Analysis of RNA Interference Lines Identifies New Functions of Maternally-Expressed Genes Involved in Embryonic Patterning in Drosophila melanogaster

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ABSTRACT Embryonic patterning in Drosophila melanogaster is initially established through the activity of a number of maternally expressed genes that are expressed during oogenesis. mRNAs from some of these genes accumulate in the posterior pole plasm of the oocyte and early embryo and localize further into RNA islands, which are transient ring-like structures that form around the nuclei of future primordial germ cells (pole cells) at stage 3 of embryogenesis. As mRNAs from several genes with known functions in anterior–posterior patterning and/or germ cell specification accumulate in RNA islands, we hypothesized that some other mRNAs that localize in this manner might also function in these developmental processes. To test this, we investigated the developmental functions of 51 genes whose mRNAs accumulate in RNA islands by abrogating their activity in the female germline using RNA interference. This analysis revealed requirements for ttk, pbl, Hip14, eIF5, eIF4G, and CG9977 for progression through early oogenesis. We observed dorsal appendage defects in a proportion of eggs produced by females expressing double-stranded RNA targeting Mkrn1 or jvl, implicating these two genes in dorsal–ventral patterning. In addition, posterior patterning defects and a reduction in pole cell number were seen in the progeny of Mkrn1 females. Because the mammalian ortholog of Mkrn1 acts as an E3 ubiquitin ligase, these results suggest an additional link between protein ubiquitination and pole plasm activity.

KEYWORDS embryonic patterning germ plasm localized mRNAs oogenesis pole cells

mRNA localization to particular intracellular regions is widespread. In the early Drosophila embryo, mRNA localization, coupled to spatially dependent translational regulation, contributes to targeting the proteins and the localized mRNAs encode to the region of the embryo that is appropriate for their developmental function (Lécuyer et al. 2007; Kugler and Lasko 2009). Hundreds of mRNAs have been identified that accumulate in the posterior pole plasm of the early Drosophila embryo, where cytoplasmic determinants specify the germ line (Lécuyer et al. 2007; Fisher et al. 2012). Although a great deal has been learned about how several of these mRNAs function in embryonic patterning and specifying the germ line, for the majority little is known about what role, if any, they have. Several maternal mRNAs that are essential for establishment of the anterior–posterior pattern and for specification of germ cells, including aret, exu, gcl, nos, orb, pge, and spir, are among approximately 50 known mRNAs that transiently accumulate in rings, sometimes termed “RNA islands,” that become apparent around the pole cell nuclei just prior to completion of their cellularization (Lécuyer et al. 2007, images publicly available at http://fly-fish.ccbr.utoronto.ca). This suggests a fundamental role for these perinuclear structures, and their constituent mRNAs, in embryonic patterning and germ cell specification. However, the functions of most mRNAs that localize to these structures in pattern formation or germ cell specification are unknown, because mutations affecting them are lethal, or because mutations block oogenesis before mature eggs that can be fertilized are formed, or because no mutants are available.

To address germine-specific functions of essential genes, genetic approaches have been developed to abrogate the functions of specific genes only in germline cells. One such approach involves inducing mitotic recombination and selecting for recombinants using a chromosome carrying a dominant female sterile mutation (Perrimon and...
This technique has been used to screen for maternal functions of many zygotically essential genes (Perrimon et al. 1984; Perrimon et al. 1989); however, it is laborious and such screens have yet to be extended to the entire genome. A more recent approach to this problem is based on the principle of RNA interference (RNAi), in which expression of a small double-stranded hairpin RNA (shRNA) including sequences homologous to a target mRNA post-transcriptionally inactivates the target through translational repression and degradation (Fire

### Table 1 Summary of visible phenotypes of RNAi knockdown lines

| Gene Name | RNAi KD at 25\a | RNAi KD at 29\a | Hatch Rate at 25\a | Hatch Rate at 29\a | Egg Laying | Cuticle Defect | Pole Cell Formation Defect | Pole Cell Migration Defect | Dorsal Appendage Defect | Bloomington Stock Number |
|-----------|-----------------|-----------------|-------------------|-------------------|------------|----------------|--------------------------|--------------------------|--------------------------|--------------------------|
| wt        |                |                 | 93%               | 84%               | Yes        |                |                          |                          |                          | 35264                   |
| Ack       | +++            | +++             | >80%              | >80%              | Yes        |                |                          |                          |                          | 43965                   |
| Ank       | ++             | +++             | >80%              | 67%               | Yes        |                |                          |                          |                          | 35394                   |
| aret      |                |                 | No                |                   |            |                |                          |                          |                          |                          |
| Bsg25D    |                |                 | >80%              | >80%              | Yes        |                |                          |                          |                          | 36828                   |
| CAH2      |                | +++             | >80%              | 62%               | Yes        | +              |                          |                          |                          | 41836                   |
| CG10077   | ++             | +++             | >80%              | 73%               | Yes        |                |                          |                          |                          | 32388                   |
| CG11597   | ++             | ++              | >80%              | >80%              | Yes        |                |                          |                          |                          | 43175                   |
| CG14322\a|                |                 | No                |                   |            |                |                          |                          |                          |                          |
| CG18446   |                |                 | >80%              | 78%               | Yes        |                |                          |                          |                          | 33735                   |
| CG2865    | ++             | ++              | >80%              | 71%               | Yes        |                |                          |                          |                          | 43165                   |
| CG31998   |                | +++             | >80%              | 12%               | Yes        | ++             |                          |                          |                          | 41828                   |
| CG3295\a |                |                 | No                |                   |            |                |                          |                          |                          |                          |
| CG4040    | +++            | 0%              |                   |                   | Yes        | +++            |                          |                          |                          | 42776                   |
| CG5292    |                | +++             | >80%              | >80%              | Yes        |                |                          |                          |                          | 32499                   |
| CG6509    | +++            | +++             | >80%              | 78%               | Yes        |                |                          |                          |                          | 41832                   |
| CG9821    | +++            | +++             | 68%               | 55%               | Yes        | ++             |                          |                          |                          | 43171                   |
| CG9977    |                |                 | No                |                   |            |                |                          |                          |                          | 43168                   |
| Charybde  |                |                 | >80%              | >80%              | Yes        |                |                          |                          |                          | 43975                   |
| Cta       |                | ++              | 5%                | 0%                | Yes        | +++            |                          |                          |                          | 39024                   |
| CycB      |                | +++             | >80%              | >80%              | Yes        |                |                          |                          |                          | 32375                   |
| Del       | +++            | 0%              |                   |                   | Yes        | +++            |                          |                          |                          | 43176                   |
| Dock      | +++            | +++             | >80%              | 77%               | Yes        |                |                          |                          |                          | 43176                   |
| elf-4G    |                |                 | No                |                   |            |                |                          |                          |                          | 33049                   |
| elf5      |                |                 | No                |                   |            |                |                          |                          |                          | 34841                   |
| Exu       | +++            | 0%              |                   |                   | Yes        | +++            |                          |                          |                          | 41816                   |
| Gap1      | +++            | >80%            |                   |                   | Yes        |                |                          |                          |                          | 41830                   |
| Gcl       |                | +++             | >80%              | >80%              | Yes        |                |                          |                          |                          | 34608                   |
| Gwl       |                | 0%              |                   |                   | Yes        | +++            |                          |                          |                          | 35212                   |
| Hip14     |                |                 | No                |                   |            |                |                          |                          |                          | 35012                   |
| Jvl       | +++            | 78%             | 36%               |                   | Yes        | ++             |                          |                          |                          | 43177                   |
| mei-P26   |                |                 | No                |                   |            | ++             |                          |                          |                          | 36855                   |
| Mlt       | +++            | 41%             |                   |                   | Yes        | ++             |                          |                          |                          | 43173                   |
| Mkn1      | +++            | +++             | 76%               | 78%               | Yes        | ++             |                          |                          |                          | 43178                   |
| Nos       | +              | +++             | 1.5%              | 0%                | Yes        | +++            |                          |                          |                          | 33973                   |
| nvr1      | +++            | 0%              |                   |                   | Yes        | +++            |                          |                          |                          | 41829                   |
| Orb       |                |                 | No                |                   |            |                |                          |                          |                          | 43143                   |
| Osk       | +++            | 0%              |                   |                   | Yes        | +++            |                          |                          |                          | 36903                   |
| pAbp      |                |                 | >80%              | 69%               | Yes        |                |                          |                          |                          | 36127                   |
| Patr-1    | ++             | +++             | >80%              | 47%               | Yes        |                |                          |                          |                          | 34667                   |
| Pbl       |                |                 | No                |                   |            |                |                          |                          |                          | 36841                   |
| Pgc       |                |                 | >80%              | >80%              | Yes        |                |                          |                          |                          | 33720                   |
| Pino      |                | +               | >80%              | >80%              | Yes        |                |                          |                          |                          | 43971                   |
| Pit3K21B  | ++             | +++             | >80%              | 59%               | Yes        |                |                          |                          |                          | 36810                   |
| Pum       | ++             | +++             | >80%              | >80%              | Yes        |                |                          |                          |                          | 41875                   |
| Rappgap1  | ++             | +++             | 72%               | 48%               | Yes        | ++             |                          |                          |                          | 42782                   |
| Sl        | +++            | +++             | >80%              | 78%               | Yes        |                |                          |                          |                          | 35604                   |
| Spir      | ++             | +               | 21%               | 8%                | Yes        | +++            |                          |                          |                          | 43161                   |
| Sra       | +              | +++             | >80%              | 66%               | Yes        |                |                          |                          |                          | 36900                   |
| Tao       | +++            | 0%              |                   |                   | Yes        | ++             |                          |                          |                          | 35147                   |
| Tm1       | ++             | +++             | >80%              | >80%              | Yes        |                |                          |                          |                          | 38232                   |
| Trk       |                |                 | >80%              | No (29\a)         |            |                |                          |                          |                          | 36748                   |
| Unr       | +++            | +++             | >80%              | >80%              | Yes        |                |                          |                          |                          | 32432                   |
| Vas       | +++            | 0%              |                   |                   | Yes        | +++            |                          |                          |                          | 38924                   |

\* RNAi stock not available.
et al. 1998). Publicly accessible libraries of Drosophila lines that express hairpin RNA targeting most protein-coding genes under the control of the upstream activation sequence (UAS) have been assembled (Mummery-Widmer et al. 2009; Ni et al. 2011). With the use of the appropriate GAL4 driver, these enable, in principle, the specific inactivation of nearly any gene in any tissue, including germline.

To investigate potential functions of mRNAs that accumulate in RNA islands in embryonic patterning or germ cell specification, in this work we conducted a comprehensive analysis of the phenotypes that result during oogenesis or in progeny embryos from maternal germ-line-specific expression of shRNA that targets each mRNA that accumulates in these perinuclear structures.

**MATERIALS AND METHODS**

**Drosophila strains**

shRNA-expressing stocks were obtained from the Bloomington stock center. Stock numbers are shown in Table 1. The full genotypes of all the lines used in this study are available on the TRiP website (http://www.flyrma.org/TRiP-HOME.html). We used the maternal triple driver MTD-Gal4 to induce expression of shRNA in germ line cells throughout oogenesis (Petrella et al. 2007), and we obtained this stock from the Bloomington stock center (stock number 31777).

**Screen setup**

Ten to 15 MTD-Gal4 males were crossed to 10–15 virgin females of each TRiP line in a vial and transferred to fresh food every 3–5 d. Crosses were incubated at 25°C throughout the experiment or, alternatively, the flies were discarded after 5 d and the vials containing larvae were transferred to 29°C to complete development. Growth of females with MTD-Gal4-driven shRNAs at 29°C sometimes produces more severe phenotypes and more effective knockdown of the target mRNA (Ni et al. 2011; this study). Progeny carrying both MTD-Gal4 and the shRNA construct were collected from these crosses, eggs were collected, and their phenotypes were assessed as described below.

**Cuticle preparation, hatch rate determination, and dorsal appendage preparation**

Cuticle preparations were performed as described in Nüsslein-Volhard et al. (1984) with the following modifications: 30–50 flies, of both sexes in approximately equal proportions, were transferred into egg-laying cages with apple juice agar plates (60-mm × 15-mm cell culture dish) supplemented with fresh yeast paste and incubated at 25°C or 29°C. Genotypes for these crosses are described in Results. Collections from the first 2 d after transfer were discarded. Subsequently, eggs were collected either overnight or for 6 hr and were allowed to develop.

![Figure 1](image-url) Dark-field photographs of cuticle preparations of RNAi knockdown embryos. Three embryos are illustrated from each knockdown line to capture the range of phenotypic severity that was observed. Embryos are oriented with anterior to the left. Control wild-type (wt) embryos are shown in the top row. The phenotypes observed for each line are discussed in Results.
for an additional 36 hr at 25°C. Hatch rate was determined by counting the number of hatched eggs and unhatched eggs for each lay. In cases where more than 20% of the eggs failed to hatch, eggs were collected for cuticle preparation as follows: first, embryos were transferred into small sieves and washed with water, then they were dechorionated in a 50% dilution of commercial bleach (12% sodium hypochlorite) for 2 min, and, finally, they were washed with water for another 2 min. The embryos were then transferred with a fine brush into a 1.5-ml microcentrifuge tube containing PBST buffer (1× PBS, and 0.1% Tween-20). Buffer was removed as completely as possible with a micropipette tip, and then 30 μl Hoyer’s medium (30 g gum arabic, 50 ml H2O, 200 g chloral hydrate, 20 g glycerol) was added. The embryos were then mounted onto a glass slide and covered with a 22×22-mm cover slip. Next, the embryos were cleared by overnight incubation at 65°C and observed under dark field illumination using a Leica DM 6000B microscope.

**Immunohistochemistry**

Ovaries were dissected from 3- to 5-d-old females in PBS and fixed in 100 μl of PBS, 1% NP-40, 600 μl of heptane, and 100 μl of 10% formaldehyde for 20 min. Samples were rinsed three times, washed three times for 10 min with PBST (PBS + 0.3% Triton X-100), and blocked in PBSTA (PBST + 1% BSA) for 1 hr at room temperature. Samples were incubated with primary antibodies overnight at 4°C in PBSTA. Samples were rinsed three times, washed three times (20 min each) with PBST, and then blocked in 1 ml of PBSTA for 1 hr at room temperature. Samples were incubated in the dark with fluorescent secondary antibody (pre-adsorbed goat anti-rat Alexa Fluor 488 and goat anti-rabbit Alexa Fluor 555; Life Technologies), final dilution 1:1000 in PBSTA overnight at 4°C, then washed four times (5 min each) and twice (15 min each) in PBST in the dark. Samples were counterstained with DAPI, mounted in anti-fade reagent in glycerol/PBS from the SlowFade Antifade Kit (Molecular Probes), and examined under a confocal microscope (Zeiss LSM510). Rat anti-Vas was used at a dilution of 1:1000. Rabbit anti-Osk was used at a dilution of 1:1000. Embryos were immunostained as described in Kobayashi et al. (1999). Primary rabbit anti-Vas was used at 1:5000. Secondary antibody was anti-rabbit Alexa Fluor 488 (Life Technologies). Images were collected on a Leica DM 6000B microscope.

**RNA isolation and RT-PCR analysis**

Total RNA was prepared from 30 embryos (0–2 hr at 25°C or 0–1 hr at 29°C) using TRIZol reagent (Life Technologies) according to the manufacturer’s protocol, followed by treatment with TURBO DNase (Ambion) for 30 min at 37°C. First strand cDNA was synthesized with Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Scientific). Quantitative differences in gene expression were determined by PCR with JumpStart REDTaq ReadyMix Reaction Mix (Sigma) using the first strand cDNA as a template. PCR products (5 μL for each) were resolved on a 1.5% agarose gel using primers that produced a product between 150 and 250 bp in length. Primers that amplify rp49 mRNA served as a positive control.

**RESULTS**

Our results are described below and are summarized in tabular form (Table 1).

**Maternal-effect loci involved in embryonic patterning**

We examined cuticle preparations from embryos produced by females expressing each RNA interference construct (henceforth referred to as knockdown embryos for brevity) that failed to hatch into larvae as a first step in characterizing their phenotype. In wild-type embryos that are about to hatch, the most prominent anterior structures are the...

**Figure 2** Embryos derived from RNAi knockdown mothers were stained for Vas protein (green) to visualize pole cells. Two embryos are shown for each knockdown line. For those that develop sufficiently, the embryo in the left panel is at the blastoderm stage, whereas the embryo in the right panel is at stage 10, which is the stage at which pole cells are in mid-migration or later. In many cases development does not progress normally beyond the blastoderm stage, and in these instances the embryo in the right panel represents what appears to be the latest stage of development achieved. In some cases, development ceases before cellularization, and then two representative embryos are shown. Wild-type embryos (wt, for comparison, are shown in the first row. The phenotypes observed for each line are discussed in Results.
mouth, which protrude from the anterior pole into the interior of the embryo (Figure 1, wt, seen most easily in the leftmost panel). Three thoracic segments and eight abdominal segments are then marked by transverse bands of short bristles called denticle belts; these are very fine and narrow for the three thoracic segments, but are broader and more prominent for the eight abdominal segments (Figure 1, wt). At the most posterior structure is a pair of structures, collectively termed the telson (Figure 1, wt, seen most easily in the rightmost panel).

Consistent with known phenotypes for the corresponding mutants (Lehmann and Nüsslein-Volhard 1986; Schüpbach and Wieschaus 1986; Nüsslein-Volhard et al. 1987; Manseau and Schüpbach 1989), most osk, nos, spir, and vas knockdown embryos exhibited a strong grandchildless-knirps phenotype (Schüpbach and Wieschaus 1986). These embryos are shorter than wild-type, lack most posterior segmentalization, and have two prominent foci of telson-derived material, surrounded by mostly naked cuticle (Figure 1, osk, nos, spir, vas). A substantial proportion (~25%) of nos embryos cultured at 29°C completely failed to develop and did not form cuticles. Interestingly, we discovered a similar grandchildless-knirps phenotype in 5–10% of mkrn1 knockdown embryos that failed to hatch (Figure 1, mkrn1, left and center panels), although posterior defects were less extreme in some of these embryos with most posterior denticile belts apparent (Figure 1, mkrn1, right panel). Severe posterior patterning defects were also observed in some Rapgap1, CAH2, Patr-1, and jvl embryos (Figure 1, Rapgap1, CAH2, Patr-1, jvl). These embryos differed from grandchildless-knirps embryos, however, in that most did not completely fill the entire volume of the egg and appeared shriveled, presumably as a result of holes in their cuticles. For CAH2 and Patr-1, these phenotypes were incompletely penetrant and many embryos appeared normal, whereas for Rapgap1 and jvl most embryos were affected. CG31998 knockdown embryos also exhibited defects in anterior-posterior patterning, but to a lesser degree than for those previously mentioned. In some CG31998 knockdown embryos, the fourth abdominal segment was partially or fully absent, or fused with the fifth (Figure 1, CG31998).

Consistent with the known phenotype for the corresponding mutant (Schüpbach and Wieschaus 1989), cta knockdown embryos failed to properly complete gastrulation. The embryos form a twisted structure with anterior holes (Figure 1, cta). Tao knockdown embryos progress through germ band extension but then do not retract, so they form U-shaped cuticles (Figure 1, Tao). These embryos also have obvious head defects. In milt knockdown embryos, various segments are partially missing or are fused and telsons are also often missing or reduced to rudiments (Figure 1, milt). del, gwl, CG4040, nrv1, and exu knockdown embryos do not progress sufficiently in development to form cuticles (Figure 1, del, gwl, CG4040, nrv1, exu); however, for exu and gwl (Figure 2), this phenotype is somewhat suppressed by a wild-type paternal copy of the gene in that cuticles form but severe anterior-posterior patterning defects are apparent, including a loss of anterior structures (Figure 1, exu with wt male). Loss of anterior structures has been reported as a maternal-effect phenotype of exu mutations (Schüpbach and Wieschaus 1989), and failure of oocytes to arrest in metaphase I of meiosis, resulting in a failure to support embryogenesis, is a phenotype of a hypomorph gwl allele (Archambault et al. 2007). Finally, in many CG9821 knockdown embryos, mouth parts are malformed and there is loss or fusion of abdominal segments (Figure 1, CG9821). Other CG9821 knockdown embryos are, however, patterned normally.

**Examination of pole cells in knockdown embryos with defects in embryonic development and assessment of dorsal appendages**

Next, we examined these knockdown embryos for their ability to form pole cells by immunostaining with anti-Vas (Figure 2). In wild-type, pole cells form at the posterior pole prior to general cellularization (Figure 2A, left panel). At gastrulation, they migrate along with the posterior midgut invagination into the interior of the embryo and then migrate as individual cells (Figure 2A, right panel) until forming two clusters in association with the gonadal mesoderm to form the two gonads. Knockdown embryos for known posterior-group genes (vas, osk, spir; Figure 2, B, E, and H) and those that did not form cuticles (del, gwl, exu, nrv1, CG4040; Figure 2, I, J, L, N, and P) also did not form pole cells, although this phenotype was completely rescued for exu (Figure 2M) and partially rescued for gwl (Figure 2K) by a paternal

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**Figure 3** Embryos derived from RNAi knockdown mothers were stained for Vas protein (green) to visualize pole cell migration defects. Wild-type embryos, for comparison, are shown in the first picture. The phenotypes observed for each line are discussed in Results.
wild-type copy of the gene. In this case, approximately 50% of gwl knockdown embryos formed pole cells in numbers smaller than those for wild-type. Consistent with the phenotype of the corresponding mutant, and that of embryos produced by females expressing antisense RNA targeting pge (Nakamura et al. 1996; Martinho et al. 2004), we observed a severe reduction in pole cell number in pge knockdown embryos; pole cells were absent in 20% of embryos and present in reduced numbers in the remaining 80% (Figure 2F). For CG31998, 15% of knockdown embryos formed 0–5 pole cells and the rest formed wild-type numbers of pole cells (Figure 2C). A similar phenotype was observed for Mkrn1, with approximately 20% of embryos forming 0–5 pole cells (Figure 2D). Pole cells were also absent in approximately 25% of Rapgap1 embryos (Figure 2G). For nos knockdown embryos cultured at 29°C, pole cells formed in normal numbers and were localized normally until the onset of pole cell migration (Figure 2O, left panel). In later-stage embryos, pole cell migration was highly aberrant and pole cell numbers diminished as development progressed, such that stage 14 and later embryos had only a few scattered pole cells (Figure 2O, right panel) or none at all. In knockdown embryos for Tao, mlt, and cta, wild-type numbers of pole cells form, but they also frequently scatter during migration. This is presumably because of the extensive somatic defects that are present in these embryos. We observed failure of pole cells to coalesce into gonads in 53% of Tao knockdown embryos, 36% of mlt knockdown embryos, and 97% of cta knockdown embryos (Figure 3). Our results differ from observations of embryos produced by a Tao hypomorphic mutant where reduced numbers of pole cells were present (Sato et al. 2007). We also observed defects in dorsal appendage structure in eggs produced by Mkrn1 and jvl knockdown females (Figure 4). For Mkrn1 knockdowns, 15% of eggs lacked dorsal appendages and 18% had a single fused dorsal appendage, whereas for jvl knockdowns 19% of eggs lacked dorsal appendages and 24% had a single fused dorsal appendage. Similar dorsal appendage

Figure 5 Ovaries derived from RNAi knockdown mothers that did not lay eggs were visualized by DAPI staining (blue). Wild-type ovaries, for comparison, are shown in the first picture, and the oocyte (oo), 15 nurse cells (nc), and follicle cells (fc) are labeled. The phenotypes observed for each line are discussed in Results. In the bottom right panel, a single wild-type ovariole and two entire ovaries from the Hip14 shRNA expressing line are photographed together to illustrate the difference in size and extent of development.
defects have been reported in eggs produced from a hypomorphic jvl mutant (Dubin-Bar et al. 2011).

**Knockdown of some genes blocked oogenesis**

Knockdown of another set of genes whose mRNAs accumulate in pole cell rings resulted in defects during oogenesis that prevented the development of mature eggs. In these cases, we analyzed the morphology of the ovaries that were produced (Figure 5). The earliest developmental blocks in oogenesis occurred in females knocked down for pbl (Figure 5C), Hip14 (Figure 5E), elF5 (Figure 5F), or CG9977 (Figure 5I). In these cases, essentially no germ line cells were observed, indicating that abrogation of function of these genes results in cellular lethality. Knockdown of orb (Figure 5D), elF-4G (Figure 5G), or aret (Figure 5H) resulted in the formation of some rudimentary egg chambers, but these did not progress beyond early pre-vitellogenic stages. The phenotypes of orb and aret knockdowns are consistent with those of known severe mutations in these genes (Schüpbach and Wieschaus 1991; Christerson and McKearin 1994). Knockdown of mei-P26 led to the formation of tumorous egg chambers similar to those described in mei-P26 mutants (Page et al. 2000). Knockdown of ttk resulted in normal oogenesis until approximately stage 6, followed by extensive cell death (Figure 5B).

**Assessment of efficacy of RNAi knockdown**

We examined the effectiveness of each RNAi construct at targeting its corresponding mRNA using RT-PCR (Figure 6). In total, we attempted to knock down the germline activities of 51 different genes that express mRNAs that localize in perinuclear rings in the precursors to pole cells. For seven of these genes [Bsg25D (Figure 6F7), CG18446 (Figure 6B7), charybde (Figure 6F6), cta (Figure 6E7), pAbp (Figure 6B9), pgc (Figure 6F9), Pino (Figure 6E8)], the effectiveness of the knockdowns appeared very poor (<40% reduction) by this assay, even when flies were cultured at elevated temperature. Although we did not observe any effects...
on oogenesis or embryonic viability from expressing shRNA targeting Bsg25D, CG18446, charidy, pAbp, or Pino, we cannot conclude that these genes play no essential role in the female germ line because targeting them in this way was inefficient. Surprisingly, despite apparently poor efficiency of the corresponding shRNA, we nevertheless obtained phe-

sra (Figure 6D7), (Figure 6E10), (Figure 6F10), and pAbp (Figure 6G6), complete (80–100%) by RT-PCR at one or both temperature condi-
tions. For the nine genes, including tik at 29°, for which knockdown pro-
duced developmental blocks in early stages in oogenesis (Figure 5), we
did not analyze the effectiveness of the knockdown in this manner
because tissue from their rudimentary ovaries was difficult to obtain
and appropriate controls were lacking.

Finally, we examined whether two pole plasm components, Osk
and Vas protein, localized normally to the posterior of the stage-10
oocyte in the knockdown lines where pole cell formation was
compromised. In flies expressing shRNA targeting del, we found that
posterior accumulation of both Osk and Vas was greatly reduced, as
was accumulation of Vas into the perinuclear nuage, but that the level
of Vas in the cytoplasm of nurse cells was comparable to that of
controls (Figure 7). Conversely, for flies expressing shRNA targeting
CG4040, exu, gw1, or nrvl, Vas and Osk accumulation appeared similar
to that of wild-type (data not shown).

DISCUSSION

In this study we analyzed a set of mRNAs that accumulate in
cytoplasmic rings within primordial germ cell precursors, sometimes
called “RNA islands,” by expressing in germ line shRNAs that target
them and examining the phenotypic consequences. This work provided
evidence for specific roles in embryonic patterning and/or germline
specification for several genes whose mRNAs localize in this way and
that have not been previously implicated in these processes. This pro-
vides further support for the conclusion that these structures accumulate
mRNAs that are involved in these developmental events.

Of particular interest to us are genes that we implicated in anterior–
posterior embryonic patterning or in germ cell specification. One of
these is mkrn1, for which no mutant phenotype had been previously
described in Drosophila. In mammals, the protein encoded by MKRN1
is an E3 ubiquitin ligase that modifies PPARγ, a transcription factor
involved in activating adipocyte differentiation, and targets it for de-
gradation (Kim et al. 2014). Several other targets have also been identified
for MKRN1, indicating it is involved in numerous cellular and disease-
related processes. Previous work has implicated post-translational mod-
ification pathways in establishing and maintaining posterior localization
of Vas (Liu et al. 2003; Kugler et al. 2010), and thus in anterior–
posterior patterning and pole cell specification. Our present obser-
vations raise the possibility that Mkrn1 may regulate the stability by

![Figure 7](image7.png)
ubiquitinating one or more proteins involved in posterior patterning and pole cell specification.

We also observed posterior embryonic defects in Patr-1 knockdown embryos. Patr-1 encodes a component of P bodies that is believed to activate mRNA decapping and miRNA degradation (Jäger and Dorner 2010; Pradhan et al. 2012; Barisić-Jäger et al. 2013; Nishihara et al. 2013), and it has also been identified as a component of the somatic piRNA pathway (Handler et al. 2013). It has been demonstrated that Patr-1 interacts with the CCR4 deadenylase at the larval neuromuscular junction (Pradhan et al. 2012), but its role in germline development has heretofore been unexplored. Given the well-established importance of decapping and CCR4-mediated deadenylation in post-transcriptional genetic regulation in the female germline (Zaessinger et al. 2006; Chicoine et al. 2007; Tadros et al. 2007; Rouget et al. 2010; Igreja and Izaurralde 2011), it is probable that the phenotype we observed in Patr-1 knockdown embryos results from effects on these processes.

The other RNA knockdown lines that produced maternal-effect anterior–posterior defects were jvl, Rapgap1, and CAH2. jvl encodes a microtubule-associated protein, and jvl mutant oocytes show defects in localization of grk, bcd, and osk mRNA, as well as disruptions of the cytoskeleton (Dubin-Bar et al. 2011). Both the mutant and our knockdown line produced embryos with dorsal appendage defects, confirming a role for jvl in the germline in producing these structures. Dorsal appendages are produced by follicle cells in response to activation of the epidermal growth factor receptor (Egfr) by its ligand Grk, which is translated in the oocyte from the localized grk mRNA and secreted over a short range (González-Reyes et al. 1995; Roth et al. 1995). Although we did not analyze grk mRNA localization in jvl (or Mkrn1) knockdown embryos because their dorsal appendage phenotypes were not fully penetrant, effects on grk mRNA localization, as observed in the jvl mutant (Dubin-Bar et al. 2011), could explain the effects we observed in these knockdown embryos on dorsal appendage formation.

Unlike in the corresponding mutant, in jvl knockdown embryos we did not observe defects in posterior Osk or Vas localization, possibly because of incomplete inactivation of the jvl mRNA. Conversely, although the defect in osk localization in the jvl mutant would be expected to lead to anterior–posterior defects in progeny embryos, this was not observed because jvl mutant eggs do not support embryogenesis beyond initial stages. In this instance, then, the incomplete knockdown (or germline specificity of the knockdown) of the target mRNA allowed the identification of a phenotype that was masked in a strong mutant allele. Another similar instance concerned del; del mutant alleles block oogenesis at an early stage (Schüpbach and Wieschaus 1991), and Del protein has recently been identified as a component of a complex that is targeted to chromatin at dual-strand piRNA clusters and is required to produce piRNAs from those clusters (Mohn et al. 2014). Although analysis of rare escape eggs that progress more completely through oogenesis indicated a later role for del in microtubule-mediated processes including localization of osk and grk mRNAs (Wehr et al. 2006), this later phenotype is much more apparent in the RNA knockdown line that produces substantial numbers of embryos.

It is more difficult to predict potential functions for the other two genes in embryonic patterning or germline specification. Rapgap1 encodes a GTPase activator involved in intracellular signaling, but a null mutant for this gene has been reported to be viable and fertile, with only minor irregularities in pole cell alignment at embryonic stage 13 (Chen et al. 1997). Further work will be necessary to determine whether the phenotype we observed results from a secondary off-target effect of the Rapgap1 shRNA. CAH2 is one of two Drosophila genes that encode a carbonic anhydrase, an enzyme that catalyzes the reversible hydration of carbon dioxide to bicarbonate (Syrjänen et al. 2013). No mutant phenotype has been reported for this gene. It is likely to be functionally redundant with CAH1 in most tissues, but high-throughput data indicate that CAH2 is by far the predominant form of the enzyme that is expressed in ovaries (Graveley et al. 2011). A role for glycolytic enzymes in germ cell development has recently been described, indicating that metabolic enzymes can have specific developmental roles (Gao et al. 2015).

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