# A zebrafish *nanos*-related gene is essential for the development of primordial germ cells

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**Asymmetrically distributed cytoplasmic determinants collectively termed germ plasm have been shown to play an essential role in the development of primordial germ cells (PGCs). Here, we report the identification of a *nanos*-like (*nanos*1) gene, which is expressed in the germ plasm and in the PGCs of the zebrafish. We find that several mechanisms act in concert to restrict the activity of Nanos1 to the germ cells including RNA localization and control over the stability and translatability of the RNA. Reducing the level of Nanos1 in zebrafish embryos revealed an essential role for the protein in ensuring proper migration and survival of PGCs in this vertebrate model organism.**

[*Key Words*: nanos; germ line; PGC; germ plasm; maternal RNA; cell migration; zebrafish]

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Two main strategies for germ cell specification have been described; in mouse (and by inference other mammals) and in urodele amphibians germ cells are thought to be specified through cellular interactions (Nieuwkoop 1969; Tam and Zhou 1996). Consistently, the formation of the founding population of PGCs in the mouse was shown to depend on the function of extracellular factors of the bone morphogenetic protein (BMP) family (Lawson et al. 1999; Tsang et al. 2001; Ying et al. 2001; Ying and Zhao 2001). In contrast to this strategy, in many other organisms from different phyla, inheritance of specific asymmetrically localized cytoplasmic components, known as germ plasm, appears to direct cells to the germ-line lineage (Houston and King 2000a; Knaut et al. 2000; Tsunekawa et al. 2000). Although the actual specification and formation of PGCs in mammals and in urodeles appear to be independent of germ plasm, material resembling nuage, germ plasm organelle, is found in germ cells of these organisms at later stages of their development (Eddy 1974; Ikenishi and Nieuwkoop 1978).

An important contribution for the understanding of the mechanisms of germ cell development in fish was the identification of the zebrafish *vasa* homolog (Yoon et al. 1997). In *Drosophila,* the *vasa* gene, which encodes an RNA helicase, is essential for the assembly of the germ plasm (Hay et al. 1988; Lasko and Ashburner 1988). In situ hybridization of four-cell stage zebrafish embryos showed specific localization of the *vasa* transcript in four stripes at the edges of the first two cleavage planes of the early zebrafish embryo (Yoon et al. 1997). At these early stages of development the *vasa* transcript was shown to reside within an electron dense, nuage-like structure characteristic of germ plasm in other organisms (Knaut et al. 2000). During gastrulation and somitogenesis stages, the vasa-positive PGCs migrate and form two bilateral rows of cells close to the position of the future gonad (Weidinger et al. 1999).

Another germ plasm component that was first identified in *Drosophila* is *nanos* RNA, which encodes an RNA binding zinc finger protein. *nanos* transcripts are enriched in the posterior of the early *Drosophila* embryo, in the region where the germ granules reside (Wang and Lehmann 1991). Furthermore, *nanos* RNA is translated exclusively in the posterior, thereby generating a protein gradient with high protein levels in the posterior (Gavis and Lehmann 1994). Interestingly, although PGCs formation does not require *nanos* activity, *nanos*-deficient PGCs develop abnormally. They fail to incorporate into the gonad, show premature activation of germ cell markers, exhibit abnormal morphology, and express mRNAs that are normally expressed in the soma (Kobayashi et al. 1996; Forbes and Lehmann 1998; Deshpande et al. 1999).

Two *C. elegans* homologs of *nanos* have been shown to be required for early development of PGCs in this organism (Subramaniam and Seydoux 1999). Both genes (*nos*-1 and *nos*-2) encode maternal RNAs that are preferentially maintained in the germ-line blastomeres. Similar to *Drosophila,* the function of the *C. elegans nanos* genes is not required for the formation of the PGCs or for

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early specification of the germ cell fate. However, PGCs lacking the function of the two *nanos* genes are not maintained, and most of them eventually die. In addition to these redundant functions, *nos*-2, independent of *nos*-1, is required for efficient incorporation of the PGCs into the gonad.

The first vertebrate *nanos*-related gene, *Xcat*-2, was identified in *Xenopus laevis* (Mosquera et al. 1993). *Xcat*-2 RNA is transported along with the germ plasm to the vegetal cortex during oogenesis where it is associated with germinal granules in a process that depends on *cis*-acting elements in the 3′UTR (Kloc et al. 2000). Although the mechanisms responsible for *Xcat*-2 localization have been thoroughly studied, the function of this conserved germ plasm component has not yet been determined. Another *Xenopus* germ plasm component is the RNA of the *DAZ*-like gene, *Xdazl*. The function of this gene is required for spermatogenesis in flies and humans (Reijo et al. 1995; Eberhart et al. 1996), oogenesis in the nematode (Karashima et al. 2000), and gametogenesis in both male and female mice (Ruggiu et al. 1997). Specific depletion of maternal *Xdazl* mRNA results in PGC migration defects, and ultimately, loss of PGCs at the tadpole stage (Houston and King 2000b).

To understand the molecular mechanisms governing PGC development in zebrafish we have isolated genes that are expressed in these cells during embryonic development. Here, we report the cloning of a germ plasm component, the zebrafish *nanos*1. We show that several levels of post-transcriptional control act in concert to restrict the function of this gene to the PGCs. Reducing the level of Nanos revealed an essential role for this molecule in early development of the PGCs in a vertebrate model organism.

## Results

### *Identification of* nanos*1, a gene expressed in the PGCs of zebrafish*

We have sought to identify new genes that are important for germ cell development in zebrafish by employing a whole-mount in situ hybridization screen approach. In the course of this screen, we isolated a *nanos*-related gene (*nos*1, for nomenclature, see Materials and Methods), which is expressed in the PGCs of zebrafish (Figs. 1, 2a–e). The zebrafish *nos*1 mRNA is provided maternally (Fig. 2a,h). During the first two mitotic divisions, the mRNA is enriched in the distal end of the cleavage furrows of the early embryo, while nonlocalized mRNA can be still detected by whole-mount in situ hybridization until late blastula stages (Fig. 2a,b). This expression pattern is similar to that described for the zebrafish *vasa* mRNA, a germ-cell specific marker. At these early stages of development, *vasa* transcrips are concentrated in an electron-dense structure, the putative zebrafish germ

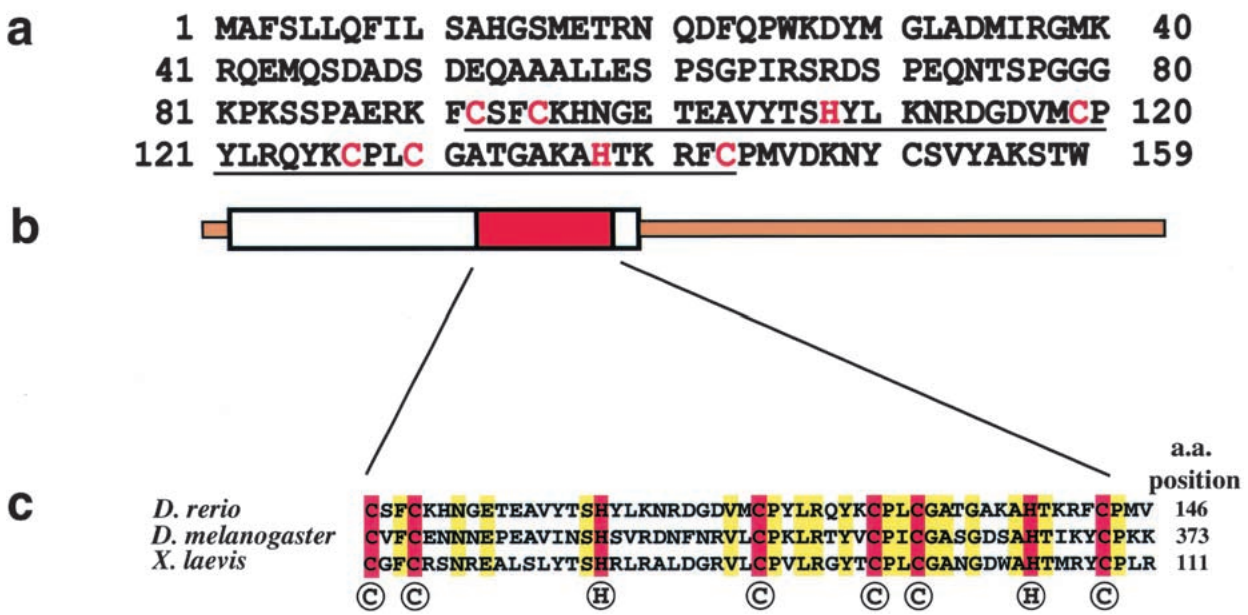


**Figure 1.** The zebrafish *nanos*1 (*nos*1) gene encodes a *nanos*-like zinc finger protein. (*a*) The predicted amino acid sequence of Nanos1 (Nos1) protein. The characteristic C-terminal CCHC CCHC zinc finger domain with its conserved residues (red) is underlined. This is the only region that shows high homology to *nanos* genes of other organisms. (*b*) Schematic representation of the cDNA of *nos*1. The cDNA contains 29 base pairs (bp) of 5′UTR (orange). The 480 bp-long open reading frame is followed by 637 bp of 3′UTR (orange). The red box within the coding region depicts the position of the zinc finger domain. (*c*) Comparison of the amino acid sequence of the zinc finger domain among Nanos-like proteins from *Danio rerio* (Nos1), *Drosophila melanogaster* (Nanos), and *Xenopus laevis* (*Xcat-2*). The amino acid residues comprising the conserved CCHC zinc finger are labeled in red, and residues that are identical in the three proteins are labeled in yellow. The total length of the Nanos1 protein is more similar to that of the *Xcat-2* protein than to the Nanos proteins of *Drosophila* and *C. elegans*.

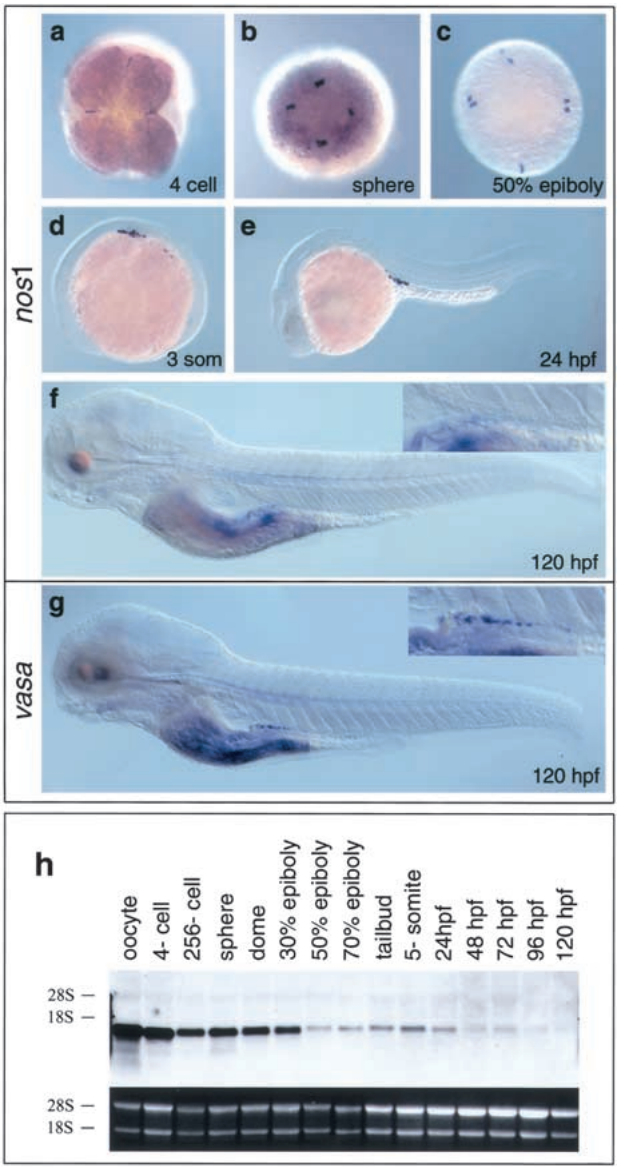


**Figure 2.** Spatial and temporal distribution of *nos*1 RNA and Nos1 protein. (*a–f*) Expression of *nos*1 mRNA during the first 5 d of zebrafish development. *nos*1 mRNA is undetectable by the fifth day of development. (*g*) Expression of *vasa* mRNA in a 5-day-old embryo. Inserts in *f* and *g* show a magnification of the region where the PGCs are normally found. (*h*) Developmental Northern blot of *nos*1. *nos*1 RNA is present in oocytes and in embryos before MBT (the 4- and 256-cell stages). The level of the transcript declines and it is barely detectable by the fifth day of development.

plasm (Yoon et al. 1997; Knaut et al. 2000). During blastula stages, *nos*1 mRNA is incorporated into the PGCs, which characteristically migrate to form two clusters during the first day of development (Fig. 2c–e; Weidinger et al. 1999). Unlike *vasa*, which continues to be strongly expressed throughout germ-cell development, *nos*1 mRNA is undetectable by in situ hybridization by the fifth day of embryonic development (Fig. 2f,g).

### *Restriction of Nanos1 activity to the PGC by posttranscriptional control mechanisms*

In the one-cell stage zebrafish embryo, nuage-like structures are evenly distributed in small particles adjacent to the actin cortex of the cell (Knaut et al. 2000). This configuration rapidly changes during the first two cell divisions. In the two- and four-cell embryos the nuage-like particles are increased in size, and are seen in close association with microtubules and mitochondria just below the distal part of the first two cleavage furrows (Knaut et al. 2000). Thus, within a period of 30 to 40 min, nuage structures that have been evenly distributed around the cortex of the large one-cell stage zebrafish embryo, accumulate in four aggregates. Several findings suggest that during the formation of these aggregates not all of the germ plasm material is transferred to the four main clusters. Although reduced in number, at the four-cell stage nonlocalized small nuage-like particles can still be observed along the actin cortex (Knaut et al. 2000). Indeed, *vasa* RNA, which labels the germ plasm, could be clearly detected up to the 64-cell stage outside of the four main germ plasm clusters (Yoon et al. 1997), and at later stages up to the sphere stage (late blastula, 4 hours postfertilization [hpf]; E. Raz and U. Wolke, unpubl.). Similarly, as mentioned above, *nanos*1 mRNA can be detected outside of the four clusters up to late blastula stages (Fig. 2a,b). Thus, although enrichment of germ plasm is observed in the positions where the PGCs will form, a significant amount of nonlocalized germ plasm particles can be found in cells that undergo somatic differentiation at later stages. These findings, coupled with the fact that up to the 16-cell stage cells of the zebrafish embryo are interconnected (Kimmel et al. 1995), call for control mechanisms that would restrict the function of germ plasm components, RNA and proteins, to the future PGCs, allowing proper development of the germ line on one hand and an uninterrupted somatic differentiation on the other.

The spatial and temporal distribution of *nos*-1 mRNA during early embryogensis indicates that, indeed, posttranscriptional control mechanisms exist that facilitate the specific expression of the gene in the PGCs. Northern blot analysis shows that the maternally provided mRNA degrades rapidly before gastrulation (up to the 50% epiboly stage in Fig. 2h) and the level of the mRNA continues to decline to a very low level by the fifth day of development (Fig. 2h). The rapidly degrading mRNA is likely to represent transcripts that were not incorporated into the PGCs, because clearance of somatic *nos*1 mRNA during these stages can be observed by whole-mount in situ hybridization analysis (Fig. 2a–c). To study the regulation of *nos*1 mRNA distribution more directly, we injected either full-length *nos*1 mRNA tagged at its 5′ end with green fluorescent protein (GFP) sequences (GFP-FL*nos*1), or GFP mRNA fused to the 3′UTR of the *nos*1 gene (GFP-*nos*1–3′UTR). We then determined the spatial distribution of the fusion-RNA molecules by in situ hybridization using GFP as an RNA probe at different stages of development. The distribution pattern of

the injected RNA derived from both fusion constructs was strikingly different from that of control RNA where GFP was fused to the 3′UTR of the *Xenopus globin* gene (GFP–*globin*). Specifically, whereas the control RNA showed slow uniform degradation in all cell types (Fig. 3m–p), *nos*1 3′UTR-containing RNAs exhibited rapid degradation in somatic tissues, while being specifically stabilized in the PGCs (Fig. 3e–h). Therefore, we conclude that the 3′UTR of *nos*1 is sufficient for directing fast degradation of the transcript in the soma, as well as for specific protection of the RNA from degradation in the germ cells. We suggest that this mechanism acts on the maternal *nos*1 mRNA to eliminate the ubiquitous distribution of the transcripts during early embryogenesis, thus leading to specific expression of *nos*1 in the PGCs.

The significance of *nos*1 3′UTR for Nos1 protein expression was determined by following the distribution of GFP in embryos injected with GFP-*nos*1–3′UTR mRNA. We found that the 3′UTR of *nos*1 is sufficient for directing specific expression of the protein in the PGCs despite the early ubiquitous distribution of the mRNA. In blastula stages, GFP was expressed uniformly, reflecting the spatial distribution of the injected RNA, but at a very low level (Fig. 3a,e). At this early stage the GFP signal derived from the similarly distributed GFP–*globin*

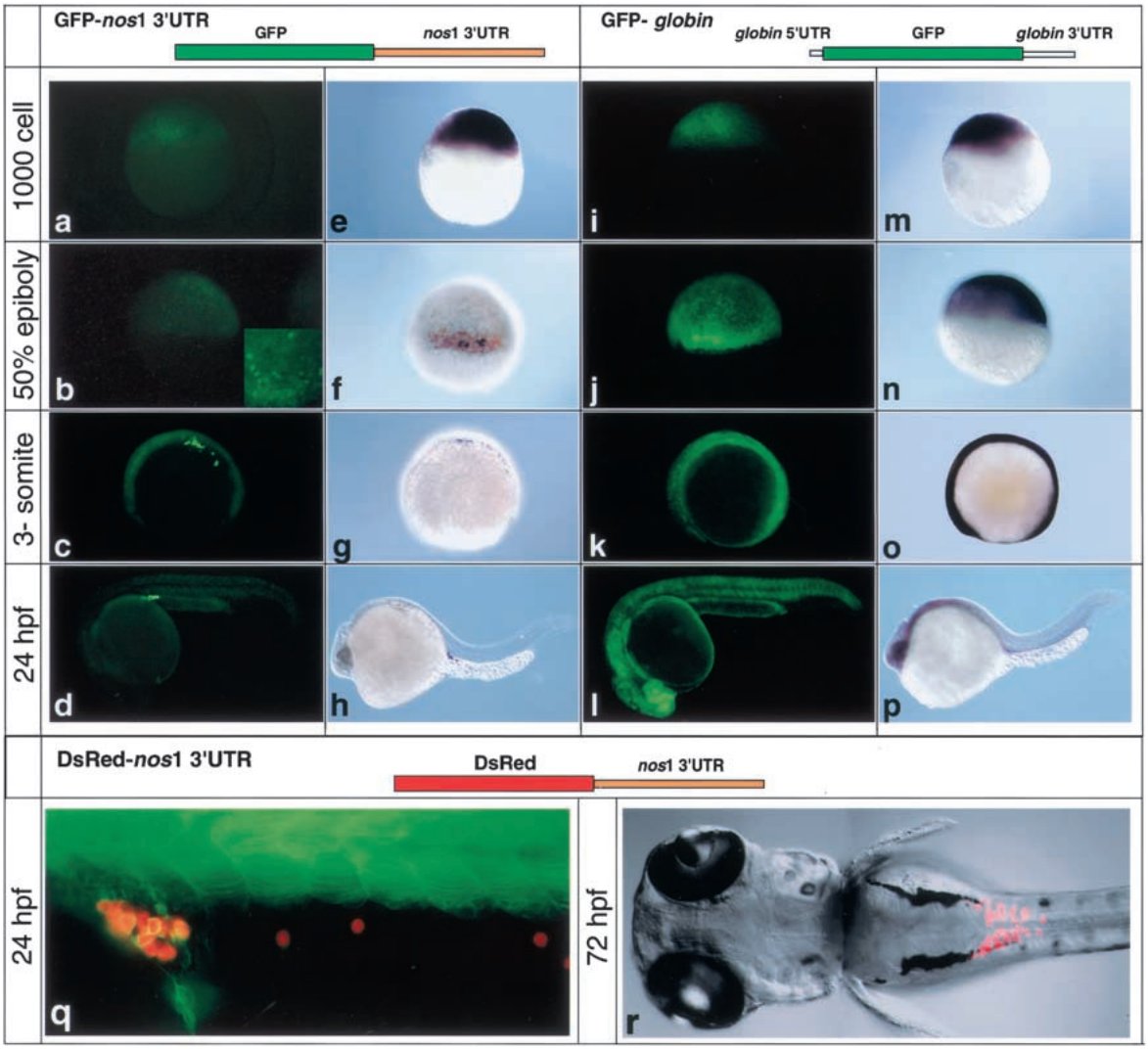


**Figure 3.** *nos*1 RNA distribution and protein expression are regulated by its 3′UTR. (*a–h*) Embryos injected with 40 pg of GFP-*nos*1 3′UTR RNA, or with 40 pg of GFP–*globin* RNA (*i–p*). (*a–d*,*i–l*) Fluorescent pictures of live embryos injected with the constructs described above. The earliest time point when the germ cells can be distinguished from somatic cells is during early gastrulation stages (insert in *b*). (*e–h*,*m–p*) Whole-mount in situ hybridization of embryos injected with the constructs described above using GFP as a probe. (*e–h*) Specific degradation in the soma and stabilization in the PGCs is observed for RNAs containing the *nos*1 3′UTR. (*q*) A 24-hour-old embryo injected with 100 pg of DsRed-*nos*1 3′UTR RNA at the one-cell stage showing red fluorescent in the PGC cluster with trailing posterior cells found along the yolk extension. Cell membranes were labeled by coinjecting 10 pg of EGFP-F-*globin* RNA. (*r*) Specific expression of DsRed in the PGCs of a 3-day-old embryo (dorsal view) injected with DsRed-*nos*1 3′UTR RNA at the one-cell stage.

mRNA is significantly higher (Fig. 3 cf. a,e and i,m, where 3a required a five times longer exposure time than 3i). A likely explanation for this phenomenon is that *nos*1 mRNA is poorly translated in somatic cells. Differential GFP expression levels that distinguish between zebrafish germ cells and somatic cells can first be seen during early gastrulation stages (Fig. 3b). Specific GFP expression in the PGCs could be observed for several days after injection (for 24 hpf, see Fig. 3d). The control mechanisms that are responsible for concentrating Nanos protein in the PGCs appear to be conserved between *Drosophila* and zebrafish, because in *Drosophila* as well, nonlocalized RNA is translationally repressed and undergoes degradation (Gavis and Lehmann 1994; Bashirullah et al. 1999). Taken together, the tight control over the distribution of *nos*1 mRNA through differential RNA stability in somatic versus germ-line cells and the control over the translation of *nos*1 mRNA, results in PGC-specific Nos1 expression despite the early widespread RNA distribution in the early zebrafish embryo.

The ability to direct expression of specific genes to the PGCs in zebrafish using the *nos*1 3′UTR has important practical implications for the study of vertebrate PGC. By using this procedure, one can now disrupt the normal function of specific genes in the PGCs (e.g., by gene overexpression or expression of dominant negative forms) to study their role in these cells. To show that proteins other than GFP can indeed be expressed in the PGCs using this method, we fused the cDNA encoding DsRed2 to the 3′UTR of *nos*1. Injection of this RNA led to specific expression of the protein in the PGCs, labeling them in red for more than 5 d (for 24 hpf and 3-day-old embryos, see Fig. 3q,r). Labeling the PGCs with GFP allows the migration to be followed at high resolution in the physiological context in live embryos to obtain information regarding the dynamics of this process. Finally, sorting GFP-labeled PGCs will constitute an important step towards efficient culturing of these cells for the purpose of in vitro studies of PGCs as well as genetic manipulations such as transgenesis (Ma et al. 2001) and mutagenesis.

### *Nanos1 is essential for proper migration and survival of PGCs in zebrafish*

As a first step in studying the function of the Nos1 protein we sought to determine its subcellular localization. In *C. elegans,* where the localization of a number of germ plasm components has been thoroughly studied, it appears these molecules are enriched in perinuclear granules that are, in turn, found in association with nuclear pores (Pitt et al. 2000). Similarly, in the zebrafish, Vasa protein is found in perinuclear granules in 1-day-old embryos (Knaut et al. 2000). The subcellular localization of Nos1 was determined by injecting mRNA containing the GFP open reading frame (ORF) fused in frame to the ORF of *nos*-1 (GFP–FL*nos*1) and following the distribution of GFP within the cell. Although the fusion protein is found throughout the cytoplasm, it is highly enriched in perinuclear granules that also contain the Vasa protein, suggesting nuclear-related functions for Nos1 (Fig. 4a).

To investigate the function of the Nos1 protein in zebrafish we inhibited its translation in early embryos using morpholino-modified antisense oligonucleotides (morpholinos) (Nasevicius and Ekker 2000). Remarkably, as will be described in detail below, embryos injected with *nos*1 morpholinos exhibited severe defects in PGC development leading to a reduced number of cells and to migration defects (Fig. 4b). The specificity of the antisense oligonucleotides was confirmed by several experiments. First, morpholinos directed against the region of the translation start site of *nos*1 efficiently inhibited the translation of synthetic *nos*1 RNA, but did not affect the translation of synthetic RNAs lacking sequences complementary to it (data not shown). Second, the severity of the phenotype correlated with the concentration of the injected antisense oligonucleotides (Fig. 4c). Third, the morpholino-induced PGC phenotype was fully reversed by coinjection of modified *nos*1 mRNA that could not be recognized by the antisense oligonucleotides (FL*nos*1mut) (Fig. 4e). Thus, uniformly supplied *nos*1 RNA that is regulated by its 3′UTR is capable of restoring Nos1 function in the germ cells. In contrast to the severe PGC phenotype induced by the morpholinos, under conditions where a PGC phenotype was observed, the development of the soma remained largely unaffected as determined by marker gene expression and morphological criteria (e.g., see Fig. 4b,f).

To elucidate the basis for the PGC phenotype in the *nos*1 morpholino-injected embryos, we followed the position and the number of the PGCs during development using *vasa* or *nos*1 probes in whole-mount in situ hybridization. Even when a very high amount of *nos*1 morpholinos was injected (2.4 ng), no change in PGC numbers at pregastrulation stages was observed (5 h of development, Fig. 4d). Therefore, we conclude that actual formation and specification of PGCs in zebrafish does not require zygotically translated Nos1 protein. Remarkably, although reducing Nos1 level did not affect PGC formation, it did deleteriously affected their migration; in morpholino-injected embryos the PGCs failed to migrate normally, reaching ectopic positions such as within the forming somites and in the head region (Fig. 4f). The migration defects are apparent at the first migration step that presumably requires directed active PGC migration, as during gastrulation the PGCs fail to align along the border between the trunk and head mesoderm and along the lateral border of the mesoderm (step III; Weidinger et al. 1999). Interestingly, although the PGCs do not reach intermediate migration targets, the cells appear to be able to migrate in a nondirectional manner, and exhibit the morphological characteristics of migratory cells. In *nos*1 morpholino-injected embryos the number of PGCs found in correct positions did not change significantly between 3-somite (11 hpf) and 24 hpf stages, whereas the number of PGCs found in ectopic positions was dramatically reduced (Fig. 4f). In this context it is noteworthy to mention that wild-type PGCs are able to survive in ectopic positions while maintaining

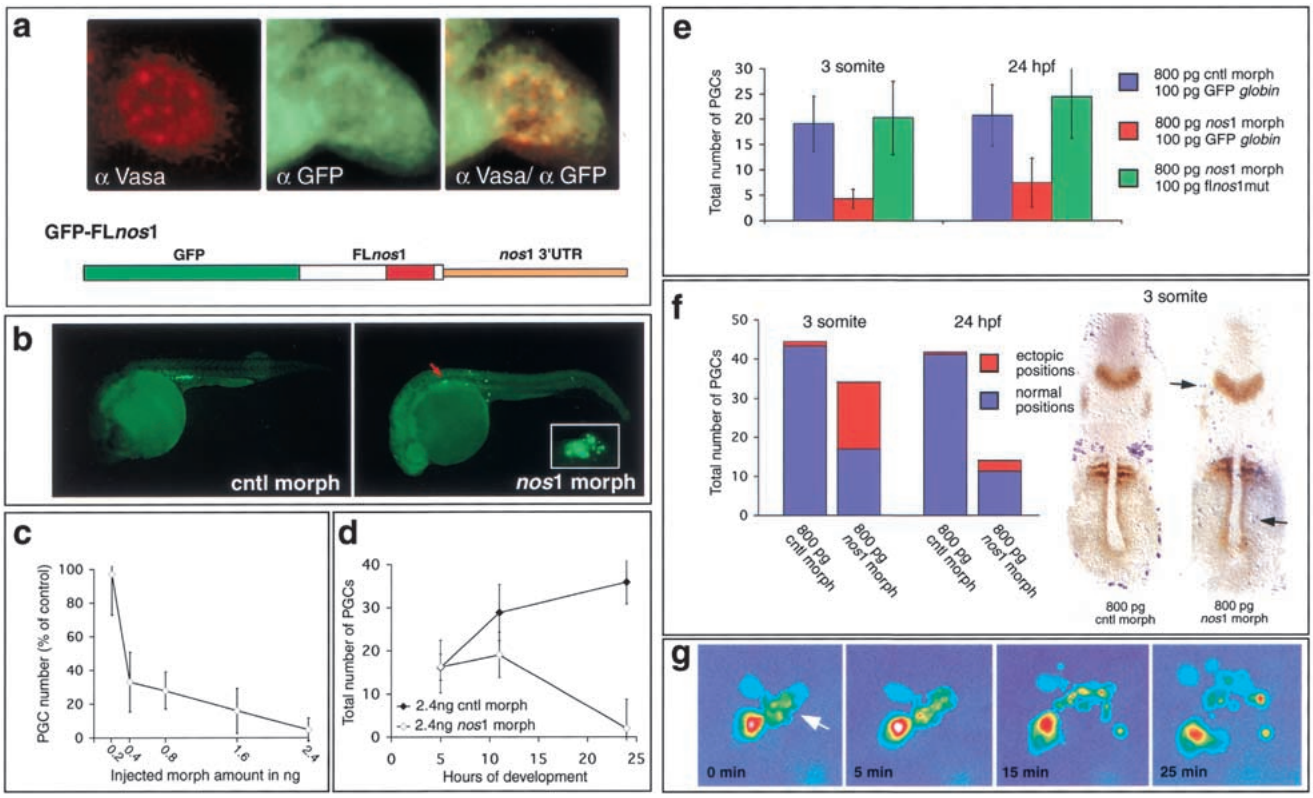


**Figure 4.** The function of Nos1 in PGC development in zebrafish. (*a*) The Nos1 protein is localized to perinuclear granules. Embryos injected with mRNA of GFP fused in frame to *nos*1 (200 pg of GFP-FL*nos*1) show localization of Nos1 to Vasa-containing granules (Knaut et al. 2000), while some of the protein appears to be distributed in the cytoplasm. (*b*) Defects in PGC migration and reduction in PGC number in *nos*1 morpholino-injected embryos. Embryos were injected with 60 pg of GFP-*nos*1 3′UTR and 800 pg of *nos*1 morpholinos (*right* panel) or with 800 pg of control morpholinos (*left* panel) and were photographed at the end of the first day of development. The insert in the *right* panel shows a magnification of a single dying ectopic PGC. The red arrow indicates the position of the cell within the embryo. (*c*) Loss of PGCs as a result of increased amounts of injected morpholinos. PGCs were visualized by whole-mount in situ hybridization using *vasa* as a probe at the 24-hpf stage. The proportion of PGC number in the *nos*1 morpholino-injected embryos relative to that of control morpholino-injected embryos is given for each experimental point (9–20 embryos were analyzed per point). (*d*) Loss of PGCs in *nos*1 morpholino-injected embryos during development. The number of PGCs was determined using *vasa* as an in situ hybridization probe at the indicated developmental stages. Even at the highest concentration used, the initial number of PGCs was not affected by the injected *nos*1 morpholinos (for each point 10–20 embryos were analyzed). (*e*) *nos*1 morpholino-injected embryos can be rescued by coinjection of *nos*1 mRNA that cannot be bound by the *nos*1 morpholino (FL*nos*1mut). (*f*) PGCs of *nos*1 morpholino-injected embryos are found in ectopic positions and die. The total number of PGCs decreases from the 3-somite stage to the 24-hpf stage, and most of the cell death can be attributed to cells found in ectopic locations (9–16 embryos were analyzed for each experimental point). Despite the severe PGC phenotype, whole-mount in situ hybridization of 3-somite stage embryos injected with morpholinos and stained with *myo*D, *pap*C, *pax*2.1, *pax* 8 (all in brown) and *vasa* (blue) show that somatic development is largely unaffected (*right* panel, ectopic cells are labeled with arrows). (*g*) A dying PGC in *nos*1 morpholino-injected embryos show the characteristic morphology of apoptotic cells. Embryos were injected with *nos*1 morpholinos as in *b* (*right* panel) and their PGCs were followed in live embryos at the end of gastrulation (1-somite stage). One of the two PGCs remains alive and exhibits normal morphology, while the other cell (arrow) undergoes cell death. Pseudocolors represent GFP intensity from blue (low) to green, yellow, red, and white (high).

typical PGC characteristics for several days (Weidinger et al. 1999). Taken together, these observations suggest that ectopic PGCs failed to migrate properly and then die due to the more severe reduction in the level of Nos1 protein relative to the cells that migrated properly. All of the ectopic cells exhibited morphological abnormalities. The dying GFP-labeled ectopic PGCs exhibited reduction in fluorescent intensity, membrane blebbing, nuclear fragmentation, followed by fast formation of apoptotic bodies (Fig. 4g). Morphological changes of this kind are characteristic of cells undergoing programmed cell death (Rich et al. 1999).

## Discussion

With mammals and urodele amphibians being an exception, vertebrates exhibit asymmetric localization of cytoplasmic determinants—the germ plasm—that was suggested to play a role in specification and development of their PGCs (Houston and King 2000a; Knaut et al. 2000;

Tsunekawa et al. 2000). In contrast to a large number of genes known to be important for germ plasm formation and PGC development in invertebrates, only few such genes have been characterized so far in vertebrates (Saffman and Lasko 1999; Wylie 1999; Houston and King 2000a). In *Drosophila*, many germ plasm components have been isolated employing genetic screens for maternal effect mutations. However in zebrafish, maternal effect screens are relatively difficult to perform and therefore are not likely to identify a large proportion of these genes (Pelegri and Schulte-Merker 1999). The identification of PGC-specific genes on the basis of sequence similarity to genes of other organisms is in some cases complicated by the low conservation of the primary sequence and by definition, such a strategy would not lead to the isolation of novel genes. In this study we utilized an alternative approach and present our results describing the first gene of several isolated as based on their specific expression in the PGCs of zebrafish (C. Thisse, B. Thisse, and E. Raz, unpubl.). Isolation of genes using this methodology coupled with the ability to efficiently block their translation in the early embryo is a powerful avenue for the identification and functional analysis of molecules important for germ plasm function and PGC development in vertebrates.

As mentioned above, germ plasm has not been identified in mammals and the pertaining evidence suggests that inherited asymmetrically-localized cytoplasmic factors have no role in PGC specification (e.g., Tam and Zhou 1996; Zernicka-Goetz 1998; Lawson et al. 1999; Tsang et al. 2001; Ying and Zhao 2001; Ying et al. 2001). Nevertheless, it appears that germ plasm-specific molecules identified in either invertebrates or vertebrates (e.g., *vasa* and *Xdazl*) do play a role at later stages of germ-cell development in mammals, as they are required for gametogenesis (Ruggiu et al. 1997; Tanaka et al. 2000). Thus, the identification and functional analysis of genes involved in early PGC development in zebrafish are relevant for the understanding of germ-line development in mammals, as well.

In this report, the function of a vertebrate *nanos* gene, a putative germ plasm component, is demonstrated for the first time. In zebrafish, *nanos* is required for the migration of PGCs and for the maintenance of their fate. These functions are strikingly similar to those of *nanos* during PGC development in invertebrates (Kobayashi et al. 1996; Forbes and Lehmann 1998; Deshpande et al. 1999; Subramaniam and Seydoux 1999). However, it is noteworthy that it is formally possible that a maternally-provided protein whose level is not affected by the antisense oligonucleotides masks an earlier role of Nanos in zebrafish PGC development. This matter could be investigated by using anti-Nanos antibodies and by generating mutations in the *nanos* locus.

In order to restrict Nos1 to the PGCs of zebrafish, several control mechanisms are operating in concert at the level of asymmetric RNA localization, differential RNA stability and translation. Interestingly, in invertebrates the spatial distribution of Nanos is similarly controlled (e.g. Gavis and Lehmann 1994 ; Bashirullah et al. 1999). Since other PGC-specific RNA molecules in zebrafish exhibit similar spatial distribution (Yoon et al. 1997; C. Thisse, B. Thisse, and E. Raz, unpubl.), it is conceivable that the mechanisms described here for *nanos* serve to restrict the function of other proteins to the PGCs as well. Taken together, our findings show that key steps in PGC development in invertebrates and in vertebrates, that is, PGC migration and maintenance of the PGC fate, require the function of related molecules, which are regulated by similar mechanisms.

## Materials and methods

### *Isolation of the* nos*-1 and nomenclature of* nanos*-related genes in zebrafish*

The *nos*1 cDNA (GenBank accession no. AY052376 ) was isolated in a large-scale screen for genes that are differentially expressed during early zebrafish development (C. Thisse and B. Thisse, unpubl.). As judged by 3′ and 5′ RACE analysis, the original clone included the full-length cDNA. Another *nanos*-related gene (*nos*2) had already been identified as an expressed sequence tag (EST, GenBank accession no. ai585000). Expression of this mRNA is first detected in the forming somites and in specific domains in the nervous system. No expression of this gene was detected in the germ cells during the first 2 d of development.

### *Whole-mount in situ hybridization and antibody staining*

RNA whole-mount in situ hybridization using the *nanos*1, *vasa*, and other probes for somatic derivatives was performed as previously described (Weidinger et al. 1999). Immunhistochemistry was performed as described by Knaut et al. (2000). The monoclonal mouse anti-GFP (B-2) antibody (Santa Cruz Biotechnology) was used at a 1:500 dilution and rabbit anti-vasa antibody (Knaut et al. 2000) was diluted 1:5000 before use. Secondary antibodies, goat anti-mouse Alexa Flour 488 and goat anti-rabbit Alexa Flour 546 (Molecular Probes) were used at a 1:200 dilution.

### *Plasmid constructs*

*FL*nos*1mut* This construct was used for rescuing *nos*1 morpholino-injected embryos. The region that is recognized by the morpholino antisense oligonucleotide was mutated by introducing nine nucleotide exchanges that do not alter the encoded protein sequence. *nos*1 cDNA served as the template for PCR amplification using the following forward primer: ATATAA GCTT**ATGGCCTTCAGCCTGCTGCAGTTTA**TCCTT TCT GCTCATGGAT (the region normally recognized by the morpholino oligonucleotide is in bold, and underlined residues designate positions of nucleotide exchanges relative to the wild-type sequence). The amplification product was cloned into pSP64T (Krieg and Melton 1984), from which the *Xenopus globin* UTRs were removed.

*GFP-FL*nos*1* The construct includes mmGFP5 (Siemering et al. 1996) cloned into the 5′UTR of *nos*1 in frame with the full *nos*1 ORF followed by the full *nos*1 3′UTR. The fused cDNA was cloned into pSP64T lacking the *Xenopus globin* UTRs.

*GFP-*nos*1–3′UTR* The construct includes the mmGFP5 ORF fused to the 3′UTR of *nos*1. The 3′UTR of *nos*1 was cloned 3′ to

mmGFP5 in pSP64T from which the *Xenopus globin* UTRs were removed.

*DsRed*-nos*1–3′UTR* This construct was cloned similarly to GFP-*nos*1–3′UTR using the DsRed2 (Clonetech) protein as a dominant marker.

*GFP*-globin mmGFP5 was cloned in between the *globin* 5′UTR and *globin* 3′UTR of pSP64T.

*EGFP-F*-globin The cDNA of farnesylated EGFP (Clonetech), which is subcellularly localized to the plasma membrane, was cloned in between the *globin* 5′UTR and *globin* 3′UTR of the RN3 RNA expression vector (Lemaire et al. 1995).

*Microinjection into zebrafish embryos*

mRNA for injection was prepared using the mMessage Machine kit (Ambion), was diluted in 10 mM HEPES (pH 7.6) and microinjected into one- to four-cell stage AB zebrafish embryos at the quantities indicated in the text and the figure legends. *nos*1 morpholino (*nos*1 morph) (TGAATTGGAGAAGAGAAAAAG CCAT) and control morpholino (cntl) (CCTCTTACCTCAG TTACAATTTATA) (Gene Tools) were injected in 10 mM HEPES (pH 7.6) at the quantities indicated in the text.

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