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Nanos Plays a Conserved Role in Axial Patterning outside of the Diptera

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Summary

Axial patterning is a fundamental event in early development, and molecules involved in determining the body axes provide a coordinate system for subsequent patterning [1, 2]. While orthologs of *Drosophila bicoid* and *nanos* play a conserved role in anteroposterior (AP) patterning within at least a subset of Diptera [3–5], conservation of this process has not yet been demonstrated outside of the flies. Indeed, it has been argued that *bicoid*, an instrumental "anterior" factor in *Drosophila melanogaster*, acquired this role during the evolution of more-derived dipterans [6, 7]. Interestingly, the interaction of *Drosophila* maternal *nanos* and maternal *hunchback* provides a system for patterning the AP axis that is partially redundant to the anterior system [8–11]. Previous studies in grasshoppers suggest that *hunchback* may play a conserved role in axial patterning in this insect [12], but this function may be supplied solely by the zygotic component of *hunchback* expression. Here we provide evidence that the early pattern of zygotic grasshopper Hunchback expression is achieved through translational repression that may be mediated through the action of grasshopper *nanos*. This is consistent with the notion that an anterior gradient system is not necessary in all insects and that the posterior pole "probably conveys longitudinal polarity on the ensuing germ anlage" [13].

Results and Discussion

Expression of *S. americana hunchback* Indicates that It May Be under Translational Regulation

Previously we have shown that *S. americana* Hunchback protein, but not mRNA, is provided maternally to the egg [12]. The maternal protein may help define the early distinction between embryonic and extraembryonic cells, but it does not appear to provide axial information. Subsequently, zygotic Hunchback protein is found in all cells of the condensing embryonic anlage but then clears from posterior tissue and continues to be expressed in anterior cells, suggesting a role for zygotic Hunchback in axial patterning (Figure 1A) [12]. On the basis of the mechanism of *hb* regulation elucidated in *Drosophila* and the presence of a Nanos response element in the *S. americana hb* 3′UTR [12], we postulated that the loss of *Schistocerca* Hb expression in posterior tissue may be a product of translational regulation and compared transcript and protein expression in order to test this. In situ hybridization with a riboprobe on stage-matched embryos (8% of development) shows that *hb* transcript is found in all cells, including posterior tissue from which protein has been cleared (Figures 1A and 1B, arrow). Expression of *hb* transcript in cells lacking protein was confirmed by double labeling embryos for both transcript and protein (Figure 1C). Absence of Hb protein in cells that contain *hb* transcript implicates translational repression in the spatial regulation of Hb expression. By 10% of development, *hb* transcript cannot be detected in posterior tissue, indicating that spatial regulation of the protein now reflects transcriptional control (Figure 1D). This suggests that initial translational repression of *hb* temporally precedes the predominantly transcriptional control of *hb*.

S. americana nos Is an Ortholog of *nanos*

A strong candidate for mediating translational repression of *hunchback* is *nanos*. Using degenerate PCR primers based on dipteran *nanos* sequences, we cloned a small fragment of *nanos* from cricket (*Gryllus domesticus*) and grasshopper (*S. americana*) cDNA (Figures 2A and 2B). Repeated amplification experiments with embryonic cDNA (0%–30% of development) as template isolated only a single *nanos* gene, supporting the suggestion that there is only one ortholog of *nos* in *S. americana*. As noted for other *nanos* orthologs, the most conserved part of the protein is found toward the C terminus of the coding region and contains two metal binding fingers of CCHC structure (residues 352–511; Figures 2A and 2B).

Since Pumilio is instrumental in the recognition of the *Drosophila hunchback* NRE and in the recruitment of Nanos into the quaternary complex that mediates translational repression, this gene should also be conserved in *S. americana* [14, 15]. Using PCR on ovarian cDNA from *S. americana*, we have isolated a *pumilio* ortholog that shows 88% identity at the amino acid level to *Drosophila pumilio* (Figure 2C). Thus, both *nanos* and *pumilio* are conserved in *Schistocerca*.

S. americana nos Is Expressed in Posterior Tissue

Having cloned *S. americana nos*, we examined the mRNA and protein distribution pattern using in situ hybridization and a polyclonal antibody raised to *S. americana* Nanos to see whether its expression is consistent with a role in the translational repression of *hunchback*. During the initial condensation of the germ anlage (5%–7% of development), we find that *nanos* transcript is found in the majority of the embryonic cells but accumulates most strongly in the posterior region of the

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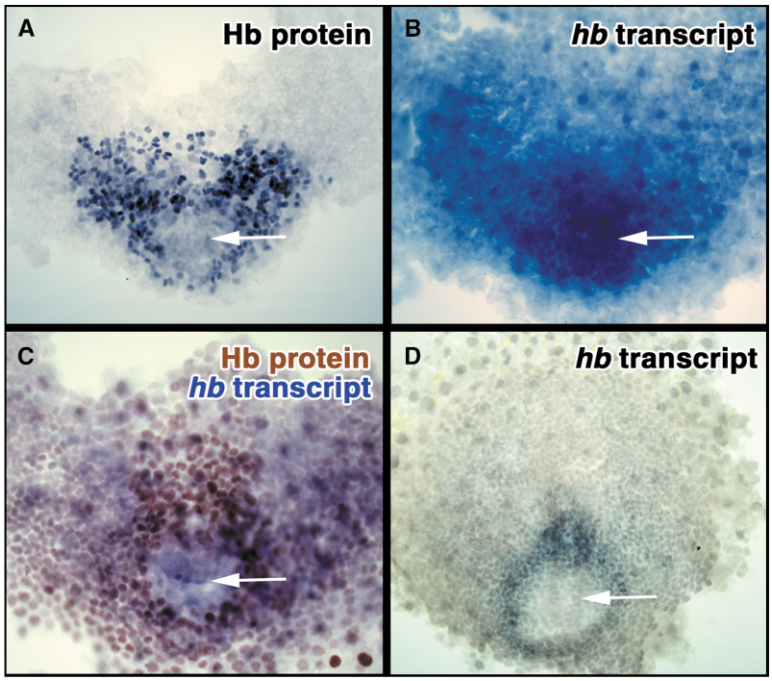

Figure 1. Hunchback Expression Indicates that It Is Translationally Repressed

Comparison of Hb protein and transcript expression in embryos at 8% of development indicates that *hb* transcript (B), but not protein (A), are expressed in the most posterior part of the embryo (arrow).

(C) Hb protein (brown) and transcript (purple) detection in a single embryo confirm that Hb protein is absent in posterior cells containing *hb* mRNA (arrow).

(D) At 10% of development, *hb* transcript is now absent from the most posterior part of the embryo (arrow). Anterior is up, and posterior is down in all panels.

embryo (Figures 3A and 3B). At about 12% of development, the asymmetry in the distribution of Nanos protein is especially striking (Figure 3C). The protein is most-highly expressed in the posterior portion of the embryo, which will go on to form all, or most, of the thorax and the abdomen. Closer inspection reveals that the immunostaining is in small dots within the cytoplasm of these cells (Figure 3D). Comparison of Hunchback and Nanos expression in the same embryo (about 7% of development) indicates that Nanos is present at highest levels in cells where Hunchback is being cleared (Figures 3E–3H), i.e., regions of reduced Hb nuclear signal (Figures 3F–3H, bracket). We do note that there is some Hunchback protein in the Nanos-containing cells at this stage, and this may, in part, be due to maternally provided Hb protein, which is still present at this time in development [12] and would not be predicted to be subject to Nanos regulation.

As development proceeds and the embryo grows and elongates, Nanos continues to be expressed in the posterior, Hunchback-negative region of the embryo (Figures 3I–3L). There is a transient overlap of expression in a posterior ridge of cells, but we believe that this is part of the extraembryonic domain of Hunchback expression, and Hunchback expression is no longer detected in this domain shortly after Nanos expression begins there. At 15% of development (Figure 3M), Nanos is expressed from anterior T1 on back, which is posterior to the strong subdomain of Hunchback expression [12]. Interestingly, both *nanos* mRNA (data not shown) and protein (Figure 3N) are undetectable by 20%–25% of development. We are able to detect expression again, by 60% of development, in the germline (data not shown). We also find that *S. americana pumilio* is expressed during early development, and both the mRNA and protein (detected with a crossreactive antisera) are expressed in all cells of the embryo (Figure 3O) during the time that we detect *nanos* expression.

Maternal *S. americana nos* Is Expressed Posteriorly

We next examined Nanos expression during oogenesis and cleavage stages in order to understand where the earliest axial information comes from in short germ insects. Nanos is seen accumulating asymmetrically in cells within the germarium of adult *Schistocerca* ovaries (the region of the ovary where oocytes arise; Figures 4A–4C). The cells expressing Nanos in the germarium are germ cells, as assessed by their nuclear morphology (Figure 4B) and expression of Vasa (Figure 4D). These cells also accumulate Pumilio (Figure 4E). Thus, *nanos* and *pumilio* may be involved in germline function in the adult ovary. Later in oogenesis Nanos protein cannot be detected (data not shown), but *nanos* transcript accumulates with a posterior focus (Figure 4F), a pattern that might arise by transcript localization or by reduced stability of the mRNA at the anterior. This pattern arises prior to the nuclear-mediated posterior localization of Hunchback protein. [12]

Consistent with the oogenesis data, *nanos* transcript, but not protein, is found at the posterior of 0–6 hr eggs (Figures 4G–4J). This asymmetry in the distribution of *nanos* transcript in 0–6 hr eggs (Figures 4I and 4J) cannot be directly correlated with the future AP axis of the embryo, which initially forms by condensation as a cap of cells at the posterior cortex, with its AP axis perpendicular to that of the egg. It is possible, nevertheless, that nuclei from the anterior end of the egg and within the yolk move extensively toward the posterior end of the egg during development, and, through such cell movement, *nanos*-expressing cells (as opposed to *nanos*-negative cells) may end up in more-posterior parts of the embryo, perhaps generating the asymmetry

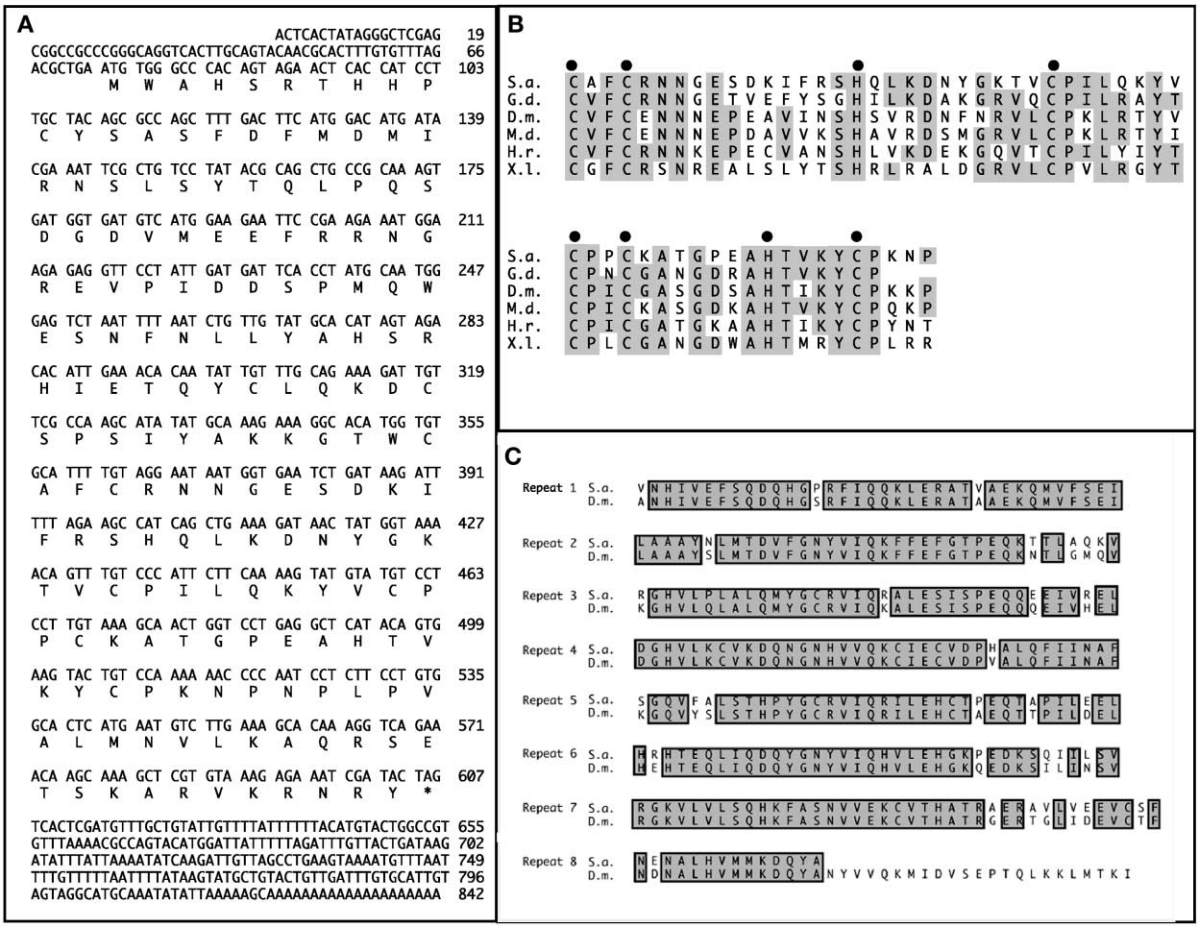

Figure 2. *S. americana nanos* and *S. americana pumilio* Are Orthologs of the *Drosophila* Genes

(A) Full-length sequence of *S. americana nanos*.

(B) Amino acid alignments of the metal binding regions of Nanos orthologs. Dots indicate metal ion contact residues. In this region of the protein, *S. americana nos* (S.a.) shows 57% amino acid identity to *Gryllus domesticus nos* (G.d.), 51% identity to *D. melanogaster nos* (D.m.), 53% identity to *M. domestica nos* (M.d.), 57% identity to *H. robusta nos* (H.r.), and 47% identity to *X.laevis X-cat2* (X.l.).

(C) Alignment of *D. melanogaster* and *S. americana* Puf repeats in the respective *pumilio* orthologs.

in transcript distribution observed in Figure 3A. Nanos protein is first detected in the embryo at 14 hr of development, when nuclei arrive cortically (Figure 4K), and, by 23 hr of development, Nanos is detected only in posterior cells of the egg (Figure 4M), although nuclei have reached the cortex at the anterior end of the egg (data not shown). Meanwhile, Pumilio is found in all cells of the egg as nuclei arrive at the cortex (data not shown).

Potential Ancestral Roles of *nanos*

Our results indicate that *nanos* mRNA and protein are expressed asymmetrically at several stages of development. Within the germarium, Nanos protein is asymmetrically distributed within the developing oocytes. During early oogenesis, *hunchback* mRNA and protein are expressed in the same pattern of cells, suggesting that there is no translational repression of *hunchback* at this stage [12] (there is also little or no Nanos protein in the Hunchback-expressing stage oocytes). Later in oogenesis, and in newly laid eggs, *nanos* mRNA is localized to the posterior pole of the egg. When cellularization begins Nanos protein is found in cells toward the posterior, but not anterior, end of the egg. While this superficially resembles the asymmetry of Nanos protein in syncytial *Drosophila* embryos, it is important to remember that we cannot, at least with our current data, correlate this expression pattern to the future AP axis of the grasshopper embryo. It should be noted that the seeming lack of correspondence of the AP egg axis with the AP embryo axis may be a derived situation in the grasshopper, as the correlation is obvious in most other insects.

Nevertheless our analysis of grasshopper *nanos* expression in the germ anlage indicates that this phase of asymmetric expression may underlie formation of the embryonic AP axis and posterior patterning of the embryo via Hunchback regulation. This suggests that an axial patterning mechanism involving translational repression of *hb* mRNA may be an ancestral feature of insect pattern formation (at least as far back as the common ancestor of *Schistocerca* and *Drosophila*). However, since maternal *S. americana* Hb is provided as protein, the target of translational repression in grass-

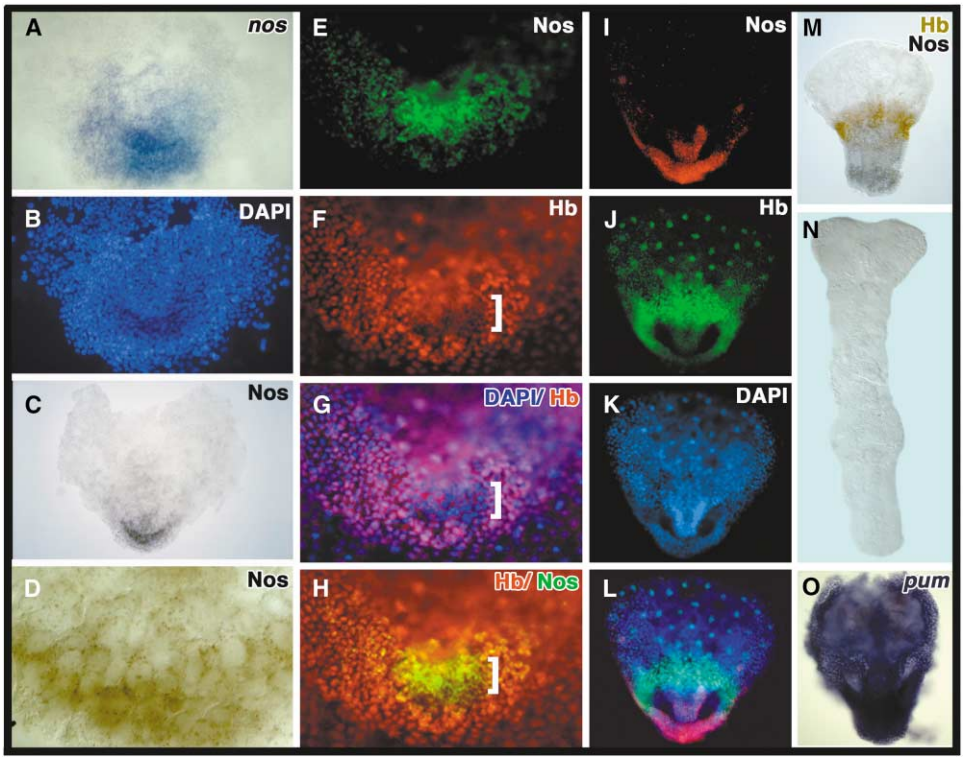

Figure 3. Nanos Is Expressed in Prospective Posterior Tissue

(A) *nanos* in situ hybridization at approximately 6%. Comparison with DAPI (B) indicates that not all embryonic cells express *nanos* and that the highest levels are in the posterior of the condensing germ disk.

(C) By 12% of development, Nanos is found only in the region that will grow to form the posterior part of the embryo, and closer examination reveals that Nanos immunostaining (brown specks) is cytoplasmic, as expected (D).

(E–H) A 7% embryo triple labeled for Nanos (Nos), Hunchback (Hb), and DAPI (nuclear stain). (E) Nos in green; (F) Hb in red; (G) Overlay of DAPI (blue) and Hb (red); (H) Overlay of Hb (red) and Nos (green). Nanos is most prominent in the posterior part of the germ disc ([E] and green signal in [H]). Hb ([F] and red signal in [H]), meanwhile, is still found in all cells of the embryo at this time but is starting to diminish in the posterior of the embryo (bracket). Overlay of Hb and Nanos ([H], red and green, respectively) and Hb and DAPI ([G], red and blue, respectively) reveals that Nanos is predominantly expressed in cells that are clearing of Hb (bracket).

(I–L) A 12% embryo showing Nanos in the posterior region (I), the gnathal Hb stripe (J), DAPI (K), and the overlay of the above channels (L).

(M) Gnathal/thoracic Hb (brown) and posterior Nanos (gray) in a 15% embryo.

(N) Nanos expression is undetectable at 22%.

(O) *pum* is expressed throughout the 14% embryo. Anterior is up, and posterior is down in all panels.

hopper would appear to be zygotic *hunchback* mRNA and not maternal *hunchback* mRNA as in *Drosophila*. It is currently unclear whether *S. americana* Nanos is acting as a switch that specifies some cells as posterior or whether it is acting in a graded fashion to permit the differentiation of different posterior identities. It is also interesting to note that work in *Tribolium* suggests that *caudal* may act as an activator of *hunchback* transcription [16] and that *S. gregaria caudal* is expressed during condensation of the germ disk and in the early germ anlage [17]. On the basis of these data, we suggest that grasshopper *caudal* (and, possibly, maternally inherited Hunchback protein) could act to promote zygotic *hunchback* transcription throughout the entire embryonic primordium and that *nanos* acts to prevent translation of zygotic *hunchback* mRNA in the posterior of the grasshopper embryo.

Drosophila nanos also has a well-studied role in germline development, and it has been suggested that the ancestral role of *nanos* in metazoans was in germline function [18, 19]. Data presented in the current paper indicate, however, that the role of *nanos* in both axial patterning and germline development is probably ancestral to at least the insects. Furthermore, *Cnnos2* is expressed in a manner consistent with a role in axial patterning of the growing buds and regenerating head, but not foot, of the cnidarian, *H. magnipapillata* [20]. Thus, the function of *nanos* in both axial patterning (not necessarily via *hb* regulation) and germline development may be ancient. Indeed *nanos* may function in situations where a specific set of cells must be set aside and protected from patterning factors. This is entirely consistent with the role of *nanos* in germline specification as well as its role in protecting cells from anterior patterning factors, such as *hunchback*, within the insects.

Experimental Procedures

Embryo Collections

Eggs were collected daily as pods from a colony of *S. americana*. Embryos develop at around 5% per day. Embryos from 5% of development to hatching are staged on the basis of morphological crite-

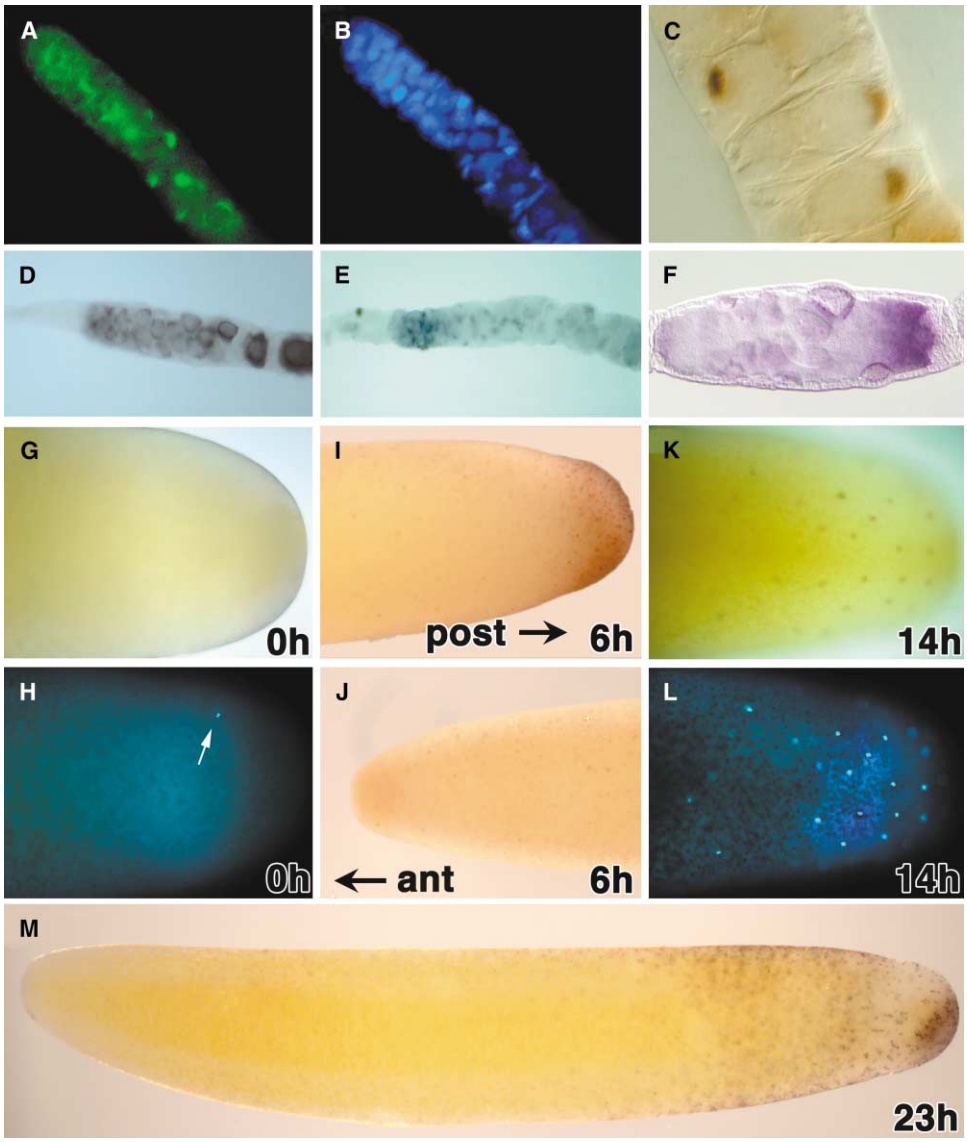

Figure 4. Nanos Is Found at the Posterior of the Oocyte and during Cleavage Stages of Embryonic Development

(A) Nanos accumulates in the germ cells within the *Schistocerca* germarium

(B) DAPI staining and Vasa expression (D) confirm that cells expressing Nanos are indeed the germline.

(C) Nanos accumulates asymmetrically in germ cells within the germarium, unlike Vasa protein (D).

(E) Pumilio is also expressed in germ cells within the germarium.

(F) In older oocytes *nanos* mRNA accumulates posteriorly.

(G and H) Nanos protein is undetectable at 0 hr of development (Nanos [G]; matching DAPI image [H], arrow indicates a nucleus within the egg).

(I and J) *nanos* transcript is found at the posterior (I), but not the anterior (J), of a 6 hr egg.

(K and L) In a 14 hr egg, Nanos protein is seen around nuclei that have arrived at the surface of the egg (Nanos [K]; matching DAPI image [L]).

(M) Nanos in a 23 hr egg. Posterior is toward the bottom right in (A)–(C) and to the right in (D)–(M).

ria. Staging before 5% (24 hr) of development is given in hours after egg laying.

Cloning of *nanos* and *pumilio*

Degenerate PCR to clone *nanos* was carried out with the following primers: 5′-aaaagaattctgygbnttytgyvrnwvnaa and 5′-aaaaggatccg grcartanyknanngtrtg. *pumilio* was cloned with the following primers: 5′-cargaycartayggnaayta and 5′-gcrtaytgrtcyttcatcat with nested primers 5′-argaycartayggnaaytayg and 5′-rttngcrtaytgrtcyttcat. 5′ and 3′ RACE were performed with Invitrogen RACE reagants.

In Situ Hybridization

In situ hybridization was performed with a digoxygenin-labeled DNA probe as previously described [12]. For whole eggs the chorion was manually peeled away, and material was not treated with xylene or Proteinase K. Hybridization was performed in a previously described SDS hybridization buffer at 55°C overnight [12]. The probe contained the sequence representing a full-length *nanos* transcript. For *pumilio* in situ hybridizations, riboprobes were synthesized, representing the 5′ (less-conserved) region of the transcript, and embryos were incubated with hydrolyzed probe overnight at 60°C. The same condi-

tions were utilized for *hb* in situ hybridizations, where the probe represents the entire *S. americana hb* 3′UTR.

Antibody Production and Immunostaining

A TrpE/*S. americana* Nos fusion protein was produced from the pATH22 vector containing the entire coding region of *nanos* cloned into the SmaI and HindIII sites [21]. Nanos immunostaining followed [22], except that serum IL68 (Rat anti-*S. americana* Nanos) was preadsorbed at a dilution of 1:50 for 15 min over *D. melanogaster* embryos. Hb double labeling was performed with the monoclonal antibody PP7C11 [12]. Fluorescent double labels utilized the TSA-Plus Cyanine 3 substrate (NEN) to detect Nanos and a secondary antibody coupled to Alexafluor 488 (Molecular Probes) to detect the PP7C11 antibody. Pumilio protein was detected with the crossreactive polyclonal antibodies rat1 and 3413 (R.P. Wharton) as well as serum 2#1 (P.M. Macdonald) raised to *Drosophila* Pumilio. Vasa immunoreactivity was detected with a polyclonal antibody against *Drosophila* Vasa, provided by P. Lasko. All embryos were imaged on a Zeiss Axiophot with a ProgRes 3012 (Kontron Electronik) digital camera.

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References

1. St Johnston, D., and Nüsslein-Volhard, C. (1992). The origin of pattern and polarity in the *Drosophila* embryo. Cell *68*, 201–219.
2. Dale, L., and Jones, C.M. (1999). BMP signalling in early *Xenopus* development. Bioessays *21*, 751–760.
3. Curtis, D., Apfeld, J., and Lehmann, R. (1995). *nanos* is an evolutionarily conserved organizer of anterior-posterior polarity. Development *121*, 1899–1910.
4. Stauber, M., Taubert, H., and Schmidt-Ott, U. (2000). Function of *bicoid* and *hunchback* homologs in the basal cyclorrhaphan fly *Megaselia* (Phoridae). Proc. Natl. Acad. Sci. USA *97*, 10844–10849.
5. Shaw, P.J., Salameh, A., McGregor, A.P., Bala, S., and Dover, G.A. (2001). Divergent structure and function of the *bicoid* gene in *Muscoidea* fly species. Evol. Dev. *3*, 251–262.
6. Stauber, M., Jäckle, H., and Schmidt-Ott, U. (1999). The anterior determinant *bicoid* of *Drosophila* is a derived Hox class 3 gene. Proc. Natl. Acad. Sci. USA *96*, 3786–3789.
7. Stauber, M., Prell, A., and Schmidt-Ott, U. (2002). A single Hox3 gene with composite *bicoid* and *zerknüllt* expression characteristics in non-Cyclorrhaphans flies. Proc. Natl. Acad. Sci. USA *99*, 274–279.
8. Irish, V., Lehmann, R., and Akam, M. (1989). The *Drosophila* posterior-group gene *nanos* functions by repressing *hunchback* activity. Nature *338*, 646–648.
9. Struhl, G. (1989). Differing strategies for organizing anterior and posterior body pattern in *Drosophila* embryos. Nature *338*, 741–744.
10. Hülskamp, M., Schroder, C., Pfeifle, C., Jackle, H., and Tautz, D. (1989). Posterior segmentation of the *Drosophila* embryo in the absence of a maternal posterior organizer gene. Nature *338*, 629–632.
11. Wimmer, E.A., Carleton, A., Harjes, P., Turner, T., and Desplan, C. (2000). Bicoid-independent formation of thoracic segments in Drosophila. Science *287*, 2476–2479.
12. Patel, N.H., Hayward, D.C., Lall, S., Pirkl, N.R., DiPietro, D., and Ball, E.E. (2001). Grasshopper *hunchback* expression reveals conserved and novel aspects of axis formation and segmentation. Development *128*, 3459–3472.
13. Sander, K. (1976). Specification of the basic body pattern in insect embryogenesis. Adv. Insect Physiol. *12*, 125–238.
14. Sonoda, J., and Wharton, R.P. (1999). Recruitment of Nanos to hunchback mRNA by Pumilio. Genes Dev. *13*, 2704–2712.
15. Murata, Y., and Wharton, R.P. (1995). Binding of pumilio to maternal *hunchback* mRNA is required for posterior patterning in Drosophila embryos. Cell *80*, 747–756.
16. Wolff, C., Schroder, R., Schulz, C., Tautz, D., and Klingler, M. (1998). Regulation of the *Tribolium* homologues of *caudal* and *hunchback* in *Drosophila*: evidence for maternal gradient systems in a short germ embryo. Development *125*, 3645–3654.
17. Dearden, P.K., and Akam, M. (2001). Early embryo patterning in the grasshopper, *Schistocerca gregaria*: *wingless*, *decapentaplegic* and *caudal* expression. Development *128*, 3435–3444.
18. Forbes, A., and Lehmann, R. (1998). Nanos and Pumilio have critical roles in the development and function of *Drosophila* germline stem cells. Development *125*, 679–690.
19. Parisi, M., and Lin, H. (2000). Translational repression: a duet of Nanos and Pumilio. Curr. Biol. *10*, R81–R83.
20. Mochizuki, K., Sano, H., Kobayashi, S., Nishimiya-Fujisawa, C., and Fujisawa, T. (2000). Expression and evolutionary conservation of *nanos*-related genes in *Hydra*. Dev. Genes Evol. *210*, 591–602.
21. Koerner, T.J., Hill, J.E., Myers, A.M., and Tzagoloff, A. (1991). High-expression vectors with multiple cloning sites for construction of trpE fusion genes: pATH vectors. Methods Enzymol. *194*, 477–490.
22. Patel, N.H. (1994). Imaging neuronal subsets and other cell types in whole-mount Drosophila embryos and larvae using antibody probes. In Methods in Cell Biology, L.S.B. Goldstein and E.A. Fyrberg, eds. (San Diego, CA: Academic Press), pp. 445–487.

Accession Numbers

The *S. americana nanos* and *pumilio* sequences have been deposited in GenBank under accession numbers AY179887 and AY179886, respectively.