

Germ Line and Somatic Cell Divergence in Animal Development

by

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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
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May 2015

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This dissertation by S. Zachary Swartz is accepted in its present form by the Department of Molecular Biology, Cell Biology, and Biochemistry as satisfying the dissertation requirement for the degree of Doctor of Philosophy.

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Dedication and Acknowledgements

I dedicate this work to Michael McCoy, who if here today, would have finished his doctoral thesis by now, in surely a much more organized fashion than I have done myself. I will not forget our shared scientific beginnings in Rochester.

I thank Gary Wessel, and all past and present members of the Wessel lab and MCB village both for the constant support and for making the past years memorable and transformative. I am also fortunate to have been welcomed into the broader sea urchin community, which has been encouraging and generous.

I am grateful to my parents for helping to cultivate my innate interests and for supporting me this far.

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Chapter 1: Introduction

Multicellularity allows for complex division of labor. Different cellular functions often results from a progressive loss of developmental potency, as uncommitted cells terminally differentiate into diverse fates. These developmental decisions are controlled by both maternal information as well as by regulatory decisions engendered during embryogenesis. Differentiation involves not just the acquisition of gene function, but also the repression of recent gene activity and the degradation of previously important mRNAs. Generally speaking, in the embryos of many species, the earliest patterning decisions are programmed by maternally-supplied information. As development progresses, patterning controls shift increasingly to those newly made in the embryo itself. This passing of the controls involves several conserved features which occur coordinately, including: **1)** an increase in the **transcriptional** regulatory activity of embryonic blastomeres, **2)** an extensive **degradation** of maternally-supplied mRNA, and **3)** remodeling of the **cell cycle** from rapid, often synchronous cleavages to longer, more asynchronous divisions. Here, we will collectively refer to these changes as **Maternal to Embryonic Transitions (METs)**. METs represent a conserved requirement for clearing of the maternal slate, which may facilitate cellular specialization by allowing embryonic gene regulation to take over. Eggs from different organisms exhibit vast differences in the extent that determinants are maternally supplied, and how rapidly these investments are removed in the shift to embryonic developmental control.

The extent of maternal loading of developmentally important information is intimately related to the manner in which embryos accomplish their division of labor. From the yellow crescent material in ascidians that dictates muscle formation in ascidians, to the annucleate polar lobes that direct mesodermal fates in mollusk embryos, to the germ plasm important for specifying the germ line in many organisms, differential loading of

maternal components greatly influences when and how development proceeds (Conklin, 1905; Crampton and Wilson, 1896). Here we will specifically explore differences in the segregation of germ line from somatic cells, and how maternal control and the MET may be differentially utilized for this process.

In contrast to the terminal differentiation of the soma, the germ line must retain the capacity for totipotency, as it will pass all heritable information to the next generation. From an evolutionary perspective, the establishment of the germ line is therefore of utmost importance. The result of germ line segregation is that committed **primordial germ cells (PGCs)** are developmentally sequestered from the soma and into a reproductive niche, which will be solely tasked with creating the **germ line stem cells (GSCs)**, which once in the gonad that will produce gametes. Despite the near universal requirement of the animal germ line, there exists a surprising diversity in the mechanisms by which it is segregated. In some animals, the germ line is formed very early – before the MET, whereas in others, germ line determination occurs after the MET has passed, and may even occur into adulthood. The timing of germ line segregation with respect to the MET necessitates differences in mechanism; that is, does it occur by post-transcriptional control, through induction and gene regulatory networks, or somewhere in between?

The goal of the work presented here is to unravel the relative contributions of maternal information and embryonic gene regulation in the separation of the germ line from the soma. We will explore the interrelationships between METs and germ line development across species, with particular emphasis on the echinoderms. This phylum comprises familiar examples such as the sea lily, the sea star, the brittle star, the sea cucumber,

the sea urchin, the sand dollar (**Figures 1 and 2**). Echinoderms offer readily accessible eggs and embryos for studying the mechanisms of developmental transition, and representatives within the phylum exhibit distinct mechanisms of germ line determination.

Are METs universal?

Analyses of different organisms have taught us that the individual characteristics of METs are indeed widespread. An archetypal example is provided by the **midblastula transition (MBT)** in the frog *Xenopus laevis*. In this animal, the rapidly-dividing early embryo first undergoes a morphologically apparent change: blastomeres no longer cleave synchronously. Secondly, based on radioactivity incorporation experiments as well as more recent genomics, there is a major activation of embryonic transcription (**embryonic genome activation; EGA**). Thirdly, a major portion of the maternally supplied transcriptome is actively degraded (Tadros and Lipshitz, 2009). In animals such as *Drosophila*, *Xenopus*, and the zebrafish *Danio rerio*, whose phylogenetic positions are delineated in **Figure 1**, loss of cell cycle synchrony, EGA, and maternal transcript degradation happen simultaneously. Dramatic examples like these have broadly influenced the terminology (e.g. midblastula transition or maternal-to-zygotic transition) in a way that perhaps does not accurately reflect the diversity in development. There exist remarkable differences in when, where, and how, embryonic genome activation and maternal RNA clearance occur.

General activation of the embryonic genome

Several species of sea urchins, including California's purple *Strongylocentrotus purpuratus*, have been particularly fruitful for investigating transcriptional regulation of embryogenesis. Sea urchin eggs are fertilized externally and adults can produce large cultures of synchronously developing embryos. Early cleavage stages in *S. purpuratus* are rapid (about 1 hour per cleavage), and complete. The first 3 cleavages are equal, but the fourth is asymmetric, and produces a 16-cell embryo of three tiers: the animal (top, by convention) most and mid-sized mesomeres, the large macromeres, and the small vegetal (bottom) micromeres. At this stage, germ layer fates have already begun to be specified. Subsequently, a ciliated blastula forms, which hatches from the fertilization envelope and becomes free swimming. Gastrulation initiates at the vegetal pole, and embryogenesis culminates in a feeding pluteus larva (**Figure 3A**).

Several important discoveries in the sea urchin embryo were transformative to the field of activation of development: **1)** the early embryo can develop independently of transcription and even independently of a nucleus, **2)** the fertilized egg (zygote) begins transcriptional activity as quickly as can be measured, and **3)** protein synthesis begins at fertilization and begins independent of new transcriptional activity (Davidson, 1986). Development independent of transcription was shown in a variety of ways. One early indication was by E.B. Harvey, when she stratified the egg into nucleated and non-nucleated fragments and then tested developmental potentials in the resultant pieces. Using a highly pigmented species for this work, the local *Arbacia punctulata* at the Marine Biological Laboratory in Woods Hole, she was able to visualize stratification of major organelles with isopycnic sucrose gradients, and even separate intact halves and quarters of eggs. From this approach, she learned that each egg fragment was capable

of fertilization and development regardless of whether it contained the egg pronucleus, either as a diploid organism (male and female pronuclei contributions), as a merogone (an enucleated egg fragment that was fertilized), or even as a parthenogenetically activated merogone (with no nucleus). These experiments documented that early cleavage and development can occur in this animal even in the absence of a nucleus (Harvey, 1940). Earlier, Theodor Boveri, while working at Stazione Zoologica di Napoli, even made use of sea urchin merogones fertilized by the sperm of other species to distinguish between contributions from the maternal stores, relative to the paternal nucleus (Boveri, 1893). Overall, these experiments introduced the nuclear theory of determination, but more importantly for our discussion here, showed that all the RNAs needed for early development in this animal, including mRNAs, rRNAs, tRNAs, and small RNAs, must already be present in the egg prior to fertilization.

Paul Gross made use of the newly-identified toxin actinomycin D as an inhibitor of DNA-dependent RNA synthesis (Gross et al., 1964; Gross and Cousineau, 1963). First, he tested how soon newly synthesized RNA was being made by incorporation of a radiolabeled uridine. Although technically limited to global RNA analysis and only by quantitation of radioactive counts, his group was able to detect significant incorporation within the first time point possible in these experiments, by 20 minutes. Further, actinomycin D treated embryos exhibited no detectable transcriptional signal, yet the embryos developed relatively normally. They therefore concluded that new transcription was not necessary for early development, and that protein synthesis was templated by RNAs stored in the egg. It was subsequently demonstrated that the egg contained such information by measuring protein synthesis in the presence of radiolabeled amino acids. David Epel learned that amino acid incorporation occurred following fertilization, also as quickly as could be measured, within 15 minutes (Epel, 1967). Thus, the egg has

substantial stored information that can support early development, and the transition from egg to embryo includes a rapid activation of both transcription and translation. While transcription is not essential for early development, the normal embryo does indeed initiate transcription with fertilization. So while some embryos (frog, fly) may not transcribe significant RNAs early in development, others (sea urchins) clearly do, supporting the concept that the orchestrated METs of different species are highly variable.

These early functional investigations into sea urchin embryonic transcription and translation guided much of the thinking in the field for decades. Recently, high-throughput RNA sequencing and other technologies documented these processes in transcript-level detail. Sampling multiple time points from 10 to 72 hours of sea urchin development onward indicated diverse genome activity (Tu et al., 2014). However, this study did not include time points between fertilization and 10 hours of development (approximately the first 9 cleavages); thus, the earliest upregulation of transcription was not captured. A further limitation of these approaches is that they do not directly distinguish between maternally supplied and embryonically transcribed RNA; embryonic activation could only be inferred by an increase in relative transcript abundance. In the future, it will be important to test additional and earlier time points with new approaches for capturing nascent RNA, such as groSEQ, or blocking of splicing to detect newly-transcribed intronic sequence (Core et al., 2008; Lee et al., 2013). For now, it can at least be deduced that there is an increasing reliance upon embryonic transcriptional activity as development progresses, that is perhaps accentuated by important milestones in embryogenesis (summarized in **Figure 4**).

Activation of regionally-specific Gene Regulatory Networks (GRNs)

Through gene regulatory analysis, it is clear that lineage specific gene expression initiates in the sea urchin by the 4th cleavage, when the micromeres express vegetal inductive signals, such as Wnt 8 (Cui et al., 2014; Wikramanayake et al., 2004). The earliest activation of this localized gene regulatory network (GRN) is downstream of the canonical Wnt/ β -catenin pathway (Logan et al., 1999; Wikramanayake et al., 1998) (**Figure 4**). Blocking nuclear β -catenin prevents Wnt8 expression, vegetal fate specification and animalizes the embryo with complete failure in gastrulation. Typically, the canonical Wnt pathway is activated by a secreted Wnt ligand binding to a Frizzled type receptor. Subsequently, membrane-associated Disheveled protein binds and disassembles a destruction complex, allowing for β -catenin to enter the nucleus and activate transcription. However, the earliest activation of the pathway in the sea urchin embryo may occur independently of a Wnt ligand. Maternally-supplied Disheveled protein is enriched in the vegetal cortex of the sea urchin egg, and based on gel mobility, is modified in a form that perhaps indicates constitutive activation (Peng and Wikramanayake, 2013). In effect, the pathway may be hard-wired, implying that the early embryo activates differential axial specification autonomously, and not until later does inductive signaling influence cell fate decisions. Indeed, when cultured in the presence of an inhibitor that blocks all Wnt secretion, embryos still establish animal-vegetal polarity and form a blastopore (Cui et al., 2014). Nuclearization of maternal β -catenin thus may be a primary maternal activator of the embryonic genome. Downstream of this initial anisotropy, regionally specific GRNs are being well-characterized to pattern the various ectodermal, endodermal, and mesodermal territories of the pluteus larva (Oliveri et al., 2008).

Loss of cleavage synchrony: a cause or effect of embryonic control?

In *Drosophila* and *Xenopus*, major activations of the embryonic genome coincide with a loss of cleavage synchrony amongst blastomeres (Farrell and O'Farrell, 2014; Tadros and Lipshitz, 2009). While cleavage patterns exhibit species differences amongst echinoderms, careful investigation of several sea urchin species reveals some commonalities. Only the first 4 cleavages are completely synchronous—that is, until the birth of the micromere lineage. This timing of synchrony loss would seem to coincide with a major activation of the differential GRN. Subsequently, embryos display “regional synchrony” – that is, particular tiers of related blastomeres divide together, but at different rates than their neighbors. Intriguingly, these regional synchronies follow an animal-vegetal polarity gradient, with vegetal cells (e.g. micromeres) dividing slower than the more animal cells. This animal-vegetal wave of cleavage is reminiscent of the metasynchronous cleavage cycles in the early syncytial *Drosophila* embryo (Edgar and O'Farrell, 1989). All cleavage synchrony in the sea urchin is lost by the 8th or 9th cycle, at the onset of ciliogenesis and hatching (**Figure 4**). Furthermore, the cell cycle is substantially lengthened at this stage (Dan et al., 1980; Masuda and Sato, 1984). The complete loss of synchrony detected in blastula stages may coincide with the arrests observed when embryos are cultured in transcriptional inhibitors. That is, even though embryos can develop without new transcriptional activity, essential gene activity is first present at the time of cell cycle loss of synchrony. More investigation is required, but it is tempting to speculate that there may be an intimate and even causal relationship between the cell cycle and EGA in the sea urchin. For example, the lagging of vegetal blastomere cleavage (e.g. the micromeres) relative to animal blastomeres may permit the early vegetal activation of GRN expression. Downstream GRNs become activated

once other blastomeres in the embryo have divided more, producing enough raw cellular material to create the other territories. The lengthening of the cell cycle at 9th division may be important to facilitate robust embryonic transcription, as occurs in *Drosophila* (Shermoen and O'Farrell, 1991). The ability of embryos to reach blastula stages with transcriptional inhibitors may simply reflect their maternally-endowed ability to divide into many cells, whereas the differentiation of those cells is under embryonic control.

The loss of synchrony, lengthening of the cell cycle, and possible relationship between cell cycle and transcription are consistent MET characters in other organisms. Cell cycle modifications in embryogenesis have been extensively characterized in *Drosophila*, which displays a dramatic extension of cleavage cycle 14—the *Drosophila* MBT. With cycle 14, a typical G2 phase is introduced – in the first ten cycles, there are no gap phases, and a short G2 only begins to be introduced at cycle 10. One mechanism for this extension is the degradation of the maternally supplied ortholog of the Cdc25 phosphatase, Twine (Farrell and O'Farrell, 2014). The lengthening of the cell cycle may be required for robust transcription, particularly from large genes. Given the extremely rapid early cleavages of the *Drosophila* embryo, transcription is limited by the ability of RNAPII to elongate before being displaced by the DNA replication machinery or by condensation in anticipation of mitosis. Taking advantage of detailed knowledge of cell cycle timing and *in situ* hybridization, it was found that *Ubx* gene transcription is interrupted by mitosis prior to cycle 14, resulting in abortive transcripts (Shermoen and O'Farrell, 1991). Thus, it is possible that the remodeling of the cell cycle observed in the sea urchin and other animals also has direct consequences for transcriptional regulation.

Degradation of the maternally supplied transcriptome

In diverse organisms, such as the zebrafish, frog, and fruit fly, degradation of the maternally supplied transcriptome is essential for developmental progression (Tadros and Lipshitz, 2009). Genomic studies strongly support a major transcriptome remodeling around the blastula to gastrula transition in the sea urchin. Temporal microarray analysis suggested that a major fraction of maternally supplied transcripts are degraded by the onset of gastrulation (Wei et al., 2006). Subsequent deep sequencing and cluster analysis indicated that the transcripts of 34% of all genes are maternally deposited into the egg, and sharply degraded by the onset of gastrulation (**Figure 4**) (Tu et al., 2014).

How these RNAs are turned over in the sea urchin remains unknown. In other animals, maternal transcriptome degradation is effected by RNA binding proteins, as well as by small RNA machinery. *Drosophila* has two phases of degradation: first, a maternal pathway that can occur in an artificially activated egg (i.e. without a fertilizing sperm) and a second pathway that requires embryonic transcription. The first pathway uses the RNA binding protein Smaug, which binds stem loop elements in the 3'UTRs of its targets, and can both repress translation and target the mRNA for degradation, typically performing both functions (Chen et al., 2014; Smibert et al., 1996). Translational repression is achieved by recruiting the *Drosophila*-specific factor Cup, which interacts with EIF4E to prevent interaction with EIF4G and mRNA looping (Nelson et al., 2004). Transcript degradation is achieved by recruiting the CCR4/POP2/NOT complex, called the **CNOT complex** in vertebrates (Semotok et al., 2005). This multisubunit complex is a major cytoplasmic deadenylase, which functions by shorting poly-A tails, making the mRNAs substrates for various RNA decay pathways, including the exosome (Collart and

Panasenko, 2012). Smaug binding to mRNA in *Drosophila* can recruit the CNOT complex and degrades over 1000 transcripts, at least 339 of which were identified as direct targets (Chen et al., 2014). While most transcripts were both translationally repressed (based on polysome association) and degraded by Smaug, many transcripts were repressed but not destabilized. This result suggests separable function for Smaug, and future work may illuminate the combinatorial control that can lead to these distinct outcomes of Smaug binding. The sea urchin contains two putative Smaug orthologs, which may be tested in the future for their role in maternal transcript turnover.

A second pathway mechanism for mRNA decay is via the micro RNA (miRNA) pathway. Together with their associated Argonaute proteins, miRNAs recognize seed sequences in 3'UTRs and promote both translational repression and transcript decay, also by recruiting the CNOT complex (Tritschler et al., 2010). In *Drosophila*, a second pathway requiring embryonic transcription further reinforces the transcript degradation initiated by Smaug. This pathway requires the mir-309 miRNA cluster. This cluster of miRNAs degrades several hundred maternally supplied mRNAs, and its deletion results in larval lethality (Bushati et al., 2008). The miRNA machinery is used in several organisms for maternal transcript clearance. In the zebrafish, miR-430 is expressed at the MBT and causes the degradation of several hundred maternal transcripts by deadenylation (Giraldez et al., 2006). The *Xenopus* ortholog of miR-430, called miR-427, is required to degrade maternally supplied cyclin A1 and B2 mRNAs; however, whether miR-427 targets a broad subset of transcripts like miR-430 is unknown (Lund et al., 2009). The miRNA pathway has also been interrogated in the sea urchin, whose genome contains at least 49 miRNAs present in the egg and embryo. Complete blockage of miRNA biogenesis prevents normal gastrulation and differentiation of the embryo, but strikingly,

this defect can be rescued by adding back four abundant miRNAs: miR-1, 31, 2012, and 71 (Song et al., 2012). This result is reminiscent of the rescue obtained when miR-430 is injected into Dicer mutant zebrafish (Giraldez, 2005). The link has not yet been directly tested, however, it seems likely that these miRNAs would play a large role in the maternal transcript degradation detected by temporal deep sequencing (Tu et al., 2014). Together, these results indicate that maternal transcriptome clearance is an essential transition in embryogenesis. However, since it occurs by different mechanisms in different species, we can infer that convergent evolution has favored multiple acquisitions of this form of MET. There must then exist a strong selective pressure for the process.

We propose that maternal transcriptome degradation is essential for clearing of the slate of the totipotent egg, allowing for specific gene regulatory processes to pattern the different cell fates of the embryo. Differences in developmental strategy that favor rapid development have influenced the evolution of the timing of the event such that it occurs later, such as in *Drosophila*, when it occurs in an embryo of several thousand syncytial nuclei. In contrast, the mouse initiates degradation of maternal transcripts in response to fertilization and is largely complete by the 2-cell stage (Hamatani et al., 2004). The segregation of the germ line is uniquely intertwined with maternal RNA acquisition, and also has evolved multiple developmental mechanisms, which we document below.

Separating the soma from the germ line: a critical fork in the road

The survival of a species depends on a means for the individual to transmit its heritable information to its offspring. In the lifecycle of most animals, important reproductive cells called the **germ line** are typically segregated away from the terminally differentiating

soma, which lacks reproductive potential and will die with that individual. Species differences in the timing of germ line/soma separation have led to ambiguity in how terminology should be applied. For example, the P blastomeres of the early *Caenorhabditis elegans* embryo are often referred to as a germ line cells because they contain characteristic germ granules (or p-granules) and will give rise to *de facto* germ line stem cells later (Seydoux and Braun, 2006). However, this blastomere will also create a number of somatic cell fates for several cleavage cycles, until finally giving rise to the Z blastomeres, which are solely germ line competent. In this work, we will therefore designate any cell as being within the **germ line** if it is capable of giving rise to a gamete, whether it is the direct progenitor of that gamete, or a general founding blastomere whose later progeny becomes gametogenic. It may be more straightforward to define the germ line by what it is not: a blastomere may be called **somatic** when its fate no longer includes germ line potential. Similarly, we will refer to a blastomere that is solely germ line competent and without somatic potential as a **primordial germ cell (PGC)**. A **germ line stem cell (GSC)** is the mature product of the PGC, which is found in the gonad and undergoes self-renewing divisions to produce meiotically capable cells that will give rise to gametes (eggs or sperm). This discussion also excludes the consideration of cells manipulated by experimental conditions that *can* make germ line by a variety of experimental or abnormal conditions.

Different roads lead to a conserved germ line program

Comparative analyses of both expression pattern and function have illuminated a set of highly conserved germ line regulators, which have been extensively reviewed elsewhere (Ewen-Campen et al., 2010). The most conserved of these factors are post-

transcriptional regulators of mRNA stability and translation. Here, we will focus our attention primarily on the translational repressor **Nanos**, but also the Argonaut family member **Piwi**, and the RNA helicase **Vasa**. In all species examined heretofore, overlapping expression of these three factors in embryonic blastomeres is predictive of germ line fate, though occasional non-germ line stem cell functions have been identified for them individually (Juliano et al., 2010a). Nanos paralogs are zinc finger-containing RNA binding proteins that act upon 3'UTRs of target mRNAs in the germ line. Nanos itself possesses only nonselective RNA binding activity, and instead derives its specificity from associating with a partner called Pumilio. Pumilio is the founding member of the PUF-family RNA binding factors, and recognizes highly conserved **Pumilio Response Elements (PREs)** of a "UGUAAAU" consensus (Chen et al., 2012). The primary consequence of Nanos/Pumilio binding a target mRNA is to effect its translational repression and/or destabilization. A conserved mechanism of Nanos action is by recruitment of the CNOT complex, similar to that described for Smaug earlier (Bhandari et al., 2014). Upon recruitment, the nuclease subunits CNOT6 and CNOT7 then degrade the mRNA's poly-A tail resulting in its destabilization. Piwi family members are nucleases guided by small RNAs (piRNAs), which are primarily thought to protect the germ line by opposing transposable element mobilization in a process called the ping-pong cycle (Mani and Juliano, 2013). The precise role of Vasa has been mysterious since its discovery, but has now been identified as an integral component of piRNA biogenesis and transposon suppression in the germ line (Xiol et al., 2014).

Despite similarities in the molecular toolkit (e.g. Vasa, Nanos, and Piwi), there exists a surprising amount of diversity in how PGCs are segregated and how germ line specifying genes come to be expressed. The mechanisms of germ line specification across species

can be considered within a continuum of what we will call here **preloaded** versus **inductive** mechanisms (sometimes referred to as preformation vs. epigenesis, respectively). In brief, induction involves a conversation of cell:cell signaling between embryonic tissues that instructs select cells to adopt germ line fate (**Figure 5A,B**). Conversely, preloaded specification involves maternally supplied determinants that are often spatially localized to one region of the egg, which when acquired early by nascent blastomeres, directs them towards a germ line fate. These dense amalgamations of protein and mRNA determinants are collectively referred to as **germ plasm** (**Figure 5C**).

PGC specification by induction

Comprehensive syntheses of available data across taxa strongly imply that induction represents the ancestral mode of germ line segregation, though the timing of the event can vary (Extavour and Akam, 2003; Extavour, 2007). Perhaps the most intensely studied example of induction is the mouse, which specifies its germ line late, at about 6.5 days post fertilization. The proximal portion of the epiblast, or embryo proper (in closest proximity to the placental attachment) contains a field of mesodermal progenitors. Synergistic signaling from the extraembryonic ectoderm to this field, notably via the secreted molecules Wnt3 and Bmp4, enacts a germ line transcriptional program in approximately 8 founding PGCs (**Figure 5A**) (Lawson et al., 1999; Ohinata et al., 2009; Tam and Zhou, 1996). The exact connections between PGC transcriptional regulators are still being elucidated, however, the mesodermal factor Brachyury/T appears to be one of the earliest activators, and a direct target of canonical signaling from Wnt3. Brachyury is then required for the activation of core mouse PGC regulators Blimp1 and Prdm14 (Aramaki et al., 2013; Magnúsdóttir et al., 2013). Together with AP2 γ , these

three factors comprise a transcriptional program termed the “tripartite network,” which is required to broadly repress somatic cell transcription but enable germ line transcription. Nanos3 is a direct target of AP2 γ as determined by ChIP analysis (Magnúsdóttir et al., 2013). It is expressed in early PGCs following their initial segregation and during their migration into the somatic gonad, and its knockout leads to loss of PGCs by apoptosis and sterility of both males and females (Suzuki et al., 2008; Tsuda et al., 2003).

While induction is mechanistically best understood in the mouse, ample morphological, gene expression, and some functional data indicate its broad usage across species. Some effort has been made into human PGC investigation, but several considerations make their isolation impractical. However, early morphological investigation revealed that human PGCs are first visible by 24 days post fertilization in a location homologous to that in the mouse, at a comparable developmental stage when adjusted for gestation time (Leitch et al., 2013). Recently, *in vitro* schemes have been devised that appear to accurately mimic the developmental course of human PGCs *in vivo*. Already such investigation has revealed some striking differences with the mouse paradigm – for example, Sox17, which was previously considered an endodermal regulator, is required to activate Blimp1; however, these PGC-like cells also upregulate Brachyury, normally associated with somatic mesoderm formation, and it will be interesting to test in the future whether Brachyury is required for Blimp1 and Prdm14 activation in human cells, or whether these are competing elements for PGC formation (Irie et al., 2015). These human PGC-like cells also upregulate Nanos3 in a Blimp1 and Sox17 dependent fashion, though it is not yet known whether it is a direct target of these regulators. A third and powerful vertebrate comparison is the axolotl or Mexican salamander, a urodele amphibian. In contrast to mice and humans, axolotl PGC specification seems to require

both Fgf and Bmp4 signaling for robust germ line gene activation. Brachyury also enhances the germ line induction in the axolotl (Chatfield et al., 2014). Intriguingly, PGC specification appears to be Blimp1 independent in the axolotl, raising the possibility that the upstream Blimp1 expression in mouse PGCs is a derived trait. Further comparisons will be required to more confidently resolve the evolutionary history of vertebrate PGCs. In any case, a conserved theme of vertebrate PGC segregation is intercellular signaling via Bmp signaling and activation of the translational repressor Nanos, whose molecular functions we will return to later.

Accumulating data outside vertebrates suggest that induction via Bmp signaling is indeed a deeply conserved mechanism for PGC segregation. The cricket *Gryllus gryllus* has been developed into a functional model organism, and has been suggested to be more representative of ancestral insect development, compared to *Drosophila melanogaster*. In the cricket, PGC clusters arise in posterior segments, and are recognized both by morphology as well as by expression of germ line factors. Knockdown of Bmp pathway components leads to a loss of PGCs, whereas overactivation of the pathway leads to supernumerary germ cells (Donoughe et al., 2014). Whether the Wnt pathway is also required, as observed in the mouse, remains to be tested. Functional PGC specification in other taxa is limited, however, expression pattern information broadly imply conservation of induction. Morphological and molecular examination indicates a lack of germ plasm or early-forming PGCs in several other arthropods, Lophotrochozoans such as the mollusk *Ilyanassa*, and the cnidarian *Nematostella*, among others (Ewen-Campen et al., 2013a; 2013b; Extavour et al., 2005; Rabinowitz et al., 2008; Swartz et al., 2008). Highly regenerative animals such as hydrazoan cnidarians like *Hydra* demonstrate a capacity to segregate germ cells

throughout their lifecycles, rather than just in embryogenesis, precluding reliance upon inherited embryonically germ plasm (Juliano et al., 2014). In these animals, a self-renewing multi- or totipotent stem cell called the i-cell continually replenishes somatic lineages, and also contains the germ line, as it will give rise to sperm and eggs upon sexual reproduction (Müller et al., 2004). The planarian *Schmidtea mediterranea* can regenerate its germ line after being dissected into very small pieces, demonstrating an impressive capacity for induction in the adult (Wang et al., 2007). Broad comparisons such as these and many others reviewed elsewhere all point to induction being the likely ancestral route for PGC segregation in Metazoa (Extavour, 2007).

PGC specification by preloading

Investigation in diverse animals has yielded multiple examples of precocious PGC segregation by preloading. In contrast to the aforementioned strategies, PGC segregation here occurs very early in development, and is thought to occur both autonomously and independently of PGC transcriptional activity. Perhaps the most famous example of germ line specification by preloading is by *Drosophila*, whose PGCs are indeed the very first cells to form, approximately 1 hour after fertilization (**Figure 5B**). In this archetypal example, a cytologically obvious germ plasm is spatially localized to the posterior of the oocyte. Germ plasm localization involves an active process of transport along microtubules, cytoplasmic streaming from the nurse cells, and anchoring to the posterior cortex (Bergsten and Gavis, 1999; Seydoux and Braun, 2006). Germ plasm assembly follows a distinct assembly hierarchy, with the insect-specific protein Oskar serving as an anchor upon which other mRNAs and proteins can aggregate. In an intriguing example of evolutionary co-option, the ancestral role of Oskar was likely in the

nervous system, while its utility as a scaffold was later employed in the germ plasms of Dipteran species (Ewen-Campen et al., 2012). Classic experiments have revealed that germ plasm is both necessary and sufficient to specify PGCs in *Drosophila*: transplant of germ plasm to an irradiated recipient can restore fertility, or even create ectopic PGCs in the anterior embryo (Illmensee and Mahowald, 1974). Recent revisiting of these experiments with genetic approaches has shown that Oskar overexpression results in ectopic, anterior PGCs (Smith et al., 1992). The nematode worm *Caenorabiditis elegans* also provides a genetically tractable example of preloaded germ line specification. In this organism, the germ plasm comprises a collection of ribonucleoprotein granules called P-granules. The P-granules contain conserved germ line factors, such as the *C. elegans* Vasa orthologs, called GLH-1 and 2 (Gruidl et al., 1996). Before fertilization, P-granules are uniformly distributed in the cytoplasm, but after fertilization and during cleavage, these granules are segregated towards the P blastomere lineage. As development progresses, P-granules become increasingly associated with the nuclei, and are eventually restricted to the Z-blastomeres, which are the *de facto* PGCs (Uptdike and Strome, 2010). While some analyses have suggested that P-granules are neither strictly required nor sufficient for the embryonic segregation of PGCs in *C. elegans*, their intimate association with the germ line is clear, and likely perform diverse tasks pertaining to RNA metabolism, post transcriptional regulation, and germ line protection (Gallo et al., 2010; Voronina, 2013).

Numerous examples of preloaded specification exist within the vertebrates as well.

Xenopus displays a prominent germ plasm, called the Balbiani body, which translocates to the vegetal cortex during oogenesis. The *Xenopus* germ plasm contains numerous germ line-associated RNAs and proteins, in addition to a collection of mitochondria,

which are commonly associated with germ granule structures in different species.

Nanos1 RNA is transcribed during oogenesis and incorporates into the germ plasm, and following fertilization is inherited by germ line fated vegetal blastomeres (Forristall et al., 1995; MacArthur et al., 1999). The zebrafish also segregates its PGCs by preloading, displaying remarkable similarities with the frog including a vegetally localized Balbiani body during oogenesis. Nanos and Vasa RNAs, as well as that of Dazl, another translational regulator, assemble into the zebrafish Balbiani body. Following fertilization, the germ plasm material translocates to the animal pole of zygote, and is becomes enriched at the distal ends of cleavage furrows. The four blastomeres that inherit this material are directed to germ line fate (Kosaka et al., 2007). A gene called *Bucky ball* is required for germ plasm assembly and transport, and while it does not bear evolutionary homology to Oskar, its protein product performs an analogous function (Bontems et al., 2009).

Evolutionary transition of PGC segregation in the echinoderms

The echinoderms provide an additional intriguing test case for the evolution of preloaded germ line specification, supported by a rich fossil record (**Figure 2**). Expression pattern and embryological analyses indicate that the ancestral mode of germ line segregation in the echinoderms was by induction (Wessel et al., 2014). In the sea star, embryological investigations suggest that a posterior coelomic pouch (**posterior enterocoel, PE**) in the larvae is required for fertility in the adult (**Figure 3B**); when this structure is removed by microsurgery, juveniles contain fewer putative PGCs by cytological criteria (Inoue et al., 1992). Patterns of germ line gene expression support an inductive mode for the sea star. Transcripts for Vasa and Piwi are ubiquitously distributed in early embryos, with no

indications of a localized germ plasm. However, these transcripts become progressively restricted to vegetal cells that form the PE as gastrulation progresses. In contrast, *Nanos* expression is activated embryonically within the PE cells, reminiscent of *Nanos* activation in the mouse PGCs after stimulation by Bmp and Wnt signaling (Fresques et al., 2014). Both *Wnt3* and *Bmp2/4* are expressed in territories close to the PE progenitors, suggesting that these pathways could have a conserved role here for germ line induction, though this premise remains to be tested functionally.

In contrast, the echinoid echinoderms display some features of preloaded PGC segregation. Echinoids comprise a relatively recently diverging clade that includes the euechinoids, or sea urchins and sand dollars, and the cidaroids, or pencil urchins (**Figure 3B**). A derived trait of this clade is the micromere lineage, which forms by an asymmetric division at the 4th cleavage. This division produces a quartet of small cells at the vegetal pole called the **micromeres**. The micromeres then divide asymmetrically again to produce the more animally-oriented large micromeres, and the vegetal **small micromeres** (hereafter abbreviated **sMics**) (**Figure 3B**). This separation of fates between the large and small micromeres is particularly dramatic, because the large micromeres are unipotent and will only construct the larval skeleton, while the sMics are the likely PGCs (Wessel et al., 2014).

Numerous lines of molecular evidence support the sMics as the bona fide PGCs with preloaded specification. Upon formation, the sMics are highly enriched for Vasa protein, which occurs via post-translational mechanisms including ubiquitination and perhaps spindle association (Gustafson et al., 2011; Yajima and Wessel, 2011a). Shortly after their formation, the sMics express *Nanos2*, which along with the forkhead transcription

factor FoxY, is one of only two known genes embryonically and selectively expressed in the sMics prior to gastrulation (Juliano et al., 2010b; Materna et al., 2013) (**Figure 6**).

Nanos is uniquely detectable in the sMics by in situ hybridization as early as the 32/64-cell stage. As in the sea star, Piwi mRNA is maternally supplied and ubiquitous in early embryos, but becomes restricted to the sMics by gastrula stages post-transcriptionally (Swartz et al., 2014; Yajima et al., 2014). Intriguingly, the vegetal egg cortex and subsequently the sMics of the sea urchin *Hemicentrotus pulcherrimus* are enriched for mitochondrial rRNAs outside of the mitochondria themselves (Ogawa et al., 1999).

Similar observations have been made for the germ plasms of *Drosophila* and *Xenopus*, but the significance of extramitochondrial rRNA is unknown (Kobayashi et al., 1998; 1993). The sMics show signs of autonomy associated with preloaded PGC segregation: when the parent lineage, the micromeres, are surgically removed and cultured in isolation, they divide again asymmetrically to form the sMics, and upregulate both Vasa protein and Nanos RNA on a time scale consistent with the intact embryo (Yajima and Wessel, 2012). After their creation, the sMics divide only once to yield 8 descendants for all of embryogenesis and early larval stages. Thus, the sMics display all characteristics associated with preloaded PGCs, specified much more precociously than in the sea star.

Given the preloaded characteristics of the sMics, the embryonic expression of Nanos2 is unique. There are three Nanos paralogs in the sea urchin (termed Nanos1-3), which are expressed at different times in the life cycle. Nanos1 is ovary-specific, while Nanos2 is detectable from shortly after the sMics are created and into the early coelomic pouches. Nanos3 is transiently expressed at the tip of the archenteron late in gastrulation (Juliano et al., 2010b). The regulation of Nanos1 and 3 are unexplored, though some traction has been gained with the early sMic paralogs Nanos2. A late requirement appears to be

FoxY, whose knockdown results in a two-fold reduction in Nanos2 levels at the onset of gastrulation (Song and Wessel, 2012). It is not clear, however, whether Nanos2 is a direct FoxY target. FoxY is directly activated by the Notch/Delta signaling pathway, which is an upstream inducer of mesoderm in the sea urchin (Materna and Davidson, 2012; Materna et al., 2013). Neither FoxY nor Delta/Notch perturbation affect Nanos2 expression in the sMics before gastrulation; therefore, the earliest inputs into this gene remain a critical open question. FoxY may instead serve a supporting role for the sMics. After its initial sMic expression, FoxY expression shifts into adjacent mesodermal precursors. Knockdown of FoxY or D/N perturbation both completely prevent coelomic pouch formation, which contain the final niche for the sMics. Delta/Notch signaling through FoxY may be important for establishing the somatic gonad required for the GSC maintenance.

Given the early activation of Nanos2 in the sMics, it seems likely that a maternally supplied factor should be responsible. A strong candidate is the maternally supplied Wnt/ β -catenin pathway (discussed above). Nuclearized beta catenin, the transcriptional effector of the Wnt pathway, is indeed highly enriched in vegetal blastomeres of the sea urchin embryo (Logan et al., 1999). Additionally, the micromere lineage expresses 3 Wnt ligands prior to gastrulation that may reinforce the maternally activated Wnt pathway (Cui et al., 2014). Lithium chloride treatment, which upregulates nuclear beta catenin throughout the embryo, results in an increase in overall Vasa protein levels throughout the embryo (Voronina et al., 2008). Future investigation should further test whether the Wnt pathway regulates Nanos expression directly, particularly in light of conserved roles for Wnt and BMP signaling in germ lines of other species.

Consistently with germ line segregation in other preloaded systems, sea urchin Nanos2 is heavily regulated post-transcriptionally. The 3'UTR contains a stability and translational control element, termed the GNARLE (global nanos associated rna lability element). The GNARLE element is sufficient to confer sMic localization of injected mRNA reporters (Oulhen et al., 2013). Intriguingly, 3' UTR mediated control of Nanos localization has also been found in zebrafish Nanos1 and mouse Nanos3 (Köprunner et al., 2001; Suzuki et al., 2010). This observation implies that transcriptional regulation alone is insufficient for selective Nanos2 expression in the sMics, and that other post-transcriptional systems are required for separation between PGC and mesodermal associated gene expression at the sea urchin vegetal plate. Instead, Nanos2 may be downstream of a more general mesodermal transcription factor (such as β -catenin), but degradation processes in the cytoplasm further refine its localization. While these processes have not yet been identified, they may involve small RNAs or RNA binding proteins as observed in zebrafish and *Drosophila*.

The cytological features of the sMics, their gene expression profile, and the fact that they do not contribute to the embryo or larva but are rather set aside for the adult rudiment, makes them candidates for PGCs in the sea urchin. However, their precise nature has been a point of controversy for some time, perhaps because the definitive resolution of their fate requires a challenging lineage trace. While transgenesis is possible in the sea urchin, the long generation times of most species (up to two years for the popular *Strongylocentrotus purpuratus*) makes the maintenance of stable lines impractical (Arnone et al., 1997). Classic embryological approaches, however, have been used to test whether the micromere lineage is required for fertility of the adult sea urchin. Surprisingly, development can proceed following removal of the micromeres before the

5th cleavage. Such embryos gastrulate, and the resultant larvae even form skeletons, by way of compensatory mesodermal cells (Ettensohn et al., 2007). When raised to adulthood, these manipulated embryos do indeed yield fertile animals (Ransick et al., 1996). It was concluded from this experiment that the micromere lineage contains no obligate germ cell determinants. However, a subsequent study found that when instead the sMics are removed at the subsequent cleavage, the resultant adults are infertile (Yajima and Wessel, 2011b). These seemingly disparate results are actually quite compatible when considering the organizing capability of the micromeres. When transplanted ectopically to the animal pole of a recipient embryo, a second axis is induced with a complete gut (Ransick and Davidson, 1993). Conversely, experiments in which sMics were transplanted to the animal cap indicated only very weak organizing activity (Kurihara and Amemiya, 2005). From these results together, one can hypothesize that the micromeres repress the germ line program in what will normally become somatic blastomeres, and upon their removal, another cell lineage can compensate for its loss. Since the sMics lack organizing capability, their removal does not induce compensation. In support of this premise, ectopic Vasa protein strongly accumulates throughout the entire embryo when micromeres are removed, but not when sMics are removed (Voronina et al., 2008; Yajima and Wessel, 2011b). Furthermore, Nanos2 mRNA accumulates in a mesodermal territory that normally neighbors the sMics, following micromere removal (Fujii et al., 2009).

Putting your germ cells in the freezer: possible advantages for preloaded specification

While there are apparent similarities in the germ plasms of different systems, mapping these characters to the phylogenetic tree strongly implies that germ line preloading evolved independently multiple times. Furthermore, examination of the genes involved reveals a lack of homology (e.g. the insect specific Oskar, and the vertebrate specific Bucky ball), pointing instead to convergent evolution. Multiple realizations of preloading imply a strong selective pressure for the strategy. A consequence of early segregation, particularly before the MET, is that the PGCs must sit and wait for the rest of the embryo to catch up. That is, the PGCs require a somatic support structure – the gonad – to migrate towards and colonize. Until colonization occurs, the PGCs remain locked down and insulated from differentiation cues. Why not specify the germ cells after the MET, in the same location where the mature GSCs will ultimately reside?

While difficult to know for certain, some hypotheses have been proposed. The work of Weismann and others leading to the Modern Synthesis suggested that early segregation of PGCs followed by cell cycle quiescence could confer a protective advantage against the accumulation of DNA replication errors (Buss, 1987). In rapidly developing organisms such as *Drosophila*, this hypotheses makes intuitive sense; complete genome replication and cleavage in the syncytial blastoderm can occur in as little as 4 minutes (Farrell and O'Farrell, 2014). *Drosophila melanogaster* can develop normally without spindle assembly checkpoint machinery, implying that its evolution has perhaps favored rapid development over absolute fidelity (Buffin et al., 2007). Over many generations, one can envision the accumulation of deleterious mutations. By setting aside the germ

line early in embryogenesis, somatic mutations are unable to gain access to the germ line and thus will not be transmitted to subsequent generations. This premise must be balanced though by the realization in many animals, mammals included, that the stem cells that give rise to eggs and sperm, will divide enormous times to give millions of oocytes and sperm. Protecting the germ line lineage early on really only delays onset of division.

Johnson and colleagues have proposed a complementary hypothesis that early germ line segregation enhances the evolvability of the species. They suggest that by establishing the germ line in isolation instead of as part of the mesoderm, constraints upon the embryo are relaxed. Consequently, somatic tissues can diversify with less risk of compromising the species' reproductive capability (Johnson et al., 2011). These authors suggest that clades with preloaded germ cell specification should speciate more rapidly than sister clades, or at least diversify in developmental programming, compared to clades with inductive specification. However, we note here that such a speciation would not occur without the imposition of appropriate selective pressures. Nature has performed this experiment for us several times, and indeed, there may be a correlation between germ line preloading and enhanced speciation. Several examples include the teleost fishes, such as zebrafish, versus inductive, more basally branching ray-finned fishes, such as the sturgeon; and anuran amphibians such as *Xenopus* that uses preloading, versus the inductive urodeles like the axolotl. In these examples, the preloaded clade does indeed appear to have greater species diversity than the inductive sister (Johnson et al., 2011). A phylogenetic analysis also suggests that genes in preloading animals tend to evolve more rapidly than their inductive sisters. Evans et al. found that gene trees between anuran and urodeles amphibians and mammals often do

not recapitulate the proper species relationships, and when they are incongruent, urodele (inductive) sequences tend to cluster with vertebrate sequences, while the anuran (preloaded) sequences are more divergent (Evans et al., 2014). This creative analysis may support the premise that germ line preloading correlates with speciation; however, incongruent trees were heavily affected by the choice of outgroup to which they were rooted. Future investigation might also seek to directly measure rates of gene evolution within the preloading-based echinoids, versus other echinoderms, for which transcriptomes in many species are becoming available. Because the sea urchin gene regulatory network is well-characterized, one could test whether particular subnetworks, such as that specifying mesoderm, display different evolutionary rates than others.

We cannot from these correlations conclude that germ line preloading is a universally optimal strategy. Instead, it is more productive to ask what unique attributes of these clades' environments influenced the convergence. One possibility influencing preloading is the need for rapid development; consider that all of the examples provided here, including *Drosophila*, *C. elegans*, *Xenopus*, the sea urchin, and zebrafish each develop through a larval form. By getting germ line specification 'out of the way' early, the embryo can then direct its investments towards reaching a motile, feeding form as quickly as possible. Precocial development may be particularly beneficial when the animal is subjected to high predation, or rapid changes in the environment.

Are METs different between the germ line and soma?

Transcriptional repression in PGCs compared to soma

Careful study of GRNs in the sea urchin has shown us that transcriptional asymmetries initiate very early in embryogenesis. The embryo is poised for these asymmetries largely thanks to the maternal β -catenin pathway, which is activated in early vegetal blastomeres (as reviewed above). The GRNs put in place by these early asymmetries prime different parts of the embryonic genome to be deployed in different embryonic territories. In comparison to the rest of the embryo, however, the sMics are poorly understood in terms of their transcriptional regulation. With a strong collective effort of the community to identify all spatially restricted developmental transcription factors, it is surprising that FoxY is the only one known to be expressed in the sMics before gastrulation (Materna et al., 2013; Song and Wessel, 2012). Furthermore, its knockdown leads to no obvious defects in the specification of the sMics. It is therefore plausible that the dearth of known transcription factors in the sMics reflects something of biological significance, such as transcriptional repression.

This premise is consistent with the PGCs of other species that undergo periods of broad transcriptional repression, including *Drosophila*, *C. elegans*, *Xenopus laevis*, and the ascidian *Ciona intestinalis* (Nakamura and Seydoux, 2008; Shirae-Kurabayashi et al., 2011). These studies have all benefitted from the generation of antibodies that specifically recognize phosphorylations of the heptapeptide repeat of the C-terminal domain (Seydoux and Dunn, 1997). Antibodies to phosphorylated Serine 2 (pSer2), which is associated with transcriptional elongation, have revealed that early PGCs transcribe at very low levels compared to somatic neighbors. Surprisingly, each of these

species achieves transcriptional repression by distinct mechanisms. *Drosophila* and *C. elegans* have both found solutions by interfering with the P-TEFb complex. This complex contains CyclinT and Cdk9, and acts by phosphorylating the C-terminal heptapeptide repeats of RNA polymerase II (RNAPII) at Serine 2. In *Drosophila*, *Pole granule component* (pgc) is localized within the germ plasm, and prevents P-TEFb recruitment to by directly binding CyclinT (Hanyu-Nakamura et al., 2008). In *C. elegans*, a protein called PIE-1 interferes with Cdk9 activity and is thought to function by mimicking the RNAPII C-terminal tail. Both Pgc and PIE-1 are species-specific genes, an example of evolutionary convergence that is intimately associated with having a preloaded germ line. In *Xenopus*, Nanos is required for pSer2 depletion, though this is almost certainly an indirect effect, given that Nanos is a cytoplasmic regulator of mRNA (Lai et al., 2012).

Each of these species uses a preloaded mode of PGC segregation, and has also acquired a mechanism for general transcriptional repression. This correlation may imply that transcriptional repression is a prerequisite for having a preloaded germ line. A possible reason for this requirement is insulation from differentiation. To be kept developmentally naïve, PGCs must not respond to the differentiation cues intended for somatic cells. When transcriptional repression is perturbed in *Drosophila*, *Ciona*, and *Xenopus*, PGCs misexpress somatic genes (Hayashi et al., 2004; Lai et al., 2012; Shirae-Kurabayashi et al., 2011). In addition to these preloaded PGC animals, the mouse (which uses induction) also shows signs of broad transcriptional repression, although there are some differences. Its PGCs are transcriptionally active at their initial segregation, but lose pSer2 intensity as they migrate toward the somatic gonad. The mechanism of this is unknown, but must involve different regulation than observed in the preloading-based systems. Mouse PGCs are arrested in G2 of the cell cycle during

migration, and their transcriptional repression thus may be cell cycle dependent (Seki et al., 2007).

The sea urchin sMics also display signs of broad transcriptional repression. Immediately after their creation, the sMic nuclei are enriched for the heterochromatin mark H3K9 trimethylation, and this enrichment persists into blastula stages. Subsequently, the sMic nuclei become depleted for elongating RNA polymerase II, based on immunofluorescence with phosphospecific antibodies (Swartz et al., 2014) (**Figure 6**).

The mechanism of this depletion is unknown, but one possibility is that a methyltransferase is either localized to, or more active within, the sMics than in the somatic blastomeres. The subsequent phase of RNA polymerase repression could be dependent upon the earlier phase of H3K9 trimethylation. There also could be cell-cycle dependency; BrdU incorporation studies have found that sMics display a prolonged S-phase from their birth until migration (Tanaka and Dan, 1990). They begin to divide at the early gastrula stage during translocation, and this mitotic activity could be a cause for their reduced RNAPII pSer2.

The sMics are also enriched for a maternally supplied form of linker histone H1 based on immunofluorescence data (**Figure 6**). While this work was carried out before the sequencing of the genome, and the precise identity of this H1 ortholog has not yet been identified, they retain this variant form from their creation through migration and into the coelomic pouches (Pehrson and Cohen, 1986). The retention of this linker histone may simply reflect mitotic quiescence of the sMics—slow cell cycling might prevent incorporation of cleavage stage histones, while somatic cells dilute out their maternally supplied histones. However, this H1 variant could also actively confer transcriptional

repression. Such a role would be consistent with the function of linker histones, which compact chromatin into higher order structure. Furthermore, a histone H1 variant called dBigH1 was recently reported to regulate embryonic genome activation in *Drosophila*. dBigH1 is exchanged for a shorter variant upon cellularization of the blastoderm and activation of embryonic transcription, but is retained in the PGCs. dBigH1 loss of function mutants precociously activate transcription in both the somatic and germ line cells (Pérez-Montero et al., 2013). Thus, histone variants may play a conserved role in regulating the timing of embryonic genome activation in PGCs and somatic cells.

In addition to these clues in the nuclei, some cell surface changes suggest that the sMics may not respond to differentiation signals. Scanning electron microscopy that suggested sMic plasma membranes are smoother than somatic neighbors (Dale et al., 1997). This observation could reflect a broad membrane rearrangement in the micromere lineage. Perhaps in support of this premise, the micromeres, and subsequently the sMics, have reduced ABC/multidrug transporter activity based on efflux of fluorescent reporter molecules (Campanale and Hamdoun, 2012). A reduction of multidrug activity in the sMics is surprising, since protection of the germ line from toxicants would seemingly confer an advantage. However, efflux activity reduction may sensitize the sMics to migration cues, as inhibition of MDRs results in improper coelomic pouch homing. Loss of efflux activity may be a consequence of a greater plasma membrane modification, hinted at by the earlier electron microscopy; perhaps, the sMics broadly internalize transmembrane proteins. Furthermore, the sMics are enriched for the mRNA of a Sprouty family member. Sprouty family proteins are negative regulators of RTK signaling and proliferation (Kim and Bar-Sagi, 2004). Taken together, these observations depict the sMics as a cell type that “covers its ears” and ignores differentiation signals.

Differential stability of mRNA in the germ line and soma

The sMics have a unique propensity for retaining inherited RNA. Transcriptomic analyses, and specific examination of Vasa and Seawi RNAs, suggest that the sMics generally inherit maternally supplied RNA, rather than transcribe them *de novo*. These RNAs are typically ubiquitously distributed throughout the early embryo, and retained in the sMics, but turn over only in somatic cells later. This pattern of acquisition is consistent with the observation that the sMics are transcriptionally repressed (Swartz et al., 2014; Voronina et al., 2008; Yajima et al., 2014). Furthermore, even exogenous mRNA injected into zygotes, while eventually degraded in somatic cells, is retained in the sMics for at least one week after fertilization (Gustafson and Wessel, 2010; Oulhen and Wessel, 2013). This RNA retention phenomenon occurs independently of sequence information, because several reporters, containing mCherry, GFP, and different UTRs, all are similarly retained in the sMics. Instead, RNA retention is probably reflective of generally increased stability for mRNA in the sMics relative to somatic cells. The sea urchin embryo achieves this differential stability at least in part by repressing accumulation of the major deadenylase CNOT6 in the sMics, while expressing it in all other blastomeres (**Figure 7**). Without its full nuclease activity, the CNOT complex would be less capable of shortening poly-A tails, making mRNAs substrates for various degradation processes. CNOT6 is directly depleted in the sMics by Nanos2 repression of its mRNA. The CNOT6 3' UTR contains two consensus PRE motifs, which are each required for CNOT6 mRNA to be degraded in the sMics. Importantly, Nanos knockdown prevents CNOT6 degradation while forcing expression of CNOT6 in the sMics disrupts their ability to retain the germ line determinants Vasa and Piwi. Conversely, broad knockdown of CNOT6 expands the domain of retention of both Piwi RNA, and

exogenous RNA. These results highlight an important difference in RNA stability between germ line and soma in the sea urchin embryo that is important for inheriting germ line molecules.

Differential RNA degradation and protection has been noted in several different species, particularly in those that use preloaded strategies. Hsp83 and Nanos RNAs provide examples in the *Drosophila* embryo. Both of these RNAs are broadly distributed throughout the oocyte, egg, and syncytial embryo; in the case of Nanos, only about 5% of its total transcripts are enriched at the early posterior pole (Bergsten and Gavis, 1999). Thus, some other process must fine-tune their localization to the PGCs. Analysis of the 3'UTRs of Nanos and Hsp83 has turned up cis elements that confer lability in somatic cells (Bashirullah et al., 1999; Dahanukar and Wharton, 1996). The RNA binding protein Smaug has been identified as the maternally supplied factor required to degrade Hsp83 RNA in *Drosophila*. Smaug targets Hsp83 for degradation by the CNOT complex, and represses translation of unlocalized Nanos (Jeske et al., 2011; Semotok et al., 2005). The piRNA pathway has been also been implicated in the turnover of maternal Nanos mRNA in *Drosophila* somatic cells by interacting with the CNOT complex (Rouget et al., 2010). In the zebrafish, Nanos1 RNA is also differentially regulated between PGCs and soma by a microRNA: miR-430 (Mishima et al., 2006).

Furthermore, specific 3' UTR elements confer protection to certain RNAs in the PGCs. Such elements have been identified in *Drosophila* Hsp83; when swapped with the Hsp70 3'UTR, Hsp83 is degraded in both somatic cells and PGCs simultaneously. Thus, the RNA degradation pathway is active in the PGCs, but certain RNAs are masked from its action. The trans-acting factors that mediate protection in the fly have not yet been

definitively identified, but protective factors have been discovered in vertebrates. Dnd1 is one such factor, an RNA binding protein that is essential for germ line development in zebrafish and mice (Weidinger et al., 2003; Youngren et al., 2005). In zebrafish, Dnd1 binds the 3'UTR of Nanos in direct proximity to miR-430 sites, and occludes access by the miRNA machinery (Kedde et al., 2007). Another conserved germ line factor, Dazl, also opposes miR-430 by enhancing polyadenylation of Nanos mRNA (Takeda et al., 2009). In each of these cases, specific mRNAs are protected by the addition of cis- and trans-acting factors. Thus, the sea urchin is the only known example which creates a general bottleneck in RNA turnover by the depletion of CNOT6. As both RNA binding proteins and miRNAs use the CNOT complex to effect target degradation, in the future, it will be important to investigate whether deadenylase depletion is a conserved phenomenon in germ cells. Expression analysis in the sea star, which uses an inductive PGC system, suggests mesodermal progenitors are indeed depleted for CNOT6 (Fresques et al., 2014).

METs are delayed in PGCs

The emerging picture is that animals that specify their germ line by preloading display a delay in both EGA and maternal transcript degradation in their PGCs. Some genomic approaches using FACS-isolated PGCs have directly tested the timing and extent of delays for EGA and maternal transcriptome degradation. *Drosophila* PGCs do not upregulate embryonically transcribed transcripts until 3 to 5 hours post fertilization, compared to the major somatic EGA, which occurs at 2 hours. A 1-3 hour delay in *Drosophila* time is substantial, as this period spans the time in which the PGCs were created, into gastrulation, until just before the PGCs begin migrating to the somatic

gonad. Furthermore, the *Drosophila* PGCs are delayed for degradation of maternal RNA by several hours. At least some mechanistic aspects of EGA and transcript degradation are similar between the germ line and soma, such as the shared requirement for the RNA binding protein Smaug (Siddiqui et al., 2012). Similarly, FACS of sea urchin sMics indicated that transcription is inhibited in the sMics from their creation, and they retain inherited mRNA substantially longer than somatic cells (Swartz et al., 2014). Future studies should test how long that repression persists; one possibility is that once the sMics have finished their migration into the coelomic pouches, transcriptional activity will resume, perhaps based on degradation of Nanos protein.

A continuum of maternal and embryonic contributions to development

We have highlighted a range of differences in maternal versus embryonic control of development amongst diverse animal species. In rapidly-developing organisms such as the fruit fly *Drosophila melanogaster*, the frog *Xenopus laevis*, and the zebrafish *Danio rerio*, the early embryo subsists entirely on maternally-provided factors for a protracted period of many cell cycles. It is only later in development that the embryonic genome activates. In contrast, organisms such as the sea urchin and the mouse initiate transcription immediately after fertilization. A more conserved feature of animal development appears to be a broad degradation of the maternal transcriptome, which is critical for cell fate specification. In animals with a classically defined midblastula transition, like the fruit fly, frog, and zebrafish, maternal transcriptome degradation and embryonic genome activation are coupled, and occur with remodeling of the cell cycle. However, in the sea urchin, maternal transcriptome degradation does not appear to

occur until many hours after the embryonic genome has activated, suggesting that these two processes can be decoupled.

Given the pervasiveness of global RNA turnover events throughout the animal tree, we have proposed that this MET may have been essential for the transition from a unicellular ancestor to a multicellular body with specialized cell fates (Swartz et al., 2014). We have highlighted the importance of miRNA and RNA binding protein-mediated turnover of maternal mRNA in the sea urchin and other organisms; blocking these processes broadly perturbs cell fate specification in development. The miRNA pathway also has a well-appreciated requirement for the differentiation of many adult stem cells in myogenesis, skin homeostasis, and hematopoiesis (Gangaraju and Lin, 2009). Global RNA turnover “clears the slate” for somatic fate specification. In contrast to the somatic cells in the sea urchin embryo, the PGCs (i.e. the sMics) protect and retain inherited mRNA and remain transcriptionally inert—in other words, they are insulated from the MET which occurs in the soma. This mechanism of germ line – soma separation, which we call the Time Capsule mode, bears striking similarity to differential RNA stability between germ line and soma observed in *Drosophila* and zebrafish, suggesting that it may be a deeply conserved strategy (Bashirullah et al., 1999; Mishima et al., 2006) (**Figure 7**). With the transition to multicellular animals, the true novelty was the soma—the specialized parts devoid of reproductive potential—as the unicellular ancestor had to play the role of both germ line and soma by itself. The diverse METs of many species documented here may be functional remnants of how that primordial separation may have occurred, by a broad degradation of RNA to allow division of labor, and insulation of a select subset of cells to remain reproductively potent.

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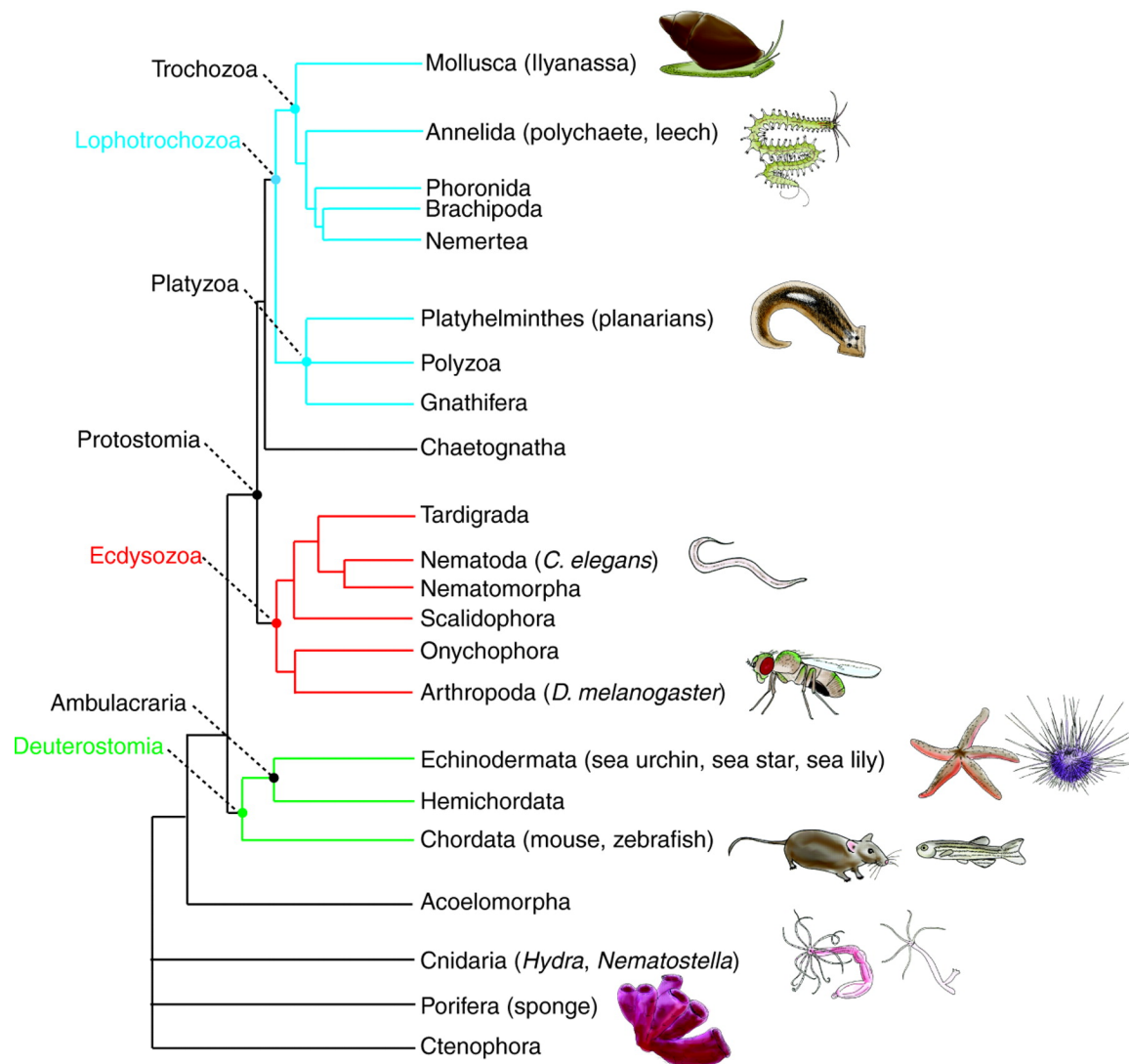


Figure 1. Metazoan Phylogenetic Tree (Reproduced from Juliano, Swartz, and Wessel, 2010). Phylogenetic relationships of major animal groups, with representative organisms highlighted within parenthesis and illustrations.

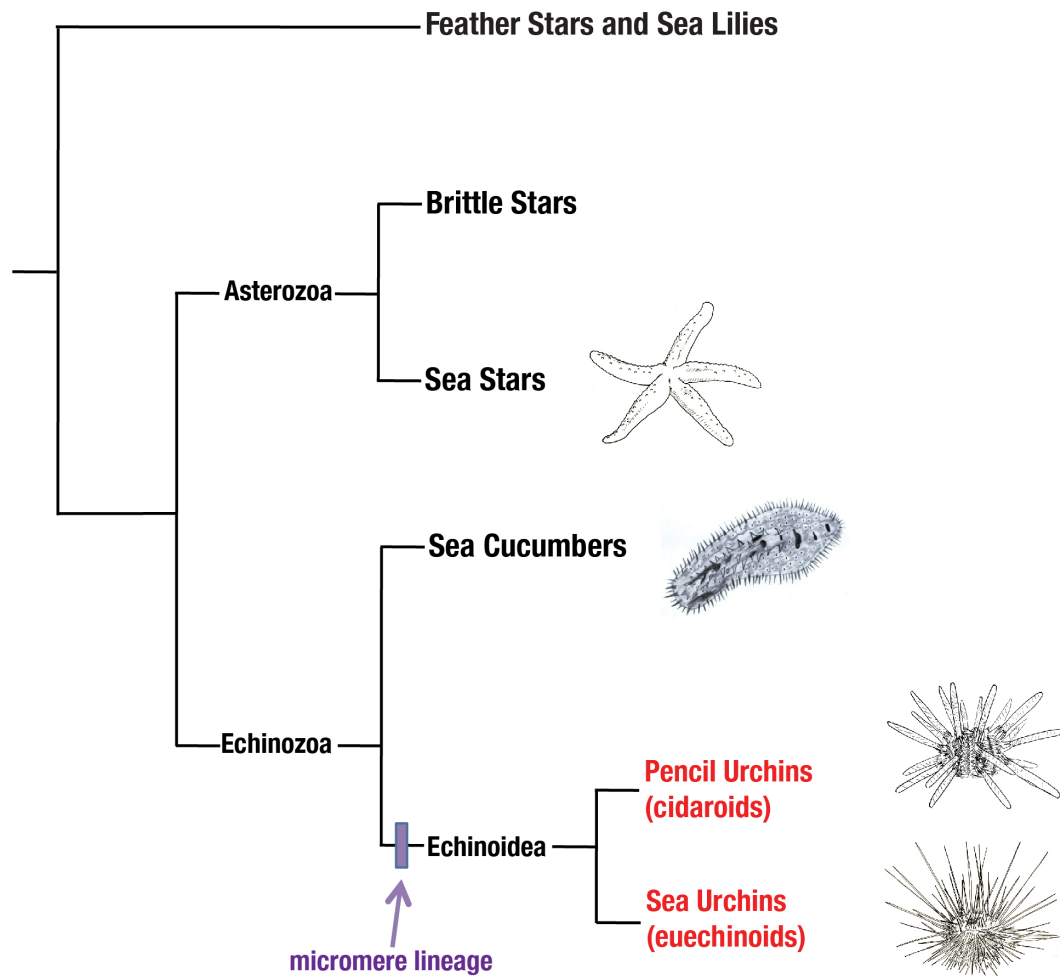


Figure 2. Species relationships of echinoderm classes (Reich et al., 2015). The relationships between major echinoderm groups are indicated, with illustrations provided for species discussed in the text. The inferred stem at which the micromere lineage was acquired is indicated in purple. Extant species with micromeres are indicated in red.

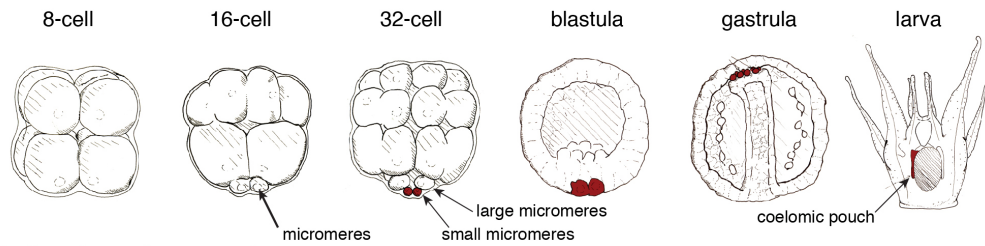
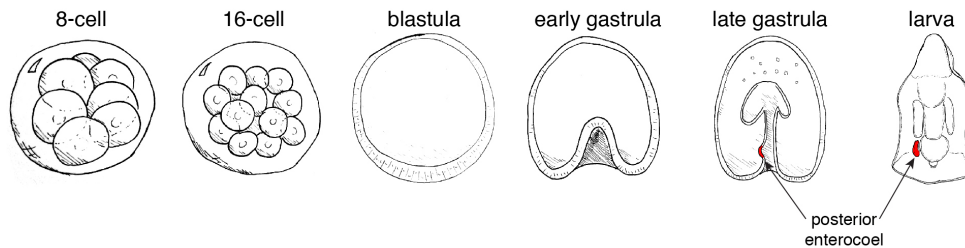
A. Sea urchin embryogenesis:**B. Sea star embryogenesis:**

Figure 3. Representative embryonic stages of echinoderms. (A) Embryogenesis in the sea urchin is distinguished by highly regular cleavages, the third of which is asymmetric, yielding a 16-cell embryo of differently sized blastomeres. At the vegetal pole, a quartet of 4 small blastomeres, the micromeres, is produced. At the next cleavage, the micromeres divide asymmetrically to produce the large and small micromeres (sMics). The large micromeres ingress into the blastocoel and form the skeleton, while the sMics (red) are the PGCs in the sea urchin. The sMics translocate along the tip of the gut during gastrulation, and subsequently assort into the two coelomic pouches. The sMics contained in the left pouch will contribute to the adult germ line. (B) Embryogenesis of the sea star, which may represent the ancestral mode of germ line segregation in echinoderms. The early cleavages are equal, yielding embryos of equally sized blastomeres with no morphologically overt polarity. A large, hollow blastula forms, and gastrulation initiates before any mesenchyme enters the blastocoel. In the later gastrula, two coelomic pouches form at the anterior tip of the gut. Subsequently, a smaller posterior pouch called the posterior enterocoel (indicated in red) forms on the left of the gut, which is visibly distinct from the gut in the larva. The posterior enterocoel contains the likely PGCs.

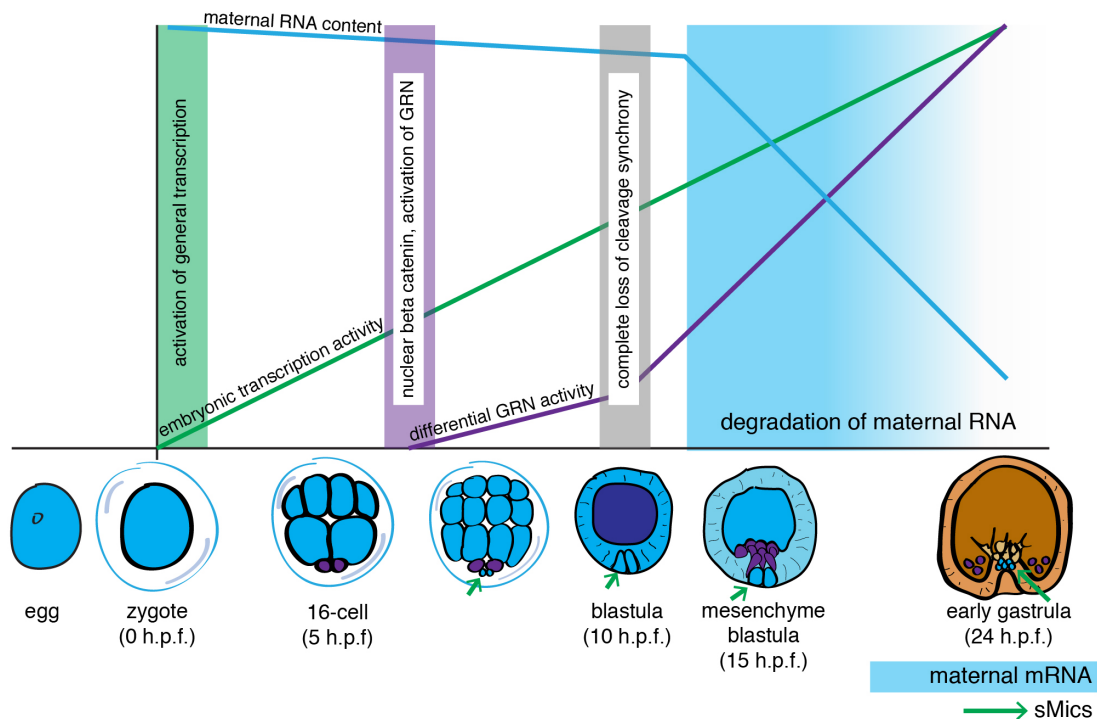


Figure 4. Summary of METs in sea urchin embryogenesis. Important embryonic milestones are denoted with embryo illustrations. General transcription based on incorporation of radioactive nucleotides initiates at fertilization and steadily increases through early development (green line). The regionally specific gene regulatory network and fate specification first occurs at the 16-cell stage, when beta catenin is nuclearized in the micromeres (purple line). We hypothesize that the differential GRN further diversifies in activity when the cell cycle is remodeled at approximately 10 h.p.f. and cleavage synchrony is lost. Based on mRNA expression dynamics, we suggest that there is a major degradation of maternal RNA that occurs as the embryo prepares to gastrulate. Maternal mRNA is represented in blue in embryo descriptions, and is lost in somatic cells in the gastrula, but retained in the sMics (green arrows).

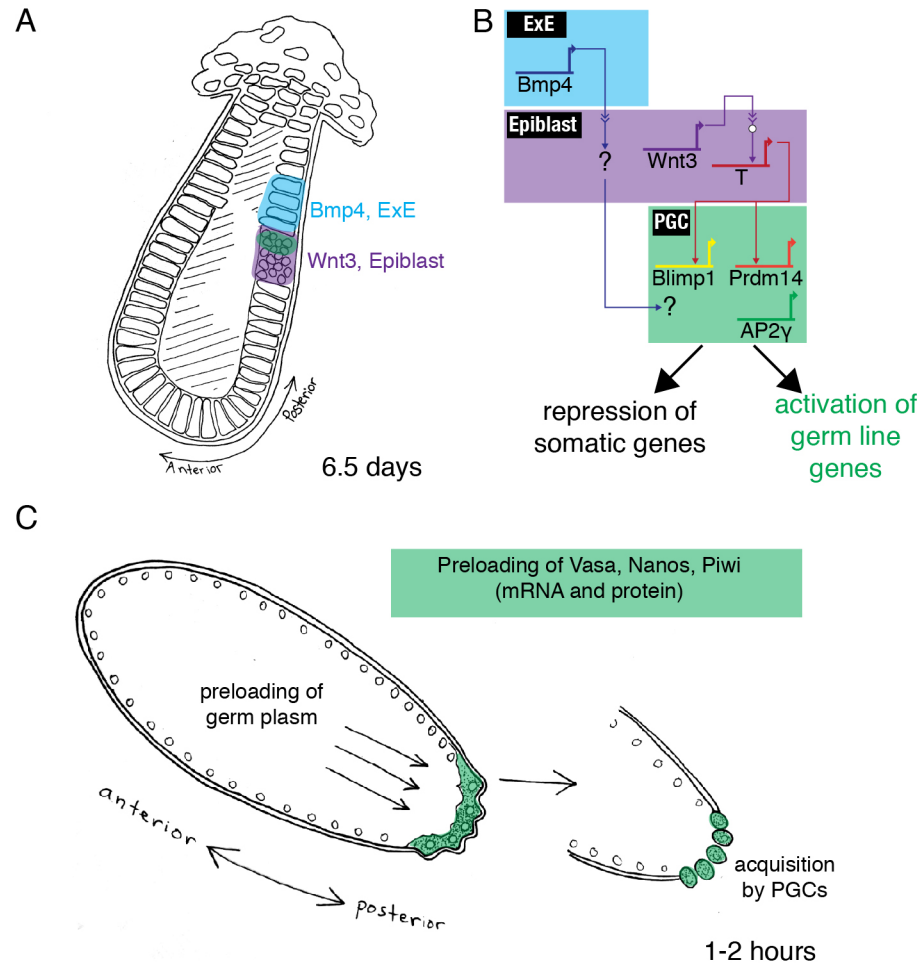


Figure 5. Strategies for germ line segregation by induction or preloading. (A) Germ line specification by induction. Shown is a simplified mouse embryo 6.5 days post fertilization. Bmp4 signaling from the extraembryonic ectoderm (ExE, blue) and Wnt3 expressed in the proximal epiblast (purple) are synergistically required to activate germ line transcriptional regulators in the PGCs (green). (B) Regulatory diagram depicting Wnt3 activating the mesodermal transcription factor T, which in turn activates Blimp1 and Prdm14, which along with AP2γ, form a tripartite network that activates germ line gene expression and represses somatic fate. The activating role of Bmp4 has not been directly delineated, but is required to license the PGCs to respond to the Wnt3 signal. (C) Germ line segregation by preloading. Shown is a syncytial blastoderm staged *Drosophila* embryo, with maternally-localized germ plasm in green. The PGCs are the first to cellularize in this embryo, and acquisition of the germ plasm directs them towards germ line fate.

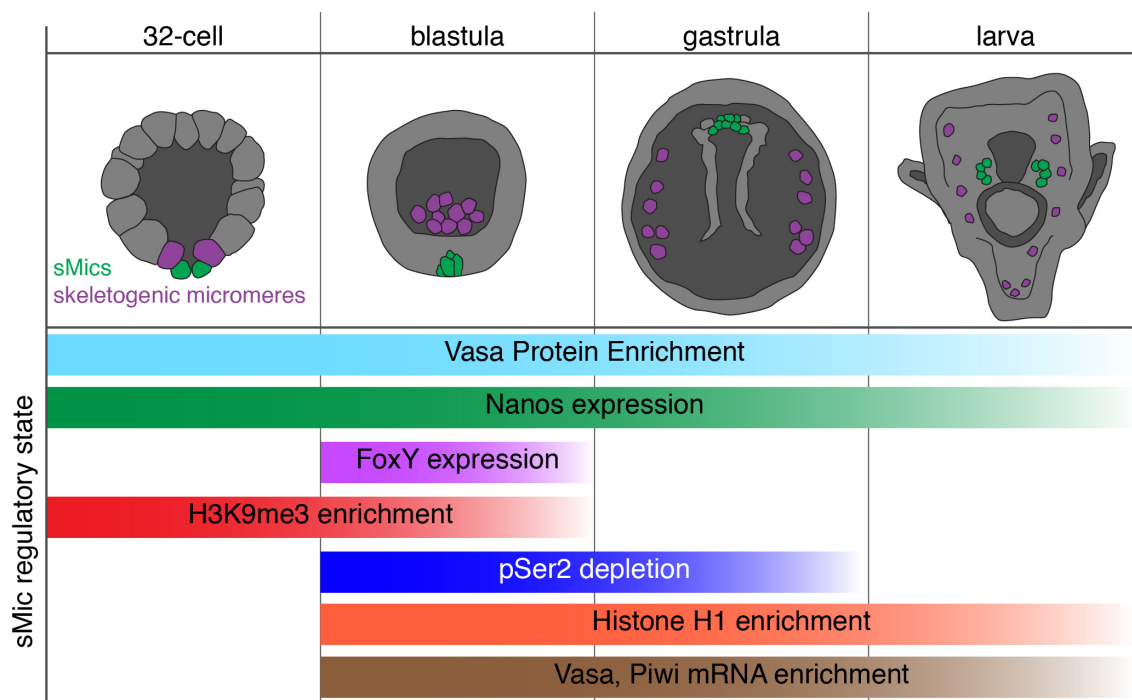


Figure 6. Temporal dynamics of sMic regulatory state (Adapted from Wessel et al., 2014). Location of the sMic lineage is indicated in green, from 32-cell stage into the larva. Colored bars indicate the presence or absence of gene expression, or enrichment or depletion for specific markers.

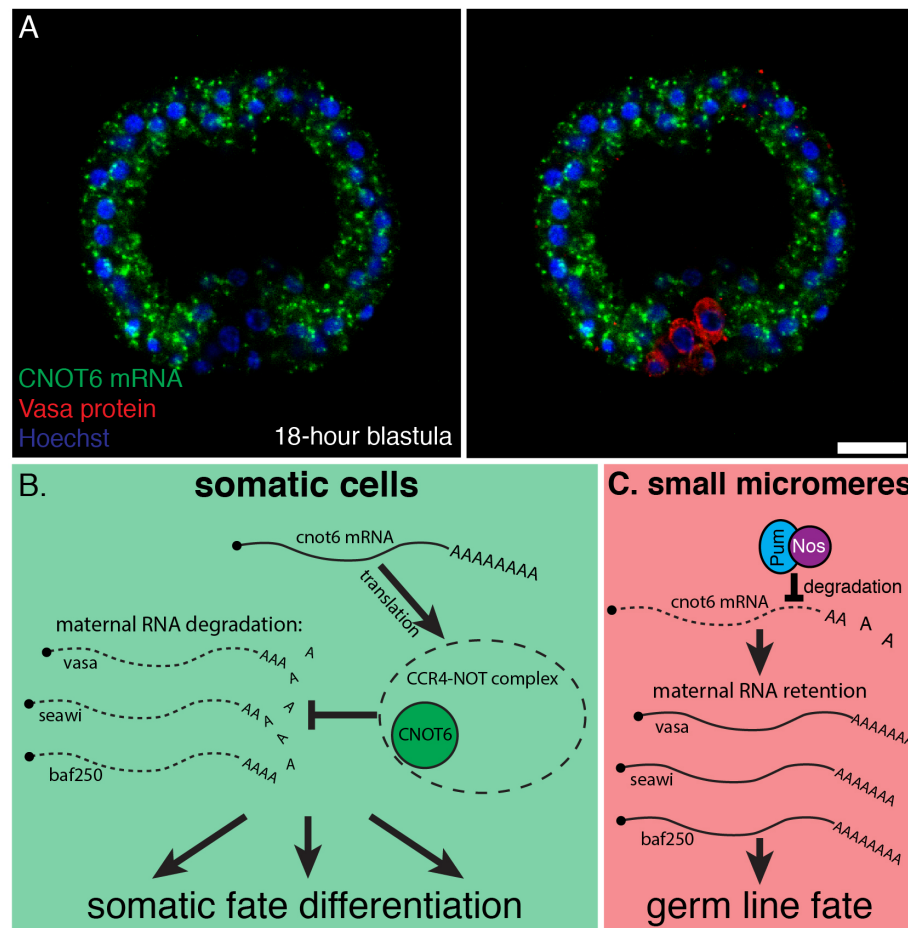


Figure 7. Time Capsule model for germ line specification (Reproduced from Wessel et al., 2014). (A) CNOT6 mRNA (green) is present in all somatic cells but depleted in the sMics (red, Vasa protein) in the 18 h.p.f. blastula. (B,C) Model for differential RNA stability between the somatic cells and sMics. (B) CNOT6 deadenylase is broadly present, and destabilizes maternal transcripts such as Vasa, Seawi, and Baf250 by shortening their poly-A tails. (B) These RNAs are protected in the sMics, because Nanos (Nos) and its partner Pumilio (Pum) repress the accumulation of CNOT6. Scale bar = 20 μ .

Chapter 2: Deadenylase Depletion Protects Inherited mRNAs in Primordial Germ Cells

Swartz, S.Z., Reich, A.M, Raz, T., Milos, P.M., Campanale, J.P., Hamdoun, A., and Wessel, G.M. (2014). *Development*, **140**: 3134-42.

I performed all experiments in this study except Helicos deep sequencing library preparation and immunoprecipitation of Nanos.

Abstract

A crucial event in animal development is the specification of primordial germ cells (PGCs), which become the stem cells that create sperm and eggs. How PGCs are created provides a valuable paradigm for understanding stem cells in general. We find that the PGCs of the sea urchin *Strongylocentrotus purpuratus* exhibit broad transcriptional repression, yet enrichment for a set of inherited mRNAs. Enrichment of several germline determinants in the PGCs requires the RNA-binding protein Nanos to target the transcript that encodes CNOT6, a deadenylase, for degradation in the PGCs, thereby creating a stable environment for RNA. Misexpression of CNOT6 in the PGCs results in their failure to retain *Seawi* transcripts and Vasa protein. Conversely, broad knockdown of CNOT6 expands the domain of *Seawi* RNA as well as exogenous reporters. Thus, Nanos-dependent spatially restricted CNOT6 differential expression is used to selectively localize germline RNAs to the PGCs. Our findings support a 'time capsule' model of germline determination, whereby the PGCs are insulated from differentiation by retaining the molecular characteristics of the totipotent egg and early embryo.

Introduction

The germ line provides an immortal link between generations by transmitting heritable information from parent to progeny. Specification of the animal germ line typically occurs during embryogenesis, when primordial germ cells (PGCs) fated to become the gamete-producing stem cells of the adult are segregated from somatic lineages. PGCs in the numerous species studied share common molecular signatures, including the RNA helicase Vasa, the translational repressor Nanos and the Argonaute family member Piwi

(Ewen-Campen et al., 2010). Surprisingly, despite this conservation of gene expression, the developmental routes that lead to it are remarkably diverse. Strategies for PGC segregation can be considered within a continuum of inherited and inductive mechanisms. An example of the inherited mode is that of *Drosophila melanogaster*, the PGCs of which are the first cells to form in the embryo. Their specification involves maternally supplied determinant mRNAs and proteins, collectively called germ plasm, which is actively transported and inherited by the presumptive PGCs. Conversely, PGCs in the mouse are specified by inductive signaling originating from the extraembryonic ectoderm. Most knowledge regarding these disparate mechanisms comes from studies in *Drosophila*, *Caenorhabditis elegans*, zebrafish and mice (Ewen-Campen et al., 2010; Pehrson and Cohen, 1986; Seydoux and Braun, 2006; Tanaka and Dan, 1990; Yajima and Wessel, 2011, 2012).

Comparatively little is known about PGCs outside these groups. Echinoderms, a large and diverse phylum, form part of the sister group to the chordates. The best-studied examples of this group are the sea urchins, including *Strongylocentrotus purpuratus*. In this species, four PGCs called small micromeres (sMics) are created by an asymmetric division at the fifth embryonic cleavage (Ewen-Campen et al., 2010; Juliano et al., 2006; Pehrson and Cohen, 1986; Seydoux and Braun, 2006; Tanaka and Dan, 1990; Yajima and Wessel, 2011, 2012) (Fig. 3C). After their formation, the sMics divide once during gastrulation to give eight descendants. These cells then assort into larval niches called the coelomic pouches, which are the major contributors to the juvenile sea urchin (Pehrson and Cohen, 1986; Tanaka and Dan, 1990). The early creation of the sMics is perhaps suggestive of inherited specification; however, sea urchins do not possess a classically defined germ plasm of aggregated germ line determinants. Instead, germline

RNAs, including those of Vasa and Seawi (a Piwi family member), are maternally deposited, broadly distributed in early embryos and later refined to the sMics during gastrulation (Juliano et al., 2006; Seydoux and Braun, 2006).

To better understand the specification of the sMics, we used a transcriptomic approach to identify sMic-enriched mRNAs. We learned that the sMics are broadly transcriptionally repressed and identified transcripts, like Vasa and Seawi, that are ubiquitous in the early embryo but are later turned over in somatic cells to result in sMic enrichment. The expression dynamics of these discovered transcripts imply a post-transcriptional mechanism by which sMics retain RNA. Transcriptome analysis identified the mRNA encoding the CCR4-related deadenylase CNOT6 as uniquely depleted in the sMics. This depletion is dependent upon the RNA-binding protein Nanos and sequence elements in the CNOT6 3' UTR that match the highly conserved binding consensus for Pumilio, the binding partner of Nanos. Depletion of CNOT6 is required for retention of Vasa protein and Seawi RNA in the sMics.

Results

Differential expression analysis and identification of sMic-enriched transcripts

We developed a method for isolating PGCs en masse. In *S. purpuratus*, the sMics selectively retain the fluorescent dye calcein due to altered multidrug transporter activity (Campanale and Hamdoun, 2012) (**Fig. 1A,B**), even when dissociated into single-cell suspensions (**Fig. 1C**). We purified sMics by fluorescence-activated cell sorting (FACS), which yielded approximately 0.5% of the total population of the embryo (**Fig. 1D**; **supplementary material Fig. S1A-F**). By qPCR, isolated calcein-positive cells were 16-

fold enriched for Nanos, a known sMic-specific transcript, but were not enriched for Spec, an ectodermal transcript (**supplementary material Fig. S1G**). With this indication of purity, total RNA was then isolated and deep-sequenced without amplification from three biological replicates: isolated sMics, non-sMics and disaggregated whole embryos. After assessing variation between samples by multidimensional scaling (MDS) analysis, we performed differential expression analysis to discover sMic-enriched and -depleted transcripts (**supplementary material Fig. S1H-J**). In summary, with a significance cutoff of 0.05 (false discovery rate, FDR), we identified a union set of 230 differentially expressed transcripts (both sMic enriched and depleted) between these comparisons (**Fig. 1E; supplementary material Fig. S1I,J; Tables S7-S9**).

The sMic-enriched transcripts included Nanos and Delta, which have previously been identified as sMic-localized, as well as SpG-cadherin, which is required for sMic fate in *S. purpuratus* and is enriched in the sMics of *Lytechinus variegatus* (Juliano et al., 2010; Miller and McClay, 1997; Oliveri et al., 2002; Yajima and Wessel, 2012). The sMic-enriched transcripts fell into diverse functional categories, but transcriptional regulation and RNA binding were overrepresented according to a gene set enrichment analysis (**supplementary material Table S1**). Several sMic transcripts lie within the same pathway. For example, we identified the DNA-binding factor Baf250 and the ATPase Brg1, which both assemble into the pluripotency-associated esBAF chromatin remodeling complex (Lessard and Crabtree, 2010). In addition to Delta, we also identified MibL, a potential regulator of Notch/Delta signaling (Le Borgne and Schweisguth, 2003).

The sMics are broadly transcriptionally repressed

Surprisingly, the majority of sMic-enriched transcripts we discovered appear to be maternally deposited. To examine temporal expression dynamics of sMic-enriched genes, we used a microarray dataset containing the whole-embryo expression level of all genes at multiple time points (Wei et al., 2006). On average, sMic transcripts were at maximum abundance at 2 and 15 hours post fertilization (hpf) (**Fig. 2A**). sMic transcripts then sharply dropped in abundance at 30 hpf, just after the onset of gastrulation. By calculating the 30 h/15 h abundance ratio, we found that sMic transcripts were statistically overrepresented for decreasing abundance compared with the whole-transcriptome average and with sMic-depleted transcripts (**Fig. 2B**). These dynamics suggest maternal loading followed by broad turnover. sMic nuclei are depleted for RNA polymerase II phosphorylated at serine 2 of the C-terminal domain, a marker of transcriptional elongation (**Fig. 2C,D**) (Seydoux and Dunn, 1997). This depletion is first apparent at blastula stage and persists through gastrulation. Furthermore, sMic nuclei are highly enriched for histone 3 lysine 9 trimethylation (H3K9me3), a heterochromatin marker (**Fig. 2E,F**). Thus, we infer that sMics are transcriptionally repressed relative to their somatic neighbors. Furthermore, we did not detect transcripts that accumulated exclusively in the sMics. In this regard, Nanos remains the unique exception (**Fig. 3A**). Rather, sMic transcripts were broadly detectable in eggs and early embryos until blastula-to-gastrula stage, when broad turnover occurs in all cells except the sMics. Such genes include Baf250, Ctdspl2/SCP2 [an RNA polymerase II (RNAPII) C-terminal domain phosphatase], z62 (a C2H2 zinc-finger protein) and MibL (**Fig. 3B**; **supplementary material Fig. S2A-F**). These observations suggest that sMics retain maternally loaded and zygotically transcribed transcripts, which are cleared from somatic

cells during the blastula-to-gastrula transition. Given this repression and the sMic transcript dynamics, we suggest that sMics inherit and retain their select mRNAs rather than actively transcribing them (with the important exception of Nanos).

CNOT6 transcript is selectively degraded in the sMics by a Nanos/Pumilio dependent mechanism

Our transcriptome analysis and in situ hybridizations imply a mechanism by which the sMics stably retain inherited RNA. In addition, we previously found that RNA microinjected into the fertilized egg is degraded in resultant somatic cells but retained in sMics, independently of its sequence (Gustafson and Wessel, 2010; Oulhen and Wessel, 2013). Our study offers an explanation for these phenomena: the top sMic-depleted transcript encodes CNOT6 (FDR=2.40E-13), an ortholog of CCR4 and a broadly conserved deadenylase subunit of the conserved CCR4/POP2/NOT complex (Collart and Panasenko, 2012). As a major regulator of RNA stability, depletion of CNOT6 could enhance RNA retention. By fluorescence in situ hybridization (FISH), CNOT6 transcript is detectable ubiquitously in eggs through 32/60-cell embryos (**Fig. 3D-F**), but the transcript is uniquely depleted in the sMics by blastula stage (**Fig. 3G-I**). Nanos is a strong candidate for mediating this depletion. In diverse species, Nanos and its binding partner Pumilio recognize highly conserved motifs in the 3'UTRs of target transcripts, termed Pumilio response elements (PREs), leading to mRNA degradation (Chen et al., 2012; Gerber et al., 2006; White et al., 2001; Wreden et al., 1997). The three *S. purpuratus* Nanos paralogs are each expressed in the sMics and are required for their survival (Juliano et al., 2010). Furthermore, we find that the 3'UTR of CNOT6 contains two PRE sequences, suggesting that it is a Nanos/Pumilio target

(**supplementary material Fig. S3**). We therefore knocked down Nanos with a translation-blocking morpholino antisense oligo (MASO) targeting the two most abundant of the three paralogs. This previously characterized MASO results in the eventual death of the sMics, and its effects were rescued by expression of a MASO-insensitive Nanos construct, thereby demonstrating specificity (Juliano et al., 2010). In morphant embryos, CNOT6 mRNA accumulated in sMics (**Fig. 4A,B**). We next tested the two putative PREs as sequence-specific targets with MASOs complementary to these two sites, which we predicted would occlude binding of Nanos/Pumilio. Consistent with Nanos knockdown, PRE-protecting MASOs caused retention of CNOT6 mRNA in the sMics (**Fig. 4C**). To further test the motifs, we used reporter constructs containing either the full-length wild-type CNOT6 3'UTR, or the full-length UTR with the PREs mutated singly or in combination (**Fig. 4D-H**). The wild-type reporter recapitulated the sMic exclusion of the endogenous transcript (**Fig. 4E**); however, mutations of the PREs resulted in sMic retention (**Fig. 4F-H**). In further support of its role, Pumilio co-immunoprecipitates with Nanos (**supplementary material Fig. S4A**). We attempted to test the effect of Pumilio knockdown on CNOT6 accumulation; however, these embryos were developmentally arrested before blastula stage, probably pointing to pleiotropic effects (data not shown). Indeed, Nanos-independent roles for Pumilio have been identified (Van Etten et al., 2012; Weidmann and Goldstrohm, 2012). Indicative of its diverse functions, Pumilio protein is detectable in granules in all cells of the early blastula and is highly enriched in the Veg2 mesodermal precursors of later blastulae (**supplementary material Fig. S4C,D**). Nanos-mediated degradation of CNOT6 transcript is surprising, especially in light of evidence that Nanos functions by recruiting the CCR4-NOT complex itself (Suzuki et al., 2012). One possibility is that the CCR4-NOT complex maintains functionality with only the Pop2-related nuclease CNOT7, which is present in sMics at

the transcript level (**supplementary material Fig. S2G**). Alternatively, maternal CNOT6 protein may be initially available in the sMics to degrade the mRNA, but then turned over later. Both possibilities are consistent with our conclusion that Nanos/Pumilio directs degradation of maternal CNOT6 mRNA in the sMics via PRE motifs in its 3'UTR.

CNOT6 repression is required for retention of germ line determinants

To test the requirement of CNOT6 repression for germline fate, we misexpressed CNOT6 in the sMics by multiple approaches. A CNOT6::mCherry fusion construct that expresses in all cells significantly reduced accumulation of a previously characterized sMic reporter, Vasa::GFP (**Fig. 5A-C**) (Gustafson et al., 2011). We next tested the accumulation of endogenous Vasa protein. Embryos were fixed at 42 hpf, following gastrulation and one division of the sMics, resulting in approximately eight descendants on average. Both CNOT6::mCherry, as well as PRE-protecting MASOs, predicted to stabilize endogenous CNOT6 in the sMics, resulted in significantly fewer Vasa protein-positive sMics (**Fig. 5D-G**). This observation could be explained by a failure in mitosis, cell death or loss of Vasa protein in the sMics. To distinguish between these possibilities, we stably labeled the sMic lineage by 5-ethynyl-2'-deoxyuridine (EdU) incorporation. Due to their slow cell cycle, sMics retain EdU pulsed before first cleavage, compared with other more rapidly dividing cells, enabling definitive lineage analysis (Tanaka and Dan, 1990). Both control and CNOT6-overexpressing embryos possessed similar numbers of sMics, indicating a loss of inherited determinants, but no other defects (**Fig. 6D**). Endogenous Vasa is probably lost later than the reporter construct (**Fig. 5A-C**) because of the abundance of maternally supplied Vasa protein, whereas injection of reporter RNA requires new translation (Voronina et al., 2008). Additionally, we tested the transcript

abundance of the endogenous Argonaute family member Seawi in the sMics with CNOT6 overexpression. Seawi transcript is normally present in all cells but is highly enriched in the sMics (Yajima et al., 2013). With CNOT6::mCherry expression, the sMics lose enrichment for Seawi RNA (**Fig. 6A-C**). We conclude that CNOT6 depletion is necessary for retention of germline determinants in the sMics.

Repression of the CNOT6 deadenylase may allow for increased background stability for inherited RNAs in the sMics. Therefore, we predicted that global CNOT6 knockdown would expand the domain of RNA retention. CNOT6 is required for normal development of the embryo; strong knockdown leads to profound endomesodermal defects (**supplementary material Fig. S4E,F**). We therefore tested the effects of weaker CNOT6 knockdown (under which development proceeds relatively normally) on Seawi transcript localization. In control embryos, Seawi transcripts are highly enriched in the sMics. However, when we globally reduce CNOT6 protein with either of two non-overlapping MASOs, Seawi transcripts are more broadly retained throughout the endomesoderm and oral ectoderm (**Fig. 6E-H**). To test the generality of CNOT6-mediated RNA retention, we used exogenous RNA encoding mCherry with an SV40 3' polyadenylation signal. As reported for other exogenous RNAs, this transcript is retained in the sMics but is degraded in somatic cells in a sequence-independent manner (**Fig. 7A**) (Gustafson and Wessel, 2010; Oulhen and Wessel, 2013). However, when CNOT6 is globally depleted, mCherry RNA is retained throughout the endomesoderm (**Fig. 7B,C**). Our results indicate that differential CNOT6 expression is crucial for proper accumulation of transcripts within the sMics and is required for normal development of somatic lineages.

Discussion

Our study reveals mechanistic insight into the divergence of germ line from soma. Uniformly dispersed mRNAs in the early embryo become highly asymmetric by the selective Nanos/Pumilio-mediated repression of CNOT6 in the germ cell precursors. This paradigm explains the localization of known sMic-enriched mRNAs, including Vasa and Seawi (Juliano et al., 2006), as well as foreign transcripts introduced in the early embryo (Gustafson and Wessel, 2010; Oulhen and Wessel, 2013). The RNA retention mechanism via CNOT6 depletion raises the question: is there specificity in the RNAs that sMics retain or is the retention completely nonselective? Although our dataset is probably incomplete, if retention were completely nonselective, one would expect to identify more than the 78 sMic-enriched transcripts we report. It is possible that the sMics possess mechanisms to exclude RNAs – Nanos/Pumilio is one such example, although there might be others that remain uncharacterized. Indeed, through bioinformatics we identified numerous transcripts that are depleted in the sMics, and it will be important in the future to investigate the mechanisms of their depletion. The fact that sMics generally retain RNA is probably necessary because they are transcriptionally quiescent. Transcriptional repression has also been documented in *Drosophila*, *C. elegans*, ascidians and mice, indicating that it is a fundamental feature of germline segregation (Nakamura and Seydoux, 2008; Shirae-Kurabayashi et al., 2011). Surprisingly, however, each organism achieves repression via distinct mechanisms. Although the precise nature of sMic repression is unknown, we find that the RNAPII phosphatase Ctdspl2 is sMic-enriched, indicating one possible mechanism.

Broad clearance of maternal RNA is a hallmark of the maternal to zygotic transition (MZT), a conserved event when developmental control is passed to the embryo. In *Drosophila*, there are two phases of degradation: the first occurs following egg activation and involves the RNA-binding protein Smaug, which recruits the CCR4-NOT complex to degrade diverse targets (Semotok et al., 2005). The PGCs possess degradation activity, but certain transcripts are protected in the PGCs by motifs in their 3'UTRs, perhaps by Oskar association (Bashirullah et al., 1999; Zaessinger et al., 2006). A second degradation process is driven by the mir-309 cluster at about 2 hpf (Bushati et al., 2008). The piRNA pathway also contributes to degradation of Nanos transcript in somatic cells and involves the CCR4-NOT complex (Rouget et al., 2010). In zebrafish, a primary effector of maternal RNA degradation at the MZT is mir-430 (Giraldez et al., 2006). It has been further observed that some mir-430 targets, such as Nanos, are degraded in the soma but protected in the PGCs (Mishima et al., 2006). In the sea urchin, the zygotic genome activates shortly after fertilization. However, the degradation aspect of the MZT in the future soma might be conserved via CNOT6 and could include small RNA mechanisms (Song et al., 2012).

Prior to this study, the only known mechanisms for stabilizing RNA in the germ line were via Dead end 1 (Dnd1) and Deleted in azoospermia-like (Dazl), which work by occluding microRNA-binding sites and by promoting deadenylation, respectively (Kedde et al., 2007; Mishima et al., 2006; Takeda et al., 2009). However, Dnd1 is not conserved outside of vertebrates. We show here in an early branching deuterostome that the deeply conserved deadenylase CNOT6 is repressed in its PGCs by Nanos/Pumilio, allowing for stable retention of inherited transcripts. As the PGCs are transcriptionally repressed, their inheritance might represent a 'time capsule' of early development; that

is, they must subsist solely on the mRNAs they retain from the egg, independently of a germ plasm (**supplementary material Fig. S5**). A consequence of this strategy might be that the PGCs remain insulated from differentiation into somatic lineages.

Furthermore, our model is consistent with an immortal cytoplasm hypothesis for the evolutionary origin of the segregated germ line at the transition from unicellular to multicellular animal life. The ancestral single-celled organism probably possessed the highly conserved factors found in animal germ lines, which were retained at the transition to multicellularity (Extavour and Akam, 2003). It is the somatic cells that acquired unique characters to diversify from the original, progenitor cell type, while sacrificing reproductive potential (Buss, 1987; Extavour, 2007). The diversification of the soma may have necessitated the evolution of global turnover events (e.g. the MZT) to eliminate RNAs associated with the egg. Instead of acquiring gametogenic capability anew, the embryonic germ cells remain protected and retain the characteristics of the egg. Downregulation of deadenylase activity provides a mechanism for understanding how cytoplasm that confers gametogenic potential is preserved in the segregated germ line.

Acknowledgements

We thank Stefanie Terrizzi and Emma Riley for assistance with FACS, Casey Dunn, Mark Howison and Jason Wood for computational expertise and helpful discussions, Geoff Williams for imaging assistance, Celina Juliano for critical feedback, TGZ for tuff feedback, and Novartis for providing PSC-833. Transcriptome analysis was conducted using computational resources and services at the Center for Computation and Visualization, Brown University.

Author Contributions

G.M.W. and S.Z.S conceived the project; S.Z.S., J.P.C., and A.H. developed FACS methods; S.Z.S. and N.O. performed experiments; A.M.R. performed bioinformatic analyses; T.R. and P.M.M. performed Helicos sequencing; S.Z.S. and G.M.W. wrote the manuscript, which was edited by A.M.R., N.O., J.P.C., and A.H.

Funding

This work was supported by the National Institutes of Health [F31 AG041637 to S.Z.S.; 2R01HD028152 to G.M.W.] and the National Science Foundation [IOS-1120972 to G.M.W.]. Deposited in PMC for release after 12 months.

Materials and Methods

Animals

S. purpuratus were maintained in aquaria containing artificial seawater at 16°C. Individuals were induced to shed gametes by shaking or by injection of 0.5 M KCl. Eggs were collected in filtered seawater (FSW) and sperm was collected dry. Eggs were fertilized in the presence of 1 mM 3-amino-1,2,4-triazole (3-AT) (Sigma) to prevent crosslinking of fertilization envelopes. Embryos were reared at 15°C at a density of about 0.2% (packed egg volume/seawater volume) in stirring culture vessels.

FACS isolation of sMics

Embryos were collected 15 hpf by straining through 45 μ m Nitex and concentrated to about 0.5% density in 50 ml FSW. PSC833 (Novartis) and calcein AM (C-AM, Invitrogen) were added to the FSW at 500 nM and 250 nM, respectively. The embryos were then incubated for 90 min at 15°C with constant rotation in 50 ml conical tubes. Embryos were

pelleted by centrifuging at 250 g for 30 s, washed twice in 50 ml of calcium-free seawater and then resuspended in a 10 ml 1 M glycine 25 mM EDTA solution. After incubating for 5 min on ice, the embryos were disaggregated by trituration through a transfer pipette 20 times. The single-cell suspension was pelleted by centrifugation at 250 g for 5 min at 4°C, washed three times in calcium-free seawater to a final sample volume of 4 ml. PSC833 was then added to 1 μ M final concentration. The cell suspension was sorted on a FACS Aria instrument (BD Biosciences) set to 4°C. For long sorts, staggered cultures were fertilized at 2 h intervals, and then labeled and disaggregated at 15 hpf to avoid cell death and changes to their transcriptional profile. Cells were first gated by forward and side scatter to remove debris and aggregates, and then sorted by fluorescence intensity versus forward scatter. Cells were sorted directly into 0.75 ml Trizol LS (Invitrogen) until the total volume reached 1 ml. Sorts, numbers of collected cells and RNA extraction yields are summarized in **supplementary material Table S2**.

Helicos sample preparation and deep sequencing

RNA was extracted using Trizol LS reagent as described by the manufacturer (Invitrogen). RNA was treated using RQ1 DNase (Promega) for 30 min at 37°C, then extracted with acid phenol:chloroform (Ambion). Three biological replicates of paired sMic, non-sMic and whole-embryo RNA were collected. Two replicate pairs were pooled from three separate sorts, whereas the third was collected from a single sort. Total RNA was stored in 100% ethanol, processed for RNA-seq without amplification or poly-A selection and sequenced by Helicos tSMS (Lipson et al., 2009). These quantitative short Helicos reads (averaging 30-35 nucleotides) were then mapped and counted to a de

novo reference transcriptome generated using Illumina technology (described below).

Helicos run statistics are listed in **supplementary material Table S3**.

Illumina sample preparation, deep sequencing and reference transcriptome assembly

RNA was extracted from several developmental stages, including ovary, 32-cell stage, 15 h blastula, 41 h gastrula and 4-day pluteus, using the RNEasy Mini kit (Qiagen) with on-column DNase. The isolated RNA was processed through standard procedures using the Illumina mRNA-Seq kit and sequenced on a single lane of a GAIIx using a read length of 105 bp with paired ends. The transcriptome was assembled using Velvet (1.0.09) and Oases (0.1.14) with a k-mer of 31 (Schulz et al., 2012). Exemplar sequences were selected from each locus based on abundance with a minimum length cutoff. The exemplar sequences were annotated with Blast2GO and compared by BLAST (minimum score of $1E-5$) with the *S. purpuratus* SPU gene predictions (Conesa et al., 2005; Sodergren et al., 2006). All Helicos reads, de novo transcriptome reads and the assembled transcriptome are available under BioProject PRJNA188114.

Whole-mount in situ hybridization (WMISH) and immunofluorescence

WMISH was performed as described previously (Juliano et al., 2006). Nanos probe generation is described by Juliano et al. (2010). Approximately 1 kb antisense probe templates were PCR-amplified from cDNA using a reverse primer tailed with the T7 promoter. Digoxigenin-labeled antisense probes were transcribed using the Roche DIG RNA labeling kit according to the manufacturer's instructions. Embryos of mixed developmental stages were fixed with MOPS-buffered PFA and hybridized for at least 5

days at 50°C with 70% formamide and 0.5 ng/ μ l probe. Hybridization was then visualized using either NBT/BCIP chromogenic detection or tyramide fluorescence amplification (TSA plus system, PerkinElmer). Vasa immunofluorescence was performed as described previously (Voronina et al., 2008). Rabbit antibodies to RNAP pSer2 and H3K9me3 were obtained from Abcam (ab5095 and ab8898, respectively; both 1:500). As the Vasa antibody was also raised in rabbit, co-labeling (e.g. Fig. 2) was performed as follows. Embryos were incubated with Vasa antibody (1:250) overnight at room temperature in PBST (0.1% Triton X-100, pH 7.4). The embryos were washed four times and then incubated 3 h at room temperature with rhodamine-labeled goat anti-rabbit Fab fragments. This procedure was then repeated sequentially with the pSer2 or H3K9me3 antibodies with FITC-labeled Fab fragments. Primer sequences used to amplify probe templates are listed in supplementary material Table S4. EdU lineage tracing of the sMics was performed with the Click-IT EdU imaging kit (Life Technologies). Briefly, embryos were soaked in 10 μ M EdU in FSW from fertilization until first cleavage, washed six times with FSW and allowed to develop until the desired stage. In situ hybridization (ISH) was then performed as described above. Following hybridization, EdU was detected with fluorescent azide according to the manufacturer's instructions. Finally, the in situ probes were detected as described above.

Cloning and reporter constructions

DNA fragments were PCR-amplified and cloned by standard methods. For overexpression studies, constructs were built in a modified pCS2 vector containing additional rare 8-base cutting restriction enzyme sites (Gökirmak et al., 2012). PRE mutant reporter constructs were generated by site-directed mutagenesis with the

QuikChange II kit (Agilent Technologies). Primers used for generating these constructs are listed in supplementary material Table S5.

MASO and mRNA microinjection

Custom MASOs were synthesized by GeneTools. The control MASO targets a divergent Nanos ortholog in a distantly related sea star species (*Patiria miniata*). MASO injection solutions contained 20% glycerol and 100 $\mu\text{g/ml}$ 10,000 MW Texas Red-Lysine or FITC dextran. MASOs were used at working concentrations listed below. Synthetic mRNAs were transcribed using the mMessage mMachine SP6 or T7 kit (Ambion) from linearized plasmid template. Eggs were dejellied by incubating for 10 min in pH 5.0 seawater and arranged in rows on protamine sulfate-coated Petri dishes. Fertilized eggs were injected with approximately 2 pl of MASO or RNA by constant pressure injection in the presence of 1 mM 3-AT (Sigma). The injected zygotes were then washed in filtered seawater and incubated at 15°C. Custom morpholino sequences are listed in supplementary material Table S6.

RNA isolation and qPCR

RNA for qPCR was isolated with the RNEasy Micro kit with on column DNase (Qiagen). First-strand cDNA synthesis was performed using MultiScribe reverse transcriptase (Invitrogen). qPCR was performed using an ABI 7900 real-time instrument with Fast Start Universal SYBR master mix (Roche). Four embryo equivalents of cDNA were used per reaction, which yielded CT values in the 20-30 range. Experiments were run in triplicate and normalized to ubiquitin. To account for variability in the amount of injected mCherry RNA, the 96 h time points were normalized to the mCherry levels tested at 18

hpf, a time point before RNA retention becomes apparent and therefore representative of the amount of RNA injected. mCherry qPCR primer sequences were as follows: F, 5'-CCCCGTAATGCAGAAGAAGA-3'; R, 5'-TCTTGGCCTTGTAGGTGGTC-3'

Supplementary material available online at:

<http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.110395/-/DC1>

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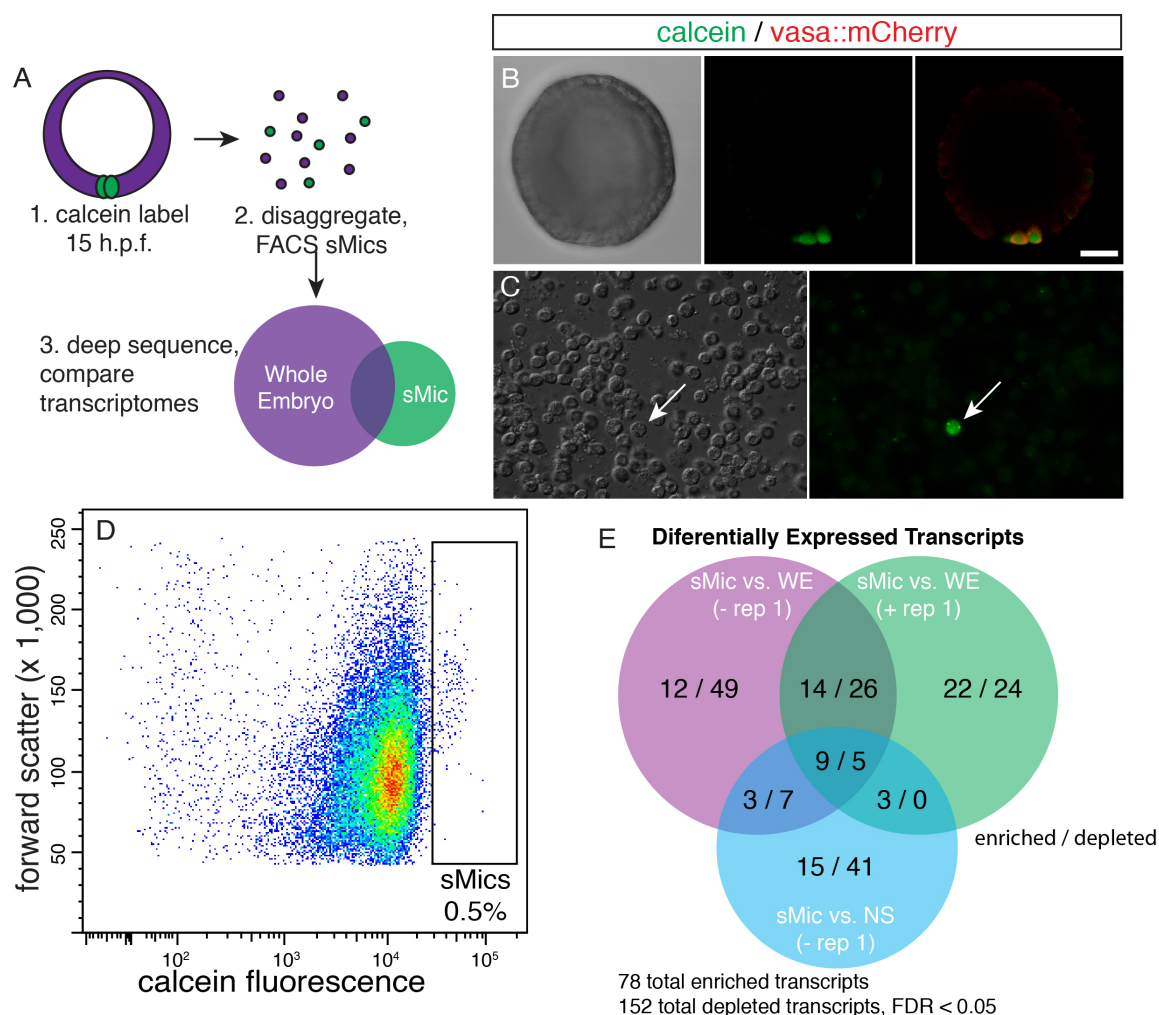


Figure 1. FACS isolation and deep sequencing of sMics. (A) Schematic diagram depicting FACS isolation, RNA deep sequencing and differential expression analysis. (B) sMics accumulate the fluorescent dye calcein at 15 hpf. sMics are co-labeled with an mCherry fusion reporter of Vasa, a conserved germline RNA helicase that localizes post-translationally. (C) Calcein labeling is retained in blastomeres dissociated at 15 hpf. Arrows indicate labeled sMic. (D) Representative scatter plot of sMics collected by FACS, plotted as forward scatter versus calcein fluorescence. Cells collected as putative sMics are indicated by the box, which represents 0.5% of the total cell sample. (E) Venn diagram summarizing the number of sMic-enriched and -depleted transcripts discovered in three differential expression comparisons with FDR<0.05: sMic versus whole embryo (WE) and sMic versus non-sMics (NS), with and without replicate 1 included. Transcript totals are given as sMic-enriched/sMic-depleted. Scale bar: 20 μ m.

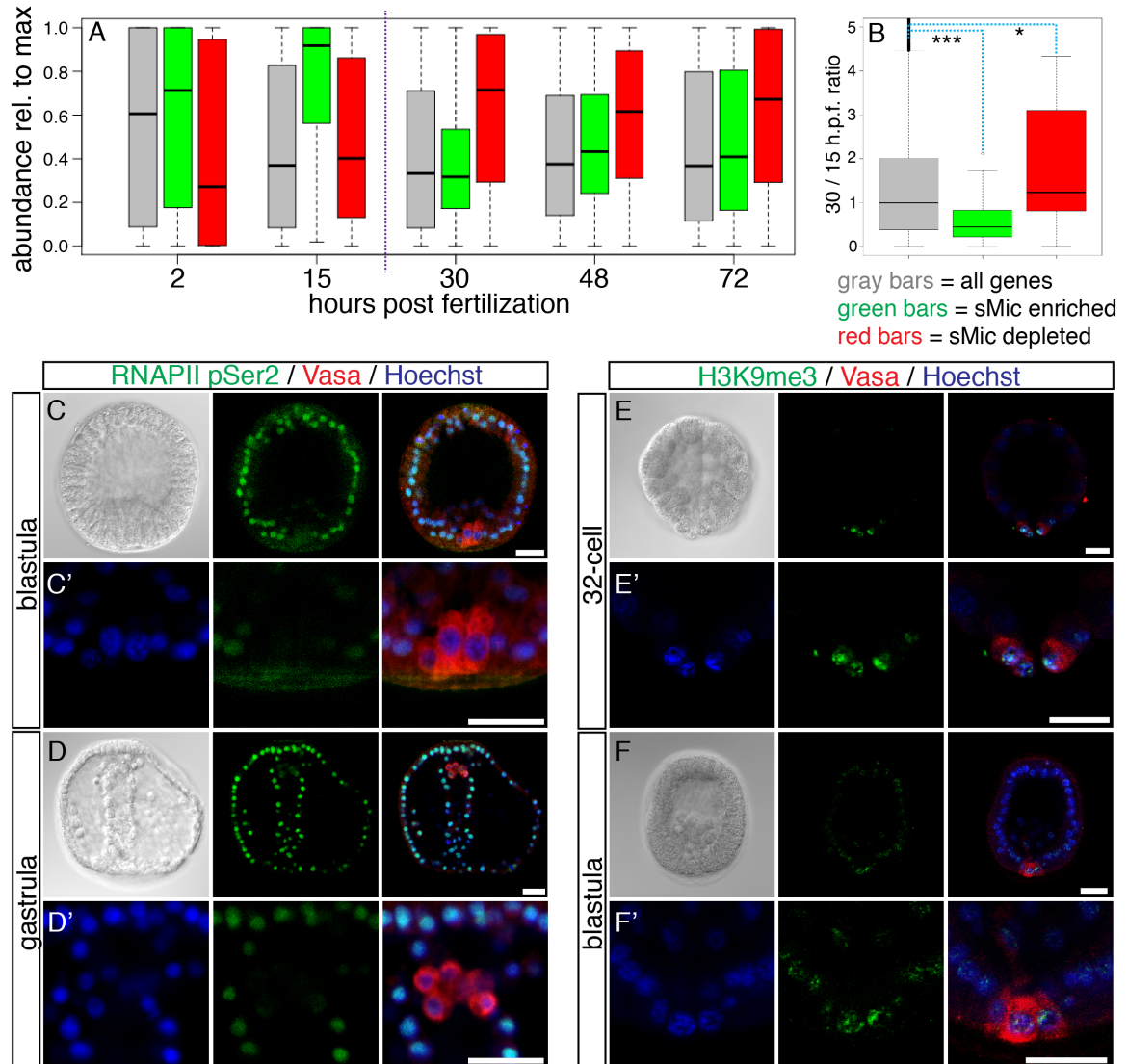


Figure 2. Transcriptional repression in sMics. (A) Box and whisker plots summarizing average relative abundance of sMic-enriched (green bars), sMic-depleted (red bars) and all transcripts (gray bars) in the microarray study at five developmental time points between 2 and 72 hpf. On average, sMic-enriched transcripts reach their maximum abundance before 30 hpf and then decrease. Conversely, sMic-depleted transcripts reach their maximum after 30 hpf. An average of all genes in the microarray study remains relatively constant through development. (B) Ratio of average expression levels from 15 to 30 hpf for sMic-enriched (green bars), sMic-depleted (red bars) and all transcripts (gray bars). *** $P=1.076e-06$, * $P=0.001357$ (Mann–Whitney U-test). (C–D') Immunofluorescence signal for RNAPII pSer2 (green) is depleted in sMics of blastula (C,C') and gastrulae (D,D'). (E–F') H3K9me3 (green) immunofluorescence is enriched in sMics in 32-cell embryos (E,E') and blastulae (F,F'). (C',D',E',F') Higher magnification views of the sMics, labeled with Vasa antibody (red) (Voronina et al., 2008). Scale bars: 20 μ m.

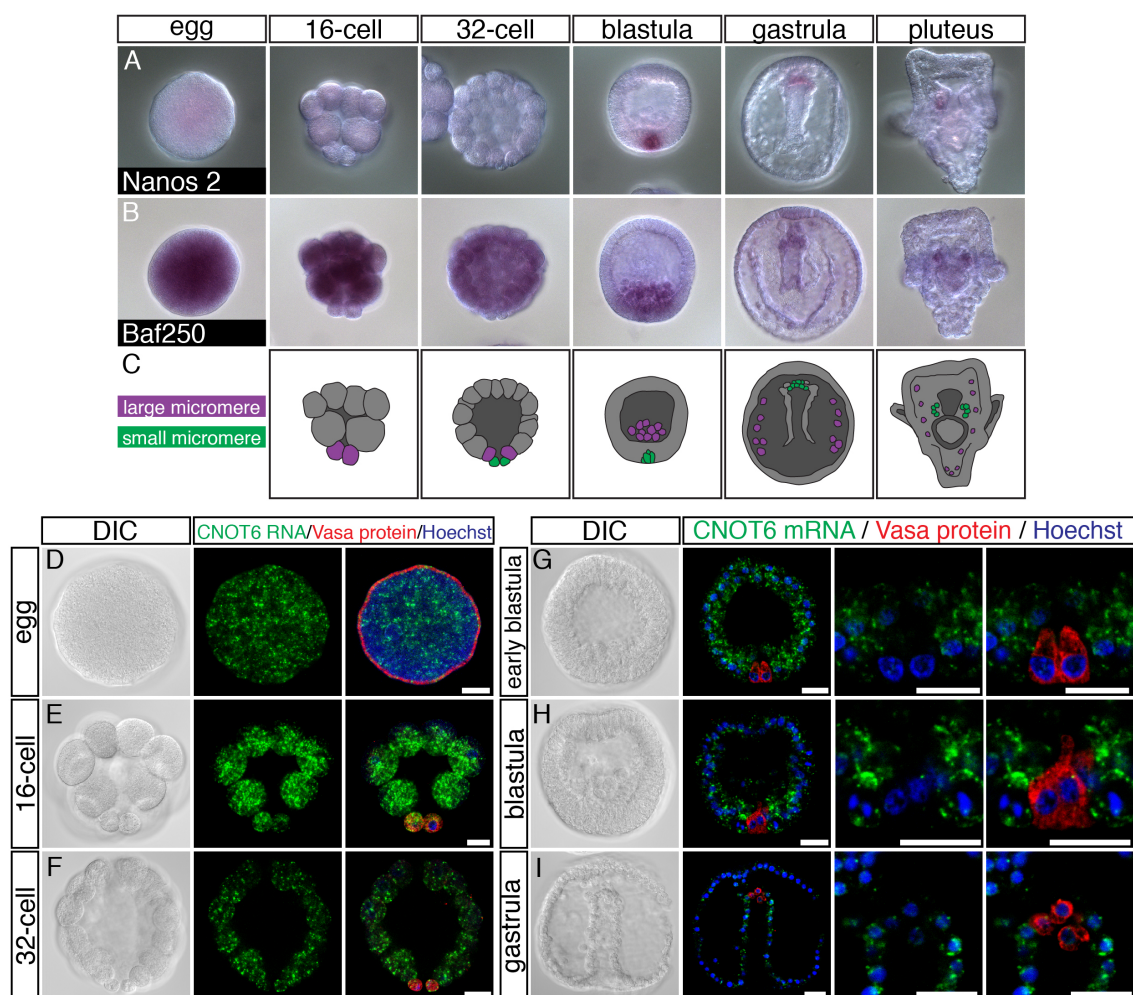


Figure 3. Localization of differentially expressed transcripts. (A,B) In situ hybridizations for sMic-enriched transcripts Nanos2 and Baf250. In contrast to Nanos, Baf250 transcript is detectable in eggs and is broadly distributed until blastula stage, when its localization is refined to the sMics. (C) Schematic depiction of the micromere lineage in early development. (D-I) FISH for CNOT6 transcript (green). CNOT6 transcript is depleted in the sMics (labeled by Vasa antibody, red) by blastula stage and remains depleted through gastrulation, after which it becomes undetectable. Scale bars: 20 μ m.

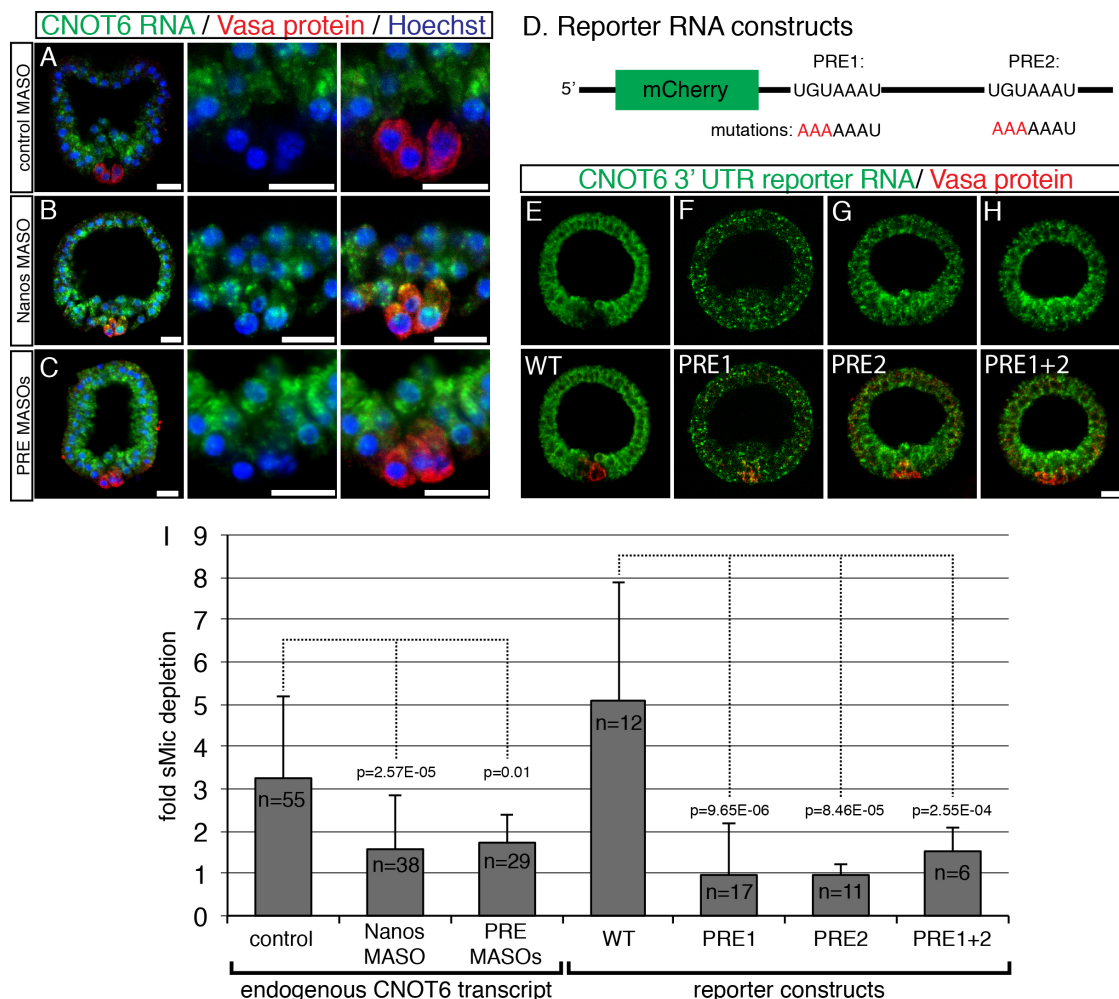


Figure 4. CNOT6 mRNA depletion in the sMics requires Nanos. (A,B) MASO knockdown of Nanos de-represses CNOT6 transcript in the sMics compared with control embryos. (C) Co-injection of MASOs targeting the two PREs also de-represses CNOT6 transcript in sMics. (D-H) A synthetic reporter construct (D) containing the full-length CNOT6 3' UTR recapitulates the localization of endogenous CNOT6 transcript (E). Mutating putative PRE sequences alone (F,G) or in combination (H) de-represses reporter accumulation in the sMics. (I) Pixel intensity quantitation of fold depletion in sMics versus the rest of the embryo for endogenous CNOT6 and reporter transcripts. P-values are calculated by two-tailed unpaired t-test. Scale bars: 20 μ m.

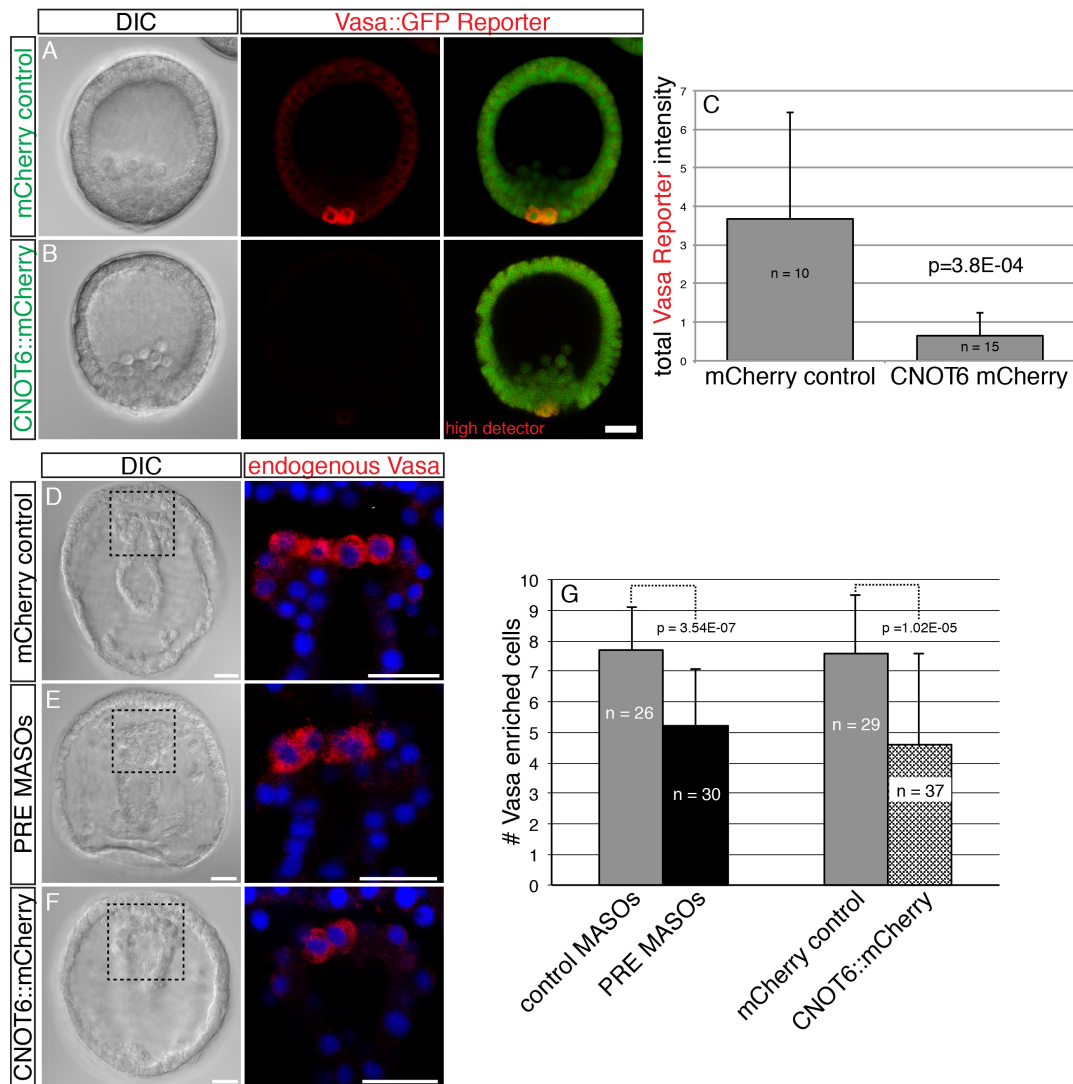


Figure 5. CNOT6 depletion is required for sMic Vasa protein expression. (A,B) Expression of CNOT6::mCherry (green) in all cells significantly reduces Vasa::GFP reporter signal (red) compared with mCherry alone (control) in 20 hpf embryos. Images at higher detector settings are provided (right hand), indicating that the sMics are still present and weakly positive for Vasa::GFP. (C) Integrated pixel intensity data for mCherry control and CNOT6::mCherry misexpression embryos. (D,E) PRE-protecting MASOs result in significantly fewer Vasa-positive cells at 42 hpf. (F) Expression of CNOT6::mCherry in all cells, including the sMics, similarly reduces the number of Vasa-positive cells compared with expression of mCherry alone. (G) Counts of Vasa-enriched cells in control, PRE-protecting MASO and CNOT6 misexpression embryos. P-values are calculated by two-way unpaired t-test. Scale bar: 20 μ m.

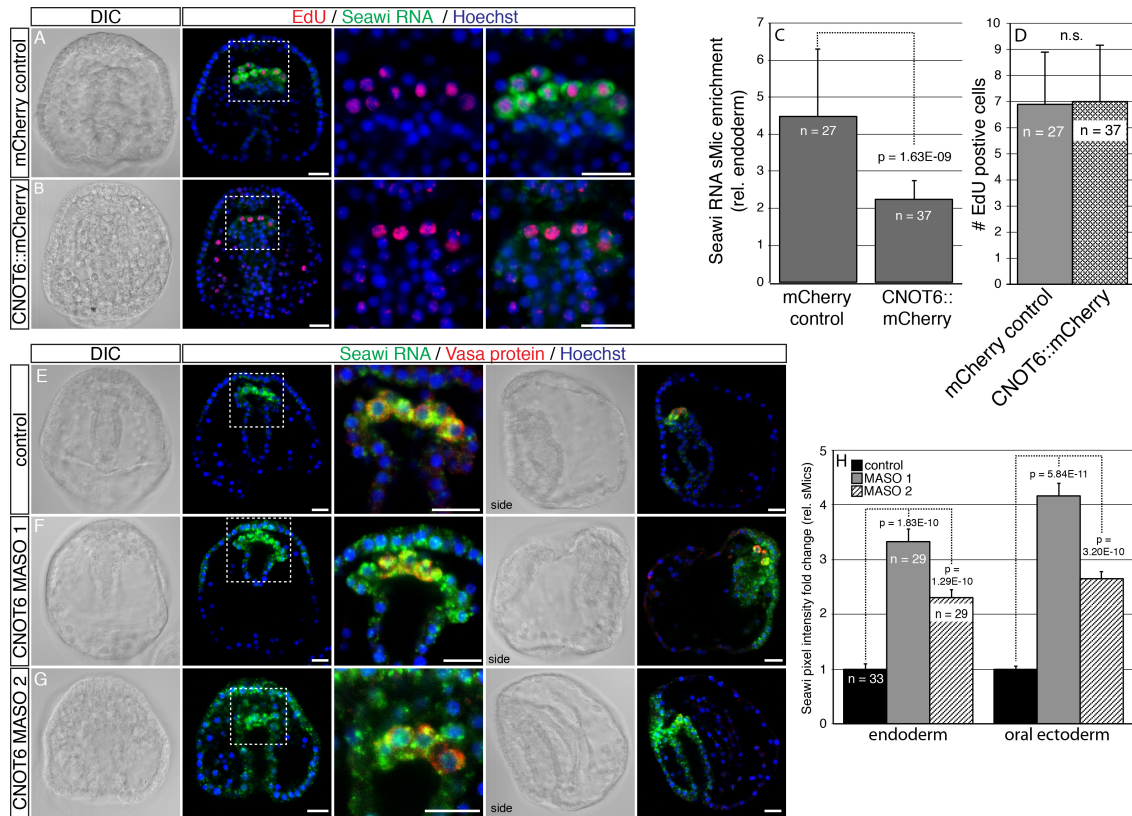


Figure 6. CNOT6 mediates selective enrichment of Seawi transcript in the sMics.

(A,B) Expression of CNOT6::mCherry in all cells reduces the enrichment of Seawi transcript in the sMics, detected by FISH (green), compared with mCherry alone controls. sMic lineage is traced by EdU incorporation (red). (C) Pixel intensity quantitation of Seawi transcript enrichment in sMics relative to the endoderm. (D) Counts of EdU-positive cells in mCherry control and CNOT6::mCherry-expressing embryos indicate no change in sMic numbers. sMics were counted by focusing through the gut tip; one representative focal plane is shown in B. (E-G) CNOT6 knockdown with either of two non-overlapping MASOs expands the domain of Seawi RNA into the endoderm and oral ectoderm at 42 hpf. (H) Fold change of Seawi FISH average pixel intensity in control and knockdown embryos, relative to sMic Seawi intensity. P-values are calculated by unpaired two-tailed t-test. Scale bars: 20 μ m.

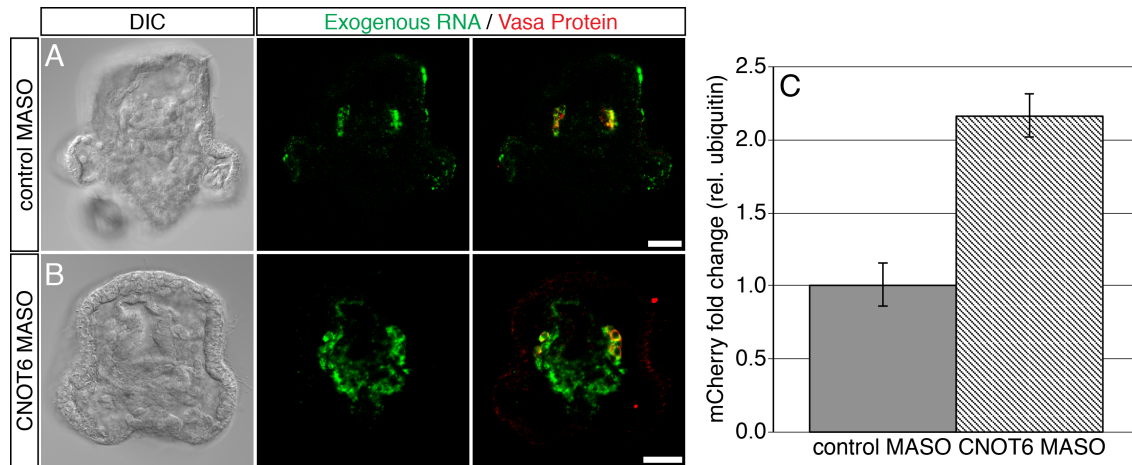


Figure 7. CNOT6 regulates general retention of exogenous RNA in the sMics. (A,B) FISH for injected RNA containing the mCherry open reading frame and SV40 3' polyadenylation signal. This transcript is normally retained only in sMics at 96 hpf (A), but is retained broadly throughout the endomesoderm with CNOT6 knockdown (B). (C) qPCR for exogenous mCherry RNA at 96 hpf. With CNOT6 knockdown, mCherry levels increase twofold. Scale bars: 20 μ m.

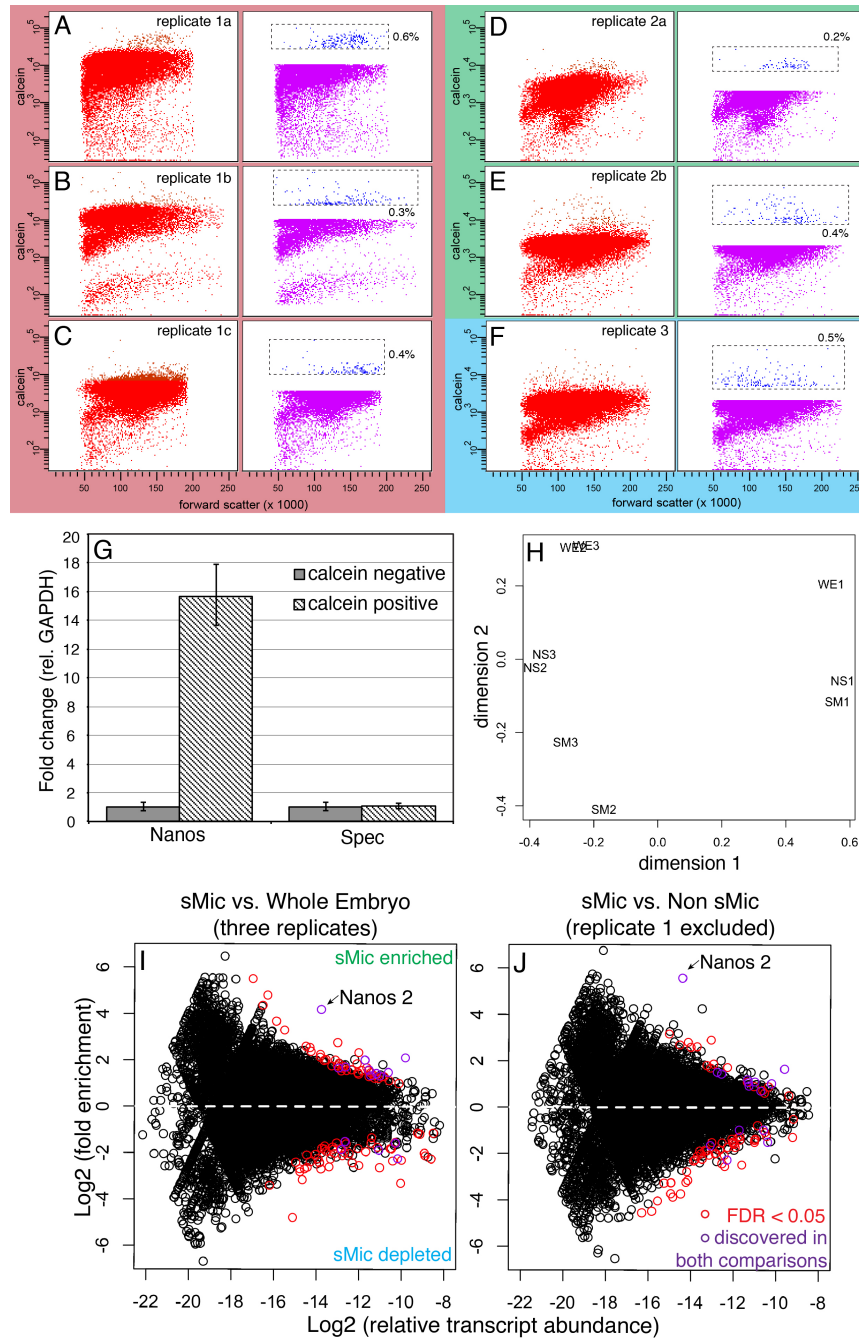


Figure S1, related to Figures 1. FACS isolation of sMics and transcriptomic analysis. (A-F) Scatter plot representations of FACS sorts for three biological replicates. Replicate 1 (A-C, red background) consisted of three sorts of embryos derived from distinct parental crosses. Replicate 2 (D-E, green background) consisted of two sorts from two parental crosses. Replicate 3 (F, blue background) was sorted from a single parental cross. Red points indicate the total cell population, blue points indicate the collected calcein⁺ cells, and purple points indicate collected calcein⁻ cells. Percentages represent

fraction of collected calcein⁺ cells relative to the whole population. (G) qPCR for Nanos indicates enrichment of sMics in the calcein⁺ fraction, as opposed for Spec, an ectodermal negative control. (H) Multidimensional scaling (MDS) analysis of replicate transcriptomes for isolated sMics (SM1-3), non-sMics (NS1-3), and whole embryo (WE1-3). Replicate 2 and 3 samples were highly related with whole embryo, sMic, and non-sMic transcriptomes clustering separately. Replicate 1 samples are outliers, showing low relatedness to the others in both dimensions, and poor separation between SM and NS samples. This variation likely points to issues with sample handling (RNA collection, or RNA-seq sample preparation) or less efficient calcein labeling. In subsequent differential expression analysis, we therefore made comparisons both with and without including Replicate 1 samples. (I,J) Smear plot depiction of differentially expressed genes. (I) sMic vs. Whole Embryo with replicate 1 included, and (J) sMic vs. non-sMic with replicate 1 excluded are considered here. Transcripts are represented by open circles, with fold enrichment in the sMics on the y-axis and relative transcript abundance on the x-axis. Transcripts meeting a significance threshold of 0.05 (false discovery rate, FDR) are in red; transcripts identified in both comparisons are in purple. Arrows indicate the previously identified sMic factor Nanos2.

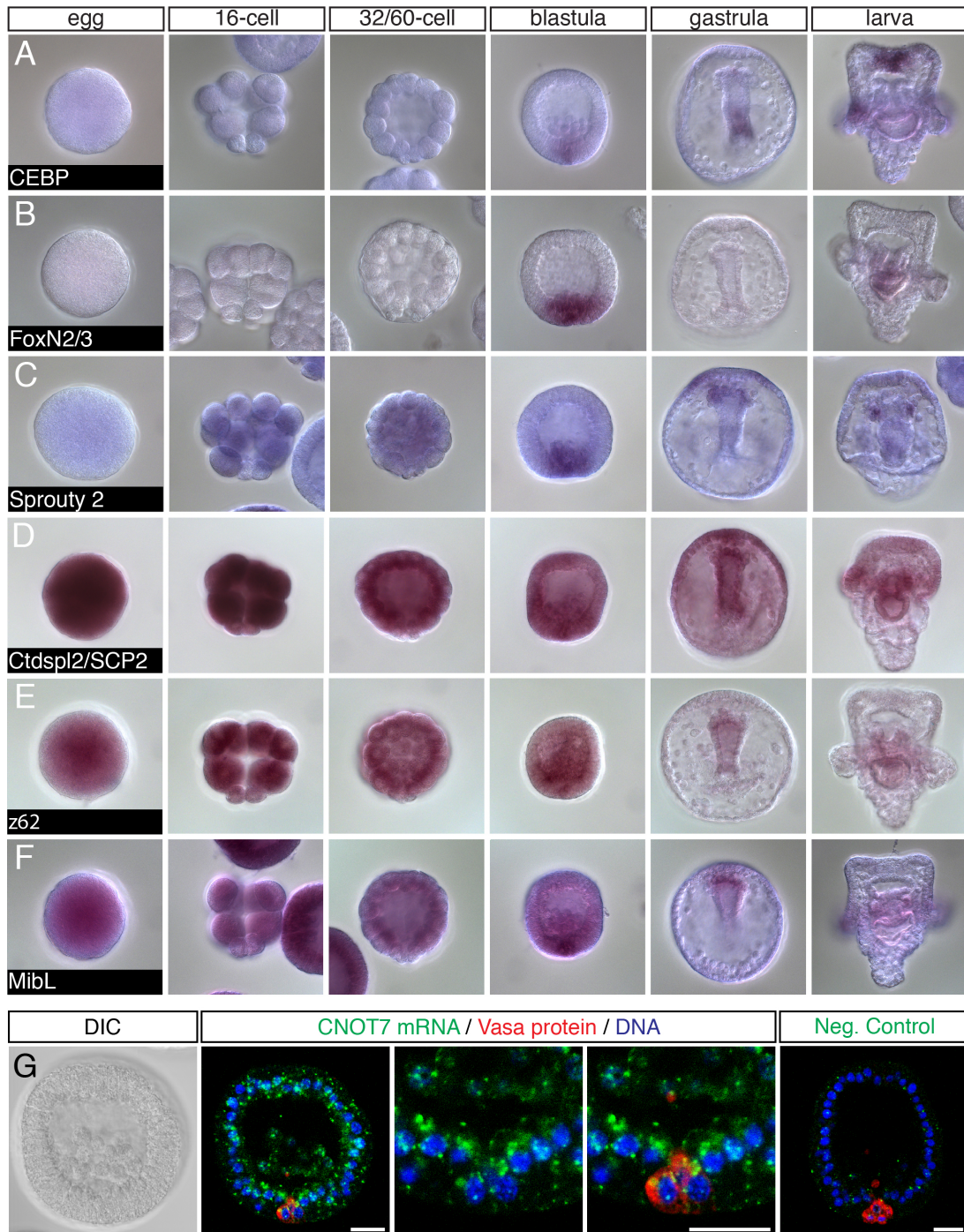
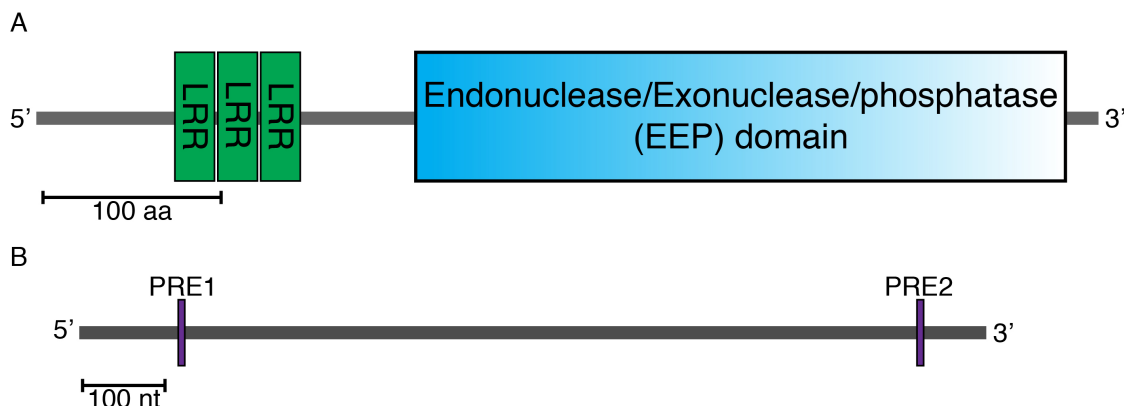


Figure S2, related to Figures 1 and 3. WMISH for selected transcripts. (A-F) WMISH for sMic-enriched transcripts identified through differential expression analysis. (G) The *S. purpuratus* genome contains two CNOT-related nucleases: CNOT6 and CNOT7. Unlike CNOT6, which is specifically depleted in the sMics, CNOT7 transcript (green) is ubiquitously distributed. sMics are labeled in red with vasa antibody.



>CNOT6 3'UTR

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CAAGCGGAGCTAGGGGACTCAGGGTGGTGTTCGTTCTTGGAACCTCGAGAATTTTAA
AGCCTCACTTGGCAATGTTTTTGTGGTAAATTTGAATTTTAA TGTAATTCGTACTTTG
TACAGTGTGTAAATTGATGCTATTTTGCCAGAGAGAAAAAACACTTGGAGCACATT
CTATTCCAAACGCTGTAGAAATCTTTATTACTGCTGTCTGATGGTACACTTCAGATAT
GCTCTATGCCAGGTCTTTAGGTATCTTCACACACATCCTATTTTTGTTGTCATTGGTG
CAACATTAATGCCAATAAAATGTTTCGAGAATAGTTAATGAATGAACCTCAATTTTAAC
TTCTTTTCTTACTAAGGGGGTTATTAATTTGATAAAATCCAGCAGTTCGGATGCAGC
TGATACATTTTCACTTGGCCCTTCTCAATGATTTTTATTTATTCATCTCTACACAAGAA
AATACTGTCTGTCTGGGCAATGTAAAGCAAAAATCCAGAAAAAGAAAAAGGGTTAAGA
AAAGCAAACATTCTTCCAGTTAGTAAGCACAAACATTTTCAAAGAATGTGACCTTTAGA
CCCTAAGAAGTAGCATTATTCAAATTGTTAGTGT TGTAATATTGACCAATAAAATCTG
CATTTTGGATAAATTATGCAATTGCTTCACTTAACCAGTTGCCAAATTGGGAAGTGG
AGCAAGTCTTTTCTTCTTTTCAATCCATGCTACTTGGCATCTTTTGTATTATGAGCCC
TTTAAAAAAAACAGTGATGATACAGTCTGCTGAGCGCCAGAGGGGCGTTACTCTTTT
ACATAGGCTTAACACCCTTCTGGCTCTCAACAGACTGTATGGCCCGAATTCACAAAG
GTTTAAGTTTGACCCAGGGTTTGAACCCTCATTTACATAGCCCATGGTTTGAATTA
GCTCAAATTAAGTGTACCTTGCCTTGCGCGTGTCTAATATTGGACCCCTGACTGTGT
CACGCTTATTTTGTGCTAATTTGAGACCATGGGTTGTGTAAATGAGGATTTGAACCA
TGGGTTGAATTTAAACCTCCTTTGTGAACTAGGGCCTATGTGTTTAGAAGCCCAACC
AATGTGCAATTCTCTGTAAAAAAAAAAAAA

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Figure S3, related to Figure 4. Organization of the CNOT6 open reading frame and 3'UTR. (A) Domain organization of CNOT6 predicted by PFAM (Punta et al., 2012). (B) Schematic of PRE motifs within the CNOT6 3'UTR and full-length sequence.

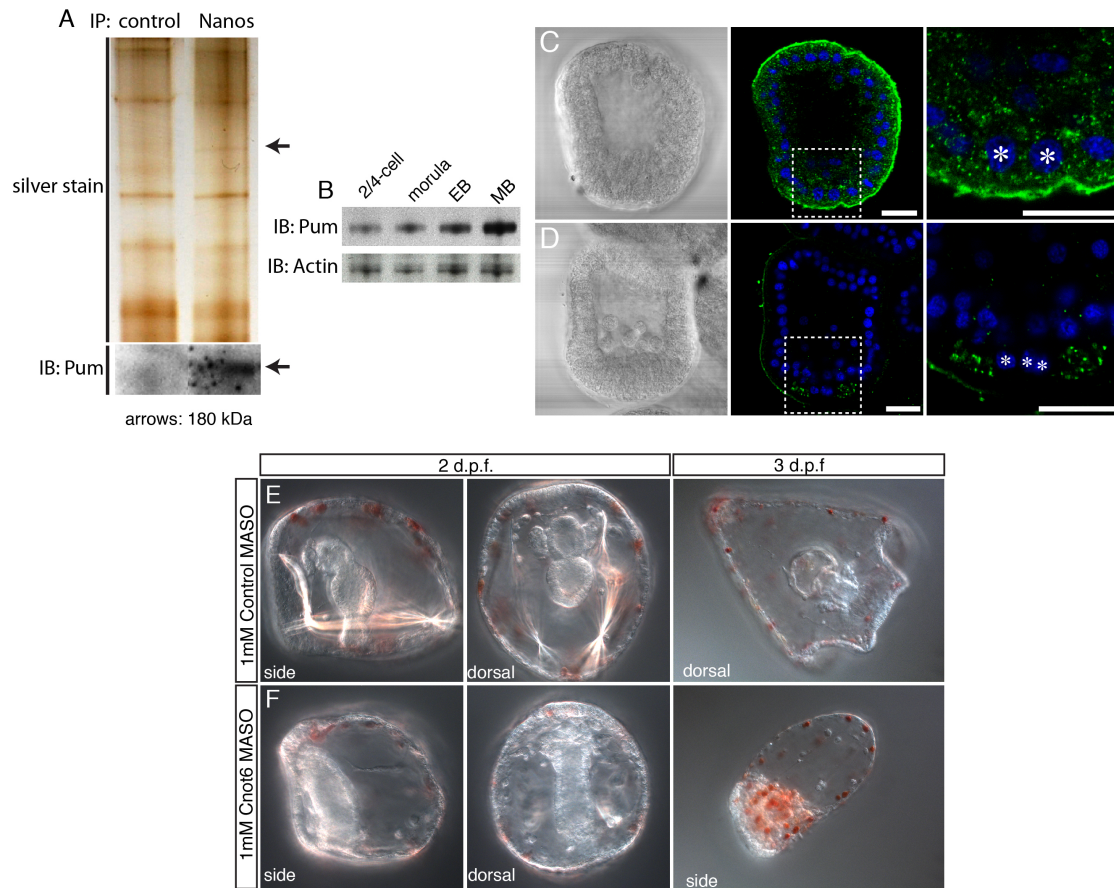


Figure S4, related to Figure 3 and 4. (A) Association of Nanos and Pumilio. Compared to Vasa antibody control, Nanos antibody specifically pulls down a 180 kDa band recognized by silver stain and Pumilio immunoblot. (B) Temporal expression of Pumilio. Pumilio is detectable in early embryos, and increases in abundance through early blastulae (EB) and mesenchyme blastulae (MB) stages. (C,D) Spatial localization of Pumilio protein. Pumilio is detectable in granule structures in all cells, including the sMics, of early blastula (C), but becomes highly enriched in the Veg2 mesodermal precursors after primary mesenchyme ingressions (D). Asterisks indicate sMic nuclei. (E,F) Injection of 1mM CNOT6 MASO 1 results in failure to produce skeletons or a tripartite gut. By 3 days, the endoderm degenerates into mesenchyme-like cells.

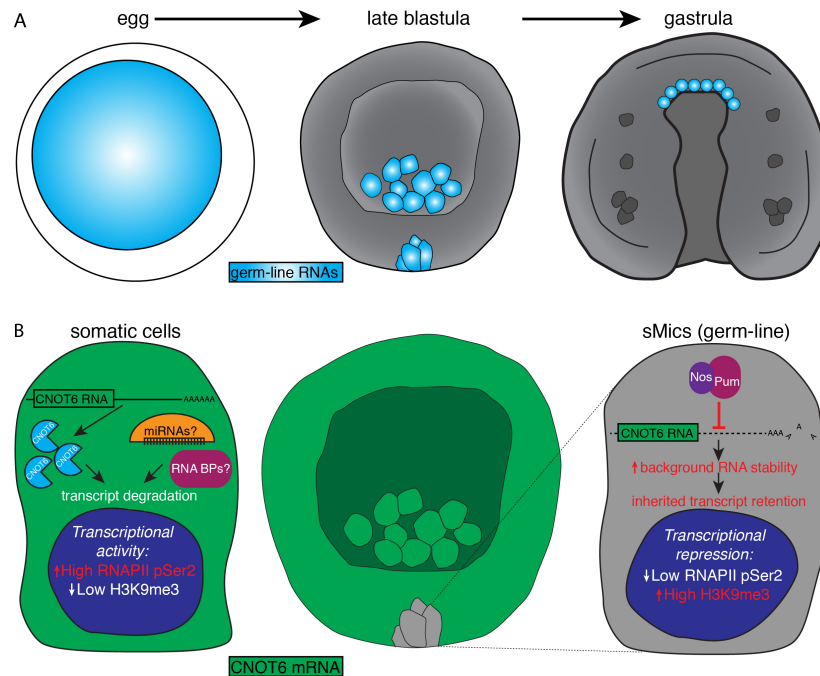


Figure S5. Model. Time capsule model for germ line development. (A) Maternally deposited mRNAs are coordinately degraded in somatic cells at blastula/gastrula stage but stabilized in the sMics. (B) Mechanism for sMic segregation. CNOT6 transcript is in green, and is present in all cells except sMics (gray), where Nanos/Pumilio (Nos/Pum) represses it. This repression creates a stable environment for inherited RNA. Conversely, CNOT6 protein accumulates in somatic cells, where it may act in parallel with and/or downstream of miRNA and RNA binding protein (BP) mediated degradation.

sMic-enriched categories			sMic-depleted categories		
GO ID	Term	p-val	GO ID	Term	p-val
0003676	nucleic acid binding	4.40E-05	0005198	structural molecule activity	4.50E-08
0000988	protein binding		0032561	guanyl ribonucleotide binding	0.0042
	transcription factor act...	0.0015			
0000989	transcription factor		0019001	guanyl nucleotide binding	0.0045
	binding transcripti...	0.0015		extracellular matrix structural	
0003712	transcription cofactor		0005201	constitu...	0.0053
	activity	0.0015		primary active	
	sequence-specific DNA		0015399	transmembrane transporter...	0.01
0043565	binding	0.0049		P-P-bond-hydrolysis-driven	
	transcription factor		0015405	transmembrane...	0.01
0008134	binding	0.006		calmodulin-dependent protein	
	MAP kinase kinase		0004683	kinase acti...	0.016
0008349	kinase kinase activity	0.0067		cysteine-type endopeptidase	
	bHLH transcription factor		0004197	activity	0.0212
0043425	binding	0.0067		active transmembrane	
			0022804	transporter activit...	0.0228
0043426	MRF binding	0.0067		protein serine/threonine	
			0004674	kinase activity	0.0237
0005488	binding	0.0086			
	steroid hormone receptor		0003723	RNA binding	0.0287
0035258	binding	0.0178			
			0016289	CoA hydrolase activity	0.0368
0003729	mRNA binding	0.0189		cell adhesion molecule	
	nuclear hormone		0050839	binding	0.0368
0035257	receptor binding	0.0224			
	hormone receptor				
0051427	binding	0.0275			
	translation regulator				
0090079	activity, nucleic ...	0.0328			
0003677	DNA binding	0.0389			
	retinoic acid receptor				
0042974	binding	0.0393			
	translation regulator				
0045182	activity	0.0393			
	cytoskeletal protein				
0008092	binding	0.0433			
	guanyl ribonucleotide				
0032561	binding	0.0446			
0046332	SMAD binding	0.0457			
	guanyl nucleotide				
0019001	binding	0.0467			

Table S1, related to Figure 1. Gene set enrichment analysis of the union sets of 78 sMic enriched transcripts, and 152 sMic-depleted transcripts. Analysis was performed using the topGO Bioconductor package. P-values are by Fisher's exact test.

	sMic	non-sMic	whole embryo
Replicate 1 <i>(3 pooled parental crosses)</i>	700 ng RNA 113,191 cells	1800 ng RNA 618,511 cells	3800 ng RNA
Replicate 2 <i>(2 pooled parental crosses)</i>	1080 ng RNA 213,174 cells	1820 ng RNA 892,329 cells	6700 ng RNA
Replicate 3 <i>(1 parental cross)</i>	350 ng RNA 55,571 cells	1278 ng RNA 438,938 cells	1508 ng RNA

Table S2. Summary of FACS replicates and RNA extraction yields.

Sample Name	Total Filtered Reads	Mean Read Length (nt)	Percent aligned reads	Sequencing Error Rate
WE1	27,035,746	31.21	49.08%	6.95%
SMM1	25,331,121	31.15	44.29%	6.86%
NSM1	30,889,594	31.27	42.25%	7.02%
WE2	14,670,195	33.75	60.12%	5.44%
SMM2	15,096,355	33.28	60.84%	5.49%
NSM2	12,043,418	34.24	57.30%	5.63%
WE3	14,450,706	33.84	54.68%	5.89%
SMM3	8,300,956	33.66	56.06%	5.62%
NSM3	10,630,234	34.15	58.47%	5.69%

Table S3. Helicos run statistics.

Primer Name	Sequence
Baf250	F: TCGACAACCACCACTACCAA R: taatacgactcactatagggTGTTGTTCACTCCACCCGTA
CEBP	F: AGGGCTGAGGTACAGCAGAA R: taatacgactcactatagggCCCTCGACACGTTTCTTTA
FoxN2/3	F: CGAATGGACAAAGGACCACT R: taatacgactcactatagggCTTGGTGATGGGGTACACT
Sprouty2	F: GCTCTGTTCCCTTGAGCAAC R: taatacgactcactatagggGCAGGGATCATCCGTACAGT
Ctdspl2/SCP2	F: AAGCCACCAATCCTGTGTTC R: taatacgactcactatagggCAGAAAGGCACAAGCAATCA
z62	F: AGTCTAGCAAATGGCGTCGT R: taatacgactcactatagggATGTAACCACATTGCGCAGCA
MibL	F: CTGGACAGACACCAGAGCAA R: taatacgactcactatagggCATCTGCTCCGTGCATAAGA
CNOT6	F: GCAGGTGCTAGGTCTGAAGG R: taatacgactcactatagggCGAGTTGGAGGAGAAGTTGC
CNOT7	F: TGCCAACTCAAACCAATGAA R: taatacgactcactatagggGCACCCTGGTTAAAAGGTCA

Table S4. Primers used for generation of template DNA for ISH probe transcription.

Primer Name (description)	Sequence
CNOT6 3'UTR (reporter construct)	F: GGGACTCAGGGTGGTGTTCT R: ACAGAGAATTGCACATTGGTTGG
PRE1 (site-directed mutagenesis)	F: TTCGTACTTTGTACAGTGaaaAAATTGATGCTATTTTGC R: GCAAAATAGCATCAATTTtttCACTGTACAAAGTACGAA
PRE2 (site-directed mutagenesis)	F: ATTTGAGACCATGGGTTGaaaAAATGAGGATTTGAACCA R: TGGTTCAAATCCTCATTTtttCAACCCATGGTCTCAAAT

Table S5. Primers used for CNOT6 reporter constructions.

MASO Name	Sequence	Concentration of injection solution
Nanos	GTGACTAAAGTGC GTGGAAACTCGA	500 μ M
Pumilio	TTTCCTTCTATGATACAGACCCGCT	100 μ M
CNOT6 1	ATTTATCTTTGGGCATCCTGGTGGC	500 μ M
CNOT6 2	GTCGGTTTTACACAGTTCAGGAGGC	500 μ M
PRE1	AATAGCATCAATTTACACACTGTAC	1000 μ M
PRE2	GTTCAAATCCTCATTACACAACCC	1000 μ M

Table S6. Custom morpholino sequences used.

Punta, M., Coggill, P. C., Eberhardt, R. Y., Mistry, J., Tate, J., Boursnell, C., Pang, N., Forslund, K., Ceric, G., Clements, J., et al. (2012). The Pfam protein families database. *Nucleic Acids Res* **40**, D290–301.

Chapter 3: Notch and Nodal Control Expression of a Forkhead Factor in the Specification Network of a Multipotent Progenitor Population in Sea Urchin

Materna, S.C., **Swartz, S.Z.**, and Smith, J. (2013). Development, 140: 1796-806.

I performed the initial characterization of FoxY spatial expression with respect to the sMics and Veg2 NSM, and helped write the manuscript.

Abstract

Indirect development, where embryogenesis gives rise to a larval form, requires that some cells retain developmental potency until they contribute to the different tissues in the adult, including the germ line, in a later, post-embryonic phase. In sea urchins, the coelomic pouches are the major contributor to the adult but how coelomic pouch cells (CPCs) are specified during embryogenesis is unknown. We here identify the key signaling inputs into the CPC specification network and show the forkhead factor *foxY* is the first transcription factor specifically expressed in CPC progenitors. Through dissection of its cis-regulatory apparatus we determine that the *foxY* expression pattern is the result of two signaling inputs: First, Delta/Notch signaling activates *foxY* in CPC progenitors and, second, Nodal signaling restricts its expression to the left side, where the adult rudiment will form, through direct repression by the Nodal target *pitx2*. A third signal, Hedgehog, is required for coelomic pouch morphogenesis and institution of laterality but does not directly affect *foxY* transcription. Knockdown of *foxY* results in failure to form coelomic pouches and disrupts expression of virtually all transcription factors known to be expressed in this cell type. Our experiments place *foxY* at the top of the regulatory hierarchy underlying specification of a cell type maintaining developmental potency.

Introduction

The ability of certain cells to resist differentiation is an important feature of animal development. Most notably, sexually reproducing animals establish germ line stem cells, which create the sperm and eggs required for fertilization (Spradling et al., 2011). In animals undergoing indirect development, embryogenesis results in a larval form that

may differ dramatically from the adult organism (Minelli, 2009). This requires that some cells are either segregated away during embryogenesis and remain multipotential, or reacquire multipotency, to contribute to the diverse tissues of the adult, including the germ line (Arenas-Mena, 2010).

In sea urchins, embryogenesis gives rise to a feeding larva, which after a prolonged period of time will produce the adult. A prerequisite for rudiment formation is the specification of coelomic mesoderm, a group of cells derived from the veg2 lineage gives rise to two pouch-like protrusions at the tip of the archenteron (Cameron et al., 1991). Rudiment formation commences with the invagination of a piece of ectodermal wall above left coelomic pouch. A complex morphogenetic process ensues during which the adult body plan with its pentameral symmetry is established (Pearse and Cameron, 1991). In *Strongylocentrotus purpuratus* each of the coelomic pouches initially contains only about 10 cells but their descendants are major contributors to various adult structures (Pearse and Cameron, 1991); at metamorphosis about 90% of the 150,000 larval cells are located in the juvenile (Cameron et al., 1989; Song and Wessel, 2012) illustrating these cells are multipotent progenitors.

In euechinoid sea urchins, CPC progenitors are specified during gastrulation as a consequence of Delta/Notch (D/N) signaling within the non-skeletogenic mesoderm (NSM) (Sherwood and McClay, 1999; Sweet et al., 2002). Interference with NSM Delta abolishes coelomic pouch formation but does not disrupt specification of other mesodermal cell types specified earlier (Materna and Davidson, 2012; Materna et al., 2013a; Sweet et al., 2002). During archenteron formation the CPC progenitors are located at the tip but then split into two groups that relocate laterally to either side of the

foregut. Little is known about the regulation of this morphogenetic process. However, expression of Hedgehog (Hh) in the adjoining endoderm and of its receptors in CPC progenitors (Walton et al., 2009) may suggest a possible involvement of this signal. The small micromeres (SMMs) are also located at the tip of the archenteron and are distributed to either coelomic pouch at pluteus stage (Pehrson and Cohen, 1986). They are set aside cells that remain mitotically quiescent during embryogenesis (Tanaka and Dan, 1990). The SMMs express orthologs of numerous germ line/stem cell markers (Juliano et al., 2010; Voronina et al., 2008), are required to form the germ line in *Lytechinus variegatus* (Yajima and Wessel, 2011), and may thus be primordial germ cells. However, whether they also contribute to other tissues remains unresolved.

The forkhead factor *foxY*, originally named *foxC* (Ransick et al., 2002; Tu et al., 2006), is the first transcription factor specifically expressed in any of the cells contributing to the coelomic pouches. At early blastula stage it is expressed in the SMMs in response to D/N signaling originating in the skeletogenic lineage (Materna and Davidson, 2012), then at the tip of the archenteron and in both coelomic pouches as they form. At late gastrula stage *foxY* becomes strongly biased to the left coelomic pouch, presumably as a consequence of Nodal signaling establishing left/right (L/R) asymmetry across the embryo (Duboc et al., 2005). Although many transcription factors are eventually expressed in coelomic pouches (Duboc et al., 2005; Howard-Ashby et al., 2006a; Howard-Ashby et al., 2006b; Luo and Su, 2012; Poustka et al., 2007; Tu et al., 2006), when CPC progenitors are first specified, *foxY* is the only gene to become expressed in direct response to NSM Delta among 182 regulatory genes, most of them with spatially restricted expression (Materna and Davidson, 2012; Ransick et al., 2002; Tu et al., 2006). This suggests *foxY* plays an important role in coelomic pouch formation.

Here we examine the regulation and significance of *foxY* expression and find *foxY* is essential for specification of CPCs. Its precise expression pattern is the result of the integration of the D/N and Nodal signals within the *foxY* regulatory region: D/N signaling directly activates *foxY* expression, first in SMMs and then in CPC progenitors, while Nodal represses *foxY* in the right coelomic pouch via *Pitx2*. Reception of the Hh signal in CPC progenitors sets in motion coelomic pouch morphogenesis, but does not directly affect *foxY* expression. However, Hh is required for proper Nodal expression on the right side of the embryo and is thus involved in the establishment of L/R asymmetry. Our results link *foxY* directly to specification of a cell type with great developmental potency en route to formation of the adult.

Results

foxY expression in sea urchin embryogenesis

At early blastula stage *foxY* is expressed in the SMMs at the vegetal pole of the embryo. During gastrulation it is expressed at the tip of the archenteron, but eventually becomes restricted to the left coelomic pouch (Ransick et al., 2002). As has been suggested by a recent study (Song and Wessel, 2012), the *foxY* expression domain may not be restricted to the SMMs during gastrulation, but instead may expand to include the NSM. We set out to obtain a better understanding of *foxY* expression.

A time series of *foxY* gene expression (Materna et al., 2010) revealed a biphasic profile through late gastrulation (**Fig. 1A**). The early expression phase lasts from about 9 hpf until 23 hpf, i.e., from when the SMMs are born to mesenchyme blastula stage. After a quick initial rise, *foxY* levels remain constant at about 140 transcripts per embryo (~35

copies in each SMMs). Staining of *foxY* message overlaps with Vasa protein, an RNA helicase involved in translational control in primordial germ cells (Fig. 1B,C) (Gustafson and Wessel, 2010). Vasa is specific to SMMs (Gustafson and Wessel, 2010; Voronina et al., 2008) confirming *foxY* expression is restricted to this cell type. The four SMMs are located at the vegetal pole and are in direct contact with the cells of the skeletogenic lineage. When the skeletogenic cells ingress into the blastocoel, the SMMs stay behind and come into contact with the NSM cells, which replace the skeletogenic cells in the vegetal plate (Fig. 1B,C).

During its second expression phase, *foxY* transcript levels increase significantly and reach a peak of ~500 transcripts/embryo at 30 hpf (Fig. 1A) followed by a steady decline. The rise of *foxY* transcript levels coincides with an expansion of the *foxY* expression domain (Fig. 1D–F); at 30 hpf *foxY* is expressed in the mesodermal cells, i.e., the veg2-derived CPC progenitors directly surrounding Vasa positive SMMs (Fig. 1D). *foxY* is expressed throughout the entire cap of the elongated archenteron, whereas the SMMs remain located at the center (they have divided once; there are now eight Vasa positive cells). Towards the end of gastrulation the cap bulges laterally to form the coelomic pouches; *foxY* expression becomes strongly biased to the left coelomic pouch (Fig. 1E). SMMs are still located at the center of the archenteron tip (Fig. 1F), but staining for *foxY* message in the SMMs becomes progressively weaker and is barely visible at 48 hpf (Fig. 1F). This may indicate expression of *foxY* is not maintained in these cells, in agreement with the diminished number of six to eight *foxY* positive cells at early larval stage observed by Ransick et al. (2002). After about 3 days postfertilization (dpf) *foxY* will be restricted to the left pouch (Ransick et al., 2002).

NSM Delta signaling specifies CPC progenitors

Delta/Notch signaling occurs in three instances during sea urchin embryogenesis: Delta is expressed in skeletogenic cells starting at ~9 hpf, then in NSM once skeletogenic cells have ingressed. It is also expressed in the apical plate although its function there has not been studied in detail (Materna and Davidson, 2012; Smith and Davidson, 2008; Sweet et al., 2002; Walton et al., 2006). Delta expression in skeletogenic cells activates gene expression in the NSM and SMMs, which are both in direct contact with the Delta source. Importantly, *foxY* is the only known target specifically activated in the SMMs (Materna and Davidson, 2012). Disruption of the second, i.e., NSM phase of D/N signaling with the Notch inhibitor DAPT causes failure to form coelomic pouches. But among 182 regulatory genes the only gene to be severely depleted at 30 hpf was *foxY* (Materna and Davidson, 2012)(Fig. S1). As we have shown above, the strong increase in transcript levels between 23 hpf and 30 hpf is due to activation of *foxY* in the mesodermal cells directly adjacent to the SMMs constituting the CPC progenitor population. The likely cause for this expansion is activation of *foxY* by D/N signaling originating in the NSM.

The time course data in Fig. 1A reveal that other genes restricted to CPCs (*foxF*, *pitx2*, *soxE*) are transcribed only at 30 hpf or thereafter. To better understand the role of D/N signaling in specification of CPCs we extended the analysis of NSM Delta function into gastrulation. We added DAPT at 17 hpf, i.e., just prior to the activation of Delta in NSM, and quantified the perturbation effects on transcript levels with the NanoString nCounter (Geiss et al., 2008). We made use of a code set containing probes for all relevant transcription factors known to be expressed in a spatially restricted fashion, plus probes

for several signaling ligands and marker genes. Probe sequences and accession numbers for all 205 genes included in our code set are available from the Dryad Digital Repository (Materna et al., 2013b).

Fig. 2A displays the quantitative perturbation data collected for a selection of genes. A table with perturbation data for all genes is available from the Dryad Digital Repository (Materna et al., 2013b). As expected from the failure to form coelomic pouches, all genes specific to and uniquely expressed in CPCs were strongly depleted in perturbed embryos compared to controls. At mid gastrula (38 hpf) this included the forkhead factors *foxC*, *foxF*, and *foxY* (Tu et al., 2006), the Sox/HMG factor *soxE* (Duboc et al., 2005; Howard-Ashby et al., 2006a), and the zinc finger gene *scratchX* (Materna et al., 2006). The *gataE* and *pax6* genes are expressed in CPCs, but are also expressed in the gut and ectoderm (Figs 3E,F; S2B–B'',C–C''). Independent expression in these compartments obscures the perturbation effects in our quantitative analysis. The spatial expression pattern of *scratchX* was unknown but it is first expressed in CPC progenitors at the tip of the archenteron, and later in both coelomic pouches (Figs 3G, 5E). These perturbation effects remain strong until late gastrulation (48 hpf) with the exception of *foxY*, which is no longer significantly affected, nor highly expressed at this time (Fig. 1A). The homeobox gene *pitx2* and the bHLH gene *myor2* have become activated by 48 hpf and are also depleted. *pitx2* is expressed in CPCs, but strongly biased to the right (Duboc et al., 2005; Luo and Su, 2012). The expression level of *myor2* is extremely low and we were unable to determine its spatial expression pattern. Endoderm genes are not activated by NSM Delta throughout gastrulation (Fig. 2A) (Materna and Davidson, 2012), despite strong expression of the Notch receptor in the archenteron (Walton et al., 2006).

To better understand the timing of the D/N signaling input into CPCs, we attempted to determine the spatial expression of *delta* during gastrulation. Similar to results reported by Walton et al. (2006), we observe *delta* expression in dispersed cells in the ectoderm and in the apical plate (not shown), where it is an ongoing input into neurogenic genes (Fig. 2A). But due to its low abundance (150 transcripts/embryo at 48 hpf) (Materna et al., 2010) and complex expression pattern we cannot rule out low level expression persists in single cells at the archenteron tip. To assess if active Notch signaling is required for maintenance of CPC gene expression, we added DAPT at 30 hpf and quantified transcript levels. Our results show some apical genes are strongly elevated at 38 hpf and 48 hpf, indicating D/N signaling is active in this domain. In contrast, no CPC gene is affected (Fig. 2B). These data suggest the mesodermal Delta signal does not function as a continuous input into CPC genes during gastrulation. However, most CPC genes are activated after 30 hpf; in fact, *foxY* and *foxC* are the only known genes expressed in this compartment at 30 hpf (Fig. 1A). Thus, our findings imply Delta activation of most CPC genes is indirect.

FoxY function is essential for coelomic pouch formation

foxY is the first known transcription factor specifically expressed in CPC progenitors downstream of Notch signaling. To test if it is a potential regulator of other CPC genes we knocked down its expression with a MASO. FoxY MASO injection has no adverse effect on the health of the embryo and does not interfere with the general progression of development. The overall anatomy of the larvae is normal, including the skeleton and partitioning of the gut (Fig. 3A'). Observation of the larvae revealed very robust defects: All treated embryos did not form coelomic pouches and their derivative structures (Fig.

3A') including the water canal normally emerging from the left coelomic pouch and connecting to the hydropore in the ectodermal wall. FoxY MASO treated larvae were unable to swallow, although food particles accumulated in their esophagus. Contraction of circumesophageal muscles is responsible for transport of food into the stomach. Rhodamine phalloidin staining of F-actin in 3.5 day old larvae revealed these muscles do not form following FoxY MASO injection (Fig. 3B'). Muscle cells are thought to migrate out of the coelomic epithelium (Burke and Alvarez, 1988a) although their exact origin remains unknown. Our results indicate they derive from the group of *foxY* positive CPC progenitors at the tip of the archenteron. Thus, *foxY* is required for all known functions of NSM Delta signaling, i.e., specification of CPCs and muscle cells (Materna and Davidson, 2012; Sweet et al., 2002).

At blastula stage (15 hpf) when *foxY* expression is restricted to SMMs, Nanostring analysis revealed no genes are affected by FoxY MASO injection. However, our code set does not contain any gene besides *foxY* restricted only to SMMs. A recent study examined the relationship between *foxY* and the *nanos* gene (Song and Wessel, 2012), a stem cell/germ line marker specific to SMM until pluteus stage (Juliano et al., 2010). We supplemented the Nanostring data with QPCR for *nanos* (not shown) and confirmed the previous finding that at 15 hpf loss of FoxY function does not affect *nanos* abundance (Song and Wessel, 2012).

If FoxY is the effector of NSM Delta signaling in the CPC progenitors, its knockdown should affect a similar set of genes as observed in our DAPT experiments (Fig. 2A). At 38 hpf and 48 hpf our quantitative analysis identified a sharply limited set of genes affected in FoxY morphants restricted to CPCs (Figs 2C; S3A). The changes were

indeed similar to those caused by disruption of NSM Delta signaling (Fig. 2A). As in our DAPT treatments the responsive gene set included all genes known to be specifically and uniquely expressed at the tip of the archenteron at 38 hpf, i.e., *foxC*, *foxF*, *foxY*, *scratchX*, and *soxE*. At 48 hpf some differences became apparent: *scratchX* was more strongly affected by FoxY MASO than DAPT treatment. In contrast, *pitx2* was significantly depleted in DAPT treated embryos but not affected in FoxY MASO morphants. Although *foxY* is initially expressed in all CPC progenitors, at 48 hpf it becomes strongly biased to CPCs on the left side, as a result of *pitx2* repression on the right, as we show below. The perturbation results thus reflect the progressive regionalization at the archenteron tip. This divergence notwithstanding, the striking agreement at 38 hpf argues *foxY* is the immediate early effector of Notch signaling within the tip of the archenteron/CPC progenitor population.

We further tested FoxY responsive genes by WMISH, including *six1/2*, its co-factor *eya*, and *scratchX*, undetectable in all FoxY MASO treated embryos (Fig. 3C,G,H). Transcript levels of the *gataE* and *pax6* genes were only slightly affected in our Nanostring analysis (Fig. 2C) but WMISH confirms expression of both genes is lost specifically in CPCs (Fig. 3E,F). As mentioned above, *gataE* is strongly expressed in the mid- and hindgut and this expression masks its loss in CPCs in our quantitative analysis (Fig. S2B–B''). Similarly, *pax6* is also expressed in the ectoderm (Fig. S2C–C''); it becomes predominantly expressed in CPCs only relatively late (Luo and Su, 2012; Yankura et al., 2010). Our Nanostring data showed *foxY* message is no longer significantly depleted at 48 hpf following FoxY MASO injection. WMISH confirmed *foxY* is detectable at the tip of the archenteron. However, following FoxY MASO treatment the *foxY* positive CPCs are dispersed and not restricted to the sides of the archenteron (Fig. 3D,D'). Despite this

exception, these results confirm that essentially all known genes specifically expressed in the CPC compartment are severely depleted when FoxY function is lost.

Pitx2 is the likely effector for establishment of left/right asymmetry

Towards the end of gastrulation *foxY* expression becomes biased to the left coelomic pouch where eventually the adult rudiment will form. Establishment of L/R asymmetry across the embryo is widely conserved and is dependent on Nodal/Tgf- β signaling (Duboc et al., 2005; Luo and Su, 2012). During embryogenesis Nodal is first expressed in the oral ectoderm and induces oral cell fate in recipient cells (Duboc et al., 2004; Li et al., 2012; Materna et al., 2013a). Later in gastrulation Nodal expression ceases in the oral ectoderm and is instead activated in the right lateral ectoderm and right coelomic pouch. Interference with this second phase of Nodal signaling results in loss of L/R asymmetry, most pronounced in the coelomic pouches where genes restricted to the left side are ectopically expressed on the right, resulting in the formation of two rudiments (Duboc et al., 2005; Luo and Su, 2012).

To identify more comprehensively the gene set responsive to late Nodal signaling, we performed Nanostring analysis of embryos treated with the Nodal inhibitor SB431542 at 24 hpf, after the oral/aboral axis is firmly established. Among all 205 genes in our analysis only six were significantly depleted (Figs 4A; S3B). This included the conserved regulators of L/R asymmetry (*nodal*, *lefty*, *pitx2*) expressed in the right ectoderm and coelomic pouch complementary to *foxY* (Fig. 5B,C,D) (Duboc et al., 2005; Luo and Su, 2012). *myor2* is depleted following Nodal perturbation as are *chordin* and the homeobox gene *not*. However, the effect on *chordin* and *not* is due to Nodal signaling within the oral ectoderm that is still ongoing by the time Nodal inhibitor was added, where these genes

are expressed during gastrulation (Li et al., 2012; Materna et al., 2013a). Although we cannot exclude having missed perturbation effects on genes with more complex expression patterns, the primary candidate as the downstream effector of late Nodal signaling in establishment of L/R asymmetry is *pitx2*.

Hedgehog signaling triggers coelomic pouch morphogenesis

Hedgehog (Hh) signaling is known to regulate developmental processes as diverse as patterning, proliferation, and morphogenesis (Ingham and McMahon, 2001) and has been linked to establishment of L/R asymmetry (Tsiarlis and McMahon, 2009). In sea urchin embryos the Hh ligand is expressed throughout the endoderm and archenteron starting at mesenchyme blastula stage (Fig. 1A) concurrent with its downstream effector *gli1* (Walton et al., 2006). The receptors *ptc* and *smo* are present throughout embryogenesis and are expressed prominently at the tip of the archenteron and in coelomic pouches (Walton et al., 2006; Walton et al., 2009). Experimental activation of Hh signaling has been shown to randomize laterality and cause defects in muscle patterning (Walton et al., 2009), but the function of endogenous Hh signal with regard to coelomic pouch development has not been studied systematically.

We disrupted Hh signaling by addition of cyclopamine, a highly specific inhibitor of Smo (Chen, 2002), to embryos at 22 hpf, prior to the onset of *Hh* transcription (Fig. 1A). We determined a final cyclopamine concentration of 0.5 μ M is sufficient to cause reproducible effects (Fig. S4) without causing severe developmental delay or death common at concentrations of 5 μ M or higher. Phenotypic inspection revealed several defects, but the most relevant here is the disruption of coelomic pouch morphogenesis. In control embryos the CPC progenitors at the tip of the archenteron split into two groups

that relocate laterally to form pouches on either side of the foregut. But in cyclopamine treated embryos these cells remained located atop the archenteron as a uniform group and formed one pouch-like structure (Fig. 5). Cyclopamine treatment also resulted in failure to form the mouth and a short gut; whether this is due to improper elongation of the archenteron or defective gut patterning will have to be addressed elsewhere.

Nanostring analysis of the perturbation effects identified a number of genes reacting strongly to the loss of Hh signaling (Fig. 4B). Some of the strongest effects are found for endoderm genes highlighting the importance of Hh signaling in gut development. In contrast, and despite the strikingly different morphology, expression levels of CPC genes were virtually unaltered, except for some minor effects (e.g., *foxF*, *soxE*) (Fig. 4B). In situ staining of *foxY* transcript at 66 hpf, more than 30 hours after pouches separate in controls, revealed strong expression at the tip of the archenteron in agreement with our quantitative analysis (Fig. 5A',A''). At this time *foxY* is restricted to the left coelomic pouch in controls (Fig. 5A). Staining of *scratchX* and *soxE* message was strong at the tip of the archenteron confirming their continued expression following treatment as well (Fig. 5E',E'',F',F'').

One CPC gene strongly depleted in cyclopamine treated embryos is *pitx2* (Figs 4B, 5D',D''). *nodal* transcript levels were also strongly reduced following cyclopamine treatment suggesting the cause for the aberrant *pitx2* expression is loss of Nodal signaling (Fig. 4B). Indeed, in situ staining of *nodal* and *lefty* confirmed their expression, like *pitx2*, is lost in coelomic pouches and also the right lateral ectoderm (Fig. 5B',B'',C',C''). In contrast to our Nodal perturbation, the *not* gene is not affected in cyclopamine treatments, supporting the conclusion that loss of Hh signaling specifically

affects the late expression phase of *nodal*, which is discontinuous from its earlier expression in the oral ectoderm; it also confirms that the *not* gene is not expressed in coelomic pouches at late gastrulation (it is eventually expressed in the right coelomic pouch during larval development (Luo and Su, 2012)). In sum, wildtype levels of Hh signaling are required for coelomic pouch morphogenesis, unilateral expression of *nodal*, and, as a consequence, for asymmetric gene expression in the coelomic pouches once they have formed.

Ni/Su(H) and Pitx2 are direct regulators of foxY

foxY expression is closely linked to Delta expression. To test whether *foxY* is a direct target of Suppressor of Hairless (Su(H)), the downstream effector of D/N signaling, we generated reporter constructs of the *foxY* regulatory region. We identified a *foxY* BAC of ~145 kb and inserted the GFP coding region into the second exon by homologous recombination downstream of the start codon to create the *foxY*:GFP BAC reporter (Fig. 6A). This construct replicates endogenous *foxY* expression, albeit in a mosaic fashion as is common in sea urchins (Smith, 2008). GFP expression is first observed in one or two of the small micromeres, then in the vegetal plate prior to gastrulation (Fig. 6B), then at the tip of the archenteron, and eventually primarily in the left coelomic pouch (Fig. 6D). Reporter expression in the right pouch occurs with low frequency (see below) as expected based on its weak expression in in situ stainings.

We amplified successively smaller fragments using the BAC as template and examined reporter expression (Fig. 6A). Construct #3 contains the sequence located between -778 bp upstream of the transcription start site (TSS) and intron 2; its expression was identical to the full BAC reporter. Deletion of intron 1 did not alter its expression behavior

(Construct #6; Fig. 6C,D). Further shortening at the 5' end resulted first in equal expression between left and right pouches (Construct #4, Fig. 6E), and then in complete loss of activation (Construct #5). Thus, the region between -400 bp and the TSS contains the regulatory element necessary for expression, and the region between -778 bp and -400 bp contains an element repressing expression in the right coelomic pouch.

We identified a potential Su(H) binding site in the proximal region (Figs 7A; S5). To test if this site is required for activation of *foxY*, we mutated it in Construct #3 and co-injected it together with an *alx* reporter driving GFP expression in skeletogenic cells (Damle and Davidson, 2012) as a marker of integration. Injection of wild type Construct #3 results in GFP expression within the vegetal plate (100%, 44/44) (Fig. 7C,C'). Expression of Construct #3 is clearly distinguishable from GFP positive skeletogenic cells within the blastocoel. In contrast, when the Su(H) site was mutated (#3-ΔSu(H)), GFP expression was absent from the vegetal plate (95%, 57/60) (Fig. 7D-D''). Similarly, we observed no *foxY* reporter expression during archenteron formation (Fig. 7F,G). Thus, mutation of the Su(H) site within the *foxY* regulatory region confirms *foxY* is a direct Notch target.

Our data suggest Nodal may repress *foxY* in the right coelomic pouch via *pitx2*.

Unfortunately, two *Pitx2* MASOs had toxic side effects resulting in abortive development 24 hours before *pitx2* is first transcribed. We therefore took a more direct approach and set out to identify potential *Pitx2* binding sites. *Pitx2* binding sites are similar to those of other homeobox factors, most notably *gsc* and *otx*, with a consensus motif of GGATTA (Berger et al., 2008). However, of these three genes only *pitx2* is expressed in the coelomic pouches at the appropriate time (Li et al., 2012; Morris et al., 2004); neither *gsc* nor *otx* are affected in our Nodal perturbations. We identified two potential *pitx2* binding

sites in the distal fragment (Figs 7A, S5). Mutation of these sites in Construct #3 (#3- Δ Pitx2; Fig. 7A) resulted in equal reporter expression in left and right coelomic pouches, similar to Construct #4 (Figs 6A, 7B,B'). In controls, 76% of embryos (40/53) expressed GFP reporter exclusively in the left coelomic pouch, 9% (5/53) in both pouches, and 15% (8/53) expressed it exclusively in the right pouch at 3 dpf. We never observe endogenous *foxY* transcript exclusively in the right pouch indicating the fraction of embryos expressing GFP reporter on only one side is an overestimate due to mosaic incorporation. In contrast, mutation of both putative Pitx2 binding sites (#3- Δ Pitx2) caused a significant shift to the right: 32% of embryos (22/68) showed expression of GFP exclusively in the left pouch, 25% (17/68) in the right pouch, and 43% (29/68) expressed the reporter in both pouches. This result resembles bilateral expression of genes otherwise restricted to the left pouch (e.g., *soxE*, *six1/2*) as is observed following Nodal perturbation (Duboc et al., 2005; Luo and Su, 2012), thus providing evidence that *pitx2* is the repressor of *foxY* in the right coelomic pouch.

Discussion

The experiments presented in this work are aimed at elucidating the earliest regulatory events required for adult formation in sea urchin larvae. Although much remains to be discovered, our results afford several new and important insights. First, we show the primary function of NSM Delta expression is to directly activate *foxY* expression in a subset of mesodermal cells constituting the pool of CPC and muscle cell progenitors. Second, our work demonstrates *foxY* is the principal effector of the NSM Delta signal. It is essential for specification of the two late mesodermal derivatives and thus a central node in the regulatory network underlying this process. Third, we find Hh signaling drives

coelomic pouch morphogenesis and Hh is essential for establishing L/R asymmetry by activating Nodal expression in the right side of the larva. As a consequence, *foxY* is repressed in the right coelomic pouch by *Pitx2*. Our results show initial specification of CPCs, morphogenesis of the pouches, and establishment of laterality are three intertwined processes. But despite their concurrent unfolding, they are regulated independently and thus separable.

foxY Expression and Mesodermal Patterning

The exact origin of late mesodermal derivatives has remained elusive. Fate mapping experiments resolved the origin of pigment and blastocoelar cells within the vegetal plate, but the results were less clear for CPCs and muscle cells (Ruffins and Ettensohn, 1996). The demonstration that NSM *Delta* is required for specification of both (Sherwood and McClay, 1999; Sweet et al., 2002) led to the identification of *foxY* as a potential downstream effector (Materna and Davidson, 2012). As our expression analysis shows, *foxY* is likely the first transcription factor specifically expressed in the entire *veg2* derived progenitor pool of CPCs and muscle cells. At early mesenchyme blastula stage *delta* transcript is present throughout the entire NSM (Materna and Davidson, 2012; Sweet et al., 2002; Walton et al., 2006) including the cells directly adjacent to the SMMs that go on to activate *foxY* expression. This contradicts the idea that *Delta* presentation and active Notch signaling are mutually exclusive (del Álamo et al., 2011) and suggests *Delta* expression may be more dynamic within the NSM than is currently assumed. The exceedingly low transcript levels of *delta* during its expression in NSM (Materna et al., 2010) and the rearrangements following ingression of pigment and blastocoelar cells make disentangling the different mesodermal lineages challenging.

The dramatic phenotype caused by FoxY MASO injection, i.e., the complete lack of coelomic pouches as well as muscle cells, demonstrates the importance of FoxY. Our observation that expression of essentially all CPC genes is abolished, identifies *foxY* as the linchpin in the regulatory network underlying specification of CPCs. Exceptions are few: *pitx2* is affected only in D/N perturbations but not following injection of FoxY MASO. But this is not surprising given the likely role of Pitx2 as the repressor of *foxY* in the right coelomic pouch. Nevertheless, the fact *pitx2* indirectly requires D/N signaling but not FoxY indicates the functions of NSM Delta signaling and FoxY, while largely overlapping, are not perfectly congruent. The transient expression of Delta in the NSM requires that *foxY* receives additional inputs to assure its continued expression. *foxY* is likely part of a positive feedback loop involving genes it first activates as is common for pioneering transcription factors establishing a new regulatory state (Davidson, 2010). Such recursive wiring may be the cause for the mostly normal, albeit low, transcript levels of *foxY* following its own perturbation at late gastrulation, i.e., only after a long delay. Additionally, *foxY* may also be activated by Bmp/Tgf- β signaling as has been suggested for other CPC genes (Luo and Su, 2012). Nevertheless, restriction of *foxY* expression to the left side, where it remains expressed (Ransick et al., 2002), is one of the first events in the progressive regionalization at the tip of the archenteron and presages where the adult rudiment will form. Although muscle cells are derived from the same group of *foxY* positive progenitor cells they give rise to between 20 and 40 fully differentiated cells in grown larvae (Burke and Alvarez, 1988b; Cox et al., 1986). In contrast, the CPCs are a major contributor to the adult; at metamorphosis their offspring numbers in the tens of thousands.

Coelomic Pouch Morphogenesis and Symmetry Breaking

Separation of CPC progenitors and their relocation to the sides of the foregut is dependent on Hh signaling. This morphogenetic process bears resemblance to the separation of midline structures in vertebrates, which is strongly dependent on Hh signaling (Schachter and Krauss, 2008). Improper regulation of Hh signaling leads to severe defects such as cyclopia and incomplete separation of brain structures (Roessler et al., 1996). In sea urchins, loss of Smo activation results in formation of only one centrally located coelomic pouch. However, none of the transcription factors expressed specifically at the tip of the archenteron are affected by loss of Hh signaling. The only exception in our dataset is *pitx2*, due to interference with late Nodal expression. Thus, the CPCs now positioned atop the foregut display the regulatory state that is normally reserved for the left coelomic pouch only. Additional Hh responsive transcription factors may await discovery but Hh signaling may also directly regulate morphogenetic effector genes, such as cell motility or adhesion molecules. When misregulated, these genes cause similar craniofacial defects as loss of Hh signaling (Chen et al., 2006).

Our data reveal Hh signaling is required for establishment of laterality in sea urchins. Loss of Hh signaling causes failure to activate *nodal* transcription in the right coelomic pouch and the right lateral ectoderm. The importance of Nodal signaling in L/R asymmetry has been studied extensively (Bessodes et al., 2012; Duboc et al., 2005; Luo and Su, 2012; Nakamura and Hamada, 2012) and a close link between Hh and Nodal signaling is well established (Zhang et al., 2001). Nevertheless, how both signaling systems relate to each other mechanistically remains unknown. Laterality defects in mice caused by loss of Hh signaling can be rescued by expression of a constitutively active Smo receptor in lateral plate mesoderm resulting in unilateral expression of *nodal* (Tsiarris and McMahon, 2009). In sea urchins overexpression of a constitutively active

Smo receptor randomizes the position of the adult rudiment (Walton et al., 2009). It has recently been shown that unilateral expression of Nodal at the archenteron tip, and thus in close proximity to the Hh source, determines the sidedness of Nodal expression in the ectoderm (Bessodes et al., 2012). However, in contrast to our Hh perturbations, disruption of Nodal signaling specifically within the archenteron leads to randomization of *nodal* transcription in the ectoderm and not loss of expression; if Hh itself is received in the ectoderm is currently unknown. Regardless, while the initial cause of symmetry breaking remains elusive, it is well recognized that Nodal signaling is part of the conserved downstream mechanism establishing laterality across the embryo (Duboc et al., 2005; Grande and Patel, 2009; Nakamura and Hamada, 2012). Our experiments demonstrate that in sea urchins this function includes repression of *foxY* in the right coelomic pouch by Pitx2, thus confining *foxY* expression, and adult rudiment formation, to the left side.

Coelomic Pouch Cells as Multipotent Progenitors

Coelomic pouches are a conserved feature of echinoderm embryos (Chia and Walker, 1991; Pearse and Cameron, 1991), but if the molecular mechanisms controlling their formation are conserved is currently unknown. Nevertheless, in the distantly related starfish *Patiria miniata* coelomic pouch formation is dependent on D/N signaling within the mesoderm just as it is in euechinoids (V. Hinman, personal communication). But whether starfish and other echinoderms possess a *foxY* ortholog and if it is employed downstream of D/N signaling is an open question. A second conserved feature is localized expression of Vasa protein. Although initially localized to SMMs, Vasa eventually becomes expressed throughout the coelomic pouches (Juliano et al., 2010).

Despite its connection to germ cell specification, recent evidence indicates Vasa is more broadly connected to pluripotency (Gustafson and Wessel, 2010) and is expressed in mammalian embryonic stem cells (Ingham and McMahon, 2001; Ramalho-Santos, 2002), and adult stem cells in planarians and *Hydra* (Rebscher et al., 2008). It is also expressed throughout the mesodermal posterior growth zone of polychaete annelids, a group of multipotent stem cells that, similar to coelomic pouches in echinoderms, gives rise to adult structures and germ cells (Rebscher et al., 2007). Coelomic pouch development thus provides a unique opportunity to understand how developmental potency is maintained throughout successive patterning events in cells embedded in an environment that otherwise promotes quick differentiation.

Acknowledgements

We are grateful for support and advice from Eric Davidson in whose lab most of the work was done. Many thanks to Celina Juliano and Andy Cameron for numerous insights and to Gary Wessel for Vasa antibody. This work was supported by NIH grant HD-037105 to Eric Davidson, a CIRM fellowship and NIH grant 1P30GM092374-01 to JS.

Materials and Methods

Perturbation experiments

FoxY morpholino antisense oligonucleotide (MASOs) (CATGGCTCCAAGTGCAGAACTAC) was obtained from Gene-tools LLC (Pilotown, OR, USA) and injected at 300 μ M in 0.12 M KCl. A random MASO (N₂₅) was injected as a control; injection volumes were 5 μ l. DAPT and SB431542 were applied as reported previously (Materna and Davidson, 2012; Materna et al., 2013a). Cyclopamine was

dissolved in ethanol and added to a final concentration of 0.5 μ M. A corresponding amount of solvent was added to controls.

RNA extraction and transcriptional profiling

Sea urchin embryos were cultured at 15°C. RNA was extracted using the Qiagen (Hilden, Germany) RNeasy Micro Kit according to manufacturer's instructions.

Expression levels were quantified with the Nanostring nCounter (NanoString, Seattle, WA, USA) using a probe set for 205 genes. Samples were processed according to manufacturer's instructions and data processed as described (Materna and Davidson, 2012). Data were supplemented by QPCR for *nanos* and fold changes calculated using *poly-ubiquitin* and *hmg1* as references (Materna and Oliveri, 2008). A threshold of 2-fold difference was chosen as a significant change (Materna and Oliveri, 2008). QPCR primer sequences are included in Table S1. Code set information and all perturbation data have been submitted to the Dryad Digital Repository (Materna et al., 2013b).

Whole mount in situ hybridization

Whole mount in situ hybridization was conducted following standard procedures (Materna and Davidson, 2012). In brief, templates were amplified by PCR (for primers see Table S1) and used for transcription of DIG labeled probes. Embryos were fixed in 2.5% glutaraldehyde, 32.5% sea water, 32.5 mM MOPS (pH 7) and 162.5 mM NaCl on ice overnight. Embryos were Proteinase K treated for 5 min at room temperature (25 ng/ μ l in TBST) followed by 30 min fixation in 4% paraformaldehyde, 32.5% sea water, 32.5 mM MOPS (pH 7), 162.5 mM NaCl at room temperature. Probes were hybridized over night and detected using anti-DIG Fab fragments conjugated to alkaline

phosphatase. Color was developed using NBT/BCIP reagents; fluorescent in situs were developed using TSA amplification.

Immunofluorescence

In situ stained embryos were co-labeled by immunofluorescence for vasa protein. Following a 30 min blocking step (TBST, 10% sheep serum), embryos were incubated overnight with Vasa antibody (Voronina et al., 2008) diluted 1:250 in TBST buffer with serum. Embryos were washed 3 times for 15 minutes with TBST buffer without serum, and incubated for 2 hours with Cy3 goat anti-rabbit secondary antibody diluted 1:250 in TBST buffer with serum. Embryos were washed 3 times in TBST buffer and imaged.

Phalloidin staining

F-actin was visualized by rhodamine phalloidin staining (Strickland et al., 2004). In brief, embryos were fixed for 1 hour in Millonig's phosphate buffered fixative followed by 2 washes in 50 mM HEPES, 50 mM PIPES, 0.6 M mannitol, 3 mM MgCl₂, 0.1 % Triton X-100, pH 7.0. Embryos were transferred to TBST and stained with rhodamine phalloidin in TBST (2U/ml) for 1 hour. Embryos were washed three times and then imaged.

Reporter assays

About 1500 molecules of reporter construct in ~4pL were co-injected with a 6-fold molar excess of carrier DNA in 0.12 M KCl. BAC reporters were constructed as reported previously (Smith, 2008) and injected with 400 copies of BAC per 4 pL without carrier. Spatial activity was assessed by fluorescence microscopy. All experiments were repeated in different batches of eggs. Cis-regulatory regions were identified as described

(Smith, 2008). For target site disruptions, C's were mutated to T's and A's to G's, and vice versa, for invariant bases in consensus sites. 5'-(C/T)(A/G)TG(A/G)GA(A/G/T)-3' was used to identify candidate Su(H) sites (Ransick and Davidson, 2006). The TG at positions 3/4, and the GA at positions 6/7, were mutated to CA and AG. 5'-GGATTA-3' was used to identify candidate Pitx2 target sites, and mutated 5'-GATT-3' to 5'-AGCC-3'.

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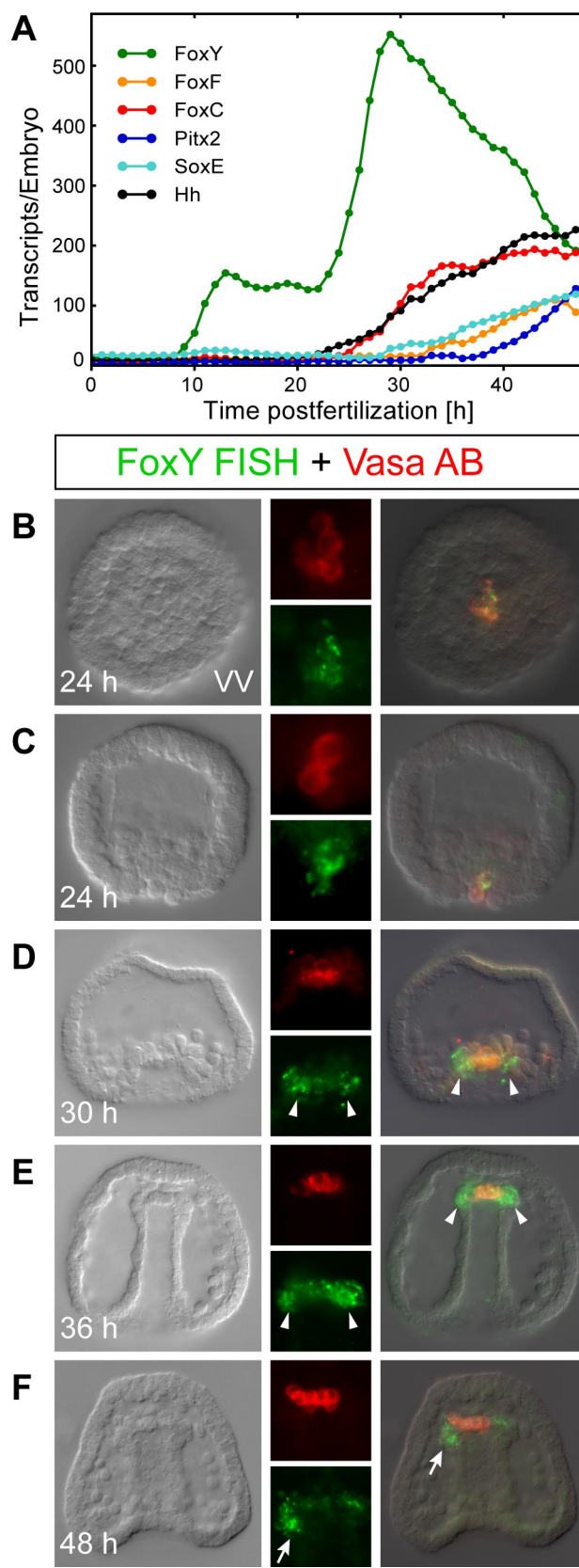


Figure 1. Expression profiles of CPC genes. A) Temporal expression from fertilization to late gastrulation. Levels are given in molecules per embryo. B–F) *foxY* mRNA/Vasa protein staining. Left panel—DIC, central panels—*foxY* signal (green) and Vasa signal (red), right—merge. Enlarged central panels capture all fluorescence. Initially, *foxY* message is restricted to SMM and overlaps perfectly with Vasa. D) *foxY* expression expands into mesodermal cells during gastrulation (arrowheads); SMMs are located at the archenteron tip. E) By mid-gastrulation the entire archenteron tip expresses *foxY* (arrowheads). F) *foxY* expression is biased towards the left coelomic pouch by 48 hpf (arrow).

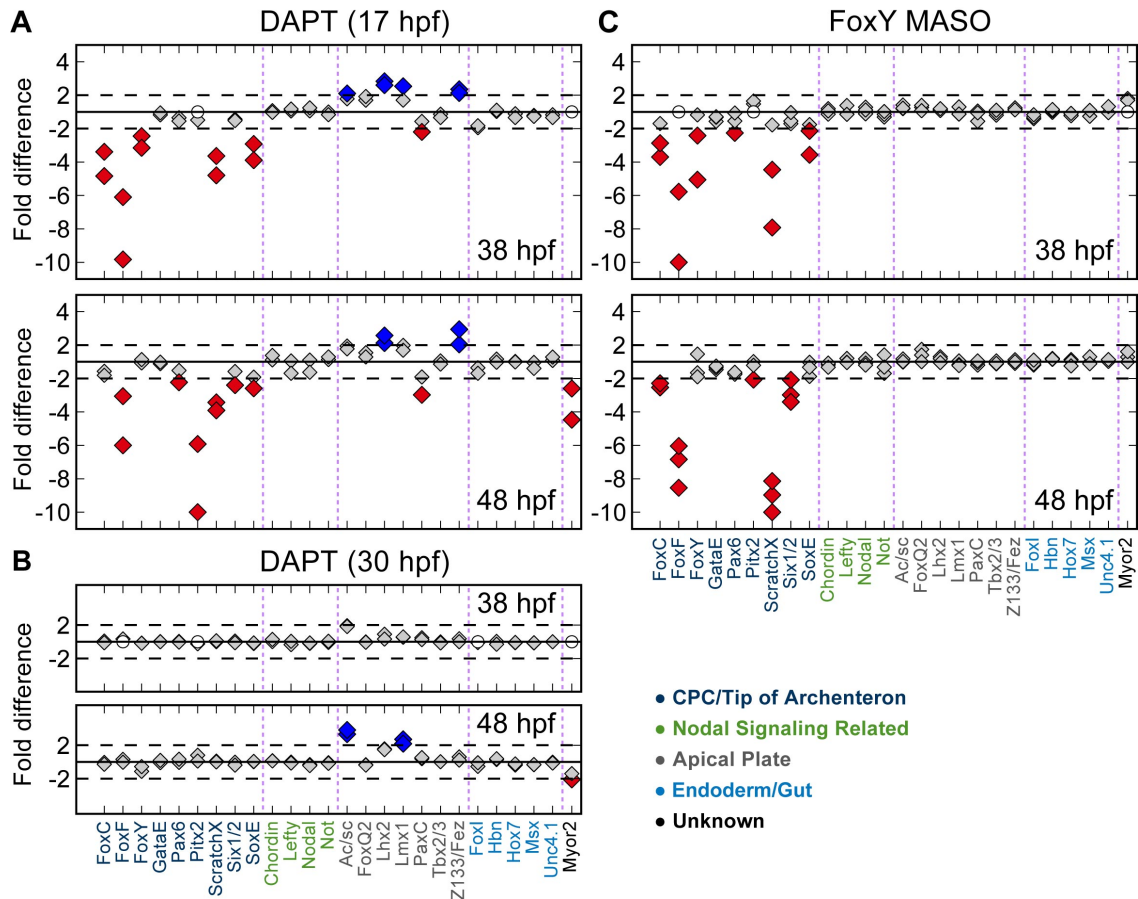


Figure 2. Effects of Delta/Notch and FoxY perturbations on transcript levels for select genes. A) DAPT treatment at 17 hpf causes strongly reduced transcript levels of CPC genes and significantly affects abundance of apical plate genes. B) DAPT treatment at 30 hpf affects apical plate genes but not CPC genes. C) Injection of FoxY MASO causes strong depletion of CPC genes only. Each diamond represent a single experiment; red indicates a significant reduction, blue an increase, and grey no significant change of transcript levels following treatment. Genes not significantly transcribed in experiment *and* control are marked with an open white circle. We estimated transcript levels by comparing our data to prior absolute measurements (Materna et al., 2010). A level of ~25 transcript/embryo was chosen as significant expression. Black dashed lines indicate threshold for significant changes following perturbation.

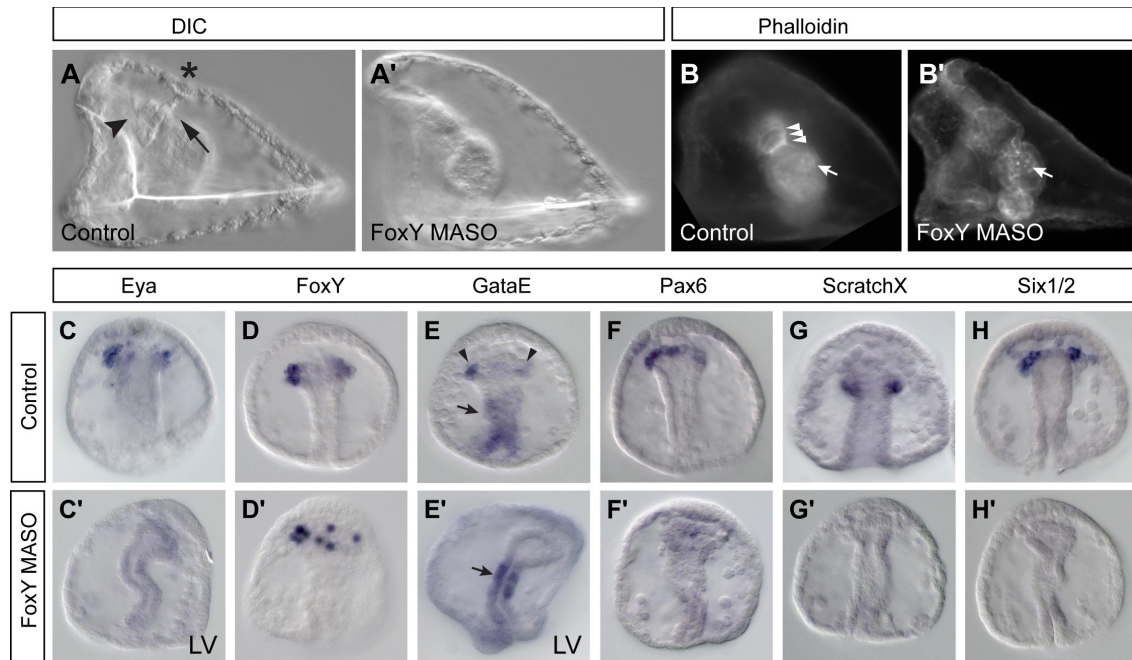


Figure 3. Loss of FoxY function disrupts coelomic pouch development. A) At 3.5 dpf the left coelomic pouch (arrowhead) lies alongside the esophagus and gives rise to the water canal (arrow) connecting to the hydropore (asterisk) in the ectodermal wall. A') FoxY MASO injection disrupts formation of all coelomic pouch derived structures. B) Rings of muscles form around the esophagus (arrowheads). B') FoxY MASO application abolishes muscle formation but does not affect rhodamin phalloiding staining in ciliated gut cells (arrow). C, C', E, E'–H, H') CPC gene expression is abolished following FoxY MASO treatment; *gataE* in the gut is not affected (arrow in E'). D, D') *foxY* expression persists but positioning of *foxY* positive CPCs is altered. Embryos in C–H are 48 hpf and oriented in an aboral view unless otherwise noted. LV—lateral view.

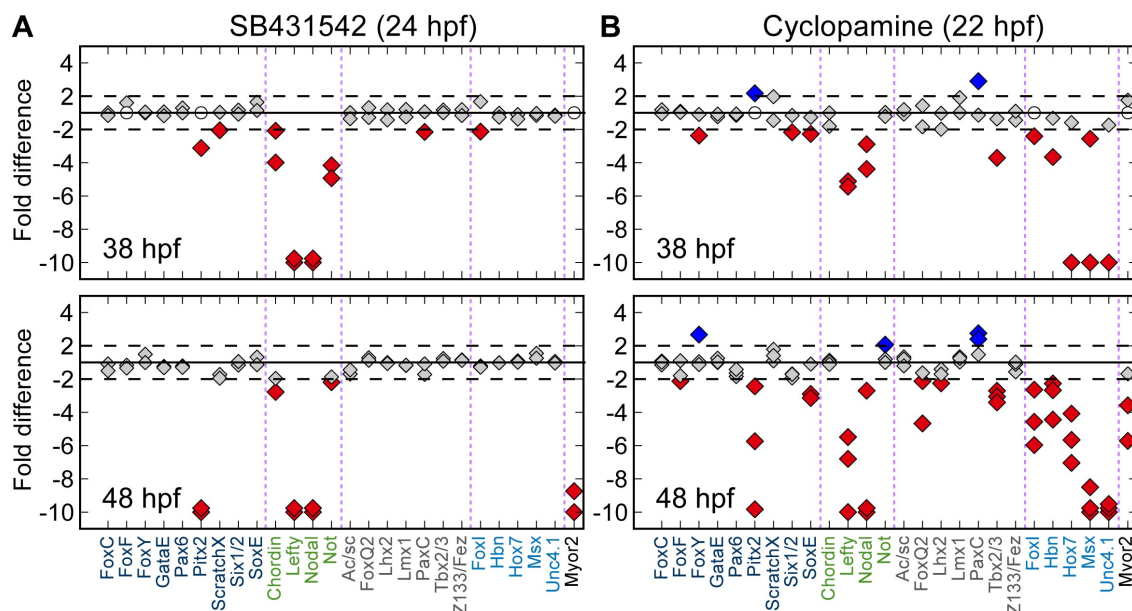


Figure 4. Effects of Nodal and Hedgehog perturbations on transcript levels. A) Application of SB431542 causes strongly reduced transcript levels of *lefty*, *nodal*, *pitx2*, *myor2* but not of endoderm and apical genes. B) Loss of Hh signaling after cyclopamine treatment affects endoderm genes (*foxl*, *hbn*, *hox7*, *msx*, *unc4.1*) in addition to *nodal*, *lefty*, *pitx2*, and *myor2*. Symbols and labels as in Fig. 2.

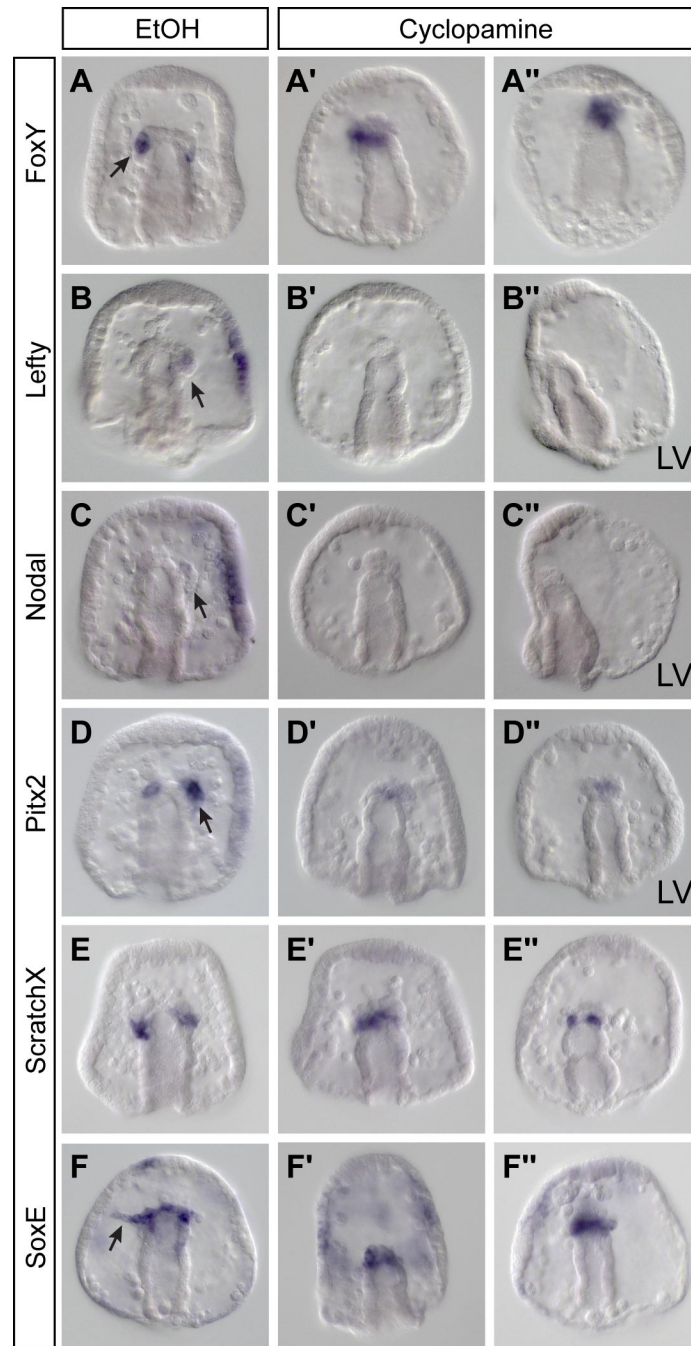


Figure 5. Spatial effects of Hedgehog perturbation. A–A'') At 66 hpf *foxY* expression is restricted to the left pouch (arrow). After cyclopamine treatment *foxY* expressing CPCs remain at the tip of the archenteron and form one pouch-like structure. B–B'', C–C'', D–D'') *lefty*, *nodal*, and *pitx2* are expressed in the right lateral ectoderm and right coelomic pouch (arrows in B,C,D); loss of Hh signaling disrupts their expression. E–E'', F–F'') *scratchX* and *soxE*, like *foxY*, remain strongly expressed following cyclopamine treatment. *soxE* is expressed in the water canal (arrow in F) and in the ciliated band (Fig. S2J''). All embryos are oriented in an aboral view unless otherwise noted. LV—lateral view.

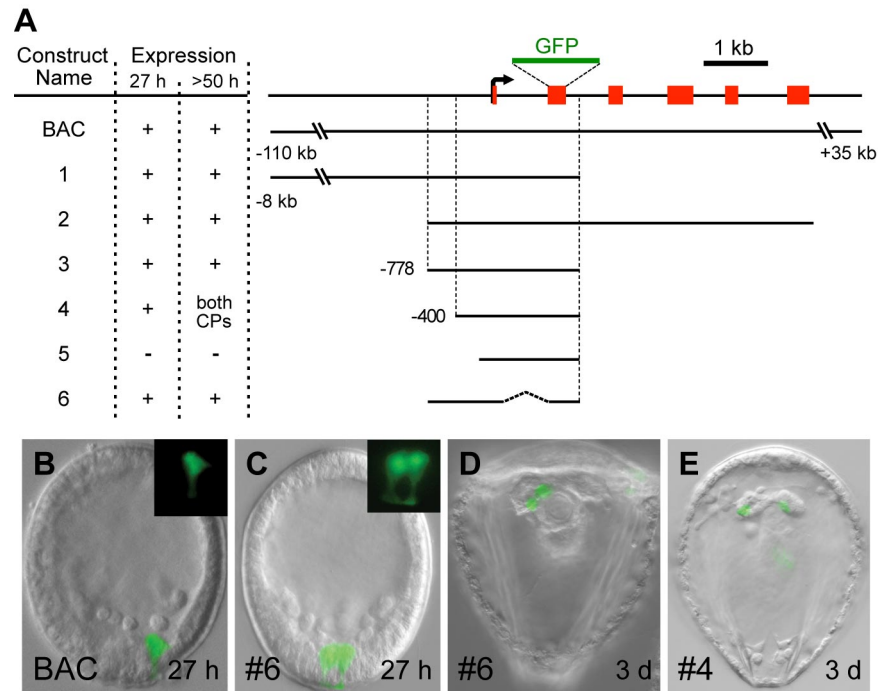


Figure 6. Identification of regulatory elements driving correct *foxY* expression. A) Constructs and expression, (+) — >85% (of GFP+ embryos) expressed GFP correctly (small micromeres/mesoderm at 27-28 hpf, left coelomic pouch past 50 hpf); (–) — loss of expression; “Both CP’s” — expression in both pouches. B) Example of FoxY BAC-GFP expression within the vegetal plate; inset shows bottle cell shape of GFP+ cells prior to gastrulation. C) Construct #6 expression is identical to the full BAC. D) Expression of Construct #6 is strongly biased to the left coelomic pouch. E) Construct #4 drives continuous expression in both pouches.

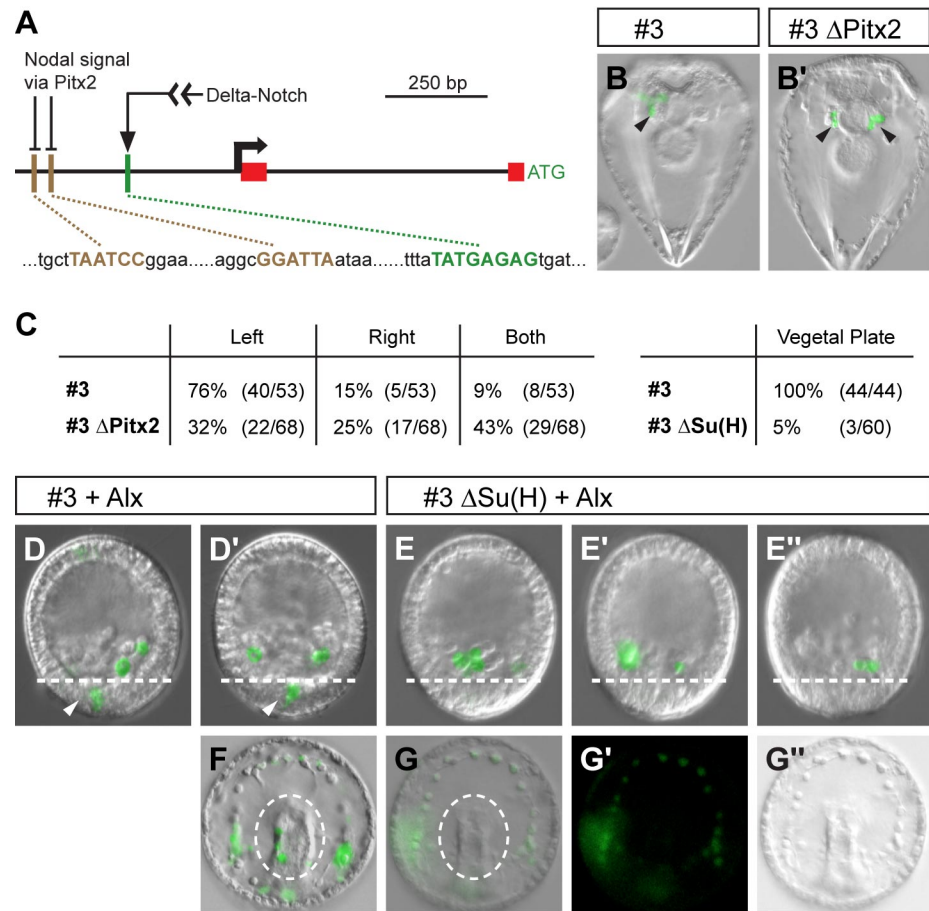


Figure 7. Identification of functional binding sites. A) Schematic view showing tested Pitx2 sites (tan) and Su(H) sites (green) upstream of the *foxY* transcription start site (bent arrow). B, B') Construct #3 shows reporter expression in the left coelomic pouch at 3 dpf. Mutation of the homeobox sites (#3-ΔPitx2) results in robust expression in both coelomic pouches. C) Frequencies for spatial expression of #3 vs. #3-ΔPitx2 at 3 dpf and #3 vs. #3-ΔSu(H) at 27-28 hpf. D, D', F) Construct #3 is expressed first within the vegetal plate (arrowhead) at 27-28 hpf, and at the tip of the archenteron at 48 hpf (within dashed circle in F). Co-injected Alx BAC-GFP is expressed in ingressed skeletogenic cells (above dashed line). E-E'', G-G'') Disruption of the Su(H) site (#3-ΔSu(H)) abolishes reporter expression in the vegetal plate and in the archenteron (within dashed circle in G); Alx BAC-GFP expression in skeletogenic cells is not affected. GFP channel is shown in G', DIC in G''.

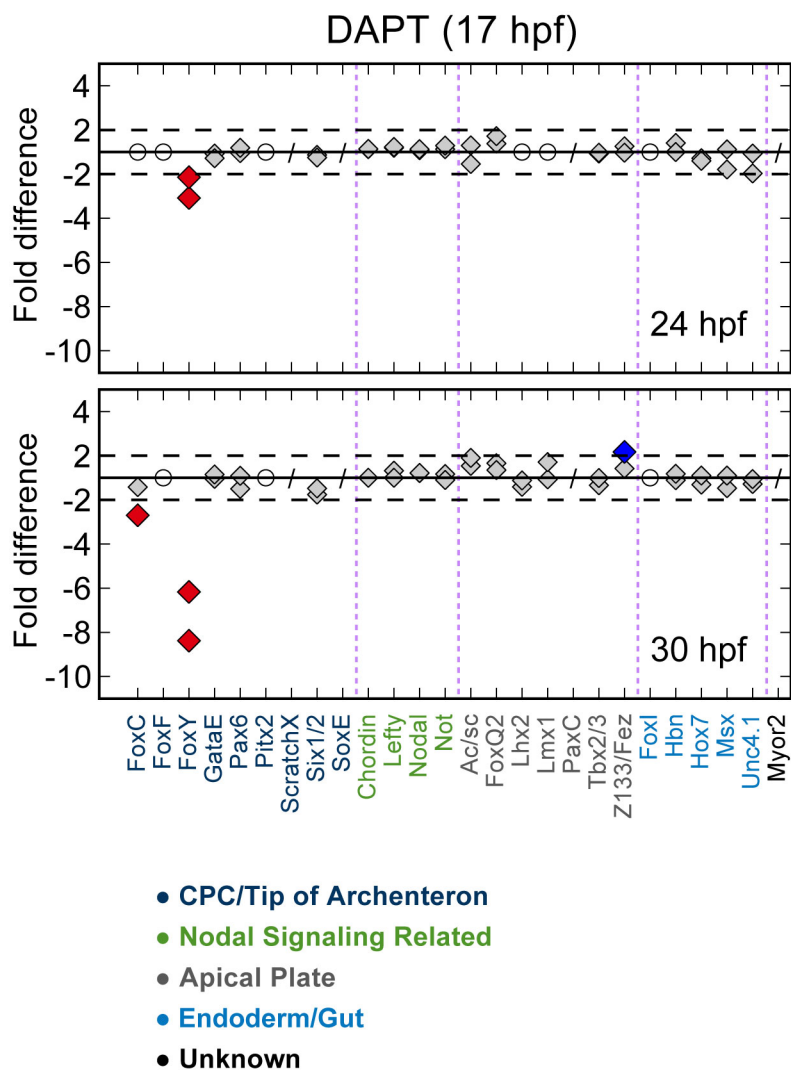


Figure S1. Effects of Delta/Notch perturbations on transcript levels at 24 hpf and 30 hpf for the same selection of genes as shown in Fig. 2. DAPT treatment at 17 hpf causes strong and consistent depletion of only foxY among the 182 genes included in this dataset. The data are reformatted from Materna and Davidson (Materna and Davidson, 2012). Each diamond represents a single experiment; red indicates a significant reduction, blue an increase, and gray no significant change of transcript levels following treatment. Genes not significantly transcribed in experiment and control are marked with an open circle. We estimated transcript levels by comparing our data with prior absolute measurements (Materna et al., 2010). A level of 25 transcripts/embryo was chosen as significant expression. Genes not tested are marked with a solidus. Black dashed lines indicate the threshold for significant changes following perturbation.

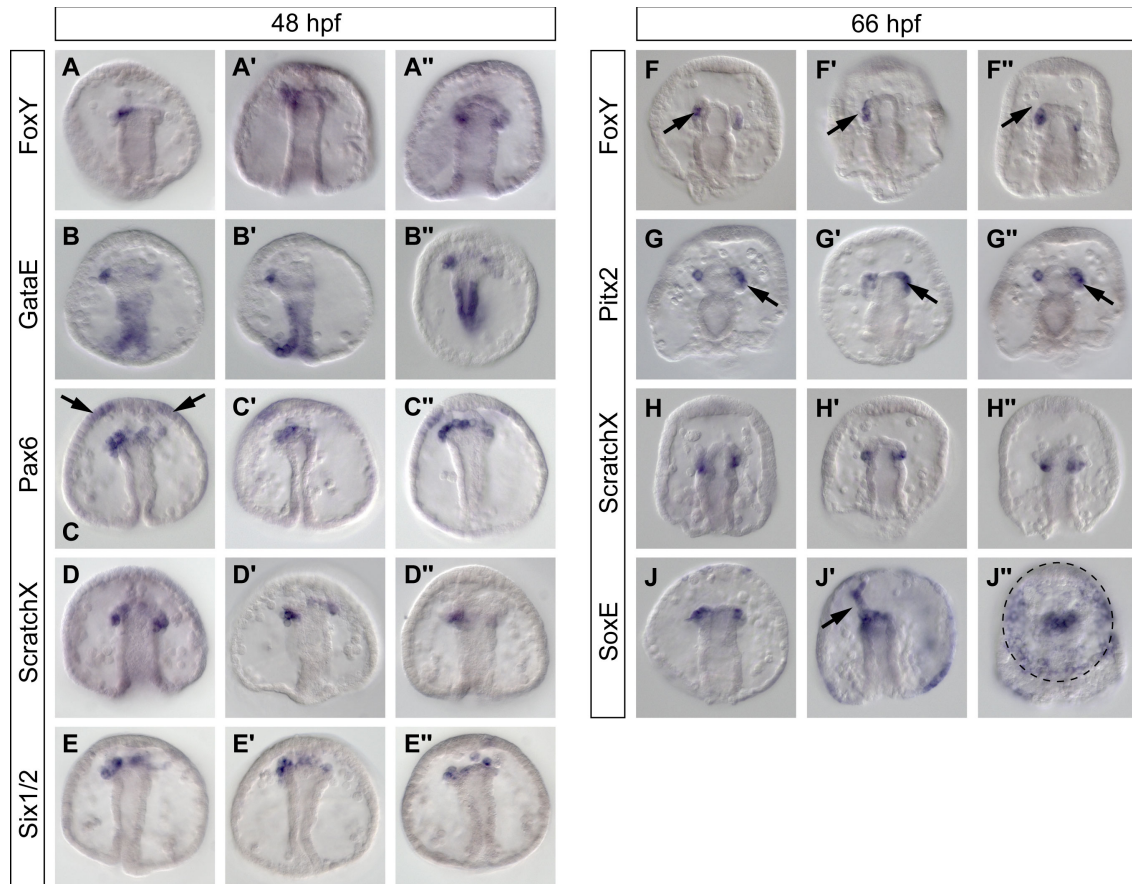


Figure S2. Additional spatial expression patterns of genes with a specific coelomic pouch expression compartment. (A-A'', F-F'') *foxY* expression is biased to the left side at 48 hpf, but becomes restricted to the left side after 66 hpf (**arrows in F-F''**). (**B-B''**) *gataE* is expressed in the gut and the coelomic pouches, and is biased to the left side. (**C-C''**) *pax6* is expressed at the tip of the archenteron, but also in the ectoderm surrounding the apical plate (arrows in C). (**D-D'', H-H''**) *scratchX* is expressed in both pouches and remains bilaterally expressed until at least 3 dpf. (**E-E''**) At 48 hpf *six1/2* is expressed at the tip of the archenteron. (**G-G''**) *ptx2* is preferentially expressed in the right coelomic pouch (arrow). *ptx2* and *foxY* expression patterns are complementary; overlap is minimal at best (compare G-G'' with F-F''). (**I-I''**) *soxE* is expressed in the coelomic pouches and the water canal as it emerges from the left coelomic pouch (arrow in I'). It has a second expression domain in the ciliated band (dashed line in I'').

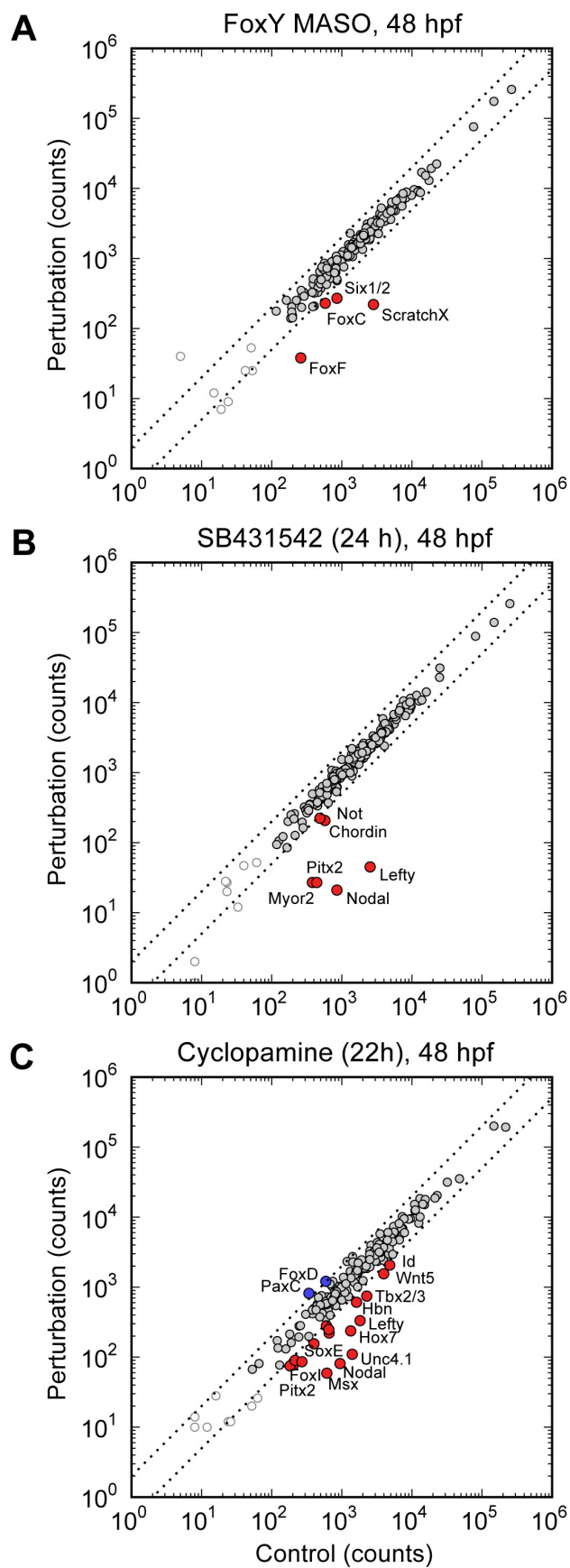


Figure S3. Global evaluation of perturbation effects by transcript quantification.

RNA from treated embryos was extracted and quantified using the NanoString nCounter and the resulting counts were normalized. Counts from perturbed embryos were plotted against those of control embryos. **(A)** FoxY MASO injection at fertilization causes depletion of transcript levels for genes specifically expressed in the coelomic pouch progenitors. Out of 205 genes only a few, none of them outside the foxY expression compartment, are affected by the perturbation, indicating that the MASO does not cause nonspecific delays in development. **(B)** Nodal perturbation by addition of the inhibitor SB431542 at 24 hpf causes lower transcript levels of only six genes included in the NanoString code set. **(C)** Disruption of Hh by application of cyclopamine has more widespread effects and also causes depletion of several endoderm genes. Labels for some weakly affected genes that were not substantiated in repeat experiments were left out of C for clarity. The dotted lines indicate a threshold of 2-fold change. A red dot indicates significant depletion following treatment, a blue dot a significant increase, and a gray dot indicates no significant change. Genes present with ~25 transcripts or fewer per embryo are marked with an open circle; transcription levels were estimated from prior absolute measurements (Materna et al., 2010).

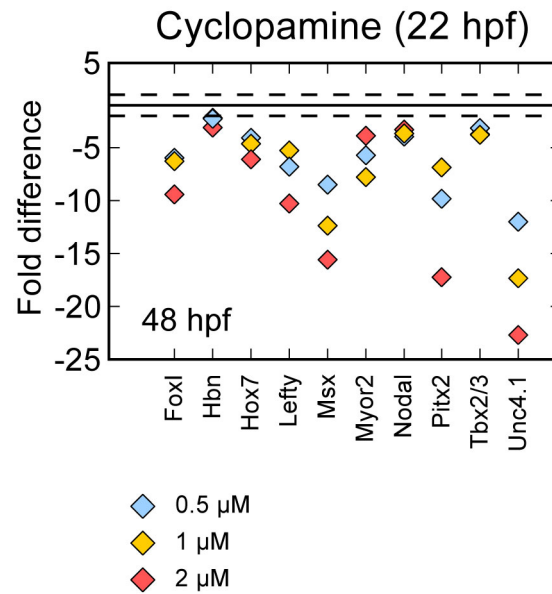


Figure S4. Effects of cyclopamine treatment are dose dependent. Sea urchin embryos from the same batch were treated with the indicated concentrations of cyclopamine to disrupt Hh signaling. RNA was extracted and quantified with the NanoString nCounter and fold differences were calculated between controls (ethanol only) and cyclopamine-treated embryos. This plot shows the genes that are most strongly affected by the perturbation (compare with Fig. 4B and Fig. S3C). In our experiments, cyclopamine concentrations of 5 μ M and higher caused severe developmental delays and death.

FoxY Construct #3 upstream region (-778 to ATG/GFP in second exon)

TTCGCAGGCTAGAGACACCTCTCTGCTTAATCCGGAAGCTTAAACAAAGAGCCCTCATTGTCAATGGTGTAGTG
 TACGAGGC GGATTAATAAATCCAATTAGAAGGGTATCACCAGGCGTGGGAAAAGGAATGGGTCTGACTTGGCTA
 GTCTTATCATTTTCATCATCAGACCGTTTTGAAAACAATCCATAATAGATTAGAGTCAATAAATTAGACAGATGA
 TAAAATGGGTCTTGATTTGCTATAGATCGTTGGCATAACCCATGCATTGGGTATGATGTCTTTTAAAGCCATTCG
 AACAAAACACTATATATGCAATCATGCACAATCTGATTTCTTCTTCTCTGTCTTTCTCTCCATGCCCCCTCCCC
 GCCCCCCC CCCCTCTCTCTCTCTCTCTCTATCTCAGAGAGTTTCTGTCTCTATATAGAACTCTCTAGACATTA
 TTTGAACTTCTATGATTGTACAATATGCGTTTGGACAATTGTCATGTTTGTGAATTTTGAAGTATCATTTTTAT
 ATGAGAGTGATATTTTGTGATCGACGGTATGATTGAAAAAGATCAACTTGGTCTGATTTCAAAGACTACCATA
 TAGAAAAAGATTGTTTCGCGCATGCGTCGCCCTGATCCTTTCTTTTCGCATCTCGATTACGTAACACCTCGCTCTC
 CTGTAATTGGCTTAGAGTTGGTAAGAACGGTGAGAACAGATCAGGTATAAAGTAAGATAGAAGTCCGATGAAGA
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 TCTCGCCTCATCGATTGCCTTACTCCTCATTTTCCATAACCATTGTAGGTGAGTATCTCACGTTAAATTGTAAT
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 AGTCTTGTTGGTTGAATGGTGACTTGCGTTTGTCTTCTTATCGATGAGAGGGGAATTCGTGTGAAGGGTTTTTA
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 TACTTCGGAATATTCTTTTGTCTTCTTGATTATGAACATTCTGGCCAGAAAGATATACGGCTAGGCCCAGAGC
 ATGATCAGGTATATTTTGTCTGTTATACGTGAGCTTTGAGTGTCTGATACATAATATATGTATATTCTTTGTAA
 ATATTTTTCGCTGGAATTCGTTTGGCCAAAATGATTATATTAGTGTGGTTTTATGAATGTCTATACAATCGA
 TACCTTTTTATGATTGTTATTTCATGAATCAAAGATGAAATAAAGAAGGAAAAAATGAACCCGTTGTTTTCCGGA
 GATGTGGGAGGTAAATTTATGACCAAAAATCTACTTGTCTTTTTTTTCACTTTTCATGAATAGTGTCTGCACTT
 GGAGCCATGAGCAAGGGCGAGGAACCTGTTCACTGGCG...

- Pitx2 binding sites
- Start of Construct #4
- Su(H) binding sites
- Exon
- Splice Site
- Start codon
- GFP sequence

Figure S5. Annotated sequence of the regulatory region upstream of the foxY transcription start site. This sequence corresponds to the region contained in Construct 3; Construct 4 is nested within, starting at position 379 (marked in blue). 59-(C/T)(A/G)TG(A/G) GA(A/G/T)-39 was used to identify candidate Su(H) sites (Ransick and Davidson, 2006). The TG at positions 3 and 4, and the GA at positions 6 and 7, were mutated to CA and AG. We used the sequence 59-GGATTA-39 to identify candidate Pitx2 target sites, and mutated 59-GATT-39 to 59-AGCC-39.

Table S1. Primers for WMISH templates and QPCR

Gene	Forward primer	Reverse primer	Use	Accession No.
<i>eya</i>	GTATTGGAAGAGGGCGTCAA	AGGAGGGGTGGATAGCATCT	WMISH template	SPU_013869
<i>foxY</i>	ACTGGATGATCAACCCAAGC	AGAAACCTTTGGTGGTGTGCG	WMISH template	AF517552.1
<i>foxY</i>	TGCACTGCACTGACTCTGC	CTTTCCATTCCGTGGTGAAG	QPCR	AF517552.1
<i>gataC</i>	GCTGGAGTAGCAAGCAGTCAAG	TGTTACCCATTACCCAGGATG	WMISH template	NM_214539.1
<i>gataE</i>	TCTTACCCATCAGCCAGGAC	TCGAGTGGAACAATGGAACA	WMISH template	NM_001005725.1
<i>hmg1</i>	GGACCGAGACAGTTCAAAGC	CTTCTCCTCCAGAGCCTTCC	QPCR	SPU_027981
<i>lefty</i>	GAACTTCATACGACAAACATCTCCTGGC	TTTCGATTATATCATATTATTCATTTTATTATG	WMISH template	SPU_009911
<i>nanos</i>	GCAAGAACAACGGAGAGAGC	CCGCATAATGGACAGGTGTA	QPCR	DQ286228.1
<i>nodal</i>	CACAAAGTGTGTTTGTGCAAG	GTGATGAAATTGAAAATATCATGA	WMISH template	NM_001098449.1
<i>not</i>	ACGATTGGAGGGTTTAAAGC	GCAACTTACCCGTCATCACC	WMISH template	NM_214562.1
<i>pax6</i>	GACAGTGTTCTCATCGTGGTG	ACCTCGCGAGAGAAAATCAA	WMISH template	SPU_006786
<i>pitx2</i>	ATCCCAAATTTCCTCGTCTTC	TTGGTGGTGTCATGTGGT	WMISH template	SPU_004559
<i>scratchX</i>	CGGCCTAACAAGGAGAAAGC	ACGGTTTAAATGGTGGAGTGC	WMISH template	SPU_15640
<i>six1/2</i>	TGAAACACCGTCAAAACAAGG	AACTTGTCGTGGTCAGTCC	WMISH template	SPU_017379
<i>soxE</i>	GGAGTAGCCCAAGAACACCA	TGACTGTACCTGCCAAGGAG	WMISH template	SPU_016881
<i>ubq</i>	CACAGGCAAGACCATCACAC	GAGAGAGTGCACCATCCTC	QPCR	SPU_021496

Chapter 4: Wnt and Delta/Notch Regulation of the Nanos Gene in the Sea Urchin Primordial Germ Cells

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I performed all experiments in this study. L.W. and A.H.W. provided the Axin construct, morpholinos that allowed me to rule out other factors not discussed herein, and significant guidance.

Abstract

Segregation of primordial germ cells (PGCs) is a critical event for sexually reproducing animals. Various Nanos orthologs, which are RNA binding proteins, are required for the development of PGCs in all species where they have been tested. However, the mechanisms by which Nanos genes are transcriptionally regulated remain elusive. We report here that activation of Nanos requires the maternal Wnt pathway components. β -catenin is highly nuclearized in the sea urchin PGCs, the small micromeres (sMics), within the domain where Wnts 1, 8, and 16 are expressed. Initial Nanos expression in the sMics requires nuclear β -catenin. Nanos expression is sustained then by overlapping action of Wnts 1 and 16. During gastrulation, following the initial segregation of the sMics, Nanos expression expands into adjacent mesodermal cells. This secondary Nanos expression requires Delta/Notch signaling. Knockdown of Wnt8, which is expressed in the micromeres, results in a large upregulation of Nanos in the adjacent mesodermal precursors adjacent to the sMics, suggesting an evolutionary origin for the germ line in echinoderms lacking the micromere lineage. Chromatin immunoprecipitation revealed a direct association between β -catenin and the Nanos2 locus, centered around a consensus Tcf site. These results uncover a germ line gene regulatory network acquired in the evolution of echinoids, initiated by maternal β -catenin, and sustained by embryonically produced Wnt and Delta/Notch signaling. Given the importance of Nanos in this animal, we believe the hardwired initiation of Nanos in the early sea urchin embryo reflects a major transition in the echinoid reproductive mechanism.

Introduction

All sexually reproducing animals, at some point in their lifecycle, specify cells capable of creating eggs or sperm. In most studied bilaterians, primordial germ cells (PGCs), progenitors of gamete-producing germ line stem cells (GSCs), are functionally segregated from somatic cells during embryogenesis. The timing and mechanisms of PGC segregation varies greatly between species, but requires either inheritance of maternally supplied determinants or induction from cell-cell communication and embryonic transcriptional networks. PGCs of *Drosophila* and *C. elegans* are largely specified by inheritance of germ plasm, a conglomerate of instructive RNA and protein factors. However, broad molecular comparisons indicate that these inherited mechanisms likely evolved secondarily, whereas inductive PGC specification may represent the ancestral mode of germ line determination (1, 2). For example, in the mouse, PGCs are induced at 6.5 days post fertilization by intercellular communication. This induction requires secretion of Bmp4 and Wnt3 ligands from the extraembryonic ectoderm. Presumptive PGCs in the distal mesoderm respond by expressing Brachyury/T, a direct target of β -catenin, and initiating a tripartite gene regulatory network. This network comprises Blimp1, Prdm14, and AP2 γ , which broadly represses somatic gene expression and initiates expression of select germ line genes (3). The axolotl, a urodele amphibian, also requires Bmp signaling and T for germ line induction (4). Recent investigation in the cricket *Gryllus gryllus* has revealed a potentially deeply conserved role for Bmp signaling in inductive germ line specification (5). Whether Wnt/ β -catenin signaling also plays a conserved partnering role in this process is unknown.

The PGCs of the sea urchin are created by two early, asymmetric cleavages. The 4th cleavage results in a 16-cell embryo, which includes a vegetal quartet of small cells called micromeres. The subsequent 5th cleavage divides the micromeres asymmetrically to produce two cell lineages. The more animal-positioned large micromeres solely give rise to larval skeletogenic cells. In stark developmental contrast, the more vegetal small micromeres (sMics) are the PGCs. Following their creation, the sMics remain mitotically quiescent, dividing only once before larval stages. They remain at the vegetal pole, until gastrulation, when the sMics passively translocate with the developing archenteron and then actively assort into two bilateral coelomic pouches, which are the major contributing structures to the juvenile sea urchin (6). Throughout this developmental progression, the sMics display signs of broad transcriptional repression. Through blastula stages, the sMic nuclei are enriched for H3K9me3, and during gastrulation, are depleted for elongating RNA polymerase II. These repressive features are similar to what has been reported in *C. elegans*, *Drosophila*, and mice (7). As a result, the sMics are likely specified largely by post-transcriptional mechanisms. Indeed, the sMics inherit and retain the RNAs and proteins of conserved germ line factors such as Seawi and Vasa, as well as a suite of other factors (8, 9). Several aspects of specification can occur autonomously; e.g. isolated micromeres in culture divide asymmetrically to produce sMics, which asymmetrically inherit Vasa and upregulate Nanos expression (10). This observation implies that the sMics inherit factors that hard-wire them to activate a germ line program. While the germ line factors are degraded in somatic cells, they are retained in the sMics at least in part by depletion of the CCR4-related deadenylase CNOT6. Depletion of CNOT6 requires the RNA binding protein Nanos, which targets CNOT6 mRNA for degradation by recognizing sequence motifs in the CNOT6 3'UTR (9). Furthermore, Nanos is essential for PGC survival in this animal; Nanos-depleted PGCs

undergo premature cell division, followed by apoptosis (11, 12). Despite being refractory to transcription in a general sense, Nanos is not maternally supplied but rather embryonically activated in the sMics shortly after their creation (11). Expression of Nanos is, therefore, an essential and upstream event in PGC segregation.

The post-transcriptional control of Nanos has been extensively characterized (13-15).

However, the transcriptional regulation of this critical germ line stem cell factor remains poorly understood. In the *Drosophila* embryo, embryonic Nanos expression is not detectable until the PGCs have arrived in the somatic gonads. A microarray study of isolated PGCs identified Ovo as a transcriptional regulator required for Nanos expression, but whether it directly regulates the Nanos promoter is unclear (16).

Potential cis regulatory elements have been identified in a minimal region of the *Drosophila* Nanos promoter that is sufficient for its expression in adult GSCs (17). In vitro RNA-seq and ChIP studies in mouse PGC-like cells suggest a direct interaction between AP2 γ and the Nanos3 promoter, which becomes active during PGC migration, well after initial specification (3). In the sea urchin, Nanos2 expression begins in the sMics after the 5th cleavage and its mRNA persists until larval stages, when the PGCs arrive into the coelomic pouches. There are three Nanos paralogs in the sea urchin, designated Nanos 1, 2, and 3. Nanos 1 and 2 are highly similar in sequence, but PCR analyses indicate that Nanos1 is specific to early oocytes, while Nanos2 is expressed embryonically. Nanos 3 is more divergent and is expressed in the sMics by late gastrula stages (11).

The upstream transcriptional activators of Nanos2 are unknown in the sea urchin; however, the forkhead transcription factor FoxY has been suggested to be required sustained Nanos2 later, at the onset of gastrulation. FoxY expression is initiated in the

sMics at around 10 hours post fertilization (h.p.f.) in response to Delta/Notch signaling. Around the onset of gastrulation, FoxY expression expands into adjacent cells called the Veg2 mesodermal progenitors (18). Knockdown of FoxY during its early sMic expression phase has no impact on early Nanos2 expression, however, a moderate reduction of Nanos2 is observed around the time of gastrulation (19). FoxY is broadly required for establishment of the Veg2 mesodermal lineage, which is adjacent to the sMics in the blastula. Its knockdown results in the loss of several mesodermal derivatives, including the coelomic pouches (18). It is therefore unclear whether FoxY's effect on Nanos expression is direct; instead, it may be required to create a permissive niche for the sMics. Collectively, however, these observations do imply differential phases of Nanos regulation.

The canonical Wnt/ β -catenin pathway has been identified as a major regulator of vegetal fate specification in the sea urchin and other embryos (20). Maternally supplied Disheveled protein is pre-localized to the egg vegetal cortex and is thought to induce nuclearization of β -catenin in vegetal blastomeres. Disheveled inhibits the function of a destruction complex containing Axin, GSK3, APC, CK1 α , and PP2A, allowing the stabilization of β -catenin. Nuclear β -catenin associates with Tcf family transcription factors and initiates transcription (21). The earliest activation of the Wnt pathway in the early sea urchin embryo may occur independently of a Wnt ligand; the localized Disheveled may be specifically modified, perhaps to an active form that hardwires the pathway (22). Furthermore, animal-vegetal patterning is established even when all Wnt secretion is inhibited by perturbing Porcupine, which is critical for Wnt palmitoylation (23). Nuclear β -catenin initiates a cascade of organizing activity within the micromeres, including expression of Wnt8, a direct β -catenin target. The sMics are also highly

enriched for nuclear β -catenin, and while its role there is unknown, it is candidate for regulation of Nanos transcription (24). Here we investigate the function of the Wnt pathway with respect to germ line segregation in the sea urchin *Strongylocentrotus purpuratus*, and link germ line specification into the sea urchin gene regulatory network for the first time. This link occurs via highly conserved signaling pathways, now exposed for understanding the striking evolutionary transition that echinoids likely underwent for their precocious germ line specification.

Results

Initial Nanos expression in the sMics requires nuclear β -catenin

The canonical Wnt/ β -catenin pathway is a major regulator of vegetal cell fates in the sea urchin embryo. Maternal localization of Disheveled protein leads to the nuclearization of the transcriptional effector β -catenin in vegetal blastomeres, particularly in micromeres, a process critical for animal-vegetal polarity. The role of nuclear β -catenin in activating organizer activity in the micromeres is well-established, however, its function in the sMics is unknown. As in other sea urchin species, β -catenin is nuclearized in the micromeres from the 4th cleavage onward, and in the sMics and other vegetal cells from the 5th cleavage through blastula stages (Fig. **S1A**) (24, 25). Additionally, three Wnt ligands, 1, 8, and 16, are expressed in the micromere lineage before gastrulation. Because Wnt signaling in the sea urchin is short range, we limited our subsequent analysis to these three most relevant candidates (23).

We next tested whether nuclear β -catenin, the effector of canonical Wnt signaling, is required for Nanos expression in the sMics. Overexpression of the destruction complex

scaffolding molecule Axin both strongly animalizes the embryo and eliminates detectable nuclear β -catenin (**Fig. S1B**) (26). We therefore injected increasing concentrations of Axin and tested Nanos transcript levels by qPCR, along with a known target of maternal β -catenin, Wnt8. Importantly, nuclear β -catenin is no longer detectable with Axin overexpressed (**Fig. S1B**). Indeed, we detected a dose-responsive decrease in Nanos levels with increasing Axin concentration, as with the Wnt8 positive control (**Fig. 1A**). To test whether a secreted Wnt ligand was required for initial Nanos2 expression, we treated embryos with the small molecule antagonist c59, which has been shown to effectively prevent Wnt secretion by inhibiting Porcupine, with identical effects to Porcupine knockdown. In contrast to β -catenin perturbation, c59 did not strongly affect Nanos2 or Wnt8 levels, suggesting initial Nanos2 activation is more reliant on a maternal pathway in pregastrular embryos (**Fig. S1C**).

To determine whether the decrease we observed in Nanos levels by qPCR was a result of sMic loss or lack of expression, we performed fluorescent in situ Nanos2 hybridization with the sMic lineage traced by pulsing the zygote with EdU. Because of their slow cell cycle, the sMics retain EdU, whereas somatic cells divide and dilute the label, thus allowing confident visualization of the sMics independently of their gene expression profile (9, 27). We observed four sMics in Axin expressing embryos, as in controls; however, we were consistently unable to detect Nanos RNA (**Fig. 1B**). These results indicate an early requirement for nuclear β -catenin in the expression of Nanos2.

Nanos2 is expressed in the PGCs and then the somatic Veg2 mesoderm

It has been previously reported that the forkhead transcription factor FoxY is required for expression of Nanos2 at 24 hours post fertilization (h.p.f.), but not earlier (19). We

therefore sought to clarify the temporal and spatial relationship between Nanos2 and FoxY. We find that Nanos2 mRNA is first detectable exclusively in the sMics following their creation through blastula stages. Surprisingly, however, at the onset of gastrulation (24 h.p.f.), we found that Nanos2 mRNA is also expressed in the adjacent Veg2 somatic mesoderm, the progenitors to the coelomic pouches, which will provide a niche for the sMics (**Fig. 2A**). This dynamic localization follows that of FoxY, which is first expressed in the sMics and subsequently in Veg2, downstream of Delta/Notch signaling in both cell types (18). To test whether Nanos2 and FoxY are expressed in the same cells, we performed double fluorescent in situ hybridization along with immunofluorescence for Vasa to label the sMics, and confirmed that Nanos2 and FoxY are indeed co-expressed in both the sMics and the Veg2 domain (**Fig. 2B**).

Previous knockdown studies have suggested that Nanos2 is a FoxY target, a possibility supported by the overlapping spatial domains we report here. To further test this premise, we performed qPCR for Nanos2 at multiple times surrounding the expansion of Nanos2 into the Veg2 domain. Rather than the Nanos2 increase trailing that of FoxY, we observe similar expression kinetics for both transcripts (**Fig. 2C**). This result is perhaps more consistent with Nanos2 and FoxY responding to the same inductive signal at the onset of gastrulation, rather than Nanos2 being downstream of FoxY. With this hypothesis, we next set out to determine what signals regulate the Nanos2 increase across the vegetal plate in late blastulae/early gastrulae.

Nanos2 is differentially regulated by the Wnt and Delta/Notch pathways at gastrulation

The surprising expansion of Nanos2 into the somatic Veg2 territory suggested that its later expression might be downstream of Delta/Notch (**D/N**) signaling. The D/N pathway is required for specification of mesodermal progenitors and several mesodermal derivatives in the larva, including pharyngeal muscles, pigment cells, and the coelomic pouches. We therefore perturbed the D/N pathway with the small molecule antagonist DAPT, and observed a roughly 2-fold reduction in transcript levels by qPCR (**Fig. 3A**). DAPT treatment selectively eliminated the Veg2 Nanos2, but did not affect the sMic expression (**Fig. 3F**). Consistent with previous analyses, we also observed a strong decrease in FoxY transcript levels, a direct target of D/N signaling. Also reduced were the transcription factors FoxC and Maf, which are expressed in distinct subsets of the FoxY positive cells (**Fig. S2**) (18, 28, 29). In contrast, DAPT did not affect Nanos2 levels in 15-hour blastulae, while FoxY was reduced as predicted (**Fig. S3A**). Thus, D/N regulation of Nanos2 is unique to Veg2 cells at gastrulation. FoxY was found to be required for all known functions of D/N signaling in mesoderm specification, and for full Nanos expression at 24 h.p.f. (18, 19). Consistently with these findings and similar to DAPT treatment, we observed a 2-fold reduction in Nanos levels with FoxY knockdown (**Fig. S3B**). Nanos2 expression was selectively lost in the Veg2 cells, but persisted in the sMics (**Fig. S3C**). While it is possible that FoxY directly regulates Nanos2 expression in the Veg2 cells, we favor the model that FoxY is broadly required for the fate of the Veg2 lineage. In support of this premise, FoxY knockdown results in a complete loss of several mesodermal fates, including the coelomic pouches, which contain the final niche for the sMics (18).

We next asked whether sustained Wnt signaling was required in this later phase of Nanos2 regulation. We blocked Wnt secretion again using c59, and observed an

approximately 40% reduction in Nanos levels by qPCR (**Fig. 3B**). As predicted, we also detected a similar decrease in Wnt8 levels, and an approximately 4-fold decrease in the Veg2 and sMic factor FoxC. These results indicate that by 24 h.p.f., the Wnt pathway activating Nanos2 has shifted from maternal to embryonic control, and that persistent Wnt signaling is necessary for protracted expression. With spatial examination, we found that Nanos2 was specifically lost in the sMics with c59 treatment, but unaffected in the Veg2 cells, which is in agreement with the decrease detected by qPCR (**Fig. 3G**). We therefore conclude that one or more secreted Wnts are required for sustained Nanos2 expression in the sMics.

We then sought to identify the specific Wnt ligand(s) required for sustained Nanos expression. Wnts 1, 8, and 16 are all expressed in the micromere lineage in pregastrular embryos. Subsequently, leading up to gastrulation, Wnts 1 and 16 remain in the Veg2 territory, while Wnt8 shifts further into the endoderm and is thus no longer in proximity for Nanos2 regulation in the sMics (23). Surprisingly, single knockdown of each of these Wnts led to an increase in Nanos2 levels. sMic and Veg2 candidate gene expression changes in Wnt1 and Wnt16 knockdown were highly similar, and we note that Wnt8 levels are decreased following both perturbations, consistently with previous findings (**Fig. S4**) (23). Therefore, Wnt1 and Wnt16 function may overlap as activators, whereas Wnt8 may act repressively. Consistent with this premise, Wnt8 knockdown resulted in about a 20-fold increase in Nanos2 levels (**Fig. 3C**). Spatial examination of Nanos2 transcripts following Wnt8 knockdown revealed that the extra Nanos2 was within both the Veg2 mesodermal cells and the sMics (**Fig. 3H**). Knockdown of Wnts1 and 16, along with Wnt8, partially suppressed the Nanos2 upregulation, reducing it to a 4-fold increase, and supporting the repressive Wnt8 effect (**Fig. 3D**). In the triple knockdown embryos,

we detected Nanos2 in Veg2 cells, but not in the sMics, indicating their essential function for sustaining Nanos2 expression in the PGCs. (**Fig. 3I**).

β-catenin directly interacts with the Nanos promoter

The above results suggest that the Nanos2 gene may be a direct target of the canonical Wnt and D/N pathways. To test for possible Wnt pathway involvement, we scanned the Nanos2 genomic locus and found multiple putative HMG-box motifs, which are recognized by the Tcf transcription factors with which β-catenin associates (**Fig. 4**). To test the potential interaction between β-catenin and the Nanos2 promoter, we performed chromatin immunoprecipitation (ChIP) followed by qPCR using primer pairs that scan from 2kb upstream to 1kb downstream of the Nanos2 transcriptional start site. We observed a peak of approximately 10-fold enrichment above background toward the 3' end of the Nanos2 open reading frame. While it was surprising to find β-catenin association within the coding region, we observe a consensus Tcf binding site precisely within the peak. We note that Nanos2 is unusual in that it lacks introns, which often contain transcription factor binding sites. Therefore, given its proximity to the transcriptional start site within the small, single exon, the β-catenin peak is consistent with direct Wnt pathway regulation of the Nanos2 gene.

Discussion

Nanos orthologs have been appreciated as essential factors for germ line development in many organisms for over 30 years (30). Our work here, provides some of the first insight into the direct transcriptional regulation of this important gene. We find that early expression of Nanos2 in the sMics, the sea urchin PGCs, requires the maternally

activated β -catenin pathway. Later Nanos2 expression in the sMics into gastrulation requires overlapping action of Wnt1 and Wnt16. Additional Nanos2 expression is activated in the Veg2 somatic mesoderm by Delta/Notch signaling, and likely reinforced by Wnt1 and Wnt16. ChIP for β -catenin strongly supports its direct regulation of the Nanos2 locus, which contains candidate Tcf consensus sites. Surprisingly, knockdown of Wnt8 leads to a dramatic increase in Nanos2 levels. We hypothesize that Wnt8 acts through an unidentified repressor to attenuate Nanos2 expression in the vegetal plate. Both Wnt1 and Wnt16 are required for full Wnt8 expression, suggesting a feedback mechanism to keep Nanos levels fine-tuned (**Fig. 5**).

A transition in germ line segregation strategy in echinoderms

Comparative analyses within the echinoderms suggest that germ line specification with the micromere lineage may be a relatively new evolutionary acquisition (31). The ancestral echinoderm probably specified its PGCs later in embryogenesis from a mesodermally derived coelomic pouch called the posterior enterocoel (PE) (6). In the sea star larvae, the PE cells accumulate both Vasa protein and selectively expresses Nanos (31, 32). Surgical removal of the PE leads to fewer morphologically apparent germ cells (33). In contrast, the micromere lineage is a derived trait unique to the echinoids, a clade that comprises the euechinoids, or sea urchins and sand dollars, and the cidaroids, or pencil urchins. Numerous lines of embryological and molecular evidence indicate that the sMics are the primordial germ cells in the euechinoids (6). How Nanos2 came to be expressed precociously (relative to the sea star) is an intriguing evolutionary question. We propose that changes to the Nanos2 sequence allowed it to directly respond to the early activating maternal β -catenin pathway, which is indeed

active in the sea star (34). The later D/N dependent Nanos2 in the sea urchin Veg2 cells may then be a holdover of the ancestral inductive strategy, homologous to the Nanos expression seen in the sea star embryo. Novel Wnt8 organizing activity from the micromeres represses strong expression of Nanos2 in the Veg2 cells, which may represent a sort of blastomere competition for becoming the PGCs.

A prediction that follows this logic is that removal of the micromeres may cause the embryo to revert to a more ancestral program. This prediction is appealing, given the observation that sea urchin embryos whose micromeres were removed can, in fact, produce fertile adults (35). Thus, the micromeres contain no obligate germ line determinants in the urchin. It was subsequently found that micromere removal results in a large ectopic upregulation of Vasa protein, and it was hypothesized that this upregulation is essential for germ line recovery (36). The broad localization of Vasa in recovering sea urchin embryos is similar to what is seen in normal sea star embryos, where it is not refined to the PE until larval stages (31). Removal of the sMics rather than the parent micromeres, however, yields no Vasa induction and an infertile adult, indicating that the micromeres are the critical organizers (37). Nanos RNA is still detectable in the gut tips of gastrulae whose micromeres were removed, and while the expressing cells were not definitively identified, we suggest that they were the Veg2 mesodermal descendants (12). We propose that Wnt8 is the micromere organizing signal for germ line recovery in the sea urchin, and future investigation will test its involvement in Vasa upregulation as well. The positive input of Wnt1 and Wnt16 (Nanos activators) into Wnt8 create a reinforced circuit: loss of Wnt1 or Wnt16 also results in a decrease in Wnt8, which relieves repression of Nanos in the Veg2 cells (**Fig. 5**).

The small micromeres: primordial germ cells specified by inheritance?

Accumulating embryological and molecular evidence strongly support the notion that the sMics are *de facto* primordial germ cells specified by inheritance, added upon an ancestral architecture for segregating the germ line by induction. The sMics do not possess a morphologically apparent germ plasm like in *Drosophila* or *Xenopus*, however, they retain maternally supplied Vasa protein and various RNAs by differential stability mechanisms (9, 38). Furthermore, they are mitotically quiescent, show signs of broad transcriptional repression characteristic of PGCs in other organisms, and are required for fertility (6, 7). While these other observations suggest an inheritance based strategy, Nanos is not maternally supplied in the sea urchin, as it is in other organisms. The embryonic activation of Nanos2 in the sMics immediately after their creation is indeed unique. However, we note that the activators of Nanos2 expression are maternally supplied, and spatially localized. Modified Disheveled protein bound to the vegetal egg cortex is inherited by the large and small micromeres, directly initiating Nanos2 expression and the cascade of supporting Wnt ligand activity. In support of inherited autonomy, isolated sMics in culture upregulate Nanos2 expression on a time course similar to the intact embryo (10).

Evolutionary pressure towards early germ line specification

The apparent acquisition of an inherited germ line from an ancestral inductive strategy in the echinoderms is one of many examples of evolutionary convergence. Within the vertebrates alone, germ plasm appears to have been independently acquired within birds, anuran amphibians, and teleost fishes (39). Within insects, the germ plasm mechanisms used by *Drosophila* are likely novelties of Diptera, whereas ancestrally,

PGCs were produced by induction, as observed in the cricket (5). Frequent acquisition of germ plasm implies a strong selective pressure for the strategy. Several hypotheses have been proposed, including the possibility that segregating the germ line early could confer a protective advantage against replication based mutation (40). Another possibility is that immediate segregation of the PGCs liberates constraints upon the soma to evolve without compromising the reproductive fidelity of the organism.

Consistent with this hypothesis, the genes of animals with germ plasm may evolve more rapidly than sister clades with inductive germ lines (2). A second novelty in the echinoids is the larval skeleton, which evolved by precocious activation of adult skeletogenic genes in the micromere lineage chassis (41). The larval skeleton facilitates feeding and dispersal of the larva, which along with ensured reproductive capability from the sMics, may extending the reproductive output of the individual (42). Very early specification of the germ line may allow the embryo to invest its energy towards reaching a feeding, motile larvae more quickly.

An incipient reality of germ line research is that it is perhaps no longer productive to think only within the binary paradigm of “inherited vs. inductive.” Instead, there exists a remarkable continuum of germ line developmental strategy, which has produced numerous heterochronies in the time and manner of PGC segregation. Despite these differences, conserved pathways operate in germ lines across species, which activate terminal germ line genes such as Nanos, Vasa, and Piwi (43). In the present work, we have shown that the canonical Wnt pathway is required for Nanos activation in the sea urchin PGCs, which bears similarity to the mouse germ line. In the mouse, Wnt3 activates Brachyury/T, which is required for activation of the PGC transcriptional network (44). One downstream component of this transcriptional network, AP2 γ , has been shown

to directly bind the Nanos3 promoter in mice (3). By instead responding to β -catenin directly, the sea urchin PGCs experience earlier activation of germ line gene expression. Future investigation will further evaluate how Wnts, other signaling machinery such as the Bmp pathway, and maternal determinates intersect in the production of germ cells in this important phylum.

Acknowledgements: We are grateful to Miao Cui and Isabelle Peter for sharing Wnt morpholinos, and to Ryan Range for Wnt1 and Wnt8 in situ probes. We thank Geoff Williams for valuable imaging assistance, Totino Srolls for double FISH advice, and Carmen Andrikou for helpful discussions at DBSU.

Materials and Methods

Animals

Strongylocentrotus purpuratus were kept in artificial seawater at 16°C. Individuals were spawned by shaking or injection of 0.5M KCl. Eggs were fertilized in the presence of 1 mM 3-amino-triazol (3-AT) (Sigma) to prevent crosslinking of fertilization envelopes. All embryos were reared at 15°C by standard methods.

Whole mount in situ hybridization and immunofluorescence

WMISH was performed as described previously, with some modification for double fluorescent in situs (9). Briefly, probes templates were produced by either amplifying 1kb of sequence with reverse primers tailed with the T7 promoter (e.g. Wnt8 and Wnt16 probes), or by linearizing cloned fragments (all others). Labeled RNA probes were transcribed from these linear templates with the Roche RNA Labeling kit, using

nucleotides labeled with either DIG or fluorescein according to the manufacturer's instructions. Embryos were fixed with MOPS buffered PFA and hybridized for 1 week at 50°C with 70% formamide and 0.1 ng/ul of each probe. Hybridizations were first detected with peroxidase-conjugated antibodies to DIG, and Cy3 tyramide fluorescence amplification (Roche). For double FISH, the first antibody was quenched with 1% hydrogen peroxide treatment for 30 minutes, followed by washing, application of an second antibody to fluorescein, and then visualized with fluorescein tyramide amplification. Immunofluorescence for Vasa protein and β -catenin was performed as described previously (25, 36).

Cloning and Reporter constructions

DNA fragments were PCR amplified and cloned by standard methods. FoxY, Nanos2, and Wnt1 ISH probes were cloned into PGEM-T easy (Promega). For overexpression of Axin, constructs were built in the pCS2 vector, linearized with NotI, and transcribed using mMessage mMachine SP6 (Ambion) as described previously (26).

Morpholino antisense oligo (MASO), mRNA microinjection, and drug treatment

Custom MASOs were synthesized by Gene-Tools. For all experiments, a control MASO against a divergent Nanos ortholog in the sea star was used. MASOs were injected with 20% glycerol and 10,000 MW fluorescent dextran. MASO sequences and working concentrations are listed in **Table S1**. Eggs were dejellied by quickly washing in pH 4.0 seawater and rowed on protamine sulfate-coated petri dishes. Zygotes were injected with 2 picoliters of MASO or RNA by constant pressure injection in the presence of 1mM 3-AT (Sigma). DAPT (Tocris) was added at fertilization to 8 μ M, a concentration

previously determined to effectively perturb D/N signaling but not grossly affect development (45). c59 (Cellagen) was added at 0.5 μ M at fertilization as described previously (23).

RNA isolation, cDNA synthesis and qPCR analysis of gene expression

RNA was isolated with the RNEasy Micro kit (Qiagen). First strand cDNA synthesis was performed using Maxima reverse transcriptase (Life Technologies). qPCR was performed using an ABI 7900 real time instrument with Maxima SYBR master mix and 25 μ l reaction volumes (Life Technologies). All primers were previously characterized and listed in **Table S2**. Four embryo equivalents of cDNA were used per reaction. Experiments were run in triplicate and normalized to ubiquitin.

Chromatin preparation

Chromatin from sea urchin embryos was prepared as described by (46) with some modification. Embryos were cultured at 0.5% (egg over seawater volume) in 50 mL tubes at 15°C until 18 h.p.f. and then washed twice with calcium free sea water (CFSW). Embryos were then resuspended in dissociation buffer (1M Glycine, 25 mM EDTA, pH 8) and incubated on ice for 10 minutes. The embryos were then washed 3 times with CFSW, resuspended in 5ml CFSW, and disaggregated by pipetting. Disaggregated cells were fixed with 1% formaldehyde for 10 minutes at room temperature, and stopped by adding glycine to 125 mM. Cells were then washed with PBS-EDTA (PBS, 0.5 mM EDTA, 0.2 mM PMSF), Wash Buffer A (10 mM Hepes pH 7.6, 10 mM EDTA pH 8.0, 0.5 mM EGTA pH 8.0, 0.25% Triton X-100, 1x Roche Complete PI cocktail, 0.2 mM PMSF), and resuspended in Wash Buffer B (10 mM Hepes pH 7.6, 100 mM NaCl, 1 mM EDTA

pH 8.0, 0.5 mM EGTA pH 8.0, 0.01% Triton X-100, 1x PI cocktail, 0.2 mM PMSF). Cells were then counted, pelleted, and snap frozen for future use. For lysis, cells were resuspended at 1×10^8 cells/ml in Lysis Buffer (20% SDS, 10 mM EDTA pH 8.0, 50 mM Tris-HCl pH 8.0) and incubated at 4°C for 10 minutes. Lysate was aliquoted to 300µl in Protein Low Bind Safelock tubes (Eppendorf) for sonication. Sonication was performed using 6 rounds of 5 minutes, cycling between 30 seconds on and 30 seconds off, in a Bioruptor bath sonicator. The sheared lysate was then pooled and centrifuged at 13,000 rpm for 10 minutes at 4°C to remove cellular debris. Supernatants were transferred to 15ml tubes and diluted 5-fold with ChIP dilution buffer (0.01% SDS, 1.2 mM EDTA pH 8.0, 16.7 mM Tris-HCl pH 8.0, 1.1% Triton-X 100, 167 mM NaCl, 1x PI cocktail, 0.2 mM PMSF), aliquoted to 1 ml, and snap frozen in liquid nitrogen.

Clean input DNA was prepared from 400µl chromatin preparation, to which 21.5µl 20% SDS, 15µl NaCl, and 1µl RNase A (10mg/ml) were added, and incubated overnight at 65°C. An additional 1µl RNase A was then added, followed by a 30 minute, 37°C incubation. Protein was then digested by adding 20µl 1M Tris-HCl, pH 6.8, 10µl 0.5M EDTA, and 3µl 20mg/ml proteinase K, and incubating 90 minutes at 42°C. The DNA was then phenol/chloroform extracted, and resuspended in 100µl of water.

Immunoprecipitation (IP)

β-catenin IP was performed with rabbit polyclonal antibodies raised to the n-terminus of *Hemicentrotus pulcherrimus* β-catenin, which bears complete sequence identity to *S. purpuratus* (25). 1µl of anti beta catenin serum or nonspecific rabbit IgG were added to 1ml of chromatin preparation and incubated at 4°C overnight with rotation. 50µl of Protein A magnetic Dynabeads (Invitrogen, pre-blocked with yeast tRNA and BSA) were

added and rotated for 2 hours at 4°C. The beads were then washed twice with 1ml RIPA 150 Buffer (50mM Tris-HCl pH 8.0, 1% NP-40, 2mM EDTA, 0.1% Sodium Deoxycholate, 0.1% SDS, 150mM NaCl), and once with RIPA 300 Buffer (as previous, but with 300mM NaCl). The beads were then washed once with 1ml LiCl/TE Buffer (0.25M LiCl, 1% NP-40, 1% Sodium Deoxycholate, 10 mM Tris-HCl pH 8, 1 mM EDTA pH 8), and twice with TE (10mM Tris-HCl pH 8, 1 mM EDTA, 0.01% SDS, 1x PI cocktail, 0.2 mM PMSF). Beads were finally eluted twice with 250µl Bicarbonate Elution Buffer (1% SDS, 0.1M Sodium Bicarbonate). DNA was then recovered as done for the input, and resuspended in 100µl water for qPCR analysis.

qPCR analysis of ChIP

Primers were designed that spanned the entire Nanos2 locus and tested for specificity by dissociation curves and gel electrophoresis. For each 25µl reaction, either 2ng of input DNA or 2ul of immunoprecipitated DNA was used as a template. All reactions were run in triplicate using Maxima SYBR (Life Technologies). Data was analyzed using the percent input recovery method.

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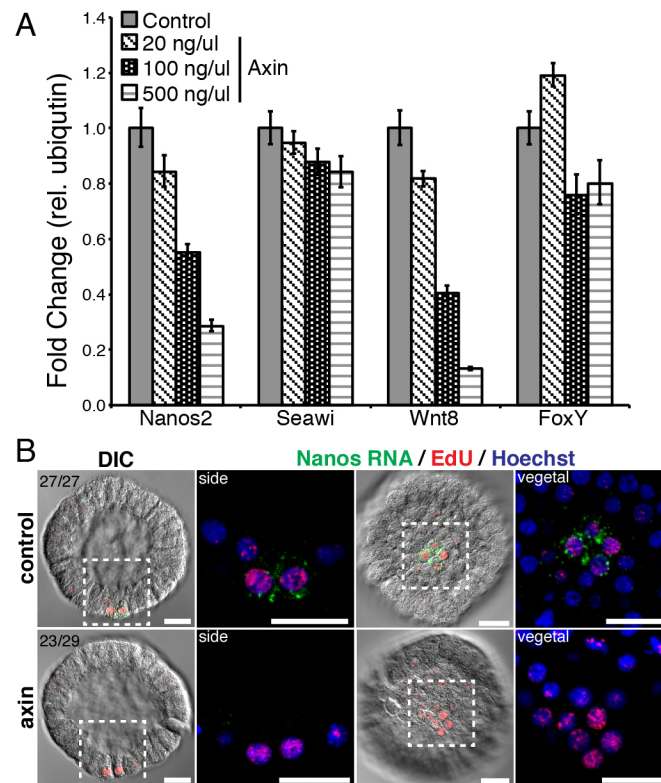


Figure 1. The Canonical Wnt pathway is required for Nanos2 expression in the sMics at 15 h.p.f. (A) qPCR for Nanos2 following microinjection of increasing Axin dosage. Expression of both Nanos2 and Wnt8, a known target of beta catenin, decrease with Axin injection. Seawi is a sMic enriched RNA whose levels do not change significantly, as it is maternally-supplied (47). FoxY is also expressed in the sMics in blastulae downstream of D/N signaling, and is not substantially affected by Axin expression. (B) Fluorescent in situ hybridization for Nanos2 RNA following Axin expression. Nanos2 transcripts (green) are expressed in the sMics in control embryos, but undetectable with Axin expression. The sMic line lineage is stably labeled by EdU incorporation (red). Scale bars = 20 μ .

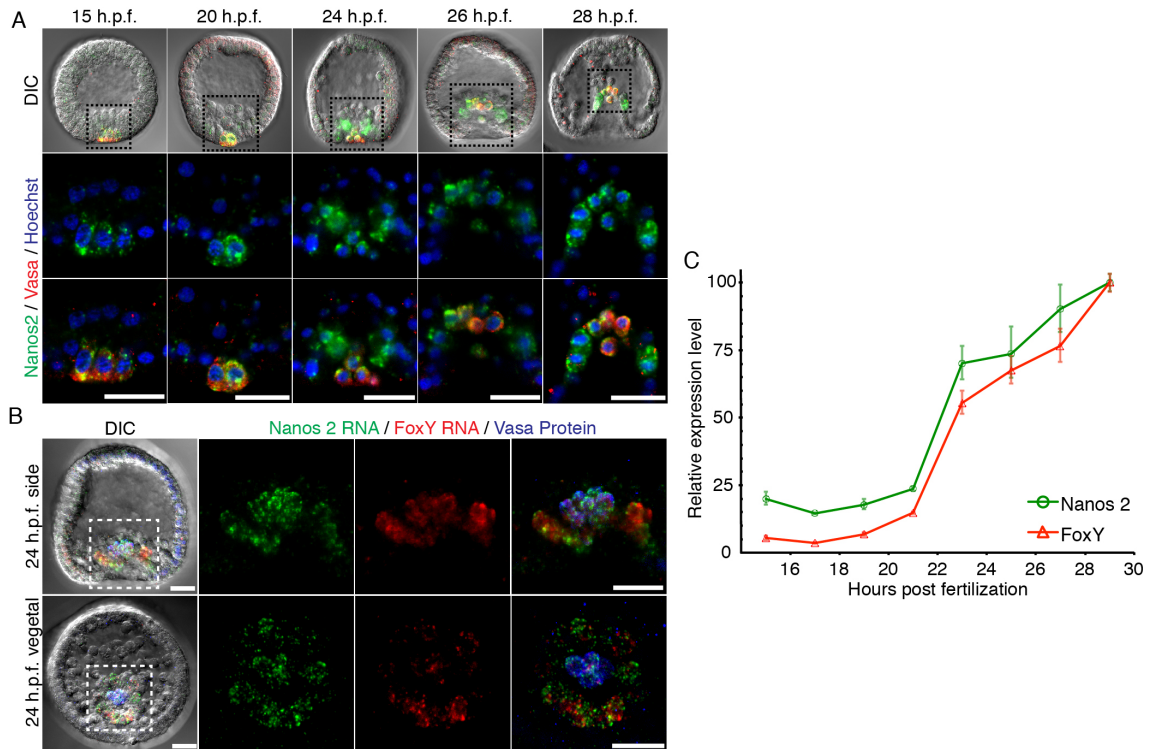


Figure 2. Nanos2 expression expands into mesodermal progenitors during gastrulation. (A) Nanos transcript localization at multiple developmental stages. The sMics are labeled by immunofluorescence to Vasa protein (red). Prior to primary invagination, Nanos expression is limited to the sMics (15 and 20 h.p.f. timepoints), but expands into adjacent cells by 24 h.p.f. (B) Colocalization of Nanos2 and FoxY mRNA. Nanos2 (green) and FoxY (red) transcripts are expressed in both the sMics and Veg2 mesoderm at 24 h.p.f. sMics are labeled by Vasa immunofluorescence (blue). (C) Nanos2 and FoxY transcript abundance at multiple developmental stages, relative to ubiquitin and normalized to maximum level. Scale bars = 20 μ .

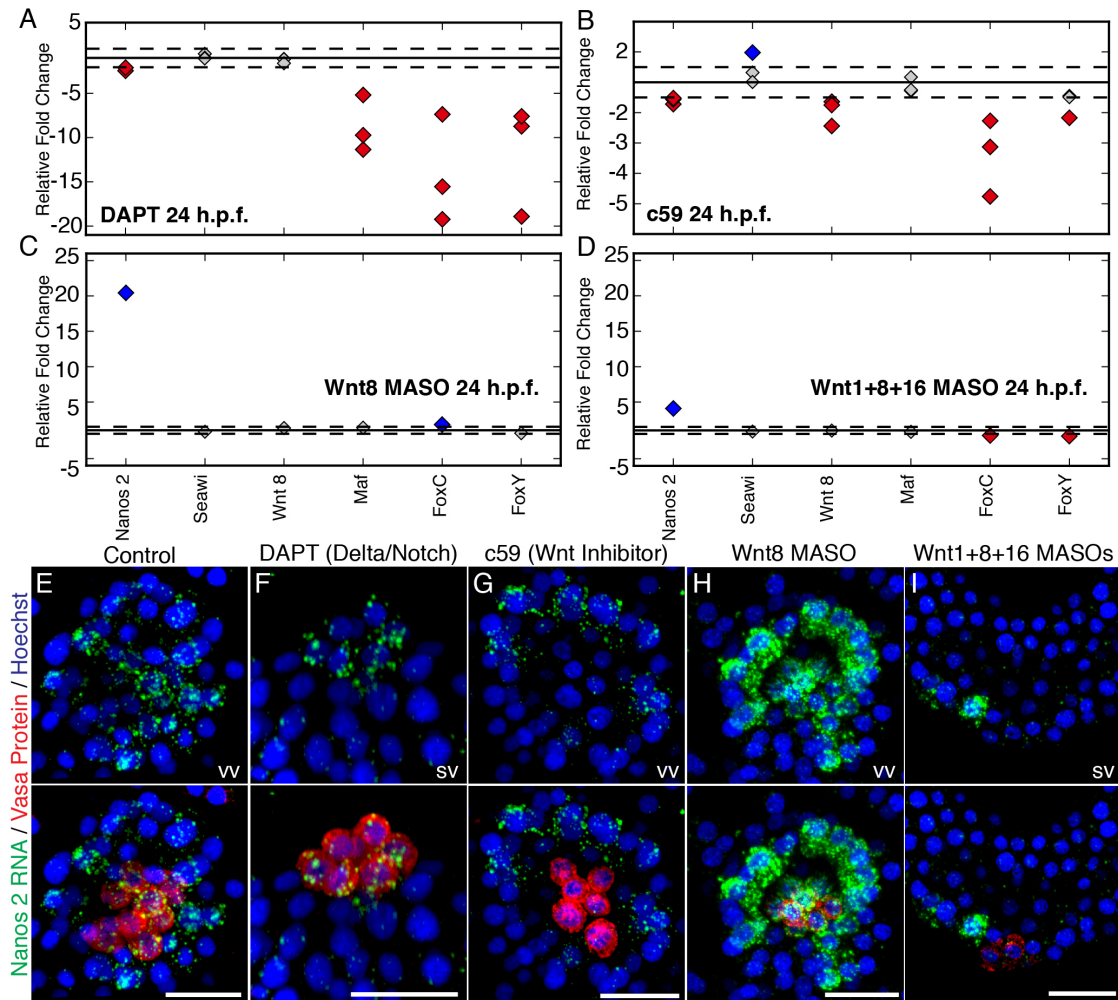


Figure 3. Nanos2 regulation by D/N and Wnt during Veg2 specification. (A-D) qPCR for Nanos2 and other select factors. Each diamond represents a biological replicate, with red/blue diamonds exceeding a fold-change threshold (2-fold in A; 1.5-fold in B-D). (A) D/N inhibition with DAPT results in an approximately 2-fold reduction in Nanos2 levels, along with substantial reductions in the Veg2 factors Maf, FoxC, and FoxY at 24 h.p.f. (B) Wnt pathway perturbation with c59 results in a consistent decrease of about 1.6-fold in Nanos2 and Wnt8 levels, and about a 3-fold decrease in FoxC. (C) Single knockdown of Wnt8 results in about a 20-fold increase in Nanos2, and a 2-fold increase in FoxC. (D) Knockdown of all micromere associated Wnts, 1, 8, and 16, results in about a 4-fold increase in Nanos2 levels. (E-I) Visualization of Nanos2 transcript localization at 24 h.p.f. following D/N or Wnt perturbation. (F) Inhibition of D/N signaling eliminates Veg2 Nanos2, but does not affect sMic Nanos2 expression. (G) Inhibition of all secreted Wnt activity prevents Nanos2 expression in the sMics, but does not affect Veg2 Nanos2. (H) Wnt8 single knockdown results in a substantial increase in Nanos levels in the sMics and Veg2 cells. (I) Triple knockdown of Wnt1, 8, and 16, like c59 treatment, selectively prevents sMic Nanos2 expression. Side view, “sv”; vegetal view, “vv”. Scale bars = 20 μ .

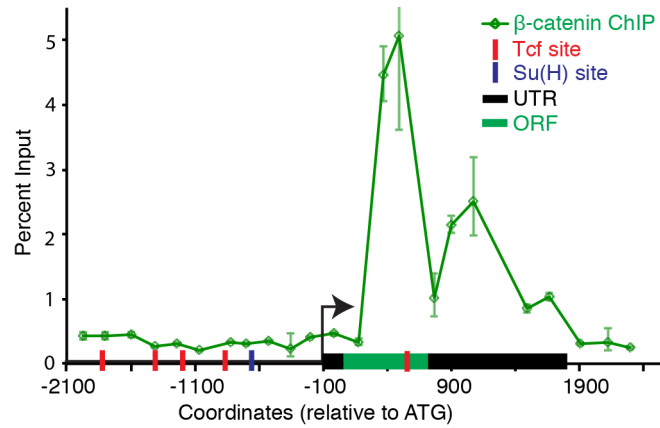


Figure 4. Direct interaction between beta catenin and the Nanos2 gene. Chromatin immunoprecipitated with beta catenin antibodies from 18 hour embryos, tested by qPCR. Primer pairs scanning across the entire Nanos2 genetic locus indicate substantial beta catenin occupancy within the Nanos2 gene body. The strongest peak coincides with a potential Tcf consensus site (5'-TACAAAG-3').

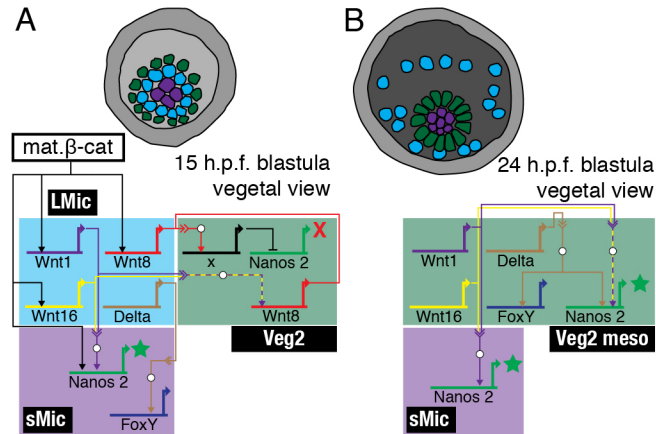


Figure 5. Integrated model for Nanos 2 transcriptional regulation. (A) Regulation of Nanos2 in the pre-mesenchyme blastula. Maternally activated beta catenin drives Nanos2 expression in the sMics, and also activates the reinforcing Wnt1 and Wnt16 in the micromere lineage. Wnt1 and 16 signaling from the large micromeres (LMic) to small micromeres (sMic) reinforces Nanos2 expression. Wnt8 in the LMics and Veg2 cells represses Nanos2 accumulation via an unidentified repressor. Full, sustained Wnt8 expression requires Wnt1 and Wnt16. Delta signaling to the sMics activates FoxY, whose function is unknown there. (B) Regulation of Nanos2 in the late blastula and early gastrula. With the ingression of the LMics and removal of the repressive Wnt8 signal, Nanos2 is activated in the Veg2 mesoderm by Delta/Notch signaling and possibly reinforced by Wnt1 and Wnt16. Nanos2 continues to be expressed in the sMics downstream of Wnt1 and 16.

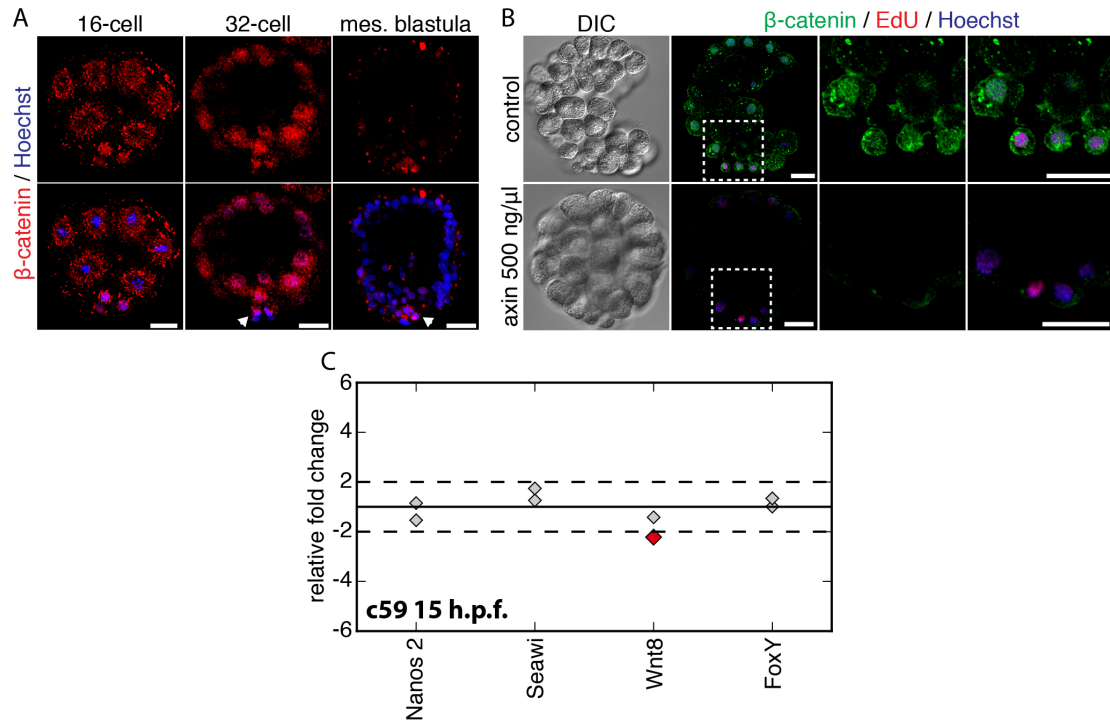


Figure S1. Characterization of beta catenin localization. (A) Beta catenin (red) is present in the large and small micromeres from their creation into late blastulae. sMics are indicated with white arrows. (B) Nuclear beta catenin (green) is eliminated when Axin is overexpressed, including in the sMics, labeled with EdU (red). Scale bars = 20 μ . (C) c59 inhibition of Wnt secretion does not affect Nanos2 expression in the 15 h.p.f. blastula.

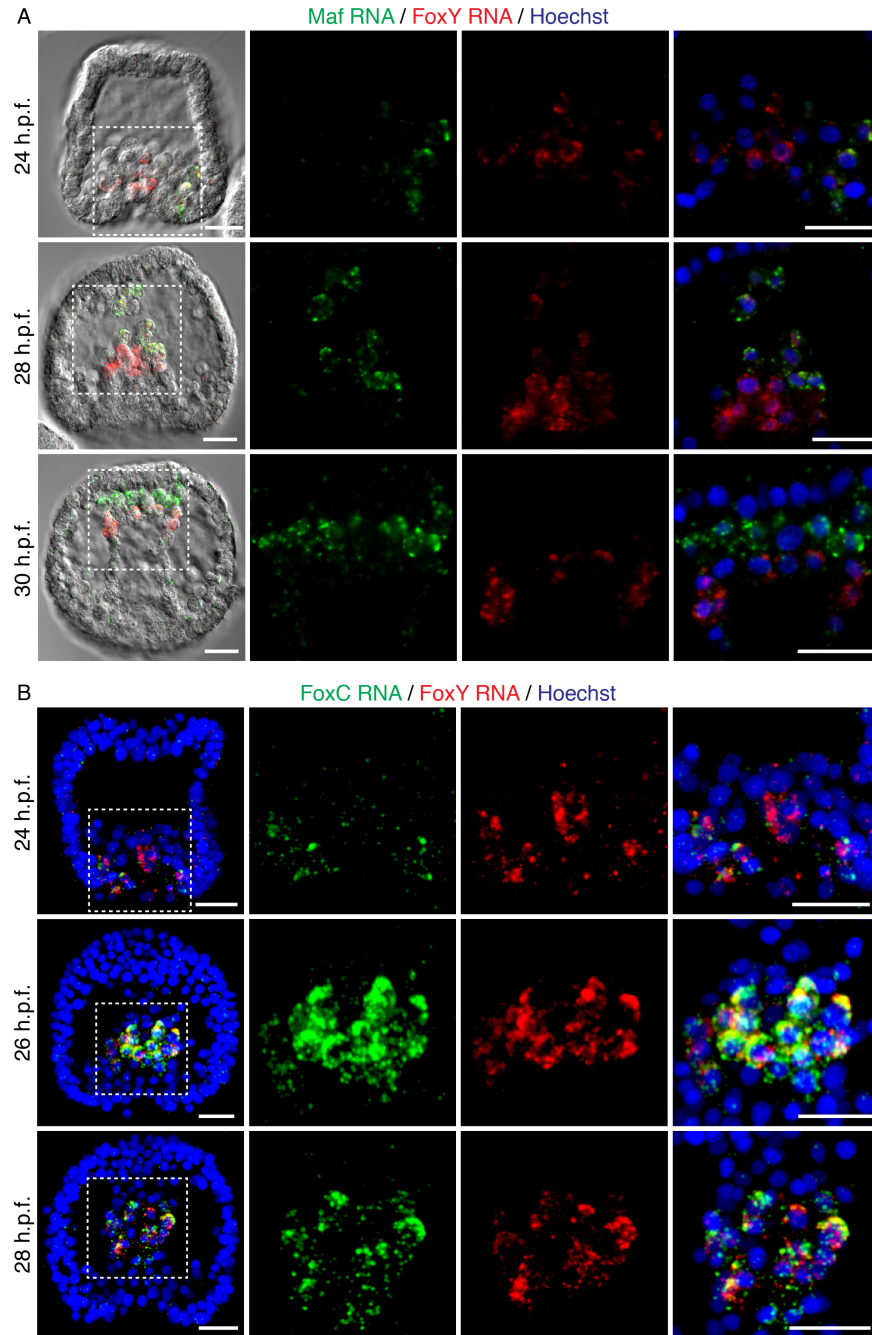


Figure S2. Localization of putative FoxY targets. (A) Maf transcripts (green) are first detectable in a subset of the FoxY-positive Veg2 mesoderm (red) at 24 h.p.f. As gastrulation proceeds at 28 h.p.f., the Maf positive cells leave the tip of the archenteron and lose FoxY expression. By 30 h.p.f., The Maf positive cells have completely separated from the remainder of the FoxY cells. (B) FoxC transcripts (green) are consistently co-localized with the FoxY positive cells, and do not leave the tip of the elongating archenteron. This localization is consistent with the sMics and somatic coelomic pouch progenitors. Scale bars = 20μ.

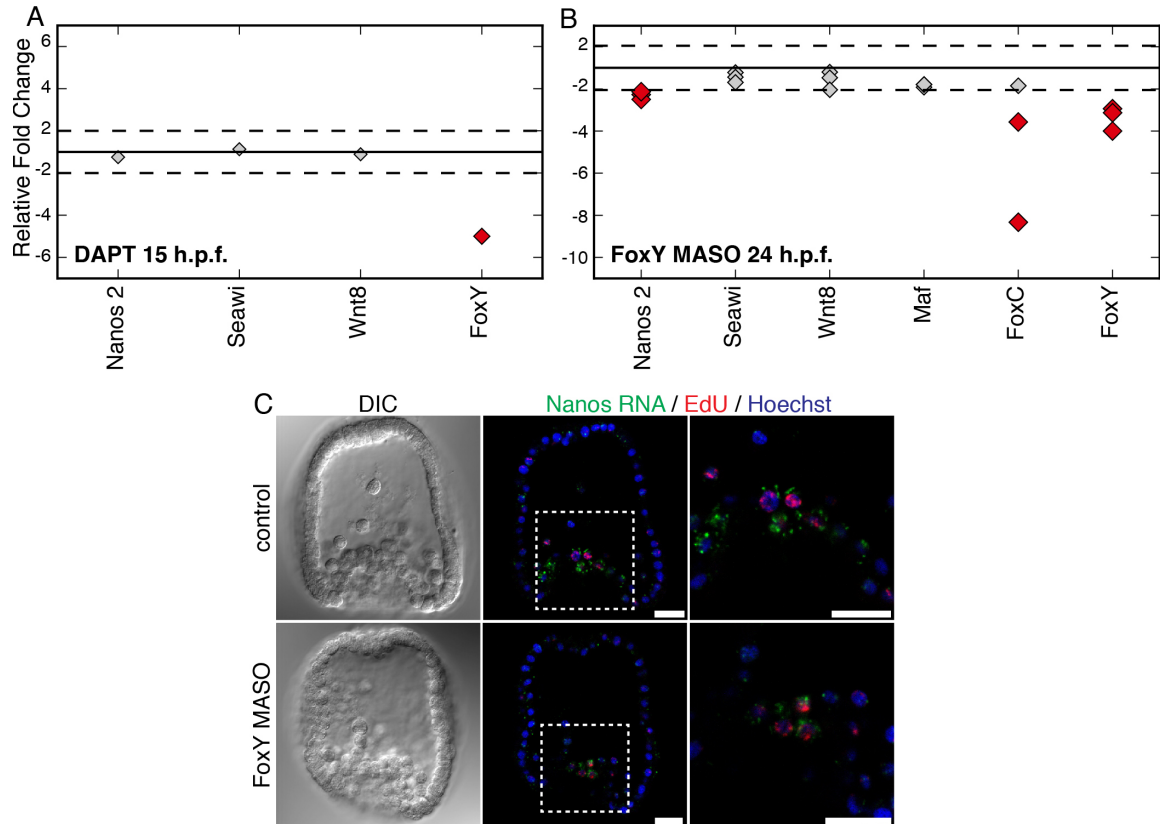


Figure S3. Delta/Notch and FoxY regulation of Nanos expression. (A,B) qPCR for Nanos2 and other targets; each diamond is a biological replicate, and red diamonds exceed a fold change of 2-fold. (A) Inhibition of Delta/Notch signaling with DAPT does not affect Nanos2 expression at 15 h.p.f., though FoxY RNA levels are reduced, as previously reported. (B) FoxY knockdown results in an over 2-fold reduction in Nanos2 RNA levels, as well as decreases in FoxC, and FoxY itself. (C) Spatial examination indicates that FoxY knockdown specifically eliminates the Veg2 Nanos2 expression (green), but does not affect expression in the sMics (labeled with EdU, red). Scale bars = 20 μ .

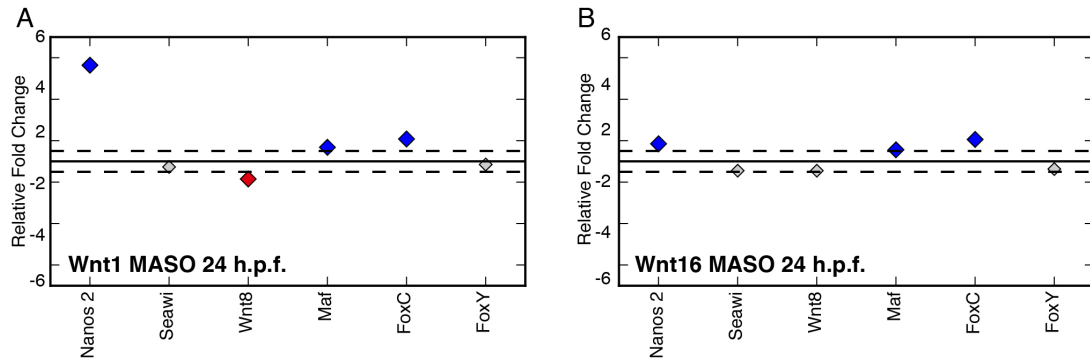


Figure S4. Gene expression following knockdown of Wnt1 and Wnt16. (A,B) qPCR for target genes following knockdown. Fold changes greater than 1.5 are colored blue or red. (A) Wnt1 knockdown results in a nearly 5-fold upregulation of Nanos2 levels, a slight decrease in Wnt8, and a slight upregulation of Maf and FoxC. (B) Wnt16 knockdown has similar, but less severe effects as compared to Wnt1 knockdown.

Morpholino	Sequence 5'→3'	Conc.	References
Nanos2	GTGACTAAAGTGCGTGGAAACTCGA	500 μ M	(11)
Wnt1	ACGCTACAAACCACTCAAGTTTCAT	250-500 μ M	(48)
Wnt8	GTAAAGTGTTTTCTTACCTTGGAT	500 μ M	(26)
Wnt16	TCTCAACAAACTCGATAGTTCAACC	250-500 μ M	(23)
FoxY	CATGGCTCCAAGTGCAGAACTACTAC	500 μ M	(18)

Table S1. MASO sequences used in this study. Morpholino sequences and working concentrations are provided, along with the references in which they were originally tested for specificity.

Primer	Sequence 5'→3'	References
Nanos2	F: GCAAGAACAACGGAGAGAGC R: CCGCATAATGGACAGGTGTA	(8)
Seawi	F: GTGATGGTGTGGTGACAGC R: TATTGATGCGCTTCTTGACG	(8)
Wnt8	F: TGTCGTTCAATTCAAGCCATC R: TATCACTCGCCATTCGTTCA	(49)
Maf	F: GTCGAGATTGAAAAGCCAGC R: AGAACTCGGCAGAGTCAGGA	(50)
FoxC	F: GAAAATCACCTCAACGGAAT R: CCCCTTGCCTGGTTTCTTAT	(28)
FoxY	F: TGCACTGCACTGACTCTGC R: CTTTCCATTCCGTGGTGAAG	(18)
Ubiquitin	F: CACAGGCAAGACCATCACAC R: GAGAGAGTGCGACCATCCTC	(8)

Table S2. qPCR primers used in this study.

Chapter 5: Conclusions, Perspectives, and Future Directions

I have explored the developmental of sea urchin primordial germ cells (**PGCs**) called the small micromeres (**sMics**) at several regulatory levels. Transcriptomic and functional analyses of isolated sMics revealed their tendency to broadly retain maternally supplied and exogenous mRNA, via a novel post-transcriptional mechanism requiring Nanos2 (**Chapter 2**). I learned that the FoxY transcription factor is essential for formation of the coelomic pouches, the somatic niches for the sMics and major constructors of the adult rudiment (**Chapter 3**). Given the central role of Nanos2 in sMic segregation, I interrogated its transcriptional regulation, and found that it is a direct target of maternally supplied β -catenin. Into gastrulation, it is sustained by embryonically secreted Wnts, and becomes secondarily activated in the Veg2 mesoderm downstream of Delta/Notch (**Chapter 4**). A model summary of these processes is integrated in **Figure 1**, starting from early activation of Nanos expression that ultimately results in RNA retention in the sMics. The perspective I have gained from these studies presents the sMics as PGCs whose fate is initiated by maternally supplied information and then regulated by embryonic cues, influenced by differential RNA stability and transcriptional repression associated with maternal to embryonic transitions (**METs**).

The METs I reviewed in **Chapter 1** encompass several milestones in embryonic development: 1) **embryonic genome activation (EGA)**, 2) **maternal transcriptome degradation**, and 3) **loss of cleavage synchrony** (Tadros and Lipshitz, 2009). These events have been extensively examined in several model organisms, including *Drosophila melanogaster*, *Xenopus laevis*, and *Danio rerio*, and I added to this picture a synthesis of available data, as well as my own new results, in the sea urchin *Strongylocentrotus purpuratus*. The frog *Xenopus* is a particularly dramatic example of the METs, because in in this organism, they occur simultaneously at the **midblastula**

transition (MBT) (Farrell and O'Farrell, 2014). However, investigation in other animals indicates that this developmental mode may be the exception and not the rule. In fact, different aspects of the METs can be decoupled, and occur at vastly different embryonic times. For example, even between the closely related *Caenorhabditis elegans* and *Drosophila melanogaster*, there is a striking difference in EGA; in the former, it occurs at the 2-cell stage, while in the latter, it is not strongly activated until the 14th cleavage (Tadros and Lipshitz, 2009). To sharpen our final discussion, I will therefore focus particularly on one aspect: degradation of the maternal investment, as it relates to the establishment of the germ line. Maternal transcriptome degradation has been observed in many distantly related organisms, and while it appears to sometimes involve divergent mechanisms, it may be a conserved requirement for differentiation of the soma relative to the germ line.

Below, I will briefly review several previously known aspects of maternal transcriptome degradation (see **Chapter 1** for further review), speculate regarding potential gaps in our understanding (e.g. is it only transcript degradation, or is there something else?), and suggest a possible evolutionary origin for this widespread phenomenon. I will then introduce a “time capsule” hypothesis for PGC development in the context of this MET. Next, I will argue that the sMics are in fact *bona fide* PGCs specified by preloading, and will offer an hypothesis for the molecular changes that supported their precocious segregation. Finally, I will speculate regarding the deep origins of PGCs in animal evolution.

Degrading the maternal investment: when, how, and why?

Timing maternal transcriptome degradation with miRNAs

The embryos of different species enact specific programs to degrade their maternal transcriptome at varying stages of development. Multiple examples of miRNAs as tools for broadly remodeling the transcriptome speak to their utility in regulating many possible mRNAs through imperfect base pairing. For example, in zebrafish, miR-430 is activated at the MBT and is required to degrade at least several hundred maternal transcripts (Giraldez et al., 2006). In *Drosophila*, the miR-309 cluster of miRNAs is activated at the MBT and also degrades many maternal transcripts (Bushati et al., 2008). How miRNA remodeling of the transcriptome is coordinated at the MET is an intriguing open question. Is transcription of the miRNA precursor RNA the rate-limiting step for activation of the turnover, or alternatively, is the activity temporally triggered by a post-transcriptional mechanism? The Hippo pathway, which is sensitive to mechanical forces associated with cell density, has recently been implicated as a sensor capable of regulating miRNA biogenesis in cultured human keratinocytes. At low cell densities, Yap, the nuclear effector of the pathway, binds to and inactivates Ddx17, inhibiting miRNA biogenesis. At confluence, Yap translocates to the cytoplasm, and miRNA biogenesis can occur (Mori et al., 2014). In the future, it would be interesting to test whether the Hippo pathway coordinates miRNA clearance at the MET, perhaps by mechanosensation of compaction in the embryo. The Hippo pathway has already been recognized to regulate inside/outside fate decisions in the early mouse embryo (Nishioka et al., 2009). Furthermore, Hippo pathway components are conserved in the sea urchin, and one could therefore be perturbed by morpholino knockdown. If this model held true, with

Hippo perturbation, I would expect a failure in miRNA genesis at the MET, and a concomitant failure to degrade maternal transcripts. Abrogation of miRNA biogenesis by Drosha knockdown causes sea urchin embryos to arrest at gastrulation; therefore, I predict that if the Hippo pathway regulated the event, its perturbation would cause a similarly timed arrest (Song et al., 2012).

Could the proteome also be extensively degraded?

Previous studies of METs have largely focused on degradation of mRNA, perhaps because there are many excellent tools for broadly characterizing the transcriptome. However, the proteome is truly where the “rubber meets the road”, and in the future, it would be valuable to characterize whether the proteome is also remodeled during the MET by proteolysis or post-translational modification. For example, ubiquitination and the proteasome are important for localization of Vasa protein to the germ line in *Drosophila* and the sea urchin (Gustafson et al., 2011; Liu et al., 2003; Styhler et al., 2002). Proteolysis of Vasa is fine-tuned by direct binding of two competing E3 ubiquitin ligases called Fat facets and Gustavus. Binding of Fat facets protects Vasa in the PGCs, whereas Gustavus targets its ubiquitination in anterior somatic cells. This is, however, an example of a single protein turnover. With improving mass spectrophotometry (MS) capabilities, we may find that differential stability of protein between somatic and germ line cells is as widespread as it is for the transcriptome. One could perform temporal MS analysis in the sea urchin at multiple points between blastula and gastrula stages, which is where transcriptome degradation appears to occur (**Chapter 1**). With current technology, however, MS would likely only detect the most abundant proteins and may therefore miss the nuance of the transition. Alternatively, one might take a more targeted

approach by systematically knocking down proteolytic factors, such as E3 ubiquitin ligases. One could then test whether different embryonic territories fail to differentiate.

Such investigations have been undertaken previously in other organisms, and revealed that proteolysis is indeed essential for development and differentiation in several contexts. One recent example is in *C. elegans*, which follows an invariant developmental pattern where blastomeres divide to give increasingly restricted, well-characterized fates. Knockdown screening of nearly 700 genes with predicted involvement in ubiquitination identified several E3 ligases that result in “reiteration” phenotypes. For example, the EMS blastomere in the 4-cell embryo normally divides to yield an E daughter, that produces endoderm, and an MS daughter, that produces mesoderm. However, when ubiquitination components are knocked down, the normally mesodermally fated daughter retains endodermal capability for an additional cell cycle (Du et al., 2015). Furthermore, components of the ubiquitination machinery are enriched among the first transcripts expressed at the 4-cell stage EGA in *C. elegans*. Emerging evidence also implicates other proteolytic pathways in processes of terminal differentiation. For example, non-apoptotic functions for several caspases, particularly caspase-3, have been identified as important for neuronal, osteoclast, and muscle differentiation, as well as spermatogenesis (Arama et al., 2003; Barnoy and Kosower, 2003; D'Amelio et al., 2009; Fernando et al., 2002; Szymczyk et al., 2006). It therefore seems plausible that the proteomes of different species may be broadly degraded at the MET, in addition to their transcriptomes.

Why degrade the maternal investment?

With the amount of investment the mother places into the egg, it is somewhat surprising that the embryo makes such a concerted effort to degrade that contribution. A sensible possibility is that differentiation cannot occur until mRNAs and proteins associated with totipotency are erased – a sort of developmental “clearing of the slate”. In support of this premise, perturbing the miRNA machinery in different animals leads to severe differentiation defects, or in some cases, complete developmental arrest (Bernstein et al., 2003; Giraldez, 2005; Song et al., 2012). Both *in vivo* studies such as these, and *in vitro* cell culture systems have indicated many contexts in which miRNAs are required for differentiation, including myogenesis, hematopoiesis, neurogenesis, skin homeostasis, and others (Shenoy and Blelloch, 2014). With several different species providing examples of broad maternal transcriptome clearance, it is possible that such METs are an ancient requirement for animal development.

The evolutionary history of METs presents a fascinating evolutionary question, though the orthology of METs events described in mice, sea urchin, *Drosophila*, *C. elegans*, zebrafish, and others are not immediately clear. For example, the miR-309 cluster in *Drosophila* and miR-430 in zebrafish do not bear sequence orthology, however, they perform an analogous function at the MBTs in those organisms. MicroRNAs are now appreciated to have complicated evolutionary histories, and it is reasonable to expect that they too would co-evolve with their targets (Thomson et al., 2014). Ctenophores, possibly the earliest diverging animal clade, appear to be missing the microRNA machinery entirely, though it is possible that this resulted from a secondary loss (Maxwell et al., 2012). However, as other non-miRNA mechanisms of clearing the

maternal load have been discovered, the ctenophore could enact an RNA-degradation MET using another pathway. Thus, while the tools of mRNA degradation may have changed through evolutionary time, the requirement for “clearing the slate” may indeed be ancient. We are now in the position to begin testing this hypothesis – the choanoflagellate, part of the sister group to animals, has been recently been gaining traction as a useful lab organism. Several choanoflagellate species can exist either as a solitary individual, or form colonies with cell specialization under certain conditions (Dayel et al., 2011). These observations have led some to believe that choanoflagellates may share important similarities with animal multicellularity which were retained from the last common ancestor between themselves and (Dunn et al., 2015). While the recently sequenced genomes reveal no siRNA machinery in the choanoflagellates, it should now be possible to test whether they undergo a transcriptome remodeling event upon differentiation and colony formation (King et al., 2008). The colony forming algae of the Volvocaceae family, such as Volvox, could provide another opportunity to test whether transcriptome remodeling occurs at the switch from solitary to colonial life (Nematollahi et al., 2006). Maybe the widespread METs in multicellular development have incredibly ancient roots in a process that preexisted in their unicellular, colony-forming ancestors.

Resisting the MET in the germ line: the Time Capsule hypothesis

PGCs must resist participating in METs associated with somatic cell differentiation. In **Chapter 1**, I reviewed transcriptional quiescence in the preloaded PGCs of different species. I further documented their tendency to protect certain RNAs from degradation. Our own work reveals a process by which the sea urchin sMics stabilize inherited RNA, as well as their own transcriptionally repressive features (**Chapter 2**). Additional

examples, particularly within the zebrafish, indicate that select inherited mRNAs are protected from degradation at the MET. While the zebrafish and sea urchin PGCs accomplish protection via different mechanisms, a commonality is that in both systems, the poly-A tail is a critical site of regulation. Furthermore, if we look earlier into embryogenesis – back to the zygote – we can find an extensive mechanism for stabilizing maternal mRNA before it is later degraded. Perhaps this initial phase of RNA stabilization could bear some relationship with that observed specifically in the PGCs?

Broad transcriptome regulation via poly-A tails

In **Chapter 2**, I uncovered a novel process by which the sea urchin PGCs broadly stabilize their inherited mRNAs. In brief, Nanos2 protein expressed in the sMics binds to the 3'UTR of the mRNA encoding CNOT6, leading to its degradation. Depletion of CNOT6 stabilizes inherited RNA in the sMics, allowing those RNAs to be retained well past the MET (**Figure 1, and Chapter 1, Figure 7**). CNOT6 is an ortholog of the conserved CCR4 deadenylase, which assembles into the CCR4-NOT or **CNOT** complex, a major cytoplasmic regulator of RNA stability (Collart and Panasenko, 2012). As I reviewed in **Chapter 1**, the CNOT complex is a shared effector of several RNA degradation pathways: it is recruited to mRNAs by the miRNA/Argonaut machinery, and required for degradation by the RNA binding protein Smaug at the *Drosophila* MBT (Semotok et al., 2005; Tritschler et al., 2010). Therefore, I predict that its depletion would cause a bottleneck for both miRNA and RNA binding protein-mediated transcriptome turnover at the MET. While this is the only known mechanism for generally enhancing the stability of RNA in germ cells, several other specific mechanisms have been identified, particularly in zebrafish. The PGC expressed factor Dnd1 binds near miR-430

target sites in the Nanos 3'UTR, and occludes access by the Argonaute machinery, which would prevent recruitment of the CNOT complex (Kedde et al., 2007). The consequence of this protection is that the poly-A tail remains extended, thus stabilizing the mRNA.

Extension of poly-A tails is an important mechanism for the initial priming of the maternally supplied transcriptome. The early cleavages of many species require a substantial pool of maternally supplied mRNA which is primed for translation immediately after fertilization. In *Drosophila*, egg activation leads to a transcriptome-wide extension of mRNA poly-A tails by Wispy (Cui et al., 2008; 2013). Wispy is an ortholog of the *C. elegans* GLD-2, part of a family of conserved cytoplasmic polyadenylation factors. Nearly 3,000 Wispy target transcripts were identified in oogenesis, and over 4,000 were identified at egg activation. These transcripts fall within diverse functional classes, but are enriched for cell cycle progression, consistent with a requirement for early cleavage before EGA. In contrast to vertebrates and echinoderms, mechanical and osmotic pressure associated with egg laying activates the egg, rather than fertilization (Horner and Wolfner, 2008). Fertilization in many species, including echinoderms and vertebrates, triggers a dramatic release of an intracellular calcium wave (**Figure 2A**). The *Drosophila* oocyte has been recently found to also produce a calcium wave, and it will be important to test whether calcium triggers Wispy-dependent extension of poly-A tails (Kaneuchi et al., 2015). In the sea urchin, translation is detected within 20 minutes of fertilization, and requires the dephosphorylation of the general translation factor eIF2 (Costache et al., 2012). Future investigation may also find that calcium and cytoplasmic polyadenylation is important for stabilization and translation of the maternal transcriptome in echinoderms. There is a GLD-2 related factor in the sea urchin genome

(NCBI reference XP_003724909.1). Furthermore, it was observed nearly 40 years ago that the amount of poly-A-containing mRNA doubles from the unfertilized egg to the 2-cell stage, while the amount of total RNA remains constant (**Figure 2A**) (Wilt, 1977). The implication is that maternal deposition of the RNA is not sufficient on its own to power embryogenesis; that RNA must be primed for translation by the extension of the poly-A tail, a modification which also confers increased stability. An open question is whether there exist mechanisms within the PGCs that, like within the zygote, can extend the poly-A tails of inherited RNAs. Such an extension could offer additional protection to germ line RNAs, in addition to the stability conferred by CNOT6 depletion. While it has not yet been tested directly, some features of the micromere lineage hint that embryonic poly-A extension may occur.

A reprisal of poly-A extension in the micromere lineage?

The dynamic calcium transients, discussed above, are not limited to the activating egg. The fourth cleavage that produces the micromeres results in suggestive asymmetries in calcium regulation (**Figure 2A**). First, the vegetal pole of the asymmetrically dividing 8-cell blastomere becomes enriched for endoplasmic reticulum (**ER**). The ER serves as a major intracellular store of calcium and it can release calcium ions through channels gated by various cues. Using fluorescent dyes, a localized release of calcium was observed at the vegetal pole of the 8-cell blastomere as it began dividing to produce the micromere. After the division, the micromere maintains higher free cytoplasmic calcium concentration than the animal blastomeres with periodic spikes (Yazaki, 2001). The mechanism of micromere calcium release also remains unknown, though some pharmacology such as gadolinium treatment suggests it may require stretch-dependent

ion channels (Yazaki et al., 2012). In an interesting parallel, gadolinium treatment also prevented robust egg activation in *Drosophila* (Horner and Wolfner, 2008). An additional possible player is the Wnt calcium pathway, a non-canonical effect of Wnt reception. In this signaling variant, a Wnt ligand binds to Frizzled and a coreceptor, activates PLC which releases IP_3 , which then releases calcium from ER stores (De, 2011).

The secondary calcium release in the nascent micromeres has been suggested to participate in the vegetal Wnt pathway by facilitating nuclearization of β -catenin. Inhibition of PKC, a calcium responsive kinase, reduces nuclear accumulation of β -catenin, though the requirement for calcium release has not been directly tested (Yazaki et al., 2014). An intriguing possibility, however, is that calcium release in the micromere is required for the activation of fate specification and the regionally specific GRN in the micromeres (Reviewed in **Chapter 1**). Given the enhanced stability of inherited mRNA in the sMics (**Chapters 1 and 2**), it is also tempting to speculate that calcium may trigger another extension of Poly-A tails via a Wispy-like mechanism (**Figure 2B**). Specific regulation of polyadenylation within the germ line has been previously reported in several contexts. One important regulator is Dazl (deleted in azoospermia-like), a widely conserved germ line specific RNA binding protein required for fertility in numerous animals. In the frog, Dazl associates with poly-A binding proteins (PABPs) to enhance translation of target mRNAs (Collier et al., 2005). It has also been found to be important for the egg to zygote transition in the mouse (Chen et al., 2011). In the zebrafish, Dazl counteracts the effects of the microRNA miR-430 by enhancing polyadenylation through an unknown mechanism (Takeda et al., 2009). The sea urchin genome encodes a related factor called *Boule*, whose RNA is present in oocytes and eggs (Juliano et al., 2006). It is possible that Boule is required for translational regulation at both the egg to

embryo transition, as well as mRNA protection in the germ line. This putative function can now be readily tested by *Boule* morpholino knockdown, followed by spatial examination of known sMic-enriched transcripts such as *Seawi* and *Vasa*. If *Boule* plays a stabilizing role, I predict that its knockdown would prevent long-term retention of germ line RNAs in the sMics. The mechanism of action could be further tested by measuring poly-A tail lengths using PCR-based assays. With various pharmacological inhibitors now available, it should also be possible to test whether calcium release orchestrates polyadenylation of target mRNAs in the micromere lineage.

The time capsule model for germ line segregation in the sea urchin

I refer to the sum of these observations as the “time capsule” mechanism for germ line segregation (**Figure 1, and Chapter 2, Figure S4**). Rather than clearing the slate at the MET and re-instituting a germ line program *de novo* by induction, the sMics instead retain the characteristics of the egg and early embryo. Conversely, the somatic cells in the embryo participate in a MET that broadly degrades maternally-supplied RNA, and possibly protein. Degradation of inherited RNA via CNOT6 is important for differentiation of the endomesoderm, as its broad knockdown completely disrupts gastrulation (**Chapter 2, Figure S4**). The presence of CNOT6 in the soma, but absence in the sMics, is a critical bottleneck step for this degradation, resulting in enhanced stability in the sMics. While the sea urchin embryo does not contain a morphologically obvious, spatially-localized germ plasm, the differential stability mechanism described here does accomplish a similar function—namely, specific acquisition of germ line associated materials in the sMics.

Preservation of maternally supplied material within the sMics could be a more efficient means for segregating the germ line quickly, rather than relying upon embryonic mechanisms. If a mother invests heritable germ line determinants into an egg, she increases the likelihood that her offspring will be fertile, and in turn produce offspring of their own. Germ line preloading is a highly homoplastic trait, and appears to be frequently gained. This strategy may be particularly favored with pressures like high predation or rapidly changing environment (conditions experienced by, for example, a fruit fly), when enhanced evolvability would be beneficial. Sea urchins, and other animals that rely upon preloaded mechanisms (e.g. *Drosophila*, *Xenopus*, *zebrafish*) all develop indirectly through a larval form; thus, it is conceivable that segregating the germ line immediately allows the embryo to invest more effort into reaching a motile, feeding form as quickly as possible (see **Chapter 1** for further discussion).

So what are the small micromeres, really?

The developmental origin and mode of germ cells in echinoderms has been controversial for at least 20 years. The controversy has been exacerbated both by the unique lifecycle of echinoderms, as well as semantic differences within the research community. Most sea urchins and other echinoderms are indirect developers; that is, embryogenesis results in a feeding larval form that can exist for weeks or months and before metamorphosing to produce the juvenile. Because a seemingly small portion of the larva actually produces the adult, whereas the rest of the larva dies, some have categorized sea urchins as “maximally indirect” (Peterson et al., 1997). However, these distinctions are somewhat blurry and maybe not relevant to the main point, which is that in this biphasic lifecycle, PGCs could either be segregated in the embryo or the larvae. After my

own investigation, as well as that of others in the field, I now argue that the sMics are the *bona fide* PGCs, whose fates are absolutely separated from the soma in the 32-cell embryos. Below, I will review the experimental lines that have led up to this conclusion.

The historically ambiguous developmental fate of the sMics

Previously, the prevailing hypothesis was that the sMics are not PGCs but rather multipotent cells, set aside within the larva, to produce the adult sea urchin. Such “set-aside” cells have been proposed to be broadly used as the raw material for indirect developers, which need to rebuild themselves into the adult form (Arenas-Mena, 2010). In sea urchins, this notion largely derives from earlier observations that the sMics comprise ~40% of the nascently formed coelomic pouches (e.g. 3-5 out of 10 total cells), whereas the remainder are macromere descendants (Cameron et al., 1991). The logic that followed was that since the coelomic pouches construct the “majority” of the adult rudiment, the sMics must therefore produce 40% of that rudiment. I find this argument to be untenable, particularly because there exists no lineage tracing data to link any coelomic pouch constituents to the adult sea urchin. Furthermore, development of the adult rudiment is a complicated morphogenetic process, many aspects of which are refractory to normal light microscopy (Smith et al., 2008). The ambiguity in phraseology that has been used in the literature to describe the quantity of coelomic pouch contribution to the adult, owes to, I believe, the fact that we just do not know it for certain. Furthermore, we cannot assume that the ratio of sMics and macromere descendants (4/6) in the nascent coelomic pouches in any way represents their final contribution to the adult at metamorphosis, as these events are separated by 4 weeks of development.

Ablation experiments have sought to test the fate of the micromere lineage, and while informative, these studies created additional ambiguities. Namely, when micromeres were removed by microsurgery, the resultant larvae metamorphosed into fertile adults (Ransick et al., 1996) (**Figure 3B**). From this result, the authors reasonably concluded that there are no obligate germ line determinants in the micromeres. While their results did not preclude a segregation of PGCs in the later blastula or gastrula stage embryo, they instead contended that the germ line must be segregated in the larva. When Juliano and colleagues found that germ line associated RNAs are enriched in the sMics by blastula or gastrula stage, they were therefore seemingly presented with a paradox (Juliano et al., 2006). While some non-germ line functions have been identified, overlapping expression of factors like Piwi, Nanos, and Vasa is strongly indicative of gametogenic potential (Ewen-Campen et al., 2010).

For several years, a sensible compromise was to call the sMics germ line competent multipotent cells—that is, they produce the germ line stem cells in the adult, as well as somatic fates. This hypothesis was favored by many, perhaps because it presented a potential system to study a persistent pluripotent cell type in nature, as an alternative to man-made vertebrate embryonic stem cells. Such a scheme has been suggested for indirect animals such as the snail *Ilyanassa obsoleta*, which segregates an endomesodermally competent blastomere called 4d. This blastomere divides once symmetrically to produce two founding stem cells, which then divide asymmetrically to populate the endomesoderm. Embryological analysis in a related clam suggests that after performing this somatic role, the 4d stem cells then assume a PGC fate (Woods, 1931). These stem cells express Vasa and Nanos, and Nanos is required not just for fertility, but the production of larval somatic fates (Rabinowitz et al., 2008; Swartz et al.,

2008). Similarly, Nanos knockdown in the sea urchin prevents rudiment formation and metamorphosis, rather than just eliciting a fertility defect (Juliano et al., 2010b).

However, when Nanos-deficient sMics were transplanted onto a normal recipient, a proper rudiment formed, though the sMics did not incorporate within it, based on fluorescent lineage tracing. These results indicate that Nanos may have functions outside the sMics, and indeed, we have now identified broader mesodermal expression of Nanos2 (**Chapter 4**).

Firmer resolution of their fate was later provided when the sMics, rather than the parent micromeres, were removed (Yajima and Wessel, 2011). With this manipulation, the resultant juveniles are apparently infertile, but are otherwise normal (**Figure 3C**).

Conversely, when the parent micromeres were removed, the animal was fertile, as previously found by Ransick and colleagues. These results indicated that the micromeres provide an organizing signal, that when lost, can trigger a germ line recovery program. My own results in **Chapter 4** suggest that Wnt8 may serve as the organizing signal (**Chapter 4**). Knockdown of Wnt8 resulted in a 20-fold increase in Nanos levels in the Veg2 mesoderm. Physically removing the micromeres, the Wnt8-presenting cells, would result in the same effect. There exists, however, a critical window for this signal – after their division to the large and small micromeres, the embryo has irreversibly committed. Formally, this experiment does not exclude the possibility that the sMics *can* produce other somatic fates in a wild type animal; definitively testing this possibility will require a genetic – based lineage trace. With the development of Crispr/CAS9 genome editing, it should now be possible to definitely fate map the sMics, particularly in a faster developing sea urchin species such as *Lytechinus variegatus* (Hale et al., 2009).

Nevertheless, the observations presented here and previously strongly argue that the

sMics are *bona fide* early-forming PGCs, as observed in *Drosophila*, *Xenopus*, *Zebrafish*, and elsewhere.

An evolutionary transition of germ line segregation mode in echinoderms

I have documented multiple species examples in which the germ line segregation strategy evolved from a later inductive mode to an earlier, more preloaded mode (**Chapter 1**). Such a transition appears to have occurred within the echinoderms, particularly the echinoids, which acquired the novel micromere lineage. Our analyses, while at this time limited to only several species, argue that the ancestral mode of germ line segregation in the echinoderms was by induction, meaning intercellular signaling cues direct a subset of cells toward germ line fate in the later embryo (**Chapter 1**). In the sea star *Patiria miniata* and the sea cucumber *Apostichopus japonicus*, we first detect Nanos transcripts in mesodermal progenitors. In larval stages, Nanos is expressed in a posterior coelomic pouch called the posterior enterocoel (**PE**) in both species (**Figure 4A,B**). The PE is a structure widespread amongst sea star and sea cucumber species, and based on microsurgical ablation, is thought to give rise to germ cells (Inoue et al., 1992). In neither example are Nanos transcripts detectable in the egg or pregastrular embryo. Vasa protein has also been found to be enriched in the sea star PE, but not in the earlier embryo, further suggesting that germ line segregation occurs later in the sea star (Juliano and Wessel, 2009).

The sister group to the euechinoids, which contains sea urchins and sand dollars, is the cidaroids, or the pencil urchins. Together with the euechinoids, they straddle the last common ancestor to all sea urchins, and are therefore critical to examine for evaluating the evolution of the micromere lineage. In the pencil urchin *Eucidaris tribuloides*, the best

studied cidaroid, variable numbers of micromeres (typically 1-3) are produced at the fourth cleavage. In contrast to the euechinoids, the micromeres have not been observed to divide asymmetrically to produce large and small micromeres. The cidaroid micromere descendants do produce the larval skeleton, though they ingress into the blastocoel after gastrulation has initiated, whereas in the euechinoids this occurs in the blastula (Schroeder, 1981; Wray and McClay, 1988). In *E. tribuloides*, we detected Nanos RNA in the likely micromeres in the early blastula, which persisted at the gut tip through gastrula stages, and expanded to encompass the entire gut tip in late gastrula stages in the region where the coelomic pouches form (**Figure 4C**). This expansion could represent proliferation of the micromeres, or alternatively, could be Nanos induced in adjacent mesoderm, as seen in the sea urchin. Similar to observations in the sea urchin, we ultimately detect Nanos expression in the left coelomic pouch. In contrast with the sea urchin, Vasa protein is not enriched in the micromere lineage until the larval stage of the pencil urchin, when Vasa antibodies give enriched staining in the coelomic pouches (Juliano and Wessel, 2009). I note that the Nanos positive micromere descendants remain in the endomesoderm, whereas other micromere descendants leave the gut and form the skeleton (Wray and McClay, 1988). Perhaps the pencil urchin micromeres do indeed divide asymmetrically to form large and small micromeres, but we have not detected this division because it occurs much later than in the sea urchin. Alternatively, the division could be morphologically symmetric but result in gene expression asymmetry. In either case, the pencil urchin micromeres appear to be a bifunctional lineage, and in the future, it would be valuable to also test expression of skeletogenic genes to determine when fate separation occurs. I propose that the pencil urchin bifunctional micromere represents the ancestral state within the echinoids, and later in

evolutionary time, the micromere acquired early and asymmetric division capability to produce the sMics.

An interesting challenge that now follows is understanding what molecular changes drove the heterochronic shift of Nanos activation from a sea star / sea cucumber like embryo to the precocious sea urchin / pencil urchin embryo. My investigation into the transcriptional regulation of sea urchin Nanos2 provides some clues. The earliest activation of Nanos2 in the sMics occurs autonomously and requires nuclear β -catenin, and is likely independently of an embryonically expressed Wnt ligand (**Chapter 4**). The sea urchin egg is pre-polarized with vegetally localized Dishevelled, which, based on gel migration, is uniquely modified compared to anterior Dishevelled. Dishevelled is an upstream transducer in the canonical Wnt pathway, and its modification could indicate that the maternally supplied and localized form is constitutively active. This localized Dishevelled is required for the earliest nuclearization of β -catenin in the micromere lineage (**Figure 5A**). A plausible scenario is that Nanos2 in the sea urchin acquired a cis regulatory element that allowed it to respond to this maternal β -catenin input (**Figure 5B**). The subsequent Nanos2 expansion into the Veg2 mesoderm in the sea urchin may then be homologous to the vegetal ring of Nanos observed in the sea star early gastrula. If this is the case, then the sea star Nanos expression should be downstream of D/N signaling, a hypothesis that is now readily testable (**Figure 6**).

Are the sMics inductive or preloaded PGCs?

In contrast to the preloaded PGCs of *Drosophila*, *Xenopus*, zebrafish, and others, Nanos is embryonically transcribed in the sMics rather than being maternally supplied. This peculiarity goes against what are typically called preloaded PGCs, which are

transcriptionally quiescent. However, the activators of Nanos expression (e.g. Dishevelled and β -catenin) are indeed maternally provided, and in vitro culture experiments indicate that Nanos2 is activated autonomously (Peng and Wikramanayake, 2013; Yajima and Wessel, 2012). In a sense, then, maternal Dishevelled plays a similar role as germ plasm in other organisms. We presented evidence that the sMics, like other preloaded PGCs, experience a protracted period of transcriptional quiescence, based on H3K9me3 enrichment and RNA polymerase II depletion (**Chapter 2, Figure 2**). It is therefore somewhat surprising that Nanos would be embryonically transcribed in the sMics. A uniqueness of Nanos2 is that it is a small, single exon gene (**Chapter 4, Figure 4**). This gene organization may facilitate its early transcription in a generally repressed nucleus. FoxY is the only other gene known to be selectively activated in the sMics before gastrulation (**Chapter 3, Figure 1**), however, its mRNA accumulates there at lower levels than it does in the Veg2 mesoderm. In the future, one could test whether genes expressed in quiescent cell types, such as the sMics, evolved to favor few introns. Indeed, it has been noted that genes pertaining to the germ line are under different evolutionary pressure (Vacquier and Swanson, 2011). If so, scanning the genome for intron deficient genes could provide an additional means for discovering new germ line or stem cell factors. Furthermore, as predicted by Johnson and colleagues, inherited germ line specification may accelerate the rate of evolution for developmental genes (Evans et al., 2014). With a now rich dataset of transcriptome from inductive sea stars and preloaded sea urchins, we are well-positioned to directly test this premise (Reich et al., 2015).

Activation of Nanos2 in the sMics is essential to enact their other preloaded characteristics. We transcriptomically identified 78 sMic-enriched RNAs, which are

generally maternally supplied and degraded by gastrula stage, a process that likely broadly requires Nanos function (Swartz et al., 2014). Nanos directly represses the accumulation of the deadenylase CNOT6 mRNA in the sMics, which endows them with enhanced RNA stability. This deadenylase depletion creates a safe place in the sMics where maternally-provided germ line RNAs, such as Vasa and Seawi, can persist. The generality of this process is underscored by the fact that exogenous injected RNA is retained in the sMics for at least 1 week after fertilization, independently of sequence (Gustafson and Wessel, 2010; Oulhen and Wessel, 2013; Swartz et al., 2014). In effect, while the sMics do not appear to acquire spatially localized mRNA, they stably retain inherited mRNAs, which are instead degraded in somatic cells.

Are you suggesting ~~coconuts~~ germ cells migrate?

Whether specified by preloading or induction, a widespread aspect of PGCs is that they are specified far from the presumptive somatic gonad. This arrangement requires that the PGCs of *Drosophila*, mice, zebrafish, chick, and sea urchin migrate a substantial distance, often in association with the embryonic gut structures, and in response to guidance cues (Campanale et al., 2014; Richardson and Lehmann, 2010; Wylie, 1999). This journey could pose some risks for the embryo: namely, having a highly potent cell type migrating through the body cavity. While germ cells are normally unipotent—that is, they will give rise to only a sperm or an egg—it is well appreciated that germ cells have a potent capacity to give rise to teratomas (tumors containing derivatives of multiple germ layers) when removed from their restrictive niche (Juliano et al., 2010a). Given this possible risk, convergent mechanisms for repressing transcription and resisting the MET, thereby insulating germ cells from differentiation, are sensible precautions.

One such protective mechanism is programmed cell death—PGCs are prone to apoptosis. Migrating PGCs must undertake a journey to the gonad, and as mentioned above, there could be some dangers inherent to a rogue PGC becoming tumorigenic. In multiple species, Nanos directly represses apoptotic pathways, and its perturbation results in apoptosis of the PGCs (Lai et al., 2012; Sato et al., 2007; Suzuki et al., 2008; Tsuda et al., 2003). When apoptotic pathways are suppressed, however, these altered PGCs can acquire somatic fates and express somatic genes, indicating their broader developmental potency (Hayashi et al., 2004). Thus, migrating PGCs may be on a hair trigger to apoptose, which provides a protective advantage to the organism and helps ensure that PGCs only populate the gonad. Apoptosis appears to occur even in the wild-type embryo; for example, mutation of p53 in an otherwise normal background results in numerous ectopic PGCs outside of the gonad in *Drosophila* (Yamada et al., 2008). In the sea urchin, the 8 sMic descendants migrate into the bilateral coelomic pouches, but asymmetrically: typically, 5 sMics arrive in the left pouch, whereas 3 arrive in the right. This migration requires ABC transporter activity, hinting at an involvement for chemoattraction (Campanale and Hamdoun, 2012). Subsequently, the sMics in the right coelomic pouch apoptose downstream of Nodal signaling (Luo and Su, 2012). We note that the right coelomic pouch loses Nanos expression, which may confer protection from apoptosis. The left coelomic pouch is thought to be the primary contributor to the adult rudiment, and so, there may be a competition for the sMics to reach that pouch and thereby contribute to the germ line (Smith et al., 2008).

Why not instead specify the germ cells directly in the future gonad, thereby eliminating the need to migrate? Such an arrangement has been observed in the cricket *Gryllus gryllus*, but there are not yet other clear examples (Ewen-Campen et al., 2013). One

possibility is that migration imposes a selective pressure at the cell type level. In the mouse, for example, 50-100 germ cells enter at the migratory phase at embryonic day 8.5, and by day 13.5, they have amplified to over 20,000 (Tam and Snow, 1981). An attractive possibility is that some of the original founder PGCs are outcompeted because of their lesser migratory capability. The result consequence would be a “higher quality” pool of germ cells to populate the gonad and therefore a more fertile individual. Selection among the PGCs has been previously considered, particularly with respect to mitochondrial inheritance. In embryos such as the frog, the germ plasm contains a large concentration of mitochondria invested for inheritance by the next generation. Based on biochemical and cytological assessment, these germ line mitochondria also appear to be metabolically attenuated, which could lower their production of reactive oxygen species, thus offering some protection to the germ line (Kogo et al., 2011).

It takes a village: the reproductive niche

A germ line cannot develop in complete isolation, even when specified in the preloaded mode. Instead, multiple aspects of PGC development and self-renewal of GSCs require contact with somatic support tissue. The PGCs, together with the somatic gonad, form the reproductive niche that will make gametes. PGC development has been heavily investigated, and perhaps the somatic cell development has received less attention. Our work here took a first step into delineating the somatic contribution to the reproductive niche in the sea urchin (**Chapter 3**). We found that in response to Delta/Notch signaling from the micromeres, and subsequently from the mesoderm, the Veg2 progenitors express the forkhead factor FoxY. Perturbation analysis revealed that FoxY is the immediate early effector of Delta/Notch signaling, and its knockdown essentially

phenocopied Delta/Notch inhibition. In such perturbed embryos, the coelomic pouches completely fail to form, and genes selectively expressed in the Veg2 mesoderm are depressed (Materna et al., 2013). The Veg2 mesoderm also gives rise to lineages outside the coelomic pouches, such as pharyngeal muscles and pigment cells in the ectoderm. In the future, it will therefore be important to delineate which subdomains within the Veg2 mesoderm are specifically responsible for associating with the sMics in the coelomic pouches. Some effort has already been made into this, and one candidate domain expresses SoxE, a transcription factor related to the vertebrate sex determining gene Sry (Luo and Su, 2012). By perturbing genes specific to the different subdomains of the Veg2 mesoderm, we may find the specific population of somatic cells required to support the sMics in the coelomic pouches.

The Delta/Notch pathway may be a conserved requirement for establishing the reproductive niche. In *Drosophila*, the somatic gonad derives from progenitor cells of the dorsolateral mesoderm (Jemc, 2011). At least in the case of the male, the hub cells, which form the innermost aspect of the niche in contact with the GSCs, require Delta/Notch signaling from the endoderm (Okegbe and Dinardo, 2011). It is not yet known whether Delta/Notch is required for establishing the vertebrate somatic gonad; however, it has been implicated numerous other stem cell niches, including the intestinal crypt, the hair follicle, and the hematopoietic system (Chiba, 2006; Koch et al., 2013). Future investigation in other tractable model organisms may find that Delta/Notch signaling is widely important for patterning the gonad. Now, with available transcriptomic evidence for somatic ovary-specific genes in the sea star, it will be possible to begin dissecting the developmental control of this critical niche (Adrian Reich, unpublished results).

Were PGCs the first cell type in animal evolution?

One cannot imagine animal life as we know it without sexual reproduction. While examples of asexual propagation have arisen, the pervasiveness of sex tells us that the ability to produce gametes must have existed in the last common animal ancestor (**LCA**). The unicellular proto-animal would have had to perform both as a meiotically capable “germ line” cell, as well as a somatic, motile feeding cell. The switch to multicellularity would have allowed the primitive animal to feed, swim, and make gametes simultaneously. The question is, then, when and how did the LCA produce those gametes? With ample comparative data across animals, we can infer that it was probably by an inductive mechanism (Extavour and Akam, 2003; Extavour, 2007). We have focused most of our previous discussion on bilaterian animals; however, there is growing molecular data in the cnidarians (jellyfishes), ctenophores (comb jellies), and poriferans (sponges). *Hydra* is a popular laboratory cnidarian which has a multipotent adult stem cell population called the i-cell. These i-cells continually segregate germ cells throughout the adult life, but also populate the somatic interstitial lineage, which includes nerves, nematocytes, and gland cells (David, 2012). Investigation of *Hydra*, and the colonial relative *Hydractinia* have shown that i-cells express the germ line suite of Vasa, Nanos, and Piwi (Juliano et al., 2014; Mochizuki et al., 2000; Rebscher et al., 2008). Expression analysis in a ctenophore indicates that Vasa and Piwi are expressed in both germ line and somatic stem cell compartments (Alié et al., 2011). Piwi expression has even been detected in the archeocytes, putative pluripotent stem cells, in a sponge (Funayama, 2010). These collective observations speak to the deep conservation of these genes, but an important question is whether they are required for the general potency of the stem cells in which they are expressed, or instead just for their

gametogenic potential. Hydra has gained substantial molecular tractability, and its Piwi ortholog was globally knocked down. In these animals, the defect is not simply reproductive, but rather complete inviability of the animal (Juliano et al., 2014). This observation implies adult somatic functions for the Piwi pathway, and in the future, tissue specific knockdown should further delineate that requirement. Future investigation into the requirement for Nanos and Vasa for somatic functions in the stem cells of cnidarians would be very valuable, not just for understanding their basic molecular function, but the evolution of stemness.

The existence of these germ line factors in cells with somatic fate has led to the proposal of the “primordial stem cell hypothesis”. In this model, the LCA segregated a stem cell in development that was capable of populating the germ line, and also somatic fates. This suggestion is supported by the developmental modes of extant animals such as hydrazoan cnidarians, with the i-cell lineage, spiralian, such as *Ilyanassa* with its multipotent 4d lineage, and *C. elegans*, whose germ plasm containing P-blastomere also gives rise to somatic fates (Solana, 2013). However, whether these diverse cell types truly fit the same definition of a “stem cell” has not been rigorously analyzed, and given so much evolutionary distance, it is difficult, if not impossible, to assign any cell type homology between them. Despite these overt differences, germ line segregation uses a conserved molecular toolkit, whose functions we have now begun to untangle.

With these markers, the emerging pattern is that evolution has repeatedly toyed with the timing of germ line-soma separation. It has been stated that “*a hen is only an egg’s way of making another egg*” (Butler, 1878). At the switch to multicellularity, the true novelty may have been the soma—that is, the loss of gametogenic potential. This terminal

differentiation may have required a transcriptome and proteome degradation event, as seen in the METs and differentiation contexts in numerous extant animals. In an animal like the fruit fly, or the sea urchin, germ line-soma separation occurs extremely early in the embryo. In contrast, that terminal separation does not occur until significantly later in the mouse embryo. In an animal with stem cells like the Hydra, the ultimate separation does not occur until adulthood. It therefore may not be productive to think simply in the binary terms of inductive versus preloaded; instead, germ line-soma separation across species occurs within a continuum of timing within the lifecycle. We might instead use term such as Germ Line Index, or GLI, which we have suggested may provide a useful means to compare germ line segregation in different species (Wessel and Swartz, 2015). The GLI may be calculated simply by dividing the total number of cells in the organism at the time of separation (whether it is an embryo or an adult) by the number of committed PGCs. With this metric, the vast range in timing of germ line segregation becomes readily apparent (**Figure 7**). In this work, I have provided some molecular insight into how such heterochronies can occur, with particular focus on how Nanos is activated by the Wnt pathway in the sea urchin sMics. I have characterized a critical function for Nanos, which is to enact a post-transcriptional program of protecting inherited RNA. Finally, I identified a function for Delta/Notch signaling and FoxY in establishing the larval somatic niche for the sMics. It is my hope that this investigation will inspire future exploration into the molecular regulation and evolution of the ancient relationship between germ line and soma.

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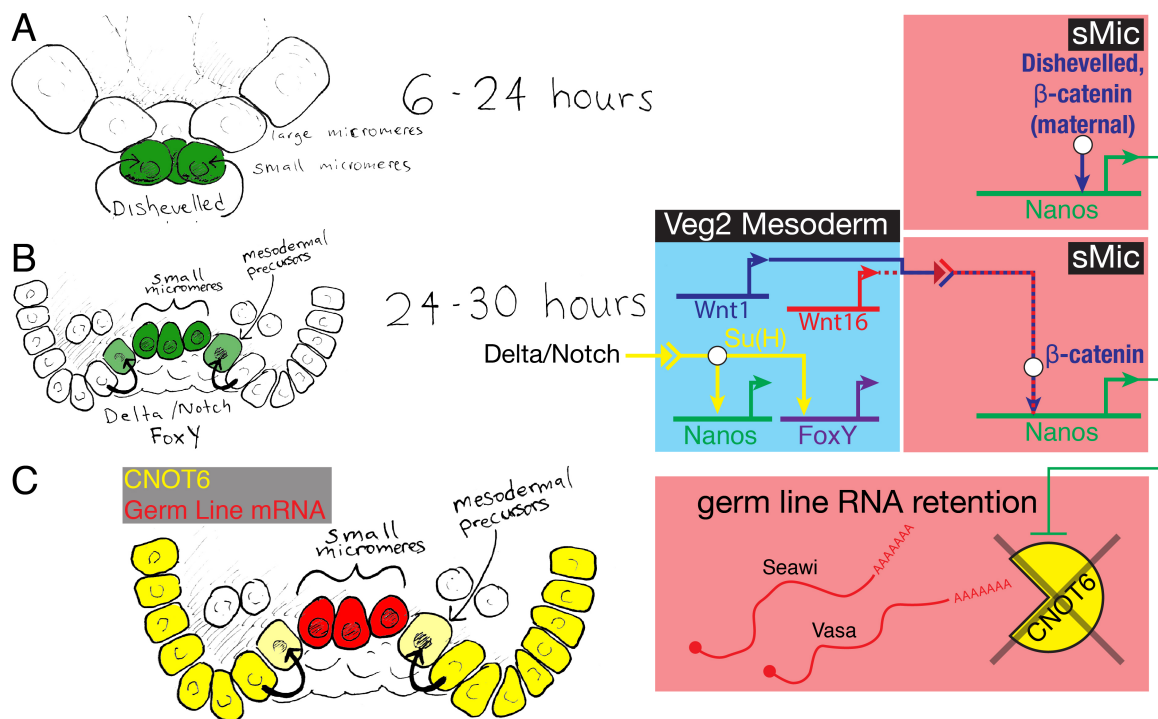


Figure 1. Mechanisms for germ line segregation in the sea urchin. (A,B)

Transcriptional regulation of Nanos expression in the sMics and Veg2 mesodermal precursors. (A) The sMics are created in the 32-cell embryo at about 6 hours post fertilization. Maternally-provided Dishevelled (localized to the micromere lineage) and β -catenin protein activate expression of Nanos. (B) Nanos expression is reinforced by embryonic expression of Wnt1 and Wnt16 in the Veg2 mesoderm. Delta/Notch signaling activates Nanos and FoxY expression in the Veg2 mesoderm. (C) Function of Nanos in the sMics. Nanos protein expression post-transcriptionally represses the accumulation of CNOT6 deadenylase in the sMics, which is present in all somatic cells (indicated in yellow). Reduced deadenylase activity allows for stable retention of inherited mRNAs such as Seawi and Vasa (indicated in red).

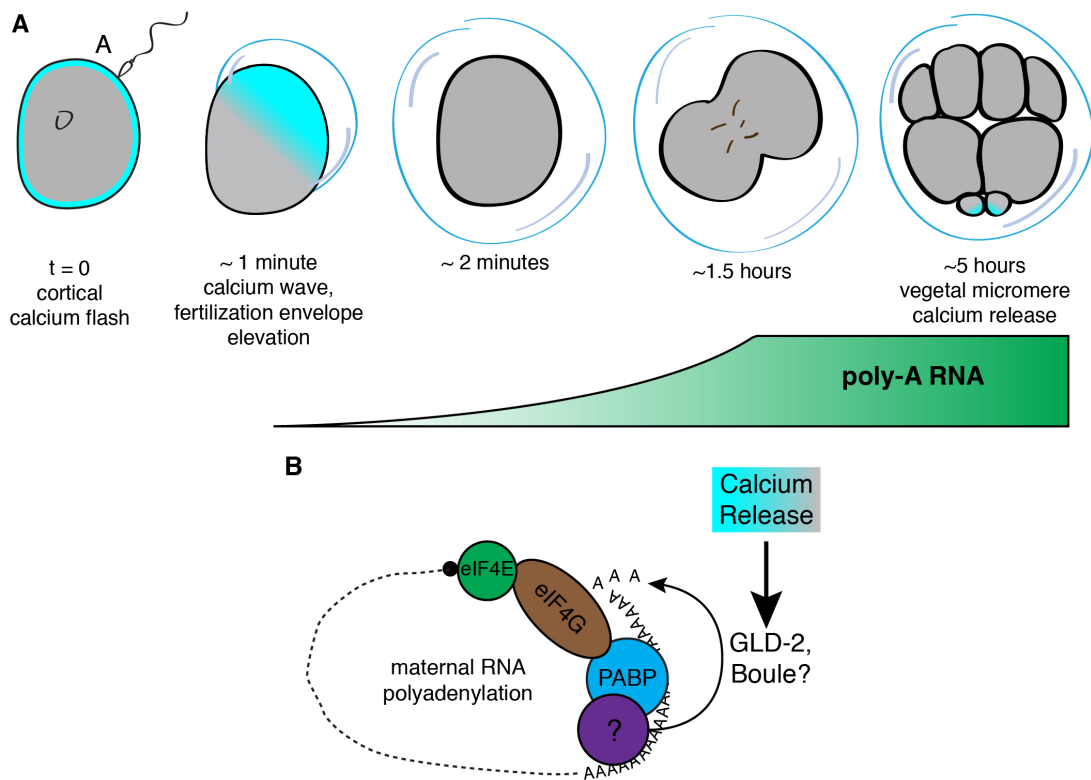


Figure 2. Hypothesis for calcium triggered poly-A tail extension of maternal RNAs. (A) Schematic of early development and calcium dynamics. Calcium release (blue) is triggered at fertilization, and first visible as a simultaneous cortical flash near the cortex. Within 1-2 minutes, a calcium wave propagates across the zygote, initiating at the site of sperm entry. A secondary calcium release occurs in the micromeres in the 16-cell embryo. (B) These calcium releases may trigger the extension of poly-A tails by an unknown mechanism. Possible effectors could a GLD-2 family member, or the germ line-associated factor Boule.

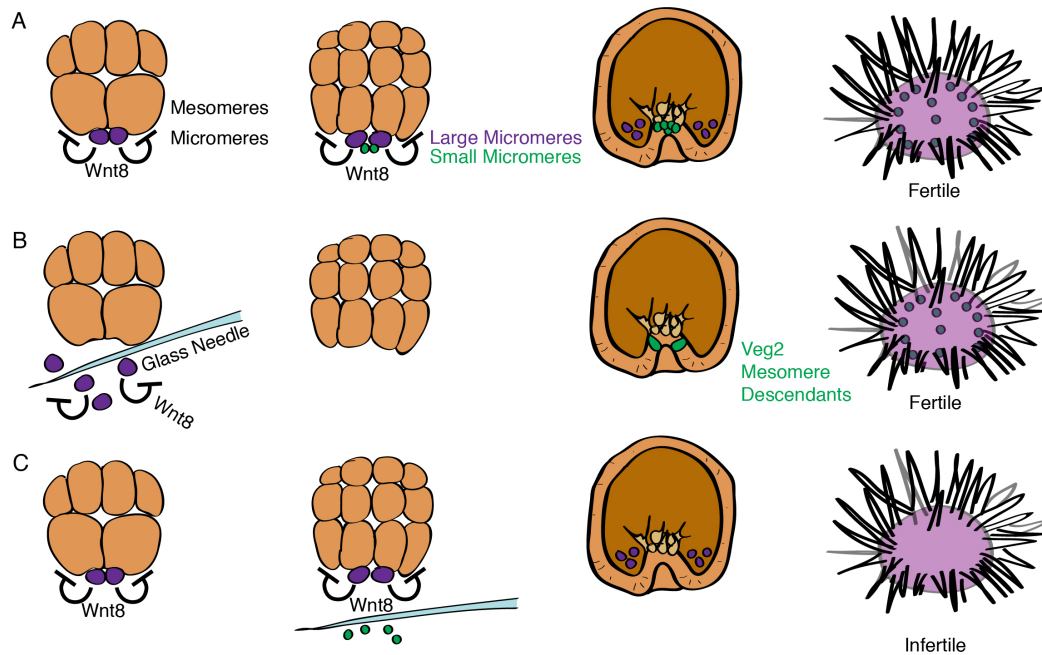


Figure 3. Summary of embryological manipulations of the micromere lineage. (A)

In an intact embryo, I hypothesize that Wnt8 signaling from the micromeres restricts robust Nanos accumulation to the sMics by repressing its expression in the mesomere descendants. The result is a fertile adult populated by sMic descendants (B) When the micromeres are surgically removed, the repressive Wnt8 signal is lost, and compensatory Nanos is robustly expressed in the Veg2 mesomere descendants, resulting in a fertile animal. (C) When the sMics are instead removed, the repressive Wnt8 signal remains in place, preventing compensatory germ line expression. The result is a sterile animal.

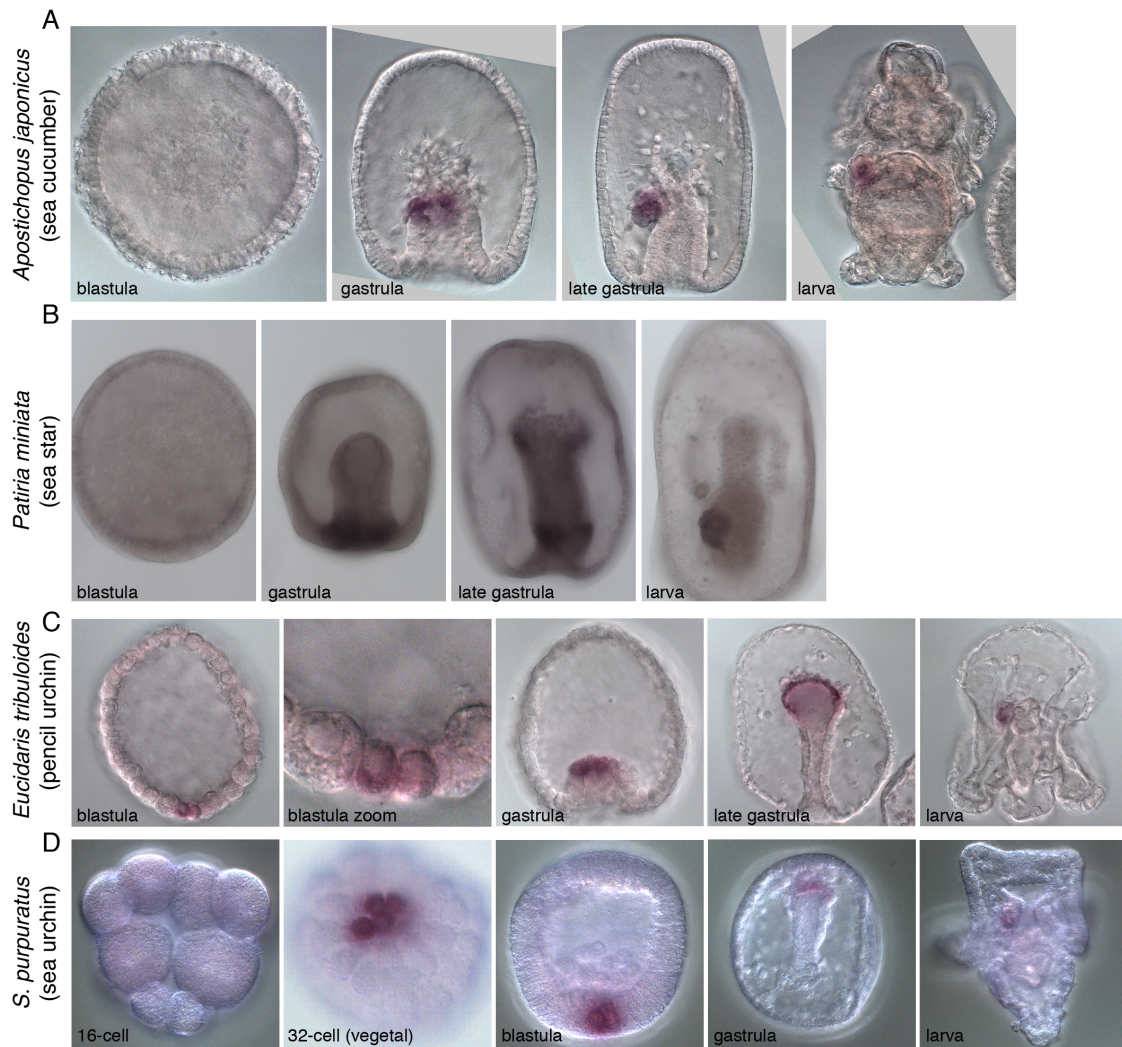


Figure 4. Embryonic Nanos localization in diverse echinoderm embryos. (A) In the sea cucumber *Apostichopus japonicus*, Nanos is first detectable at the tip of the early archenteron in the nascently forming PE, which buds off as a discrete unit. Nanos signal persists into later larval stages. (B) In the sea star *Patiria miniata*, Nanos RNA is first detected in a ring at the vegetal pole of the early gastrula. These Nanos positive cells then leave the endodermal epithelium and form the PE on the left side of the larva. (C) In the pencil urchin *Eucidaris tribuloides*, Nanos RNA is first detectable in 1-3 cells, the micromeres, in the early blastula (a zoom view is provided). Nanos signal persists at the tip of the archenteron in the gastrula, but subsequently becomes expanded at the gut tip in later gastrulae. In larvae, Nanos is detected only in the left coelomic pouch. (D) In the sea urchin *Strongylocentrotus purpuratus*, Nanos2 is first detected in the sMics at the 32-cell stage, persists in the sMics through gastrulation, and is finally detected in the left coelomic pouch. *Patiria miniata* (B) images by Tara Fresques; *Strongylocentrotus purpuratus* images are adapted from Swartz et al., 2014 and Juliano et al., 2010.

Method summary: In situ hybridization templates were amplified from cDNA using *de novo* transcriptome assembly. PCR fragments were then transcribed using T7 polymerase with DIG labeled NTPs, hybridized, processed, and imaged by standard methods (Swartz et al., 2014).

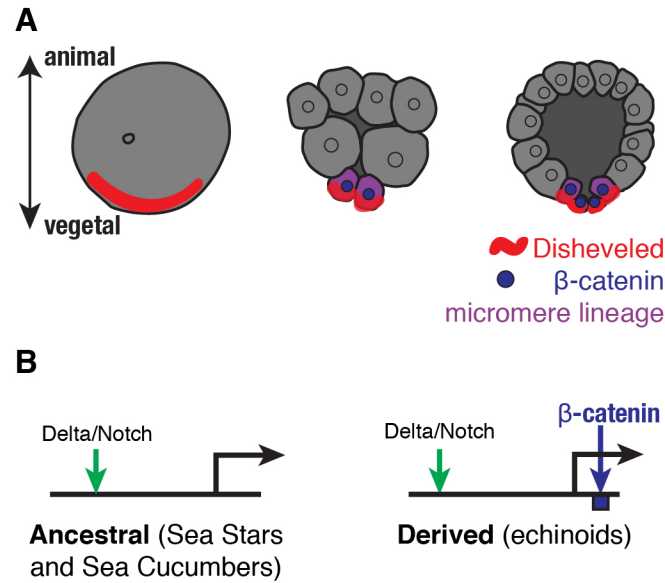


Figure 5. Model for precocious germ line expression of Nanos2 in the sMics. (A) Maternal Disheveled protein is localized to the vegetal cortex of the egg and inherited by the micromere lineage, resulting in nuclearization of β -catenin. (B) I hypothesize that the echinoid (e.g. sea urchin) Nanos2 promoter acquired a cis regulatory element (blue) that enabled it to respond to early nuclear β -catenin. In the ancestral condition, Nanos was activated later in the mesoderm by Delta/Notch signaling, which is retained in the echinoids.

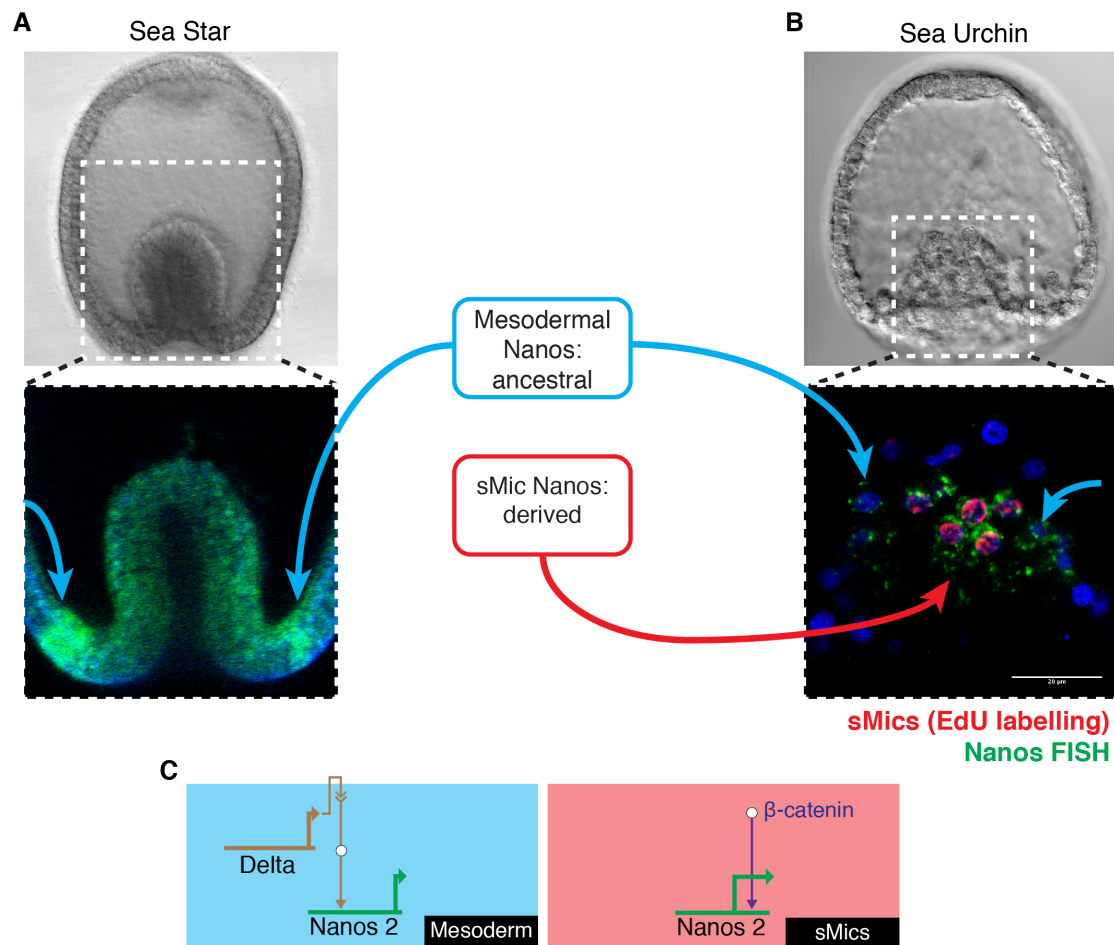


Figure 6. Cell type relationships between sea star and sea urchin embryos. (A,B) Localization of Nanos RNA in the sea star *Patiria miniata* and the sea urchin *S. purpuratus*. I hypothesize that the Nanos first detectable in the sea star vegetal pole is homologous to the Veg2 mesodermal Nanos expressed in the urchin (blue arrows). sMic Nanos is a derived feature unique to echinoids. (C) Simplified regulatory diagram, highlighting the Delta/Notch input into Nanos2 in the mesoderm, and β -catenin activation in the sMics. Sea star fluorescent in situ by Tara Fresques (unpublished results).

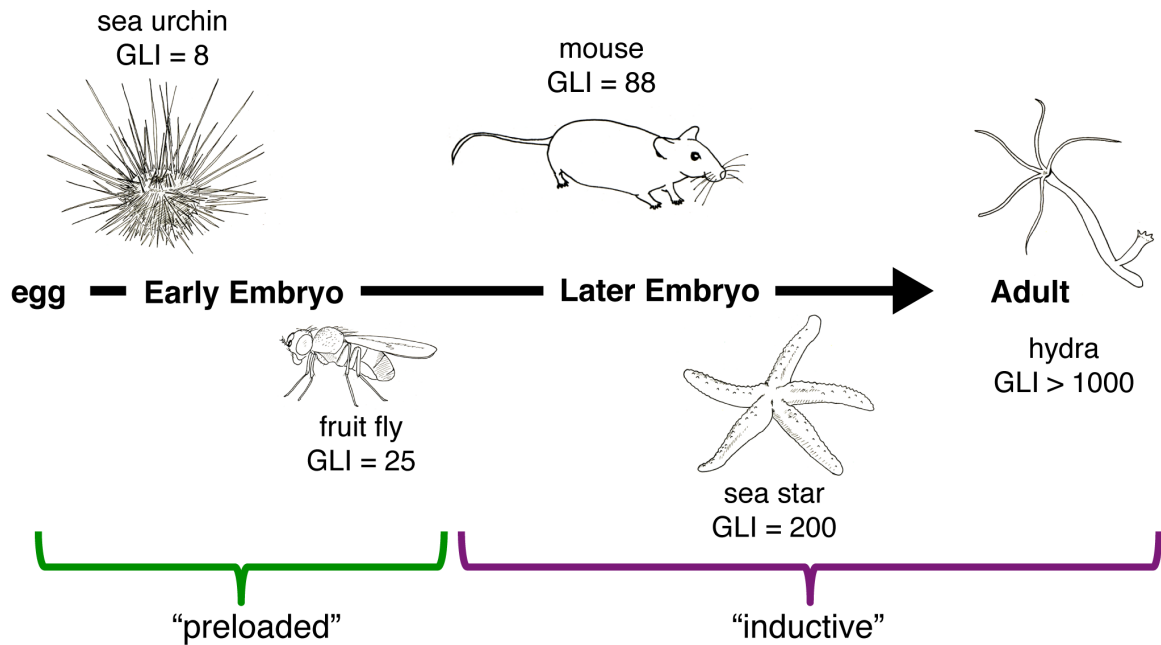


Figure 7. Relative timing of germ line separation from the soma. Representative animals typically considered to use preloaded or inductive strategies are presented in rank order based on their Germ Line Index (GLI). This calculation is a ratio of the total number cells in the embryo divided by the number of PGCs when the germ line completely separates from the soma. Numbers used are as follows: sea urchin, 32 somatic cells divided by 4 PGCs; fruit fly, 512 syncytial nuclei divided by about 24 PGCs; mouse, 700 epiblast cells divided by 8 PGCs; sea star, 2000 cells divided by 10 PGCs; and hydra, estimated from an animal containing several thousand cells and relatively few PGCs.

Appendix A: z426 Localization and Function in the Micromere Lineage

I performed all experiments in this study.

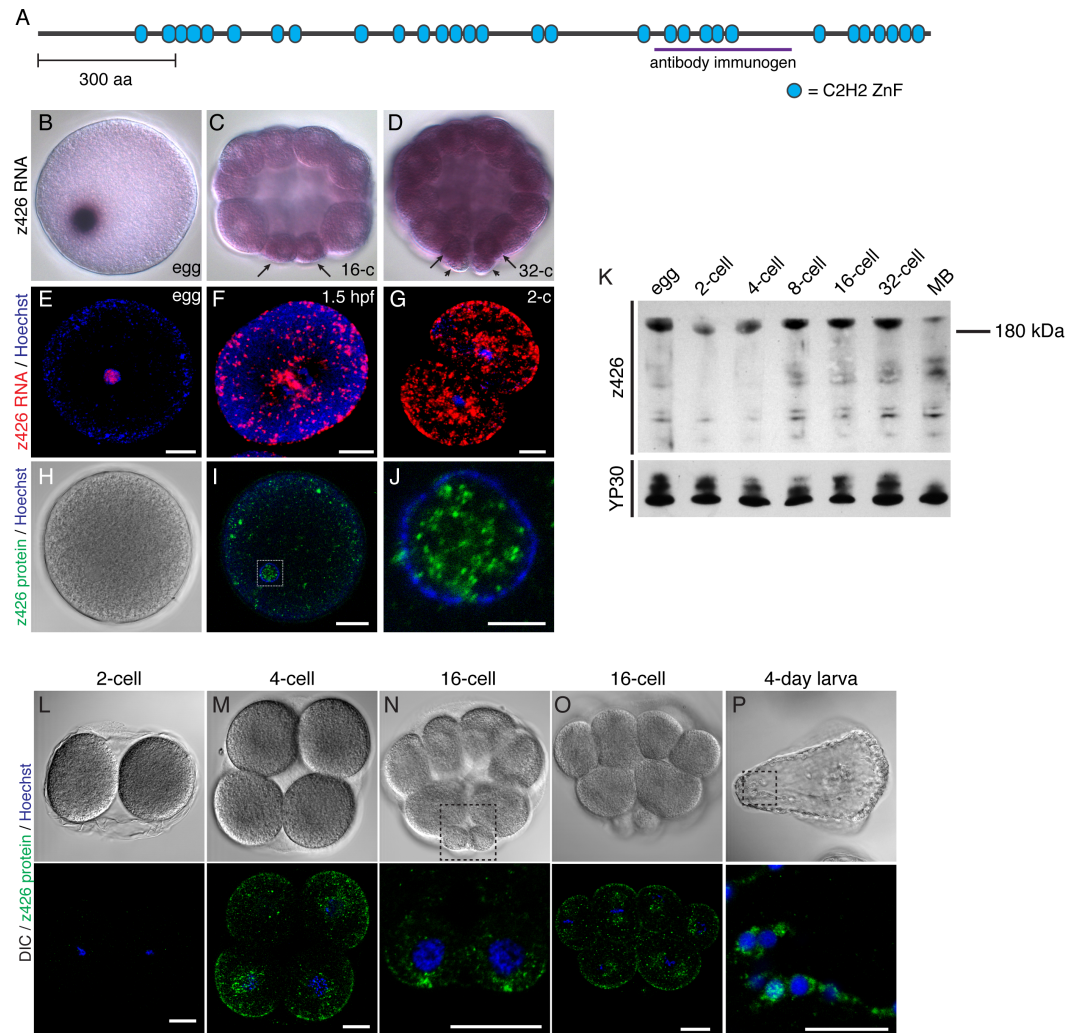


Figure 1. Expression of z426. z426 (echinobase.org gene ID: SPU_026390) was identified as a small micromere enriched mRNA in an early analysis of the small micromere transcriptome (**Chapter 2**). (A) Domain organization of z426. This 1,984 amino acid protein contains 31 putative zinc finger motifs for a total molecular weight of 225 kD. (B-D) Colorimetric in situ hybridization indicating the spatial localization of z426 mRNA in early embryos. (B) z426 mRNA is completely restricted to the pronucleus of the unfertilized egg. (C) In the 16-cell embryo, z426 mRNA is broadly distributed, but slightly enriched in the micromeres (arrows). (D) At the 32-cell stage, z426 remains ubiquitous, but enriched in the micromeres (arrows), but not in the small micromeres (arrowheads). (E-G) Fluorescent in situ hybridization for z426 mRNA. (E) z426 RNA (red) is restricted to the pronucleus in the egg, but following nuclear envelope (F) breakdown just prior to first cleavage, is released into the cytoplasm. (G) In the 2-cell embryo, z426 mRNA is enriched at the cell cortex and near the chromosomes. (H-J) z426 protein (green) is enriched in the egg pronucleus, shown in a magnified view in (J). (K) Immunoblot for z426 at multiple cleavage stages and mesenchyme blastula (MB). A strong band of about 200kb (consistent with the predicted size) is strongly present in the egg, at lower levels in 2 and 4-cell embryos, at higher levels in 8-32 cell embryos, and markedly reduced at MB stage. Yolk protein 30 (YP30) is included as a loading control. (L-P)

Immunofluorescence for z426 at multiple stages. (L) z426 protein is not detectable in the 2-cell embryo. (M) In the 4-cell embryo, z426 is present at the cell cortex and near the mitotic apparatus. (N) A zoom view of the micromeres at 16-cell stage is provided, showing enrichment of z426 at the vegetal spindle pole. (O) An equatorial slice of the mesomeres and macromeres of a 16-cell embryo with strong cortical and spindle-associated z426. Given the cytoplasmic localization of z426 and the presence of many zinc finger domains, I hypothesize that it serves as an RNA-binding scaffold. Scale bars = 20 μ .

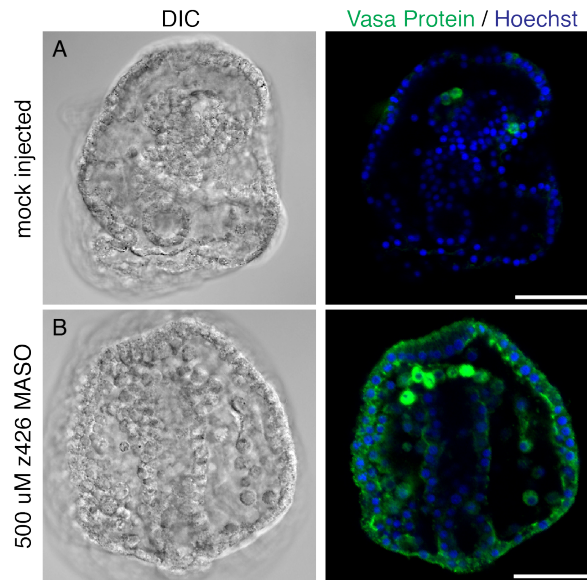


Figure 2. Vasa protein accumulation in z426 morphant 3-day larva. In the 3-day larva, Vasa protein is normally enriched in the sMics, in the bilateral coelomic pouches (mock injected control). With z426 knockdown, coelomic pouches do not form, and instead the sMics remain scattered at the tip of the archenteron. The gut does not acquire tripartite organization, and skeletogenesis does not occur. Furthermore, Vasa protein accumulates more strongly in the sMics themselves, and is expressed ectopically throughout the endoderm and ectoderm. Scale bars = 20 μ .

Appendix B: Baf250 Localization and Possible Role in Small Micromere Determination

I performed all experiments in this study.

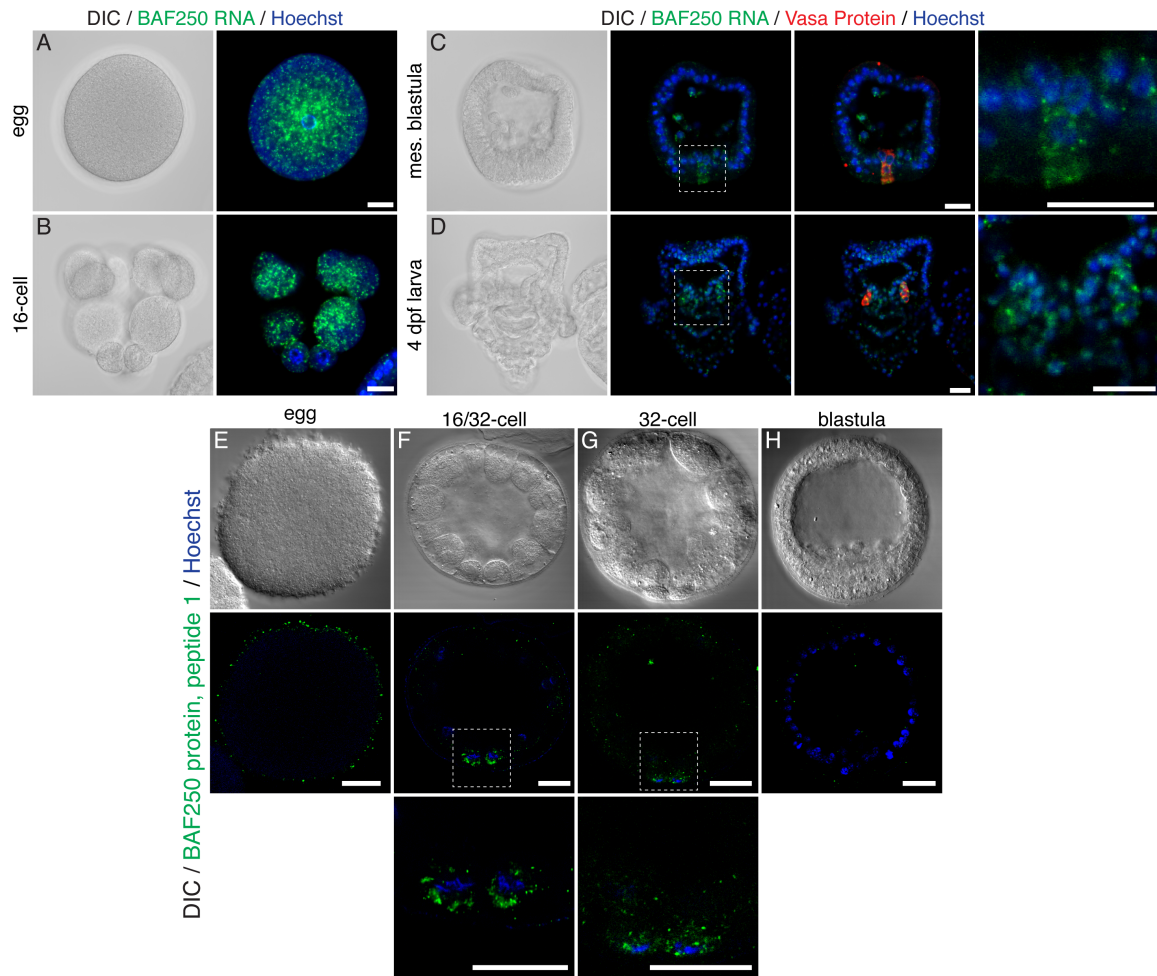


Figure 1. Localization of BAF250. Baf250 was identified in **Chapter 2** as a small micromere enriched transcript by deep sequencing. It assembles into the Swi/Snf related esBAF nucleosome remodeling complex, and is required for pluripotency maintenance in several contexts (Lessard and Crabtree, 2010). (A-D) Fluorescent in situ hybridization for Baf250 mRNA. (A) Baf250 mRNA (green) is maternally provided in the egg and broadly distributed in the 16-cell embryo (B). By mesenchyme blastula stage (C), it is enriched in the small micromeres, labeled with antibody to Vasa protein (red). Baf250 mRNA is present in the sMics of both coelomic pouches in the 4-day larva (D). (E-H) Immunofluorescence for Baf250 protein with a custom peptide antibody and methanol fixed embryos. (E) Baf250 is undetectable in the egg. (F) In the 16-cell embryo, Baf250 protein is enriched in the micromeres. (G) In the 32-cell embryo, Baf250 protein is enriched in the small micromeres. (H) Baf250 is undetectable by mesenchyme blastula stage. Scale bars = 20 μ .

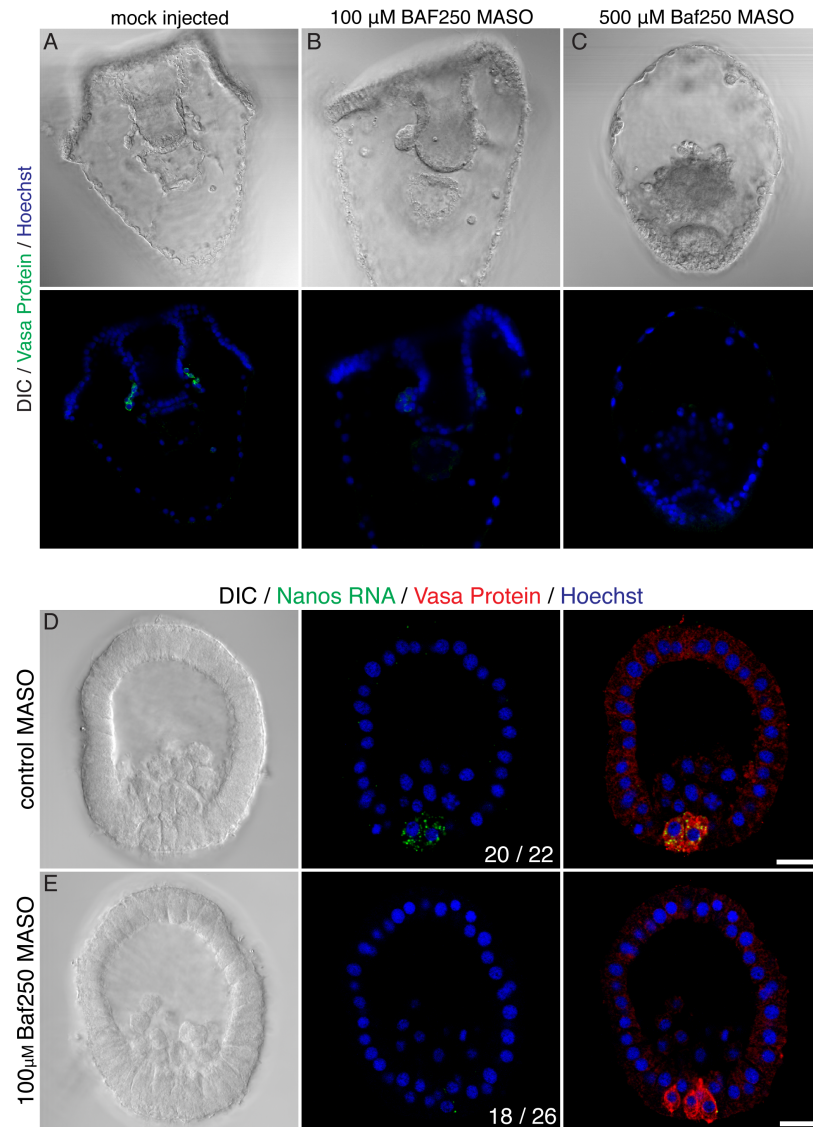


Figure 2. Possible role for Baf250 in small micromere development. (A-C) 4-day larvae with varying level of Baf250 knockdown. (A) Control larvae have strong Vasa staining in both coelomic pouches, which have a thread-like morphology. (B) With 100 μ M Baf250 morpholino, coelomic pouch morphology is altered and more compact. Vasa accumulation is strongly reduced. (C) With 500 μ M Baf250 morpholino, endomesoderm differentiation fails, and instead the blastocoel is filled with granular, mesenchyme-like cells. No Vasa protein is detected. (D,E) Nanos2 expression following Baf250 perturbation. (A) Nanos2 is strongly detected by fluorescent in situ hybridization (green) in the small micromeres, which are labeled in red with Vasa antibody. (B) With 100 μ M Baf250 antibody, Nanos2 expression is reduced or eliminated in about 70% of embryos. Scale bars = 20 μ .

Appendix C: MibL Localization and Possible Role in Preventing Small Micromere Differentiation

I performed all experiments in this study.

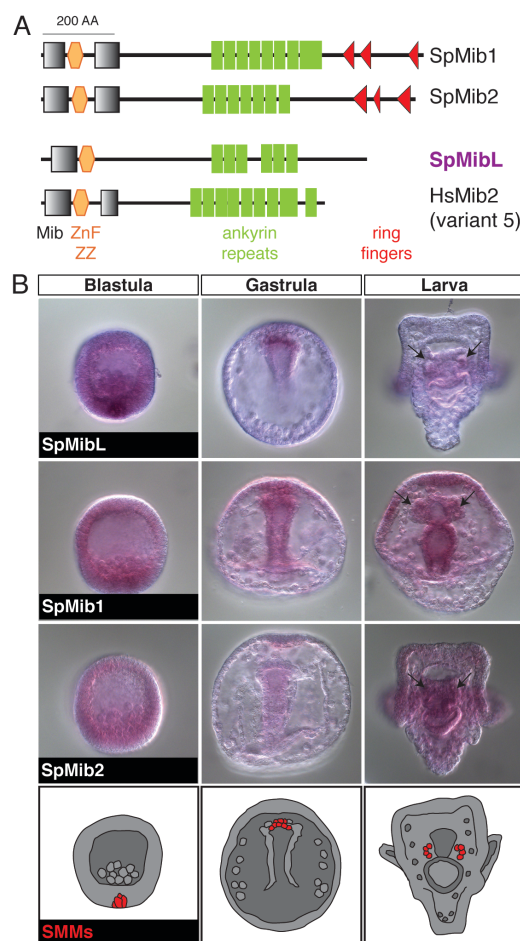


Figure 1. Domain organization and transcript localization of sea urchin Mind bomb family members. SpMibL was identified as a small micromere enriched transcript by deep sequencing in **Chapter 2**. Mind bomb genes are E3 ubiquitin ligases that modulate the Delta/Notch pathway by ubiquitinating the intracellular domain of Delta ligand, enhancing its endocytosis, which is required for robust signaling to the notch receptor (Le Borgne and Schweisguth, 2003). (A) Domain organization of sea urchin and human Mind bomb paralogs. SpMib1 and SpMib2 contain c-terminal ring finger domains, which are required for ubiquitin ligase activity. In contrast, SpMibL lacks ring fingers but contains the N-terminal Mib domain; thus, I predict that it can bind Delta but not recruit the ubiquitin ligase machinery, conferring a dominant negative function. A human Mib splice variant (HsMib2) also lacks the ring finger domain, suggesting this conformation may be conserved. (B) In situ hybridization for sea urchin Mib family members. SpMibL is enriched in the small micromeres by blastula stage, and remains enriched in the small micromeres in gastrula and coelomic pouches in larvae (arrows). By contrast, SpMib1 and SpMib2 are broadly distributed. I hypothesize that the truncated organization of SpMibL would prevent Delta/Notch signaling in the small micromeres, perhaps insulating them from differentiation into somatic mesodermal fates.

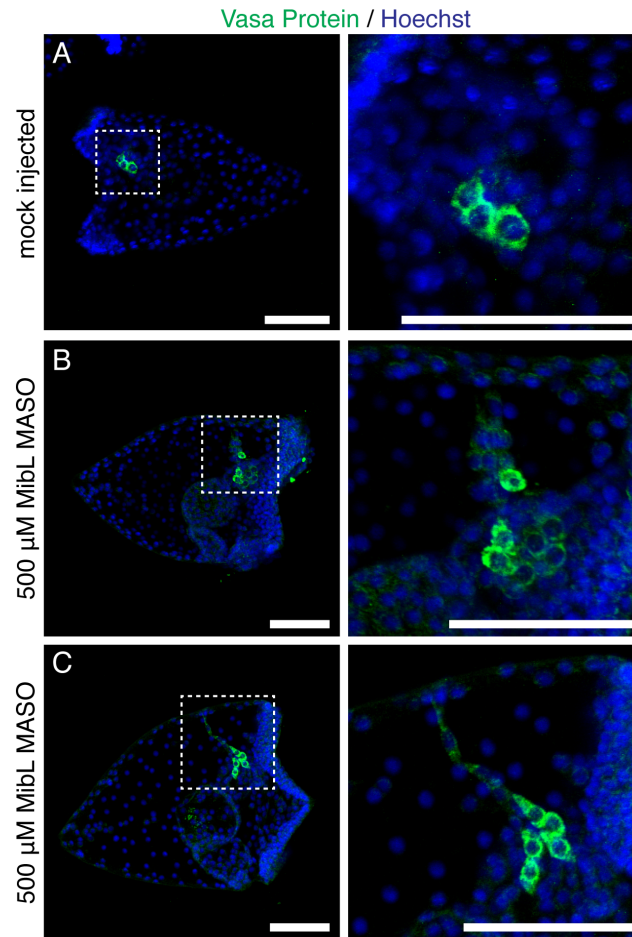


Figure 2. Possible role for MibL in preventing small micromere differentiation. (A) In 5-day old-mock injected larvae, the small micromeres (labeled with Vasa antibody, green) sit at the ventral side of the coelomic pouch. (B,C) With knockdown of MibL, Vasa positive cells were present in the hydropore canal, a somatic structure. This change in localization could indicate differentiation of the small micromeres into surrounding mesodermal structures; alternatively, ectopic Vasa protein may have accumulated outside of the small micromeres. Scale bars = 50 μ .

References

- Le Borgne, R., and Schweisguth, F. (2003). Notch signaling: endocytosis makes delta signal better. *Current Biology* 13, R273–R275.
- Lessard, J.A., and Crabtree, G.R. (2010). Chromatin regulatory mechanisms in pluripotency. *Annu. Rev. Cell Dev. Biol.* 26, 503–532.