RESEARCH ARTICLE

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The Forkhead Transcription Factor FoxY Regulates Nanos

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SUMMARY

FoxY is a member of the forkhead transcription factor family that appeared enriched in the presumptive germ line of sea urchins (Ransick et al. *Dev Biol* 2002;246:132). Here, we test the hypothesis that FoxY is involved in germ line determination in this animal. We found two splice forms of FoxY that share the same DNA-binding domain, but vary in the carboxy-terminal trans-activation/repression domain. Both forms of the FoxY protein are present in the egg and in the early embryo, and their mRNAs accumulate to their highest levels in the small micromeres and adjacent non-skeletogenic mesoderm. Knockdown of FoxY resulted in a dramatic decrease in Nanos mRNA and protein levels as well as a loss of coelomic pouches in 2-week-old larvae. Our results indicate that FoxY positively regulates Nanos at the transcriptional level and is essential for reproductive potential in this organism.

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INTRODUCTION

Forkhead proteins are transcription factors characterized by their highly conserved forkhead (fkh) DNA-binding domains (Kaufmann and Knochel, 1996). They have diverse functions in a wide variety of animals and fungi, including cell fate specification and differentiation, regulation of the cell cycle, and metabolism (reviewed in Carlsson and Mahlapuu, 2002). Forkhead proteins are thought to bind to DNA as monomers, with binding sites consisting of 15–17 bp (reviewed in Carlsson and Mahlapuu, 2002). The number of forkhead-box genes, or Fox genes, correlates with the anatomical complexity of the organism. For example, 4 forkhead transcription factors are found in the yeast Saccharomyces, 22 in the purple sea urchin *Strongylocentrotus purpuratus* (Tu et al., 2006), and 39 in humans (reviewed in Carlsson and Mahlapuu, 2002).

The *Strongylocentrotus purpuratus* FoxY (FoxC-like) gene was first identified by Ransick et al. (2002) in a differential screen for endomesoderm effectors. Ransick et al. compared the mRNA of embryos treated with LiCl, known to

Additional supporting information may be found in the online version of this article.

Abbreviations: hpf, hours post-fertilization; MASO, morpholino antisense oligonucleotide; QPCR, real-time, quantitative PCR; UTR, untranslated region.

"vegetalize" the embryo, to mRNA from "animalized" embryos over-expressing a dominant-negative cadherin that blocks the nuclearization of β-catenin (Miller and McClay, 1997). This approach was used to search for transcription factors involved in endomesodermal specification. The FoxY mRNA localized prominently with small micromeres of the early embryo and in the coelomic pouch of the larvae, a pattern of special interest to us because it overlaps with the localization of both *nanos* and *vasa* mRNAs and proteins (Juliano et al., 2006, 2010b; Voronina et al., 2008). *Nanos* and *vasa* are genes involved in germ line function and in multipotency (Juliano et al., 2010a).

Nanos contains two CCHC zinc fingers and acts with Pumillio as a translational repressor by binding the Nanos response element (NRE) located in the 3′-untranslated regions (3′-UTRs) of Nanos-regulated mRNAs. The role of Nanos was first described in *Drosophila*, where Nanos and Pumilio are required together to translationally repress *hunchback* mRNA to promote posterior patterning (Wharton and Struhl, 1991; Murata and Wharton, 1995). In the sea urchin, Nanos is required to maintain the small micromeres and to form the adult rudiment (Juliano et al., 2010b). While many studies focus on the function of Nanos as a translational repressor, its transcriptional regulation is not well understood. We tested FoxY as a potential regulator of *nanos* based on the previously reported FoxY in situ hybridization pattern (Ransick et al., 2002) and found that FoxY positively regulates *nanos* transcription.

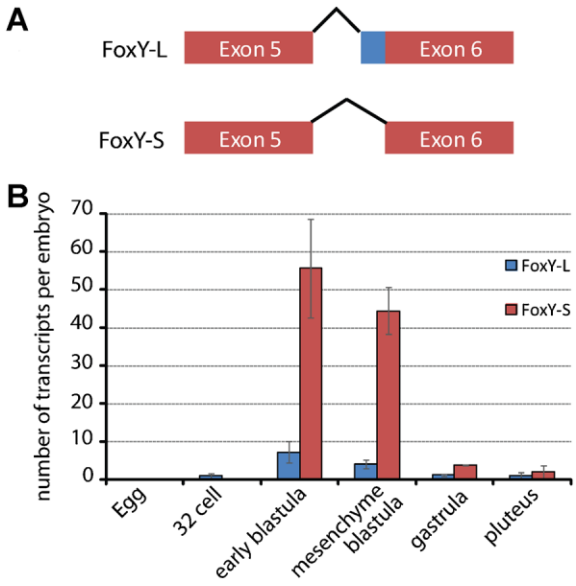

Figure 1. Two splice forms of *foxy*. **A**: *foxy* splice forms both share the same conserved forkhead DNA-binding domain, but differ in the sixth exon near the C-terminal domain of the protein. **B**: QPCR quantitation of FoxY-L and FoxY-S transcripts. Calculation of the estimated number of transcripts is based on ubiquitin levels measured during corresponding developmental stages (Materna et al., 2010).

RESULTS

Two Splice Forms of FoxY

We found two splice forms of *foxy* mRNA (Fig. 1, S1) that share the same DNA-binding region in the amino-terminus. Within the sixth exon, the short form has a segment of 76 nucleotides not present in the long form, resulting in a frame shift of the carboxy-terminus of the protein. As a consequence, the isoelectric point and molecular weight of FoxY-S (short splice form) and FoxY-L (long splice form) differ significantly: 6.56/62.7 kDa and 8.3/71 kDa, respectively.

Both splice forms are most abundant in early blastula and mesenchyme blastula and decrease in gastrula (Fig. 1), which is similar to a previous study detailing a high resolution time course for all forms of *foxy* mRNA (Materna et al., 2010). Using probes against both forms of *foxy*, we observed *foxy* mRNAs accumulate in cells of the small micromere lineage, as indicated by Vasa protein immunolabeling, and in the adjacent non-skeletogenic mesoderm (Fig. 2). By the larval stage, *foxy* mRNA decreases in abundance by real-time, quantitative PCR (QPCR), and is not detectable any longer by RNA in situ hybridization (Fig. 1 and data not shown).

FoxY Protein Is Present Maternally

To identify FoxY protein, we generated two different peptide antibodies against the FoxY splice forms (N- and C-term antibodies) and tested them by both immunoblotting and in situ immunolabeling. By immunoblot analysis, we detected both forms of the FoxY proteins of the expected sizes present in the egg and early developmental stages (Fig. 3). Unfortunately, these antibodies do not label embryos in situ, so we do not know the spatial distribution of the FoxY protein. At this point, we cannot decipher if FoxY-L and FoxY-S have different mRNA and/or protein localizations, since the in situ RNA probes of whole mount embryos cannot distinguish against these splice forms and the antibodies do not work in whole mount immunolabeling.

The peptide sequences used for antibody generation were designed outside of the conserved Forkhead domain. We blasted these peptide sequences in the annotated genome databases (SpBase.org) and do not find similarities to any other protein, including other Fox proteins (data not shown), suggesting that it's unlikely that these antibodies recognize other Forkhead protein family members. By immunoblot, we observed a 48% decrease in the level of FoxY protein in *foxy* morpholino antisense oligonucleotide (MASO)-knockdown embryos (see below), indicating that the antibodies are largely selective, that the knockdown is not complete, and/or that any maternally loaded FoxY protein is stable in the embryo (Fig. 4A). We also tested the efficacy of a *foxy* MASO with reporter constructs containing a complementary sequence to the *foxy* MASO (Fig. 4B,C). Results indicate that only the mCherry reporter construct with the antisense *foxy* MASO sequence was repressed by the *foxy* MASO, whereas a control eGFP

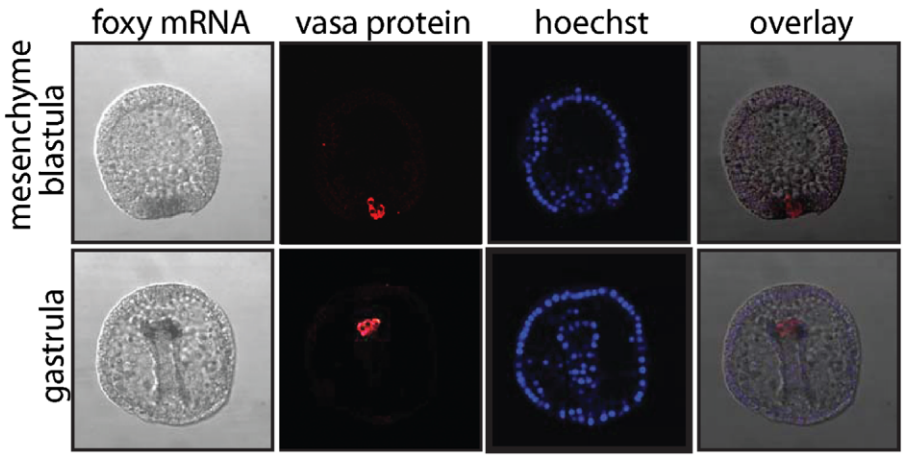

Figure 2. *foxy* mRNA has broader accumulation than the Vasa protein. Embryos were first labeled with a *foxy* mRNA in situ probe, followed by immunostaining for Vasa protein (red). *foxy* mRNA has broader expression in the non-skeletogenic Vegetal-1 (Veg1) and Veg2 regions.

reporter construct without the target *foxy* sequence co-injected into the embryo was not affected by the presence of the MASO (Fig. 4B,C). This result suggests that the injected *foxy* MASO is specifically binding to the target sequence and is functional in the embryo.

Knockdown of FoxY Leads to a Decrease in *nanos* mRNA and Protein

To test the function of FoxY, we knocked down FoxY in embryos. We first assayed the level of *foxy* mRNAs in these embryos at 11-, 17-, and 24-hr post-fertilization (hpf) by QPCR, and observed that FoxY positively regulates its own mRNA (Fig. 5).

Next, we examined the effect of knockdown on Nanos2, which is present exclusively in the small micromere lineage. *nanos* mRNA was not significantly altered with FoxY knockdown at 11 and 17 hpf; by 24 hpf, however, *nanos* mRNA was significantly decreased by QPCR (Fig. 5A). A fluorescent dye was coinjected with the *foxy* MASO as a proxy of the amount of *foxy* MASO injected into the newly fertilized embryo, so we were also able to determine by in situ hybridization that *nanos* mRNA quantities remaining in an embryo depended on the amount of *foxy* MASO introduced (Fig. 5B). *foxy* knockdown embryos also showed a significant decrease in Nanos protein by 24 hpf (Fig. 6).

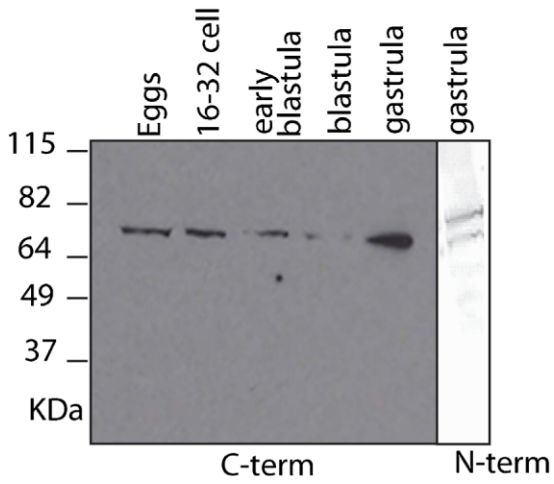

Figure 3. FoxY protein expression. FoxY protein isoforms are detected in the egg and embryos of various developmental stages. The C-term antibody specifically detects FoxY-S, whereas the N-term antibody detects both forms.

FoxY Knockdown Leads to a Loss of Coelomic Pouches

FoxY depletion in the embryo resulted in no obvious developmental defects up to the pluteus stage. After 2 weeks of culturing, however, the overall size of the larvae was significantly smaller than the mock-injected embryos of the same age (Fig. 7). In addition, coelomic pouches that were present in the pluteus stage regressed and eventually were lost. This *foxy*-knockdown phenotype is similar to the *nanos*-knockdown phenotype (Juliano et al., 2010b), suggesting that FoxY is epistatic to Nanos.

DISCUSSION

FoxY Splice Variants May Have Different Downstream Regulatory Effects

Results indicated that the two splice forms of *foxy* are present in the egg and all early developmental stages.

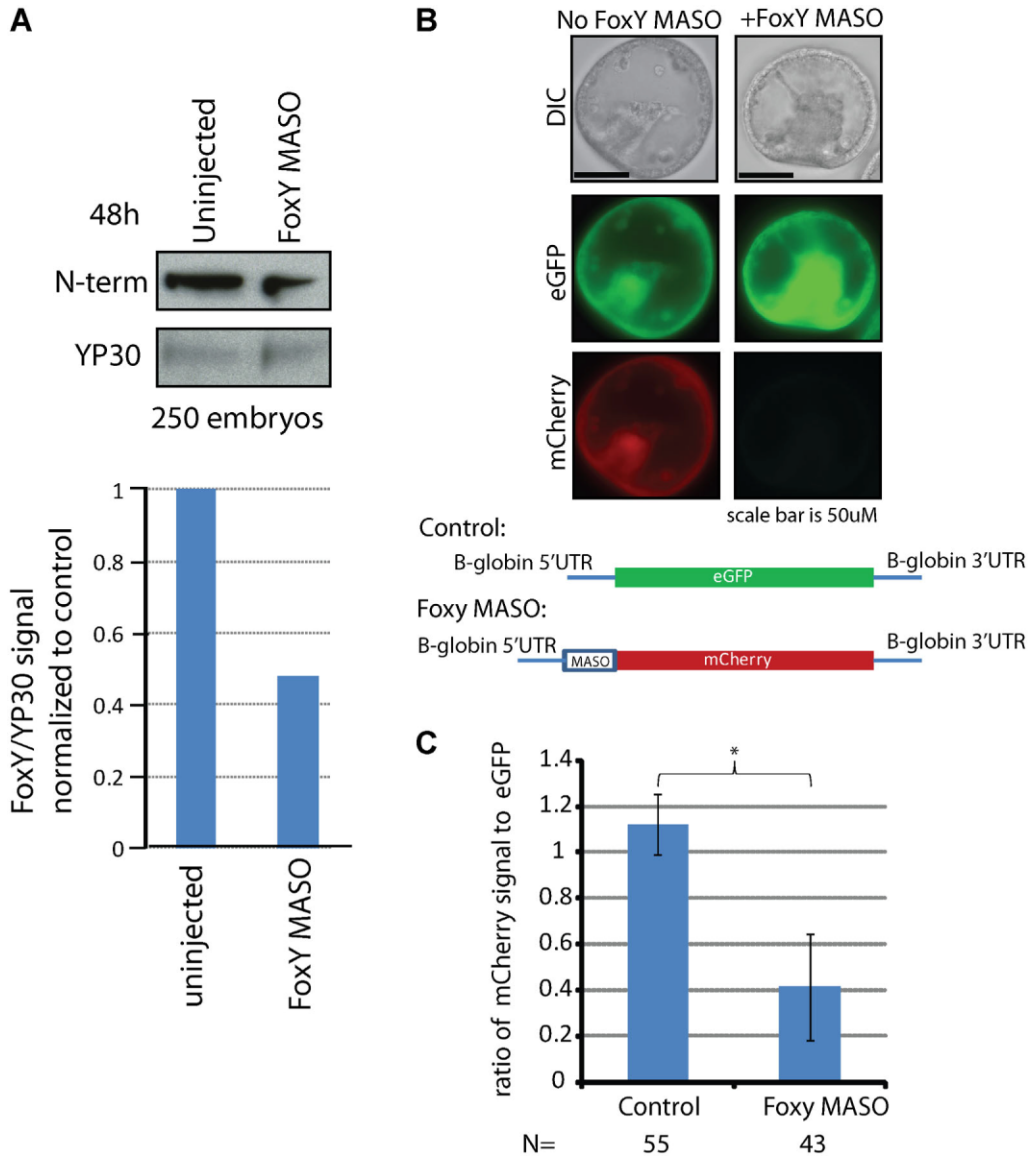

Figure 4. *foxy* MASO control experiment. **A**: FoxY protein levels decreased by approximately 50%, as measured by densitometry using imageJ. FoxY protein was normalized to YP30 yolk protein. **B**: mCherry reporter transcript containing complimentary sequence to the *foxy* MASO was repressed in the presence of *foxy* MASO, whereas the control eGFP reporter, whose coding sequence was flanked by β-globin UTRs, was expressed in the presence or absence of *foxy* MASO. **C**: The fluorescence level of mCherry was normalized to eGFP signals to adjust for the amount of injected mRNAs. The difference in mCherry/eGFP fluorescence measurements observed in control versus *foxy* MASO-treated sets was statistically significant ($P = 1.89658 \times 10^{-24}$, Student's *t*-test).

These two splice variants have different carboxy-terminal amino acid composition, thus each variant has its own *trans*-activation/repression domains and may differentially regulate its target genes. The functional difference between these two FoxY isoforms is currently not known, although previous studies have shown that the function of forkhead proteins may depend on their phosphorylation sites (Tan et al., 1998; Chen et al., 2009; Singh et al., 2012). As FoxY-L has 7 additional predicted serine phosphorylation sites and 1 additional threonine and tyrosine phosphorylation sites, we speculate that the FoxY transcription factor isoforms may recruit different cofactors or have its activity modified, depending on the status and location of its phosphorylation sites.

FoxY Knockdown Results in a Decrease of *nanos2* mRNA and Protein

Both forms of *foxy* mRNA peak around the blastula stage (Fig. 1), and FoxY protein is present in great abundance in

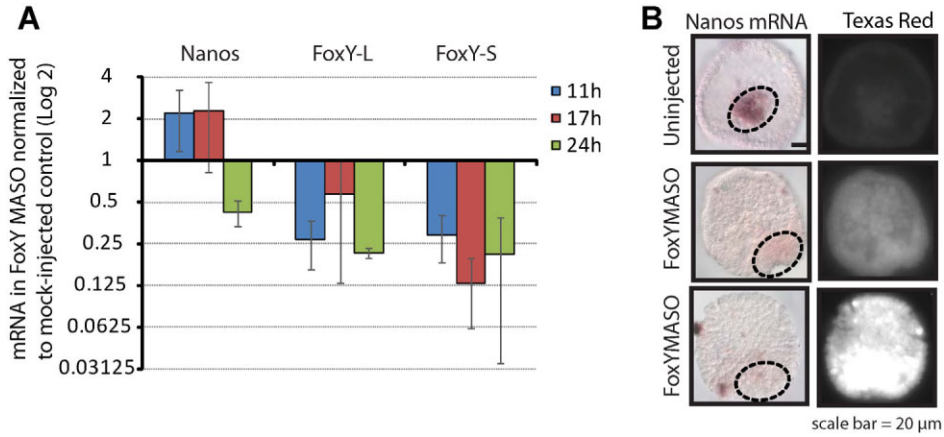

Figure 5. *foxy* knockdown results in a decrease of *nanos* mRNA at 24 hpf. **A**: QPCR was used to measure the level of transcript levels in *foxy* MASO-injected embryos. Transcript levels of *nanos*, *foxy-L*, and *foxy-S* were assayed at 11, 17, and 24 hpf. Results are normalized to mock-injected (Texas Red) controls. **B**: Whole mount RNA in situ hybridization was used to detect the level of *nanos* mRNAs in control and *foxy* MASO-injected embryos at 24 hpf. The depletion in *nanos* mRNA is dependent on the amount of *foxy* MASO injected (indicated by the dashed circle), as reflected by the intensity of co-injected Texes Red dextran.

the egg (Fig. 3). From previous studies, *nanos* mRNA and protein are also abundant in the oocytes and only later are detected in the small micromere descendants of the embryo (Juliano et al., 2006, 2010b). Although several studies have focused on the post-translational regulation of Nanos, the *cis*-regulatory transcriptional control of the *nanos* gene is mostly unknown. Recent study of *Drosophila nanos* identified an enhancer element located between −108 and +97 of its transcription start site that is sufficient to drive Nanos-GFP expression in the germline stem cells (Ali et al., 2009). Maternal transcription factors Ovo and initiation complexes bip2 and Trf2 are required for germline-specific gene expression of *nanos* and *vasa* in the germ plasm in *Drosophila* (Yatsu et al., 2008). Ovo is a transcriptional activator involved in female germline maintenance in *Drosophila* (Oliver et al., 1987; Mevel-Ninio et al., 1991; Staab and Steinmann-Zwicky, 1996). The sea urchin *ovo* mRNA is localized to the vegetal plate of the mesenchyme blastula, but is undetectable later (Juliano et al., 2006). Since *nanos* mRNA is upregulated in the early blastula stage

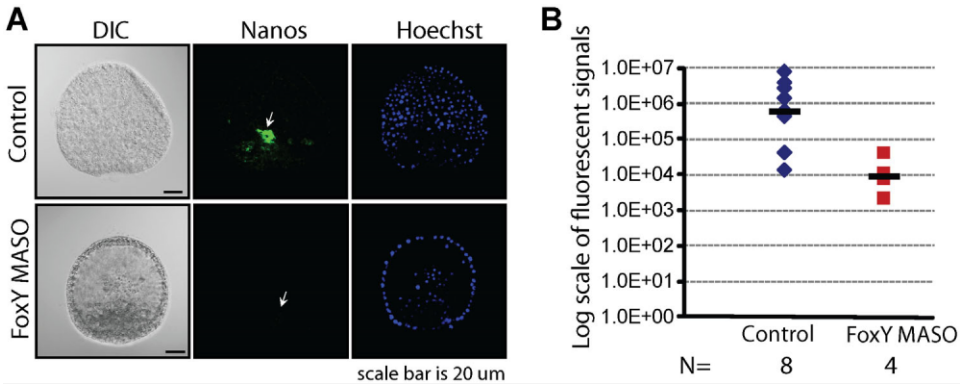

Figure 6. *foxy*-knockdown embryos have decreased Nanos protein. Embryos injected with *foxy* MASO were immunolabeled with the Nanos antibody at the blastula stage. **A**: Fluorescent signals using the Nanos antibody (green) were quantitated by pixel intensity in uninjected and *foxy* MASO-treated blastulae. The Hoescht dye indicates nuclear staining. **B**: Fluorescent signals were quantified with Metamorph as described in the Methods and Materials Section. The horizontal bar indicates average fluorescent signals ($P = 0.06$ by the Student's *t*-test).

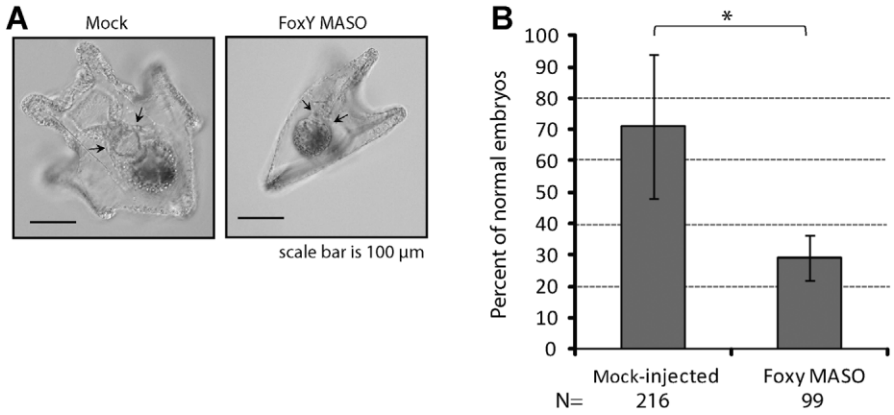

Figure 7. *foxy* MASO-injected embryos are smaller and lose their coelomic pouches in the late larval stage (2 weeks after fertilization). **A**: *foxy* MASO-injected embryos were smaller in size and have a loss of coelomic pouches. **B**: Three independent sets of embryos were injected with Texas Red control or *foxy* MASO and cultured for 2 weeks. The *foxy* MASO-injected embryos have significantly fewer normal embryos. Normal embryos are defined as having six arms, skeletal structures, a functional gut, and coelomic pouches. All abnormal embryos have a loss of ceolomic pouches. Student's *t*-test was used to determine the significance of the knockdown compared to the mock control ($P = 0.038$).

and maintained at the same levels until the pluteus stage, other transcription factors are likely involved in the transcriptional activation of *nanos*. The majority of forkhead proteins bind to a seven-nucleotide core consensus sequence (RYMAAYA [(R = A or G; Y = C or T; M = A or C)]) that is found several times in the *nanos* promoter, suggesting that forkhead transcription factors can directly regulate *nanos* (Overdier et al., 1994; Pierrou et al., 1994; Kaufmann et al., 1995). *Nanos* is also subjected to post-transcriptional control, such as translational regulation by RNA localization, as observed in *Drosophila* (Gavis and Lehmann, 1994) and by Dead end RNA-binding protein in zebrafish (Kedde et al., 2007); microRNA-mediated deadenylation (Mishima et al., 2006); and translational repression by a RNA secondary-structure element, as in *Xenopus* (Luo et al., 2011).

Functions of FoxY in Adulthood and Embryogenesis

The small micromere descendants that become part of the coelomic pouches contribute to the adult rudiment. In adult tissues, *foxy* mRNA is present in the gonads and its protein is present in the ovary and the egg (data not shown and Fig. 3). FoxY may function during oogenesis (in adult tissues) and in early development by regulating *nanos*. It may also regulate endomesodermal genes. Previous studies indicated that *foxy* mRNA is found only in the small micromeres (Ransick et al., 2002; Materna and Davidson, 2012); however, using Vasa protein as a reference for small micromeres (Voronina et al., 2008), we find that *foxy* mRNA has a broader distribution within the small micromeres and in the adjacent non-skeletogenic mesoderm in the blastula and gastrula stages (Fig. 2). This suggests that FoxY may potentially regulate other genes within other lineages, although one dominant function appears to be the transcriptional regulation of *nanos*.

MATERIALS AND METHODS

Animals

Adult *Stronylocentrotus purpuratus* were obtained from Patrick Leahy (Point Loma Invertebrate Lab, Lakeside, CA) and kept in 15°C artificial seawater until needed. Artificial seawater was generated from Instant Ocean (Aquarium Systems, Mentor, OH). Eggs and sperm were collected by intracoelomic injection of 0.5 M KCl. Eggs were collected in filtered seawater and sperm was collected dry.

Cloning of *foxy* Spliced Forms

Nested *foxy* primers were used in the cloning of full-length *foxy* in a PCR reaction using gastrula cDNA. Primers used in the first and second rounds of PCR were: FoxY-5′-103For (5′-CTGCACTGACTCTGCCTACA) with FoxY-3′ + 2038Rev (5′-TGTCTGTTGGATCCAGCAGT) and FoxY5′-77For (5′-GCTTCACAAATCTCGCCTCA) with FoxY-TGAend (5′-TCACATACTGTGTATTCGTGT). Splice forms were detected using nested primers FoxY-F1 (5′-CATCCTAACTTGCCATGCAC) with 3′FoxY-3′end (5′-TCACATACTGTGGTATTCGTGT) and FoxYF2 (5′-TTTGATCAGTGCGATCGACTC) with FoxY R1 (5′-CTTGAGATCGTCCTGATGTC) using cDNAs from the ovary, egg, 4-cell, 32-cell, early blastula, mesenchyme blastula, gastrula, and pluteus. PCR products were run on 1.5% agarose gel. Bands were cut out, gel purified using QiaQuick columns (Qiagen, Inc., Valencia, CA), and cloned

into pGEM T-easy (Promega, Madison, WI). Recombinant clones were sequenced.

Whole Mount In Situ Hybridization

foxy antisense probes were labeled with digoxigenin (DIG RNA Labeling Kit (SP6/T7); Roche Diagnostics Corporation, Indianapolis, IN). PCR primers used to clone the RNA probe were Fox-1F (5′-ATGGAGGACCAGGAGGATGATG) and Fox3′Rev (5′-GAATTCACTTTGATGATGTACGCTCAGGTC). The plasmid was linearized with *Bam*HI and in vitro-transcribed with Sp6 RNA polymerase. This probe was used to detect native transcript in eggs and embryos at a concentration of 0.1 μg probe/ml, according to previously published protocols (Minokawa et al., 2004).

Real-Time, Quantitative PCR (QPCR)

A total of 500 uninjected or 100 injected embryos were collected at various time points, and total RNA were extracted from them using the Qiagen RNeasy microkit according to manufacturer's instructions (Qiagen, Inc.). cDNA was amplified using the TaqMan Reverse Transcription Reagents kit (Applied Biosystems, Foster City, CA). QPCR was performed using the 7300 Real-Time PCR system (Applied Biosystems). QPCR primers were designed using the Primer3 program (Rozen and Skaletsky, 2000). Ten uninjected or two injected embryo-equivalents were used for each QPCR reaction with the SYBER Green PCR Master Mix (Invitrogen, Carlsbad, CA). The estimated numbers of transcripts are calculated based on the level of ubiquitin in various developmental stages, as described previously (Materna and Davidson, 2012). For injected embryos, results were first normalized to ubiquitin levels and expressed as fold-difference compared to the uninjected eggs. Primers QPCR-L-For (5′-CTTCTGATCGAATCCATGCC) and QPCR-exonRev (5′-TTGTCGGATGTGTGTCTGGA) were used to detect FoxY-L and primers QPCR-S-F (5′-GATGATGAGAGACAACCAAC) and QPCR-exonRev were used to detect FoxY-S.

Antibody Generation

Antiserum was raised in rabbits against polypeptides indicated in Figure S1. N-term (DAIRATDVTAESRHC) and C-term (CPPSPFTTVSPPDTH) peptide polyclonal antibodies were generated by Sigma Genosys (Woodlands, TX). For affinity purifications, peptides were immobilized using the Pierce AminoLink Plus Immobilization Kit (ThermoFisher Scientific, Rockford, IL) according to the manufacturer's instructions. Heat-inactivated serum was passed over the antigen-immobilized column, and bound antibodies were eluted with 1 ml of 100 mM glycine (pH 2.5) into 50 μl of 1 M Tris (pH 9.5).

Fluorescence Quantification

Confocal Z-stack images were collected on an LSM 510 laser scanning confocal microscope (Carl Zeiss, Inc., Thornwood, NY). Projection images of the Z-stacks were generated for quantitative analysis. Quantitative pixel-intensity analysis of whole embryos was conducted using the Metamorph program (Molecular Devices, Downingtown, PA) to determine the levels of Nanos2 immunolabeling in untreated, mock, and *foxy* MASO-injected embryos.

Western Blotting

Embryo extracts were prepared in 2× sample buffer with 1 mM dithiothreitol and boiled for 5 min. Samples were separated by one-dimensional polyacrylamide gel electrophoresis (PAGE) in pre-cast 4–20% polyacrylamide Tris–glycine gels (Nu-Sep Incorporated, Lawrenceville, GA). Total protein was transferred onto nitrocellulose membrane (Fisher Scientific, Pittsburgh, PA) and blocked with Blotto [3% non-fat dry milk (w/v), 170 mM NaCl, 50 mM Tris, 0.05% Tween20 (v/v) pH 8]. Ten micrograms of each affinity purified N-term and C-term primary antibody were diluted separately in 3 ml Blotto, followed by either alkaline phosphatase- or horseradish peroxidase-conjugated secondary antibodies diluted in Blotto [1:5,000 dilution (v/v); Jackson Immunoresearch Laboratories Incorporated, West Grove, PA]. Blots were washed two times with Blotto for 15 min, followed by one wash in 1× phosphate-buffered saline (PBS)—0.5% Tween20. Immunocreactivity was detected in developmental series by the alkaline phosphatase colormetric reaction using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) for the N-term antibody blot (Fig. 3). For the C-term antibody blot (Fig. 3) and the *foxy* MASO Western blots, proteins were detected by horseradish peroxidase-dependent chemiluminescence using luminol/hydrogen peroxide. The FoxY protein was normalized to the yolk protein YP30 and quantified by densitometry using Image J (Abràmoff et al., 2004).

Microinjection

Morpholino antisense oligonucleotides (MASO) against *foxy*, (5′-CATGGCTCCAAGTGCAGAACACTAC) was ordered from GeneTools (Philomath, OR). Microinjections were performed as previously described (Cheers and Ettensohn, 2004), with modifications. MASO oligonucleotides were resuspended in sterile water and heated to 60°C for 10 min prior to use. Injection solutions contain 20% sterile glycerol, 2 mg/ml 10,000 MW Texas Red lysine charged dextran (Molecular Probes, Carlsbad, CA), and varying concentrations of MASO. Eggs were dejellied in acidic seawater (pH 5.2) for 10 min on ice, followed by seawater washes. Dejellied eggs were rowed onto protamine sulfate-coated (4% w/v) $60 \times 15\,mm^2$ petri dishes. Eggs were fertilized with sperm in the presence of 1 mM 3-amino-triazol (Sigma, St. Louis, MO). Injections were performed using a Femto Jet® injection system (Eppendorf, Hamberg, Germany). Injection needles were pulled from $1 \times 90\,mm^2$ glass capillaries with filaments (Narashige, Tokyo, Japan) on a vertical needle puller (Narashige, Tokyo, Japan). The injected *foxy* MASO corresponds to approximately 12 nM.

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