings of larval stages indicating different tissues for each organism. **A:** A 3.5-day, non-feeding auricularia larva. **B:** A 6-day, eight-armed ophiopluteus. **C:** A 5-day bipinnaria larva. **D:** A 4-day aspidochirotid auricularia. **E:** A 4-armed pluteus larva. Dorsal views of the larvae are shown. Members of the class Echinozoa, which have a micromere lineage, are split into the subclasses Euechinozoa (sea urchins and sand dollars) and Cidaroida (pencil urchins).

reliability, a feeding larval-development stage is most likely the primitive mode of development in echinoids. The variability between direct developmental modes also suggests that they emerged independently in several lineages (Holland, 1991). As all of the crinoids described so far undergo direct development (Holland, 1991; Nakano et al., 2003), our analyses of coelomogenesis and PGC specification will focus on indirect developers. The only other echinoderm phylogenetic clade in which the specification of the PGCs has been functionally tested is that of the Asteroidea (sea stars). For this reason we will outline what is known about germ-cell specification in sea stars first.

Asteroidea (Sea Stars)

The origin of germ cells in the sea star *Asterina pectinifera* was traced back to the posterior enterocoel of 2-day-old bipinnaria larvae. Inoue et al. (1992) removed the posterior enterocoel from 2-day-old bipinnaria larvae and observed a decrease in the number of germ cells in the

cellular cluster of 6-week-old brachiolariae juveniles. Germ cells in this case were identified through morphological characteristics: large cells, large nuclei, mitochondrial aggregations, and electron-dense, nuage-like structures (Inoue et al., 1992). Furthermore, the germ-line factor Vasa accumulates in the posterior enterocoel of the sea stars *P. miniata* and *Asterias forbesi* (Juliano and Wessel, 2009). Older morphological studies that identified PGCs with classical germ-cell characteristics are consistent with these recent data because the origin of the germ line was described to be in the "dorsal horn of the left somatocoel of the bipinnaria larvae," which we interpret to be derived from cells in the posterior enterocoel (Gemmill, 1914; Chia and Walker, 1991). Together, these observations suggest that the posterior enterocoel is the origin of PGCs in the Asteroidea clade.

So what is the left posterior somatocoel/enterocoel? Coeloms are fluid-filled cavities of mesoderm that, in the case of echinoderms, arise from the archenteron. Chia and Walker (1991) outlined that asteroids use three general strategies to develop their coeloms. In the first type, two

coelomic pouches arise from the anterior of the archenteron, and then grow posteriorly, the left much faster than the right. The left pouch also gives rise to the water-vascular system. In the second type, a single coelom arises from the anterior of the archenteron and grows posteriorly on both sides as a U-shaped coelom. Later in development, two septa form on each side of the "U" to form the anterior coelom and two posterior coeloms (left and right). In the third type, the coeloms arise from both the anterior and posterior ends of the archenteron (Chia and Walker, 1991). Recently, Morris et al. (2009, 2011) found the second mode of coelomogenesis to be used for the development of *Parvulastra exigua* larvae whereas the third mode of coelomogenesis seems to be used for the development of *P. miniata*, *Asterina pectinifera*, *Pteraster tessellatus*, and

Asterina glacialis (*Marthasterias glacialis*) because the left posterior coelomic pouch in these animals arises by budding off the left posterior side of the archenteron (Fig. 18; Gemmill, 1914; Heath, 1917; Kuraishi and Osanai, 1992; David et al., 1994; Hinman et al., 2003). Deviations from this mode of coelomogenesis were noted in earlier descriptions of other Asterozoa larval development. For example, in 1914, Gemmill noticed that the posterior enterocoel in the sea star *Asterias rubens*, although usually developing from the dorsal left side of the stomach, was also rarely seen to arise from the right side of the gut and also medially (Gemmill, 1914). He also noticed the posterior enterocoel in *Porania pulvillus* usually developed on both sides of the dorsal stomach (Gemmill, 1915).

Because the left posterior coelom is believed to be important for PGC specification in sea stars, we will consider larval coelomogenesis in our analysis of what is known about PGC specification in the other echinoderm clades to determine if a similar structure to the Asterozoa left posterior coelom is also found in other echinoderm clades. This analysis is important because the literature reveals a marked lack of knowledge of PGC specification in other echinoderm clades.

Crinoids (Feather Stars, Sea Fans, Sea Lillies)

The only studies of crinoid germ-line specification that we are aware of were outlined nicely by Holland (1991). In this article, the author states that the most detailed morphological study of the origin of crinoid germ line, although incomplete and controversial, was accomplished by Achille Russo (Russo, 1902). In Russo's studies of the developing *Antedon mediterranea* larvae, he found that PGCs were first detected morphologically in the cystidean larval stage "from the mesentery separating the left and right somatocoels in the anal interradius." Then these cells accumulated in the "mesenchymal layer of the mesentery" to make up the primary gonad. Russo states that this is homologous to the development of the Holothuroid gonad, yet in crinoids, this primary gonad then vanishes! Later in development, Russo detected "secondary" PGCs in the pentacrinoid larval stage "from the coelomic epithelial cells of the ventral mesentery crossing the right somatocoel," and these cells form the secondary gonad "near the oral end of the axial organ" (Russo, 1900). Holland (1991) outlines serious doubts of a vanishing gonad in crinoid development, and concludes by saying the identification of PGCs in crinoids begs serious reinvestigation. A recent study of the development of *Antedon bifida* by Sabine Engle reinstates this confusion (Engle, 2013). In this animal, morphologically defined germ cells form in the horizontal mesentery. Yet, morphological germ cells also form in the left extension of the left somatocoel. Again, Engle concludes by looking to future studies for clarification of PGC specification.

The larval development of many stalked crinoids has been studied. Because these descriptions are similar and *Oxycomanthu japonicas* is the most extensively studied species for larval development, we will use Holland's (1991)



Figure 18. Late gastrula stage from *P. miniata*. This dynamic sequence shows mesenchyme migrating from the apical plate (ap) and coming in contact with the anterior vesicle (av). 1: The archenteron bears the usual two enterocoel pouches and a single, left posterior one. 2: Anterior end of bipinnaria showing the completion of the mesenchyme formation at the apical pole and its migration to the esophageal region. The left hydrocele is provided with a pore canal and hydropore. lp, left posterior vesicle. 3: Section of gastrula showing the apical plate with segms of mesenchyme formation. 4: Bipinnaria showing mesenchyme migrating past the anterior vesicle (av) to the esophagus. The left hydrocele has severed its connection with the archenteron. lp, left posterior vesicle in process of formation. 5: Surface overview of gastrula immediately before mesenchyme development from the apical plate. Reprinted with permission from Heath (1917).

description of *O. japonicas* larval development to outline the development of stalked crinoid larval coeloms. Gastrulation produces two concentric spheres, the outer ectodermal layer, and the inner endo-mesodermal with little blastocoel space. The endo-mesodermal archenteron sphere then constricts in the center, which yields a dumbbell-shaped “mesenteric sac.” The posterior lobe of the dumbbell buds off first, which later buds into the left and right somatocoels. The anterior lobe of the dumbbell then buds to form the enteric sac, axocoel, and hydrocoel. While other echinoderms have right and left axocoels and hydrocoels, only crinoids have the equivalent of the left axocoels and hydrocoels; the right are absent (Chia and Walker, 1991).

The early larval development of the stalkless crinoid, *Metacrinus rotundus*, has recently been described by Nakano et al. (2003) and Omori et al. (2011). After gastrulation the archenteron is similar to *O. japonicas* gastrulation. The archenteron begins by constricting between the anterior and middle wall to yield a dumbbell-shaped sac. The anterior lobe is the first to bud from the dumbbell, which corresponds to the axo-hydrocoel. The posterior lobe and middle part of the dumbbell then separate from each other. The posterior lobe, or somatocoel, then separates into left and right somatocoels. Meanwhile, the middle part, or enteric sac, elongates towards the posterior and separates the left and right somatocoels (Nakano et al., 2003; Omori et al., 2011).

Ophiuroidea (Brittle Stars)

The development of morphologically distinct PGCs in Ophiuroidea is reported to occur during the final stage of metamorphosis, in a cluster in the wall of the left posterior coelomic sac (Cuenot 1891; Russo, 1893, 1903a, 1903b; Bury, 1896; Hamann, 1901; McBride, 1903; Narasimhamurti, 1932, 1933; Smith, 1940; Olsen, 1942a; Austin, 1966; Hendler, 1991). Embryogenesis in Ophiuroidea species is also varied. Hendler (1991) summarizes that one coelomic cavity forms from the tip of the archenteron. This original sac then splits into left and right coelomic sacs, and these sacs divide a second time to form two left coelomic sacs (anterior and posterior) and two right coelomic sacs (anterior and posterior). From there, hydrocoels form between the anterior and posterior sacs, and in different cases either arise from anterior or posterior sacs. Deviations from this “general” scheme of Ophiuroid early development are common (Hendler, 1991). In some cases, the right anterior coelomic sac does not develop or disappears during metamorphosis (Müller, 1846a,b, 1851, 1852a; McBride, 1903; Mortensen, 1921; Narasimhamurti, 1933; Olsen, 1942a; Hendler, 1991). In others, the left posterior sac grows directly from the archenteron rather than from the anterior sac (Grave, 1900, 1916; Hendler, 1991).

In our analysis of the literature, Ophiuroid embryos generally contain two anterior coeloms and two posterior coeloms. The left anterior coelom becomes the axocoel while the left posterior coelom buds to form the hydrocoel and somatocoel (Bury, 1889, 1896; McBride, 1903; Narasimhamurti, 1933; Mortensen, 1937; Yamashita,

1985; Hendler, 1991). The confusion and lack of knowledge come in regards to the origin of each coelom. In a report of *Amphipholis kochii* development, the author was unsure how the left and right posterior coeloms were formed, especially because the right posterior coelom formed later in development than the left posterior coelom (Yamashita, 1985). In a report of *Amphiodia occidentalis* development, the archenteron was split into a left pouch and an anterior/medial pouch. The left pouch gave rise to the left coelom while the medial pouch definitively gave rise to the left axocoel and the anterior region of the gut. Also, the origin of the right coelom and posterior gut was uncertain and there was only an account of one coelom on the right side (Emlet, 2006). In a report of *Ophiodaphne formata* development, the left and right anterior coelomic pouches divided to form anterior and posterior coeloms on each side (Tominaga et al., 2004).

Holothuroidea (Sea Cucumbers)

Information regarding germ-cell specification in holothuroids is very scarce. Smiley states that, given the available evidence, PGC's have not been identified in holothuroids prior to metamorphosis (Smiley, 1986). Coelomogenesis in *Parastichopus californicus* starts from a single out-pocket on the dorsal aspect of the archenteron. This sac then divides, with and the anterior portion becoming the future hydrocoel while the posterior portion will divide once more to become the left and right somatocoels. Furthermore, development of the coeloms is similar in direct developers, except for the time of appearance of the structures (Ohshima, 1921; Hyman, 1955; Smiley, 1986; Smiley et al., 1991). In other studies, such as that for *Holothuria grisea*, coelomogenesis starts from a single out-pocket on the apex of the archenteron; the rest is similar to *P. californicus* in that the somatocoel precursor buds from the left posterior of the anterior sac and later gives rise to both the left and right somatocoel (Balser et al., 1993). Recently, McCauley et al. (2012) described the development of *Parastichopus parvimensis* and found only a posterior enterocoel, but no obvious anterior enterocoels.

Echinoids (Sea Urchins, Sand Dollars, Pencil Urchins)

We refer the reader to the text above for the most modern view of PGC specification in echinoids; here we only mention coelomogenesis. In the most recent account of sea urchin development, paired enteric sacs form from the tip of the archenteron. They then extend posteriorly on both sides of the esophagus where they give rise to the left and right axocoel, hydrocoel, and somatocoel, which extend anterior to posterior, respectively (the tooth sacs in the adult arise from evagination of the left posterior somatocoel (Smith et al., 2008). In older accounts of sea urchin coelomogenesis, the left and right enteric sacs divided into anterior and posterior sacs, and the left anterior sac was reported to give rise to the axocoel and hydrocoel. Whether the differences between these two accounts are a result of

the analysis of different species or simply a modern technique remains uncertain.

Overall Conclusions

We hypothesize that the left posterior enterocoel represents the ancestral mode of germ-line specification in the echinoderm clade because the sMic lineage is not conserved among echinoderms (the location of PGC specification in sea urchins) although the posterior coelomic pouches (the location of PGC specification in sea stars) are conserved in all echinoderm clades. As mentioned above, germ-line molecular markers accumulate in the posterior enterocoel of sea stars, and its removal decreases the number of germ cells in older larvae (Inoue et al., 1992; Juliano and Wessel, 2009). In order to test this hypothesis, more experimental analyses are required, including posterior enterocoel explants or photoablation studies in species from several clades, to test if this structure is the origin of germ cells in these other echinoderm clades. In addition, molecular analyses need to be performed to determine if conserved germ-line determinants start to accumulate in the left posterior enterocoel in the early larvae stage of development in these other echinoderm clades. Future studies should include lineage-tracing analyses where cells from each coelom are labeled early in development to determine if they take on germ-cell characteristics later in development and to test which cells of each coelom the PGCs originate from.

It is important to note that the ancestral mode of germ-line specification in all metazoans is hypothesized to be inductive (cell interactions) instead of inherited (acquisition of maternal determinants) (Extavour and Akam, 2003). If the posterior enterocoels represent the ancestral mode of germ-line specification within the echinoderm clade, it is possible to postulate that the sea urchin sMic lineage represents a transition from the specification of the germ line in later larval development to an earlier embryonic time point, the 32-cell stage. The sMics are cell-autonomous in their ability to express *nanos* in culture (Yajima and Wessel, 2012). Sea urchins have many derived features in development, including micromeres, hyaline layers, and endoskeletons in larvae. As such, the Echinoidea lineage within the echinoderm phylum may represent a transition from an inductive mechanism for germ-line specification to a more inherited-like mechanism. If this is the case, we can compare germ-line specification mechanisms between echinoderm clades to determine what is required for this shift from one mechanism to another.

Johnson et al. (2011) hypothesized that a benefit of the inherited mode of PGC specification is the relinquishing of restrictions on signaling mechanisms (formerly required for germ-cell specification) so that the embryo can develop more derived larval characters. Coincidentally with the birth of the sMic lineage in sea urchins, a variety of factors in sea stars localize instead to the larval skeleton in sea urchins (Hinman et al., 2003; Koga et al., 2010). Did the shift allow for diversification of gene regulatory networks and transcription factors at earlier time points in the sea urchin

larvae, resulting in more diversity in larval form and formation of a larval skeleton? And do the holothuroids, animals without a sMic lineage but with a less morphologically complex larval skeleton, represent another point on the continuum between sea stars and sea urchin mechanisms of PGC specification? Future experiments that test the timing of PGC specification in sea cucumbers are necessary—for example, an accumulation of germ-line markers at a developmental time between those of the sMic in sea urchins and the posterior enterocoels in sea stars would support this hypothesis.

The adult rudiment forms in the anterior left side of sea urchins but in the entire posterior of sea star larvae. Are L/R signaling molecules restricted to PGC specification in sea stars? In order to address these questions, the inductive signals required for PGC specification in sea stars need to be elucidated. Only then we can analyze the function of the same signaling molecules in sea urchins, testing if they have become unrestricted and contribute to diversification of body structures in early development. Also, we can test if inductive signals required for PGC specification in sea stars are conserved in a broader sense with other metazoans that use inductive mechanisms for PGC specification, such as in the mouse.

Glossary:

Adult rudiment: The larval structure that contains the adult primordial tissues. It will develop throughout the larval stage and eventually evert at metamorphosis to form the juvenile sea urchin.

Large micromeres (LMics): Sister cells to the small micromeres that give rise solely to the larval skeleton.

Micromeres: The parent cells of the large and small micromeres, formed by an asymmetric cell division at the 4th cell cycle of the sea urchin embryo.

Primordial germ cells (PGCs): The lineage of an embryo/larva that is committed to producing the germ line in animals. These cells migrate to the gonad and multiply to eventually form the eggs or sperm in the adult.

Small micromeres (sMics): Cells formed at the 5th cell division of embryogenesis by an asymmetric cell division that contribute to the germ line of the adult sea urchin.

ACKNOWLEDGMENTS

We gratefully acknowledge grants from the NIH HD028152, NSF IOS-1120972, the PEW Foundation, and CONACyT to make this work possible. We thank all members of PRIMO for feedback on this research and for a rich work environment.

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Appendix E: Origin and development of the germ line in sea stars

Wessel G, **Fresques T**, Kiyomoto M, Yajima M, and Zazueta V. (2014) Origin and development of the germ line in sea stars. *Genesis*. 52(5):367-377.

I contributed to the research involved in Figure 6.

REVIEW

Origin and Development of the Germ Line in Sea Stars

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Received 2 January 2014; Accepted 14 March 2014

Summary: This review summarizes and integrates our current understanding of how sea stars make gametes. Although little is known of the mechanism of germ line formation in these animals, recent results point to specific cells and to cohorts of molecules in the embryos and larvae that may lay the ground work for future research efforts. A coelomic outpocketing forms in the posterior of the gut in larvae, referred to as the posterior enterocoel (PE), that when removed, significantly reduces the number of germ cell later in larval growth. This same PE structure also selectively accumulates several germ-line associated factors—*vasa*, *nanos*, *piwi*—and excludes factors involved in somatic cell fate. Since its formation is relatively late in development, these germ cells may form by inductive mechanisms. When integrated into the morphological observations of germ cells and gonad development in larvae, juveniles, and adults, the field of germ line determination appears to have a good model system to study inductive germ line determination to complement the recent work on the molecular mechanisms in mice. We hope this review will also guide investigators interested in germ line determination and regulation of the germ line into how these animals can help in this research field. The review is not intended to be comprehensive—sea star reproduction has been studied for over 100 years and many reviews are comprehensive in their coverage of, for example, seasonal growth of the gonads in response to light, nutrient, and temperature. Rather the intent of this review is to help the reader focus on new experimental results attached to the historical underpinnings of how the germ cell functions in sea stars with particular emphasis to clarify the important areas of priority for future research. *genesis* 52:367–377, 2014. © 2014 Wiley Periodicals, Inc.

Key words: sea star; starfish; posterior enterocoel; germ line; primordial germ cell

INTRODUCTION

Sea stars are some of the more recognizable animals for the general public. From Patrick on “*Spongebob Square Pants*”[†], to decorative icons on dishware and shower curtains, sea stars hold a special place in the world. The generic body plan linked to this animal, that is, of a central body disc with five symmetric arms emanating from the disk is in fact a prevalent body plan for this popular order of Echinoderms. But morphology in the order is greatly diverse; some adults have upwards of dozens of arms (e.g., the sun-star, *Heliaster belianthus*), arms that are fused (e.g., the batstar, *Pateria miniata*), arms that are barbed (e.g., the crown of thorns sea star, of the *Acanthaster* genus), and arms of a myriad of colors (the reader is referred to the excellent website of echinoderms Echinoblog [www.echinoblog.com] and the World Asteroidea database (http://www.marinespecies.org/asteroidea/) for a view of this diversity).

Sea stars[‡] also have a special place in the ecosystem. They are common predators in a variety of marine

[†]http://spongebob.nick.com/[‡]The terms sea stars and starfish refer to the same group of organisms. Since starfish are not actual fish with a backbone, the community is more often referring to them as sea stars, a nomenclature parallel to other some of the other organisms in this phylum, e.g. sea urchins, sea cucumbers, sea lilies, and yet still distinguishable from others, e.g. brittle stars.

Abbreviations: dpf, days post-fertilization; GFP, green fluorescent protein; GSC, germ-line stem cells; hpf, hours post-fertilization; iPS, induced pluripotent stem; MASO, morpholino antisense oligonucleotides; PE, posterior enterocoel; PGC, primordial germ cells; qPCR, quantitative Polymerase chain reaction; sMics, small micromeres; TEM, transmission electron microscopy.

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This work was supported by grants from the NSF and the NIH.

Published online 19 March 2014 in

Wiley Online Library (wileyonlinelibrary.com).

DOI: 10.1002/dvg.22772



FIG. 1. Adult sea star (*Asterina miniata*) shedding several million eggs. These cells are spawned having begun the process of meiosis and are fertilizable for the next few hours. The gonads line the sides of each arm and the gonopores (site of gamete release, asterisk) from each gonad is at the base of the body. Thus, the gonopores from adjacent arms are close to each other but shed oocytes from distinct gonads (double arrow pointing to the sites of the two adjacent gonopores). From (Wessel *et al.*, 2010).

habitats. They usually occupy the top level of the food chain in the benthic ecosystem and are in some cases keystone species (e.g., *Pisaster ochraceus*) defined as consumers having a large effect on communities relative to their abundance (e.g., Menge and Sanford, 2013). Sea stars have several characteristics that underlie this importance in the ecosystem. They are relatively large compared to most prey of the ecosystem, they have indeterminate growth and are thereby “rewarded” for high prey consumption, their digestive systems can handle a variety of foods, they have relatively rapid reproduction and development, high fecundity, and their mode of locomotion enables their stability in a variety of physical challenges in the environment of currents, intertidal motions, and wave actions (Menge and Sanford, 2013). Many animals are prevalent in varied habitats but seldom are they as successful as sea stars.

Echinoderms are an ancient taxon, first appearing in the fossil record ~550 Mya (Erwin *et al.*, 2011; Greenstein, 1993; Kroh and Smith, 2010; Kroh, 2007; Porter, 1977; Smith *et al.*, 2013). The ancient echinoderms appeared to diversify rapidly, with four distinct body plans appearing within the first 10–15 million years following their first appearance (Smith *et al.*, 2013). This rapid diversification was then amplified over the subsequent 500 million years, making it difficult to accurately determine the phylogenetic relationship between extant echinoderms, especially within sea stars.

In the midst of this wonderful diversity are many common characters for which sea stars are used for basic

research in cellular, molecular, and developmental biology. The broadcast spawning of large numbers of oocytes is valuable for easy laboratory culturing of single to millions of synchronous embryos (Fig. 1). Many of the species yield large eggs (>200 microns) of optical clarity enabling micromanipulations/microinjections and in vivo analysis (see e.g., Foltz *et al.*, 2004; Jaffe and Terasaki, 2004, for more details on these procedures). Uniquely though in the sea stars are the experimental controls possible over oocyte maturation. Adult females store gametes as germinal vesicle staged oocytes (prophase of meiosis I) which can be stimulated to re-initiate meiosis by the natural inducer, 1-methyl-adenine (commercially available). This means that oocytes can be studied effectively for mechanisms of meiotic progression, or the oocytes can be preloaded with biological compounds to examine developmental mechanisms in the resulting embryo. More recently it was shown that the abundance of specific proteins in the oocyte can be reduced (knocked down) by conventional morpholino antisense oligonucleotides (MASO) injection into the oocyte followed by culturing for 12–48 h (Oulhen *et al.*, 2014). In this way, sea star oocytes can be studied more effectively than even sea urchins for meiosis or early embryogenesis, and coupled with the ability to reactivate meiotic progression with high synchrony, enable a special research tool into basic biological features in the oocyte-to-embryo transition. Another feature that is special in these echinoderms is regeneration; the embryos, larvae, and adults are remarkably capable of replacing missing pieces and developing compensatory cellular arrangements and fates into a normal structure.

SEA STARS AS AN IMPORTANT MODEL OF REGENERATION

Sea stars are renowned for their strong regenerative abilities. Most, if not all sea stars are able to replace body parts following their injury or removal (Fig. 2). Many sea stars are capable of autonomously casting off or detaching an arm following stress or injury to the limb and then growing the structure back with correct proportionality. In some species, such as the sea star from the *Linckia* genus, the self-detached (autotomized) arms may even regenerate to form a completely new adult (Cuenot, 1948). Most species, however, require an intact central disk (body) for their complete regeneration. Then, they are capable of regenerating most of the normal functional appendage including its spines, tube feet, and pedicellariae as well as its internal organs such as the gut, nervous system, and even gonad, complete with germ cells (Emson and Wilkie, 1980; Hyman, 1955).

Sea star larvae also have regenerative properties and were the first larvae reported in deuterostomes to completely regenerate all missing body structures, tissues, and organs at the occasion of amputation (Vickery and

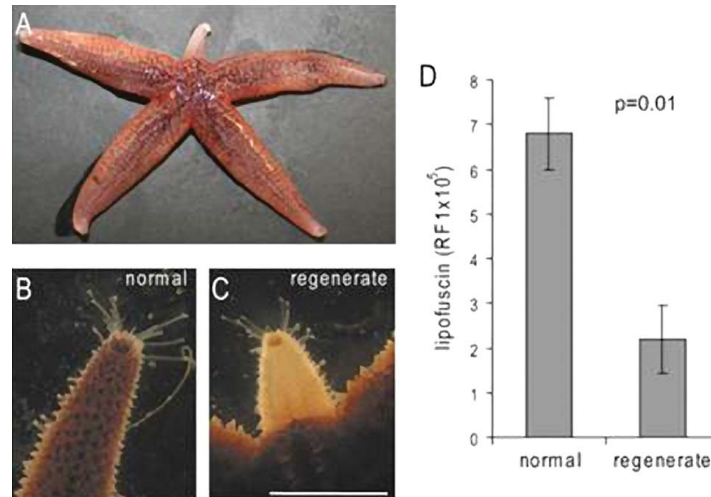


FIG. 2. *Asterias rubens* with an arm regenerating from the base (a–c). The early regenerate (c) is less pigmented than an intact arm of the same animal (b). Fluorometrical analysis of extracted lipofuscin content shows lower levels of lipofuscin in regenerating arms than in the tips of corresponding intact arms of the same animals (d). Lipofuscin is expressed as mean $\pm$ s.e.m. Relative fluorescence intensities (RFI). R, regenerate; N, normal/intact arm. Light micrographs and data were obtained from animals with approximately 1 cm regenerates. Data obtained from animals with 1.5–2 cm regenerates. Scale bar: 1 cm (b, c). From (Hemroth *et al.*, 2010).

McClintock, 1998) (Fig. 3). This remarkable regeneration process appears to be also supported by an intrinsic mechanism, and the sea star larvae were also first reported for its cloning capability (asexual budding) in natural conditions (Jaekle, 1994; Knott *et al.*, 2003; Vickery and McClintock, 2000). Followed by those findings in sea star larvae, other echinoderm larvae were identified to have remarkable abilities of asexual reproduction through cloning (Evaes and Palmer, 2003). Despite the contributions of starfish larvae initially in regeneration research, knowledge of the cellular and molecular mechanisms of regeneration/cloning is very limited. Several genes have been identified that are differentially expressed during early larval sea star regeneration (Vickery *et al.*, 2001), yet functional analysis and the contributions of each gene during the regeneration process has not been tested. Further, it is not known whether regenerated larvae recover any germ cell function.

ENVIRONMENTAL IMPACT ON REPRODUCTION

Development of gametes (gametogenesis) in sea stars is highly dependent on the environmental conditions of the animal. These conditions include temperature, light, food availability, age of the animal, salinity of the media, and parasite load. While many of these conditions have been replicated and tested under laboratory

conditions, overall the influences of each as they contribute to gametogenesis and gonad growth are only beginning to be resolved. The consensus model is that these conditions likely coordinate the adult physiology and behavior through hormonal regulation. Some of these hormones and mechanisms have been identified (see e.g., Mercier and Hamel, 2013). It is clear that the genetic makeup of the animal also has a significant impact on this process. Different species of sea stars may exhibit markedly different reproductive cycles even when living in the same environment, and different populations of the same species may also exhibit different reproductive cyclicities within the same environment. Sea stars can be shifted experimentally in their gonadal cyclicity under laboratory conditions by altering the light, temperature, and nutritional conditions (Chia and Walker, 1991). This approach has been successfully applied in adults to understand and to prioritize the environmental conditions that impact the animal's reproduction. Not surprisingly, the hierarchy of environmental conditions is different for various species and for different times of the year. From these studies it is also obvious that both physiology and behavior are influenced by the environmental conditions. Of particular interest is the fluctuation of steroidal hormones during the seasonality changes (Hines *et al.*, 1992; Lawrence, 2013). Several reviews on environmental impact of reproduction in asteroids are available that very

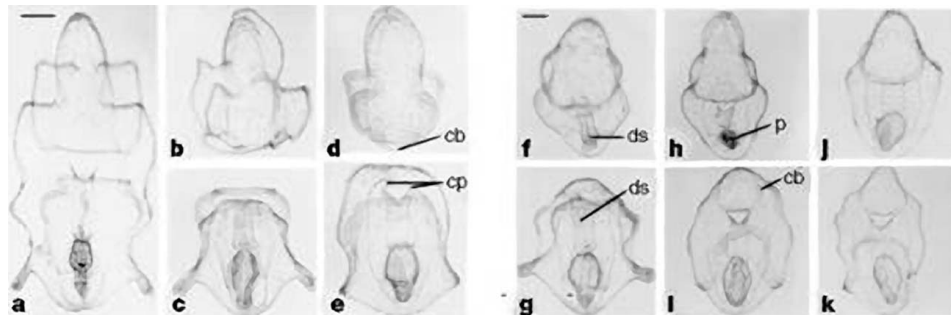


FIG. 3. Bipinnaria larvae of *P. ochraceus* at various stages of regeneration after surgical bisection. The anterior larva is shown in the top row, and the posterior larva in the bottom row. (a) Bipinnaria before surgical treatment. (b, c) Day 1. Anterior (b) and posterior (c) larvae after surgical division. The gut of the posterior larva is intact enough for feeding. (d) Day 3. (e) Day 4. Elongation along the larval axis has taken place. (f, g), Day 7. The anterior larva (f) has regenerated a functional digestive system. (h) Day 9. (i) Day 10. Complete regeneration of ciliary bands has occurred in both anterior and posterior larvae. (j, k), Day 12. Both larvae have regenerated and are similar to control larvae. cb, ciliary band; cp, coelomic pouch; ds, digestive system; p, phytoplankton. a–e, Scale bar (in a), 200 μ m. f–k, Scale bar (in f), 200 μ m. From (Vickery and McClintock, 1998).

effectively document this biological–environmental relationship [reviewed in e.g. (Chia and Walker, 1991; Mercier and Hamel, 2013)]. The results of these studies also suggest the important role of germ line stem cells within the adult. As a broadcast spawner, many sea stars are capable of releasing millions of oocytes (Fig. 1), and countless sperm, during their reproductive life. This robust fecund capability implies a very large and constantly renewing source of germ line stem cells, although such cells have not been identified. These (anticipated) cells are likely a target of the hormonal fluctuations impacted by the environment and, were they identified and isolated, would be an excellent to study regulatory mechanisms of germ line stem cells and the hormonal influences on their proliferation.

ORIGIN AND DEVELOPMENT OF THE GERM LINE IN ADULTS

The germ cells appear to accumulate in adult sea stars within the aboral aspect of the haemal system (Fig. 4). Prior to development of this structure, and prior to the development of the gonad, the presumptive germ cells are present within the haemal tissue, in a structure called the genital bud (Boulard and Jangoux, 1990), which is located in the most aboral part in the haemal system (in *Pateria pectinifera*, Fig. 4). The genital bud is a candidate of the source of germ-line stem cells in the adult because they appear in this structure throughout the year, even in post-spawning stages. Since complete regression of the gonad occurs after spawning in many species, annual neo-formation of the gonad must occur from cells within the arms of the adult prior to gametogenesis (Boulard and Jangoux, 1990). During regression and then subsequent proliferation, the

gonads remain connected to the haemal sinuses, which are not lost after spawning (in *Asterias pectinifera*, now *Pateria pectinifera*, (O'Loughlin and Waters, 2004)). This connection is a viable source of germ-line stem cells for the next breeding season, in addition to any remaining gonia within the spent gonads (Inoue and Shirai, 1991). Thus, the replenishment of stem cells for each round of gametogenesis likely includes germ cell migration into the regenerating gonads from the stem of the haemal system, and proliferation of remaining gonia within the gonadal remnant.

Inoue and Shirai examined the appearance of germ cells during ontogeny of the sea star, *P. pectinifera*, by electron microscopic histological approaches (Inoue and Shirai, 1991). Germ-line stem cells (gonia) were first identified in gonads following spawning: the gonia are larger than somatic cells, have large nuclei with electron-lucent nucleoplasm, and contain mitochondrial aggregates associated with electron-dense bodies (much like nuage). Similar cells were detected in young sea stars before gonad formation (6 mm) or even in the final stage of larval development (4 weeks) and in metamorphosed juveniles (10 weeks). They could detect germ cells as a cellular cluster in the coelomic epithelium near the hydroporic canal (see Fig. 4). Quantitation of the germ cells in each region indicated that the migratory path of these putative PGCs is through the haemal system to the gonads during normal development (hence also a model for populating regenerating/cycling gonads with gonia). The central aspect of the haemal system is an oral and aboral ring connecting the axial sinus running along the hydroporic canal. Ten genital branches (two for each arm) extend from the aboral ring to the gonads. Germ cells migrate in a cluster from the aboral haemal sinus near the hydroporic canal (a

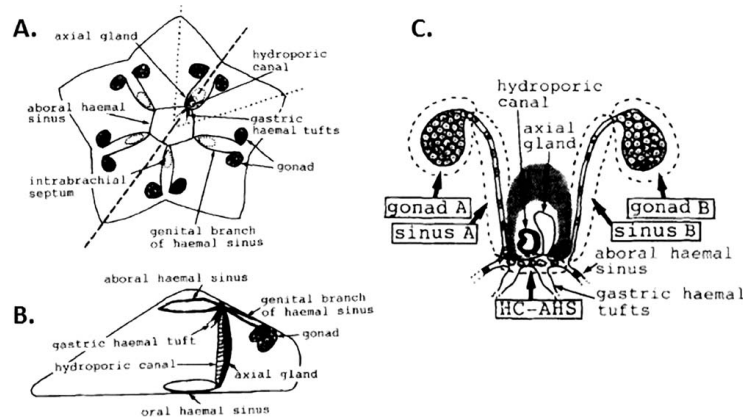


FIG. 4. Schematic diagrams of the relationships between the gonad and various haemal sinuses in *A. pectinifera*. (a) Oral view. The gonads are connected to each other by genital branches of the aboral haemal sinus. Gastric haemal tufts connect with pyloric caeca. The broken line shows the position of the semi-cross section for a lateral view. The dotted line shows the regions shown in part (c). (b) Lateral view. The aboral haemal sinus connects with the oral haemal sinus through the axial gland. (c) Schematic diagram of the genital region. Aboral view. The arrangement of the areas in dotted lines show many cells left out of this diagram for clarity. HC-AHS; aboral haemal sinus near the hydroporic canal, sinus A and B, genital branches of haemol sinus. Shaded area surrounding HC and axial gland indicates the intrabrachial septum. From (Inoue and Shirai, 1991).

part of aboral haemal ring), through the genital branches, and to the gonads. In other starfish (e.g., *Asterina gibbosa*), gonads were also observed in the vicinity of the hydroporic canal a short time before metamorphosis (Delavault, 1966). Thus, the germ-line stem cells of the organism appear to undergo seasonal migration from a distant site into the growing gonad. The genital bud appears to be formed from the accumulation of these cells in the juvenile and the germ cell cluster observed in the late larvae of *P. pectinifera* is thought to correspond to this genital bud. In *Leptasterias hexactis*, which has large eggs (a mean of diameter 0.8 mm) and exhibits modified development through a non-feeding larva compared to indirect developing species, germ cells were observed first above the dorsal horn of the left posterior coelom near the aboral portion of the stone canal before metamorphosis, then they subsequently invade the haemal sinus (Chia, 1968). It is still not clear what the origin is of these germ cells but the classical morphological appearance of germ cells in late larvae is consistent in asteroids.

ORIGIN OF THE GERM LINE DURING EMBRYOGENESIS—THE POSTERIOR ENTEROCOEL

The origin of the germ line in many sea stars appears to be within the posterior coelom (Inoue, 1992). In sea star embryos, coelomic pouch formation begins during late gastrulation. Chia and Walker (1991) distill the diverse developmental patterns used by asteroids to

develop their various coeloms into three general systems (Fig. 5). In Type 1 (our terminology to facilitate this discussion) two coelomic pouches arise from the anterior of the archenteron and grow posteriorly, one on the left side and one on the right side of the gut (Fig. 5a). The left coelom grows much faster and opens to the outside via a hydroporic canal, the hydrocoel, which gives rise to the water-vascular system during larva development. This is the most familiar type of coelom where popular research species such as *Luidia*, *Astropecten*, *Asterias*, and *Pisaster* are found. In Type 2 coelom development, coeloms arise from the anterior of the archenteron and grow posteriorly on both sides as a U-shaped coelom. Later in development, two septa form on each side of the "U" to form the anterior coelom and two lateral posterior coeloms (left and right). The left lateral coelom grows much faster and a part of it will become the hydrocoel (Fig. 5b). Species as *Parvulastra exigua* (formerly *Patiriella exigua*), *A. gibbosa*, and *L. hexactis* belong to this group. In the Type 3 system, the coeloms arise from both the anterior and posterior ends of the archenteron (Chia and Walker, 1991). This mode of coelomogenesis is used in the development of *Patiria miniata*, *Patiriella regularis*, *Pateria pectinifera*, *Pteraster tessellatus*, and *A. glacialis* (*Marthasterias glacialis*), some of the more intensively studied species for molecular mechanisms of germ-line determination. In these animals, the left posterior coelomic pouch (posterior enterocoel (PE)) arises first from the dorsal side of the archenteron and then shifts to the left side and buds off the left

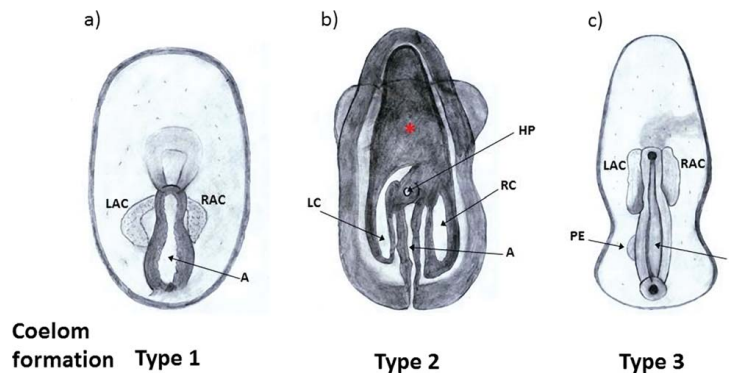


FIG. 5. Different patterns for coelomic development in Asteroids. Schematic representation of the three different types of coelomic pouch formation. Dorsal view of larvae. (a) 4-Day bipinnaria larva from *Pisaster ochraceus* shows two lateral bulges derived from the anterior part of the archenteron which become the right and left enterocoels. (b) In 4-Day brachiolaria larva of *P. exigua*, coeloms are conformed by three parts, an anterior coelom which extends from the archenteron, and two septa formed from the anterior coelom which become the left lateral coelom and the right lateral coelom. The anterior coelom and the archenteron are formed by a continuous cavity which can be seen in a ventral view of the larva. The left lateral coelom is larger than the right lateral coelom at the level of their junction with the archenteron. The hydropore is close to where the left lateral coelom joins the archenteron cavity. A red asterisk represents the localization of the anterior coelom in a ventral view of the larva (Morris *et al.*, 2009). (c) In 2.5-Day bipinnaria larva from *Patiriella regularis*, right and left enterocoels form from the expanded tip of the archenteron and a small group of cells forms the posterior enterocoel (Byrne and Barker, 1991). A: archenteron; LAC: left anterior enterocoel; RAC: right anterior enterocoel; LC: left lateral coelom; RC: right lateral coelom; HP: Hydropore; PE: Posterior enterocoel.

endodermal epithelium (Fig. 5c), in a manner independent and physically separated from the anterior coeloms (Bury, 1889; David *et al.*, 1994; Gemmill, 1914; Heath, 1917; Hinman *et al.*, 2003; Kuraishi and Osanai, 1992; McEdward, 1995; Newman, 1925). Observations made by Newman described PE formation as a thick-walled evagination of the wall of the archenteron, which did not expand into a thin-walled vesicle, but merely constricted off a small solid ball of cells that later expanded and formed a lumen (Newman, 1925). Deviations from this mode of coelomogenesis were noted in earlier descriptions of other Asteroidea larval development. For example, in 1914 Gemmill noticed that the PE in the sea star *Asterias rubens* also arose periodically from the right side of the gut and also medially (Gemmill, 1914). These observations were in contrast to observations in *Porania pulvillus*, where the PE arose dorsally on both sides of the median line, but sometimes in the median line, or on one side only, occurring rather more often on the right than on the left side (Gemmill, 1914). Lineage analysis of each of the blastomeres of an eight cell embryo documented the formation of the PE from the mid-dorsal surface of the archenteron in *P. pectinifera*. These cells originated from two different eight cell blastomeres and shifted in concert to the left side of the archenteron (Kuraishi and Osanai, 1992). The expression pattern resulting from an endoderm (or archenteron) marker shows that the PE never expressed this marker suggesting that the

mechanism of commitment of the PE is different than that of the endoderm (Kuraishi and Osanai, 1994). On the other hand, the direct action of toxic contaminant agents affected coelomic formation in *P. miniata* (Child, 1941). The effect of temporary exposure to moderate concentration of these chemical agents (such as lithium chloride, Nile blue sulfate, acidified sea water, hypotonic sea water) was development of several posterior coeloms (from two to six), at the same level as the original PE and equally spaced. A longer exposure of these agents inhibited posterior coelom development completely (Child, 1941). These results suggest that formation of the PE is dependent on inductive cellular interactions and suggests that distinct from the sea urchin, the germ line may originate from cellular communication during gastrulation.

EVIDENCE THAT THE PE IS A SOURCE OF GERM CELLS IN THE SEA STAR

Several complementary lines of evidence suggest that the PE contributes to the germ line of the sea star. These include: (1) PE removal experiments that result in larvae with significantly fewer germ cells (Inoue, 1992), (2) Vasa immunolabeling experiments show the PE is the first restricted site of Vasa protein localization (Juliano and Wessel, 2009), (3) conserved germ line factors accumulate selectively in the PE, for example nanos and piwi in addition to vasa (Fig. 6) (Fresques

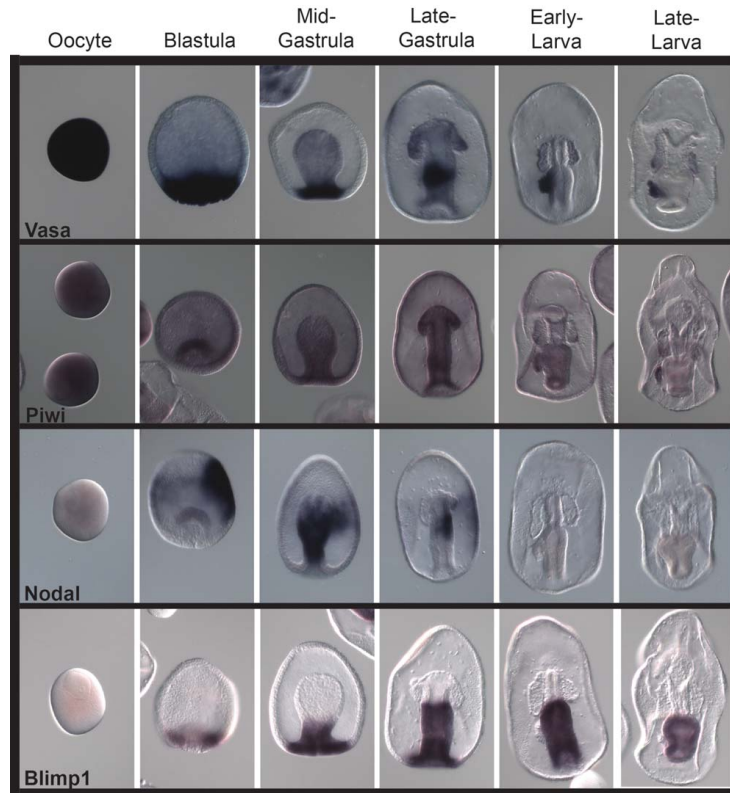


FIG. 6. Genes associated with germ-line determination. *Vasa* and *piwi* are factors often associated with germ cell development, and these markers become enriched in the PE in early larval stages. *Nodal* is a TGF- β signaling model and is present in the right side of the embryo, and gut, opposite of the PE. We hypothesize that *nodal* signaling may direct *vasa* and *piwi* localization, and perhaps the asymmetry of the PE. *Blimp1* is a transcription factor important for somatic endomesoderm formation and is markedly enriched in the gut during gastrulation. Importantly, it is excluded from the PE when it is formed, at a time when *vasa* and *piwi* are enriched in the PE, suggesting that clearance of somatic determinants is important in PE formation. Oocytes of this sea star (*P. miniata*) are ~200 microns in diameter. From (Fresques *et al.*, in press).

et al., 2014), and (4) the PE exhibits rapid depletion of mRNAs encoding factors involved in somatic cell fates, for example *Blimp1* (Fresques *et al.*, 2014). None of this evidence on its own is sufficient to conclude that the PE is the germ line, or that only primordial germ cells are in the PE, but in concert, the evidence is sufficiently compelling to conclude that the PE contributes to the germ line.

Critical analysis of each aspect of this evidence helps identify shortcomings in this conclusion. In the work by Inoue *et al.* (1992), the investigators removed cells of the PE from the larvae (at 48 h) and analyzed the resultant larvae several weeks later. Previous evidence (see above) suggested that the germ cells are present in

the coelomic epithelium near the hydroporic canal and contained characteristic features of germ cells in the transmission electron microscope: large nuclei, mitochondria aggregates, distinctive chromatin condensation along the nuclear envelope, less cytoplasmic organelles than nearby somatic cells. Older morphological studies that identified PGCs with classical germ cell characteristics are consistent with this recent data because the origin of the germ line was described to be in the "dorsal horn of the left somatocoel of the bipinnaria larvae," which is interpreted to be derived from cells in the PE (Chia and Walker, 1991; Gemmill, 1914). In larvae resulting from PE removal, the number of these phenotypic cells in the hydroporic region was

significantly reduced, and retained only about 35–40% the value of the control larvae. While the loss did not reach 100% of the cells, several qualifiers should be considered: (1) The assay for detection was microscopic (TEM) analysis of cellular morphology. This assay is highly detailed, but less quantitative than current approaches of detecting germ line markers by immunolabeling, by in situ RNA hybridization, by GFP-recordings, or by qPCR analysis. Further, the tissue sections needed for TEM may have missed displaced cells as a result of the surgical operation for PE removal. (2) The effectiveness of PE removal—PGCs have strong proliferative abilities especially later in development and were the investigators to have missed removing a single PE cell, it may have had the ability to proliferate and even compensate the lack of sibling cells. (3) The PE is not the sole source of PGCs and may continually accumulate over larval development. Mice have (at least) two phases of primordial germ cell determination and although much closer in developmental time, it does suggest that PGC may be fated more broadly than at a distinct point in time (Hayashi *et al.*, 2007). (4) Compensatory PGCs. The sea urchin has an ability to make new germ cells following removal of the precursor cells of the PGC (Ransick *et al.*, 1996; Voronina *et al.*, 2008; Yajima and Wessel, 2011). Perhaps trans-fating of multipotent cells within the larvae to a PGC is one way that the larvae maximizes its germ line potential, and/or retains multipotency beyond the initial period of specification. This explanation is particularly relevant in sea stars, an animal with remarkable regenerative capacities (see Section “Sea stars as an important model of regeneration”).

The PE is the site of enriched germ line markers. This too is suggestive of the germ line originating in the PE. In the past decade though, many non-model organisms have been shown to have germ line markers in many non-germ line cells. Moreover, these markers are often in multipotent cells than can (or do) give rise to the germ line. Therefore, although the results support the contention that the PE is the site of germ cells, the PE may instead be a highly multipotent cell that has the developmental capabilities to give rise to many cell types. A fine lineage analysis is important to accomplish on the PE cells, all ~25 of them, in order to understand what diversity of fates these cells may contribute. The point of understanding the fates of all the cells (not necessarily each individual cell) is because recent evidence suggests the cells in the PE are heterogeneous, at least by SoxE accumulation (Fresques *et al.*, in press). Thus, the fate(s) of the PE in general should be tested.

Finally, the rapid turnover of mRNAs of genes involved in somatic differentiation in the PE is consistent with the PE fate of germ cells. Blimp is an important germ line factor in mice (Ancelin *et al.*, 2006) but is a somatic factor in sea urchins and sea stars (Hinman and

Davidson, 2003; Livi and Davidson, 2006) for endomesodermal cell fates, not germ line fates. As recently shown in mice, an organism relying strongly on inductive mechanisms of germ line determination, the cells that become the germ line first exclude mRNAs and gene activity responsible for somatic cell fates. Were the cells to not receive the critical germ line signals, these cells in the mouse instead would develop into mesoderm. Much of this transition in the future germ cell is in changes in the epigenetic landscape, making areas of the genome encoding somatic cell fates heterochromatic and shifting the balance to inductive-based gene expression important for these germ cells. Currently, we do not know how Blimp is excluded from the PE so rapidly, but it is hypothesized that it includes a change in the epigenetics of the PE cells, and a selective turnover of its mRNA (Fresques *et al.*, 2014). Blimp is not the only somatic factor excluded from the PE but it is a robust marker making this analysis more tractable.

Most animals reveal one or another of two broad and overlapping mechanisms of germ line determination, inductive or inherited (also referred to as *acquired*) (Ewen-Campen *et al.*, 2010; Extavour and Akam, 2003; Juliano and Wessel, 2010; Seervai and Wessel, 2013). Some animal embryos acquire partitioned information stored in the egg that directs the fate of the cells along the path of the germ line, the inherited mechanism. The fruit fly, *Drosophila*, and the roundworm *C. elegans* are good examples of this mechanism in play. Although only a few molecular candidates are known to be important in the acquired material, the structures within which they reside are apparent. These are referred to as nuage, perinuclear granules, or germ plasm, depending on the animal. The mechanism in the opposite extreme is where cells signal amongst themselves and germ cells are somehow diverted from somatic fate to become the germ line. This is usually referred to as inductive germ line determination, and the sea star appears to utilize this mechanism in the cells of the PE. We hypothesize that the PE forms by inductive mechanisms based on the timing of PE formation—it forms relatively late in development, during late gastrulation. It would be hard to imagine how maternal factors could be so tightly partitioned during this prolonged development (from one to thousands of cells at the time of PE formation) to be a consideration in an acquired germ line mechanism. This conclusion of inductive germ line behavior would be important experimentally since currently very few animals are amenable to study that use inductive mechanisms, the mouse being the most prominent. Yet in mice, the embryo is embedded in the uterus during this important time of PGC generation, making embryonic, cellular, and molecular analysis more challenging. The genetic applications in mice, along with stem cell

analysis in vitro (e.g., iPS, GSC) do offer remarkable benefits, each of which may be complemented by another organism tractable for more direct cellular, molecular, and visual analysis. The sea star may be important for more direct visualization, biochemical analysis of isolated germ cells, and molecular manipulations in germ cells whose fate is determined largely by inductive mechanisms.

MOLECULAR MECHANISM OF PE FORMATION IN THE SEA STAR AND EVOLUTIONARY TRANSITIONS FROM THE SEA URCHIN

In *P. pectinifera* and *P. miniata*, the two most intensively used species for studies in PE formation, the PE first arises as a bump on the dorsal gut which then appears to transition to the left side of the gut (Kuraishi and Osanai, 1992), (Fresques “unpublished observations”). The cellular dynamics of PE formation, however, remain poorly understood. Do these cells undergo an Epithelial to Mesenchymal Transition (EMT) to break away from the dorsal side of the gut and undergo active migration as a cohort to the left side of the gut? Or do they first outpocket as a sheet that migrates leftward and pinches off from the dorsal gut? Detailed microscopic and lineage studies are necessary to understand the molecular mechanisms of PE formation and morphogenesis and a live marker of PE cells would be valuable to answer these questions using live-fluorescence microscopy.

Although the mechanism of the transition of the PE is poorly understood, conserved molecules involved in Left/Right asymmetry may be involved. For example, at the same time that Vasa mRNA is restricted to the left side of the gut, Nodal mRNA is restricted to the right side of the gut (Fresques *et al.*, 2014). This is significant because Nodal signaling is required for the correct specification of Left/Right asymmetries in many animals (e.g., Vandenberg and Levin, 2013). How Nodal signaling on the right side of the gut inhibits the accumulation of Vasa positive cells in the right remains unclear. Nodal signaling may cause the PE cells in the right (1) to migrate to the left, (2) to lose Vasa mRNA, or (3) to undergo programmed cell death. These hypotheses may not be mutually exclusive as is the case with the impact of L/R asymmetry on the accumulation of putative PGCs in sea urchins.

In sea urchins, Nodal signaling appears to influence the sMic's (putative PGCs), as to whether they accumulate in either the left or right coelomic pouch. Sea urchins do not have posterior coelomic pouches and only have two anterior coelomic pouches. During gastrulation, the sMics translocate from the vegetal pole, to the tip of the archenteron, and finally accumulate in the coelomic pouches (Luo and Su, 2012; Pehrson and Cohen, 1986; Yajima and Wessel, 2012). Normally, they

exhibit a Left/Right asymmetry in regards to how many germ cells first migrate to each coelomic pouch and normally an average of five cells migrate to the left coelomic pouch and three cells migrate to the right coelomic pouch (Campanale and Hamdoun, 2012; Pehrson and Cohen, 1986; Tanaka and Dan, 1991). In addition, by the pluteus stage, all of the sMics that have translocated to the right coelomic pouch undergo apoptosis while many (all?) of the sMics that have ended up in the left coelomic pouch survive (Campanale and Hamdoun, 2012; Luo and Su, 2012). This Left/Right asymmetry is dependent on Nodal signaling. Nodal is active in the right coelomic pouch of the sea urchin embryo and is required for the apoptosis of sMic cells in the right (Luo and Su, 2012).

Were Nodal to have similar effects in both sea urchins and sea stars we hypothesize that Nodal first inhibits germ cells from accumulating in the dorsal-right by influencing their directional translocation and then clears any remaining right-sided germ cells via programmed cell death. However, since Vasa accumulates in an entire structure (the PE) in the sea star and only 8 cells (the sMics) in the sea urchin, it is likely that Nodal has an extra role in sea stars in regards to posterior coelomic pouch (PE) formation and translocation that was lost in sea urchins with the loss of the posterior coelomic pouches.

It is important to note that disruption of Left/Right asymmetry signals in sea urchins does not affect the accumulation of Nanos, Vasa, or Piwi in sMics (Luo and Su, 2012), a testament that these cells have already been committed to a germ lineage. It will be important to test whether Left/Right asymmetry signaling molecules have a similar effect in sea stars. If these signals are involved in acquisition or loss of the germ cell fate and not just migration then we can use this system to understand how signals actively promote or inhibit the germ cell specification network.

CONCLUSIONS AND FUTURE DIRECTIONS

The utility of studies on reproduction in the sea star is broad. From regeneration of their gonads and germ cells, to the environmental impact on the germ line growth, to its formation in larvae and replenishment in cycling adults, to the more recent molecular revelations in the PE formation, the sea star has many unique and complementary biological features that make them valuable for studies in reproduction. The diversity of these animals is also a distinct asset—especially in the reproductive mechanisms in which a comparative approach has many revelations for extracting consensus mechanisms that can be used for prioritization of more detailed studies. With the rapid expansion of genomic resources in this group of animals, coupled with the advanced imaging capabilities for embryos with optical

clarity and the new methods in transcriptional and post-transcriptional gene disruption, sea stars throughout the world have a great deal of excitement for future work in development and reproduction.

ACKNOWLEDGEMENTS

We are grateful to the anonymous Reviewers for their detailed feedback to promote the improvement of this manuscript, and to Laurinda Jaffee for first exposing us to this remarkable animal.

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Appendix F: Regeneration in bipinnaria larvae of the bat star *Patiria miniata* induces rapid and broad new gene expression

Oulhen N, Heyland A, Carrier TJ, Zazueta-Novoa V, **Fresques T**, Laird J, Onorato TM, Janies D, Wessel G (2016). Regeneration in bipinnaria larvae of the bat star *Patiria miniata* induces rapid and broad new gene expression. *Mech. Dev.* Nov; 142:10-21

I performed part of the experiments that contributed to Figure 9.



Regeneration in bipinnaria larvae of the bat star *Patiria miniata* induces rapid and broad new gene expression



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ARTICLE INFO

Article history:

Received 23 May 2016

Received in revised form 12 August 2016

Accepted 16 August 2016

Available online 20 August 2016

Keywords:

Vasa

SRAP

Dysferlin

Vitellogenin

Transcription

Repair

Patterning

ABSTRACT

Background: Some metazoa have the capacity to regenerate lost body parts. This phenomenon in adults has been classically described in echinoderms, especially in sea stars (Asteroidea). Sea star bipinnaria larvae can also rapidly and effectively regenerate a complete larva after surgical bisection. Understanding the capacity to reverse cell fates in the larva is important from both a developmental and biomedical perspective; yet, the mechanisms underlying regeneration in echinoderms are poorly understood.

Results: Here, we describe the process of bipinnaria regeneration after bisection in the bat star *Patiria miniata*. We tested transcriptional, translational, and cell proliferation activity after bisection in anterior and posterior bipinnaria halves as well as expression of SRAP, reported as a sea star regeneration associated protease (Vickery et al., 2001b). Moreover, we found several genes whose transcripts increased in abundance following bisection, including: Vasa, dysferlin, vitellogenin 1 and vitellogenin 2.

Conclusion: These results show a transformation following bisection, especially in the anterior halves, of cell fate reassignment in all three germ layers, with clear and predictable changes. These results define molecular events that accompany the cell fate changes coincident to the regenerative response in echinoderm larvae.

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"If there were no regeneration there could be no life. If everything regenerated there would be no death. All organisms exist between these two extremes. All things being equal, they tend toward the latter end of the spectrum, never quite achieving immortality because this would be incompatible with reproduction."

[Richard Goss, *Principles of Regeneration* 1969.]

1. Introduction

Regeneration enables an organism to restore lost tissue and organ functions following damage or removal. This process first requires wound healing, which is then followed by cell fate changes, migration,

cell proliferation, and differential signaling to compensate for the lost region(s) of the organism (Goss, 1969). The mechanisms underlying regeneration are predicted to be under strong selection, as they provide the organism with the capacity to return to its full reproductive and developmental potential after injury (Bely and Nyberg, 2010). Most organisms do not have an ability to regenerate significantly and a major question in biology is whether this phenomenon could be induced in an otherwise non-regenerative species. To answer this question, we require a deep understanding of the mechanisms underlying this phenomenon.

In both planarians and *Hydra*, a stem cell population in the adults is set aside for growth of adult tissue, as well as for replication and pluripotency of a regenerating individual. Planarians use neoblasts as their sole replicative cell type capable of replacing all other cells of the adult, even the germ line (Alvarado and Yamanaka, 2014). So too in *Hydra*: the stem cells of ectodermal and endodermal epithelium and the I-cells are the source for the replicative cells of the adult. One or another of their progeny contributes to all cell types of the adult, including the germ line (Technau and Steele, 2011; Tomczyk et al., 2015). These highly proliferative and pluripotent (or totipotent in the case of the planarian) stem cells are responsible for the vast regenerative capacity of

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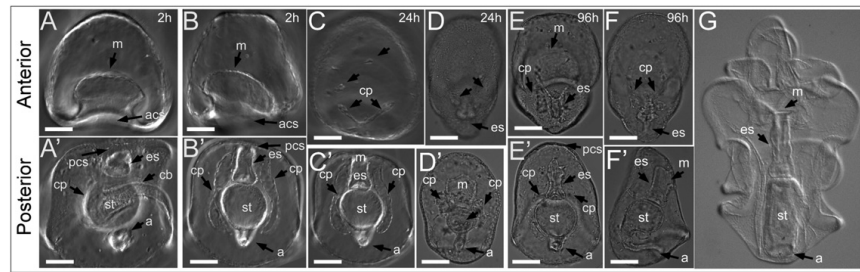


Fig. 1. Documentation of regenerative process over the course of the first 96 h after bisection. We bisected bipinnaria larvae (2 weeks old) and performed a detailed morphological analysis of anterior (A–F) and posterior (A'–F') parts 2 h, 24 and 96 h post-cutting. Posterior parts clearly regenerate a gut morphologically within 96 h while anterior parts require more time (see text for details). (G) Uncut sibling larva. a: Anus; acs: anterior cut site; cp: coelomic pouches; es: esophagus; m: mouth; pcs: posterior cut site; st: stomach. Scale bars A, A', B, B', C, C' (100 µm); D, D', E, E', F, F' G (75 µm).

these adults. It is not clear though whether this reliance on specialized stem cells devoted to tasks of replenishment and regeneration is a shared strategy amongst metazoans.

Sea stars (Echinodermata: Asteroidea) have been used extensively as a classic example for regeneration studies in invertebrates (Anderson, 1962; Goss, 1965; Mladenov et al., 1989). Adults may lose an arm, or multiple arms, but are capable of regenerating all lost tissues and appendages as long as they still have a portion of the central body disk. The regenerated tissues include the many cell types of the dermis and epidermis, the gut, nerves, and even the gonads and germ cells (Huet, 1974). Significant efforts are being made to understand this complete replacement of each arm in a sea star, but this regeneration process may take several months, depending on the species.

Interest in experimental testing of regeneration in echinoderms was reinvigorated when larval budding was observed in four of the five echinoderm classes: ophiuroids (Balser, 1998), echinoids (Eaves and Palmer, 2003; Vaughn and Strathmann, 2008), asteroids (Rao et al., 1993) and holothuroids (Eaves and Palmer, 2003). In general, a significant percentage of larvae bud off clones from the parent larva, which serves to develop a new, fully functional larva. This cloning characteristic in echinoderm larvae led to experimentation in regeneration following bisection of sea star larvae that resulted in the observation of wound healing, and even complete regeneration of lost body parts. For example, the posterior halves would develop features of the anterior, including new coelomic pouches, foreguts, and mouth openings, and the anterior halves would develop posterior features, e.g. a new stomach,

intestine, and anal openings (Vickery and McClintock, 1998; Vickery et al., 2002). In an attempt to uncover underlying mechanisms of sea star larval regeneration, SRAP (sea star regeneration associated protease) emerged as a potential candidate gene from a differential cDNA library screen by Vickery and colleagues (Vickery et al., 2001b). This protease was suggested to be functionally involved in modifications of the extracellular matrix needed for wound healing and regeneration.

Here we explore the wound healing and regenerative capacity of larvae from the bat star *Patiria miniata*, and document general mechanisms of the regeneration phenomenon based on spatial and temporal gene expression patterns, transcription and translation activity and general morphological changes after bisection. Enormous genomic, transcriptomic, antibody, and experimental resources are available for this species, thus making it an excellent model to explore regeneration using a molecular and cellular approach.

2. Results

2.1. Morphological changes during regeneration

We performed a detailed morphological analysis of regeneration in bisected bipinnaria larvae in this species by documenting anterior and posterior halves for 96 h post cutting (Fig. 1). Posterior fragments can regenerate the mouth within 96 h (Fig. 1E,F) while anterior pieces require more time to regenerate the digestive tract (up to ~15 days; see also Fig. 2, but this largely depends on the rearing conditions. Under

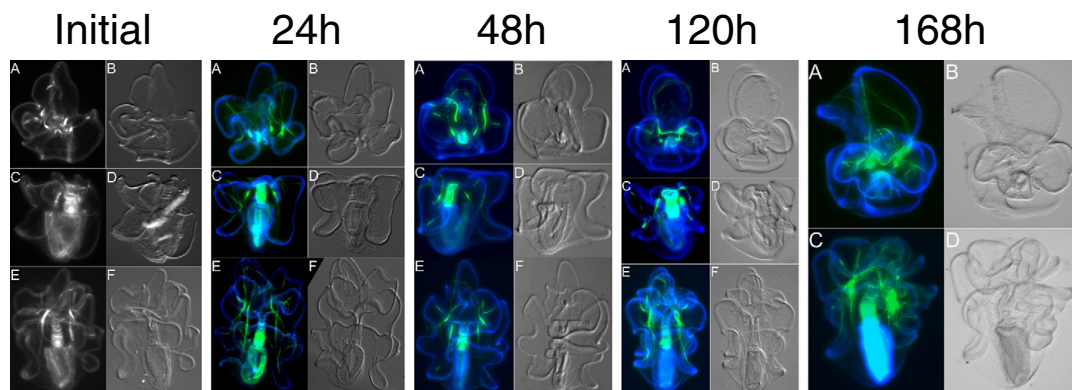


Fig. 2. Muscular regeneration of anterior and posterior pieces after bisection of two week old larvae. Bisected larvae were stained using phalloidin 24, 48, 120 and 168 h post bisection. We observed the generation of new muscle fibers post-bisection in the cut area. Blue: DRAQ-5 nuclear stain, green: phalloidin.

high food conditions (>12 cells *Rhodomonas lens* per μl of culture; see, Experimental procedure), anterior parts can regenerate a functional digestive tract (new anal opening through the ectoderm) in <12 days (Fig. 1). We also observed several cell types migrate to the wound healing site, but these cells will require further identification for relevance to the regenerative process. One very obvious change over the first 96 h were substantial changes in musculature. We provide a complete time series of muscle regeneration using phalloidin stain (Fig. 2). Larvae regenerate their musculature over a seven day period (~168 h). Immediately after bisection, the injury sites are visible as phalloidin stain shows slightly stronger signal in the areas of injury. Over time, muscle strands regenerate by creating web-like extensions at the site of injury (Fig. 2A, 24 h). In subsequent days muscle strands develop similar phenotypes to the control larvae. Note however that seven days is not a sufficient time to see a complete regeneration of musculature. Together these processes (i.e., cell movement and muscle regeneration) may be critical for the wound healing and regeneration process. Observations presented here are based on >100 larvae and we found that the sequence of regeneration events is largely independent of cutting site and method.

2.2. Cell proliferation during regeneration

To determine if changes in cell replication are induced by bisection, sea star larvae were bisected three days post-fertilization, incubated with EdU and fixed 2 h and 24 h later. Following the Click-IT reaction for fluorescence labeling (red – Fig. 3) and staining the nuclei with Hoechst (blue – Fig. 3), these specimens were analyzed on the confocal microscope for cell proliferation (EdU incorporation) using uncut larvae as a reference. Cell proliferation was quantified as a ratio of positive cells [RPC: Normalized # cells (EdU/DNA)]: the ratio of number of cells labeled with EdU over the number of nuclei (Fig. 3). Using time post-cut (2 h, 24 h), body part (Whole larva, anterior, posterior) and cut (no cut, cut) as factors in a three-way ANOVA we found that all three factors had a significant effect on cell proliferation (Time: $F_{1,24} = 170.97$; $p < 0.01$; Cut: $F_{1,19} = 6.53$; $p = 0.01$; Part: $F_{2,9} = 20$; $p < 0.01$). We then analyzed whether cutting the larva had an effect on cell proliferation in anterior and posterior halves using a two-way ANOVA. We found that 2 h post cutting cell proliferation was not different between cut and uncut larvae ($F_{1,9} = 3.10$; $p = 0.10$) but we found a significant effect of body part on cell proliferation ($F_{1,9} = 21.53$; $p < 0.01$). Specifically we found that cell proliferation was higher in anterior than in posterior parts. We also found a significant interaction between cutting and body part ($F_{1,19} = 8.32$; $p = 0.01$), indicating cell proliferation patterns

in anterior and posterior parts are different at 2 h post-cut between cut and uncut larvae. Specifically, we found that proliferation increases in anterior parts compared to uncut controls, while it stays the same in posterior parts compared to controls. 24 h post cutting we found that cutting significantly affected cell proliferation ($F_{1,9} = 60.96$; $p < 0.01$) and cell proliferation in anterior and posterior parts were significantly different from each other ($F_{1,9} = 15.87$; $p < 0.01$). Specifically, cutting reduced cell proliferation and cell proliferation was higher in anterior parts than in posterior parts. We also found a significant interaction between cutting and body part ($F_{1,19} = 11.92$; $p < 0.01$), indicating cell proliferation patterns in anterior and posterior parts are different at 24 h post-cut between cut and uncut larvae. Specifically, we found that cutting larvae reduced cell proliferation in both anterior and posterior parts.

2.3. General transcription and translation during regeneration

Cellular activity may be altered during regeneration and may not be uniformly represented along the wound healing sites. To test if transcription is affected during wound healing and regeneration, the bisected larvae were incubated with EU for 6 h or 24 h following the cut. The nuclei were labeled with Hoechst (Fig. 4A and B) and overall transcriptional activity was measured as a ratio of fluorescence intensity: the ratio of fluorescence intensity obtained with EU over the fluorescence intensity obtained with Hoechst. Using time post-cut (6 h, 24 h), body part (Whole larva, anterior, posterior) and cut (no cut, cut) as factors in a three-way ANOVA we found that no factor had an effect on transcription (Time: $F_{1,19} = 2.94$; $p = 0.10$; Cut: $F_{1,15} = 0.18$; $p = 0.67$; Part: $F_{2,7} = 1.01$; $p = 0.38$). We therefore did not perform any further analysis.

To test if protein synthesis is altered during wound healing and regeneration, sea star larvae and bisected halves were similarly prepared and following a 30 min incubation with HPG to visualize protein synthesis *in situ*, were fixed at 6 h, and 24 h following bisection (Fig. 4C and D). The nuclei were labeled with Hoechst and uncut larvae were used as a control. The overall translational activity was measured as a ratio of fluorescence intensity: the ratio of fluorescence intensity obtained with HPG over the fluorescence intensity obtained with Hoechst. Using time post-cut (6 h, 24 h), body part (Whole larva, anterior, posterior) and cut (no cut, cut) as factors in a three-way ANOVA we found that time and part had a significant effect on protein synthesis (Time: $F_{1,24} = 7.09$; $p = 0.01$; Part: $F_{2,9} = 25.34$; $p < 0.01$). Cutting did not affect protein synthesis ($F_{1,19} = 2.26$; $p = 0.14$). We then analyzed

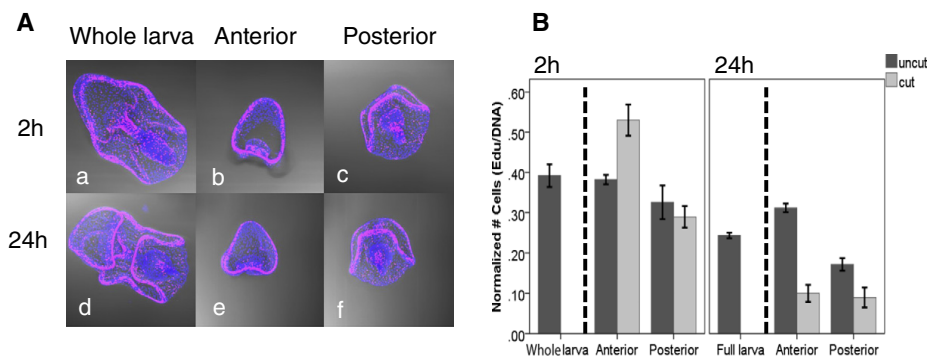


Fig. 3. Cell proliferation in anterior and posterior pieces of three-day-old larvae. A) Representative pictures of cut and uncut bipinnaria larvae. B) quantitative analysis of cell proliferation activity. Sea star larvae were cut in half and fixed 2 h and 24 h later, after a 75 min incubation with EdU (red). The nuclei were labeled with Hoechst (blue). Uncut larvae were used as a reference.

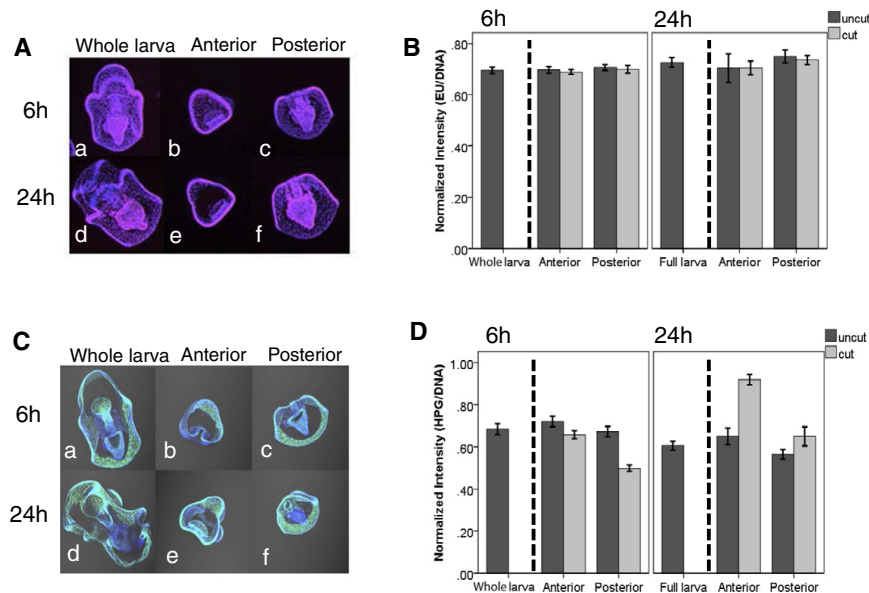


Fig. 4. Transcriptional (A,B), and translational activity (C,D) in anterior and posterior pieces. A) Representative images of transcriptional activity in cut and uncut bipinnaria larvae (whole larvae), three days of age, B) quantification of transcriptional activity. Sea star larvae were cut in half and incubated with EU (red), for either 6 h or 24 h before being fixed. The nuclei were labeled with Hoechst (blue). Uncut larvae were used as a reference. C) Representative images of translational activity in cut and uncut bipinnaria larvae, D) quantification of translational activity. Sea star larvae were cut in half and fixed 6 h and 24 h later, after a 30 minute incubation with HPG (green) to visualize protein synthesis *in situ*. The nuclei were labeled with Hoechst (blue). Uncut larvae were used as a reference.

whether cutting the larva had an effect on protein synthesis in anterior and posterior halves using a two-way ANOVA. We found that 6 h post cutting protein synthesis was different between cut and uncut larvae ($F_{1,9} = 30.27$; $p < 0.01$) and anterior and posterior parts ($F_{1,9} = 23.07$; $p < 0.01$). Specifically, protein synthesis was reduced in cut larvae and lower in posterior parts than in anterior parts. We also found a significant interaction between cutting and body part ($F_{1,1,9} = 6.46$; $p = 0.02$), indicating protein synthesis patterns in anterior and posterior parts are different at 6 h post-cut between cut and uncut larvae. Specifically, we found that the decrease in protein synthesis from the control was more pronounced in posterior parts compared to anterior parts. 24 h post cutting we found that cutting significantly affected protein synthesis ($F_{1,9} = 26.32$; $p < 0.01$) and protein synthesis in anterior and posterior parts were significantly different from each other ($F_{1,9} = 26.27$; $p < 0.01$). Specifically we found that cut larvae showed higher protein synthesis levels than uncut larvae and anterior parts showed higher protein synthesis patterns than posterior parts. We also found a significant interaction between cutting and body part ($F_{1,1,9} = 7.13$; $p = 0.02$), indicating protein synthesis patterns in anterior and posterior parts are different at 24 h post-cut between cut and uncut larvae. Specifically, we found that while protein synthesis increased in anterior parts compared to the control, it stayed the same in posterior parts.

2.4. Specific gene expression during regeneration

2.4.1. SRAP

The sequence of SRAP (sea star regeneration associated protease; (Vickery et al., 2001b)) was originally identified in a differential cDNA screen of mRNAs over-expressed in bisected larval halves, but its location in the animal and developmental dynamics of expression were not tested. To test the model that SRAP is involved in regeneration, we

first localized its transcript accumulation in embryos and larvae and found that it is not present in embryos of *P. miniata*. Only in 4-day larvae and beyond was SRAP detectable (Fig. 5). This expression was found localized to individualized cells within the epithelium of the gut, and mostly in the mid-gut region of the digestive system. We then tested its role in regeneration by quantitating its mRNA levels before and after bisection (Fig. 6). Three-day-old larvae were bisected, cultured and each half was frequently tested for appearance of SRAP mRNA. We first quantified the level of SRAP mRNA during regeneration in *P. miniata*, using the same developmental milestones as were used to identify it in the first place in *Luidia foliolata* (Vickery et al., 2001a). We found no significant difference in SRAP levels in the posterior regenerates during the 6 h incubation period (Fig. 6; $F_{1,5} = 0.02$; $p = 0.89$) while no SRAP expression was detected in anterior halves in this time frame. In these experiments the initial cut of the bisection at three days results in all of the SRAP partitioning to the posterior region, as concluded from bisections for *in situ* RNA hybridization, but no change in SRAP mRNA levels was seen in the window of regeneration tested here.

We then tested SRAP expression during regeneration over prolonged periods by *in situ* RNA hybridization. In the posterior halves, SRAP expression appeared on time in four-day-old posterior gut regions much as seen in intact larvae (Fig. 7). This expression was temporally and spatially comparable to the normal intact larvae. In the posterior halves, SRAP appeared in individualized cells of the gut epithelium as normally seen in whole larvae, even though the posterior halves undergo significant cellular and tissue rearrangement. More surprisingly, SRAP appeared in the anterior halves, as well. Even though the cut site of bisection is well anterior of the normal expression of SRAP in the mid-gut epithelium, SRAP appeared within the regenerating gut of anterior halves, and within the “equivalent” mid-region of the regenerates. SRAP expression in this newly regenerated region was equal to that

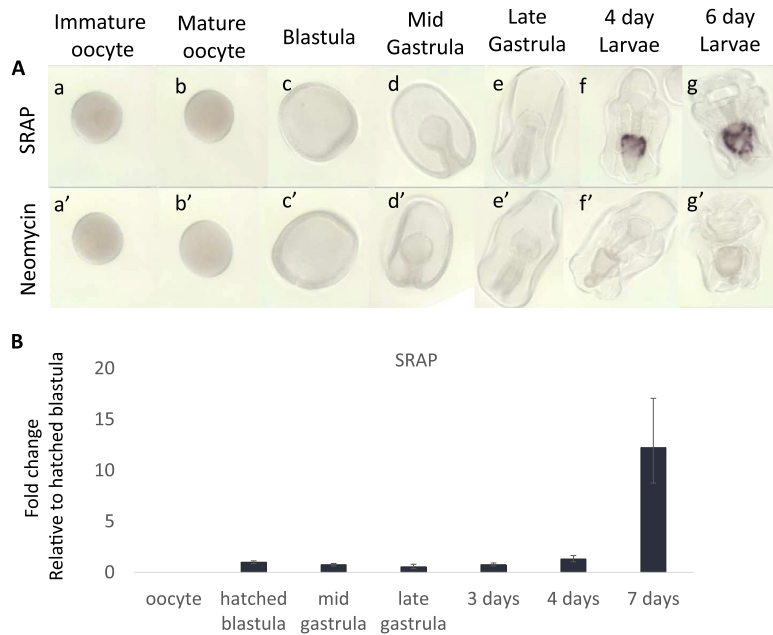


Fig. 5. SRAP expression in normal development of *P. miniata*. To determine the temporal expression of the SRAP transcript in *Pm*, an RNA probe was synthesized for *in situ* hybridization and a probe against the neomycin-resistance gene was used as a negative control (A). The expression was tested in immature (a) and mature (b) oocytes, blastula (c), mid (d) and late gastrula (e), four day larva (f), and six day larva (g). The strongest expression was detected in the stomach of the larvae. qPCR was used to measure the RNA levels of SRAP at the indicated developmental stages: immature oocytes, hatched blastula, mid and late gastrula, three day, four day and seven day old larvae (B). All values were normalized against the 18S mRNA and represented as a fold-change relative the amount of RNA present in hatched blastula.

seen in the intact larvae; that is, SRAP was found selectively in the anterior most region of the mid-gut of the 15 day and older regenerating larvae, and in individualized cells of the epithelium. Thus, SRAP expression in the anterior halves of bisected larvae identifies newly regenerated

mid-gut tissue in the anterior halves of the bisection, a region of the intact larvae that would not normally serve as mid-gut or SRAP positive cells. This is the first molecular marker of re-specified cell fates within the regenerating anterior halves. This result provides a molecular marker of regenerating events in this animal, and shows that entire regions of the animal re-specify tissues in comparable spatial and temporal sequences.

We then tested the putative function of SRAP, a secreted serine protease, during wound healing and regeneration by use of a specific protease inhibitor for serine proteases, soybean trypsin inhibitor (SBTI). SBTI was selected because its history of successful vital use in echinoderms (e.g. (Haley and Wessel, 1999, 2004)) and its inability to cross the plasma membrane and thereby access only secreted trypsin proteases. When regenerating anterior and posterior halves of a three-day-old larvae were exposed to $100 \mu\text{g} \cdot \text{ml}^{-1}$ SBTI, neither regions demonstrated altered regenerative progression, positively or negatively. Thus, based on the expression pattern of SRAP, its slow response to the regeneration phenotype especially in the anterior half, and the lack of effect on regeneration in targeted inhibition of its function, we conclude that SRAP is not directly involved in regeneration in this animal. Instead it may be involved in feeding and digestion, since its expression appears limited to a set of epithelial cells dispersed throughout the gut. We do not, however, see SRAP expression induced by feeding (data not shown). Thus, we conclude that SRAP in *P. miniata* is constitutively expressed in larvae following development of the digestive system. This result does not preclude the potential functionality of SRAP in regeneration or other activities in *L. foliolata*, the species of sea star for which it was initially identified as a regeneration-responsive gene product during larval development (Vickery et al., 2001b). Additional results suggest that SRAP expression may instead be induced by infection in posterior

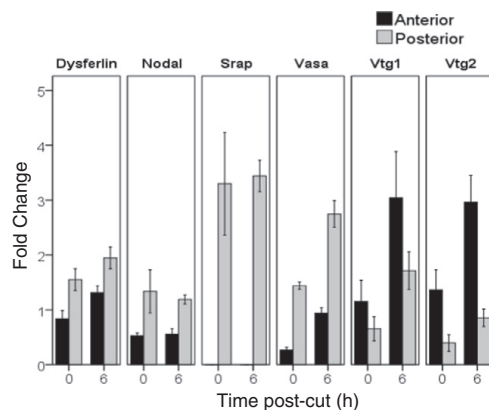


Fig. 6. Expression of dysferlin, Nodal, SRAP, Vasa, vitellogenin 1 and 2, in anterior and posterior pieces relative to uncut larvae. Expressions of dysferlin, Vasa, vtg1 and vtg2 increase 6 h post bisection while SRAP and Nodal are not. Error bars show one standard error from three independent biological replicate samples.

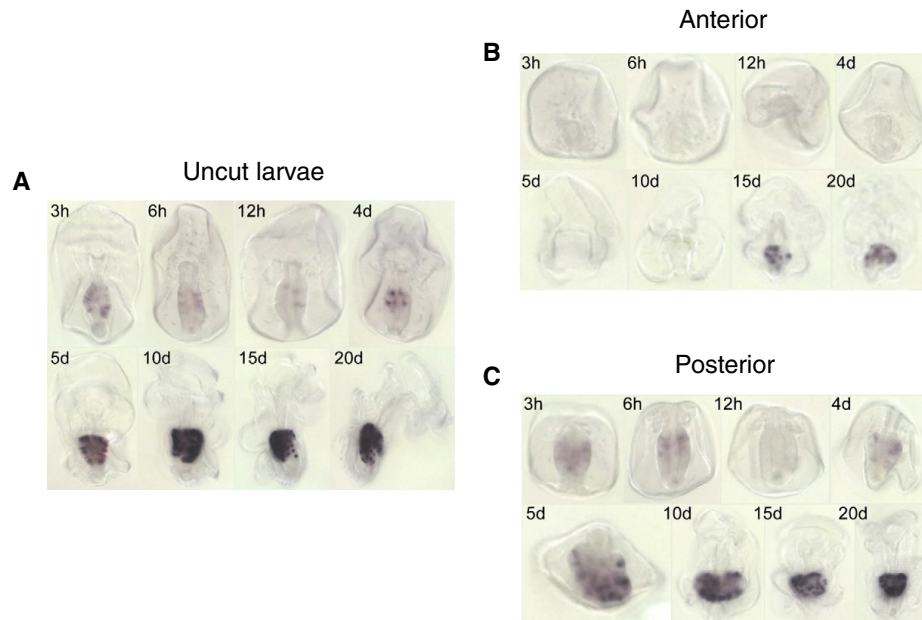


Fig. 7. Uncut larvae (A), anterior (B), and posterior (C) pieces monitored for SRAP expression post bisection. SRAP expression in the stomach is strongly correlated with stomach regeneration in both pieces.

parts after bisection (data not shown), which might explain differences in the results seen here, and in the initial identification of SRAP in *L. foliolata*.

2.4.2. Alkaline phosphatase activity as a metric of gut regeneration

We identified and tested alkaline phosphatase (AP) activity during the regeneration process in *P. miniata* in order to monitor digestive activity of the gut as the larva regenerates. We found that alkaline phosphatase activity, much like in sea urchins (Livingston and Wilt, 1989; Lyons and Weaver, 1962), is not detectable in early embryos, but much like for SRAP, first appears in gastrulae restricted to the hind and mid-gut endoderm (Fig. 8). Remarkably, although alkaline phosphatase activity is robust in the developing gut, this activity is immediately excluded from the posterior enterocoel (PE) upon its formation. The PE is believed to be the site of primordial germ cell formation in this animal and opposite from what is seen in vertebrates, this activity is excluded from the germ line in the sea star (Kuraishi and Osanai, 1994).

Bisecting three-day-old larvae and testing for AP activity following culture demonstrated that the larval fragments were capable of re-specifying cell fates during regenerative phenomena. While the posterior halves containing the major gut tissues expressed robust activity after 20 days of culture, the anterior fragments, which started the recovery period with only a small portion of the foregut, regenerated a complete gut with distinct fore-, mid-, and hindgut segments and with robust AP activity appropriately represented in the hind-, mid-gut sections. Although SRAP and alkaline phosphatase have regions of overlapping activities, they are readily distinguishable in both the intact larva and in the regenerate; SRAP accumulates in distinct, and individualized cells, whereas alkaline phosphatase accumulates broadly in all cells of the gut region, and in the induced regenerate. We conclude from these first two molecular markers that during regeneration new cell fate

acquisition is rapid, broad, and as a consequence, likely utilizes multiple different developmental pathways.

2.4.3. Induction of other gene products during regeneration

We tested other candidate genes of interest to determine how broadly new gene expression was during regeneration of the bisected halves. Dysferlin is a calcium-binding protein shown previously to be important in wound healing of cells (e.g. (Cheng et al., 2015)), even in other members of this phylum (Covian-Nares et al., 2010) and in endocytosis of sea star (*P. miniata*) oocytes and embryos (Oulhen et al., 2014), a feature perhaps important during cellular rearrangements in response to regeneration. Dysferlin is found throughout the larva, and in both the anterior and posterior regions of bisected larvae (Fig. 6; (Oulhen et al., 2014)). Bisection, however, had a significant effect on dysferlin mRNA expression 6 h after cutting ($F_{1,5} = 6.55$; $p = 0.03$) wherein both anterior and posterior halves increased dysferlin mRNA steady state levels. We also found a significant difference in dysferlin response between anterior and posterior parts ($F_{1,5} = 15.60$; $p < 0.01$) although we see no significant interaction between time (0 vs. 6 h) and part (anterior vs. posterior) ($F_{1,15} = 0.02$; $p = 0.89$). We detected a significant difference in nodal expression (Fig. 6) between anterior and posterior parts ($F_{1,5} = 11.82$; $p < 0.01$) but expression levels did not change over the 6 h period ($F_{1,5} = 0.08$; $p = 0.79$).

We found significant stimulation of Vasa mRNA accumulation in both the anterior and posterior halves ($F_{1,5} = 117.20$; $p < 0.01$) over a 6 h period. Vasa is an RNA helicase thought to be involved in broad translational regulation in echinoderms (Yajima and Wessel, 2015b). Vasa was initially found in the germ line of *Drosophila* (Schupbach and Wieschaus, 1986) but has since been seen in the germ line of all animals tested, and in somatic cells of many of those (Alie et al., 2011; Mochizuki et al., 2001; Pek and Kai, 2011; Pfister et al., 2008; Renault, 2012; Swartz et al., 2008; Yajima and Wessel, 2015a; Yajima and Wessel, 2011). It is also postulated to be involved in wound healing and regeneration:

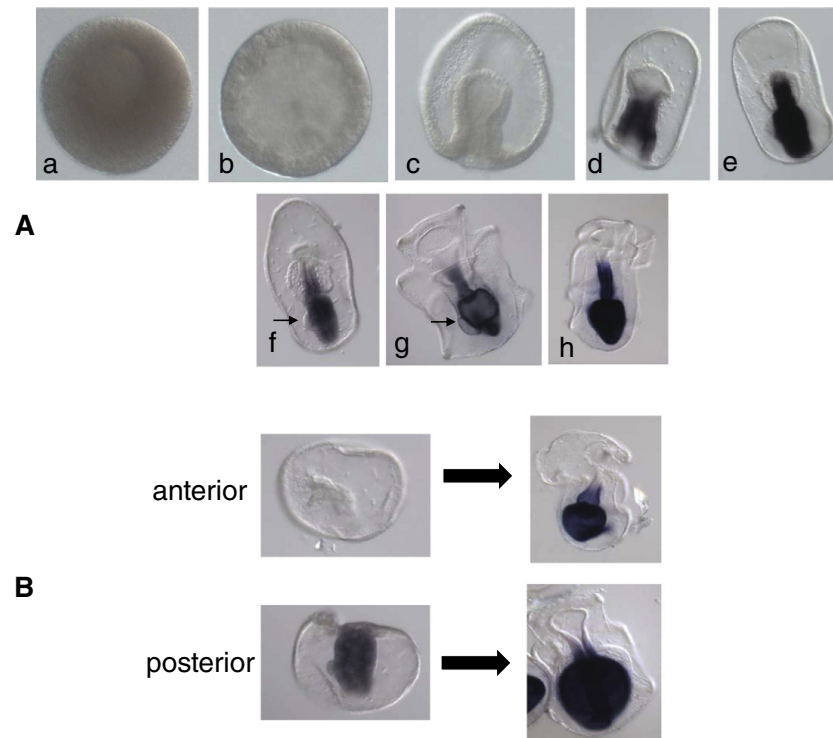


Fig. 8. Expression of Tissue Nonspecific Alkaline Phosphatase (TNAP), during the normal development of *P. miniata* (A). The TNAP activity was tested in egg (a), blastula (b), early gastrula (c), mid gastrula (d), late gastrula (e), three day (f), eight day (g), and 20 day (h) old larvae. To test the regeneration of the gut, three day-old larvae were cut in half, and cultured for 20 days (B). The expression of TNAP was tested at 20 days in the anterior and posterior pieces. Arrow points to the posterior enterocoel (PE).

damaged arms of sea urchin larvae increase the accumulation of Vasa protein, even though the mRNA does not change (Yajima and Wessel, 2015b). This behavior is consistent with Vasa being highly regulated post-transcriptionally. In bisected sea star larvae, Vasa mRNA accumulated significantly during a 6 h window of regeneration ($F_{1,5} = 51.80$; $p < 0.01$) and we detected a significant interaction between time and body part suggesting that the increase of expression over time (6 h) is more pronounced in anterior parts ($F_{1,5} = 5.34$; $p = 0.05$). Spatially, Vasa accumulated more intensively in areas already populated by Vasa at bisection, but the accumulation in anterior halves was more instructive. In cases where the bisection was directed more posteriorly, such that the anterior half included more of the coelomic pouches of the larva, Vasa accumulation increased during regeneration, but only in the pouches, where it is normally present. In cases, however, where the bisection was more anteriorly disposed, which minimized the amount of coelomic pouch tissue, then Vasa mRNA was induced both more robustly, and throughout the anterior half (Fig. 9A).

We also tested temporal and spatial expression patterns of vitellogenin (vtg). This yolk protein of animals is present in *P. miniata* in two copies, vtg1 and vtg2. Upon bisection, both vitellogenin genes are activated and the mRNAs of each transcript accumulate significantly after 6 h (vtg1: $F_{1,5} = 8.45$; $p = 0.02$; vtg2: $F_{1,5} = 10.12$; $p < 0.01$) as detected both by quantitative PCR (Fig. 6), and by *in situ* RNA hybridization (Fig. 9). We also found higher expression of vtg2 in anterior than in posterior parts ($F_{1,5} = 22.58$; $p < 0.01$), a difference that we observed qualitatively for vtg1 as well but was not statistically supported

($F_{1,5} = 3.25$, $p = 0.11$). In the case of vtg2, induction of transcript accumulation recapitulates the mRNA accumulation in normal larvae; transcripts are concentrated in the coelomic pouches and individualized, overlying cells of the ectoderm.

Interestingly, immunoblots using antibodies against Vasa, vtg1 and vtg2 indicate that the expressions of these 3 proteins are not affected during regeneration (Fig. 10). Even though, the corresponding transcript levels increase in the early steps of regeneration (6 h after the cut), their protein expressions appear unaffected during the first 24 h after the cut.

3. Discussion

As non-chordate deuterostomes, echinoderms represent excellent models for studying the evolution of developmental mechanisms. While sea stars and sea cucumbers have been studied for decades to better understand regeneration in adults, relatively little knowledge on underlying molecular mechanism of this process exists. Herein, is the first report of molecular markers *in situ* that are seen in regeneration sea star larval phenotypes, and which are now useful for analysis of the mechanism of regeneration and the accompanying cell fate changes.

With the discovery and documentation of larvae regeneration and cloning in several echinoderm taxa including sea stars, excellent histological and morphological analyses have been reported (Vickery and McClintock, 1998; Vickery et al., 2002). Our study complements this

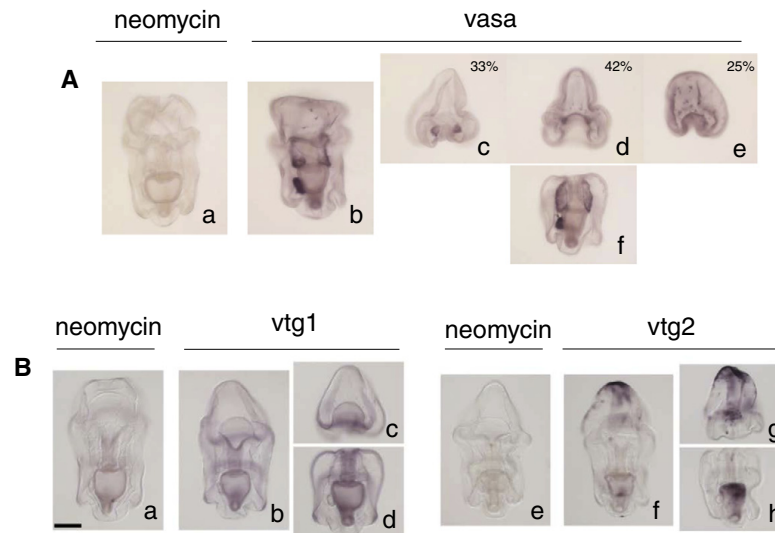


Fig. 9. Expression of Vasa (A) transcript in the anterior (c,d,e) and posterior (f) pieces was tested 6 h after being cut, and compared to uncut larvae three days of age (b). Three expression profiles were obtained in the 24 anteriors that were visualized, the corresponding percentages of representative Vasa expression are indicated in the right corner. If the percentage is not indicated, it means that the image represents 100% of the observed embryos. A probe against the neomycin resistant gene was used as a negative control (a). Expression of vitellogenin 1 and 2 (B) transcripts in the anterior (c,g) and posterior (d,h) pieces was tested 6 h after being cut, and compared to uncut larvae (b,f). A probe against the neomycin resistant gene was used as a negative control (a,e).

data by providing a detailed and quantitative molecular description of the regeneration process in *P. miniata*. Both anterior and posterior halves are capable of regenerating gut tissue and eventually form a

functional and morphologically complex digestive system. The process involved considerable restructuring of muscular tissue creating a temporary digestive system for the larvae.

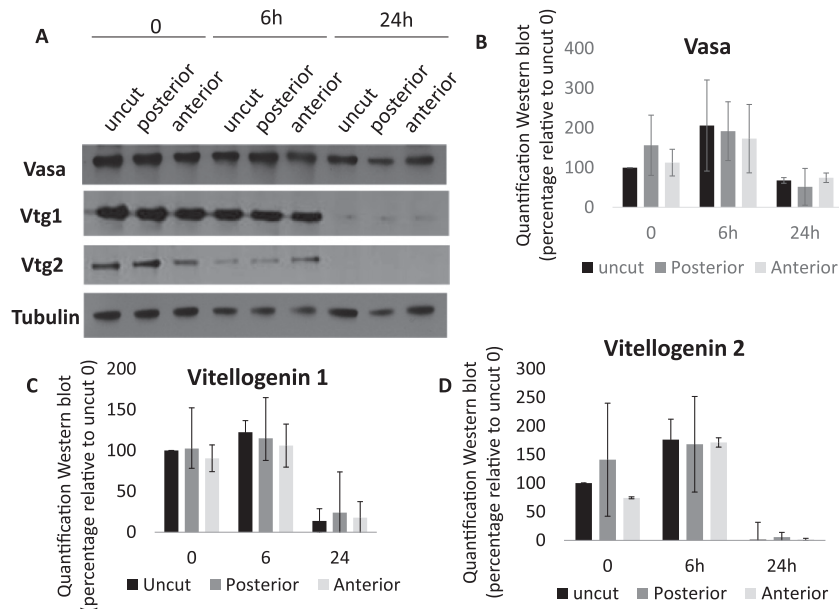


Fig. 10. Immunoblot of Vasa and vtg1 and 2. Three day old larvae were cut and fixed at time 0 (just after the cut), 6 h or 24 h after the cut. Samples were loaded on a gel for western blot. (A) Tubulin was used as a loading control. The protein bands were quantified using Image J (B,C,D). The protein expressions of vtg1, vtg2, and Vasa are not affected during regeneration.

3.1. Broad transcriptional changes during regeneration

SRAP (sea star regeneration associated protease; (Vickery et al., 2001b)) was originally identified in a cDNA screen of mRNAs over-expressed in bisected larvae halves. The hypothesis was that SRAP, based on its predicted protease sequence, may be involved in cellular rearrangements, and modifications to the extracellular matrix needed for wound healing and regeneration. In contrast to these data from *L. foliolata*, we did not find evidence for up regulation of SRAP in *P. miniata*. However, SRAP expression in the anterior halves of bisected larvae identifies transformation of mid-gut tissue in the anterior halves of the bisection, a region of the intact larvae that would not normally serve as mid-gut or contain SRAP positive cells. This is the first identified molecular marker of re-specified cell fates within the regenerating anterior halves. This experiment demonstrates that regenerating events do indeed occur in the sea star *P. miniata*, and that entire regions of the animal re-specify tissues in a manner comparable to the uncut larvae.

Based on the response time for the regeneration phenotype, especially in the anterior half, and the targeted inhibition of its function, we conclude that SRAP is not directly involved in regeneration in this animal. Instead, it may be involved in feeding and digestion, since its expression appears limited to a set of epithelial cells dispersed throughout the gut. However, when we tested this hypothesis we did not see SRAP expression responsive to feeding either (data not shown). Thus, we conclude that SRAP in *P. miniata* is constitutively expressed in larvae following development of the digestive system likely used for digestion of food. This result does not preclude the potential functionality of SRAP in regeneration or other activities in *L. foliolata*, the species of sea star for which it was initially identified as a regeneration-responsive gene product during larval development (Vickery et al., 2001b). Furthermore, our preliminary data on SRAP up-regulation in response to infection may suggest a function in innate immunity, a function that may be shared with *L. foliolata*. Similarly, in planarians, the expression of a trypsin like serine protease, detected in the gut during normal development and during regeneration, increases after bacterial challenges, but remains unaffected after addition of food (Zhou et al., 2012).

Using a large transcriptomic database, sequence alignment, and phylogenetic analyses we find that SRAP and closely related serine

proteases occur in two main groups of the gene tree (Fig. 11). The first group contains echinoderm sisters of the genes identified by Vickery et al. (2001b) in *L. foliolata* (Genbank accession AAK15274) and the sister gene we sequenced from *Patiria miniata* (Reich et al., 2015). This echinoderm-only group forms a clade that includes genes from all five extant classes of echinoderms and is thus hypothesized to have recently evolved in the phylum.

Similar to SRAP, alkaline phosphatase (AP) activity is also induced in the regenerating gut of the anterior halves, in cells that otherwise would not have this activity. It was rapidly cleared though from the cells of the posterior enterocoel (PE) cells. The PE is believed to be the site of primordial germ cell formation in *P. miniata* (Fresques et al., 2014; Inoue et al., 1992; Inoue and Shirai, 1991; Wessel et al., 2014) and opposite from what is seen in vertebrates, this activity is excluded from the germ line in the sea star. We do not know if the alkaline phosphatase protein, and/or its mRNA, is rapidly turned over in the PE, or if the PE contains some inhibitory activity for the enzyme, but the speed with which the alkaline phosphatase activity changes in the PE suggests a post-transcriptional mechanism. Therefore, even with these two molecular regeneration markers, diverse spatial regulation appears to occur.

3.2. Transcriptional activity after bisection

The overall transcriptional activity in the regenerate appears comparable to the intact larvae at the 6 h time point. Whereas the expression of transcripts such as nodal is not affected during regeneration, several genes like SRAP, Vasa, dysferlin, vtg1 and 2, are induced to increase in disparate regions of the regenerate. Quantitatively, dysferlin, SRAP and Vasa all show higher levels of expression in the posterior than in the anterior part. In contrast, vtg1 and 2 show higher levels in the anterior part. Whether these expression levels are in any way functionally linked to regeneration remains to be tested but it further emphasizes that each of these markers will serve as valuable reagents to probe the mechanism of cell-cell signaling responsible for these changes.

The experimentation presented here is on larval stages – that is, a stage of differentiated cells and tissues of diverse functionality and developmental history, yet is still developmentally not an adult. Based on gene expression levels our results suggest a distinct mechanism

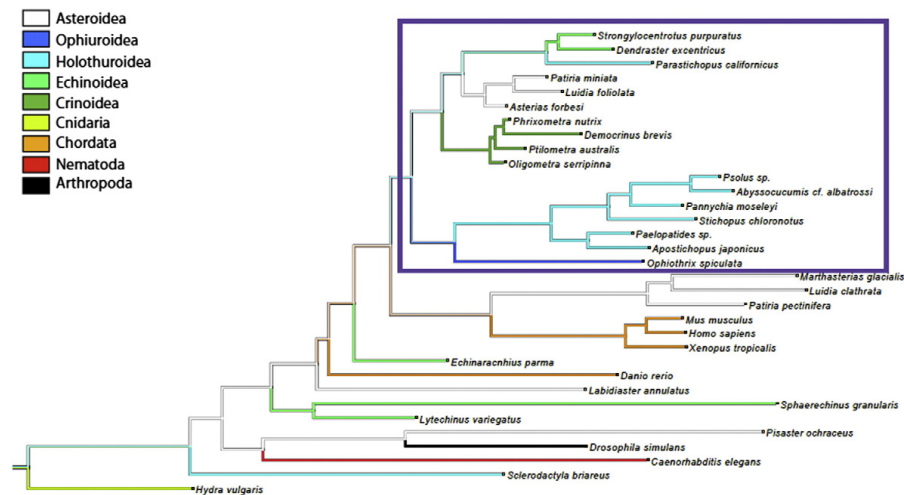


Fig. 11. Phylogenetic tree for genes related to SRAP identified from *L. foliolata* (Vickery et al., 2001a,b). The tree contains two groups 1) an echinoderm only group indicated by the purple rectangle that includes genes from all 5 extant classes of echinoderms. Another group of genes from echinoderms that are sisters to genes from Chordates, Arthropods, Nematodes, and Cnidaria. Our phylogenetic results show that SRAP orthologs in echinoderms consist of genes that occur only in echinoderms and genes that are closely related to taxonomically distant organisms (e.g., Chordates, Arthropods, Nematodes and Cnidaria).

compared to what can be found in other regenerating organisms such as *Hydra* and planarians in which a pre-determined, stem cell population is largely responsible for the regenerative capacity of those animals. We make this conclusion based on multiple time points of cellular proliferation; we do not see focal points of proliferation that appear to add to existing structures but rather a broad, continuous proliferation and transformation of cellular activity that results in new tissue functions. It is important to note that a significant amount of development has occurred in the larvae of echinoderms at this stage and cells of various tissues are expressing unique gene profiles throughout the animal (Hinman et al., 2003; Hinman and Jarvela, 2014; McCauley et al., 2013), yet they are still able to transition into other functional elements following bisection. This includes the three-part digestive system and its associated glands, a functional nervous system that coordinates swimming and eating behaviors, and multiple ectodermal and mesodermal regions of distinct functionality. Subsequent development of this stage emphasizes the formation of the adult rudiment – that portion of the larvae that will become the adult during the processes of metamorphosis and settlement. Overall then in the dramatic regenerative capabilities of this animal, we find evidence for significant and broad cellular responses for cellular fate transitions to compensate for portions of the animal lost in bisection. Use of the larva for these studies permits more rapid response and analysis than in the adult making it a tractable system for regeneration studies. We believe that the dependence on broad cell transitions of the larvae for regeneration is distinct from the mechanisms used by planarians and *Hydra*, and may more closely reflect a vertebrate model of regeneration for future studies of gene identification, logistics of cellular transitions, and the intersection of mechanisms between wound healing and regeneration.

4. Conclusions

Overall, we find that bisection of sea star larva serves as an excellent and tractable model for studying regeneration. We report for the first time, several molecular markers of disparate types and expression patterns that provide a rapid, predictable, and productive model for studies of regeneration. Here, we have documented the process in a popular sea star for studies in development, evolution, and with large transcriptomic resources (echinobase.org; Janies et al., 2016)). We hypothesize that this larva regenerates by broad cellular transitions, apparently not by pre-specified stem cell populations used to uniquely replicate and replace lost tissues. In combination with the documented genes found to significantly change their expression patterns, we believe this animal will serve as an important model for studying the mechanistic underpinnings of regeneration in a deuterostome.

5. Experimental procedures

5.1. Culture and bisection of larvae

Patiria miniata were collected from the Southern coast of California, USA from either Pete Halmay (PeterHalmay@gmail.com) or Josh Ross (info@scbiomarine.com). Fertilization and embryo/larvae culture and feeding was performed as described at 16 °C (Wessel et al., 2010). Larvae were fed *R. lens* at libitum (i.e., >12 cells·μl⁻¹). At specified times, samples from the culture were placed in a watch glass and individuals were bisected manually with a #15 sterile scalpel. Control (uncut) larvae and the anterior and posterior halves were collected by mouth pipetting and transferred to individual dishes (30 mm Petri dishes) containing the same type of specimens.

5.2. In situ RNA hybridization

Control larvae and regenerate halves were fixed and hybridized essentially as described (Arenas-Mena et al., 2000). Briefly, samples were resuspended in Fix Solution II (5% PFA, 162.5 mM NaCl, 32.5 mM

MOPS pH 7.0, 32.5% filtered sea water) and incubated overnight at 4 °C. Samples were then washed five times with MOPS Buffer (0.1 M MOPS pH 7.0, 0.5 M NaCl, 0.1% Tween 20) and stored at –20 °C in 70% ethanol until needed for hybridization. Larvae were then rehydrated by washing 3 times with MOPS Buffer and pre-hybridized for 3 h at 50 °C in Hybridization Buffer (70% formamide, 0.1 M MOPS pH 7.0, 0.5 M NaCl, 0.1% Tween 20, 1 mg·ml⁻¹ BSA). Samples were hybridized with 0.2 ng·μl⁻¹ of DIG labeled probe for 1 week at 50 °C. The probe was washed out with 5 MOPS Buffer washes, then a 3 hour incubation at 50 °C in Hybridization Buffer, and 3 more MOPS Buffer washes before the blocking step at room temperature for 20 min in Block Solution 1 (0.1 M MOPS pH 7.0, 0.5 M NaCl, 10 mg·ml⁻¹ BSA, 0.1% Tween 20). Samples were then blocked at 37 °C for 30 min in Block Solution 2 (Block Solution 1 containing 10% Sheep serum) and then incubated with antibody overnight at room temperature in Block Solution 2 (Fab anti-digoxigenin conjugated to alkaline phosphatase; 1:1500, Roche Diagnostics). Samples were then washed and stained as described for 6–24 h, and the reaction was stopped with 5 mM EDTA in MOPS Buffer. Samples were stored up to 1 day at 4 °C and imaged on a Zeiss Axiovert 200M Microscope with an AxioCam MRC5 color camera. In all cases control samples were also incubated with the same concentration of neomycin probe as a negative control. Probes were made using the following primer sets: *Pm Vasa* (Fresques et al., 2014), *Pm SRAP* (F: TATACGCCGACTGTGGTGTG and R: TCATCGACGGTGGTTTCCTG), *Pm vitellogenin1* (F: CCACCGATCCGTACTTCGAG and R: CTCATCGTCAGGTAGTCGC) and *Pm vitellogenin 2* (F: ACAGTATCCATGACGACCGC and R: CCCCTCAGGTGACTGTAGA). The sequences for vtg transcripts can be found within GenBank: vtg1 (KU569217), vtg2 (KU569218).

5.3. Testing cell proliferation, transcription, and protein synthesis

The Click-IT EdU Imaging Kit (C10340) was used to label cell proliferation (Life technologies, Carlsbad, CA). The modified thymidine analogue EdU is efficiently incorporated into newly synthesized DNA and fluorescently labeled. Briefly, 3-day-old larvae were cut and soaked in 10 μM EdU for 75 minute incubation at the indicated time point. The nuclei were labeled with Hoechst (blue). Uncut larvae were used as a reference. Transcriptional activity was visualized using the Click-IT EU kit (C10330), (Life Technologies, Carlsbad, CA). Briefly, 3-day-old larvae were cut, and incubated for either 6 h or 24 h with 0.5 mM EU. The nascent transcripts were then labeled according to the manufacturer's instructions. At the end of the click it reaction, the larvae were washed overnight in PBS at 4 °C before being imaged. The nuclei were labeled with Hoechst (blue). Uncut larvae were used as a reference. Protein synthesis was visualized using the Click-IT protein synthesis assay kit (Life Technologies, Carlsbad, CA) with HPG, L-homopropargylglycine (C10428). Briefly, the larvae were incubated for 30 min with HPG used at 1:2000, and fixed with 4% paraformaldehyde in seawater. The nascent proteins were then labeled according to the manufacturer's instructions. At the end of the click it reaction, the larvae were washed overnight in PBS at 4 °C before being imaged. The nuclei were labeled with Hoechst (blue). Uncut larvae were used as a reference. For each staining (Edu, EU and HPG), negative controls labeled with Hoechst only were used as references to define the best laser intensity required to eliminate possible background and autofluorescence. Each larvae, cut or uncut, was entirely imaged by Z stacks using confocal microscopy (Zeiss). The images presented in Figs. 3 and 4 represent the Z projection obtain for each larvae. For Edu, the number of nuclei was used to take into account the possible variations of larva thickness. The number of Edu labeled cells, and the number of nuclei, were analyzed using image J (Otsu threshold). For both EU and HPG, the overall fluorescence intensity was used as a measurement (Metamorph) and normalized to the overall fluorescence intensity obtained with Hoechst. We analyzed specific differences between time points and parts after bisection using ANOVA with *post-hoc* comparison. Note that for time, Bonferroni correction was performed to adjust for multiple comparisons.

5.4. Analysis of musculature development during wound healing and regeneration

Uncut and cut bipinnaria larvae were fixed at various times for 20 min in 4% paraformaldehyde and stained in a 0.2 μ M solution of Alexa Fluor® 488 Phalloidin (Thermo Fisher) in PBST (1 $\times$ PBS with 0.3% Triton X) for 30 min. After washing 2–3 times with PBS specimens were mounted on slides in 90% glycerol with 10 g/l DABCO (1,4-Diazabicyclo[2.2.2]octane) as an anti-fading agent and visualized on a Nikon Ti fluorescent microscope.

5.5. qRT-PCR

Three day old larvae were bisected, RNA was extracted from 50 uncut control larvae or 50 halves collected at the indicated times, using the RNeasy Micro Kit (Qiagen; Valencia, CA). cDNA was prepared using the Maxima First Strand cDNA synthesis kit for RT-QPCR (Thermo Fisher Scientific; Waltham, MA). QPCR was performed on the 7300 Real-Time PCR system (Applied Biosystems; Foster City, CA) with the SYBR Green PCR Master Mix Kit (Applied Biosystems; Foster City, CA). *Pm* dysferlin primer set is described in (Oulhen et al., 2014). The following primers were used to amplify *Pm* Vasa (F: TGGCTGATGCTCAACAAGAC and R: AAAGTTTCCGCTCCGTAAT), *Pm* SRAP (F: AAAGACTCTTGCC AGGGTGA and R: TTGGCAACGATGTTGTGAT), *Pm* vtg1 (F: GCGA CTACCTGACGATGGAG and R: GGTGGTGAAGTCTGCTCA), *Pm* vtg2 (F: CCCCAGCGTTTCATCCTCT and R: CACGGACTTGTCTCTTCCA). Experiments were run in triplicate and the data were normalized to 18S RNA levels (F: TTGGAGTGTCAAAGCAGGC and R: TCGCCATTCT CACATTCGTA). Relative expression levels (FC) were calculated using delta Ct method (Livak and Schmittgen, 2001; Schmittgen et al., 2000; Winer et al., 1999) and FC levels from at least three independent replicates were analyzed using 2 factor Analysis of Variance (ANOVA) commands in SPSS (v23). Genes were analyzed separately with time post bisection and body part as fixed factors. Statistical significance was assessed for a 5% cut-off.

5.6. Sequence analysis and phylogenetic tree construction

As a means to determine whether SRAP orthologs were present throughout Echinodermata and many non-echinoderm model systems, amino acid sequence data from Vickery et al. (2001a, 2001b) (*L. foliolata*), Reich et al. (2015) (*P. miniata*), GenBank (all non-echinoderm model systems), and Echinodb (<http://echinodb.uncc.edu>; for all other echinoderms; (Janies et al., 2016)) were combined. An alignment in MAFFT v7.215 (Katoh and Standley, 2013) using default values and tree search in (Stamatakis, 2014) v8.1.16 using 100 replicates under a PROTCATJTTF model of substitution of over 530 sequences was conducted. From the resulting phylogenetic tree, a sub-clade of sequenced related to those of interest was identified. After duplicative sequences from the Echinodb were removed, they were combined with that of *L. foliolata* (Vickery et al., 2001a), *P. miniata* (Reich et al., 2015), and the model systems, totaling 33 sequences. The 33 sequences from clade of interest were realigned in MAFFT, resulting in an alignment of 301 amino acid characters. Following this, a new tree search was conducted under the same conditions. The resulting tree was rooted on *Hydra vulgaris* (GenBank: XP_002162561.1), and a taxonomic character was traced in Mesquite (Version 2.75, build 564; (Maddison and Maddison, 2011)), resulting in a tree with branches colored to indicate major taxonomic groups containing SRAP orthologs.

5.7. Western blot

Three day old larvae were bisected, and lysed in loading buffer at times 0 h, 6 h or 24 h following bisection. Western blot analysis was performed following electrophoretic transfer of proteins from SDS-PAGE onto 0.22 μ m nitrocellulose membranes (Towbin et al., 1979).

Membranes were incubated with antibodies directed against *Pm* Vasa (1/4000), in 20 mM Tris-HCl (pH 7.6), 2% BSA, and 0.1% Tween-20, vtg1 (1/1000), vtg2 (1/1000), or tubulin (1/1000; Sigma) in Blotto; overnight at 4 °C. The antigen-antibody complex was measured by chemiluminescence using horseradish peroxidase-coupled secondary antibodies according to the manufacturer's instruction (ECL; GE Healthcare Biosciences, Pittsburgh, PA, USA). This experiment was performed twice with different parents, and the expressions of the proteins were quantified with Image J. Vasa polyclonal antibody was used as described (Juliano and Wessel, 2009). Rabbit polyclonal antibody was constructed against the peptide sequence INEPTEDMLDNILRC of vtg2 from *P. miniata* and against the peptide sequence CEKVNYPYEVTDPDR of vtg1 from *P. miniata*.

Acknowledgements

The authors thanks members of PRIMO for invigorating discussions, to Dr. Veronica Hinman for sharing pre-published data and for her insightful suggestions, and to the funding agencies for support (TRAIN 5T36GM101995 to Andrew Campbell and TO; an ASCB VP grant 5T36GM008622, to TO; NIH RO1 HD28152 to GMW; and a NSERC Discovery Grant 400230 to AH).

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