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Report

Nanos Is Required in Somatic Blast Cell Lineages in the Posterior of a Mollusk Embryo

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Summary

During animal development, blast cell lineages are generated by repeated divisions of a mother cell into a series of daughter cells, often with a specific series of distinct fates. Nanos is a translational regulator that is involved in germline development in diverse animals [1–4] and also involved in somatic patterning in insects [5, 6]. Recently, Nanos was found to be required for maintenance of stem cell divisions in the *Drosophila* germline [7, 8]. We have found that in the mollusk *Ilyanassa*, Nanos messenger RNA and protein are specifically localized in the mesendodermal blast cell lineage derived from the strongly conserved 4d cell. Nanos activity is required for differentiation of multiple tissues that are derived from the 4d cell, showing that IoNanos is required for somatic development in this embryo. At the cellular level, we show that IoNanos activity is required for the highly stereotyped cleavage pattern of the 4d lineage, the proliferative capacity of the blast cells, and the marked asymmetry of the blast cell divisions. These results suggest that IoNanos is involved in regulating blast cell behaviors in the 4d lineage.

Results and Discussion

We recovered an *Ilyanassa obsoleta* ortholog of Nanos (IoNanos) while screening unsequenced embryonic complementary DNA (cDNA) clones by in situ hybridization for patterns of localization in the early embryo [9]. The IoNanos messenger RNA (mRNA) exhibited a specific pattern of RNA distribution during cleavage stages that suggested a function in the lineage of the 4d cell (this lineage is shown in Figure 1). During the first five cleavage cycles, the IoNanos RNA is found in all cells of the embryo (Figure S1 available online). However, between the 24- and 36-cell stages, the pattern of RNA distribution becomes restricted to two cells (Figure 2C): the yolk-rich 4D macromere, which is nutritive and dispensable for normal development [10], and the 4d micromere. Among the many protostome phyla with spiralian development, the 4d micromere, or mesentoblast, is one of the most striking developmental commonalities [11]. In mollusks and most other spiralians, the 4d cell divides bilaterally, and the daughter cells become teloblasts, executing a series of asymmetric cell divisions that generate a number of smaller cells with mesodermal and endodermal fates. In the veliger larva of gastropods, this lineage develops into the visceral mesoderm (including the heart and several other organs), a portion of the main larval retractor muscle, and the intestine, or hindgut [12, 13].

The IoNanos mRNA is found in a subset of 4d derivatives during the first three rounds of division in this lineage (Figures 2C, 2E, and 2G). We generated polyclonal antibodies against the IoNanos protein and examined the patterns of protein distribution in early cleavage. The protein largely followed the pattern of RNA localization (Figure 2 and Figure S2). During the first five cleavage cycles, the protein is present in all cells, but between the 24- and 36-cell stages, the protein becomes increasingly enriched in 4d. During cleavage of 4d derivatives, IoNanos protein is detected in a subset of these cells. After the fourth division in the 4d lineage, the protein is segregated into the $4d^{L\&R112}$ cells, where it accumulates in the nuclei. After this stage, both the mRNA and the protein are undetectable for the remainder of early cleavage and gastrulation.

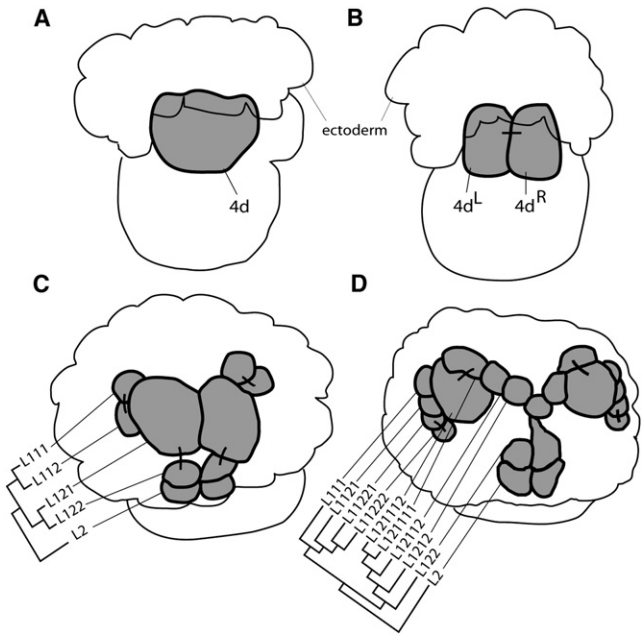

Figure 1. The Cell Lineage and Cleavage Pattern of the Mesentoblast Cell 4d in *Ilyanassa*

The cells descended from 4d are shown in gray, and the other cells in the embryo are outlined for orientation. The sister cells from the immediately preceding divisions are indicated by hatch marks.
(A) Twenty-eight-cell embryo after the birth of 4d.
(B) Two hours after the birth of 4d (4d + 2 hr), the cell has divided along the embryo's axis of bilateral symmetry to generate $4d^L$ and $4d^R$.
(C) At 4d + 8 hr, the 4d lineage has five cells on each side, and each telobast has divided three times: once toward the vegetal pole (to produce $4d^{L\&R2}$), once toward the animal pole (to produce $4d^{L\&R11}$, whose daughters $4d^{L\&R111}$ and $4d^{L\&R112}$ are shown), then once again toward the vegetal pole (to produce $4d^{L\&R122}$).
(D) At 4d + 24 hr, each teloblast has divided three more times to make a band of cells that leads back toward the future dorsal-posterior side of the embryo. In order of birth, these cells are $4d^{L\&R1212}$, $4d^{L\&R12112}$, and $4d^{L\&R121111}$. The cells $4d^{L\&R112}$ have divided, and their daughters $4d^{L\&R1122}$ have also divided, making four cells on each side that are derived from the $4d^{L\&R11}$ cells. The cleavage pattern and nomenclature is based on [29] and unpublished data (X.Y.C. and J.D.L.).

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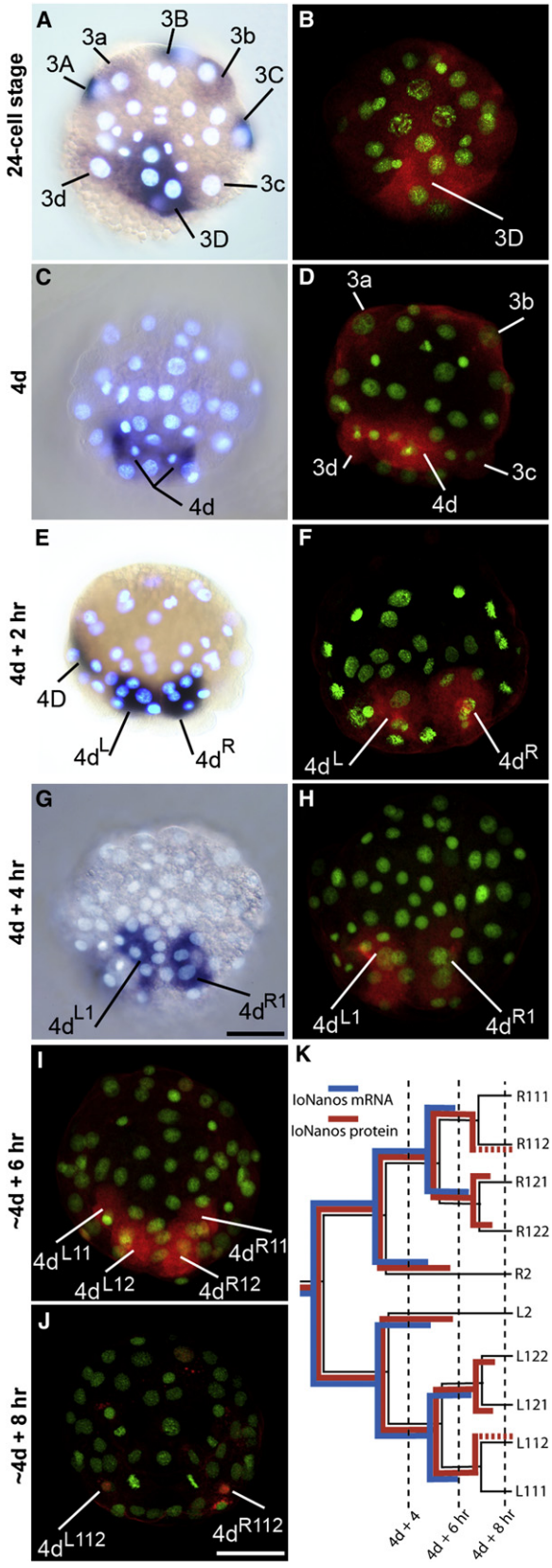

Figure 2. IoNanos RNA and Protein Are Localized to the 4d Lineage

(A, C, E, and G) RNA is detected by in situ hybridization and then chromogenic detection (blue black), and nuclei are stained with DAPI (bright blue). (B, D, F, H, I, and J) The IoNanos antibodies were detected with a fluorescent secondary (red), the nuclei were stained with YOYO-1 (green), and projections were constructed from Z stacks of each embryo. In all figures, the animal pole is toward the viewer, and the D quadrant is down.

(A) After the fifth cleavage cycle (24-cell stage) the RNA is most abundant in the cytoplasm of the 3D macromere cell and is also found on the centrosomes of the other macromeres (3A, 3B, and 3C). The message is also detected in the cytoplasm of the third quartet micromeres (3a–3d).

(B) The protein at this stage is found throughout the embryo but is most abundant in 3D.

(C) After the division of 3D into 4d and 4D, IoNanos RNA is found in 4d (in telophase in this embryo, with the DNA of the daughter cells indicated) and 4D (not visible in this view).

(D) The protein is abundant in the newly born 4d cell but is also found elsewhere in the embryo, including the third-quartet micromeres 3a–3d.

(E) After the division of 4d (4d + 2 hr), the RNA is abundant in its two daughter cells, $4d^L$ and $4d^R$, and detectable in 4D. Nuclei of multiple ectodermal cells overlie the staining in $4d^L$ and $4d^R$.

(F) The protein at 4d + 2 hr is largely restricted to the daughter cells $4d^L$ and $4d^R$.

(G) After the next cleavage cycle in the 4d lineage (4d + 4 hr), the RNA is again found in two cells of the 4d lineage, $4d^{L1}$ and $4d^{R1}$. Again, nuclei of multiple ectodermal cells overlie the staining in these two cells.

(H) Like the RNA at 4d + 4 hr, the protein is restricted to $4d^{L1}$ and $4d^{R1}$.

(I) After another cleavage cycle in the 4d lineage (~4d + 6 hr), the protein is found in $4d^{L11}$, $4d^{L12}$, $4d^{R11}$, and $4d^{R12}$.

(J) After an additional cleavage cycle in the 4d lineage (~4d + 8 hr), the protein is largely restricted to the nuclei of the cells $4d^{L112}$ and $4d^{R112}$. The scale bar represents 50 μm.

(K) Summary diagram of IoNanos RNA and protein distribution in the 4d lineage. The dotted red lines indicate protein localization in the nucleus.

To test the function of IoNanos in development, we devised for the *Ilyanassa* embryo a robust pressure-injection method, which has been lacking in this system, and designed a morpholino oligo against the predicted translational start site of the mRNA. Injection of IoNanosMO into zygotes caused reproducible dose-dependent phenotypes in the larvae (Figure 3 and Figure S3; Table 1). Injection of 0.1 mM solutions (approximately 1 uM after injection) always resulted in larvae that lacked all of the derivatives of 4d that can be reliably scored. These animals never had hearts, normal larval retractor muscles, or intestine. In contrast to these endomesodermal derivatives, ectodermal structures were largely normal. IoNanosMO larvae had two velar lobes, and most had two eyes. They developed relatively normal shells, but these were smaller than controls and rotated counterclockwise relative to the head and foot. Development of the larval foot was variable, with many larvae having foot structures like statocysts or operculum but poor organization of the foot as a whole. Several lines of evidence indicate that this phenotype results from specific knockdown of IoNanos activity in the embryo. First, injection of 10-fold higher concentrations of a five-mismatch control morpholino oligo results in wild-type larvae (Figure 3 and Table 1), and morpholinos targeted to other RNAs give different phenotypes (J.S.R, X.Y.C., and J.D.L., unpublished data), indicating that these phenotypes are not generic effects of morpholino oligos. Second, levels of IoNanos protein were strongly reduced in embryos injected with 0.1 mM IoNanosMO at time points after the birth of 4d, indicating that IoNanos translation was repressed (Figure S4). Finally, zygotic injection of RNA antisense to the IoNanos transcript generated an identical phenotype to the 0.1 mM IoNanosMO injection (Figures S3 and S5; Table 1). Injection of this RNA results in the loss of detectable IoNanos protein but not mRNA, suggesting that it inhibits translation. Injection of RNAs that are antisense to other transcripts give different phenotypes (J.S.R and J.D.L., unpublished data). Taken together, these results suggest that loss of IoNanos activity in

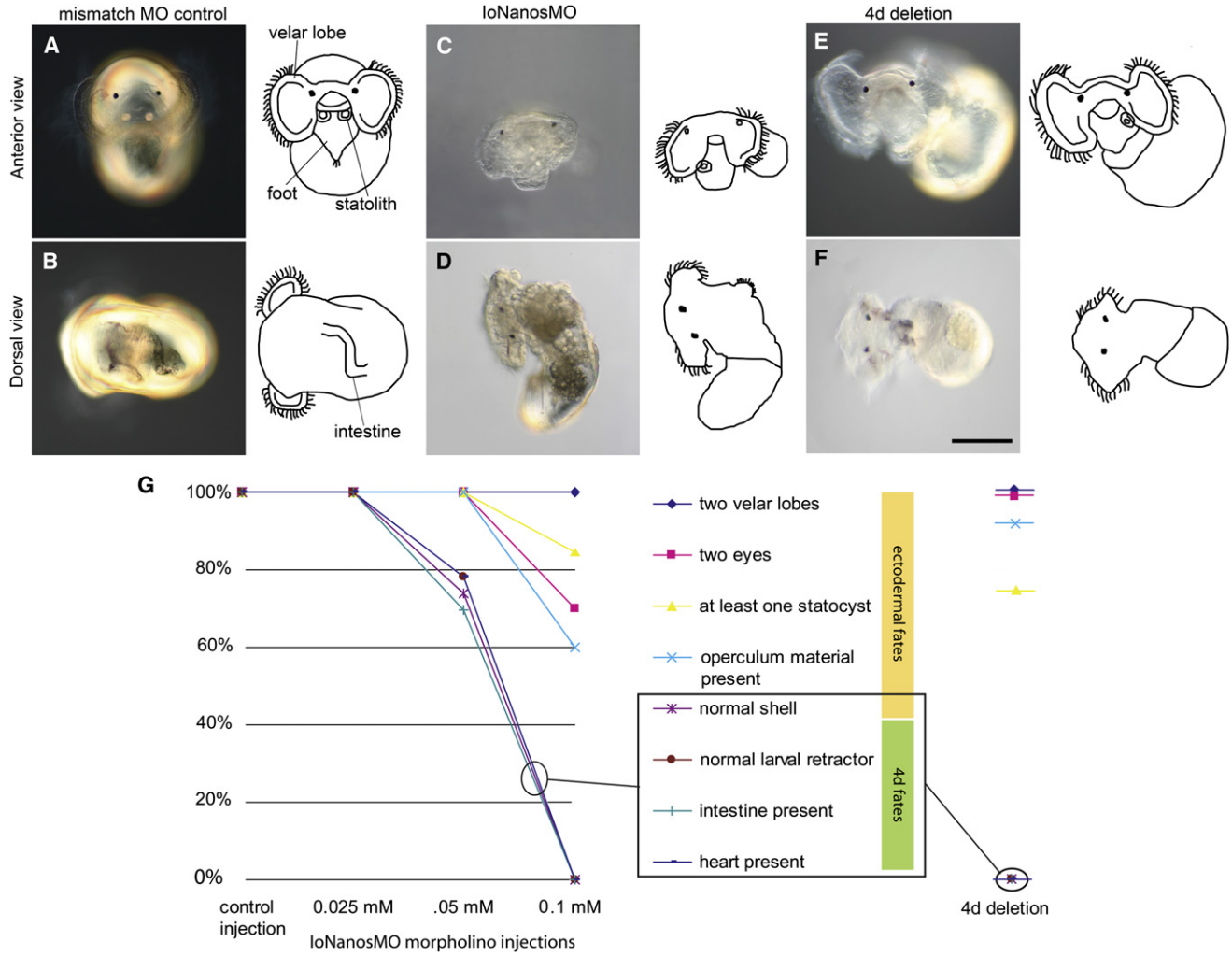

Figure 3. The Effects of IoNanos Knockdown and 4d Deletion on Various Larval Organs

(A and B) Anterior (A) and dorsal (B) views of 7-day-old larvae showing normal development after zygotic injection of 1 mM 5-mismatch control morpholino. The intestine is naturally pigmented.

(C and D) Larvae from zygotes injected with 0.1 mM IoNanosMO. These animals are lacking intestine and heart (scored in live animals) and have larval retractor muscles that are reduced compared to controls (Figure S3). They have relatively normal heads, except that the velar lobes are smaller than controls and eyes are sometimes missing. They have small shells that are rotated to the left side compared to controls and variable development of foot structures.

(E and F) Larvae that developed after ablation of the 4d micromere also lack intestine, heart, and normal retractor muscles. They also have variable development of head and foot structures, as well as shells that are rotated to the left side. The scale bar represents 100 μm.

(G) All organs that can be reliably scored in the larva were examined in 20–23 larvae from three different clutches for each treatment, and the fraction of larvae with the indicated tissue or condition was plotted. The velum and eyes derive mainly from the first quartet of micromeres. The shell derives mainly from the second quartet, and the foot structures like operculum and statocysts derive from the third quartet. The heart and retractor muscle are mainly derived from 4d, and the intestine is derived completely from 4d. Because the heart data was recorded before fixation and larvae were sometimes lost during fixation or processing, the fraction with hearts for each treatment is normalized to the number of animals scored after fixation.

the embryo blocks normal development of 4d derivatives and has less severe and less penetrant effects on some ectodermal organs, especially the foot.

Because the IoNanos knockdown seemed to have specific effects on 4d-derived organs, we directly compared this phenotype to the effects of 4d ablation. We killed 4d with a glass needle, removed the cell, and scored the larvae in parallel to the IoNanosMO animals. As expected from lineage tracing and ablation data [13, 14], 4d deletion embryos were always lacking heart, intestine, and normal retractor muscle (Figure 3 and Figure S3; Table 1). More surprisingly, these larvae also had some mild ectodermal defects that would not be predicted from the clonal contribution of 4d. Their shells were sometimes smaller and rotated counterclockwise relative to the head, and the development of the foot mass was often perturbed. Thus, the 4d ablation phenotype is very similar to the effect of 0.1 mM IoNanosMO injection and shows that 4d is required for the normal development of some ectodermal structures. Some of the effects on ectodermal structures might reflect events in organogenesis. The loss of retractor muscle might cause the abnormal shell orientation by perturbing the process of torsion [15], and other defects in the shell might reflect requirements for 4d-derived tissues like intestine in normal shell development [16]. However, the effects of 4d ablation on the foot structures are not easily explained as a secondary effect of losing 4d-derived structures and are more likely caused by a failure of 4d to complete organizer signaling in the early embryo. Organizer signaling is a well-established

Table 1. Differentiation of Larval Tissues after IoNanos Knockdown or 4d Deletion

	Two Velar Lobes	Two Eyes[a]	Normal Foot	No Statocysts	One Statocyst	Two Statocysts	Operculum Material Present	Normal Shell	Small, Cup-Shaped Shell or Better	Retractor Muscle Present	Retractor Muscle Normal Size	Heart[b]	Intestine
Control injection	20/20	20/20	20/20	0/20	0/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20
1 mM 5-mismatch MO[c]	16/16	16/16	16/16	0/16	0/16	16/16	16/16	16/16	16/16	16/16	16/16	16/16	16/16
0.025 mM IoNanosMO	21/21	21/21	21/21	0/20	0/20	21/21	21/21	21/21	21/21	21/21	21/21	21/21	21/21
0.05 mM IoNanosMO	23/23	23/23	16/23†	0/23	0/23	23/23	23/23	17/23[d]	23/23	23/23	16/23[d]	18/23[d]	16/23[d]
0.1 mM IoNanosMO	20/20	14/20	1/20	3/20	12/20	5/20	12/20	0/20	20/20	20/20	0/20	0/20	0/20
4d deletion	24/24	24/24	4/24	6/24	6/24	12/24	22/24	0/24	24/24	24/24	0/24	0/24	0/24
IoNanos antisense RNA	23/23	17/23	0/23	3/23	15/23	5/23	22/23	0/23	23/23	22/23	0/23	0/23	0/23

[a] All larvae had at least one eye.
[b] Larvae were scored for hearts while living, and the same larvae were not necessarily scored for other organs after fixation.
[c] One animal was completely abnormal; it lacked all structures scored, and was not counted in this series.
[d] All of the animals with missing or abnormally developed tissues in the 0.05 mM injections series were from one of the three capsules used.

role for the mother cell of 4d, the 3D macromere [10, 17], but has not been generally appreciated for 4d. However, one report of 3D ablations done shortly before the cell divides indicates that it does not complete induction, implying that induction might be completed by 4d [18]. Also, ERK1&2 MAPK activation, which is required for organizer function, is observed in 4d [17]. In the closely related snail *Crepidula fornicata*, all of the organizer signaling seems to be carried out by 4d [19].

There are differences between the effects of IoNanos knockdown and 4d deletion. Most notably, knockdown results in smaller velar lobes and loss of eyes in some cases. These effects are not seen after 4d deletion (Figure 3 and Table 1). One possible explanation is that in the deletion experiments, 4d is intact for 10–15 min after its birth (to ensure that we do not kill its sister cell 4D), and 4d could be signaling in this interval. Another possible explanation is that IoNanos expression observed before 4d in other lineages might be required for normal development.

The patterns of RNA and protein localization, in combination with the congruence of the IoNanosMO phenotype with the 4d fate map and deletion data, indicate that IoNanos is required for normal development of the 4d lineage in *Ilyanassa*. This is striking because clear roles for Nanos in the development of somatic tissues have not been found outside of insects. Our results are similar to those recently reported in the leech *Helobdella robusta*, where Nanos knockdown causes embryonic arrest during gastrulation and defects in the behavior of the ectodermal and mesodermal germinal bands, which are derived from teloblast cells [20]. Thus, it might be that the role of Nanos in somatic patterning dates to the base of the spiralian clade, which makes up a large part of the lophotrochozoan assemblage [21]. The similarities between the role of Nanos in spiralians and insects [5, 6] suggest that it might have been in place at the base of the protostome bilaterians. Notably, the 4d lineage roughly corresponds to the posterior of the fate map during gastrulation in spiralians.

Nanos expression is associated with the germline across the Metazoa, and in some cases, Nanos function has been shown to be required for germline development [1–4]. In some spiralians, the germline appears to derive from 4d [22, 23]. However, it is not clear whether the germline is specified during embryogenesis in *Ilyanassa*. Primordial germ cells have not been identified in the larva, and expression of a germline marker, Vasa, does not persist after the first few divisions of 4d [24]. Because we currently cannot assess the differentiation of germline derivatives, the question of the effect of Nanos knockdown on the germline remains open.

The effect of IoNanos knockdown on multiple organs derived from 4d suggests that the protein might be required for fundamental properties of this lineage rather than simply acting as a switch for a particular fate or subset of fates. By 24 hr after the birth of 4d, the highly asymmetric and stereotypical divisions of the two large 4d teloblast cells have generated ten smaller cells on each side of the embryo in a regular arrangement (Figure 1). To examine the effects of the IoNanos knockdown on the behavior of this lineage, we labeled 4d with the lineage tracer DiI in embryos that had been injected with 0.1 mM IoNanosMO as zygotes (Figure 4). In these embryos, 4d is born on schedule, 3.5 hr before the other fourth-order micromeres (4abc). However, after knockdown, the ensuing divisions in the lineage lack the regularity and the cell-size asymmetry observed in wild-type embryos. At 24 hr after the birth of 4d, the arrangement of 4d lineage cells in knockdown embryos is highly variable and does not resemble that of wild-type lineages. The large size disparity in control lineages between teloblasts and other cells was reduced by IoNanos knockdown, indicating that the asymmetry of the divisions in the lineage is reduced (Figure 4E). The IoNanosMO lineages have around 40% fewer cells than the controls (Figure 4F), indicating that the proliferative capacity of the teloblasts is impaired. Together, these results indicate that the cleavage program and division asymmetry of the teloblast cells of the 4d lineage are disrupted after IoNanos knockdown. This is similar to the effects of Nanos knockdown in the leech *Helobdella*, disrupting the proliferation of the teloblasts and the behavior of the teloblast progeny cells. These results imply that Nanos has a conserved role in blast cell function in spiralians and suggest that Nanos orthologs might be involved in teloblast function in other protostomes. In the *Drosophila* ovary Nanos is required for the maintenance of self-renewal of germline stem cells [1, 7, 8]. The requirement for IoNanos for normal blast cell proliferation, division asymmetry, and progeny cell differentiation suggests that this protein might be involved in maintaining stem cell-like properties in some somatic lineages, as well.

Experimental Procedures

Animal Collection and Husbandry

Adult *Ilyanassa obsoleta* were collected at Western Bay, near Mount Desert Island, Maine, or Peconic Bay, New York, or obtained from the Marine Resources Center at the Marine Biological Labs, Woods Hole, Massachusetts. Animal care and embryo collection have been described [25].

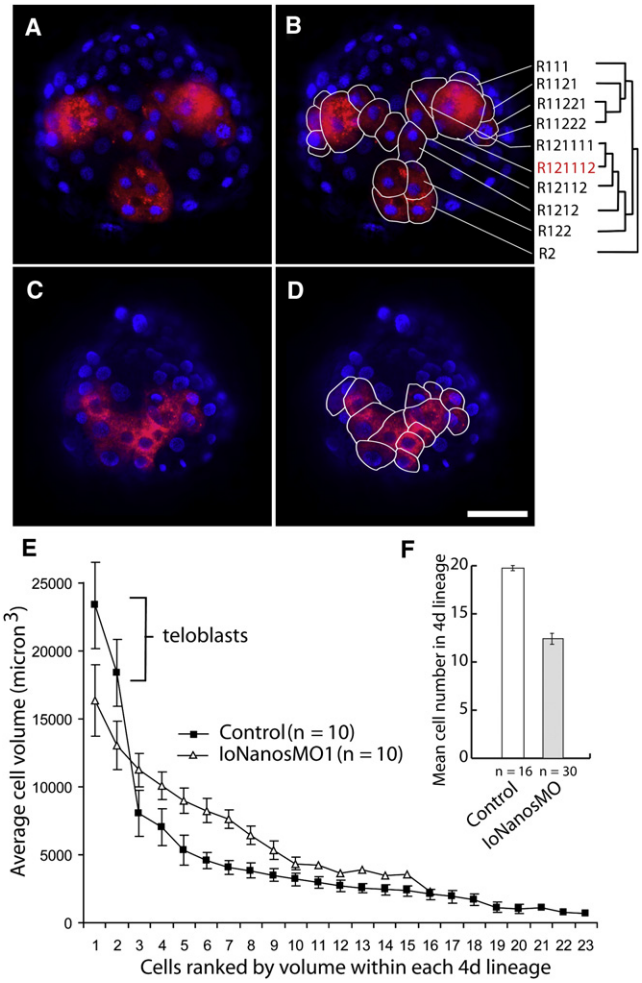

Figure 4. The Effects of IoNanos Knockdown on the Teloblastic Cleavage Pattern

(A–D) The 4d lineage was labeled by DiI injection of 4d in control embryos (A and B) or IoNanosMO1-injected embryos (C and D). Embryos were fixed at 4d + 24 hr. DiI is red, and nuclei are stained with DAPI (blue). The cells in the lineages are shown in (B) and (D). The cells are identified in (B) (with the teloblast highlighted in red), but the cleavage pattern is irregular after IoNanosMO injection, so cells could not be identified.

(E) Cell volumes were measured by confocal microscopy of IoNanosMO and control embryos that were labeled with DiI in 4d as above. After injection, they were reared to 4d + 24 hr, fixed, and stained with phalloidin for the visualization of cell boundaries. Each labeled cell was traced in those Z sections where it appeared, and sections were summed so that the total volume could be estimated. Because the teloblasts were not identifiable in knockdown embryos, we ranked cells in each lineage by volume and plotted the mean size across embryos for each size rank. In control embryos, the teloblasts were always the largest two cells. IoNanos knockdown reduces the size asymmetry of cells within the 4d lineage by reducing the size of the largest cells and increasing the size of the smaller cells. Measurements are from ten embryos of each type, and error bars show 95% confidence intervals around the mean. These error bars were calculated for all size ranks where n = 10; so size ranks 21–23 in the control and size ranks 11–16 in the IoNanosMO curves do not have error bars because not all ten embryos in the respective samples had the requisite number of cells.

(F) Mean cell number in the 4d lineage at 4d + 24 hr, in control and IoNanosMO-injected embryos. Error bars show 95% confidence intervals around the mean. Controls ranged from 17 to 23 cells in the 4d lineage and had a mean number of 19.9 cells, with 11/16 embryos having 20 cells. IoNanosMO embryos ranged from 10 to 20 cells in the 4d lineage and had a mean number of 12.2 cells. The embryos where cell volumes were calculated for (E) are a subset of those where cell numbers were determined for (F).

Nanos Cloning

The IoNanos clone recovered in a previously described in situ screen [9] was 2236 bp. Northern blotting showed that the mRNA was approximately 3.5 kb, so we performed 3′ rapid amplification of cDNA ends (RACE) on a poly-T-primed cDNA pool, obtained a 3′ fragment that extends the original clone 1238 bp, and performed 5′ RACE on a pool primed from a ligated linker and recovered 14 additional bases; the final clone is 3490 bp (GenBank EU087572).

In Situ Hybridization and Antibody Staining

In situ hybridization was performed as described [9]. Polyclonal antibodies were generated to a peptide corresponding to a portion of the IoNanos amino acid sequence and affinity purified against the peptide (details about antibody generation and purification are in the Supplemental Data). Embryos were fixed for 20 min in Pipes-EGTA-Magnesium (PEM) (100 mM PIPES [pH 6.9], 10 mM EGTA, and 1 mM $MgSO_4$) with 4% paraformaldehyde and 0.1% Triton X and then washed and stored in phosphate-buffered saline with 0.1% Tween (PBTw). Antibody staining and YOYO-1 (Molecular Probes) staining of nuclei were carried out as described [17]. Z stacks of stained embryos were captured on a Leica SP confocal microscope and projected with Leica software. For the fluorescent images presented, all collection and processing parameters were kept constant, allowing for comparisons between panels.

IoNanos Knockdown, 4d Ablation, and Scoring

Zygotes were injected with the indicated concentrations of IoNanosMO (Gene Tools) in 1× injection buffer and 1% fluorescein isothiocyanate (FITC)-dextran. (Details of the *Ilyanassa* injection protocol are in the Supplemental Data). Full-length antisense IoNanos RNA was synthesized with T3 RNA polymerase, and 5′ caps were added (mScript, Epicenter). Antisense RNA was injected at 1 ug/ul. Control sense RNA was a fragment corresponding to positions 2013–3490 in the IoNanos transcript (a portion of the 3′ untranslated region [UTR]) and was injected at 525 ng/ul (roughly equimolar to the antisense RNA). For ablations, a 4d cell was pricked freehand with a pulled glass needle, causing it to detach from the embryo. Manipulated or injected embryos and controls were reared to the larval veliger stage in separate dishes of 0.22 μm filtered artificial sea water (FASW) for 7 days (hatching stage). While still alive, 7-day-old veliger larvae were individually scored for beating hearts, relaxed in a mixture of two parts FASW saturated with trichlorobutanol and one part FASW at 4°C, and then fixed together with 3.7% formaldehyde in FASW. Veligers were fixed overnight at 4°C and then washed twice with PBTw. The fixed veligers were costained with DAPI (500 ng/ml) and phalloidin (33 nM) and mounted in 80% glycerol in PBS. Veligers were then individually scored at 400× for each of the structures noted in Figure 3 and Table 1. Sketches of larvae were made by the tracing of images taken at several focal planes of the same sample.

Lineage Tracing and Cell-Volume Analysis

The fluorescent dye DiI was resuspended in EtOH at 100 mg/ml then diluted 1/20 in soybean oil [26]. For confocal analysis, we mixed DiI 1:1 with DiD, which emits in the far-red range. We pressure injected 4d with 1–2 small drops of oil and then fixed embryos in 3.7% formaldehyde in FASW with 100 mM EDTA to preserve the label [27]. To calculate the cell volumes, we stained embryos with phalloidin-Alexafluor-488 (33 nM) to visual cell boundaries and then optically sectioned embryos at 1 μm intervals on a Leica SP confocal. Cross-sectional areas of stained cells were measured in sections where they appeared with ImageJ [28], and cell volumes were estimated as the sum of the areas of sections for a cell.

Supplemental Data

Experimental Procedures and five figures are available at http://www.current-biology.com/cgi/content/full/18/5/331/DC1/.

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