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Distinct transcriptional regulation of Nanos2 in the germ line and soma by the Wnt and delta/notch pathways

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ABSTRACT

Specification of the primordial germ cells (PGCs) is essential for sexually reproducing animals. Although the mechanisms of PGC specification are diverse between organisms, the RNA binding protein Nanos is consistently required in the germ line in all species tested. How Nanos is selectively expressed in the germ line, however, remains largely elusive. We report that in sea urchin embryos, the early expression of Nanos2 in the PGCs requires the maternal Wnt pathway. During gastrulation, however, Nanos2 expression expands into adjacent somatic mesodermal cells and this secondary Nanos expression instead requires Delta/Notch signaling through the forkhead family member FoxY. Each of these transcriptional regulators were tested by chromatin immunoprecipitation analysis and found to directly interact with a DNA locus upstream of Nanos2. Given the conserved importance of Nanos in germ line specification, and the derived character of the micromeres and small micromeres in the sea urchin, we propose that the ancestral mechanism of Nanos2 expression in echinoderms was by induction in mesodermal cells during gastrulation.

1. Introduction

All sexually reproducing animals specify cells devoted to making eggs or sperm. In most studied bilaterians, the primordial germ cells (PGCs) are segregated functionally from somatic cells during embryogenesis. The timing and mechanisms of PGC segregation vary greatly between species, but usually follow a mechanism of either inheritance of localized, maternally supplied determinants or by induction from cell-cell communication. Broader evolutionary comparisons support a model in which ancestrally, PGCs were specified by inductive mechanisms. For example, in the mouse, PGCs are induced at 6.5 days post-fertilization by a combination of Bmp4 and Wnt3 ligands from the extraembryonic ectoderm. Presumptive PGCs in the mesoderm proximal to the extraembryonic tissue respond by expressing Brachyury/T, a direct target of beta-catenin, and initiating a tripartite gene regulatory network. This network, which includes Blimp1, Prdm14, and AP2γ, broadly represses somatic gene expression and initiates expression of select germ line genes (Magnusdottir et al., 2013). As in the mouse, the axolotl, a urodele amphibian, requires specifies germ cells by induction via Bmp signaling and T for germ line induction (Chatfield et al., 2014). Recent investigation in the cricket *Gryllus gryllus* has revealed a potentially deeply conserved role for Bmp signaling in inductive germ line specification (Donoughe et al., 2014). However, multiple evolutionary lineages appear to have independently evolved more precocious PGC segregation by inheritance of maternal determinants. For example, the PGCs of *Drosophila* and *C. elegans* are specified largely by inheritance of germ plasm, a conglomerate of instructive RNA and protein factors (Seydoux and Braun, 2006). Similarly, anuran amphibians such as the frog *Xenopus laevis*, are proposed to have independently acquired an inheritance-based mechanism distinct from the axolotl (Johnson and Alberio, 2015).

The PGCs of the sea urchin also display characteristics of inheritance-based specification, and are formed following two early asymmetric cleavages. The 4th cleavage results in a 16-cell embryo, which includes 4 macromeres and 4 small cells called micromeres at the vegetal pole. The subsequent 5th cleavage divides the micromeres asymmetrically to produce two distinct cell lineages. The large micromeres solely give rise to

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larval skeletogenic cells. In stark developmental contrast, the small micromeres (sMics) are the PGCs. Although the lineage of the small micromeres has not been traced to sperm and eggs, the plethora of data support the conclusion that these cells are a PGC population of the animal. Following their creation, the PGCs remain mitotically quiescent, dividing only once before larval stages. The PGCs are also quiescent transcriptionally and translationally during blastula and gastrula stages (Oulhen et al., 2017; Swartz et al., 2014). The PGCs remain at the vegetal pole until gastrulation when they passively translocate along the developing archenteron and then actively assort into two bilateral coelomic pouches (Campanale et al., 2014; Yajima and Wessel, 2012).The coelomic pouches consist of cells derived from the small micromere lineage and from the Veg2 mesoderm of the macromere lineage (Cameron et al., 1987, 1991). The PGCs in the left coelomic pouch proliferate while those in the right undergo apoptosis (Luo and Su, 2012) reflective of the major contribution of the left pouch structures to the rudiment of the adult.

Nanos is a translational inhibitor essential for PGC formation and survival. It functions with its partner Pumilio to bind a specific element in the 3'UTR of its target RNAs (Sonoda and Wharton, 1999; Wharton and Struhl, 1991). Only six direct targets of nanos/pumilio binding have been identified so far: Drosophila *hunchback, cyclin B and hid* (Asaoka-Taguchi et al., 1999; Dalby and Glover, 1993; Kadyrova et al., 2007; Murata and Wharton, 1995; Sato et al., 2007; Wreden et al., 1997), *Xenopus VegT* (Lai et al., 2012), and sea urchin *CNOT6* and *eEF1A* (Oulhen et al., 2017; Swartz et al., 2014). In the sea urchin *Strongylocentrotus purpuratus* (Sp), three nanos homologs have been found and each of them is expressed with different timing (Juliano et al., 2010b). *Nanos1* mRNA is only present in the ovaries (Juliano and Swartz, unpublished data). *Nanos2* and *Nanos3* accumulate in the PGCs (Juliano et al., 2010b) but *Nanos2* is the first *de novo* mRNA to accumulate selectively in the PGCs, and is required for their translational quiescence and survival (Fujii et al., 2009; Juliano et al., 2010b; Oulhen et al., 2017). The post-transcriptional control of Nanos2 accumulation has been extensively characterized in this animal (Oulhen and Wessel, 2014, 2016; Oulhen et al., 2013). However, the transcriptional control of this critical germ line stem cell factor remains poorly understood.

The canonical Wnt/beta-catenin pathway has been identified as a major regulator of primary axis fate specification in the sea urchin and other embryos (Martindale and Hejnol, 2009). Maternally-supplied Dishevelled protein is pre-localized to the sea urchin egg vegetal cortex and directs the nuclearization of beta catenin preferentially in the vegetal blastomeres (Weitzel et al., 2004). Dishevelled inhibits the function of a destruction complex containing Axin, GSK3, APC, CK1alpha, and PP2A, allowing the nuclearization of beta-catenin. Nuclear beta catenin associates with Tcf family transcription factors and initiates transcription. The earliest activation of the Wnt pathway in the early sea urchin embryo may occur independently of an embryonically-expressed Wnt ligand; the localized Disheveled may be modified based on gel mobility, perhaps to an active form that hardwires this part of the pathway (Peng and Wikramanayake, 2013). Furthermore, animal-vegetal patterning is established even when all Wnt secretion is inhibited by perturbing Porcupine, which is critical for Wnt palmitoylation and its subsequent function (Cui et al., 2014). Beta-catenin in the nucleus initiates a cascade of organizing activity of the micromeres. The PGCs also contain nuclear beta-catenin, and while its role there is unknown, it is a candidate for regulation of Nanos2 transcription (Logan et al., 1999).

The forkhead transcription factor FoxY may also influence Nanos2 expression. FoxY expression begins in the PGCs at around 10 h post-fertilization (h.p.f.) in response to Delta/Notch signaling (Materna et al., 2013). At the onset of gastrulation, FoxY expression expands into adjacent somatic cells, derived from the Veg2 mesodermal progenitors (Materna et al., 2013). FoxY is broadly required for establishment of the Veg2 lineage, and its knockdown results in the loss of several mesodermal derivatives, including the coelomic pouches (Materna et al., 2013). Knockdown of FoxY during its early PGC expression phase has no impact on early Nanos 2 expression, however, our prior work indicated a moderate reduction of Nanos2 around the time of gastrulation, when FoxY is expressed in the non-skeletogenic mesoderm (NSM) (Song and Wessel, 2012). Similarly, Nanos2 expression was found to be sensitive to FoxY perturbation in sea urchin mesodermal precursors (Andrikou et al., 2013, 2015). Collectively, these observations imply differential phases of Nanos regulation, but whether they indicate direct transcriptional inputs into the Nanos2 gene, or rather indirect effects of perturbing the mesodermal lineage remains unresolved.

Prior work indicates exquisite regulation of Nanos2 expression at the transcriptional and post-transcriptional levels. However, the mechanisms responsible for the earliest activation of Nanos2 expression remain unknown. Furthermore, whether FoxY regulates Nanos2 directly or indirectly is unclear. Here, we investigate the signaling inputs and transcriptional mechanisms of Nanos2, and integrate our findings into the sea urchin gene regulatory network.

2. Material and methods

2.1. Animals

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

2.2. Whole mount in situ hybridization and co-immunofluorescence

WMISH was performed as described previously, with some modification for double fluorescent in situs (Swartz et al., 2014). Briefly, probe templates were produced by either amplifying 1 kb of sequence with reverse primers tailed with the T7 promoter, or by linearizing cloned fragments. Labeled RNA probes were transcribed from these linear templates with the Roche RNA labeling kit, using nucleotides conjugated to 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 in the presence of 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 in situ hybridizations, the first antibody was quenched with 1% hydrogen peroxide treatment for 30 min, followed by washing, application of a second antibody to fluorescein, and then visualized with fluorescein tyramide amplification. Immunofluorescence for Vasa and β-catenin was performed as described previously (Voronina et al., 2008; Yazaki et al., 2015).

2.3. FoxY antibody purification, Western blot and immunofluorescence

Rabbit polyclonal antibodies against the N-terminal region of FoxY (N377 and N378 (Song and Wessel, 2012);) were purified using the Amino link Plus Immobilization kit (Thermo Fisher). Immunoblot analysis was conducted on 60 gastrulae lysed in 15 μl of sample buffer, incubated for 10 min at 95 °C and subjected to SDS-PAGE and blotted onto 0.22 μm nitrocellulose membranes (Towbin et al., 1979). Membranes were incubated with antibodies against FoxY (1:1000). The antigen–antibody complex was measured by chemiluminescence using horseradish peroxidase-coupled secondary rabbit antibodies according to the manufacturer's instructions (ECL).

For immunofluorescence, embryos or larvae were fixed with 90% methanol overnight at −20 °C, and washed 3 times with PBS-Tween (PBST). The embryos were blocked with PBST sheep serum 4% for an hour at room temperature. FoxY antibody (N377) was used at 1:200 dilution in the blocking buffer overnight at 4 °C. After 3 washes in PBST, an anti-rabbit Alexa Fluor 488 was used as the secondary conjugated antibody (Life Technologies), diluted by 1:500 in blocking buffer, for 3 h at room temperature.

Hoechst (1:1000) was incubated with the secondary antibody to co-label the DNA. Images were captured using a LSM 800 laser scanning confocal microscope (Carl Zeiss).

2.4. *DNA cloning and reporter constructions*

DNA fragments used in this study were PCR amplified and cloned by standard methods. FoxY and Nanos2 in situ probes were cloned into pGEMT easy (Promega). For overexpression of Axin, constructs were built in the PCS2 vector, linearized with Not1 and transcribed using mMessage mMachine SP6 (Ambion) as described previously (Range et al., 2013). The Axin mRNA was then polyadenylated using the PolyA tailing kit (Thermo Fisher Scientific).

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

Custom MASOs were synthesized by Gene-Tools (http://www.gene-tools.com/). 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. Two MASOs against FoxY were used in this study, and they both gave similar results: 5’-TGCACACCAGGACAACGATGTTTCC-3 and FoxY MO2: 5’-CATGGCTCCAAGTGCAGAACACTAC-3’. Both MASOs were injected at 500 µM. Eggs were dejelled by washing in pH 4.0 sea water and rowed on protamine sulfate-coated Petri dishes. Zygotes were injected with 2 pL of MASO or RNA by constant pressure injection in the presence of 1 mM 3-AT (Sigma). DAPT (Tocris) was added at fertilization to 8 µM, a concentration previously determined to effectively perturb the Delta/Notch signaling without affecting overall development (Materna and Davidson, 2012). C59 (Cellagen) was added at 0.5 µM at fertilization as previously described (Cui et al., 2014).

2.6. *CRISPR/Cas9*

The plasmid pCS2-3xFLAG-NLS-SpCas9-NLS (a gift from Yonglong Chen, Chinese Academy of Sciences; Addgene plasmid #51307) was linearized with *Not*I and transcribed with SP6. Eleven gRNA templates (Table 1) directed against *Sp* FoxY were designed according to CRISPRscan priorities (CRISPRscan.org). The gRNAs were synthesized by T7 RNA polymerase using the MegaShortScript T7 transcription kit (AM1354, ThermoFisher) and purified using the miRNeasy mini kit (217004, Qiagen). These RNAs were mixed (400 ng/µl of each gRNA and 500 ng/µl of Cas9 mRNA), injected into freshly fertilized eggs, and cultured until the desired stage. The genomic DNA of injected embryos was extracted with 10 µl of QuickExtract DNA Extraction Solution (http://www.epibio.com/), according to the manufacturer's instructions. Regions targeted by gRNAs were cloned following PCR amplification and sequenced individually.

Table 1
gRNA templates used to mutate Sp FoxY.

taatacgactcactataGGGATGGAGTTTGCGGGCCGgttttagagctagaa
taatacgactcactataGGGGAGTTCTCGCTGGGAAgttttagagctagaa
taatacgactcactataGGGGATGAGTGTCGCGACTgttttagagctagaa
taatacgactcactataGGCAGCCTTTGTAGGATGTTgttttagagctagaa
taatacgactcactataGGTTTCTTTCCATTCCGTGGgttttagagctagaa
taatacgactcactataGGTCAGCGTAGGTAAAACCCgttttagagctagaa
taatacgactcactataGGCGATCACGAAAGAACTGTgttttagagctagaa
taatacgactcactataGGGCGACGACTGAATCGCCAgttttagagctagaa
taatacgactcactataGGGCAACGGTCTTATGCAGGgttttagagctagaa
taatacgactcactataGGCGAGTGAGGTAGGTCGTTgttttagagctagaa
taatacgactcactataGGCCCAGTCAGACCCTTGATgttttagagctagaa

2.7. *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 µl reaction volumes (Life Technologies). All primers were previously characterized and listed in Table 2. Four embryo equivalents of cDNA were used per reaction. Experiments were run in triplicate, normalized to ubiquitin, and controls were set to 1. When an error bar is presented in a control, it represents the technical replicates. In cases where no error bar is present in the controls, multiple experiments were combined and normalized as above.

2.8. *Chromatin preparation*

Chromatin from sea urchin embryos were prepared as described (Larschan et al., 2007) with some modification. Embryos were cultured at 0.5% (egg over sea water 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 re-suspended in dissociation buffer (1 M glycine, 25 mM EDTA, pH 8) and incubated on ice for 10 min. The embryos were then washed 3 times with CFSW, re-suspended in 5 mL CFSW and disaggregated by pipetting. Disaggregated cells were fixed with 1% formaldehyde for 10 min 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 pH8, 0.5 mM EGTA pH8, 0.25%Triton x-100, 1x Roche complete PI cocktail, 0.2 mM PMSF), and re-suspended in Wash Buffer B (10 mM Hepes pH 7.6, 100 mM NaCl, 1 mM EDTA ph 8, 0.5 Mm EGTA pH 8, 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 re-suspended at 1×10^8 cells/ml in Lysis Buffer (20% SDS, 10 mM EDTA pH 8, 50 mM Tris-HCl pH 8) and incubated at 4 °C for 10 min. Lysate was aliquoted to 300 µl in Protein Low Bind Safelock tubes (Eppendorf) for sonication. Sonication was performed in a Bioruptor bath sonicator using 6 rounds of 5 min each, cycling between 30 s on and 30 s off,. The sheared lysate was then pooled and centrifuged at 13,000 rpm for 10 min at 4 °C to remove cellular debris. Supernatants were transferred to 15 ml tubes and diluted 5-fold with CHIP dilution buffer (0.01% SDS, 1.2 mM EDTA pH 8, 16.7 mM Tris-HCl pH 8, 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.

Clear input DNA was prepared from 400 µl chromatin preparations, to which 21.5 µl of 20% SDS, 15 µl of NaCl and 1 µl of RNAse A (10 mg/ml) were added, and incubated overnight at 65 °C. An additional 1 µl RNAse was then added, followed by a 30 min incubation at 37 °C. Proteins were then digested by adding 20 µl of 1 M Tris-HCl pH 6.8, 10 µl 0.5 M EDTA, and 3 µl 20 mg/ml proteinase K, and incubated 90 min at 42 °C. The DNA

Table 2
List of primers used for qPCR.

Primers	Sequences 5’-3’
Nanos2	F: GCAAGAACAACGGAGAGAGC
	R: CCGCATAATGGACAGGTGTA
Seawi	F: GTGATGGTGTTGGTGACAGC
	R: TATTGATGCGCTTCTTGACG
Wnt8	F: TGTCGTTCATTCAAGCCATC
	R: TATCACTCGCCATTCGTTCA
Maf	F: GTCGAGATTGAAAAGCCAGC
	R: AGAACTCGGCAGAGTCAGGA
FoxC	F: GAAAATCACCCTCAACGGAAT
	R: CCCCTTGCCTGGTTTCTTAT
FoxY	F: TGCACTGCACTGACTCTGC
	R: CTTTCCATTCCGTGGTGAAG
Ubiquitin	F: CACAGGCAAGACCATCACAC
	R: GAGAGAGTGCGACCATCCTC
Delta	F: ACGGAGCTACATGCCTGAAC
	R: TCACAATGGACCGAATCAGA

was then extracted by phenol/chloroform and resuspended in 100 μl of water.

2.9. Immunoprecipitation (IP)

β-Catenin IP was performed with rabbit polyclonal antibodies directed against either the *Hemicentrotus pulcherrimus* β-catenin which bears complete sequence identity to *S. purpuratus* (Yazaki et al., 2015) or the *S. purpuratus* (Sp) β-catenin. 1 μl of *Hp* anti beta-catenin serum or non-specific rabbit IgG were added to 1 ml of chromatin preparation and incubated at 4 °C overnight with rotation. For the Sp β-catenin antibodies, 3 μg of each antibodies was used for the ChIP. For FoxY IP, 2 μl of anti FoxY antibodies (FoxY-N1 or FoxY-N2), or 2 μl of non-specific IgG were added to 1 ml of chromatin preparation (50 μg of chromatin for each IP) and incubated at 4 °C overnight with rotation. 50 μl of Protein A magnetic Dynabeads (Invitrogen), were added and rotated for 2 h at 4 °C. The beads were then washed twice with 1 ml of RIPA 150 Buffer (50 mM Tris-HCl pH 8, 1% NP-40, 2 mM EDTA, 0.1% Sodium Deoxycholate, 0.1% SDS, 150 mM NaCl) and once with RIPA 300 Buffer (as previous but with 300 mM NaCl). The beads were then washed once with 1 ml LiCl/TE buffer (0.25 M LiCl, 1% NP-40, 1% Sodium deoxycholate, 10 mM Tris-HCl pH8, 1 mM EDTA pH8) and twice with TE (10 mM Tris-HCl pH 8, 1 mM EDTA, 0.01% SDS, 1x PI cocktail, 0.2 mM PMSF). Beads were finally eluted twice with 250 μl of Bicarbonate elution buffer (1% SDS, 0.1 M Sodium bicarbonate). DNA was then recovered as for the input, and re-suspended in 100 μl of water for qPCR analysis.

2.10. qPCR analysis of the ChIP

Primers were designed against the entire Nanos2 locus (Table 3) and tested for specificity by dissociation curves and gel electrophoresis. For each 25 μl reaction, either 2 ng of input, or 2 μl of immunoprecipitated DNA was used as a template. All reactions were run in triplicate using Maxima SYBR (Life Technologies). Data were analyzed using the percent input recovery.

Table 3
Primer sequences for ChIP - qPCR.

Nos2ChIP F12	GCCAATCAAAACACGACATCT
Nos2ChIP R12	CGTCGTAGAGCTAGTCAAGAATGA
Nos2ChIP F13	TCCACGCACTTTAGTCACCA
Nos2ChIP R13	GACGTTGACGCCGTTAGAAT
Nos2ChIP F14	GTCTCTGGTCTGCCCGTCT
Nos2ChIP R14	ACCACCTCTGCCGTTGTT
Nos2ChIP F15	GCAAGAACAACGGAGAGAGC
Nos2ChIP R15	CCGCATAATGGACAGGTGTA
Nos2ChIP F16	CCGGGCACACACAATAAAAT
Nos2ChIP R16	GCTGAGACTTGAAGTACGACGA
Nos2ChIP F17	AACGACAACCGACTTTGGAC
Nos2ChIP R17	TCTCATCAACCTCGAGACGA
Nos2ChIP F3	GTTTTGTCCGCAATGCATAA
Nos2ChIP R3	ATGATTAGTACGAAGCCCAAATC
Nos2ChIP F4	CCCCCTTTAGGGATTAGCAG
Nos2ChIP R4	CGGCGATTAAAACACCTTTC
Nos2ChIP F5	TTGCCAACTTTTCCCTTTTC
Nos2ChIP R5a	TCGAGACGCAGATAAAATCTCA
Nos2ChIP F6	ACACATGGAATGGACCGATTA
Nos2ChIP R6	AGCTTTTGACAACTCTTTCTTTTCA
Nos2ChIP F7	TTCCATAACAGAAATGCGGTAA
Nos2ChIP R7a	TCCGAACACTTTCTCAACTTTGT
Nos2ChIP F8a	TGTCCAATTGTTTGAAGCACA
Nos2ChIP R8a	GCCTTTTCTGCGATGTTAAA
Nos2ChIP F9	CGCAGAAAAGGCTTTGGATA
Nos2ChIP R9	AATGCAAAACAAACAACAACAT
Nos2ChIP F10	TTTGACCCGGACTTTACCTG
Nos2ChIP R10	TTCGCATCGAATGAAAAGAC
Nos2ChIP F11	TATACCGCACGTGGTTTGAA
Nos2ChIP R11	CCTTTTCGCTAGCTTTGTCC

Nos is Nanos2, F is forward and R is reverse. All primers are shown in the 5’ - to - 3’ direction.

3. Results

3.1. Nanos2 is expressed in both the PGCs and the somatic Veg2 mesoderm

To define the dynamic nature of Nanos2 transcription in the sea urchin embryo, we tested its expression temporally and spatially using fluorescent RNA in situ hybridization for increased cellular resolution by confocal microscopy. Nanos2 mRNA is first detectable exclusively in the PGCs following their formation through blastula stages (Fig. 1A and B). However, at the onset of gastrulation (24 h.p.f.), we found that Nanos2 mRNA is also expressed in the adjacent somatic Veg2 mesoderm lineage (Fig. 1C–E).

3.2. FoxY and Nanos2 are co-expressed in the somatic Veg2 cells

The transcription factor FoxY is required for normal expression of Nanos2 at 24 h post-fertilization (h.p.f.), but not earlier (Song and Wessel, 2012). FoxY is first expressed in the PGCs and subsequently in Veg2, downstream of Delta/Notch signaling in both cell types (Materna et al., 2013). FoxY downstream of Delta/Notch is essential for specification of mesodermal progenitors and several mesodermal derivatives in the larva, including pharyngeal muscles, pigment cells, and the coelomic pouches. If FoxY regulates Nanos2 transcription, we reasoned that its expression dynamics should parallel those of Nanos2. We therefore tested FoxY and Nanos2 expression levels by qPCR during the expansion of Nanos2 into the Veg2 mesoderm. We observed similar expression kinetics for both transcripts (Fig. 2A). To test whether Nanos2 and FoxY mRNAs are expressed in the same cells, we performed double fluorescent in situ hybridizations along with immunofluorescence for Vasa to label the PGCs. We found that Nanos2 and FoxY mRNAs are indeed co-expressed in both the PGCs and the Veg2 somatic mesoderm early in gastrulation (Fig. 2B).

3.3. The initial expression of Nanos2 in the PGCs requires the canonical Wnt pathway

The canonical Wnt/beta-catenin pathway is a major regulator of vegetal cell fates in the sea urchin embryo. The role of nuclear beta-catenin in activating organizer activity in the micromeres is well-established, however, its function in the PGCs is unknown. As in other sea urchin species, beta-catenin is nuclearized in the micromeres from the 4th cleavage onward, and in the PGCs and other vegetal cells from the 5th cleavage through blastula stages (Logan et al., 1999; Yazaki et al., 2015).

We tested whether nuclear beta-catenin, the effector of canonical Wnt signaling, is required for Nanos2 expression in the PGCs. Overexpression of the destruction complex scaffolding molecule Axin downregulates canonical Wnt signaling. Expression of this construct both strongly animalizes the embryo and eliminates detectable nuclear beta-catenin (Fig. S1) (Range et al., 2013). We therefore injected Axin mRNA and measured Nanos2 transcript levels by qPCR, along with a known target of maternal beta-catenin, Wnt8. Indeed, we detected a decrease in Nanos2 levels as with the Wnt8 positive control (Fig. 3A). Seawi, a maternally-supplied germ line ortholog of PIWI, and FoxY were not affected by this perturbation. Delta was also significantly decreased in the embryos overexpressing Axin, supporting potential cross-talk of these two important signaling pathways (Oliveri et al., 2002, 2003).

To distinguish whether the decrease in Nanos2 transcript levels was a result of PGC loss or lack of expression, we performed fluorescent in situ Nanos2 hybridization while stably labeling the PGCs lineage by EdU pulsing in the zygote. Because of their slow cell cycle, the PGCs retain EdU, whereas somatic cells divide frequently and dilute the label, thus allowing confident visualization of the PGCs independently of their gene expression profile (Tanaka and Dan, 1991). We generally observed four PGCs in Axin expressing embryos, as in controls; however, we were consistently unable to detect Nanos2 RNA (Fig. 3B). To further test the

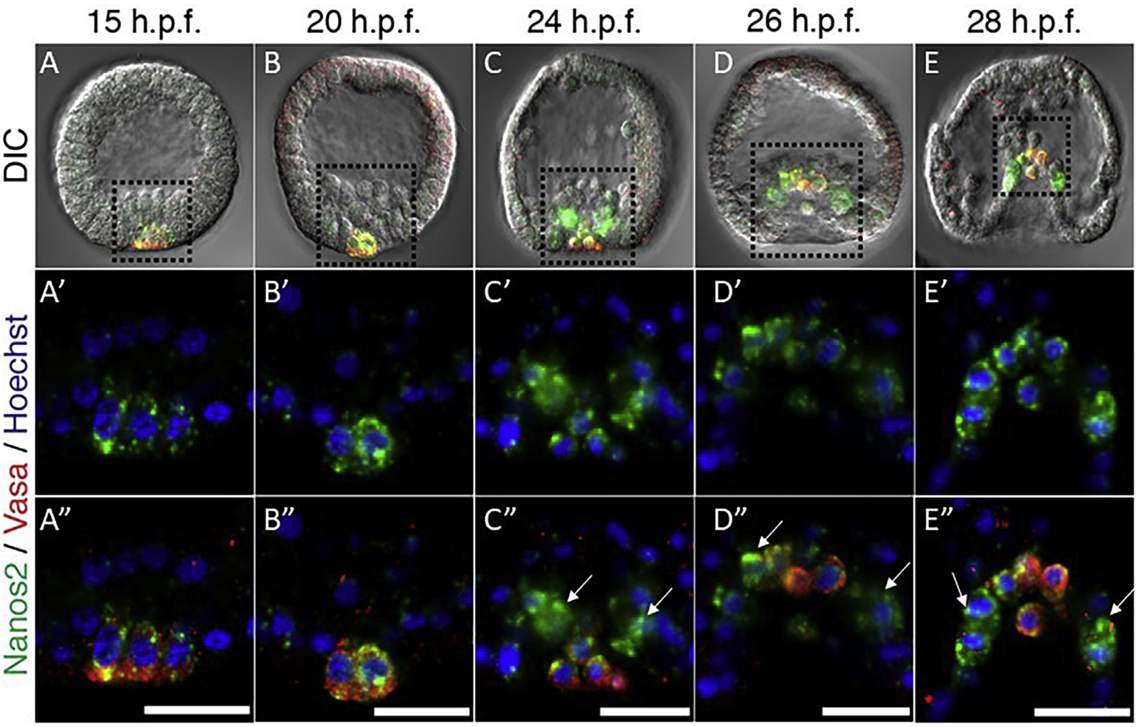

Fig. 1. Nanos2 is transcribed in both the PGCs and the somatic Veg2 mesoderm. (A–E) Nanos2 transcript (green) localization at multiple developmental stages. The PGCs are labeled by Vasa immunofluorescence (red). Images merged with DIC are provided in the top row to provide an overall view of the embryo. (A,B) Prior to primary invagination formation, Nanos2 expression is limited to the PGCs (15 and 20 h post-fertilization). (C–E) Nanos2 expression expands into the adjacent somatic cells by 24 h.p.f. (hours post-fertilization). (A'-E') Zoom views provided of Nanos2 transcripts (green) and Hoechst (blue). (A"-E") As in the middle panel, but merged with Vasa immunofluorescence (red) to visualize the PGCs. The Veg2 cells are indicated by the white arrows. Scale bars = 20 μm.

function of the Wnt pathway on Nanos2 regulation, embryos were injected with the mRNA coding for the beta-catenin/Engrailed (Montross et al., 2000), a fusion protein in which the C-terminal transactivation domain of beta-catenin has been replaced with the active repression domain of Drosophila Engrailed. This experiment also yielded a significant decrease in Nanos2, Wnt8 and Delta mRNA, as well as FoxY mRNA. The transcripts coding for Seawi were not affected (Fig. 3C). These results indicate an early requirement for nuclear beta-catenin in the expression of Nanos2.

3.4. Beta-catenin interacts with the Nanos2 promoter

The above results suggest that the Nanos2 gene may be a direct target of the canonical Wnt pathway. To test for direct Wnt pathway involvement, we scanned the Nanos2 genomic locus (800 bp upstream and downstream of the ATG) for trans-factor binding sites computationally, which revealed 2 putative HMG-box motifs that are recognized by the Tcf transcription factors with which beta-catenin associates (Gustavson et al., 2004; van Beest et al., 2000). To test for an interaction between beta-catenin and the Nanos2 promoter, we performed chromatin

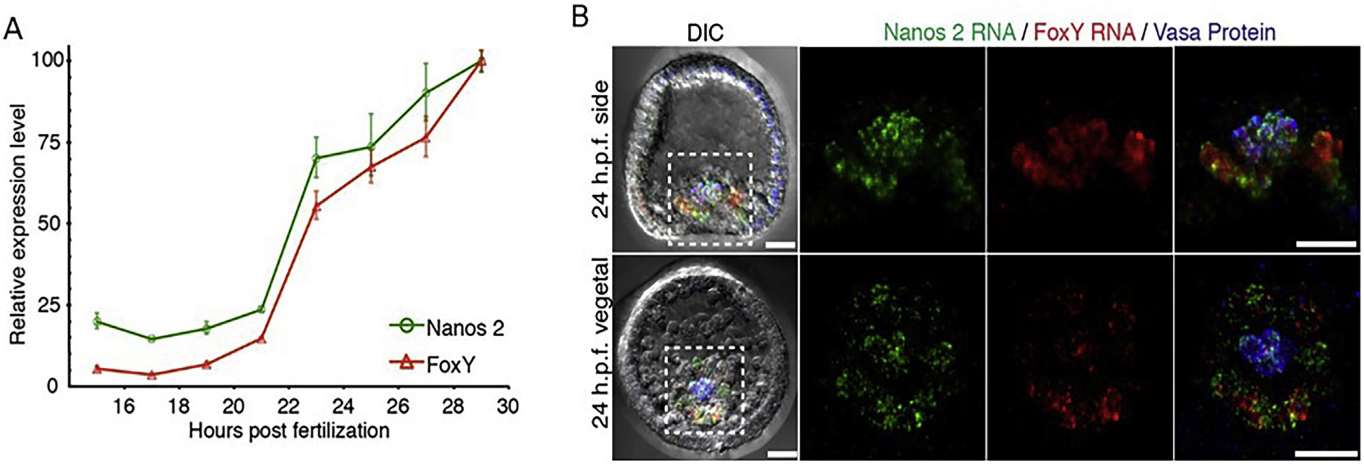

Fig. 2. Nanos2 and FoxY are co-expressed in PGCs and mesodermal cells. (A) Nanos2 and FoxY transcript abundance at multiple developmental stages, relative to ubiquitin and normalized to the maximum level. Error bars represent the mean and standard deviation of three replicates. (B) Nanos2 (green) and FoxY (red) transcripts are co-expressed in both the PGCs and the somatic Veg2 mesoderm at 24 h.p.f. The PGCs are labeled by Vasa immunofluorescence (blue). Scale bars = 20 μm.

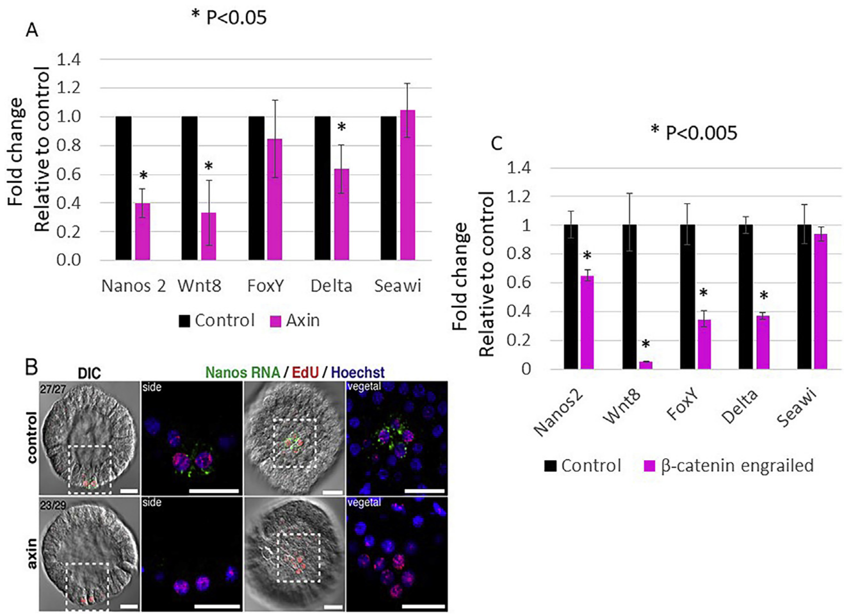

Fig. 3. The Canonical Wnt pathway is required for Nanos2 expression in the PGCs at 15 h.p.f. (A) qPCR for Nanos2 following microinjection of Axin mRNA at 500 ng/ul. Expression of both Nanos2, and Wnt8 (a known target of β-catenin), decrease with Axin expression. Seawi is a PGC-enriched RNA whose levels do not change significantly in response to Wnt signaling (Yajima et al., 2014). FoxY is also expressed in the PGCs in blastulae, downstream of the Delta/Notch signaling and is not substantially affected by Axin expression. (error bars represent the mean and standard deviation of 3 replicates, *p value < 0.05). (B) Fluorescent in situ hybridization for Nanos2 following Axin expression. Nanos2 transcripts (green) are expressed in the PGCs in control embryos, but are undetectable with Axin expression. The PGC lineage is stably labeled by EdU incorporation (red). The nuclei are labeled with Hoechst (blue). Scale bars = 20 µm. (C) qPCR for Nanos2 following microinjection of beta-catenin/Engrailed mRNA at 400 ng/ul. Nanos2, Wnt8, FoxY and Delta were significantly down-regulated (error bars represent the mean and standard deviation of 3 replicates,*p value < 0.005).

immunoprecipitation (ChIP) followed by qPCR using primer pairs that scan this Nanos2 genomic locus (Fig. 4). We observed a consistent and specific peak about 200 bp upstream of the Nanos2 ATG start codon. This peak corresponds to the location of the putative HMG-box motif GACAAAG and is decreased by inhibition of Wnt secretion with the porcupine inhibitor c59 inhibitor (Cui et al., 2014). These experiments used an antibody against beta-catenin from the sea urchin *Hemicentrotus pulcherrimus* (Yazaki et al., 2015), and a similar peak was obtained with 2 other antibodies against different sites of the sea urchin *Strongylocentrotus purpuratus* beta-catenin (Stepicheva et al., 2015) (Fig. S2).These results support the conclusion of direct regulation of Nanos2 transcription by the Wnt pathway.

3.5. Expression of Nanos2 in the somatic Veg2 mesoderm requires the delta/notch pathway

Nanos2 and FoxY are both expressed with similar kinetics in the Veg2 mesoderm (Figs. 1 and 2A). This observation could suggest that they respond to the same inductive signal at the onset of gastrulation, or alternatively, Nanos2 could be downstream of FoxY. To test if FoxY functions in Nanos2 transcription, we blocked its translation using a previously-characterized morpholino (Fig. 5A (Materna et al., 2013);) and found that the level of Nanos2 mRNA significantly decreased in a FoxY/MASO-dose dependent manner, consistent with prior results (Andrikou et al., 2013, 2015; Song and Wessel, 2012). We also tested the expression of the transcription factors Maf and FoxC, which are expressed in distinct subsets of the FoxY- positive cells (Fig. S3). Like Nanos2, FoxC mRNA was significantly reduced. In contrast, mRNAs coding for Delta, Maf, FoxY itself, Wnt8 (a direct beta-catenin target) and Seawi were not affected by FoxY knockdown. A second morpholino targeting a distinct FoxY sequence also reduced Nanos2 expression. (Fig. S4)). We also employed CRISPR/Cas9 approaches to inactivate the FoxY gene, but this gene locus was immutable by Cas9 (Fig. S5).

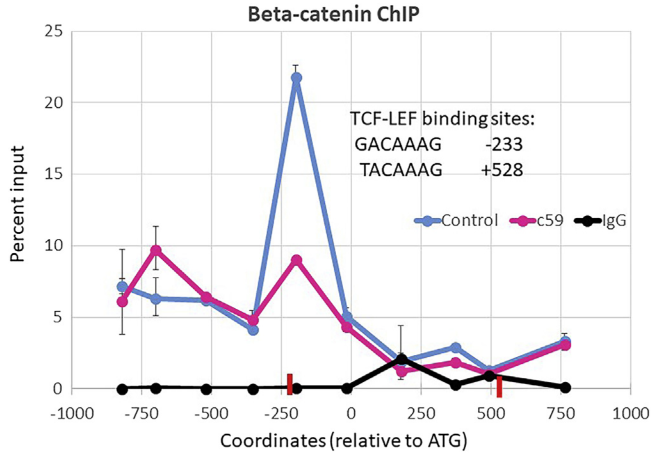

Fig. 4. β-catenin directly binds the Nanos2 genetic locus. Chromatin immunoprecipitated with β-catenin antibodies at 18 h.p.f. was tested by qPCR. Primer pairs scanning across the entire Nanos2 locus reveals a β-catenin binding peak approximately 233 nucleotides upstream of the transcriptional start site of the Nanos2 open reading frame. Error bars represent the mean and standard deviation of 3 replicates. Coordinates (x-axis) are relative to the translational start site of the Nanos2 ORF. Putative HMG-box binding motifs detected computationally are indicated upstream of the Nanos2 genomic locus.

To determine whether the decrease we observed in Nanos2 levels by qPCR was a result of PGC loss or lack of expression, we performed fluorescent in situ Nanos2 hybridization while identifying the PGCs lineage by EdU. Our results indicate that the PGCs were retained following FoxY knockdown. However, Nanos2 expression was selectively lost in the Veg2 lineage, while its expression was maintained in the PGCs (Fig. 5B). These results suggest that the Nanos2 gene has distinct transcriptional inputs in the PGCs and in the Veg2 lineage.

3.6. FoxY binds to the Nanos2 promoter

Depletion of FoxY from the embryo had a marked effect on the expression of Nanos2. It was previously observed that two splice forms of FoxY mRNA are expressed in the sea urchin embryo: FoxY-L (long) and FoxY-S (short) (Song and Wessel, 2012). To test if FoxY binds to the Nanos2 gene, we affinity purified and tested antibodies made against the sea urchin ortholog of FoxY and tested their specificity. We successfully purified two independent antibodies (FoxY-N1 and FoxY-N2) directed against the N terminal region of FoxY and tested them by Western blot using gastrula stage embryos (Fig. S6A). We detected one FoxY protein, (FoxY-S), with the FoxY-N1 antibody and two bands (FoxY-L and FoxY-S) with the FoxY-N2 antibody, all at their expected sizes. Both antibodies were highly specific in their recognition of whole embryo lysates. We then used these antibodies to test the localization of the FoxY protein during the sea urchin embryonic development. The FoxY-N1 (FoxY-S) antibody revealed that protein FoxY-S is expressed in the vegetal pole at

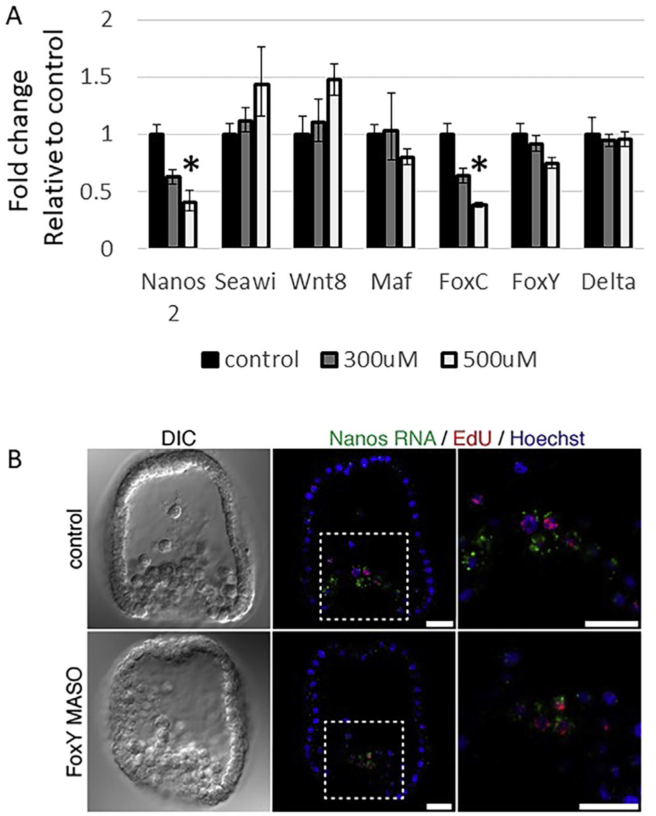

Fig. 5. FoxY regulates Nanos2 expression in mesodermal cells. (A) FoxY knock-down results in an over two fold reduction of Nanos2 transcript, as well as a decrease in FoxC. Error bars represent mean and standard deviation of 3 replicates, $^*p < 0.005$ (B) Spatial examination indicated that FoxY knockdown specifically eliminates Nanos2 expression in Veg2 mesodermal cells while its expression is maintained in the PGCs (green). The PGCs are labeled with EdU (red). Scale bars = 20 μm.

blastula, in cells surrounding the gut at gastrula stage and the left coelomic pouch at the larva stage (Fig. S6B).

To test whether FoxY interacts with Nanos2 promoter, we first searched for putative FoxY binding sites at the Nanos2 genomic locus. The majority of forkhead proteins bind to the RYMAAYA (R = A or G; Y= C or T; M = A or C) consensus, and we identified three such potential sites (Fig. 6) (Carlsson and Mahlapuu, 2002). We used both FoxY antibodies to perform a chromatin immunoprecipitation (ChIP) followed by promoter scanning using qPCR. Purified rabbit IgGs were used as a negative control (Fig. 6). Each FoxY antibody resulted in a similar peak around 800bp upstream of the ATG start codon. A second specific peak was identified 200bp upstream of the ATG site using the FoxY-N1 antibody that only recognizes FoxY-S (Fig. S6A). Both of these peaks were located in the regions including putative FoxY binding sites. We conclude from these data that FoxY interacts directly with the Nanos2 promoter.

3.7. *Nanos2 is transcribed in two distinct cell types by two distinct signaling systems*

Our results indicate that the canonical Wnt pathway is required for the early activation of Nanos2 expression in the PGCs. We next tested whether sustained Wnt signaling was required for Nanos2 expression after gastrulation, when its expression domain expands into Veg2 mesoderm. We treated embryos with the small molecule antagonist c59, which prevents Wnt secretion by inhibiting Porcupine, and observed a 40% reduction in Nanos2 mRNA levels by qPCR (Fig. 7A). These results indicate that by 24 h.p.f., ongoing Wnt signaling is necessary for continued Nanos2 expression. With spatial examination, we found that Nanos2 was specifically lost in the PGCs with c59 treatment, but unaffected in the Veg2 mesoderm, which is consistent with the 40% decrease detected by qPCR (Fig. 7C). We therefore conclude that the Wnt pathway is required for sustained Nanos2 expression selectively in the PGCs.

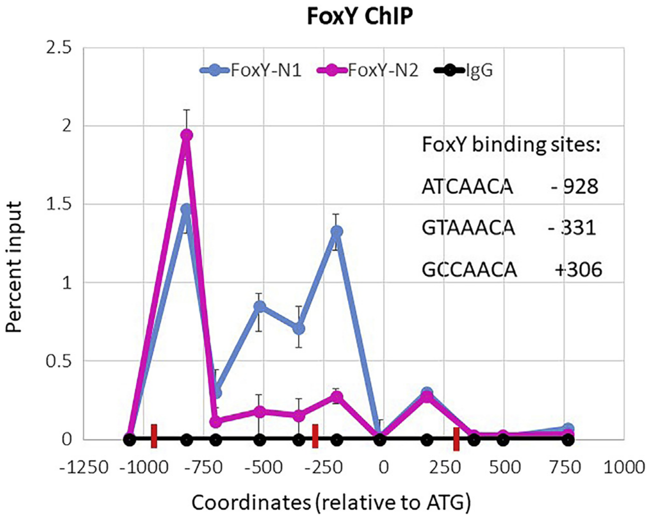

Fig. 6. FoxY directly binds the Nanos2 genetic locus. Chromatin immunoprecipitated with FoxY antibodies (FoxY-N1 and FoxY-N2) at 24 h.p.f. was tested by qPCR. Primer pairs scanning across the entire Nanos2 locus indicate a peak of FoxY binding approximately 800 nucleotides upstream detected with both antibodies. Error bars represent the mean and standard deviation of three replicates. Putative FoxY binding motifs matching a forkhead consensus in the Nanos2 genomic locus are indicated in red.

Prior work has shown that FoxY expression is downstream of Delta/Notch signaling (Ohguro et al., 2011). Since FoxY is required for full Nanos2 expression after gastrulation, we tested the effect of Delta/Notch signaling on Nanos2 transcript levels. Using the small molecule antagonist DAPT, we observed a 2-fold reduction in Nanos2 transcript levels by qPCR (Fig. 7B), similar to the results obtained with the FoxY morpholinos. Spatial examination of Nanos2 transcripts following DAPT treatment selectively eliminated the Veg2 Nanos2, but did not affect the PGC expression (Fig. 7C). These results are consistent with a model in which Delta/Notch signaling induces expression of FoxY, which in turn activates Nanos2 expression in somatic mesoderm by directly binding its promoter.

4. Discussion

Nanos orthologs have been appreciated as essential factors for germ line development in many organisms for over 30 years (Juliano et al., 2010a), and are usually thought of as germ-line specific regulators. We previously found that Nanos2 expression is regulated by a combination of selective RNA retention, distinctive translational control mechanisms (Oulhen et al., 2013), and protein stability (Oulhen and Wessel, 2016). Our work here provides some of the first insight into the direct transcriptional regulation of this important gene in both the germ line and the soma. We found that early (15hpf) and late (24hpf) expression of Nanos2 in the sea urchin PGCs requires activity of the Wnt pathway. During gastrulation, Nanos2 expression expands outside of the germ line into the Veg2 mesoderm. This expression does not require the Wnt pathway, but instead requires Delta/Notch signaling and the transcription factor FoxY. Together, these distinct inputs into Nanos2 lead to its dynamic temporal and spatial expression. Indeed, Nanos2 protein regulates specific mRNAs essential for the correct development of the embryo. In some animals, misexpression of Nanos2 causes embryonic lethality (Luo et al., 2011) and tumor growth (Wu and Ruvkun, 2010). Thus, the function of Nanos2 is restricted to relatively few cells via regulation at multiple levels.

Accumulating embryological and molecular evidence strongly

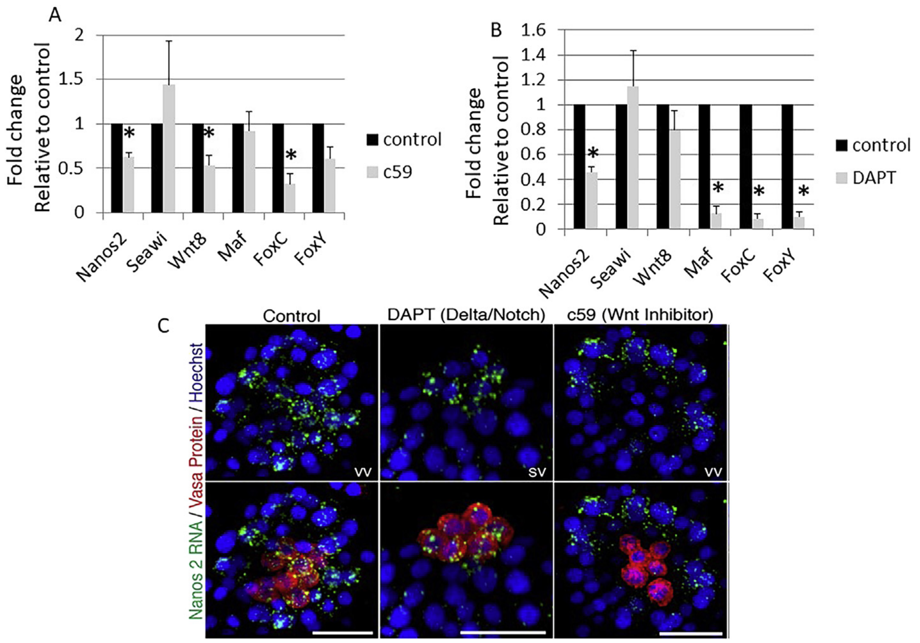

Fig. 7. Nanos2 is regulated by both Wnt and FoxY after gastrulation. qPCR following a C59 (A) or DAPT treatment (B). (error bars represent the mean and standard deviation of 3 replicates, $*p < 0.05$).(C) Spatial examination of Nanos2 transcripts (green) following Delta/Notch or Wnt pathway perturbation. Inhibition of Delta/Notch eliminates Veg2 Nanos2, without affecting PGC Nanos2. Conversely, inhibition of the Wnt pathway prevents Nanos2 expression in the PGCs without affecting the Veg2 Nanos2. Side view, "sv", vegetal view "vv". Scale bars = 20 μm.

supports an inheritance-based model for sea urchin PGC specification. While sea urchins do not possess a morphologically apparent germ plasm like in *Drosophila* or *Xenopus*, they do retain maternally supplied Vasa protein and Seawi RNA by differential stability mechanisms (Gustafson et al., 2011; Swartz et al., 2014). Furthermore, they are mitotically quiescent, show signs of broad transcriptional and translational repression characteristic of a stem cell line, and are required for fertility (Nakamura and Seydoux, 2008; Wessel et al., 2014). While such observations support an inheritance based strategy, Nanos is not maternally supplied in the sea urchin, as it is in *Drosophila* or *Xenopus*. The embryonic activation of Nanos2 in the PGCs immediately after their creation is unique, and indeed the only transcript known to be selectively expressed in the PGCs prior to gastrulation. However, we note that the activators of Nanos2 expression in the PGCs are maternally supplied, and spatially localized. Modified Dishevelled protein bound to the vegetal egg cortex is maternally inherited by the large and small micromeres, directly initiating Nanos2 expression and the cascade of supporting Wnt ligand activity. Furthermore, Nanos2 is a short, intronless gene, which we speculate could aid in its transcription despite the general transcriptional repression observed in the PGCs. In support of inherited autonomy, isolated PGCs in culture upregulate Nanos2 expression on a time course similar to the intact embryo (Yajima and Wessel, 2012).

A remarkable continuum exists in strategies of germ line developmental timing and mechanisms of PGC segregation. Despite these differences, several conserved pathways operate in germ lines across species, which activate terminal germ line genes such as Nanos, Vasa, and Piwi (Ewen-Campen et al., 2010). In the present work, we have shown that the canonical Wnt pathway is required for early 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 (Aramaki et al., 2013). One component of this transcriptional network, AP2γ, has been shown to directly bind the Nanos3 promoter in mice (Magnusdottir et al., 2013). We note that the micromere lineage is an evolutionary novelty unique to the echinoid lineage (e.g. sea urchins, pencil urchins, and sand dollars) of echinoderms. In sea stars, the germ line instead appears to be specified after gastrulation by inductive signals (Fresques and Wessel, 2018). We propose that in the evolution of the echinoids, a change in the Nanos2 cis-regulatory control apparatus enabled it to be activated earlier by the maternal Wnt pathway. This heterochronic shift in regulation resulted in a more inheritance based germ line segregation strategy in the micromere lineage. The subsequent expression of Nanos2 in the Veg2 mesoderm may be homologous to the ancestral mechanism present in sea stars, which activates Nanos after gastrulation. Future comparative investigation will evaluate how Wnts and other signaling machinery such as the Bmp pathway, along with additional maternal determinants and transcriptional factors intersect in the evolution of germ cells.

Expression of Nanos2 is regulated at multiple levels, precisely enforcing its accumulation in select cells at certain times of development. Indeed, ectopic expression of these genes often induces cell cycle and developmental defects (Luo et al., 2011; Wu and Ruvkun, 2010) and Nanos is thought to be "toxic" outside of its normal domain (Lai and King, 2013). Therefore, the expression of Nanos2 in the somatic Veg2 lineage is surprising. However, prior studies have shown that sea urchin embryos can develop normally when the micromere lineage is ablated, and even respecify the germ line, resulting in fertile adults (Ransick et al., 1996; Yajima and Wessel, 2011). Furthermore, Nanos2 RNA was detected in the archenteron tips in these micromere-ablated gastrulae, and while the expressing cells were not definitively identified, the present results do suggest that Nanos2 expression does occur in somatic cells. We propose that germ line recovery in these embryos was performed by a transfating of Veg2 mesodermal cell population, which expresses Nanos2 downstream of FoxY (Fujii et al., 2009). Whether Nanos2 binds its partner Pumilio in the Veg2 cells and performs its canonical function in translational repression is still under investigation. The somatic cell functions of Nanos in different animals, as well as the contribution of the Veg2 lineage to the fertility of the adult sea urchin, remain fascinating open questions.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ydbio.2019.04.010.

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