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Temporal and spatial control of germ plasm RNAs

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Abstract

In many species germ cells form in a specialized germ plasm, which contains localized maternal RNAs [1–5]. In the absence of active transcription in early germ cells, these maternal RNAs encode germ cell components with critical functions in germ cell specification, migration and development [6,7]. For several RNAs, localization has been correlated with release from translational repression, suggesting an important regulatory function linked to localization [3,4,8,9]. To address the role of RNA localization and translational control more systematically, we assembled a comprehensive set of RNAs that are localized to polar granules, the characteristic germ plasm organelles. We find that the 3′-untranslated regions (UTRs) of all RNAs tested control RNA localization and instruct distinct temporal patterns of translation of the localized RNAs. We demonstrate necessity for translational timing by swapping the 3′UTR of *polar granular component* (*pgc)*, which controls translation in germ cells, with that of *nanos*, which is translated earlier. Translational activation of *pgc* is concurrent with extension of its poly(A) tail length, but appears largely independent of the Drosophila CPEB homolog ORB. Our results demonstrate a role for 3′UTR mediated translational regulation in fine-tuning the temporal expression of localized RNA and may provide a paradigm for other RNAs that are found enriched at common cellular locations such as the leading edge of fibroblasts or the neuronal synapse.

Results and Discussion

Translation of germ plasm RNAs is temporally regulated by their 3′UTRs

To investigate the translational state of germ line localized RNAs, we assembled a list of RNAs localized to germ plasm using publicly available databases and published reports. We used data from the Berkeley Drosophila Genome Project (BDGP) *in situ* database, the embryo data base by Lecuyer *et al.* and literature searches to assemble a list of RNAs present in germ cells and then tested these RNAs for their mode of germ cell localization [10,11]. We based our analysis on the expression patterns of RNAs previously known to be localized to the germ plasm such as *nanos*, *germ cell less (gcl)* and *polar granule component* (*pgc)*; by electron microscopy these RNAs were shown to be localized to the polar granules, which are integral RNA-protein components of germ plasm [12,13]. *nanos*, *gcl* and *pgc* are initially localized in the form of a crescent at the posterior pole of the embryo (stage 1–2) and are then incorporated

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into developing germ cells (stage 3–4). Our analysis suggests that about ~33% (58/171) of germ cell RNAs are localized in a manner similar to *nanos*, *gcl* and *pgc*, while the remaining RNAs are protected in germ cells by selective stabilization without prior localization (see Supplementary Tables 1 and 2). The majority of maternally synthesized RNA is not localized or protected and this RNA is degraded at the transition from maternal to zygotic gene expression (stage 4–5) [8,14–18]. Of the 58 genes with expression patterns comparable to *nanos*, *gcl* and *pgc* we selected 11 for further analysis (Table 1).

In the case of *nanos*, RNA translation in the embryo is linked to its localization to the germ plasm, and both aspects of RNA regulation are mediated by the *nanos* 3′UTR [8]. In order to determine whether this link between RNA localization and translation applies more generally to RNAs localized to the germ plasm and is mediated by 3′UTRs, we generated reporter constructs containing the 3′UTRs of selected localized RNAs and used previously described reporters for *nanos* and *orb* [19,20]. We fused the maternally active *nanos* promoter and its 5′ UTR to the green fluorescent protein (GFP) coding region, which carried a HA-tag on both the C- and N-termini, and added to this reporter cassette the 3′UTRs of selected localized RNAs (Figure 1A). We assayed the resulting transgenic lines for localization to the germ plasm by using *in situ* hybridization analysis for GFP RNA. For each of the seven transgenes generated, the 3′UTR was sufficient for germ plasm localization as well as degradation of the uniformly distributed RNA that is found throughout the embryo (Table 1,Figure 1B–D,Supplementary Figure 1B–K). To determine if the localization pattern of these hybrid transgenic constructs was germ plasm dependent, we tested a reporter construct containing the *pgc* 3′UTR (*pnos::HA-GFP-HA pgc* 3′UTR) in an *oskar (osk)* mutant background in which germ plasm is not formed (Supplementary Figure 2). GFP RNA was not localized to the posterior pole in embryos from *osk* mutant mothers. We confirmed this result by crossing the transgene into females that carried an *osk-bcd* 3′UTR transgene; in this genetic background germ plasm is formed ectopically at the anterior pole due to OSK mediated assembly of germ plasm at the anterior pole and the expression of the reporter is found at the anterior (Supplementary Figure 2) [21]. Thus in both assays, localization of the hybrid *pgc* reporter construct was dependent on a functional germ plasm.

As the reporter constructs demonstrated that 3′UTRs were sufficient to localize RNAs to the germ plasm, we wanted to analyze the translational state of these RNAs beginning at the germ plasm stage (stage 1) through stage 8 of embryogenesis, when zygotic transcription is initiated in germ cells [22]. In addition to following the expression of GFP protein translated by the respective reporter construct, we analyzed the expression of endogenous proteins when antibodies were available. The results are summarized in Figure 1E and Supplementary Figures 3–6. In general, we found that all localized RNAs tested were translationally regulated. With the exception of *cyclin B*, the reporter RNAs were not translated outside the germ plasm in the early embryo. Although all of the RNAs analyzed showed an apparently identical localization to germ plasm, the onset of translation varied and we observed distinct patterns, which we assigned to five different classes. Class I RNAs such as *nanos* and *orb* are already translated in the germ plasm (stage 1) (Supplementary Figure 3). Class II RNAs such as *gcl* are repressed in germ plasm and translated at nuclear cycle 9 just before germ cell formation (stage 2–3) (Supplementary Figure 4). Class III RNAs including *pgc*, *sra*, CG5292, CG18446 and Rapgap1 are translationally repressed in the germ plasm and translationally active concurrent with germ cell formation (stage 4) (Supplementary Figure 5). Class IV RNAs such as *bruno* and CG2774 are not translated in germ cells during embryogenesis (Supplementary Figure 6). Class V includes *cyclin B*, which is translationally repressed in germ plasm and germ cells and activated at stage 16, when germ cells have reached the somatic gonad [23,24]. By utilizing available antibodies or published protein expression patterns we found that the onset of translation was identical between the respective reporter constructs and the endogenous proteins for PGC, GCL, NANOS and BRUNO [25–28]. However, GFP protein often persisted in germ cells

beyond detection of the endogenous protein (Supplementary Figure 7), likely due to differences in protein stability. To address whether the amount of RNA localized may affect the timing of translation, we compared the onset of translation in embryos that received two copies of the *pnos::HA-GFP-HA pgc* 3′UTR transgene from their mother to embryos that had received one copy of the transgene and embryos with reduced germ plasm (derived from mothers heterozygous for *oskar*). While the amount of RNA localized to the germ plasm clearly differed, the onset of translation was not affected (Supplementary Figure 8). Taken together, our analysis of an extended number of localized RNAs suggests that RNA localization per se does not trigger translation and demonstrates that discrete information encoded by specific 3′UTRs dictates the exact timing of expression of a localized RNA.

Translation of *pgc* is associated with polyadenylation

To further explore the link between 3′UTR-mediated localization and subsequent activity, we chose to focus on Class III RNAs, namely *pgc* and four other RNAs, for which translation was repressed during the early cleavage stages of embryogenesis and was activated upon germ cell formation (Figure 1E, Supplementary Figure 5). This particular pattern suggests that these protein products may perform functions required specifically in newly formed germ cells. Indeed, *pgc*, the best-studied representative of this class of RNAs, controls transcriptional silencing in germ cells at this stage [25, 29]. To determine how translation is induced upon germ cell formation, we determined if *pgc* activation is mediated by poly(A) tail elongation, a common mechanism of 3′UTR regulation [30]. We collected cDNA from adult ovaries and timed embryo collections using progeny from wild-type and *oskar (osk)* mutant flies and performed poly(A) tail length (PAT) assays [31]. Using RT-PCR we could detect an amplicon for *pgc* RNA during oogenesis in both wild-type and *osk* mutant flies (Figure 2, Supplementary Figure 9A, B). During embryogenesis, we detected a strong *pgc* RNA signal between stages 1–4 (0–150 mins after egg deposition (AED)) and a weaker signal until stage 8 (330 mins AED) in wild-type embryos; this result is consistent with the degradation of the majority of unlocalized *pgc* RNAs during the maternal to zygotic transition of gene expression at stage 4–5 and protection of the localized RNA in germ cells [14]. Indeed, in *osk* mutants in which *pgc* RNA is not localized to germ cells, we detected *pgc* until stage 3–4 but not at later stages (Figure 2A, Supplementary Figure 9B). Using the PAT assay, we detected a prominent RNA species with a short poly(A) tail of about 100 nucleotides (nt) during oogenesis and embryogenesis in wild-type and *osk* mutant embryos; an additional, longer poly(A) tail of about 200–250 nt was present in the 0.5–1.5 hour collection from wild-type embryos (Figure 2A, Supplementary Figure 9B). Only 12% percent of the total RNA was shifted to the longer tail length (Supplementary Figure 9C). These data are consistent with previous studies showing that only a small fraction (4–18%) of total *nanos* and *oskar* RNA, respectively, are localized [32]. Since the long poly(A) tail species of *pgc* RNA were only detected in wild-type embryos, but not in *osk* mutant embryos, we conclude that the long poly(A) tail is only present when *pgc* RNA is localized to the germ plasm and translated in germ cells. We also analyzed the length of the poly(A) tail of Class IV RNA *bruno*, which is localized but not translated during germ cell formation. During oogenesis when *bruno* is translated it has a long poly(A) tail of 350–400 nt (Supplementary Figure 9D) [28]. However, during embryogenesis *bruno* is not translated and has a short poly(A) tail of about 75 nt. A short poly(A) tail is also observed in *oskar* mutants (Supplementary Figure 9D). Taken together our data show that translation of localized RNA correlates with an increase in poly(A) tail length and suggest that polyadenylation is one of the mechanisms that triggers 3′UTR-mediated translational timing.

One mechanism by which *pgc* RNA poly(A) tail length may be regulated is by regulated access of the Cytoplasmic Polyadenylation Element binding protein (CPEB) to the RNA. In neuronal granules, as well as during oocyte maturation of *Xenopus laevis* eggs, repressed RNAs are activated by poly(A) elongation via the activity of CPEBs [33]. *Drosophila* has two CPEBs;

of these the one encoded by the *orb* gene is predominantly expressed in the germ line. *orb* RNA and protein are both present in germ plasm and in germ cells (Table 1 and Supplementary Figure 10). However, genetic analysis of ORB's role in germ plasm translation is difficult. ORB plays essential roles during oogenesis including positively regulating the translation of *osk* at the posterior pole of the oocyte [34,35]. Indeed, the weak orb^{mel} allele has a phenotype similar to that of *osk*, and embryos laid by orb^{mel} mothers fail to assemble germ plasm or form germ cells, precluding the direct analysis of a later role of ORB in germ plasm or germ cells [36].

To assess if ORB is required for *pgc* RNA poly(A) tail elongation and translational activation we circumvented the necessity for *orb* in the translation of *osk* and thus the formation of germ plasm. We localized *osk* RNA to the anterior pole of the embryo by utilizing the *bcd* 3′UTR [15]. Embryos from orb^{mel}/orb^{343} mutant mothers, carrying both the *osk-bcd* 3′UTR and p*nos::GFP-HA-pgc* 3′UTR transgenes, were collected and stained for VASA, a germ cell marker, and the HA tag to detect expression from the *GFP-HA-pgc* 3′UTR transgene (Figure 2B–C). The mutant embryos had no germ cells at the posterior end, confirming the role of ORB protein in the synthesis of endogenous OSK protein. VASA positive cells, however, were detected at the anterior pole, which also stained positively for HA (Figure 2B–C). Thus, *orb* does not affect the translation of *pgc* and *germ cell less (gcl)*, which are required for germ cell specification and formation downstream of *osk* [26]. Low levels of ORB activity present in the orb^{mel} mutant could be sufficient for *pgc* and *gcl* translation but not for *osk* translation or the Drosophila poly(A) polymerase, *hiiragi* (*hrg*), which has been shown to act cooperatively with ORB for *osk* translation, acts independently of ORB for *pgc* and *gcl* regulation [37]. Alternatively, rather than polyadenylation, deadenylation may be the regulated component that controls the onset of *pgc* and *gcl* translation. Indeed, the CCR4-Not-Pop2 deadenylation complex has been shown to control *Cyclin B* RNA translational repression in early germ cells [24].

Polar granules coordinate translation of germ plasm RNAs

The role of polar granules in the regulation of germ cell RNAs remains elusive. In somatic cells, Processing (P) bodies are known centers of RNA repression [38]. As polar granules share common components with P bodies, it has been proposed that polar granules are centers of RNA repression [6,39]. However, EM studies have also shown that polar granules contain ribosomes, thereby predicting a more active role in translation [13]. Among the localized germ plasm RNAs that we investigated, *nanos*, *gcl* and *pgc* are found in polar granules by electron microscopy at the germ plasm stage [12,13]. These three RNAs are translated at different time points, namely at the germ plasm (stage 1), germ bud (stage 2–3) and germ cell stage (stage 4) respectively. If polar granules had a solely repressive or activating role, one might expect that the association of these RNAs with polar granules would change during development as each RNA becomes translated. We used a transgenic line that expressed an Aubergine-GFP fusion protein (AUB-GFP), to mark polar granules [40,41] while also assessing *nos*, *pgc* and *gcl* RNA localization by fluorescent *in situ* hybridization. We found that all three RNAs co-localize with polar granules during all stages of germ cell formation (Supplementary Figure 11, 12). While it is possible that small amounts of RNA leave the granules and are then translated, we favor the hypothesis that polar granules are dynamic centers of RNA regulation that control both RNA repression and translation.

Regulation of translation by 3′UTR is important for proper development

Our results show that germ line RNAs are translationally regulated during embryogenesis in a temporally restricted manner. We next wanted to determine whether altering the temporal expression of these RNAs by switching 3′UTRs had consequences for proper germ line or somatic development. We chose *pgc* because of its role as a global transcriptional repressor in

germ cells, a function that is required when germ cells form [29]. Furthermore, ectopic expression of *pgc* causes transcriptional silencing in somatic tissues [25]. We swapped the *pgc* 3′UTR, which restricts translation to the germ cell stage (stage 4), with the *nanos* 3′UTR, which confers translational activation earlier as oocytes mature during late oogenesis and in germ plasm (Figure 3B1–B4′) [42]. In transgenic lines that carry *pgc* under the control of the *nanos* 3′UTR, somatic cells located adjacent to the germ cells failed to cellularize properly and nuclei fell into the yolk, leaving a "pole hole" in ~50% (≥3 cells) of embryos (n = 75) compared to ~10% in wild type (n=70) (Figure 3C–D). Since PGC protein represses transcription in a global manner, we asked whether ectopic PGC protein reduces the expression of zygotically expressed genes that are required for somatic cell formation. We therefore analyzed the status of RNA Polymerase II activity by staining embryos with an antibody that recognizes phosphorylation of Ser2 (P-Ser2) in the Carboxy Terminal Domain (CTD) of RNA Polymerase II, a marker for active transcription. In embryos with precocious PGC translation, the pSer2 epitope was reduced in the nuclei of the posterior blastoderm and consequently these nuclei expressed lower levels of proteins like SLAM that are required for somatic cellularization (Figure 3E–H). We conclude that temporal regulation of germ plasm restricted RNAs like *pgc* is important to segregate the germ line program from the somatic program.

Conclusion

By systematically analyzing RNAs localized to germ plasm in the embryonic germ line we show that 3′UTRs play an instructive role in the spatial and temporal control of germ line expression, a role made especially critical due to the lack of active transcription in early germ cells. In general, sequences within the 3′UTR restrict and protect RNAs with function in germ line biology to the germ cells. Moreover, the 3′UTR also harbors a specific program to repress and activate translation at distinct times of development. Thus, contrary to previous findings with *oskar* and *nanos* RNA, which suggested a mechanism by which translational repression was relieved concomitant with localization, our results suggest that additional mechanisms regulate translation during different stages of germ line development. Our results suggest that association with polar granules may not be limited to translationally active or repressed RNAs. Since transcription is repressed in germ cells, intrinsic timing mechanisms need to control the activity of transacting factors or the accessibility of RNA structure to relieve repression within polar granules. Large scale RNA localization is not unique to Drosophila germ plasm but has also been observed in migrating fibroblasts and in neuronal dendrites [43,44]. So far only a small number of RNAs have been analyzed in detail for their regulation. A more systematic analysis of regulated RNAs should provide new insight into the logic contained in 3′UTRs that instructs specific translational outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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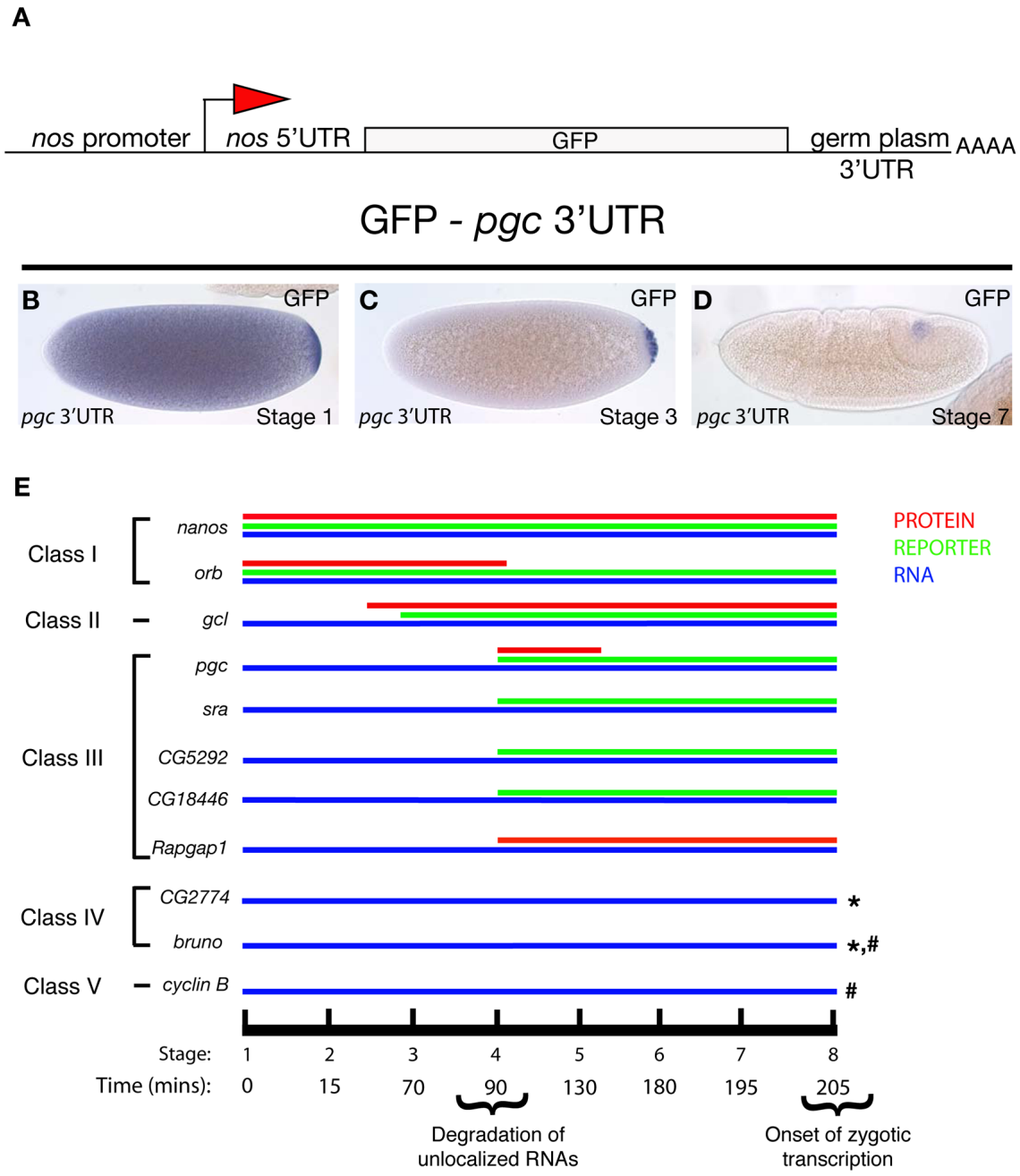

Figure 1. Translational regulation of germ plasm RNAs

A. Diagram shows the GFP-HA-3′UTR reporter cassette used in this study. For *nanos* 3′UTR, GFP was fused to moesin instead of HA [19]. *Orb* 3′UTR was fused to LacZ [20] **B–D.** *pgc* 3′ UTR recapitulates endogenous RNA localization. *In situ* hybridization for *GFP* RNA at different stages of embryogenesis shows degradation of unlocalized *pgc* RNA and protection of localized RNAs in germ cells. **E.** Classification of germ plasm localized RNAs according to onset of translation. Blue line represents endogenous RNA. Green line represents translation of the reporter construct under control of respective 3′UTRs. Red line represents endogenous protein expression when antibodies were available. Stages of embryonic development and corresponding developmental time after egg deposition are indicated by the black line. Lines

marked with (*) were tested for reporter expression only and showed no expression of GFP/HA. Lines marked with (#) were tested for expression of endogenous protein and showed no protein expression. Stages as indicated, posterior of the embryo is to the right.

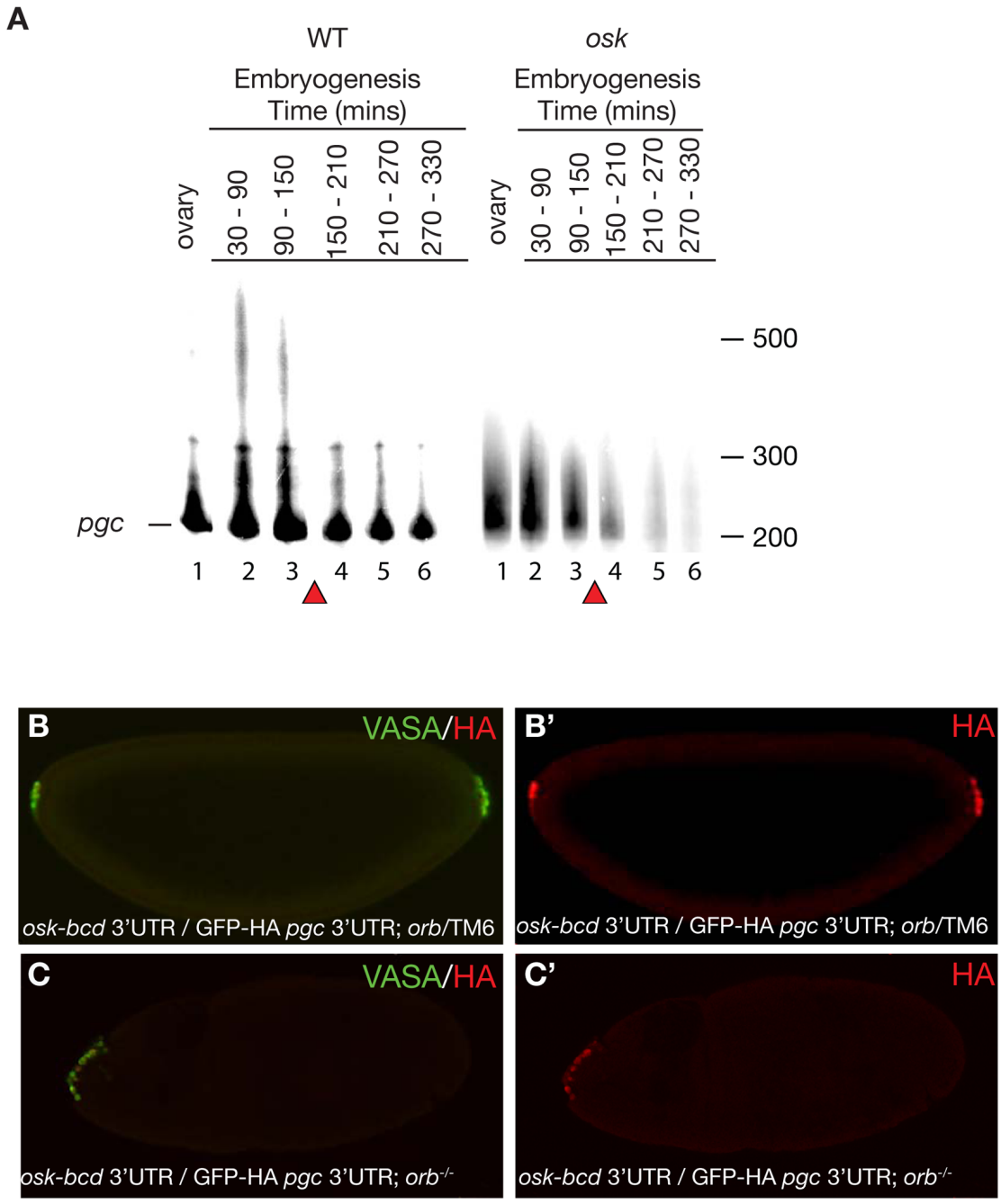

Figure 2. Translation of *pgc* is concurrent with poly(A) tail extension and is CPEB independent

A. PAT assay was performed for *pgc* RNA as indicated in materials and methods and products were run on an urea denaturing acrylamide gel. Poly(A) tail length of ovaries and embryos from wild-type and *osk* mutant females. Lane 1: Ovary; Lane 2: 30–90 mins AEL (stage 1–3); Lane 3: 90–150 mins AEL (stage 3–4); Lane 4: 150–210 mins AEL (stage 4–5); Lane 5: 210–270 mins AEL (stage 5-6-7); Lane 6: 270–330 mins AEL (stage 8–10). The baseline band indicated by a line corresponds to the shortest amplified fragment at 200nt. Poly(A) tail length is measured form this line. Red triangles mark the maternal to zygotic transition during which unlocalized maternal RNAs are degraded. A loading control for wild-type and *osk* mutant embryos is shown in Supplementary Figure 9. **B and B′.** Germ cells are formed at both anterior

as well as posterior poles of embryos from *osk-bcd* 3′UTR/ *pnos::HA-GFP-HA-pgc* 3′UTR; orb^{mel}/*TM6* mothers. (B) Merge of both VASA and HA antibody, (B′) stained for GFP-HA reporter. **C and C**′. Germ cell formation only at anterior pole and not posterior pole in embryos from *osk-bcd* 3′UTR/ *pnos::HA-GFP-HA-pgc* 3′UTR; orb^{mel} /orb^{343} mothers. (C) Merge of both VASA and HA antibody, (C′) stained for GFP-HA reporter. Posterior of the embryo is to the right.

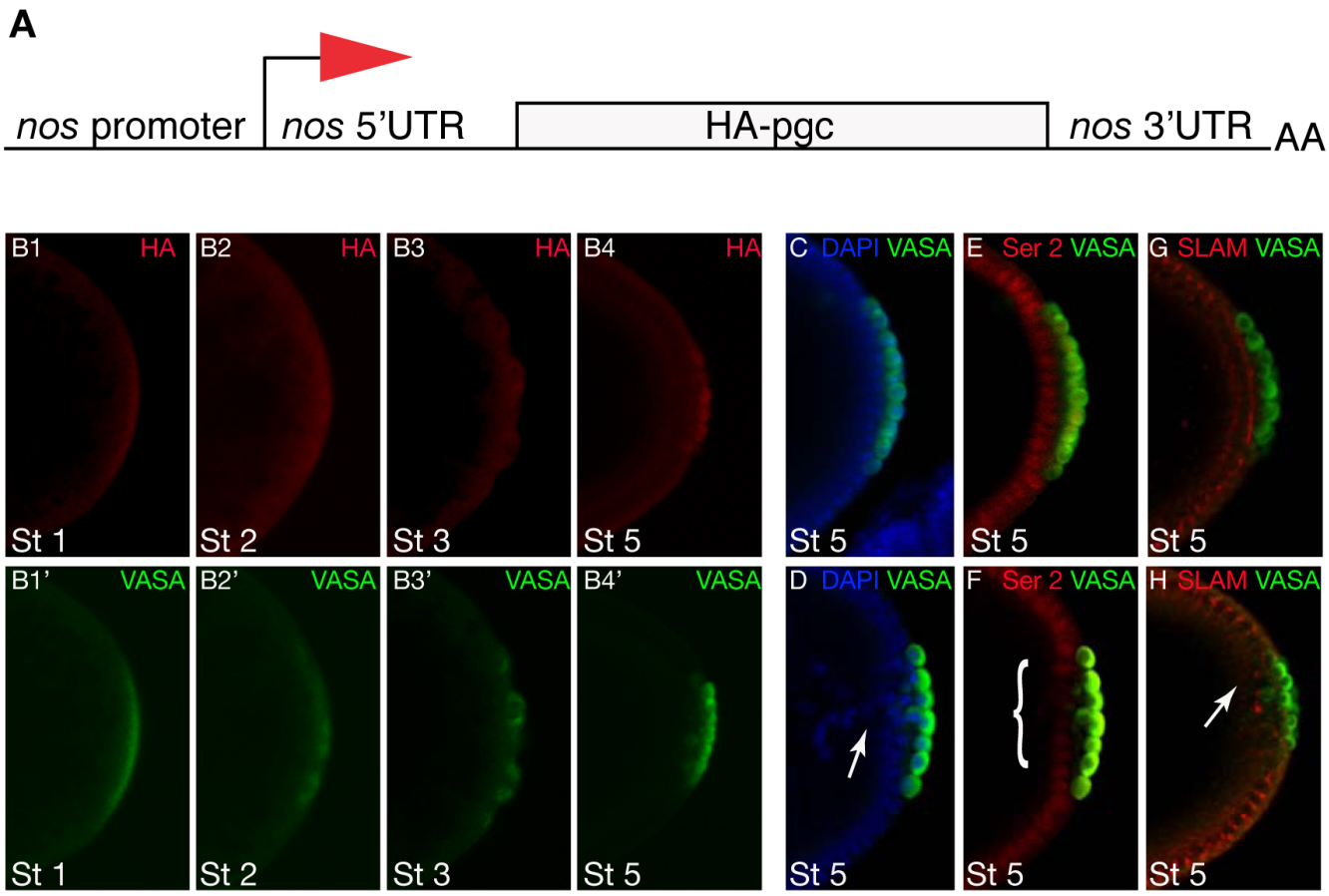

Figure 3. Precocious expression of PGC affects embryonic development

A. Diagram showing the *pnos::PGC-HA-nos* 3′UTR construct used in this experiment to translate *pgc* in the germ plasm under the control of the *nos* 3′UTR. **B.** Nos-like translation pattern of embryos from mothers carrying *pnos::PGC-HA-nos* 3′UTR reporter construct. **B1–B4** immuno-staining for HA shows translation in germ plasm and germ cells. **B1′-B4′** immuno-staining for VASA shows staging of germ line development. **C and D.** Wild-type embryo (C) and embryo from *pnos::PGC-HA-nos* 3′UTR female (D) stained for nuclei in blue (DAPI) and germ cells in green (VASA). The somatic cells adjacent to the germ cells form a continuous epithelial layer in the wild type (C) but fail to cellularize properly and fall back into the yolk ("Pole hole phenotype") in the embryo that precociously expresses PGC (arrow). **E and F.** Wild-type embryo (E) expresses high levels of active RNAPol II (detected by antibodies against the P-Ser2 epitope in the of RNAPol II CTD in red) in somatic cells adjacent to the germ cells (VASA, green); note that germ cells are transcriptionally silent due to PGC function. Embryo from *pnos::PGC-HA-nos* 3′UTR female (F) expresses reduced levels of the P-Ser2 epitope (red (marked by bracket)) in somatic cells adjacent to germ cells (VASA, green) because of expanded expression of PGC. **G and H.** Wild-type embryo (H) immunostained for Slow as molasses (SLAM) (red) a zygotically expressed gene required for somatic cellularization; germ cells stained for VASA in green. Expression of SLAM is disrupted in embryo from female carrying the *pnos::PGC-HA-nos* 3′UTR transgene (H, arrow). Stages as indicated, posterior is to the right.

Table 1

RNAs localized to germ plasm

Column 1 lists RNAs localized to germ plasm. Column 2 shows the function of these localized RNAs. Column 3 shows RNAs that were localized to the germ plasm by performing *in situ* hybridization (data not shown) and databases used to identify RNAs localized to germ plasm. Column 4 shows RNAs that form RNA islands similar to those described for *nanos* RNA [48]. Islands of germ plasm form when nuclei migrate into the germ plasm at nuclear cycle 9; RNA island formation is a characteristic feature of germ plasm localized RNAs. Column 5 lists 3′UTRs sufficiency to localize reporter construct to the germ plasm (see also Supplementary Figure 1).

RNA	Function	Germ plasm[#]	RNA islands[#]	3′UTR Sufficiency	References
nos	Translational control	+	+	+	[45]
bruno	Translational control	+	+	+	This study
pgc	Transcriptional silencing	+	+	+	This study
qcl	Germ cell formation	+	+	+	This study
CG5292	RNA binding[*]	+	+	+	This study
sra	Ca^{2+} signaling	+	+	+	This study
CG18446	Zinc ion binding[*]	+	+	+	This study
CG2774	Endocytosis[*]	+	+	+	This study
cyclin B	Cell Cycle	+	+	+	[46]
orb	Translational control	+	+	+	This Study, [20]
rapgap1	GTPase	+	+	ND	[47]

[*] Predicted function

ND: Not determined

[#] Localization Data from BDGP [11] or Lecuyer *et al.* [10] confirmed in this study.