

Nodal induces sequential restriction of germ cell factors during primordial germ cell specification

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Abbreviations:

Posterior Enterocoel (PE)

Primordial germ cells (PGCs)

Scanning electron microscopy (SEM)

Summary Statement

We identify an inhibitory signal, Nodal, required for the progressive restriction of germ cell factors in sea star embryos.

Abstract

Specification of the germ cell lineage is required for sexual reproduction in animals. The mechanism of germ cell specification varies among animals but roughly clusters into either inherited or inductive mechanisms. The inductive mechanism, the use of cell-cell interactions for germ cell specification, appears to be the ancestral mechanism in animal phylogeny, yet the pathways responsible for this process are only recently surfacing. Here we show that germ cell factors in the sea star initially are present broadly and then become restricted dorsally and then in the left side of the embryo where the germ cells form a posterior enterocoel. We find that Nodal signaling is required for the restriction of two germ cell factors, Nanos and Vasa, during the early development of this animal. We learned that Nodal inhibits germ cell factor accumulation in three ways including: inhibition of specific transcription, degradation of specific mRNAs, and inhibition of tissue morphogenesis. These results document a signaling mechanism required for the sequential restriction of germ cell factors, which causes a specific set of embryonic cells to become the primordial germ cells.

Introduction

The germ line is the only immortal cell lineage in most animals. This lineage participates in reproduction and directly contributes to the next generation while all remaining cells, somatic cells, are destined to die. As such, the proper development of the germ cell lineage, cells with the developmental potential to become an egg or sperm, is essential for the survival of sexually reproducing animals. An early step required for germ cell development is specification, when a subset of cells acquire a unique set of instructional molecules that cause them to become germ cells. At the same time, all other cells of the embryo become somatic cells.

Two distinct mechanisms for germ cell specification have been described in animals. An inherited (Fig. 1A) mechanism relies on asymmetrically localized materials in the egg and/or early embryo that directs the embryonic cells that acquire this material to take on a germ cell fate. This mechanism appears to predominate in animals such as frogs, nematode worms, flies, and teleost fish (Illmensee and Mahowald, 1974; Kawasaki et al., 1998; Kuznicki et al., 2000; Mello et al., 1992; Smith, 1966; Yoon et al., 1997). An induced (Fig. 1A) mechanism instead relies on cell – cell signaling events which instructs a subset of embryonic cells to take on a germ cell fate. Mammals, axolotls, and crickets appear to rely primarily on inductive mechanisms for specification of their germ cell lineage (Chatfield et al., 2014; Ewen-Campen et al., 2013; Tam and Zhou, 1996).

Although the inductive mechanism of germ cell specification appears to be the ancestral mechanism in animals and is widespread throughout the animal kingdom (Extavour and Akam, 2003), the mechanisms that directly induce a germ cell fate are only beginning to be discovered and from a limited repertoire of animals: mouse, cricket, and axolotl. In order to discover the breadth of this mechanism of germ cell specification and to determine which aspects are conserved between species it is necessary to determine how germ cells are induced in diverse animals.

The sea star may be a useful model to examine inductive germ cell specification for several reasons. The sea star: 1) Holds a unique position in animal phylogeny as an invertebrate deuterostome. This means comparisons can be made more effectively with chordates to understand germ cell specification in the deuterostome ancestor. 2) Preliminary data shows inductive mechanisms contribute to germ cell specification in the sea star (C. et al., 1992; Fresques et al., 2014; Wessel et al., 2014b). 3) Germ cell factors are present widely in early development and provide a useful assay to determine how signaling molecules contribute to their restriction with excellent in vivo imaging capabilities.

Here we show that Nodal signaling is required for the sequential restriction of Nanos and Vasa mRNAs in early development. Although the function of Nanos and Vasa remains to be tested in the germ line of sea stars we strongly suggest that they are required for germ cell specification because 1) these factors are usually found together in the germ cell lineage (Juliano et al., 2010) 2) these factors are required for germ cell specification in many animals (Juliano et al., 2010) and 3) these factors accumulate in the posterior enterocoel (PE), a structure that has previously been shown to contribute to primordial germ cells (Inoue et al., 1992). Although we are not able to test Vasa function specifically in the germ line by conventional means (knockdown of Vasa expression in early embryos leads to aborted development, as it does in the sea urchin; data not shown), we propose that the sequential restriction of germ cell factors is a significant mechanism involved in germ cell specification. That is, germ cell factors are present broadly in cells during early development and embryonic signals reduce the field of cells to the future germ line.

Results

Germ cell factors are sequentially restricted during early development

We noticed in previous studies in *P. miniata* that the mRNA of the germ cell factors Vasa, Nanos, and Piwi are present broadly in early development but then become restricted to the posterior enterocoel (PE) (Fresques et al., 2016; Fresques et

al., 2014). The restriction of Vasa and Nanos mRNA in particular shows a similar restriction pattern during two stages of embryonic development. That is, Vasa and Nanos accumulate in a vegetal ring at the mid-gastrula stage, and subsequently, by the late-gastrula stage, these two factors are eliminated from cells in the ventral part of the developing gut (Fig. 1C, i-vi). Then, in the transition from late-gastrula to early-larva these same germ cell factors are eliminated from cells in the right side of the developing gut, and the cells with the remaining mRNA on the left-side form the posterior enterocoel (Fig. 1C, ix-xiv). In order to test if germ factor mRNAs are decreasing or just shifting during this dynamic period, we performed qPCR. Our results show that during the dorsal and left phases of restriction Vasa and Nanos mRNA levels decrease significantly (Fig. 1C, xvii-xviii). This suggests that Vasa and Nanos mRNA is lost from cells in the ventral and right part of the developing gut. As a result, Vasa and Nanos mRNA is specifically retained in cells in the dorsal and left side of the gut.

Nodal is required for the restriction of germ cell factors

We next sought to determine what embryonic signal(s) could be involved in the dorsal and left restriction of Vasa and Nanos. Previous research in a closely related animal, the sea urchin, shows that Nodal is required for the patterning of the Dorsal/Ventral and Left/Right axes (Duboc et al., 2004; Duboc et al., 2005). In order to test if Nodal is relevant for restriction of germline factor mRNAs in the sea star we first determined where Nodal mRNA was localized during sea star development (Fresques et al., 2014). We found that Nodal is expressed in the domain opposite of germ cell factors: in the ventral side of the embryo during the blastula stage and then in the right side of the embryo during the late gastrula stage (Figs 1C, vii and xv; S1). These data suggest that Nodal expression is counter to the retention of germ cell factor mRNA's (Fig. 1C; i, ii, ix, and x dotted oval).

In order to test if Nodal is required for the dorsal and left restriction of Vasa and Nanos we perturbed Nodal signaling in two ways. First, we inhibited Nodal signaling with the pharmacological inhibitor SB-431542, previously shown to inhibit the Nodal receptor in the sea urchin (Duboc et al., 2005). Our results show that when Nodal signaling is downregulated, Vasa mRNA is poorly restricted compared to embryos

treated with the inhibitor vehicle (DMSO) as a control (Fig. S2, A-D). Vasa mRNA did not restrict in the dorsal side of the majority of embryos treated earlier in development. In embryos treated later in development Vasa mRNA did not restrict in the dorsal side of 57% of embryos and in the left side in 43% of embryos. Second, we inhibited Nodal signaling by use of a translation blocking morpholino. As embryos were injected with increasing amounts of morpholino we saw a dose dependent phenotype similar to the pharmacological inhibitor phenotype (Fig. S2, E-G). With an intermediate amount of Nodal morpholino the majority of embryos did not restrict Vasa mRNA in the left side of the embryo and with a higher concentration, the majority of embryos did not restrict Vasa mRNA in the dorsal side of the embryo. The results from the two distinct approaches, drug inhibition and morpholino treatment, are markedly similar in their phenotypic effect on the embryo. The relatively minor differences detected in these treatments may be the result of differences in timing, efficacy of inhibition, and specificity of each. For example, only 20-30% of control embryos in pharmacologically treated embryos have Vasa restricted in the left side of the embryo while it is restricted in 100% of control embryos (Fig S2, B-G). These differences are commonly seen and are likely based on differences in the females selected for these experiments, times of the reproductive season in which they are used, and natural variations in culture conditions. These are, after all, animals collected from the wild but the experiments are balanced by having large sets of control embryos to directly compare with the experimental set. Overall though, these results suggest that Nodal is required for the dorsal and left restriction of the germ cell factor Vasa.

Nodal's effect on the dorsal restriction of Vasa could also be due to a delay in development. We tested this premise by injecting a concentrated amount of Nodal morpholino and fixing embryos at an earlier developmental time point (when the controls were morphologically indistinguishable from the Nodal morphants). We found that two germ cell factors, Nanos and Vasa, failed to restrict to the dorsal half of the embryos in 97% and 94% of Nodal morphants, respectively, while they were properly restricted to the dorsal half in 98% and 95% of control morpholino injected embryos, respectively (Fig. 2C,D). These data strongly suggest that Nodal is required for the

sequential restriction of Nanos and Vasa and that Nodal knock-down is not simply causing a delay in embryonic development.

To test if embryos injected with Nodal morpholino had decreased Nodal signaling we performed several control experiments. First, we saw that a known transcriptional target of Nodal signaling in animals, Lefty, is downregulated in Nodal morphants (Fig. 3B,C) (Shiratori and Hamada, 2014). Second, we saw that sea star embryos become radialized when we inject a Nodal morpholino. In the sea urchin, Nodal signaling is required for establishment of the Dorsal/Ventral axis. As a result, when Nodal signaling is decreased early in embryogenesis the resulting embryos became radialized (Duboc et al., 2004). We found in sea star embryos that Tbx2/3 and Nodal mRNA became radialized in the majority of Nodal morphants (Fig. 2E,F). Together these control experiments suggest that sea star embryos injected with Nodal morpholino have decreased levels of Nodal signaling. We were however unable to specifically test Nodal protein or smad activity for the technical reasons of antibody incompatibility and non-specific binding.

To further test if Nodal signaling is required for the dorsal and left restriction of Nanos and Vasa RNA we experimentally increased Nodal signaling in sea star embryos in two ways. Previous research in sea urchins demonstrates that addition of the human Activin protein to sea water activates the Nodal receptor (Duboc et al., 2005; Luo and Su, 2012). When we added this protein to sea star embryos we saw Vasa expression was either significantly decreased or completely lost in the majority of embryos (Fig. S3A). The second experiment we used to increase Nodal signaling was NiCl₂. Although the mechanism is unknown, when NiCl₂ is added to sea urchin embryos they show a ventralization phenotype similar to Nodal overexpression (Duboc et al., 2004). When we added NiCl₂ to sea star embryos we saw a similar decrease in Vasa expression in the majority of embryos (Fig. S3C). In both experiments we tested if Nodal signaling was increased by analyzing the localization of brachyury, a gene product normally found in the ventral ectoderm (Shoguchi et al., 1999). We found that brachyury mRNA accumulation was radialized in the majority of Activin and NiCl₂ treated embryos (Fig. S3 B,D), demonstrating that the phenotypes we observe are consistent with overexpression of Nodal signaling. In summary, when we perturb Nodal signaling in four

distinct ways our results support the conclusion that Nodal is required for the dorsal and left restriction of Nanos and Vasa RNA.

Tangentially, our work suggests Nodal is involved in general patterning of the Dorsal/Ventral and Left/Right axes of the sea star as it does in sea urchins. For example, Nodal inhibition causes the embryo to radialize and take on a dorsal phenotype: a mouth does not form and the gut does not bend towards the ectoderm (Figs. 2 and S2). Conversely, Nodal overexpression causes the embryo to radialize and take on a ventral phenotype: a mouth attempts to form radially in the ectoderm (Fig. S3). Nodal inhibition later in development also causes radialization of the left side phenotype and the posterior enterocoel forms radially (Fig. S2 and 4). In summary, Nodal signaling is required for patterning the Dorsal/Ventral and Left/Right axes in both sea urchins and sea stars. Our data suggests significant differences, though. For example, Nodal and Tbx2/3 mRNA accumulation is radialized in sea star Nodal morphants whereas they are lost in sea urchin Nodal morphants (Duboc et al., 2004). It is important in future experiments to determine Nodal's role in Dorsal/Ventral and Left/Right patterning in the sea star to test if Nodal's restriction of the germ cell fate is a part of general Dorsal/Ventral and Left/Right patterning.

Transcriptional inhibition and post-transcriptional degradation mechanisms contribute to Nodal-dependent restriction of germ line mRNAs

Nanos and Vasa mRNAs significantly decreased in abundance during the developmental time of Nodal-dependent spatial restriction to the dorsal and left side of the embryo (Fig. 1C). To determine if this decrease is caused by decreased transcription and/or by increased turnover of the mRNAs, we used a nascent RNA capture system. We incubated control embryos and Nodal morphant embryos with the nucleotide analog EU at the mid gastrula stage. Following isolation of EU incorporated RNA, we found that Nodal morphant embryos had significantly higher levels of nascently transcribed Nanos and Vasa RNA compared to control embryos via qPCR (Fig. 3C). The results suggest that Nodal signaling decreases both Nanos and Vasa transcription. In addition, we found that “old” EU-minus Nanos and Vasa mRNA

(produced before the EU pulse) was also significantly increased in Nodal morphant embryos compared to control embryos (Fig. 3B). This suggests that Nodal is also required for turnover of Nanos and Vasa mRNA produced prior to the mid-gastrula stage. As a control, we measured the levels of Lefty RNA (a known target of Nodal signaling). As expected, we saw decreased levels of Lefty RNA in Nodal morphants compared to control embryos, reflective of decreased Nodal function (Fig. 3 B,C). Together, these results imply that areas not influenced by Nodal signaling retained germ cell factor mRNAs, whereas cells under the influence of Nodal reduced both transcription and retention of germ cell factor mRNAs. The overall result causes the selective retention of a germ cell fate in time and space.

Nanos and Vasa-positive cells are transcriptionally active during germ cell specification

In some animals, precursor germ cells have low transcriptional activity (Seydoux and Dunn, 1997; Swartz et al., 2014). The sea urchin germ cell precursors, for example, have marked quiescence that includes nearly undetectable transcription in the cell while their neighboring somatic cells were highly active in transcription, translation, and mitochondrial activity (Oulhen et al., 2017). Here we tested if the Vasa-positive cells that turnover the Vasa mRNA and become somatic cells had reduced transcriptional activity that might help explain the rapid turnover of their germ cell mRNAs. We used an RNA polymerase II phosphoserine II antibody to detect the general transcriptional activity of cells and we found that activated RNA pol II was present at similar levels in the somatic cells and in the future germ cells during this the last sequential restriction phase in the sea star (Fig. S4 C). This result indicates suggests that the decrease in germ cell RNAs is not based on a general decrease in transcriptional activity in Vasa-positive, future somatic cells. More significantly this experiment shows that germ cell precursor cells are transcriptionally active. In support of this, we also saw that the quiescence marker, H3K9me3, is not present in germ cell precursors in the sea star (Fig S4 A). This is very different from the quiescence seen in sea urchin germ cell precursors and supports the contention that sea stars depend on inductive events for germ cell specification. do not display the quiescence seen in sea urchin germ cells precursors, and supports the

contention that sea stars depend on inductive events for germ cell specification by selective germ cell factor turnover to restrict cells to a somatic fate by inductive events.

Apoptosis does not contribute to the restriction of Nanos and Vasa-positive cells

Another mechanism that could contribute to the decrease in Nanos and Vasa positive cells is programmed cell death. Cells impacted by Nodal signaling may apoptose resulting in a sculpting of the field for germ cell. To test this hypothesis we used a DeadEnd Fluorometric TUNEL System to determine if any cells in the ventral or right side of the developing gut are undergoing apoptosis. Our results show that very few cells in the germ cell field, or in other domains of the embryo, undergo apoptosis during development in the sea star (Fig. S5 A,B). In addition, the localization of cells undergoing apoptosis is stochastic and does not correlate with Nanos and Vasa positive cells. These results show that programmed cell death does not contribute significantly to the loss of Nanos and Vasa mRNA positive cells in the ventral or right side of the developing gut.

Nodal inhibits tissue morphogenetic events involved in germ line segregation

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

To determine how Nodal affects the morphogenetic events involved in germ cell pouch formation we analyzed pouch formation in Nodal morphant embryos. By performing SEM at 2 different developmental time points, we found 1) ectopic bulging in

the ventral and right side of the developing gut in early larvae (Fig. 4Bi,ii) and 2) ectopic pouch formation in the developing gut in later larvae (Fig. 4Biii). These results suggest that Nodal signaling in the ventral and right sides of the embryo inhibits the cell shape changes that lead to PE formation.

To determine what cell shape changes contribute to posterior enterocoel formation we fluorescently labelled cell membranes by injecting a mRNA encoding the PH-domain of PLC-gamma fused to GFP (gifted from Brad Shuster) into sea star zygotes. In embryos with a control morpholino, we saw that the endodermal cell volume is similar to the posterior enterocoel cell volume; no significant change in cell volume was detected as the posterior enterocoel forms (Fig. 5 A,C). However, we did see a significant increase in the Basal:Apical cell length ratio in posterior enterocoel cells compared to endodermal cells (Fig. 5 A,D). This suggests that apical constriction of posterior enterocoel precursor cells contributes to the PE outpocketing. In order to test if Nodal inhibits this apical constriction, we inhibited Nodal signaling and measured the Basal:Apical cell length ratios in normal and ectopic PEs. Nodal morphants formed at least 2 PE's that had similar Basal:Apical cell length ratios as the single PE in control embryos (Fig. 5 B,D). Together, our results suggest that Nodal signaling is required to inhibit apical constriction in the ventral and right side of the narrow band of tissue from which the germ cell field originates in the mid-gut. This combination of nodal as a repressor of the PE and another positive, but currently unknown signal that directs localized PE formation, results in the asymmetric PE selectively on the dorsal-left side of the endoderm.

Discussion

A new mechanism revealed for inductive germ cell specification

Germ cells are generally thought to be specified by either inherited or inductive mechanisms. The inherited mechanism generally describes what is known about germ cell specification in flies and worms: whichever embryonic cells inherit maternal germ cell factors will become the germ cell lineage. The inductive mechanism generally describes what has been discovered in mice, axolotl, and crickets: whichever multipotent cells receive an inductive signal will become the germ cell lineage (Extavour

and Akam, 2003). However, these classifications are based upon a limited number of animals studied. Growing evidence suggests that these classifications are too simplistic (Bertocchini and Chuva de Sousa Lopes, 2016) because they 1) cannot be generalized to other animals and 2) limit our understanding of how germ cells are specified in animals. For example, it is still unknown how germ cell promoting signals specifically induce a germ cell lineage in animals that use the inductive mechanism for germ cell specification (Gunesdogan et al., 2014). To fully understand how germ cells are specified in diverse animals it is important to characterize germ cell specification mechanisms in more and diverse animal species.

We present evidence that suggests the sea star embryo specifies its germ line by inductive events and that this mechanism requires Nodal signaling for the sequential restriction of cells that retain germ cell RNA's. We propose that this sequential restriction may be involved in inductive germ cell specification in other animals. That is, germ cell promoting factors may be initially present in broad embryonic regions and subsequent rounds of signals that inhibit these factors and narrow the field of cells in which a germ cell fate is retained. This inductive mechanism can subsequently restrict the number of cells that retain a multipotent early embryonic cell fate (as germ cell promoting factors are also often required for multipotency (Juliano and Wessel, 2010; Juliano et al., 2010)). However, most importantly, these restrictive signals can define which cells ultimately retain these factors and become primordial germ cells. We do not suggest that positive signals that promote a germ cell fate are not present during sea star germ cell specification. In fact, preliminary evidence suggests that BMP and Wnt signals may be involved in promoting a germ cell fate (unpublished results). Therefore, our results suggest that within the inductive mode of specification both negative and positive signals that promote and inhibit the germ cell fate are involved in germ cell specification.

When we apply this knowledge to other animals that use inductive germ cell specification we can predict how germ cells are specified uniquely. For example, it is known that Wnt3 and BMP4 are required to positively promote the germ cell fate during germ cell specification in mice (Aramaki et al., 2013; Lawson et al., 1999). However, it is also known that other cells in the epiblast retains the potential to become a germ cell

and will become a germ cell if they are exposed to the same signals. Our results suggest that it is likely that future somatic cells of the pluripotent epiblast receive restrictive signals that may inhibit their potential to become the germ cell lineage. Indeed previous researchers have found that restrictive signals present in the anterior of the embryo promote the secretion of negative regulators of the BMP4 and Wnt3 pathway, the domain opposite of where germ cell precursors are found (Lewis et al., 2008; Ohinata et al., 2009; Perea-Gomez et al., 2002).

Similarly, restrictive signals that inhibit a germ cell fate may also be involved in germ cell specification in the cricket. It is known that BMP's are required for germ cell specification in the cricket, however BMP's are present in large embryonic territories during germ cell specification (Donoughe et al., 2014). We predict that signals that inhibit the germ cell fate may be present in future somatic cells shifting their potential from becoming a germ cell. We also propose that restrictive signals are also present in inductive germ cell specification of the axolotl. Previously researchers proposed a "last cell standing" model for germ cell specification in this animal. In short, the germ cell lineage is the last cell lineage that retains multipotency while all other cell lineages differentiate into somatic fates (Johnson and Alberio, 2015). Restrictive signals here may shift cells from retaining the potential to become the germ cell lineage. In summary, we suggest that future studies in diverse animals consider not just the positive, but also the negative signals that influence the germ cell lineage.

Signals that contribute to germ cell specification in sea stars

Nodal signaling is required in the sea urchin embryo for Dorsal/Ventral and Left/Right patterning (Duboc et al., 2004; Duboc et al., 2005; Luo and Su, 2012). During these patterning events Nodal and BMP signals oppose each other which effectively set up a gradient of these signals across the Dorsal/Ventral and Left/Right axes. We hypothesize that a similar system is present in the sea star to pattern the same axes. In support of this hypothesis previous researchers have found that BMP signaling is active in the dorsal ectoderm of the sea star embryo (Yankura et al., 2013). An important question to investigate in the future is how the Nodal signal restricts the germ cell fate. Does Nodal signaling in the ventral and right sides of the embryo directly inhibit the

germ cell fate? Or does BMP promote the germ cell fate in the dorsal and left sides of the embryo and Nodal simply restricts BMP to those sides of the embryo?

We predict 1) germ cell RNA restriction and 2) germ cell morphogenesis respond to Nodal signaling differentially. 1) We predict Nodal acts directly on germ cell RNA restriction in parallel to Dorsal/Ventral patterning mechanisms. Nanos and Vasa mRNA are initially expressed in a radial domain. Assuming that the radial expression of Nanos and Vasa occurs prior to the establishment of the Dorsal/Ventral axis we hypothesize that once the Dorsal/Ventral axis is established and Nodal signaling becomes active in the ventral ectoderm it directly inhibits germ cell RNA retention in that part of the embryo. in those cells of the in the ventral region of the embryo. Therefore, we predict Nodal-based restriction of germ cell factor transcription and Nodal-based promotion of degradation of these RNAs to be direct and to act in parallel to Dorsal/Ventral patterning. 2) Subsequently, we predict Nodal-mediated Dorsal/Ventral and Left/Right patterning mechanisms directly influence PE morphogenesis and these do not act in parallel pathways. PE morphogenesis only occurs on the dorsal-left side of the embryo. We only see radial PE's when the Dorsal/Ventral and Left/Right axes are perturbed. Therefore, we hypothesize that the morphogenesis of the PE depends on positional/patterning information. We predict Nodal restriction of PE formation is dependent on Nodal- mediated Dorsal/Ventral and Left/Right patterning mechanisms. To address these questions in the future it will be important to determine how Nodal and BMP signals: 1) pattern the Dorsal/Ventral and Left/Right axes; 2) restrict or promote the germ cell fate; 3) act directly or indirectly on the future germ line; and 4) how they may influence the signaling of each other.

Evolution of an inherited germ cell specification mechanism in echinoids

Previous research suggests that the inductive mechanism is likely the ancestral germ cell specification mechanism and that the inherited mechanism likely evolved many times during animal evolution (Extavour and Akam, 2003). Within echinoderms we see vastly different germ cell specification mechanisms as well. A study of Nanos localization in echinoderms suggests that the inductive mechanism is likely ancestral while the inherited mechanism has evolved uniquely in the echinoid lineage (Fresques

et al., 2016). We use the sea star here as a model of the ancestral inductive germ cell specification mechanism that may represent the mechanisms of the other echinoderm taxa and be capable of exploring the embryonic transcription and cell-cell signals that contribute to germ cell specification. In contrast, the sea urchin is representative of the echinoid group in which an inherited germ cell specification apparently has evolved. Germ cell specification in the sea urchin occurs early in development, by the 32-cell stage and is dependent on the retention of maternal RNA's in the germ cell lineage (Swartz et al., 2014; Wessel et al., 2014a). By comparing germ cell specification mechanisms in the sea star and sea urchin we can begin to understand the evolutionary changes that might have happened during the evolution of the germ cell lineage in the echinoid lineage. Two main changes correlate with the evolution of an inherited germ cell lineage in the echinoid lineage. First, the left posterior enterocoel structure, or its equivalent, was lost in the echinoid lineage and germ cells instead accumulate in the left anterior coelomic pouch. Second, an evolution of early unequal cell divisions occurred in echinoids that instead produced primordial germ cells. In the future, it will be important to understand how signals that promote and restrict the germ cell fate changed during the evolution of the inherited germ cell specification mechanism in echinoids. The answer to this question will ultimately allow us to determine what evolutionary factors affect the specification of the most potent cell lineage in animals- the germ cell lineage.

Materials and methods

Embryo culture-

Adult *Patiria miniata* animals were collected by either Pete Halmay (PeterHalmay@gmail.com) or Josh Ross (info@sbiomarine.com) off the California coast. Embryos were cultured essentially as described before (Fresques et al., 2016).

In situ hybridization-

DIG-labelled RNA probes were made with a Roche DIG probe synthesis kit as described before (Fresques et al., 2016). Probe hybridized embryos were developed

either with NBT + BCIP for purple staining or with FastRed (Sigma F4648) for red staining. A list of primers used for probe synthesis can be found in Table S1. Embryos were incubated with probe for 1 week and were developed essentially as described before (Fresques et al., 2016). For statistical analysis of the localization of in situ probes in control vs. morphant embryos, experiments were performed either in duplicate or triplicate and a one-tailed t-test was used to analyze significance.

Pharmacological inhibition-

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

Morpholino injection-

Oocytes were injected with either 0.1 mM, 0.5 mM or 1.0 mM of morpholino (stock concentration, Gene-Tools) in injection solution: 10% glycerol and 1 mM Texas Red Dextran or 0.83 mM FITC Dextran (Molecular Probes). After incubation for 1 day at 16° Celsius, injected fluorescent oocytes were selected and matured with 1-methyl adenine (3.0 μ M Acros Organics). Eggs were fertilized with diluted sperm, washed, and incubated in filtered sea water at 16° Celsius until they developed into the desired embryonic stage. The Nodal morpholino sequence used is:

5' TCAAGTTCTTGGTCATTCTCGAAAC 3'.

qPCR analysis-

RNA was extracted from ~150 embryos in each desired stage by using the RNeasy Micro kit (Qiagen). A reverse transcription reaction was performed using M-MLV Reverse Transcriptase (Promega) or M-MuLV Reverse Transcriptase (Thermoscientific, Maxima first strand cDNA synthesis kit). Sybr green was used for qPCR analysis (Thermofisher). Experiments were done either in duplicate or in triplicate and ubiquitin or 18s was used to normalize RNA levels between samples. All data are represented as fold-change relative to the standards. A one-tailed t-test was used to calculate significance. Error bars reveal +/- 1 standard deviation. A list of primers used can be found in Table S2.

Nascent RNA Collection-

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

Scanning Electron Microscopy-

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

Tunel Assay-

Double stranded breaks were labelled with fluorescein via a DeadEnd Fluorometric TUNEL System (Promega). Briefly, embryos were grown to the desired stage, fixed in 4% PFA in PBS, and permeabilized with 0.2% TritonX-100. Then Terminal Deoxynucleotidyl Transferase was added to the embryos in combination with

fluorescein-12-dUTP. After 3 washes in PBS, embryos were imaged on a Zeiss LSM 800 confocal microscope.

PH-domain of PLC Gamma-GFP mRNA Injections-

Embryos were co-injected with either Control morpholino or Nodal morpholino and RNA at a concentration of 1 $\mu\text{g}/\text{ul}$. Z-stacks of embryos were obtained using a Zeiss LSM 800 confocal microscope. Volume was calculated using the polygon selection tool in Fiji. The volume of 18 individual cells were calculated from a total of 5 embryos. Apical and Basal cell lengths were calculated using the segmented line tool in Fiji. For the control, the lengths of 15 cells were counted in a total of 5 embryos. For the Nodal morphants, the lengths of 32 cells were counted in a total of 6 embryos. A 1 tailed t-test was used to calculate significance. Error bars reveal \pm 1 standard deviation.

Activin treatment-

Human Activin AB protein was ordered from Bio-technie and resuspended in 0.1% BSA in PBS. Embryos were treated by adding Activin to the sea water 30-45 minutes post-fertilization. Embryos were treated with either 120 ng/ml or 240 ng/ml of Activin or 0.1% BSA as a control.

Nickel Chloride treatment-

NiCl_2 was dissolved in filtered sea water to make a stock concentration of 10 mM. Embryos were treated with a range of final concentrations of NiCl_2 (0.25 mM, 0.5 mM, and 1.0 mM). Untreated embryos were used as controls. Embryos were treated 30 minutes post-fertilization until the early larva stage when they were fixed.

Immunofluorescence-

Embryos were fixed overnight in MOPS buffered PFA. H3k9me3 was labelled by incubating embryos with rabbit anti-H3k9me3 primary antibody (Abcam 8898, 1:250). RNA Pol2 P-Ser2 was labelled by incubating embryos with rabbit anti-RNAPol2PSer2 primary antibody (Abcam 5095, 1:250). Embryos were then incubated with anti-rabbit Alexa 488 secondary antibody (Fisher, 1:500). DNA was labelled by incubating embryos

with Hoescht (1:1000) for at least 15 minutes. Embryos were visualized with a Zeiss 510 confocal microscope.

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Competing interests

No competing interests declared.

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Figures

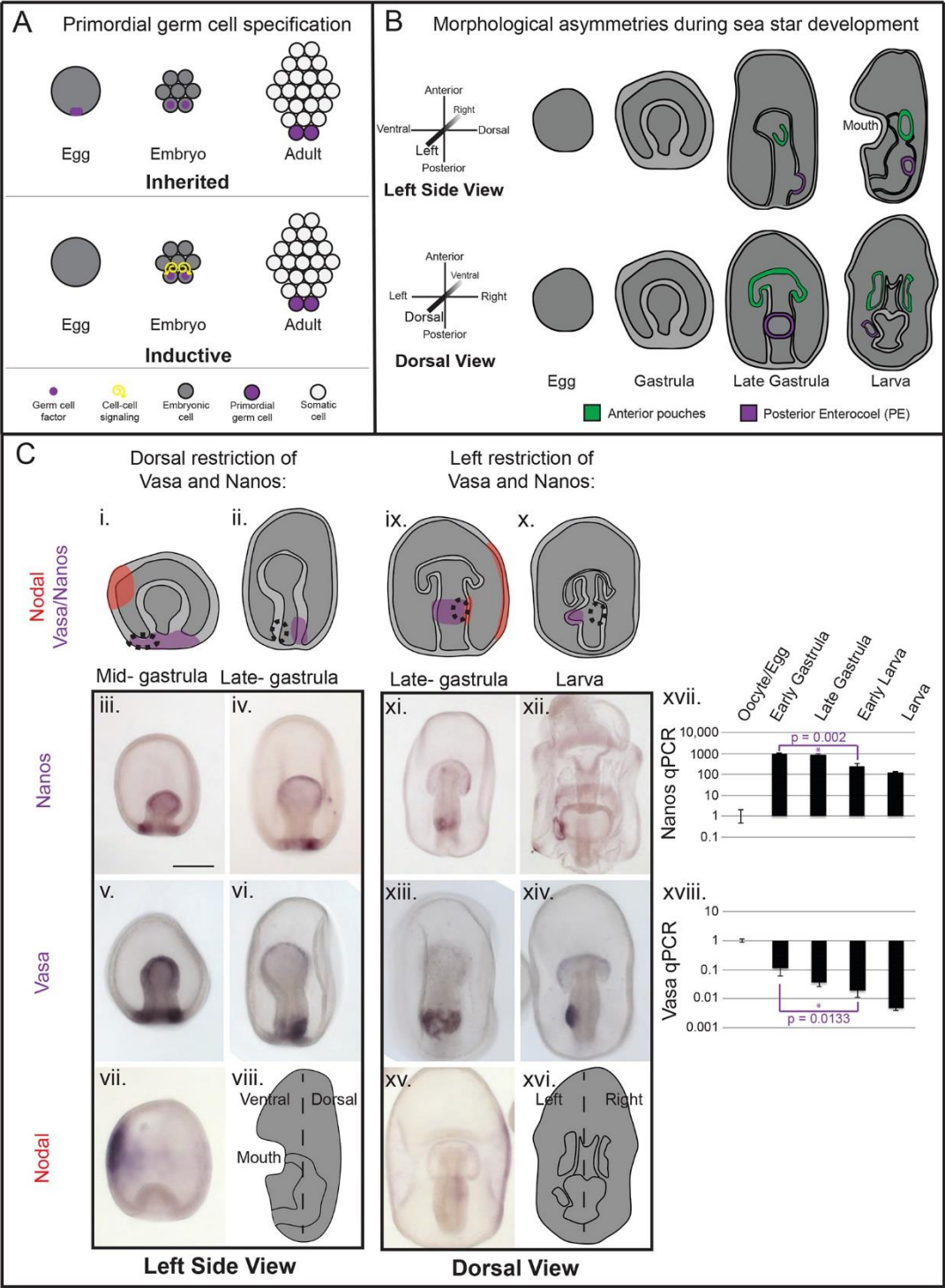


Figure 1: Germ cell factors are restricted dorsally and on the left during embryogenesis

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

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

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

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

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

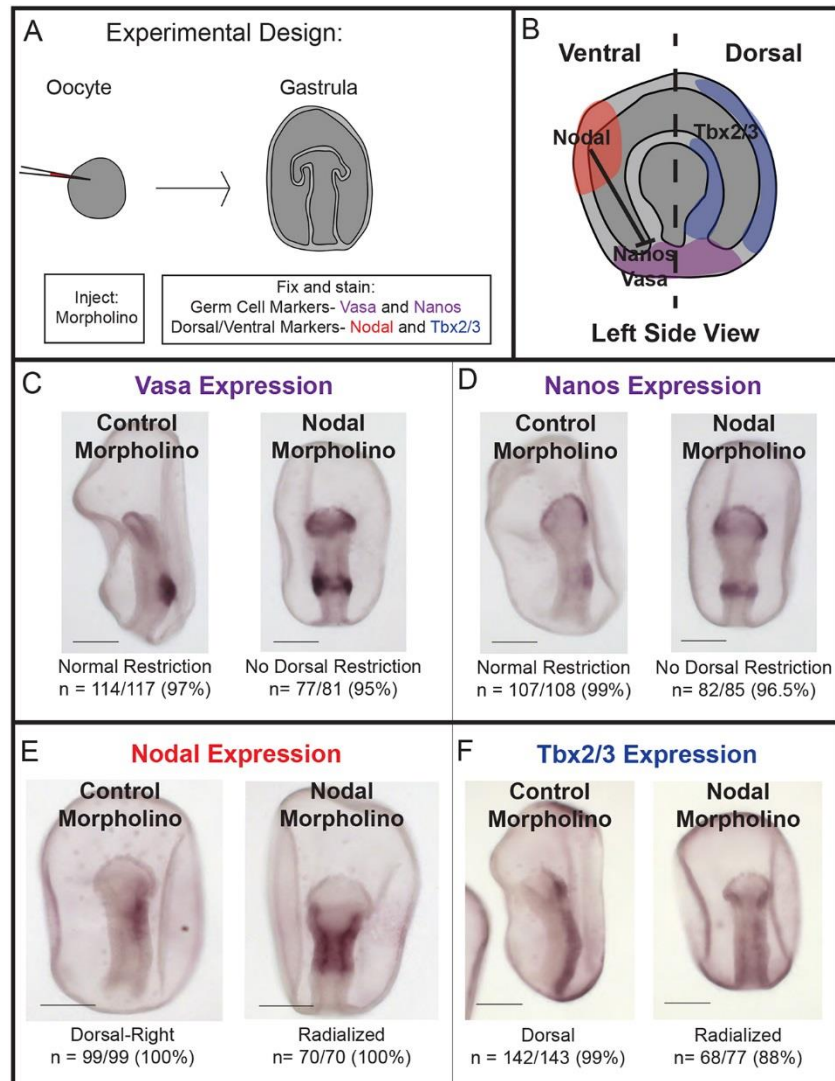


Figure 2: **Nodal restricts the germ cell factors Vasa and Nanos**

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

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

C-D) Vasa and Nanos accumulate in a dorsal patch on the gut in 95% and 98% of embryos injected with control morpholino. In contrast, Vasa and Nanos are not restricted and instead accumulate in a ring around the gut in 94% and 97% of embryos injected with a Nodal morpholino. *p= .00002, Vasa *p=.0007, Nanos.

E-F) Nodal accumulates in the dorsal-right side of the developing gut in 100% of embryos injected with control morpholino. Tbx2/3 mRNA accumulates in the dorsal side of the developing gut in 99% of embryos injected with control morpholino. In contrast, Nodal and Tbx2/3 mRNAs are not restricted and instead become radialized in 100% and 86% of embryos injected with a Nodal morpholino. *p= 2.5E-13, Nodal *p=.0002, Tbx2/3.

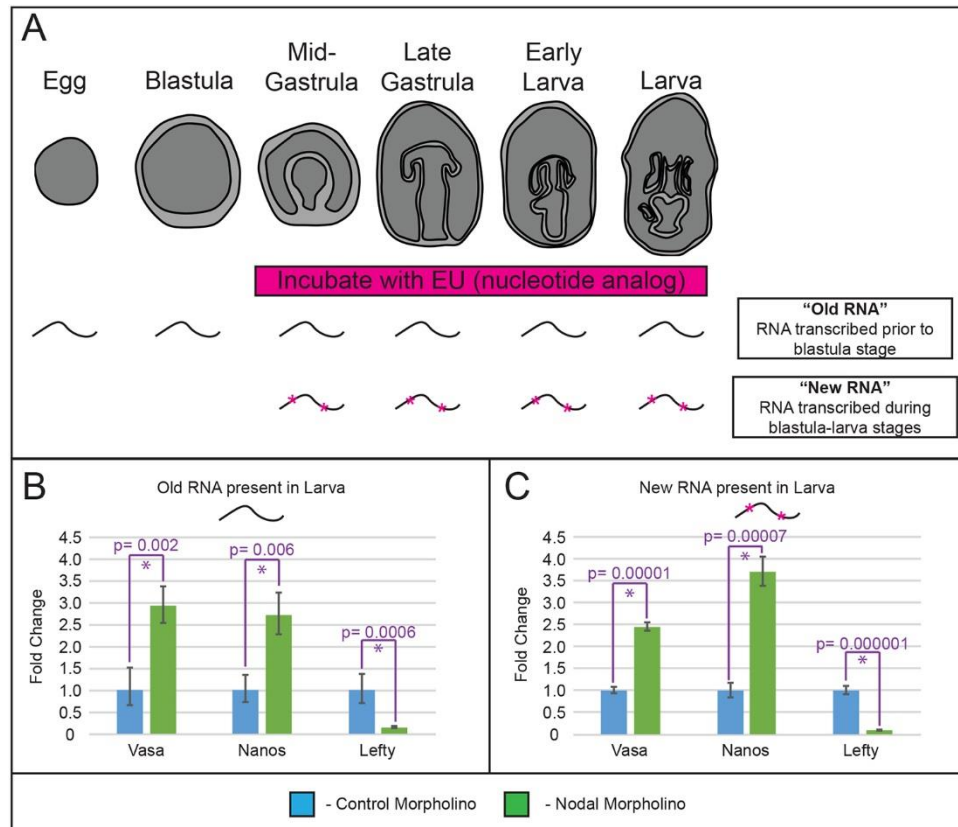


Figure 3: Nodal restricts transcription of germ cell factors and causes their degradation

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

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

C) qPCR analysis shows that germ cell factor RNA's (Vasa and Nanos) produced during EU labelling are significantly increased in embryos injected with Nodal morpholino (green) compared to embryos injected with control morpholino (blue). As a

control for inhibition of the Nodal signaling pathway, Lefty RNA levels are significantly decreased in embryos injected with Nodal morpholino (green) compared to embryos injected with control morpholino (blue).

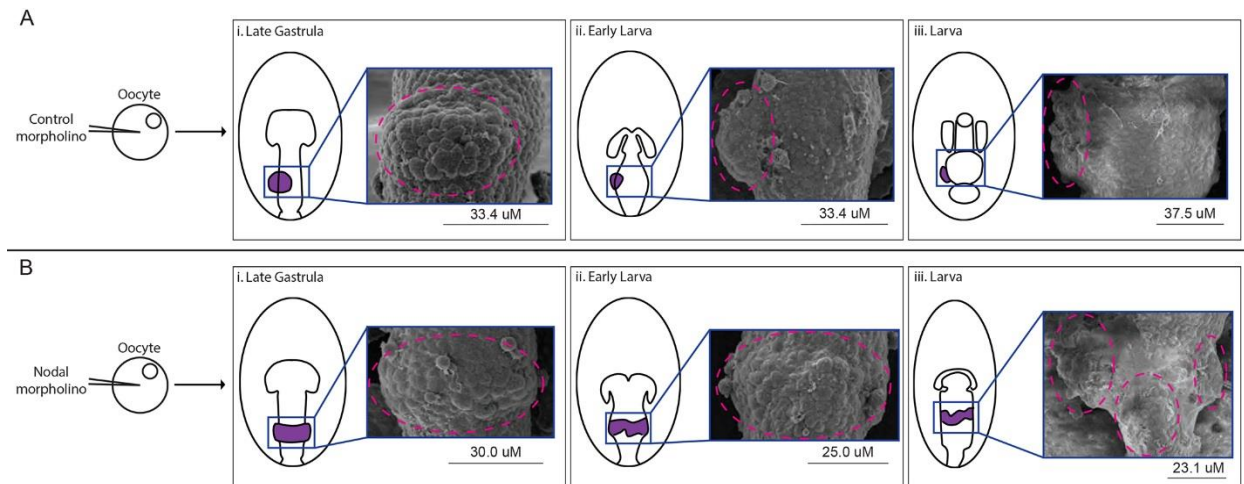


Figure 4: Nodal restricts cells from bulging out of the mid-gut.

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

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

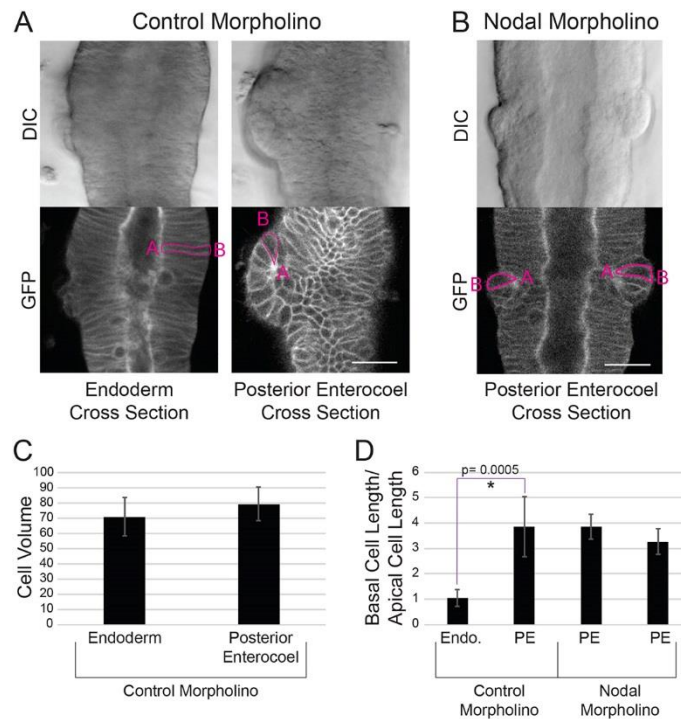


Figure 5: Nodal inhibits the apical constriction of mid-gut cells

A) DIC and GFP images of an embryo injected with a control morpholino and PH domain of PLC Gamma-GFP. The first column represents a section in which the endodermal cells are at a cross section. The second column represents a section in which the posterior enterocoel cells are at a cross section. Cells are outlined in pink and apical and basal sides of the cells are labelled A and B, respectively.

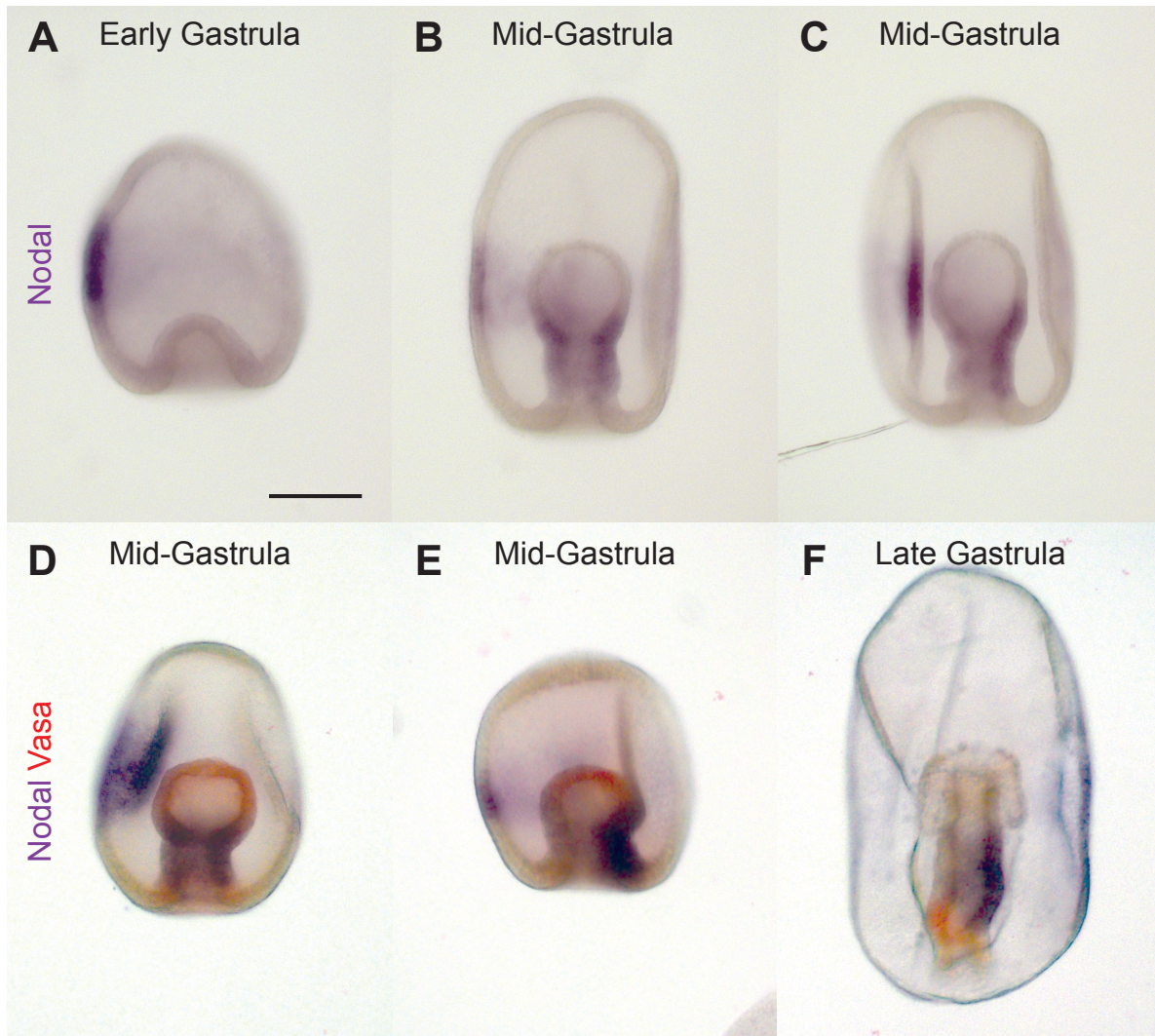
B) DIC and GFP images of an embryo injected with Nodal morpholino and PH domain of PLC Gamma-GFP. In these images the posterior enterocoel cells are at a cross section. Cells are outlined in pink and apical and basal sides of the cells are labelled A and B, respectively.

C) The cell volume of endodermal cells and posterior enterocoel cells is similar in embryos injected with control morpholino.

D) In embryos injected with control morpholino, the ratio of Basal:Apical cell length is significantly increased in posterior enterocoel cells (~4) compared to endodermal cells (~1). In embryos injected with Nodal morpholino, the ratio of Basal:Apical cell length in

multiple posterior enterocoels (~4 and ~3) is similar to that of the posterior enterocoel in embryos injected with control morpholino (~4).

Scale bars represent 25 microns.



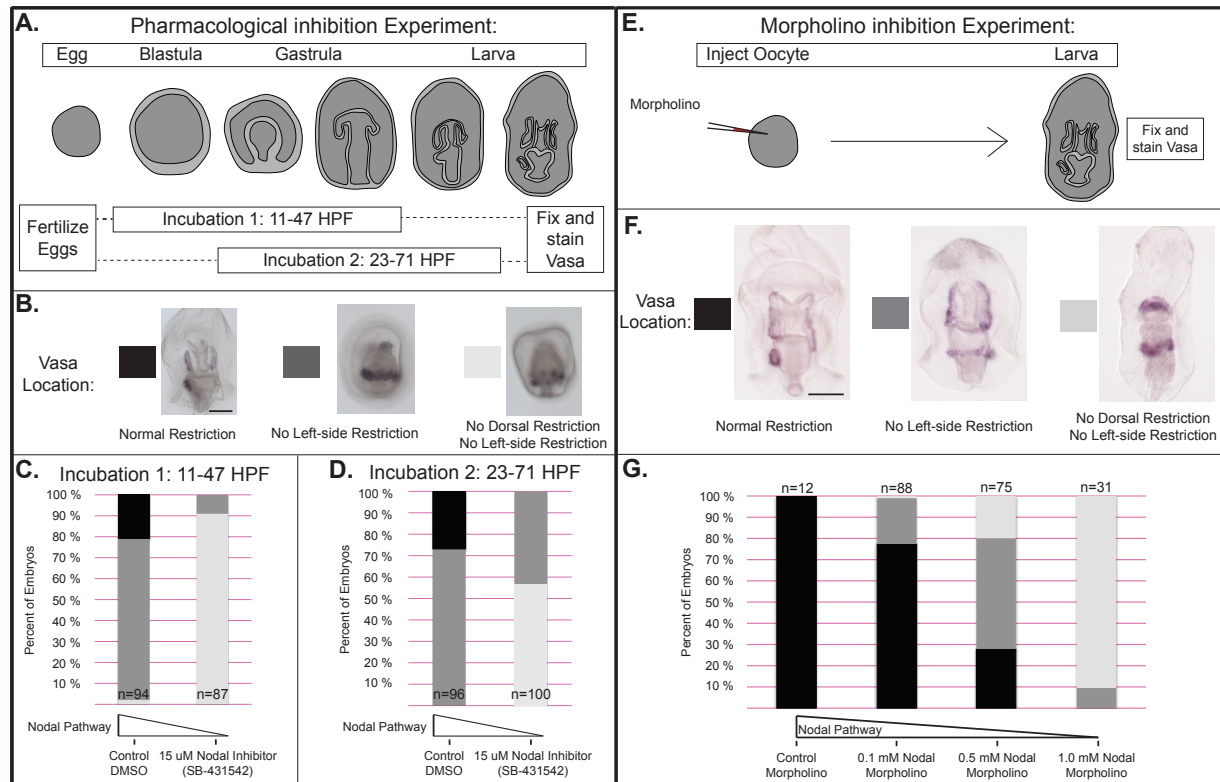
Supplemental Figure 1: Nodal and Vasa localization during sea star development.

A-C) Nodal in situ hybridization during the gastrula stage.

D-F) Nodal and Vasa double in situ hybridization during the gastrula stage.

All views are left side views except for F which is a dorsal view.

Scale bar is 100 microns.



Supplemental Figure 2: Nodal inhibition causes Vasa to fail to restrict in the dorsal and left sides of the developing gut.

A) Pharmacological experimental design: Embryos were incubated with 15 uM Nodal inhibitor (SB-431542) from either 11-47 hours post-fertilization or from 23-71 hours post fertilization. Embryos were then fixed at the larva stage and Vasa mRNA was localized via an in situ hybridization.

B) When embryos were treated with inhibitor Vasa was localized in 3 different patterns. If there was normal restriction then Vasa was localized in the left side of the gut (black). If there was no left-side restriction then Vasa was localized in the dorsal side of the gut (dark gray). If there was no dorsal or left side restriction then Vasa was localized in a ring around the gut (light gray). Scale bar is 100 microns.

C) When embryos were treated with inhibitor at an earlier time point then there was a shift in Vasa localization such that there is less restriction in embryos treated with inhibitor relative to embryos incubated with DMSO as a control.

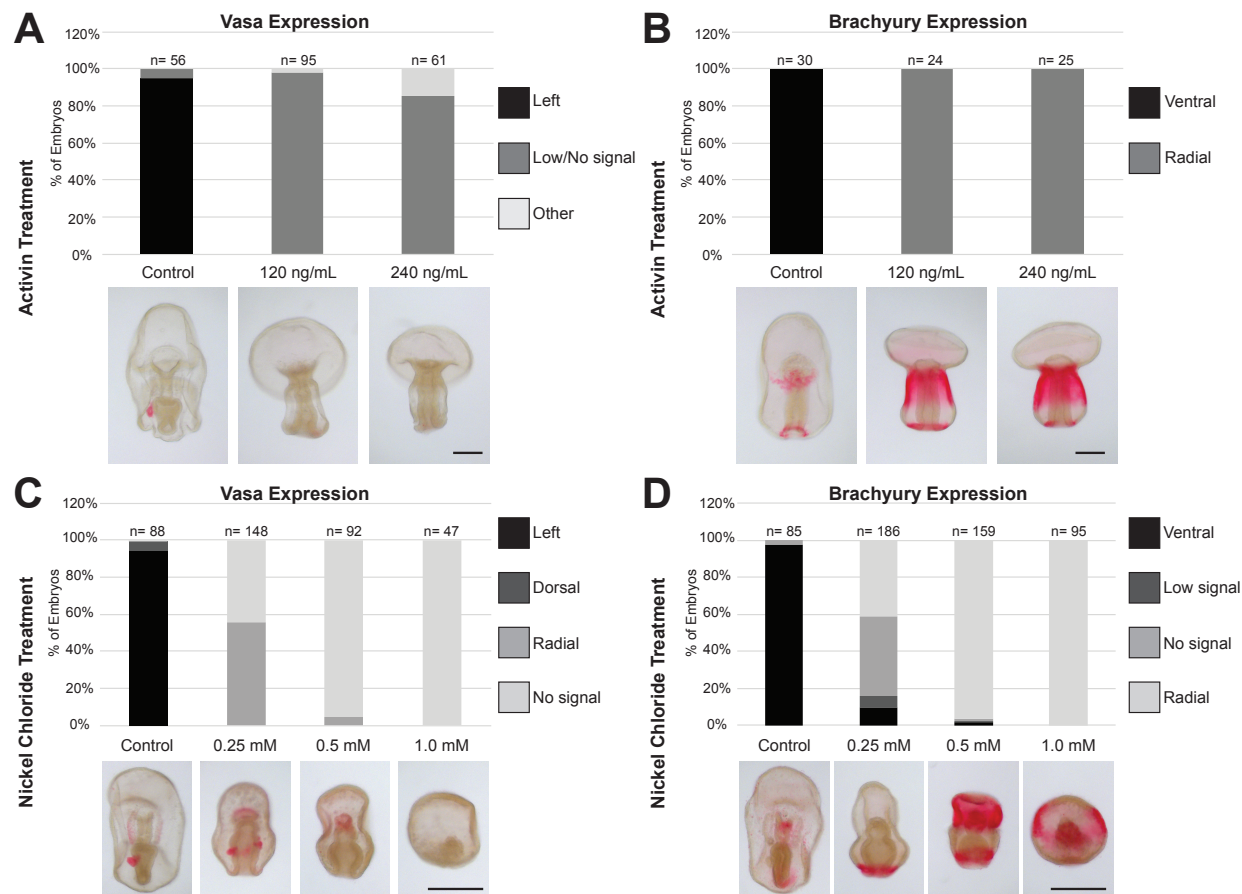
D) When embryos were treated with inhibitor at a later time point then there was a shift in Vasa localization such that there is less restriction in embryos treated with inhibitor relative to embryos incubated with DMSO as a control.

E) Morpholino experimental design: Oocytes were injected with Nodal morpholino or control morpholino at varying concentrations. The resulting embryos were fixed at the larva stage and Vasa was localized via an RNA in situ hybridization.

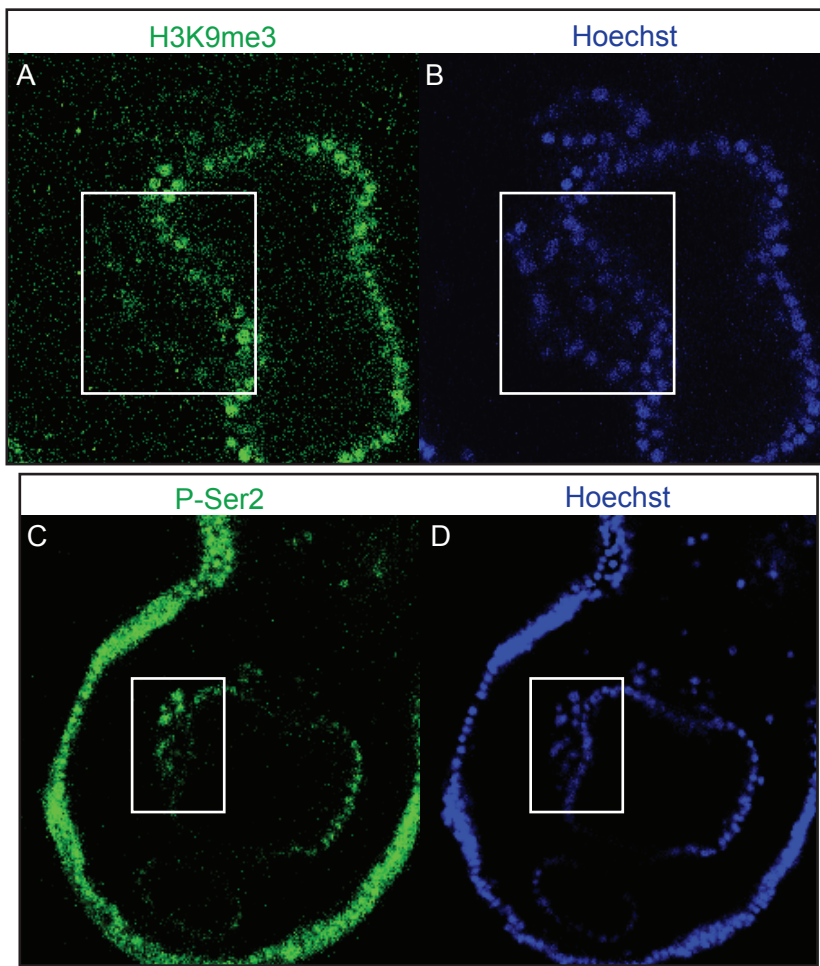
F) When embryos were injected with Nodal morpholino at different concentrations Vasa was localized in 3 different patterns. If there was normal restriction then Vasa was localized in the left side of the gut (black). If there was no left-side restriction then Vasa was localized in the dorsal side of the gut (dark gray). If there was no dorsal or left side restriction then Vasa was localized in a ring around the gut (light gray). Scale bar is 100 microns.

G) There is a dose dependent effect of Nodal morpholino upon Vasa restriction. Oocytes injected with morpholino at 0.1 mM result in 22% of embryos with a defect in left side restriction. Oocytes injected with 0.5 mM morpholino result in 52% of embryos with a defect in left side restriction and 20% of embryos with a defect in both left and dorsal side restriction. Oocytes injected with 1.0 mM Nodal morpholino result in 10% of embryos with a defect in left side restriction and 90% of embryos with a defect in both left and dorsal side restriction.

(All images are dorsal views of embryos.)



Supplemental Figure 3: Nodal overexpression causes a decrease in Vasa mRNA
A) Embryos were treated with varying concentrations of human Activin AB from 30 minutes post-fertilization to the larva stage. In control embryos, Vasa localized in the left posterior enterocoel in the majority of embryos. When embryos were treated with Activin AB Vasa expression was decreased or absent in the majority of embryos.
B) Embryos were treated with varying concentrations of human Activin AB from 30 minutes post-fertilization to the late gastrula stage. In control embryos, Brachyury localized in a ventral patch in the ectoderm. When embryos were treated with Activin AB brachyury expression was radialized.
C) Embryos were treated with varying concentrations of Nickel Chloride from 30 minutes post-fertilization to the larva stage. In control embryos, Vasa localized in the left posterior enterocoel in the majority of embryos. When embryos were treated with 0.5-1.0 mM Nickel Chloride Vasa expression was absent in the majority of embryos.
D) Embryos were treated with varying concentrations of Nickel Chloride from 30 minutes post-fertilization to the larva stage. In control embryos, Brachyury localized in a ventral patch in the ectoderm. When embryos were treated with 0.5-1.0 mM Nickel Chloride, Brachyury expression was radialized.
Scale bars represent 100 microns.



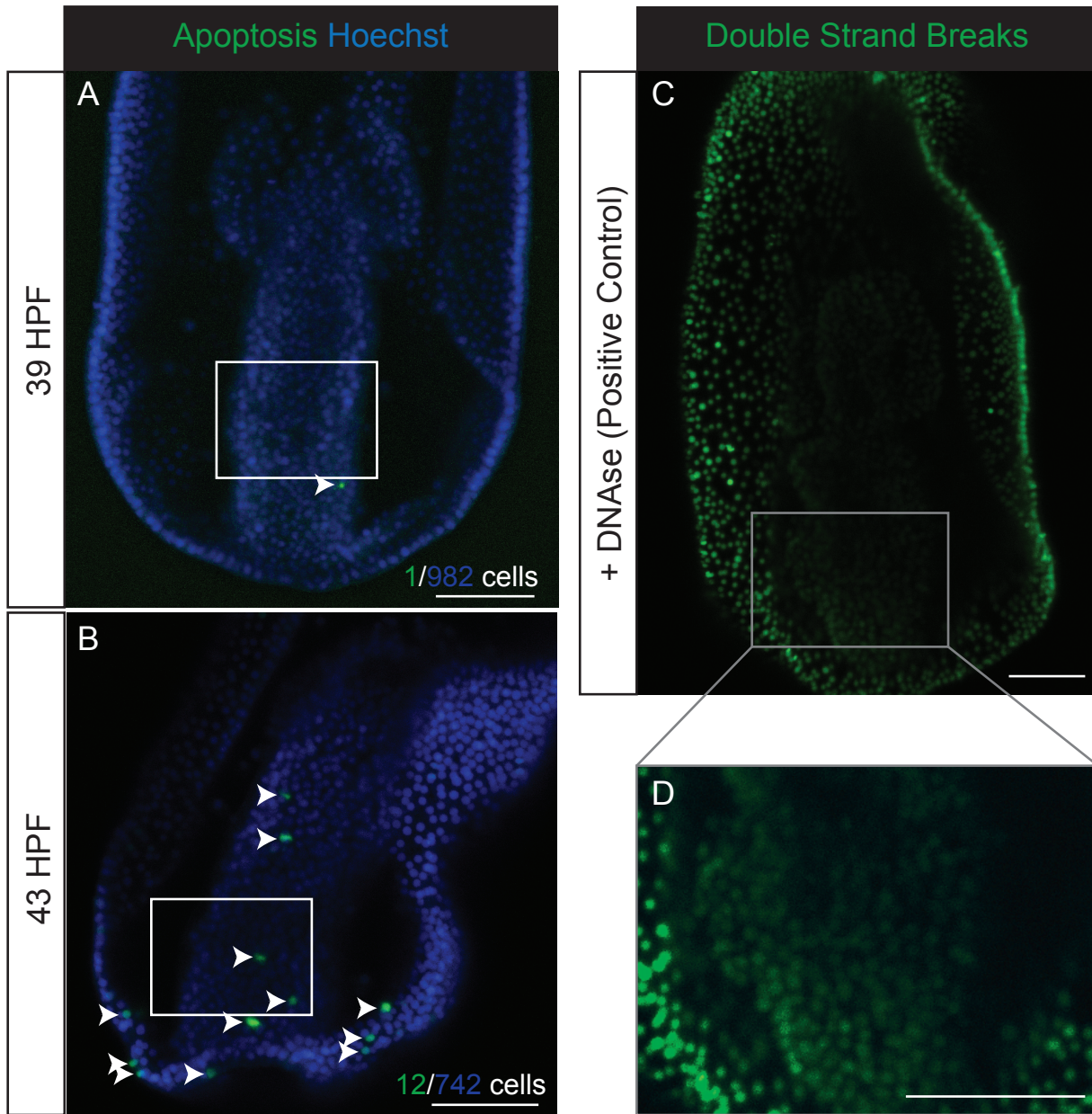
Supplemental Figure 4: H3K9me3 and RNA Polymerase II antibody staining in sea star development.

A) H3K9me3 antibody staining in the sea star larva. The white box outlines the posterior enterocoel.

B) Hoechst staining of the same embryo in A.

C) RNA Polymerase II, Phospho-Serine II antibody staining in the sea star larva. The white box outlines the posterior enterocoel.

D) Hoechst staining of the same embryo in C.



Supplemental Figure 5: Apoptosis during sea star development.

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

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

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

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

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

Supplemental Table 1- Primers used for in situ probe synthesis

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

Supplemental Table 2- Primers used for quantitative PCR