Supplemental Data

Temporal and Spatial Control of Germ-Plasm RNAs

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Supplemental Experimental Procedures

Transgene construction

The reporter constructs were generated by cloning the 3'UTRs of various RNAs mentioned in Table 1 into p{CaSpeR-2} which carries the *nos* promoter and 5' UTR to produce germline specific expression as previously described in [1]. eGFP was cloned into this vector between the SpeI site and XhoI sites. An additional HA tag was introduced on the Carboxy terminal end of GFP. The sequences of the relevant 3'UTRs were obtained from FlyBase and were amplified using primers that introduced stop codons before the start of the 3'UTR. The 3' UTR fragments were then cloned into the above vector that contained the *nos* promoter, 5' UTR and HA-GFP by replacing the *K10* 3'UTR between XhoI and KpnI sites.

Fly stocks

The following stocks (described in FlyBase) were used in this study: osk^{54}, osk^{346}, orb^{mel}, orb^{343}, *osk-bcd* 3'UTR and *Oregon R* (Bloomington Drosophila Stock Center and Lehmann Lab). Flies were raised using standard corn meal-molasses medium at 25°C.

Embryos collection and fixation

Flies of indicated genotypes were allowed to lay for 0-2 hrs or overnight and embryos were collected, dechorionated in 50% bleach for 10 minutes, fixed in a 80% heptane, 17.5% PBS and 2.5% formaldehyde solution for 30 minutes and stored in methanol at -20° C [1, 2].

***In situ* hybridizations**

Digoxigenin (DIG) labeled RNA probe for GFP and LacZ were generated using *in vitro* run-off transcription using modified nucleotides (DIG-UTP) (Roche). *In situ* hybridization was carried out as previously described with the exception that the probes for GFP and LacZ were not carbonate treated [1, 2].

Immunostaining

Immunostainings of embryos were performed using standard procedures as described in [1, 2]. The following primary antibodies and dilutions were used: Rabbit α-VASA (1:5000) (Lehmann lab), Rat α-HA (1:1000) (Roche), Rabbit α-SLAM (1:1000) (Lehmann lab), mouse α-P-Ser2 (1:500) (Covance). For detecting ORB in the germ plasm and germ cells α-ORB antibody was used at a dilution of 1:500 instead of 1:1000. Cy2, Cy3 or Cy5 conjugated to appropriate secondary antibodies were used at a 1:500 dilution (Invitrogen).

Fluorescent imaging

Immunostainings and FISH were visualized using Zeiss LSM-510 Meta confocal microscope using 10x, 20x, oil-immersed 40x. The anterior of a stage 1 embryo was used

to correct for background.

PAT assays

RACE-PAT assays were performed as described in [3] and the products were run on an 8% urea denaturing gel. 5'GCGAGCTCGGCGCCCGCGTTTTTTTTTTT 3' was used as an anchored dT primer for first strand synthesis. 5'TTGCTTTCGTGAAAACTCGCATTGT3' was used for *pgc* 3'UTR. Dried gels were exposed to Molecular Dynamics PhosphorImager screens and quantified with Image J software.

cRT-PCR for *bruno* was carried out as described in [4, 5]. 4 ug of total RNA was decapped and circularized and RT was carried out using the primer 5' CATGGAAGAGTGACGCGGTACGGCCG3', and was amplified using 5' CATGGAAGAGTGACGCGGTACGGCCG3' and 5' GGTCCCTGAAATATGTAAATAATATCCGC3'. The products were run on a 5 % agarose gel (MetaPhor, FMC Bioproducts).

Western blots

Western blots were performed following standard procedures [1]. 40 μg of protein from stage 1-2 embryo extract was loaded on the gel to detect ORB. Rabbit α-ORB primary antibody was used at a dilution of 1/1000.

Fluorescent *in situ* hybridizations

Digoxigenin labeled RNA probes were generated using *in vitro* run off transcription using modified nucleotides (DIG-UTP) (Roche). Single RNA Fluorescent *in situ*

hybridizations were visualized with Tyramide Signal Amplification (TSA) (Invitrogen, Molecular Probes) as previously described with the exception of the PBS washes which were carried out in PBS + 0.2% Tween 20 to prevent aggregation of embryos [2].

RNA-Protein Double labeling

Primary detection reagents, biotin-conjugated α-DIG and α-GFP, were incubated simultaneously for 2 hours. This was followed by an incubation with fluorescent secondary antibody with streptavidin-HRP. Lastly DAPI staining and the TSA reaction were performed as described in Lécuyer *et al*. [2]. Non-cross-reactive antibodies were used. All embryos are oriented posterior to the right.

References

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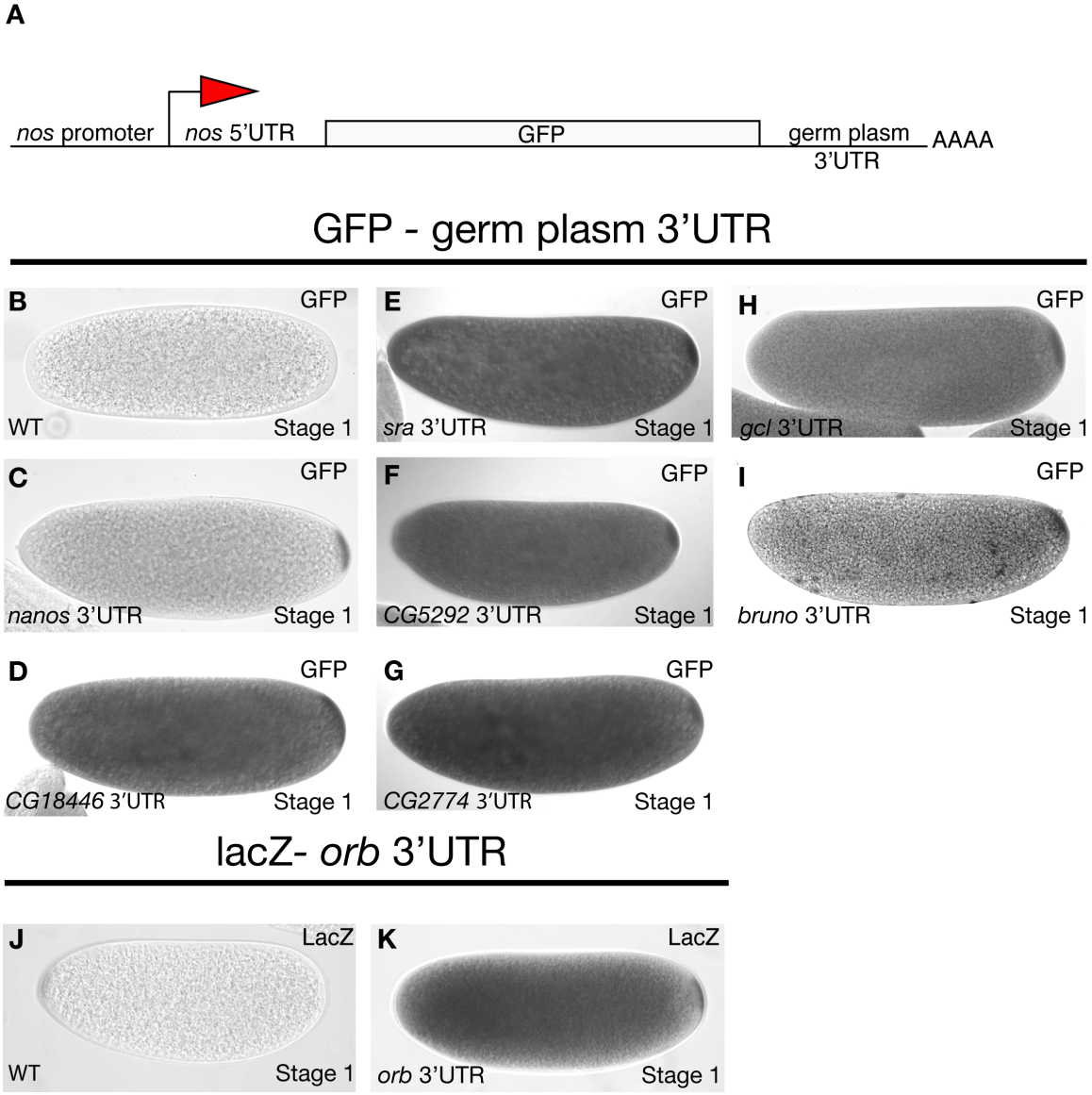


Figure S1: RNA **Localization to germ plasm is 3'UTR dependent.**
A. Diagram showing the *pnos::HA-GFP-HA-germ plasm* 3'UTR construct used in this study. **B-I.** *In situ* hybridization with GFP RNA probe to stage 1 embryos from mothers carrying the *pnos::HA-GFP-HA-germ plasm* 3'UTR as indicated. For *nanos* 3'UTR (C) GFP was fused to moesin instead of HA. **J-K.** *In situ* hybridization with *lacZ* RNA in embryos from mothers carrying a *phsp83::LacZ-orb* 3'UTR transgene [6]. All embryos are oriented posterior to the right.

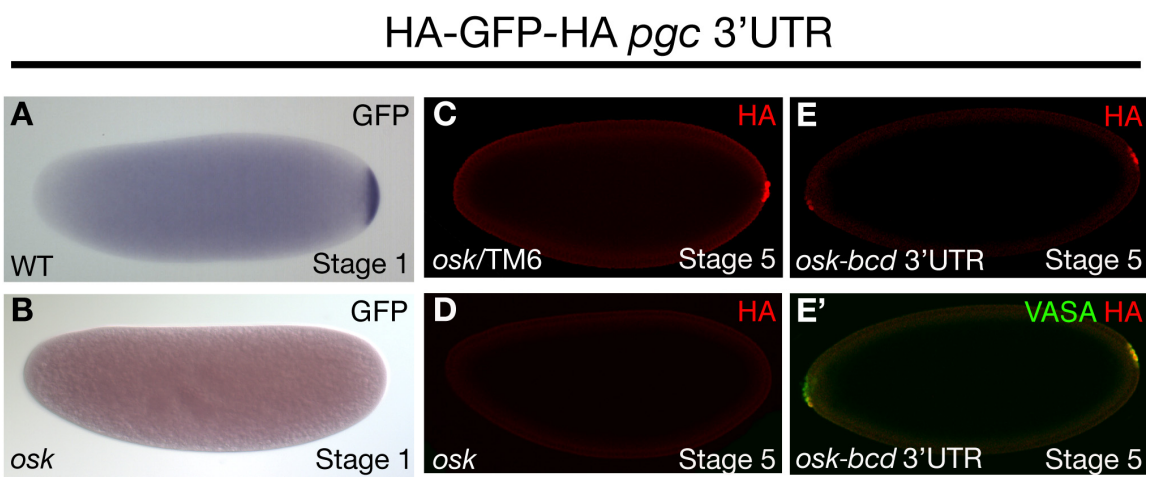


Figure S2: Localization of *pgc* 3'UTR reporter is *osk* dependent.
A and B. *In situ* hybridization with GFP RNA probe in embryos from wild-type **(A)** and *oskar* mutant **(B)** females carrying the *pnos::HA-GFP-HA-pgc* 3'UTR transgene. **C and D.** Expression of GFP-HA in embryos from wild-type (C) and *oskar* mutant (D) females carrying the *pnos::HA-GFP-HA-pgc* 3'UTR transgene stained with HA antibody. **E and E'.** Expression of GFP-HA in embryos from females carrying *osk-bcd* 3'UTR and *pnos::HA-GFP-HA-pgc* 3'UTR transgenes monitored by HA staining for GFP expression (E) and VASA (E') to mark germ cells. HA under control of the *pgc* 3'UTR is expressed in both normal posterior and ectopic anterior germ cells. All embryos are oriented posterior to the right.

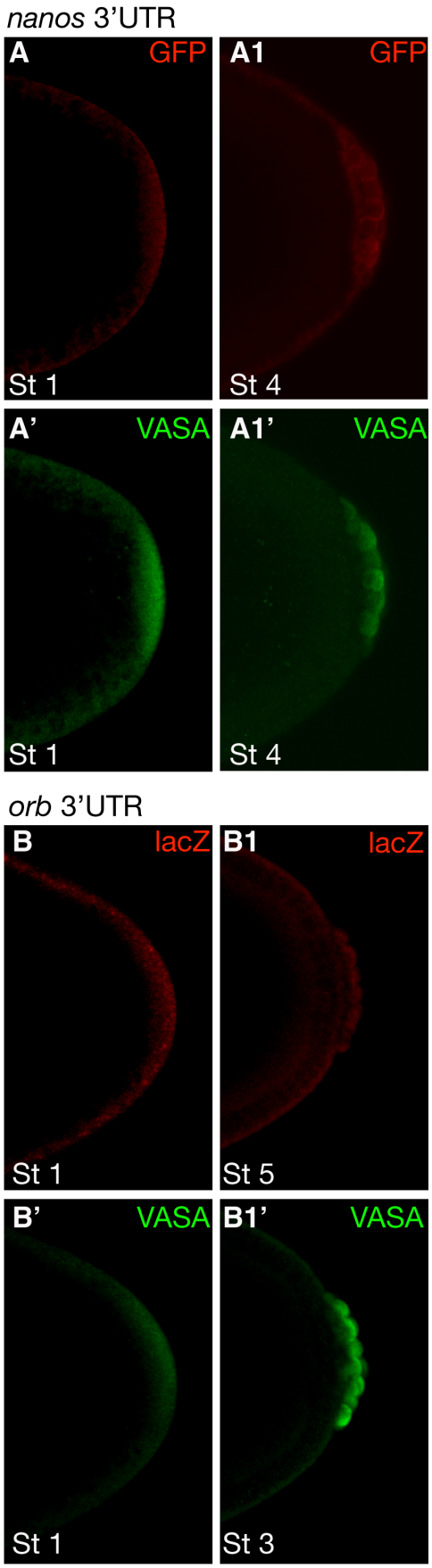


Figure S3: Class I localized RNAs, *nanos* and *orb*, are translated from germ plasm stage onwards.

A. Timing of Moesin-GFP expression fused to *nanos* 3'UTRs monitored by antibody staining against GFP. GFP (red) is expressed in germ plasm (stage 1, A) marked by VASA antibody (green) (A') and continues to be expressed in germ cells (stage 5, A1) marked by VASA antibody (A1'). **B.** Timing of LacZ expression fused to *orb* 3'UTR. LacZ (red) is expressed in germ plasm (stage 1, B) marked by VASA antibody (green) (B') and continues to be expressed in germ cells (stage 5, B1) marked by VASA antibody (B1'). Endogenous antibody staining for ORB is shown in Supplementary Figure 10. All embryos are oriented posterior to the right.

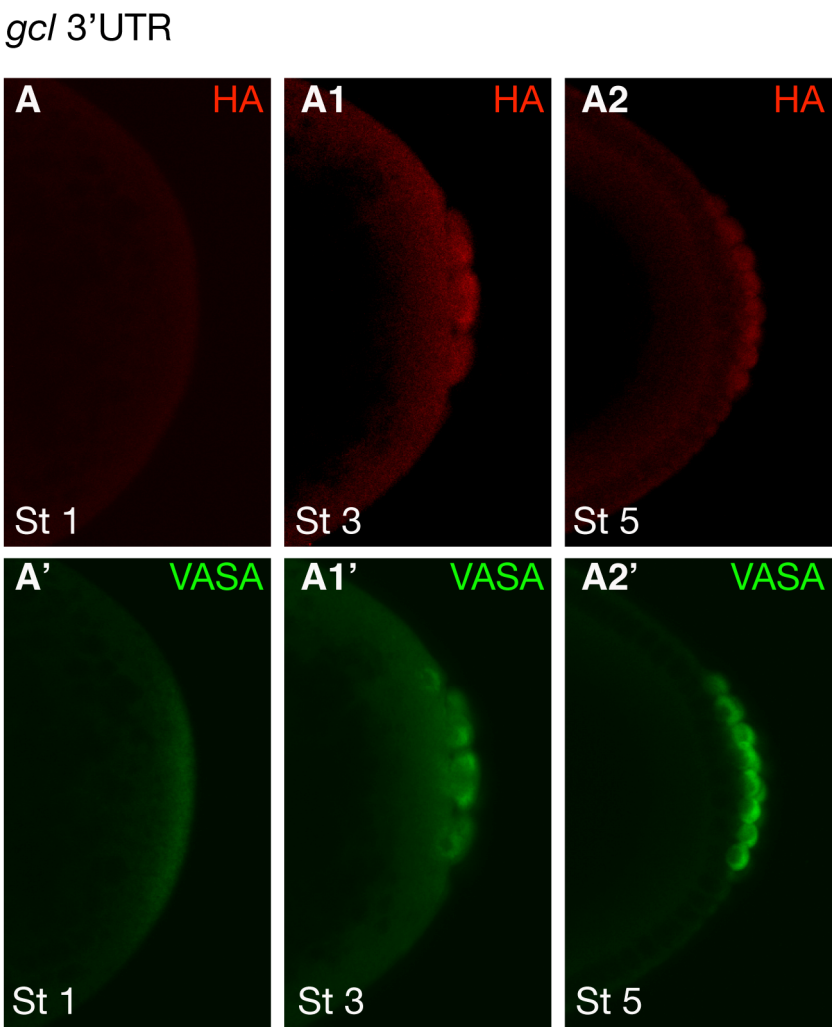


Figure S4: Class II localized RNA, *gcl*, is translated from germ bud onwards.
Timing of expression of GFP-HA fused to *gcl* 3'UTR monitored by antibody staining against HA. VASA in green marks germ plasm (stage 1, A'), germ buds (stage 3, A1') and germ cells (stage 4, A2'). HA staining (A-A2) is observed from germ bud stage onwards. All embryos are oriented posterior to the right.

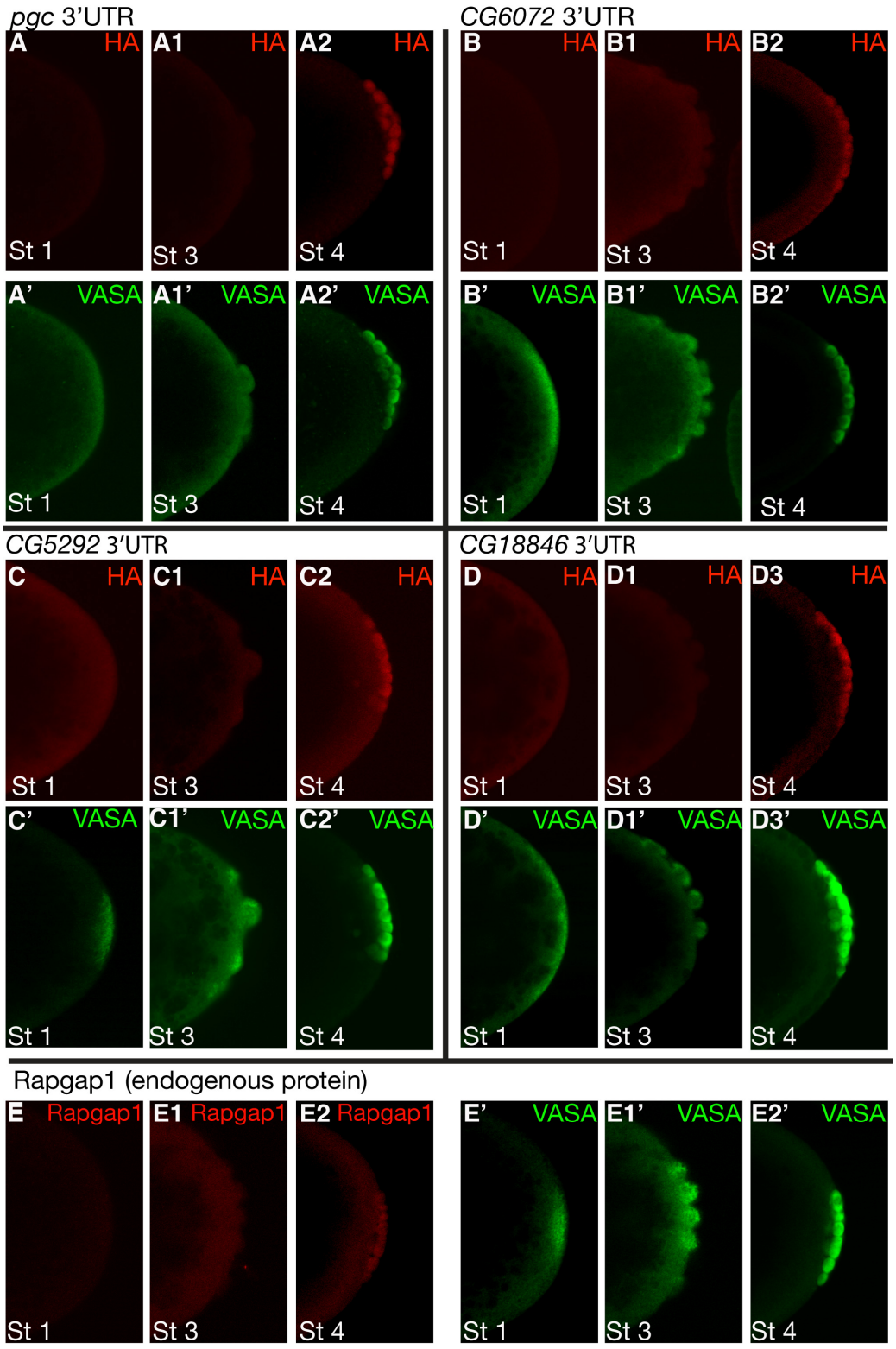


Figure S5: Class III localized RNAs, *pgc, CG6072, CG5292, CG18846* and *rapgap1*, are translated only in germ cells.

A-D. Timing of expression of GFP-HA fused to indicated 3'UTRs monitored by antibody staining against HA. **A-A2.** *pgc* 3'UTR. **B-B2.** *CG6072* 3'UTR. **C-C2.** *CG5292*

3'UTR **D-D3.** *CG18846* 3'UTR**.** The 3'UTRs are indicated on top of each panel. HA staining is in red and VASA in green. **E-E2.** Staining for endogenous protein Rapgap1. All RNAs in this class are translated as germ cells form. All embryos are oriented posterior to the right.

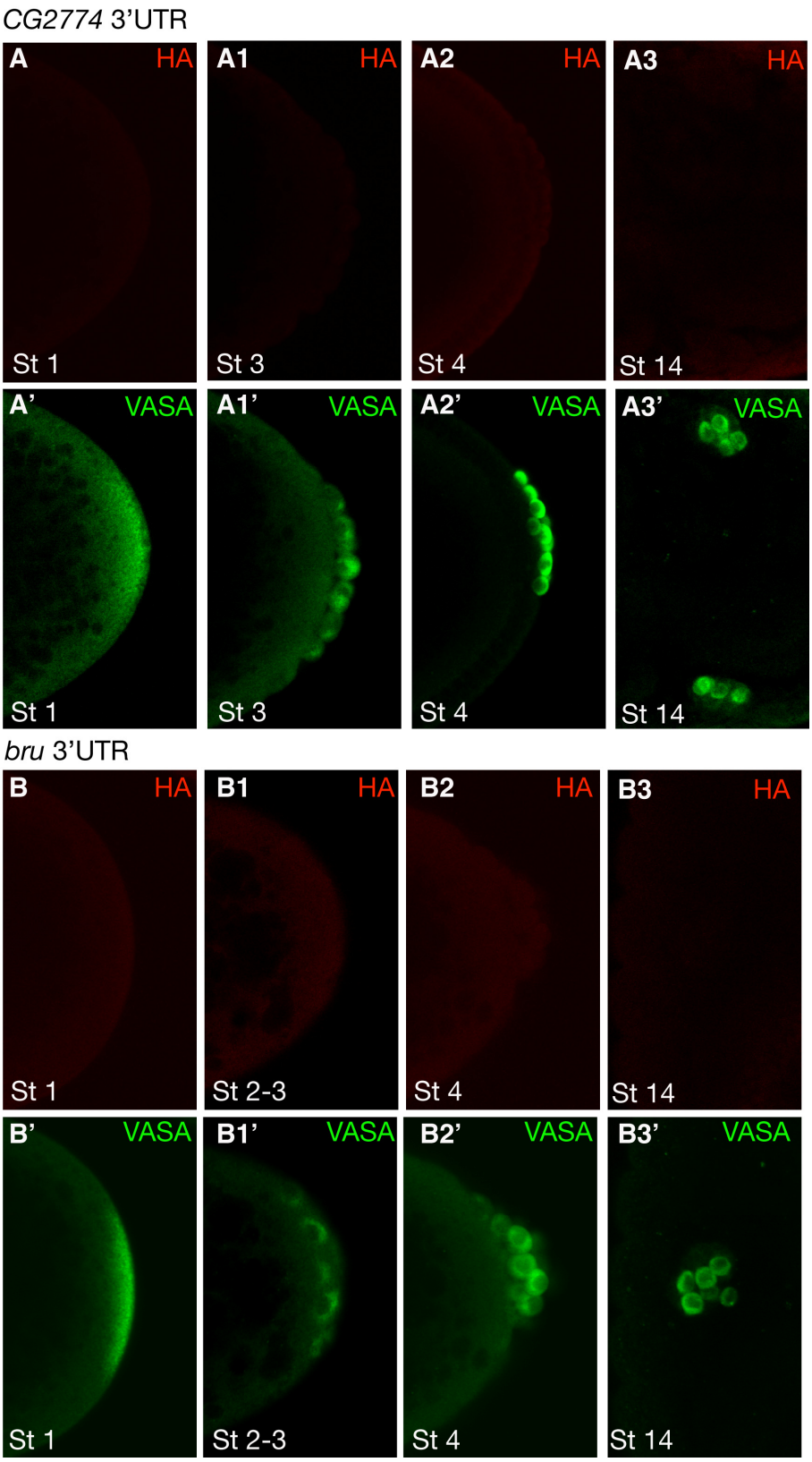


Figure S6: Class IV RNAs, *bruno* and *CG2774*, are not translated in the early embryo.

A-A3. Expression of GFP-HA fused to *bruno* 3'UTRs monitored by antibody staining against HA. **B-B3.** Expression of GFP-HA fused to *CG2774* 3'UTR monitored by antibody staining against HA. The 3'UTRs are indicated on top of each panel. No HA staining is observed in germ plasm or germ cells. Note lack of expression in stage 14 gonads (A3, B3) **A'-A3', B'-B3'.** VASA in green marks germ cell development. All embryos are oriented posterior to right.

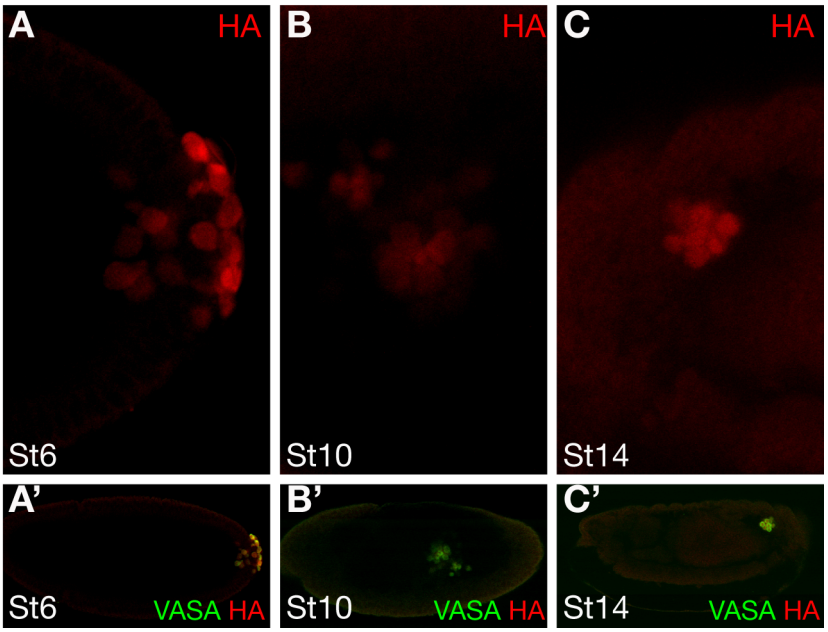


Figure S7: Expression of *pgc* reporter construct persists beyond endogenous protein expression.
GFP-HA expression in embryos from females carrying the *pnos::HA-GFP-HA-pgc* 3'UTR monitored by antibody staining against HA. HA staining is in red and VASA in green. The reporter expression can be detected in the gonad while endogenous protein expression is only observed during stage 4-5 [7]. **A-A'.** HA staining at stage 6 of embryogenesis. **B-B'.** HA staining at stage 11 of embryogenesis. **C-C'.** HA staining at stage 14 of embryogenesis showing reporter persists beyond endogenous protein expression. All embryos are oriented posterior to the right.

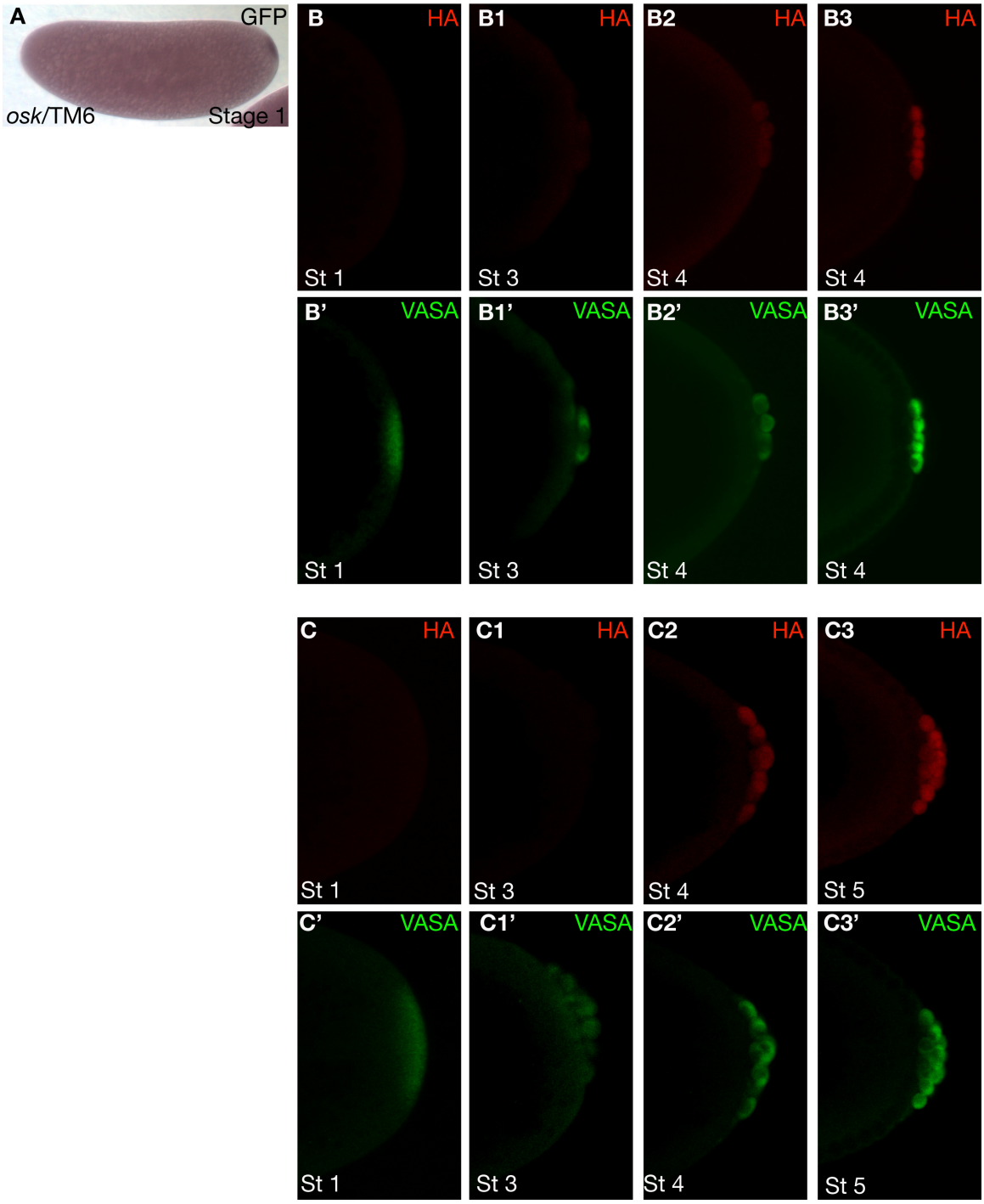


Figure S8: Timing of expression of *pgc* reporter construct is not affected by RNA levels.

A. *In situ hybridization* for GFP RNA to detect *HA-GFP-HA-pgc* 3'UTR RNA in embryo from female carrying the *pnos::HA-GFP-HA-pgc* 3'UTR transgene in *osk* heterozygous background, where only half the amount of germ plasm is present and hence less GFP-*pgc* 3'UTR RNA is localized compared to wild type (see Figure 1). **B-B3.** GFP-HA translation in embryos from females carrying the *pnos::HA-GFP-HA-pgc* 3'UTR transgene in *osk* heterozygous background. **B'-B3'.** VASA in green marks various stages

of germ cell development. Reduction in levels of localized RNAs does not affect timing of translation. **C-C3.** Reduction in copy number of *pnos::HA-GFP-HA-pgc* 3'UTR transgene does not affect timing of translation. Embryos with one copy of *pnos::HA-GFP-HA-pgc* 3'UTR transgene stained for HA. **C1'-C3'.** VASA in green, marks various stages of germ cell development. All embryos are oriented posterior to the right.

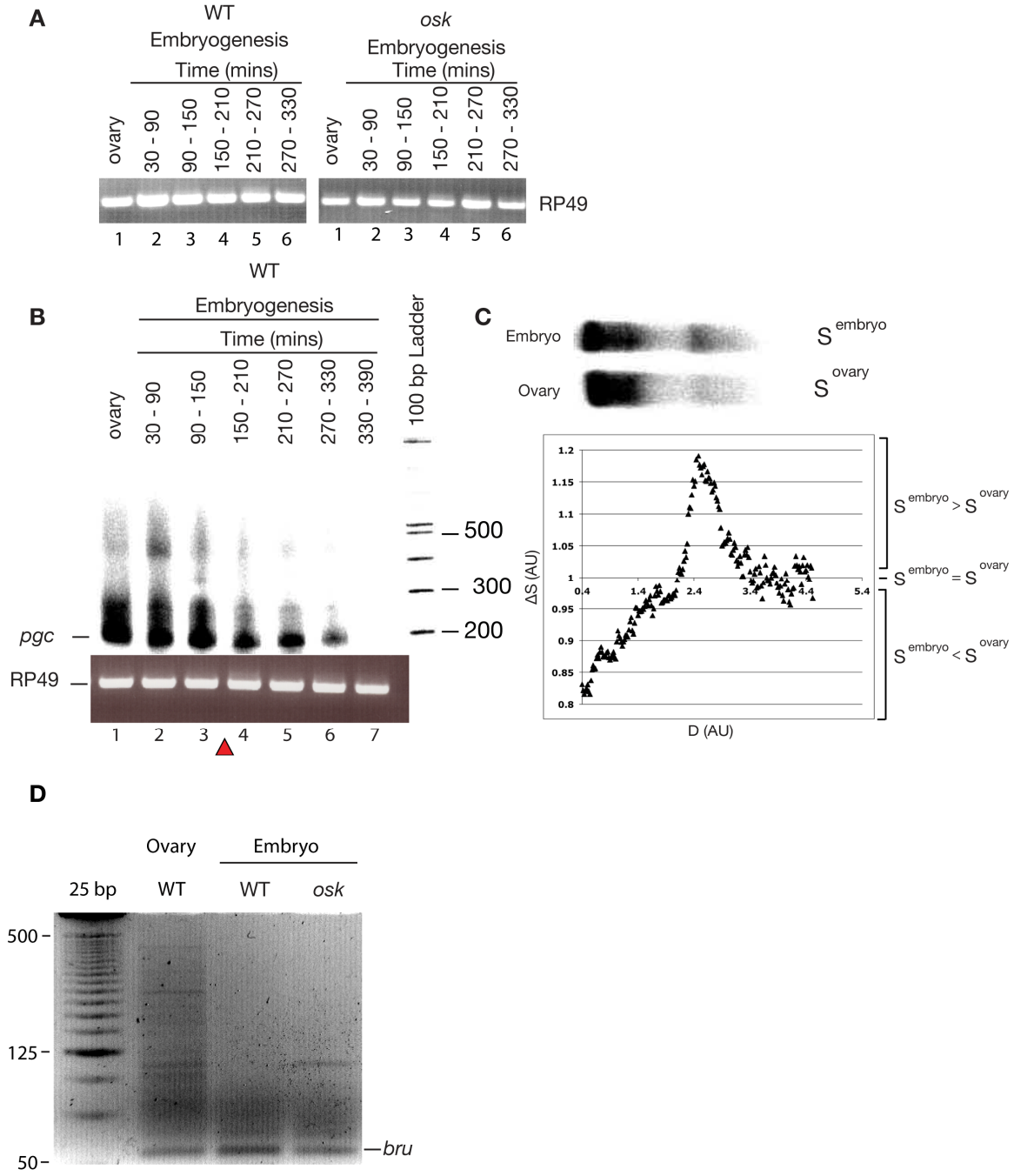


Figure S9: Poly(A) tail extension is germ plasm dependent.
A. RP49 control for PAT test of wild-type and *osk* mutant embryo extracts shown in Fig2A. **B.** Poly(A) tail length (PAT) analysis of *pgc* RNA. PAT assay was performed for *pgc* RNA as indicated in materials and methods and products were run on a denaturing acrylamide gel. Lane 1: Ovary; Lane 2: 30-90 mins (stage 1-3); Lane 3: 90-150 mins (stage 3-4); Lane 4: 150-210 mins (stage 4-5); Lane 5: 210-270 mins (stage 5-7); Lane 6: 270-330 mins (stage 8-10); Lane 7: 330-390 mins (stage 11). Red triangles mark maternal to zygotic transition during which unlocalized RNAs are degraded, Line

indicates baseline for non-adenylated RNA. **C.** Poly (A) tail length in the ovary (S^{ovary}) and embryo (S^{embryo}) was quantified as described in materials and methods. The fractional change in poly (A) tail length ($\Delta S = S^{embryo}/ S^{ovary}$) was plotted as a function of distance (D) from the main baseline band. Quantitation of signal in these lanes shows an increase in poly(A) tail length during embryogenesis. **D.** Bruno, a class IV RNA, shows a long poly(A) tail during oogenesis but not during embryogenesis. *Bruno* poly(A) tail length was measured using cRT-PCR as described in materials and methods. Bruno has a long Poly(A) tail of about 350nts during oogenesis. During embryogenesis the poly(A) tail is short ~75nt in embryos from *osk* and wild-type females.

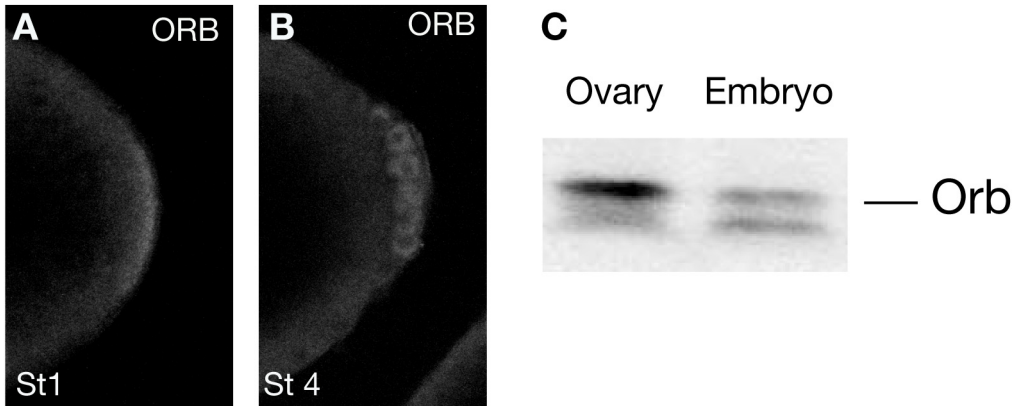


Figure S10: Translation of *pgc* is CPEB independent.
A, B. ORB staining in germ plasm and germ cells respectively. **C.** Western blot of ovary and embryo extracts probed with ORB antibody shows the presence of ORB protein during oogenesis and embryogenesis respectively.

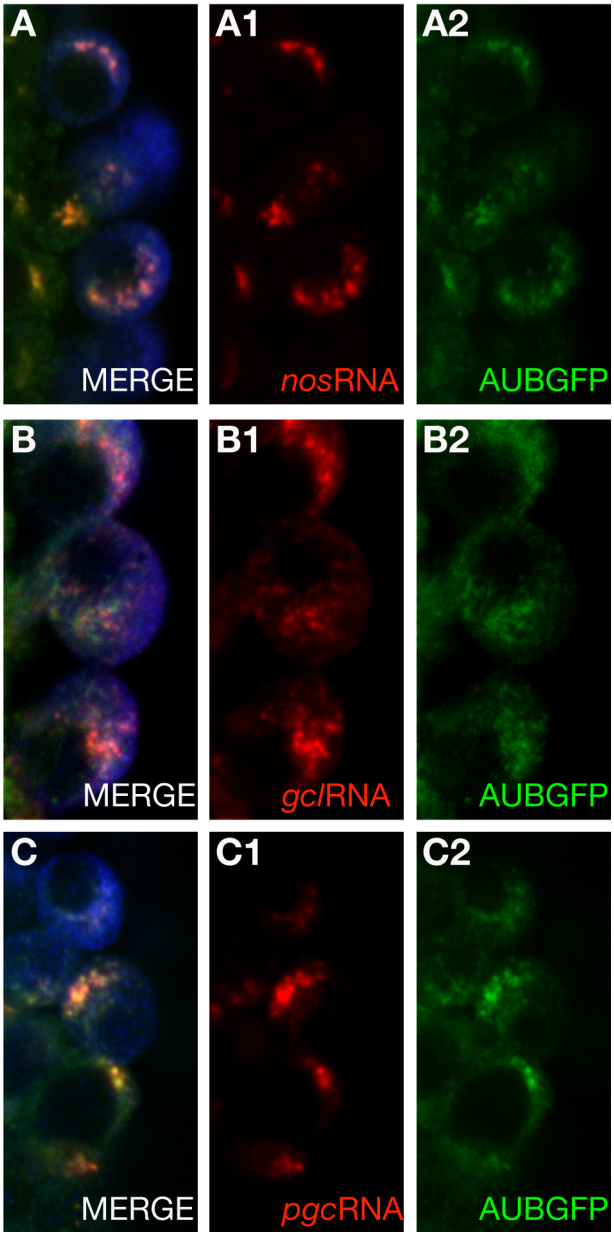
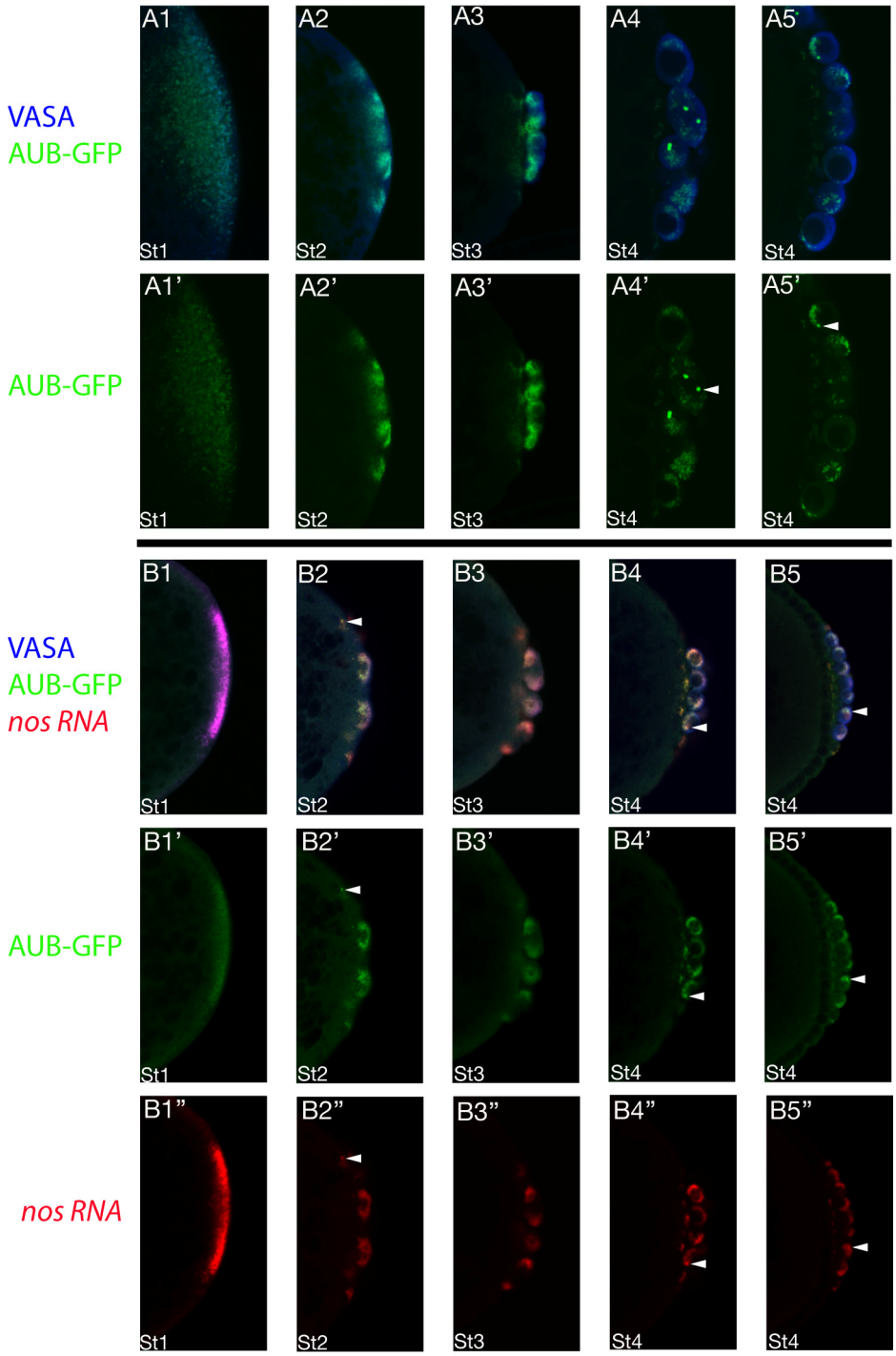


Figure S11: Polar granules coordinate germ plasm RNAs:
Embryos were stained for VASA (blue) to identify germ cells and Aubergine-GFP (green) to mark polar granules. *In situ* hybridization for *nanos, gcl* and *pgc* RNAs were carried out to determine association of these RNAs with polar granules at stage 5. **A-A2:** *nanos* RNA (red) colocalizes with polar granules (green) at stage 5. **B-B2:** *In situ* hybridization with *gcl* RNA probe (red) showing colocalization with polar granules (green). **C-C2:** *In situ* hybridization with *pgc* RNA probe (red) showing colocalization with polar granules (green). All embryos are oriented posterior to the right.


VASA
AUB-GFP
A1
A2
A3
A4
A5
St1
St2
St3
St4
AUB-GFP
A1'
A2'
A3'
A4'
A5'
St1
St2
St3
St4
VASA
AUB-GFP
nos RNA
B1
B2
B3
B4
B5
St1
St2
St3
St4
AUB-GFP
B1'
B2'
B3'
B4'
B5'
St1
St2
St3
St4
nos RNA
B1"
B2"
B3"
B4"
B5"
St1
St2
St3
St4

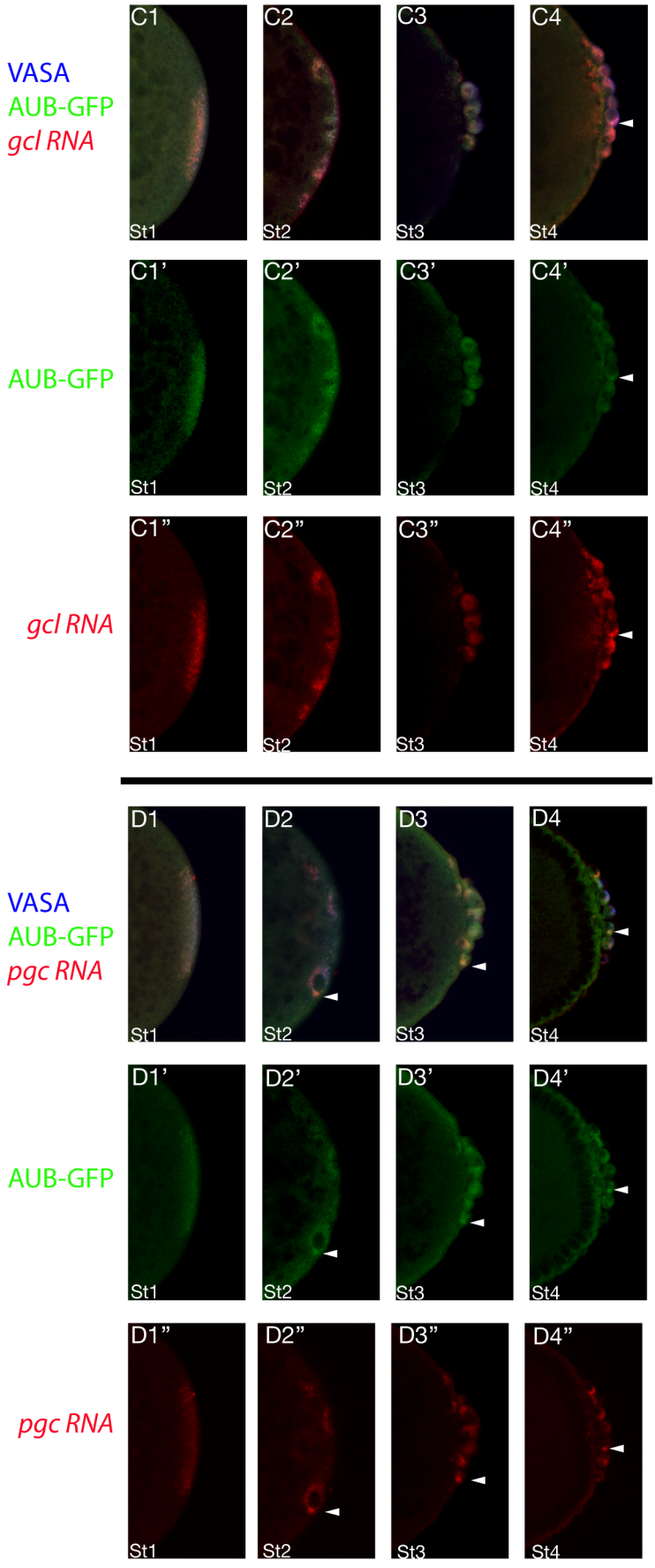

VASA
AUB-GFP
gcl RNA
C1
C2
C3
C4
St1
St2
St3
St4
AUB-GFP
C1'
C2'
C3'
C4'
gcl RNA
C1"
C2"
C3"
C4"
VASA
AUB-GFP
pgc RNA
D1
D2
D3
D4
AUB-GFP
D1'
D2'
D3'
D4'
pgc RNA
D1"
D2"
D3"
D4"

Figure S12: Polar granules are continuously associated with germ plasm RNAs.
Embryos were stained for VASA (blue) to identify germ cells and Aubergine-GFP (green) to mark polar granules. **A1-A5:** Merge of VASA and AUB-GFP channels showing polar granules as a function of time. The granules become bigger and more prominent at stage 4-5 [8]. **A1'-A5'**: AUB-GFP channel showing polar granules alone. White arrowheads point to polar granules. **B1-B5:** Merge of VASA (blue), AUB-GFP (green), *nanos* RNA *in situ* hybridization (red). *In situ* hybridization for *nanos* was carried out to determine association of *nanos* RNA with polar granules as function of time. *nanos* RNA colocalizes with polar granules from germ plasm to germ cell stage. **B1'-B5', B1''-B5'':** AUB-GFP and *nanos* RNA channels respectively. Similar experiments were carried out with *gcl* RNA (**C1-C5**) and *pgc* RNA (**D1-D5**), respectively. All embryos are oriented posterior to the right.

Supplementary Table 1

No.	CG Name	Gene Name
1	CG10076	spire (spir)
2	CG10077	CG10077
3	CG10192	Off Schedule
4	CG10868	oo18 RNA Binding Protein (ORB)
5	CG10901	Oskar (OSK)
6	CG10954	Arc P34
7	CG11597	CG11597
8	CG1308	CG1308
9	CG13933	CG13933
10	CG14025	Blastoderm-specific gene 25D (Bsg25D)
11	CG14066	La Related Protein (LARP)
12	CG14217	Tao1
13	CG14814	CG14814
14	CG17678	Concertina (CTA)
15	CG1838	Myoglianin
16	CG18446	CG18446
17	CG2677	eIF2B Beta
18	CG2699	Pi3K21B
19	CG2774	CG2774
20	CG2865	CG2865
21	CG2941	CG2941
22	CG3171	Trapped in Endoderm 1 (TRE1)
23	CG31762	Arrest (ARET)
24	CG31998	CG31998
25	CG32756	CG32756
26	CG3325	Spindle B (SPNB)
27	CG33529	Rapgap1
28	CG3727	Dreadlocks (DOCK)
29	CG4040	CG4040
30	CG4294	CG4294
31	CG4427	cabut (cbt)
32	CG4710	smell impaired 21F (smi21F)
33	CG4735	Shutdown (SHU)
34	CG4755	RhoGAP92B
35	CG4903	Misexpression Suppressor of Ras4 (MESR4)
36	CG5292	CG5292
37	CG5303	meiotic from via Salaria 332 (mei-S332)
38	CG5637	Nanos (NOS)
39	CG6017	CG6017
40	CG6072	Sarah (SRA)
41	CG6418	CG6418
42	CG6509	CG6509
43	CG6721	GTPase Activating Protein 1 (GAP1)
44	CG6783	CG6783
45	CG6805	CG6805
46	CG7015	Upstream of N-Ras (UNR)

47	CG7184	Makorin 1 (MKRN1)
48	CG7533	charybde (chrb)
49	CG7719	greatwall (gwl)
50	CG8104	nudE
51	CG8114	Pebble (PBL)
52	CG8411	germ cell-less (gcl)
53	CG8994	exuperantia (exu)
54	CG9252	Deadlock (DEL)
55	CG9699	CG9699
56	CG9755	Pumilio (PUM)
57	CG9821	CG9821
58	CG32885	pgc

Table S1: List of RNAs localized to the germ plasm.

We assembled a list of RNAs localized to germ plasm based on the expression patterns of RNAs described by Lecuyer *et al*. [9], the Berkeley Drosophila Genome Project RNA expression, Tomancak *et al*. [10] and literature searches. Criterion for inclusion in this list was a pattern of localization similar to that described for *nanos, gcl* and *pgc:* "Localized RNAs" are maternally synthesized and become localized in a crescent at the posterior pole of the early embryo (stage 1-2), once nuclei enter the germ plasm these RNAs form characteristic "RNA islands" around the nuclei at stage 3, and the RNAs are incorporated into developing germ cells at stage 4 [9]. Localized RNAs can persist in germ cells until late stages of embryogenesis, while maternal RNA not incorporated into germ cells is degraded during the maternal to zygotic transition of gene expression (stage 4-5). The RNAs in this table were included based on published reports (see above). In contrast to the eleven RNAs analyzed in this study, localization patterns were not confirmed by RNA *in situ* hybridizations.

Supplementary Table 2

No.	CG Name	Gene Name
1	CG10050	CG10050
2	CG10120	Malic enzyme (men)
3	CG10215	Ercc1
4	CG10360	refractory to sigma P (ref(2)p)
5	CG10462	CG10462
6	CG10520	Tube (TUB)
7	CG10701	Moesin (MOE)
8	CG10811	Eukaryotic Translation Initiation Factor 4G(eIF4G)
9	CG10895	loki (lok)
10	CG10981	CG10981
11	CG11181	Cup
12	CG11254	Maelstrom (MAEL)
13	CG11372	Galectin
14	CG11513	Armitage (ARMI)
15	CG11793	Superoxide Dismutase (SOD)
16	CG11987	tango (tgo)
17	CG11992	Relish (REL)
18	CG12055	Glyceraldehyde 3 Phosphate Dehydrogenase 1
19	CG12084	CG12084
20	CG1242	Heat Shock Protein 83 (HSP83)
21	CG12576	diaspora
22	CG1258	Pavarotti (PAV)
23	CG12737	Calmodulin Binding Protein (CRAG)
24	CG12819	Slender Lobes (SLE)
25	CG13344	CG13344
26	CG13349	CG13349
27	CG13777	Milton (MILT)
28	CG1417	Sluggish A (SLGA)
29	CG14226	Domeless (DOME)
30	CG14489	Olf186M
31	CG14648	Growl
32	CG14712	CG14712
33	CG1489	Pros45
34	CG15737	CG15737
35	CG1578	CG1578
36	CG1633	Thioredoxin Peroxidase 1 (JAFRAC1)
37	CG1691	IGF2 mRNA Binding Protein (IMP)
38	CG17161	Grapes (GRP)
39	CG1721	Phosphoglyceromutase (PGLYM78)
40	CG17270	CG17270
41	CG17658	CG17658
42	CG1780	Imaginal Disc Growth Factor 4 (IDGF4)
43	CG17870	14-3-3 Zeta
44	CG1793	Mediator Complex Subunit 26 (MED26)
45	CG17960	RhoGAP1A
46	CG1812	CG1812

47	CG1962	CG1962
48	CG2009	bip2
49	CG2041	legless (LGS)
50	CG3068	Aurora (AUR)
51	CG31137	Twin
52	CG31156	CG31156
53	CG3157	gamma Tubulin at 23C (gammaTub23C)
54	CG3183	Geminin
55	CG32243	CG32243
56	CG32473	CG32473
57	CG32616	Stellate 2D Orphon
58	CG3273	Scrambled (SCED)
59	CG3278	Tif-IA
60	CG33166	Stem Cell Tumor (STET)
61	CG34100	Molting Defective (MLD)
62	CG3506	Vasa (VAS)
63	CG3509	CG3509
64	CG3510	Cyclin B (CYCB)
65	CG4035	Eukaryotic Initiation Factor 4E (eIF4E)
66	CG4063	ebi
67	CG4183	Heat Shock Protein 26 (HSP26)
68	CG4193	Deadhead (DHD)
69	CG4466	Heat Shock Protein 27 (HSP27)
70	CG4581	Thiolase
71	CG4799	Pendulin (PEN)
72	CG4898	Tropomyosin 1 (TM1)
73	CG4911	CG4911
74	CG4944	Ciboulot (CIB)
75	CG5026	CG5026
76	CG5692	Rapsynoid (RAPS)
77	CG5753	Staufen (STAU)
78	CG6433	Quail (QUA)
79	CG6500	Beadex (BX)
80	CG6543	CG6543
81	CG6605	Bicaudal D (BicD)
82	CG6667	Dorsal (DL)
83	CG6875	Abnormal Spindle (ASP)
84	CG6906	Carbonic Anhydrase 2 (CAH2)
85	CG6967	CG6967
86	CG7070	Pyruvate kinase (PyK)
87	CG7271	CG7271
88	CG7369	CG7369
89	CG7413	Retinoblastoma-family protein (Rbf)
90	CG7660	peroxinectin, pxt
91	CG7869	Suppressor of Under Replication (SuUR)
92	CG7935	Moleskin (MSK)
93	CG8042	CG8042
94	CG8231	TCP1 Zeta (TCP1Zeta)
95	CG8418	Ras which interacts with Calmodulin (Ric)

96	CG8439	T-complex Chaperonin 5 (CCT5)
97	CG8440	Lissencephaly1 (LIS1)
98	CG8507	CG8507
99	CG8571	Smallminded (SMID)
100	CG8632	CG8632
101	CG8668	CG8668
102	CG8915	CG8915
103	CG8977	Cct gamma (CCTgamma)
104	CG9028	Short Spindle 2 (SSP2)
105	CG9057	Lipid storage droplet-2 (Lsd-2)
106	CG9078	infertile crescent (ifc)
107	CG9748	Belle (BEL)
108	CG9916	Cyclophilin1 (CYP1)
109	CG9925	CG9925
110	CG9984	TH1
111	Indora	Indora (IDR)
113	CG6122	Piwi

Table S2: List of RNAs protected in germ cells.

We assembled this list of protected RNAs based on the expression pattern of previously known RNAs that are protected in the germ cells such as *hsp 83*. Protected RNAs are not localized to the germ plasm as described for localized RNAs in Supplementary Table 1. Protected RNAs are maternally synthesized; a fraction of the RNA becomes incorporated into developing germ cells and can persist in germ cells until late stages of embryogenesis, while maternal RNA not incorporated into germ cells is degraded during the maternal to zygotic transition of gene expression (stage 4-5). The distribution patterns of RNAs listed in this table were not confirmed by RNA *in situ* hybridizations and are based on the expression patterns of RNAs described by Lecuyer *et al*. [9], the Berkeley Drosophila Genome Project RNA expression, Tomancak *et al*. [10] and literature searches.