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Multiple Levels of Posttranscriptional Control Lead to Germ Line-Specific Gene Expression in the Zebrafish

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Summary

An important mechanism for the specification and development of the animal germ line is the localization of specific molecules to the germ plasm [1]. Restriction of these molecules to the germ line is considered to be critical for proper development of the germ line as well as the soma. Cytoplasmic localization alone, however, may not be sufficient to achieve germ line-specific expression. While zebrafish *vasa* mRNA is localized to the germ plasm [2, 3], the Vasa protein is initially distributed uniformly in the embryo, and its expression becomes restricted to the PGCs only later in development [2, 4]. Here, we demonstrate that, in addition to *vasa* RNA localization, multiple cell type-specific posttranscriptional mechanisms act on *vasa* mRNA and Vasa protein. We show that the portion of the maternal *vasa* mRNA, which is partitioned to somatic cells, is rapidly degraded, whereas *vasa* RNA is stabilized in the PGCs in a process that is mediated by *cis*-acting elements within the molecule. Similarly, the Vasa protein is highly unstable in somatic cells, but not in the PGCs. Finally, we demonstrate that subcellular localization of Vasa protein involves *cis*-acting domains within the protein. In conclusion, we show that posttranscriptional degradation-protection mechanisms acting on RNA and protein function in a vertebrate to enrich for specific molecules in the PGCs.

Results and Discussion

Germ cells must remain totipotent, i.e., maintain the potential to differentiate into all cell types of a new organism. Consequently, in many animal species, the primordial germ cells (PGCs) are set aside from somatic lineages very early in embryonic development [5, 6]. In most cases, the PGCs are specified by inheritance of germ plasm, a specific type of cytoplasm that contains maternally provided germ line determinants and electron-dense germinal granules. The zebrafish germ plasm shows a unique pattern of localization within the early embryo. At the 4-cell stage, four large germ plasm aggregates are found at the distal parts of the first two cleavage furrows [2]. Following asymmetric partitioning of these germ plasm aggregates during subsequent cleavages, four PGCs are specified at the late blastula (late sphere) stage, 4 hr postfertilization (hpf). The first characterized molecular marker for the zebrafish germ plasm is maternal *vasa* mRNA [2, 3]; zygotic transcription of *vasa* is initiated immediately following PGC specification [2]. *vasa* belongs to a conserved family of genes encoding germ line-specific, putative RNA helicases, which are essential for germ line development and gametogenesis in several species [7].

A Significant Fraction of the Maternal *vasa* mRNA Is Not Incorporated into the PGCs and Undergoes Degradation

Northern Blot analysis has indicated a marked decrease in the level of *vasa* mRNA during the first 24 hr of development [3]. To determine the time course of this decay more accurately, we have analyzed *vasa* mRNA levels at a higher temporal resolution. Our results show that the level of this RNA remains stable for the first 6 hr of development, but drops sharply during the next 2 hr, followed by a slower, gradual decrease (Figure 1A). These data suggest that degradation of maternal *vasa* mRNA commences a short time after the specification of the PGCs.

Could all or most of the degraded fraction of maternal *vasa* mRNA represent RNA that is not localized to the PGCs? To answer this question, we reinvestigated the expression pattern of *vasa* mRNA during early embryogenesis. Analysis of strongly stained embryos generated by whole-mount in situ hybridization revealed the presence of many small, *vasa*-positive aggregates in the cortex of 4-cell-stage embryos (Figure 2A), as well as many distinct aggregates of various sizes in putative somatic cells up to 4 hpf, when the PGCs are specified (Figures 1D and 1E and data not shown). In addition, the formation of extra *vasa*-containing aggregates along the third cleavage planes could be observed in about 10% of the 8-cell-stage embryos (arrows in Figures 1B and 1C), in agreement with previous findings [3]. After 4 hpf, diffuse *vasa* staining is found in somatic cells (Figure 1F), whereas several hours later, *vasa* is detectable exclusively in the PGCs (Figure 1G).

Together, these results suggest that a large fraction of the maternally supplied *vasa* mRNA does not become localized to the main germ plasm aggregates and is rather found in aggregates in putative somatic cells up to the late blastula stage (4 hpf). Thereafter, this material appears to disintegrate, followed by the gradual degradation of *vasa* mRNA in somatic cells.

vasa mRNA Is Specifically Stabilized in the Germ Line

In the invertebrate model organisms *Drosophila* and *Caenorhabditis elegans*, the initiation of zygotic transcription in the PGCs is delayed relative to somatic cells [8]. This transcriptional quiescence is accompanied by selective maintenance of specific transcripts in the PGCs, whereas the same molecules are degraded in somatic lineages [9, 10]. In vertebrates, PGC-specific transcriptional quiescence has not been described, and

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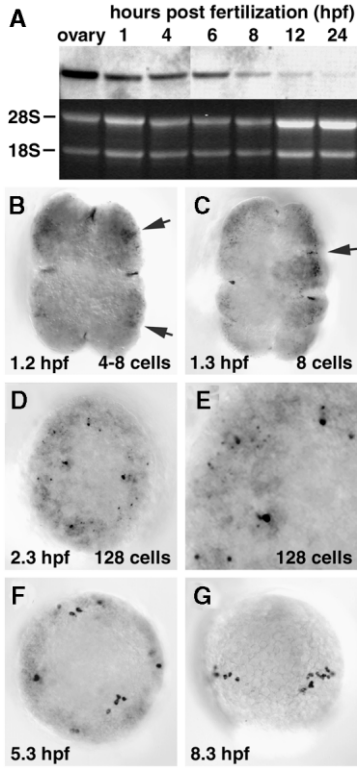

Figure 1. Maternal *vasa* mRNA Localized to Somatic Cells Is Concentrated in Aggregates and Undergoes Degradation

(A) Northern blot hybridized with a *vasa* probe (upper panel). The lower panel shows total RNA loaded on each lane.

(B–G) In situ hybridization of wild-type embryos using a *vasa* probe at the indicated stages. (B and C) Aggregation of cortical *vasa* mRNA along the third cleavage planes (arrows). (D and E) Typical distribution of *vasa* mRNA in many distinct aggregates in a blastula-stage embryo, magnified in (E). (F and G) After 4 hpf, *vasa*-containing aggregates in somatic cells are no longer visible. (B)–(F) show animal pole views, and (G) shows a dorsal view.

evidence in zebrafish argues against a similar mechanism [2]. Could degradation-protection processes acting on germ cell components nevertheless also play a role in vertebrate germ line development? We asked if *vasa* mRNA is specifically protected from degradation in the PGCs. To this end, we followed the fate of *vasa*-GFP hybrid RNAs injected into 1-cell-stage zebrafish embryos. The distribution of these RNAs at later stages of development was revealed by in situ hybridization using a GFP antisense probe. As a control, we injected RNAs containing only GFP sequences, flanked by untranslated regions (UTRs) of the *Xenopus globin* gene (construct 0, Figure 3A) or followed by the SV40 polyadenylation signal (see the Experimental Procedures). The control RNAs are degraded slowly in all tissues (Figures 2D–2F), with a slight delay in the PGCs in about 20% of the embryos (data not shown). Initially, two *vasa*-GFP constructs were used, with identical results: construct 1, an insertion of GFP cDNA into the full-length *vasa* cDNA; and construct 3, in which GFP was fused with the *vasa* 3′UTR (for maps, see Figure 3A). At the 4-cell stage (1 hpf), injected *vasa*-GFP RNAs, like the control RNAs, are uniformly distributed in the cytoplasm (Figures 2D and 2G). At the 6-somite stage (12 hpf), in striking contrast to the control RNAs, the *vasa*-GFP RNAs 1 and 3 are almost fully degraded in somatic cells, while the PGCs remain strongly labeled (compare Figures 2E and 2H). Importantly, at 24 hpf, the PGCs of these *vasa*-GFP RNA-injected embryos still contain high levels of the injected RNAs (Figure 2I), whereas the control RNAs are completely degraded (Figure 2F). *vasa*-GFP RNA can be detected in the PGCs up to 50 hpf (data not shown). These results suggest that the *vasa* mRNA is subject to a tissue-specific degradation-protection process, i.e., decay in somatic cells and, conversely, remarkable stability in the germ line. When we injected lower concentrations of *vasa*-GFP RNAs, these RNAs were already fully restricted to the germ line by the end of gastrulation (8–9 hpf; data not shown), similar to endogenous *vasa* mRNA (see Figure 1). Interestingly, several newly identified RNA markers for the zebrafish PGCs exhibit an early expression pattern similar to that of *vasa* (C. Thisse, B. Thisse, and E.R., unpublished data). One of these RNAs (*nos1*) appears to be regulated by a similar mechanism [11], indicating that the degradation-protection process described here may act on several mRNAs and could be generally important for the proper development of the zebrafish germ line and soma.

Several Redundantly Acting Elements Mediate Stabilization of *vasa* mRNA in the PGCs

To further analyze the tissue-specific *vasa* mRNA degradation and protection, we defined *cis*-acting regions involved in this process. We fused GFP RNA to smaller portions of the *vasa* mRNA and analyzed the injected embryos as described above. First, all hybrid RNAs containing portions of the *vasa* 3′UTR (constructs 4, 5, and 6) are rapidly degraded in the soma (as analyzed at the 6-somite stage) when compared to the control UTRs (Figure 3A). Second, we have defined three regions within the *vasa* transcript that independently mediate specific RNA stabilization in the PGCs, resulting in a high level of injected RNA in the PGCs at 24 hpf (Figure 3A). Two of these elements map to the *vasa* 3′UTR (constructs 5 and 6), and another element is located in the *vasa*-coding region (constructs 8 and 9). The remaining portions of the *vasa* mRNA are not sufficient for germ line-specific RNA stabilization (Figure 3A). Therefore, at least three redundantly acting elements mediate specific protection of *vasa* from degradation in the germ line.

Vasa Protein Is Specifically Degraded in the Soma but Maintained in the PGCs

Zebrafish Vasa protein is distributed uniformly in all cells of early embryos up to the late blastula stage (4 hpf), whereas at 24 hpf, Vasa expression is restricted to the PGCs [2, 4]. We asked if Vasa protein is subject to a tissue-specific degradation-protection process, similar to its RNA. To address this question, we injected RNAs encoding either GFP or Vasa-GFP fusion proteins. GFP protein is stable in all tissues for several days (see below). The Vasa-GFP fusion protein translated from construct 1-RNA (Figure 3B), however, is degraded rapidly in somatic tissues; at 24 hpf, GFP fluorescence is almost undetectable in somatic cells, whereas the PGCs remain

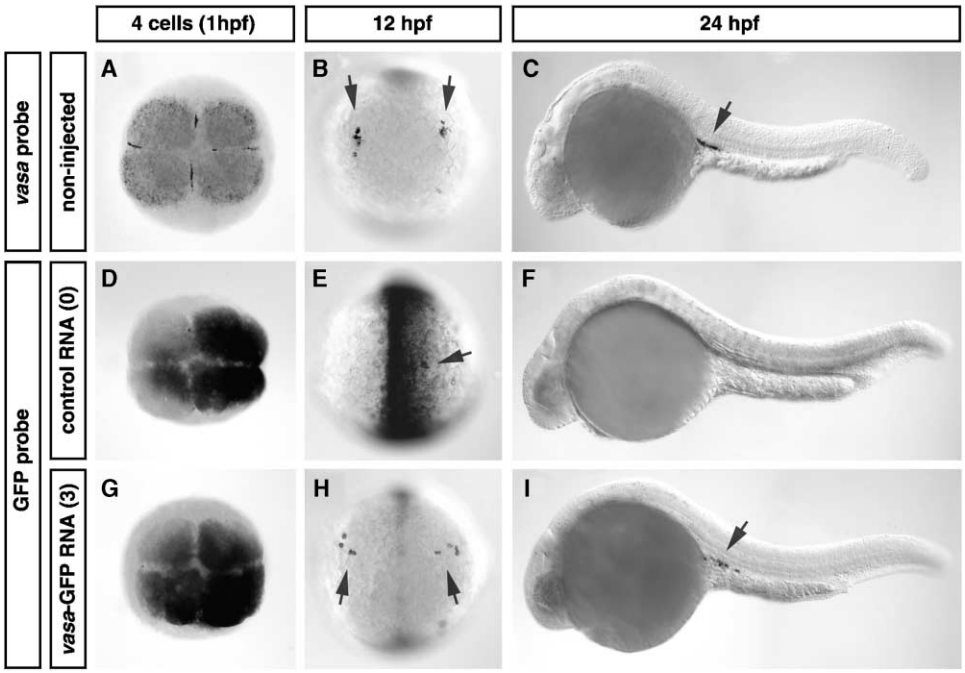

Figure 2. *vasa*-GFP Hybrid RNA Is Rapidly Degraded in Somatic Cells but Stabilized in the PGCs

(A–C) In situ hybridization of noninjected embryos using a *vasa* probe at the indicated stages. Arrows mark the PGCs.

(D–I) In situ hybridization using a GFP probe, following RNA injection at the 1-cell stage. Arrows indicate labeled PGCs. (D–F) Embryos injected with a control RNA (construct 0), which is slowly degraded in all tissues. (G–I) Embryos injected with *vasa*-GFP RNA (construct 3). This RNA is rapidly degraded in the soma but maintained in the PGCs. (A), (D), and (G) show animal pole views; (B), (E), and (H) show dorsal views; and (C), (F), and (I) show lateral views.

strongly labeled (Figure 4B, arrow). Importantly, this Vasa-GFP protein displays approximately the same time course of degradation as nonmodified Vasa protein: we injected RNAs encoding these proteins into embryos depleted of endogenous Vasa (see the Experimental Procedures), which allowed us to detect only the experimentally introduced proteins using a Vasa antibody. Our results (Figures 4I–4L) demonstrate that Vasa and Vasa-GFP proteins are degraded similarly in somatic cells but are maintained in the germ line (arrows in Figures 4J and 4L).

One obvious caveat is that the observed tissue-specific protein expression could simply reflect the underlying RNA pattern or translational control rather than differential protein degradation. The following observations argue against these options. First, a Vasa-GFP fusion protein regulated by the *Xenopus globin* control UTRs (construct 2, Figure 3B) is also unstable in the soma but maintained in the PGCs, similar to construct 1 (see Figures 4A–4D). Second, we used two control RNAs (constructs 3 and 11), which encode only GFP but are regulated by *vasa* RNA sequences. As described above (and data not shown), all RNAs containing the *vasa* 3′UTR are degraded in exactly the same pattern (see Figures 2G–2I and 3A). GFP protein translated from these control RNAs is detectable in somatic cells for 2–3 days after the RNA is fully degraded (Figures 4F and 4G), in contrast to about 10 hr for Vasa-GFP protein (Figure 4B). In the PGCs, however, GFP as well as Vasa-GFP protein are stable for up to 5 dpf (days postfertilization) (Figures 4D and 4H, arrows), i.e., 2–3 days after the RNAs are fully degraded (data not shown). These results were also confirmed for an earlier stage of development: we measured the GFP fluorescence levels in the soma and PGCs at 24 hpf for a different set of constructs (12 and 13, Figure 3B). Whereas both RNAs were degraded in the same pattern (data not shown), the two proteins displayed different stabilities in the soma but were both stable in the PGCs (for details, see Table S1 in the Supplementary Material available with this article online).

We were not able to define specific elements required for this differential Vasa protein degradation, as fusions of GFP with smaller parts of Vasa gave inconsistent results. We also observed that, unlike endogenous Vasa protein, fusions of GFP to the N terminus or C terminus of Vasa were surprisingly stable in all tissues.

Taken together, our results suggest that, in addition to differential degradation of *vasa* mRNA, Vasa protein is subject to a degradation-protection process: Vasa protein in somatic cells is rapidly degraded, whereas it shows significantly higher stability in the PGCs. Interestingly, a very similar cell type-specific degradation process has been described for the *C. elegans* germ line factor PIE-1 [12].

Domains Mediating Subcellular Localization of Vasa Protein

Beginning at 4 hpf, Vasa protein is found in defined perinuclear structures within the PGCs, which resemble nuage particles described in other animals [2, 4]. We addressed the question of whether specific domains within the Vasa protein are involved in this subcellular localization, which concentrates the protein in the posi-

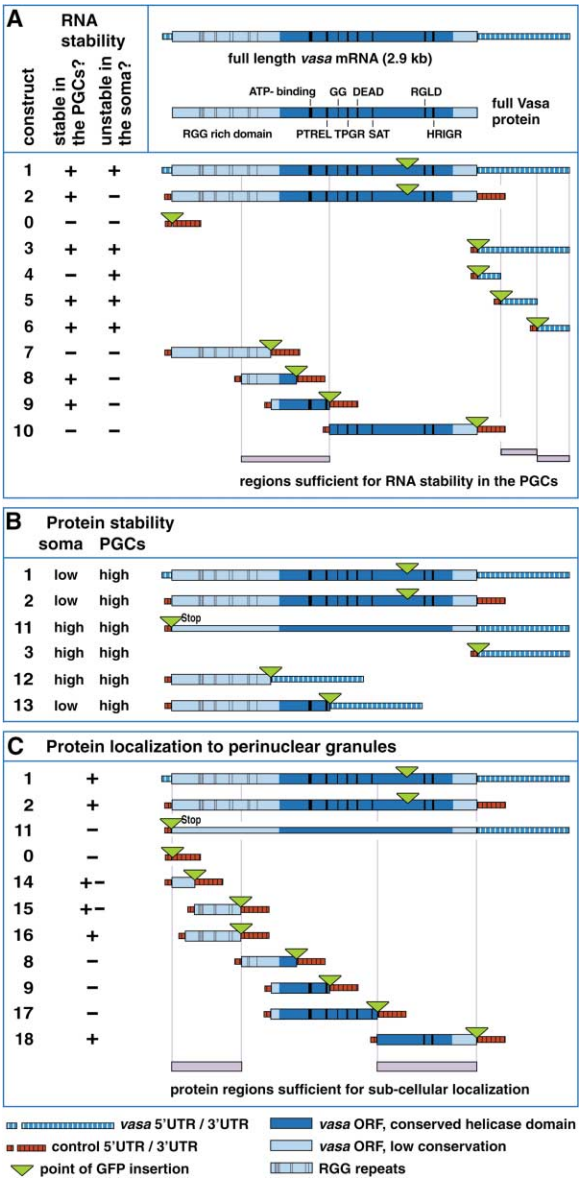

Figure 3. Maps of the RNA Expression Constructs

(A) RNA stability. High (+) or low (−) stability in the PGCs was determined at 24 hpf, whereas rapid (+) or slow (−) somatic degradation of the injected RNAs was determined at 12 hpf, as shown in Figure 2. Elements sufficient for RNA stability in the PGCs are indicated at the bottom.

(B) Protein stability in the soma and PGCs was analyzed as shown in Figures 4A–4H.

(C) Elements sufficient for protein localization to perinuclear granules within the PGCs. We distinguished complete (+), incomplete (+−), and no localization (−). The corresponding phenotypes are shown in Figures 5A–5C. ORF, open reading frame.

tion where its function is presumably required. We first analyzed if Vasa-GFP fusion proteins can become localized to these structures. Indeed, the GFP fusion protein derived from construct 1 and endogenous Vasa protein colocalize to the same granules (data not shown), whereas GFP protein alone is not localized (constructs 0 and 11, Figures 3C and 5C). To identify domains within the protein responsible for this localization process, we injected RNAs encoding partial Vasa-GFP protein fusions (Figure 3C). We identified two redundantly acting protein regions of Vasa, which are able to direct localization of the fusion proteins (constructs 14–16 and 18, Figure 3C; Figure 5A). Interestingly, one of these regions is the N-terminal domain typical for all Vasa proteins, which is characterized by a very low level of primary sequence conservation and the presence of several RGG (arginine-glycine-glycine) repeats. Smaller parts of this domain mediate partial localization (+−, Figure 5, and constructs 14 and 15 in Figure 3C). Intriguingly, the two domains described here overlap with the two regions of the *Drosophila* Vasa protein implicated in subcellular localization [13], raising the possibility that the underlying molecular mechanisms for Vasa localization are conserved between these organisms.

Conclusion

Our study, together with previous results, demonstrates that at least five levels of control function to regulate the distribution of the zebrafish *vasa* mRNA and protein. The earliest step of regulation takes place before the specification of the PGCs: maternal *vasa* mRNA is enriched in four large germ plasm aggregates [2, 3]. Once the PGCs are specified, four additional processes act differentially in the soma and PGCs: selective maintenance of *vasa* mRNA as well as Vasa protein in the PGCs (this study), together with zygotic transcription of *vasa* [2], result in a high level of Vasa protein in the PGCs. On the other hand, the same molecules are specifically degraded in the soma (this study). In addition, Vasa protein is localized to perinuclear structures within the PGCs. Our data suggest that differential posttranscriptional regulation of germ line factors may be generally important in the development of the vertebrate germ line.

Finally, our study has interesting technical implications. So far, no method was available for visualization of PGCs in live zebrafish embryos. Injection of *vasa*-GFP RNAs enabled us to easily identify the PGCs from the beginning of gastrulation up to several days of development (see the Supplementary Material for a time-lapse movie). This method will greatly facilitate various approaches including transplantation, culturing, and in vitro manipulations of germ cells.

Experimental Procedures

Zebrafish Maintenance

Zebrafish (*Danio rerio*) (AB wild-type strain) were maintained as described previously [14]. Embryos were kept at 28.5°C.

Construction of Plasmids, Preparation of Sense RNA, and Injection

Full-length *vasa* cDNA was amplified as described in [15]. As control UTRs, we used the *Xenopus globin*-5′ and -3′UTRs, derived from pBluescriptRN3 [16] or the Simian Virus 40 late polyadenylation site [17]. The GFP variant mmGFP-5 was used [18]. All recombinant cDNAs were cloned into the vector pBluescriptRN3 [16]. An XbaI site was added to the vector for the linearization of templates. For base positions of the *vasa*-GFP constructs, see Table S2 in the Supplementary Material.

Capped sense RNA was synthesized using the T3-Message Machine kit (Ambion). Equimolar amounts (3×10^{-17} moles) of RNA

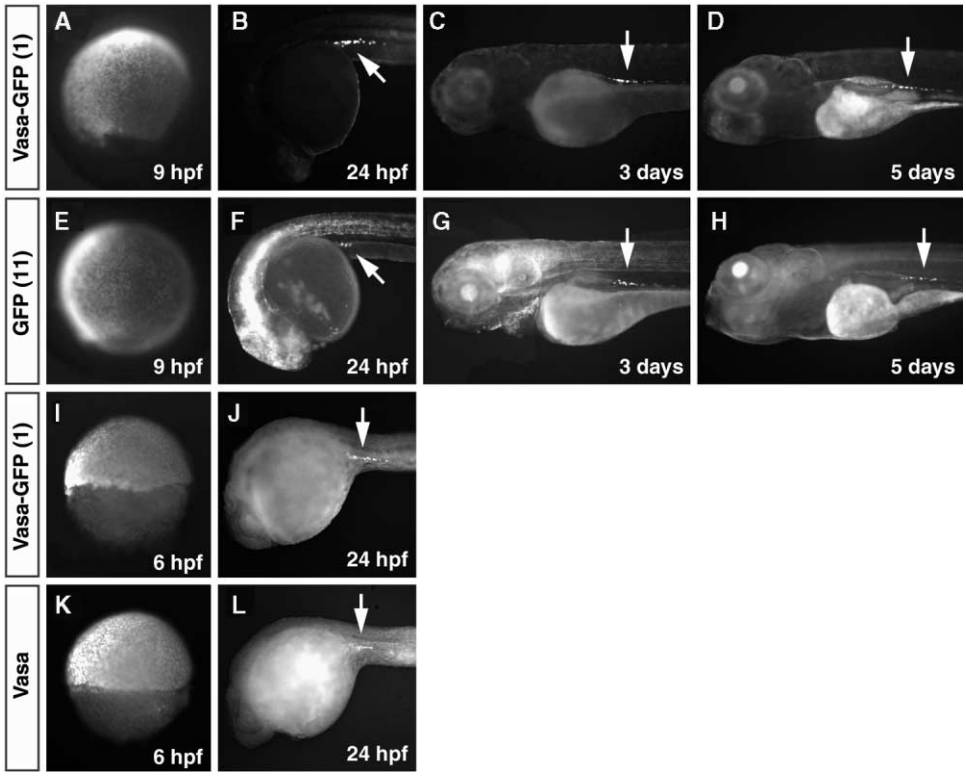

Figure 4. Vasa Protein Is Rapidly Degraded in the Soma but Maintained in the PGCs

(A–H) GFP fluorescence in injected embryos; arrows mark labeled PGCs. (A–D) Embryos injected with construct 1-RNA. (A) Vasa-GFP protein is uniformly expressed at 9 hpf; (B) at 24 hpf, this protein is no longer detectable in the soma, (C and D) whereas it is maintained in the PGCs for up to 5 dpf. (E–H) GFP protein translated from injected construct 11-RNA is detectable for (E–G) 3 days in somatic tissues and for up to (H) 5 days in the PGCs.

(I–L) Immunostainings reveal a similar, cell type-specific degradation behavior of Vasa and Vasa-GFP proteins in embryos depleted of endogenous Vasa protein. (I and J) Embryos injected with construct 1-RNA. (K and L) Embryos injected with full-length *vasa* mRNA. All embryos are shown in lateral view; in some cases, the yolk ball shows nonspecific background fluorescence.

per embryo were injected for all constructs. For each RNA, unless mentioned otherwise, at least 20 embryos were analyzed.

Whole-Mount In Situ Hybridization

Whole-mount in situ hybridization was performed as described by Jowett and Lettice [19].

Depletion of Endogenous Vasa Protein and Immunostainings

We injected 800 pg of a *vasa* morpholino antisense oligo, which blocks the translation of endogenous *vasa* mRNA but does not affect other aspects of germ line development [20]. The coinjected experimental RNAs carried silent mutations that rendered them insensitive to the morpholino oligo. A Vasa antibody was used for immune histology as described in [2].

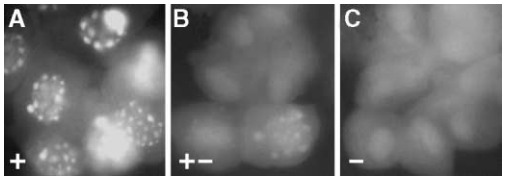

Figure 5. Localization of Vasa-GFP Fusion Proteins to Perinuclear Granules within the PGCs

(A–C) GFP fluorescence in clusters of PGCs at the 12 hpf stage. (A) The Vasa-GFP fusion protein derived from construct 1 is fully localized (+) to the perinuclear granules. (B) PGCs in embryos injected with construct 14-RNA; the perinuclear granules are only faintly labeled (+−). (C) PGCs in embryos injected with the construct 0-control RNA; GFP protein is not localized to these granules (−).

Supplementary Material

Supplementary Material including detailed information regarding the differential degradation of Vasa-GFP protein fusions and a detailed description of the DNA constructs used in the different experiments is available at http://images.cellpress.com/supmat/supmatin.htm. The movie that is included in the Supplementary Material demonstrates labeling of PGCs in live embryos, made possible by the method described in the manuscript.

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