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RESEARCH ARTICLE

Pgc suppresses the zygotically acting RNA decay pathway to protect germ plasm RNAs in the *Drosophila* embryo

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ABSTRACT

Specification of germ cells is pivotal to ensure continuation of animal species. In many animal embryos, germ cell specification depends on maternally supplied determinants in the germ plasm. *Drosophila polar granule component* (*pgc*) mRNA is a component of the germ plasm. *pgc* encodes a small protein that is transiently expressed in newly formed pole cells, the germline progenitors, where it globally represses mRNA transcription. *pgc* is also required for pole cell survival, but the mechanism linking transcriptional repression to pole cell survival remains elusive. We report that pole cells lacking *pgc* show premature loss of germ plasm mRNAs, including the germ cell survival factor *nanos*, and undergo apoptosis. We found that *pgc*$^{-}$ pole cells misexpress multiple miRNA genes. Reduction of miRNA pathway activity in *pgc*$^{-}$ embryos partially suppressed germ plasm mRNA degradation and pole cell death, suggesting that Pgc represses zygotic miRNA transcription in pole cells to protect germ plasm mRNAs. Interestingly, germ plasm mRNAs are protected from miRNA-mediated degradation in vertebrates, albeit by a different mechanism. Thus, independently evolved mechanisms are used to silence miRNAs during germ cell specification.

KEY WORDS: Germ cell, Germ plasm, microRNA, Transcriptional repression, Maternal-to-zygotic transition

INTRODUCTION

In many animals, including *Drosophila*, *Xenopus* and zebrafish, germ cell specification and maintenance depends on maternally provided RNAs and proteins located in the germ plasm (Voronina et al., 2011). An important and conserved feature of the germ plasm is the active repression of RNA polymerase II (RNAPII)-dependent transcription. This mechanism is thought to ensure germ cell fate by preventing induction of the somatic transcriptional program (Nakamura and Seydoux, 2008; Nakamura et al., 2010).

In *Drosophila*, mRNA transcription is quiescent in germline progenitor cells (pole cells) until late gastrulation, whereas activation of mRNA transcription in somatic nuclei starts from the syncytial blastoderm stage, prior to pole cell formation (Seydoux and Dunn, 1997; Van Doren et al., 1998). As these early zygotic genes play crucial roles in body patterning and sex determination in the soma (Pritchard and Schubiger, 1996; ten Bosch et al., 2006), it is proposed that they must be repressed in newly formed pole cells (Nakamura and Seydoux, 2008; Nakamura et al., 2010).

The key factor for the repression of mRNA transcription in newly formed pole cells is encoded by *polar granule component* (*pgc*), a maternally supplied mRNA that localizes to the germ plasm (Martinho et al., 2004; Hanyu-Nakamura et al., 2008). *pgc* encodes a small 71 amino acid protein that is transiently expressed in pole cells. The Pgc protein inhibits mRNA transcription by interfering with positive transcription elongation factor b (P-TEFb), a component of RNAPII-dependent transcription (Hanyu-Nakamura et al., 2008). During the transcription cycle, RNAPII is recruited to the gene promoter region, forms the pre-initiation complex and initiates RNA synthesis. RNAPII often stalls immediately downstream of the transcription start site after initiation, and its release into productive elongation requires the recruitment of P-TEFb, a complex of Cyclin-dependent kinase 9 (Cdk9) and Cyclin T (CycT), to transcription sites (Zhou et al., 2012; Jonkers and Lis, 2015). Pgc binds Cdk9 and prevents the recruitment of P-TEFb to transcription sites, by an unknown mechanism. Pole cells lacking Pgc, or overexpressing P-TEFb, fail to repress mRNA transcription of genes that are normally expressed in the adjacent somatic cells (Hanyu-Nakamura et al., 2008). As Pgc is expressed in pole cells during the blastoderm stage (stages 4-5) and disappears during early gastrulation (stages 6-7), Pgc-mediated transcriptional repression is transient. After maternally deposited Pgc runs out from pole cells during gastrulation, chromatin-based mechanisms appear to contribute to keeping transcription silent (Martinho et al., 2004).

In embryos lacking *pgc*, normal numbers of pole cells are initially formed. However, these cells degenerate before they reach the embryonic gonads and >80% of *pgc* mutant embryos develop into sterile adults (Nakamura et al., 1996). Similarly, overexpression of P-TEFb in pole cells induces their degeneration. Therefore, Pgc-mediated transcriptional repression appears to be essential for pole cell maintenance (Hanyu-Nakamura et al., 2008). Pgc is also implicated in the maintenance of *nanos* (*nos*) transcript (Nakamura et al., 1996; Deshpande et al., 2012), which encodes an evolutionarily conserved factor essential for germ cell survival (Kobayashi et al., 1996; reviewed by Cinalli et al., 2008). Therefore, Pgc most likely has a critical target whose repression in pole cells is required for *nos* maintenance and pole cell survival. However, the mechanism by which Pgc maintains *nos* in pole cells remains unknown.

Here, we report that pole cells lacking *pgc* show precocious degradation of multiple germ plasm mRNAs, including *nos*. Furthermore, we show that Pgc prevents the misexpression of multiple miRNA genes in pole cells, thereby restricting miRNA action to the somatic region during the maternal-to-zygotic transition (MZT). We provide evidence that Pgc protects germ plasm mRNAs from miRNA-mediated degradation in pole cells, thereby promoting pole cell survival. Intriguingly, it has been reported that miRNA-mediated degradation of germ plasm mRNAs is also blocked in vertebrates, although by a different mechanism

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(Mishima et al., 2006; Kedde et al., 2007; Takeda et al., 2009; Koebernick et al., 2010). Therefore, our results provide insight into convergent evolution of the mechanisms by which blocking miRNA action contributes to maintaining germ plasm integrity during the establishment of the germ cell fate.

RESULTS

Pgc-mediated transcriptional repression stabilizes Nos in pole cells

To examine the role of Pgc in pole cell development in more detail, we first tested whether the distributions of Vasa (Vas) and Nos proteins were affected in embryos from *pgc*-null mothers (hereafter termed *pgc*$^{-}$ embryos). Vas and Nos serve as markers for the germ plasm and germ cells. The distributions of Vas and Nos in *pgc*$^{-}$ embryos were both normal during the blastoderm stage (stage 5) (Fig. 1A,B). However, although Vas levels were unaffected, Nos levels decreased in pole cells from stage 10 onward, compared with controls (Fig. 1D,E). We next examined whether the reduction of Nos in *pgc*$^{-}$ pole cells was caused by the failure to repress RNAPII-dependent transcription. When P-TEFb components, *Cdk9* and *CycT*, are simultaneously overexpressed in pole cells in a *pgc* heterozygous background, active mRNA transcription is induced in newly formed pole cells even in the presence of Pgc, and these cells degenerate later in development (Hanyu-Nakamura et al., 2008). When P-TEFb was overexpressed in pole cells, Nos was detected in these cells during the blastoderm stage but disappeared from stage 10 onward (Fig. 1C,F), similar to what was observed in the *pgc*$^{-}$ embryos. This suggests that a reduction in Nos levels depends on *de-novo* transcription in *pgc*$^{-}$ pole cells.

Nos is known to prevent pole cells from entering mitosis and apoptosis (Asaoka-Taguchi et al., 1999; Hayashi et al., 2004). Consistent with the loss of Nos, both *pgc*$^{-}$ and P-TEFb-overexpressing pole cells entered mitosis precociously, visualized by staining with an antibody against phospho-Histone H3 (pH3), a marker for mitosis (Fig. 1G-I). During gastrulation, pH3-positive pole cells were not observed in *pgc*$^{-}$ embryos through stage 9. However, at stage 10, 15.8% of *pgc*$^{-}$ embryos had pH3-positive pole cells, coincident with the decrease in Nos levels (Fig. 1C,F and Table S1). Intriguingly, this was very similar to the occurrence of pH3-positive pole cells in stage 9-10 *nos*$^{-}$ embryos (~20%) (Asaoka-Taguchi et al., 1999). Similarly, when P-TEFb was overexpressed in pole cells, 9.9% of embryos had pH3-positive pole cells at stage 10 (Table S1).

We also observed signals for cleaved Caspase-3 in both *pgc*$^{-}$ and P-TEFb-overexpressing pole cells, as in *nos*$^{-}$ pole cells, which indicates that they were undergoing apoptosis (Fig. 1J-L). When the zygotic activities of the apoptosis inducers *head involution defective* (*hid*), *reaper* and *grim* were all removed by introducing *Df(3L)H99* deficiency, *pgc*$^{-}$ pole cells did not enter apoptosis (Fig. S1A-C). In these embryos, Nos levels decreased in pole cells from stage 10

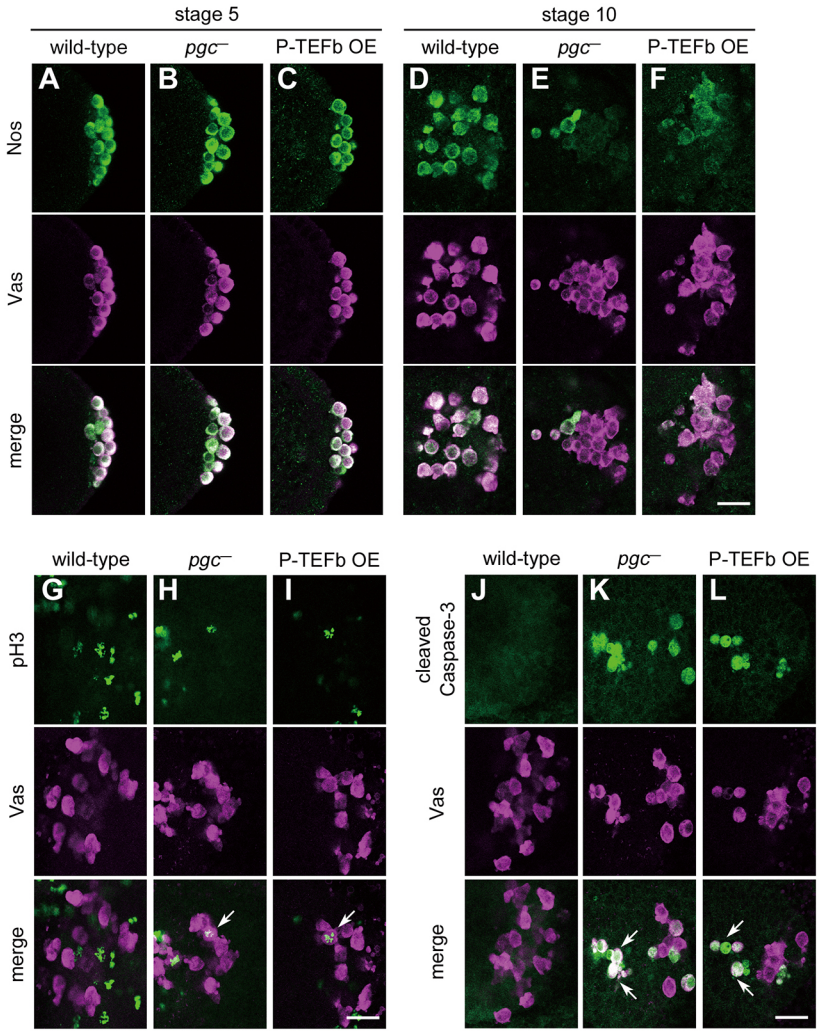

Fig. 1. *pgc* is required for the maintenance of Nos in pole cells. (A-F) Stage 5 or 10 embryos were stained using anti-Nos (green) and anti-Vas (magenta) antibodies. Embryos were derived from wild-type, *pgc*$^{\Delta 1}$*/Df(2R)X58-7* (*pgc*$^{-}$) and *pgc* heterozygous females overexpressing the P-TEFb components *Cdk9* and *CycT* (*UASp-Cdk9-nos* 3′ *UTR*/*pgc*$^{\Delta 1}$*; UASp-CycT-nos* 3′ *UTR*/*nos-GAL4-VP16* – P-TEFb OE in the figure). (G-I) Stage 10 embryos were stained using anti-phospho-Histone H3 (pH3, green) and anti-Vas (magenta) antibodies. Arrows indicate the pH3-positive pole cells. (J-L) Stage 10 embryos were stained using anti-cleaved Caspase-3 (green) and anti-Vas (magenta) antibodies. Arrows indicate the cleaved Caspase-3-positive pole cells. Scale bars: 20 µm.

Development (2019) 146, dev167056. doi:10.1242/dev.167056

onward, as observed in the *pgc*⁻ pole cells (Fig. S1D-F). Thus, the precocious reduction of Nos in *pgc*⁻ pole cells is independent of apoptosis. These results are consistent with previous suggestions that pole cell death in *pgc*⁻ embryos is caused, at least in part, through a reduction in Nos levels (Nakamura et al., 1996; Deshpande et al., 2012).

To test whether the loss of Nos is sufficient to explain the death of *pgc*⁻ pole cells, we examined whether increasing Nos levels in pole cells could suppress the *pgc* mutant phenotype. Expression of *nos* in the germ plasm depends on the *nos* 3′ untranslated region (3′ UTR), which contains regulatory elements for the localization and translation of *nos* mRNA (Gavis et al., 1996a,b; Dahanukar and Wharton, 1996; Bashirullah et al., 1999). As only 4% of maternal *nos* mRNA is localized in the germ plasm through a diffusion and entrapment mechanism (Bergsten and Gavis, 1999; Forrest and Gavis, 2003), the amount of *nos* mRNA in the embryo would not be a rate-limiting step for the expression of Nos in the germ plasm. To express Nos in pole cells independently of the mechanisms that regulate endogenous *nos* mRNA, we generated a transgene in which the *nos*-coding sequence was fused to the *pgc* 3′ UTR, which targets transcripts to the germ plasm (Rangan et al., 2009). Ectopic Nos expression can affect embryonic patterning (Gavis and Lehmann, 1992). Consistent with this, embryos exhibited severe patterning defects when the *nos-pgc 3′ UTR* transgene was expressed during oogenesis under the control of the germline-specific *nos-GAL4-VP16* driver. The resulting somatic defects made it difficult to analyze the effect of *nos* overexpression on pole cell development. In contrast, when a weaker *otu-GAL4-VP16* driver (Rørth, 1998) was used to express the *nos-pgc 3′ UTR* transgene, 75% of embryos were morphologically normal and could be analyzed for pole cell development. These embryos contained significantly more gonadal pole cells (3.3±2.6, *n*=100) than did the *pgc*⁻ embryos (1.0±1.3, *n*=100) (Fig. 2A-D and Table S2). The rescued pole cells developed into functional germ cells, as indicated by the restored fertility of the adult progeny (Table S2). These results indicate that restoring Nos expression can, at least partially, rescue the *pgc* mutant phenotype.

Nos has been reported to be involved in transcriptional repression in newly formed pole cells, although the underlying mechanism remains unknown (Deshpande et al., 1999; Schaner et al., 2003). This raises the possibility that *nos* overexpression might restore transcriptional quiescence, resulting in pole cell survival. To test this possibility, we examined the status of Ser2 phosphorylation in the C-terminal domain (CTD) of RNAPII (CTD pSer2), a hallmark of active transcription. Although CTD pSer2 signals were absent in most wild-type pole cells (Fig. 2E and Fig. S2), they were easily detected in transcriptionally derepressed *pgc*⁻ pole cells during the blastoderm stage (Fig. 2F and Fig. S2) (Martinho et al., 2004; Hanyu-Nakamura et al., 2008). The pattern of CTD modification in *pgc*⁻ pole cells remained unchanged even when *nos* was overexpressed (Fig. 2G and Fig. S2), suggesting that phenotypic rescue did not depend on restoration of transcriptional repression.

We noted that restoration of pole cell survival by *nos* expression was quantitatively incomplete (Fig. 2D and Table S2), which might reflect insufficient production of Nos in the rescued pole cells to fully compensate for the reduced endogenous Nos levels. Alternatively, other germ plasm factors, which play a role in pole cell survival, might be lost in *pgc*⁻ pole cells. Although additional factors may participate in regulation of pole cell survival, our findings suggest that a reduction of Nos levels contributes to pole cell apoptosis in *pgc*⁻ embryos.

Pgc-mediated transcriptional repression is required for the maintenance of germ plasm mRNAs in pole cells

We next examined the effect of removing *pgc* on the distribution of *nos* and other germ plasm mRNAs. We previously reported that antisense RNA-mediated *pgc* knockdown results in the precocious downregulation of two germ plasm mRNAs, *nos* and *germ cell-less* (*gcl*), in pole cells at the cellular blastoderm stage (Nakamura et al., 1996). We confirmed that *nos* and *gcl* mRNAs

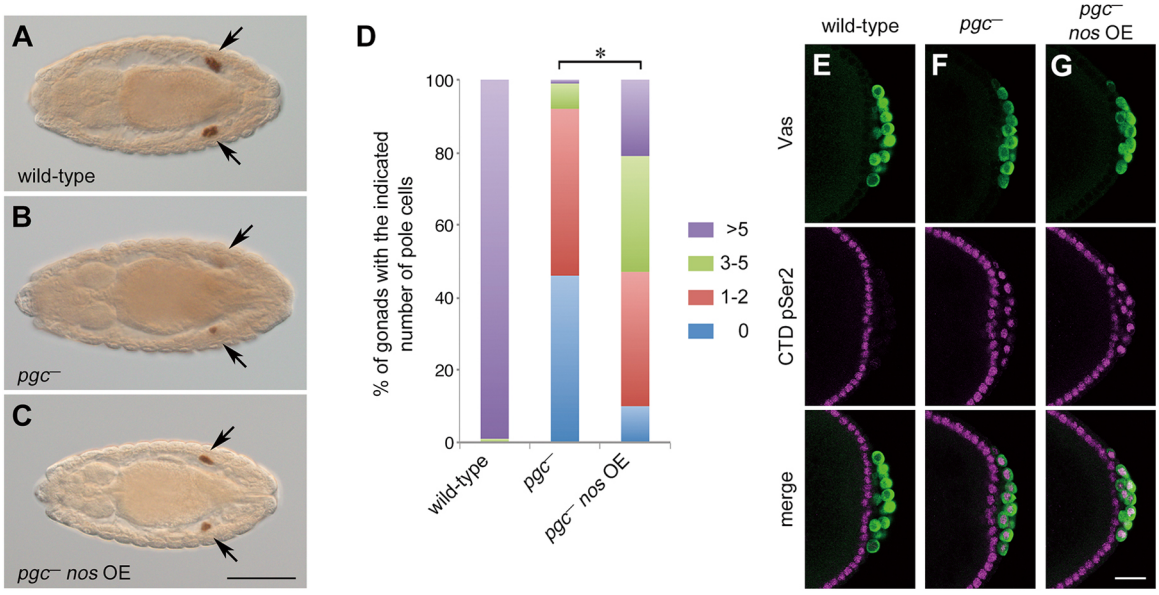

Fig. 2. *nos* overexpression in pole cells suppresses the *pgc* mutant phenotype. (A-C) Wild-type, *pgc*⁻ embryos and *pgc*⁻ embryos overexpressing *nos* with the *otu-GAL4-VP16* driver (embryos from *otu-GAL4-VP16/+; pgc*$^{\Delta 1}$*/Df(2R)X58-7; UASp-nos-pgc 3′ UTR/+* females – *pgc*⁻ *nos* OE in the figure) were stained using an anti-Vas antibody. Arrows indicate the gonads. Scale bar: 100 µm. The percentages of gonads with the indicated pole cell numbers in stage 14-15 embryos are shown in D. One hundred gonads of each genotype were scored. Significance was calculated using the Wilcoxon rank sum test (*P<0.001). (E-G) Syncytial blastoderm embryos (stage 4) were stained using anti-Vas (green) and anti-RNAPII CTD pSer2 (magenta) antibodies. Scale bar: 20 µm.

Development (2019) 146, dev167056. doi:10.1242/dev.167056

were normally localized to the germ plasm during the cleavage stage (stage 2) in *pgc*⁻ embryos (Fig. 3A,B,E,F), but their levels subsequently dropped in pole cells during the cellular blastoderm stage (stage 5) (Fig. 3C,D,G,H).

We also examined the distributions of two other germ plasm mRNAs implicated in pole cell survival, *Tao* and *wunen2* (*wun2*). *Tao* is implicated in pole cell formation and/or maintenance, as embryos from *Tao*-depleted females have reduced numbers of pole cells (Pflanz et al., 2015). *Tao* encodes two different transcripts. The longer mRNA is maternally expressed and accumulated in the germ plasm and pole cells (Sato et al., 2007; Pflanz et al., 2015) (Fig. 3I,K). In *pgc*⁻ embryos, *Tao* mRNA was localized normally to the germ plasm during stage 2, but disappeared precociously from pole cells during stage 5 (Fig. 3J,L). *wun2* regulates pole cell migration to the gonads as well as their survival (Hanyu-Nakamura et al., 2004; Renault et al., 2004; Slaidina and Lehmann, 2017). Notably, the distribution of *wun2* mRNA in early embryos differs from that of *nos*, *gcl* and *Tao* mRNAs: maternal *wun2* mRNA is initially distributed throughout the cleavage-stage embryos. Subsequently, it remains in pole cells in stage 5 embryos, presumably by protection of its degradation specifically in pole cells (Hanyu-Nakamura et al., 2004) (Fig. 3M,O). In *pgc*⁻ embryos, *wun2* mRNA signals were initially detected throughout the embryos, but disappeared from the entire embryo, including pole cells, during stage 5 (Fig. 3N,P).

To quantify mRNA levels more precisely, we visualized *nos* mRNA using the single-molecule fluorescent *in situ* hybridization (smFISH) technique (Raj et al., 2008). In this system, multiple fluorescent oligonucleotides hybridize to the target mRNA, and these hybridized signals are detected without further signal amplification. In *pgc*⁻ embryos, the intensity of *nos* mRNA signals in the germ plasm was equivalent to that of wild-type embryos during stage 2. However, *nos* mRNA signals in *pgc*⁻ pole cells were reduced to 53% of that in wild-type pole cells during

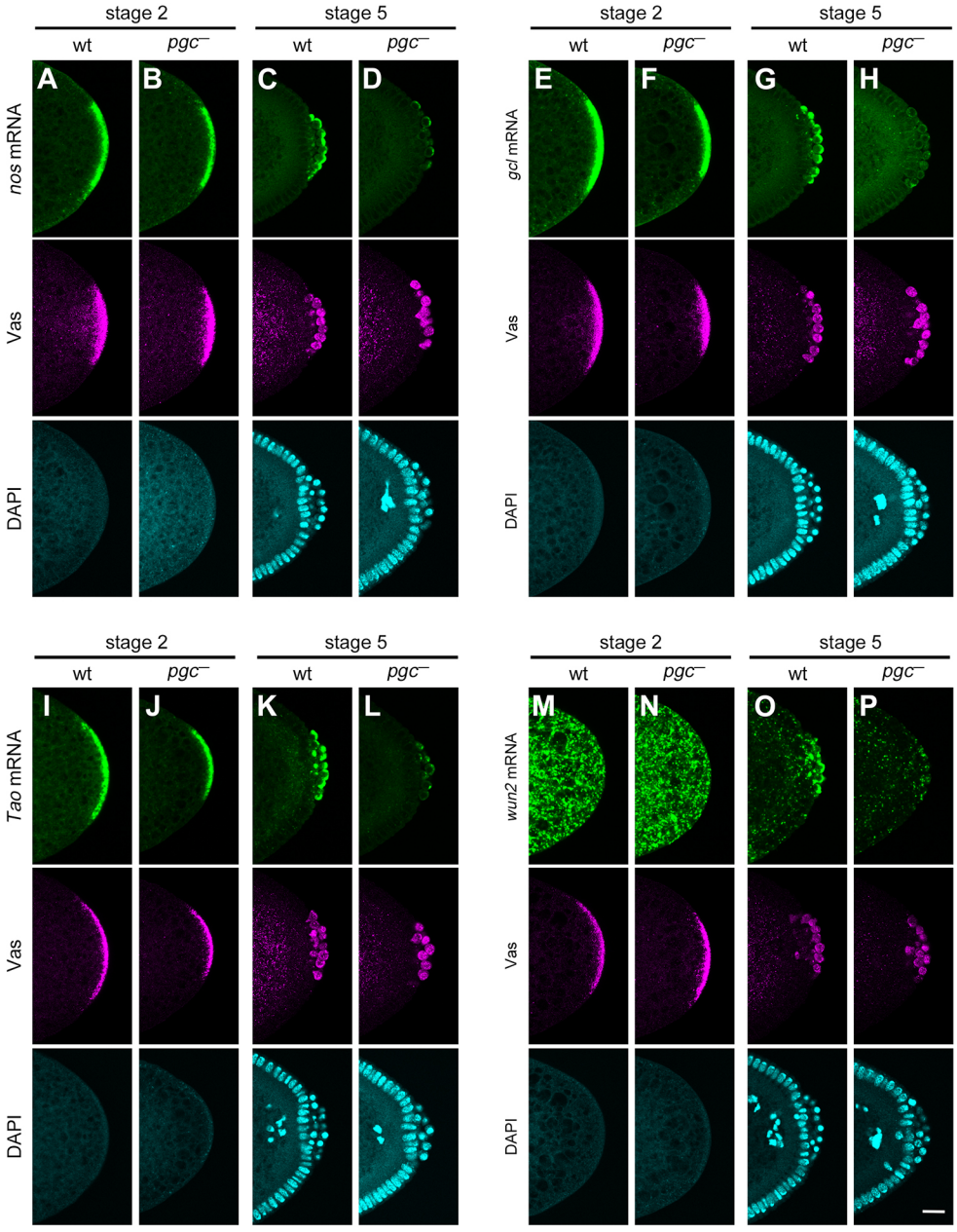

Fig. 3. *pgc* is required for the maintenance of germ plasm mRNAs in pole cells. (A-P) Stage 2 or 5 embryos were analyzed by *in situ* hybridization to visualize *nos* (A-D), *gcl* (E-H), *Tao* (I-L) and *wun2* mRNAs (M-P) (green), and stained using an anti-Vas antibody (magenta). Nuclei are counterstained with 4,6-diamidino-2-phenylindol (DAPI; cyan). Embryos were derived from wild-type and *pgc*⁻ females. Scale bar: 20 µm.

Development (2019) 146, dev167056. doi:10.1242/dev.167056

stage 5 (Fig. S3). These results indicate that maternal *nos* mRNA enriched in the germ plasm are precociously degraded in *pgc*⁻ pole cells, and suggest that misexpression of genes that are involved in RNA degradation destabilize germ plasm mRNAs in *pgc*⁻ pole cells.

Pgc-mediated transcriptional repression prevents the expression of multiple miRNAs in pole cells

It has been shown that many maternal mRNAs in the somatic region are eliminated during the blastoderm stage, as a part of the process known as the MZT (De Renzis et al., 2007; Tadros et al., 2007; Thomsen et al., 2010), whereas a subset of maternal mRNAs escape from degradation in pole cells (Bashirullah et al., 1999). For example, the majority of maternal *nos* mRNA is distributed throughout the cytoplasm of cleavage-stage embryos (Bergsten and Gavis, 1999) and is actively degraded during the blastoderm stage, but a small proportion of *nos* mRNA is localized to the germ plasm and remains in pole cells until the pole cells start to migrate toward the future gonads (Bashirullah et al., 1999). The degradation of maternal mRNAs during the MZT is mediated by the combined action of two pathways that are driven by maternal and zygotic factors (Bashirullah et al., 1999; Thomsen et al., 2010). We reasoned that these degradation mechanisms should be repressed in pole cells to protect germ plasm mRNAs, and that Pgc-mediated transcriptional repression might suppress the zygotic degradation pathway in pole cells.

The *mir-309* cluster encodes eight miRNAs, which start to be expressed during the syncytial blastoderm stage, and has been proposed to degrade hundreds of maternal mRNAs, including unlocalized germ plasm mRNAs, in the somatic region during the MZT (Bushati et al., 2008). We therefore examined the expression of the primary transcript for the *mir-309* cluster (*pri-miR-309*) in *pgc*⁻ embryos. As reported (Bushati et al., 2008), *pri-miR-309* was detected in all of the somatic nuclei but was barely detected in pole cells of wild-type embryos during the blastoderm stage (Fig. 4A and Table S3). However, in the absence of *pgc*, the *pri-miR-309* was ectopically expressed in the pole cells (Fig. 4B and Table S3). Misexpression of *mir-309* cluster miRNAs might contribute to the *pgc* mutant phenotype. We therefore tested whether removing the *mir-309* cluster could rescue the *pgc* mutant phenotype. However, there was no difference in the number of pole cells between the *pgc*⁻ mutant embryos and those simultaneously lacking zygotic *mir-309* (Table S4).

Given that the removal of the *mir-309* cluster alone had no impact on pole cell survival in *pgc*⁻ embryos, we hypothesized that, in addition to *miR-309*, other zygotic miRNAs might be misexpressed in *pgc*⁻ pole cells and contribute to the degradation of germ plasm mRNAs. Recent genome-wide studies, such as microarray and RNA-seq, have shown that many miRNAs are expressed zygotically in the somatic region during the blastoderm stage (Fu et al., 2014; Luo et al., 2016), although these miRNAs have not been tested for whether they target maternal mRNAs. We focused on

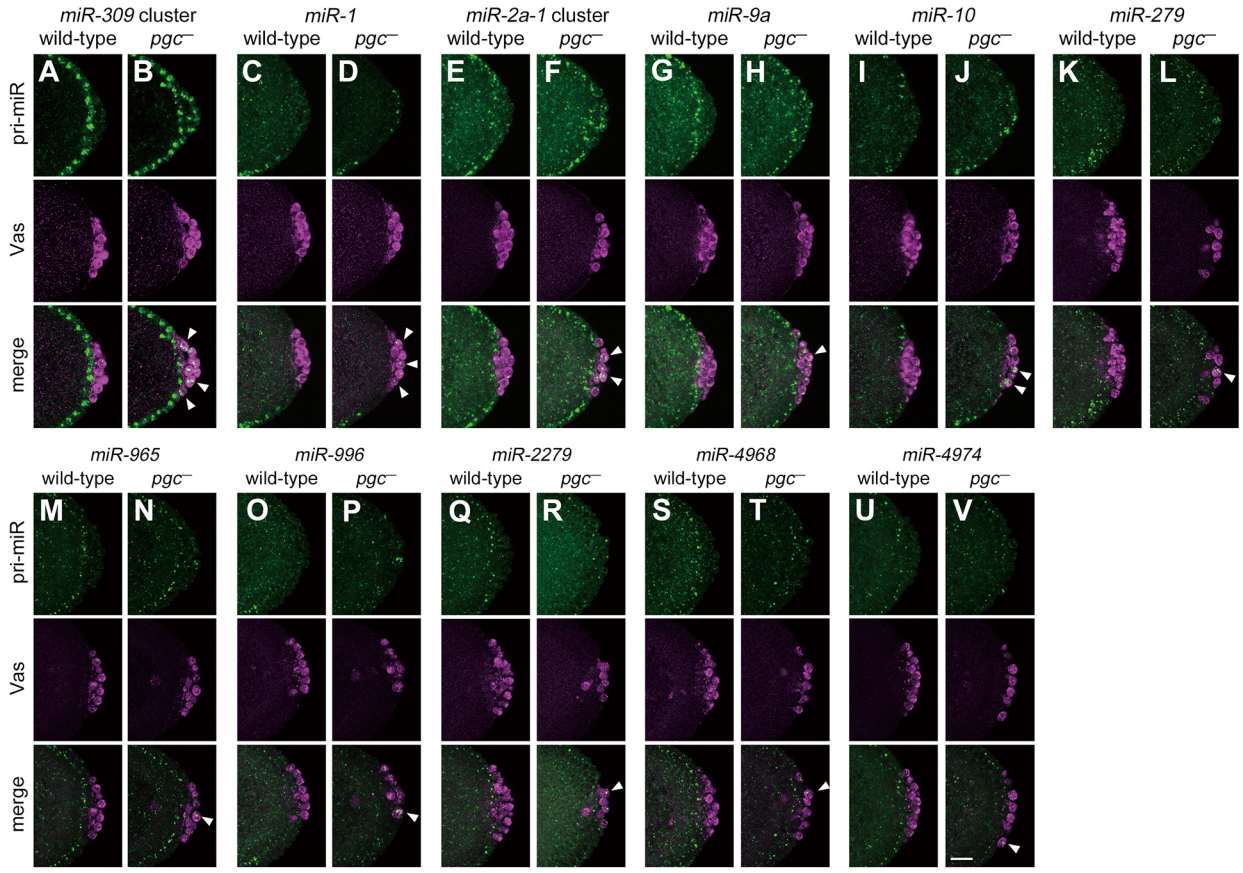

Fig. 4. *pgc* represses the expression of multiple zygotic miRNAs in pole cells. (A-V) Wild-type and *pgc*⁻ embryos were analyzed by *in situ* hybridization to visualize the primary transcripts of miRNAs (pri-miRs, green) and stained using an anti-Vas antibody (magenta). The primary transcript signals were localized to the nucleus. Stage 4 (A-J) or stage 5 (K-V) embryos were analyzed. The *miR-309* cluster consists of eight miRNAs (*miR-3*, *miR-4*, *miR-5*, *miR-6-1*, *miR-6-2*, *miR-6-3*, *miR-286* and *miR-309*). The *miR-2a-1* cluster consists of three miRNAs (*miR-2a-1*, *miR-2a-2* and *miR-2b-2*). Arrowheads indicate pole cells expressing pri-miRs. Scale bar: 20 µm.

Development (2019) 146, dev167056. doi:10.1242/dev.167056

miRNAs that are known to be present (Leaman et al., 2005; Aboobaker et al., 2005; Ruby et al., 2007) or be expressed (Fu et al., 2014; Luo et al., 2016) in early-stage embryos, and examined their primary transcripts by fluorescent *in situ* hybridization. We also tested miRNAs that are predicted to target germ plasm mRNAs, such as *nos*, *pgc* and *gcl*, from publicly available databases, including TargetScanFly, microRNA.org and microT-CDS (Ruby et al., 2007; Betel et al., 2008; Paraskevopoulou et al., 2013). Among 98 miRNAs (68 miRNA loci) tested, 17 miRNAs (12 loci) were transcribed in somatic nuclei during the syncytial blastoderm stage (stage 4) and another five miRNAs (five loci) initiated expression at the beginning of cellularization (stage 5) (Fig. S4 and Table S5). Although none of these zygotic miRNAs was expressed in wild-type pole cells during stages 4-5, 12 miRNAs (10 loci) were ectopically transcribed in *pgc*$^{-}$ pole cells (Fig. 4C-V and Table S3). These results indicate that Pgc represses the transcription of multiple miRNAs in pole cells. Because a large number of miRNAs are expressed zygotically in blastoderm embryos (Luo et al., 2016), we assume that many other miRNAs would be misexpressed in *pgc*$^{-}$ pole cells.

The misexpressed miRNAs can target the *nos* 3′ UTR

We next examined the ability of miRNAs that were misexpressed in *pgc*$^{-}$ pole cells to downregulate germ plasm mRNAs. Among the misexpressed miRNAs, *miR-1* and *miR-10* were predicted to target *nos* mRNA (Ruby et al., 2007; Betel et al., 2008), and neither these miRNAs nor miRNAs in the same family were reported to be expressed in *Drosophila* S2 cells (Ruby et al., 2007). To examine whether these miRNAs target *nos* mRNA, we performed luciferase reporter assays in S2 cells. The *nos* 3′ UTR has two predicted binding sites for *miR-1* and three for *miR-10* (Fig. 5A). Expression of *miR-1* or *miR-10* repressed the expression of a reporter gene fused to the *nos* 3′ UTR, but not to a control 3′ UTR (Fig. 5B). When endogenous *Argonaute-1* (*AGO1*), a crucial component of miRNA-mediated RNA silencing (Huntzinger and Izaurralde, 2011), was depleted by RNA interference (RNAi) beforehand, neither *miR-1* nor *miR-10* suppressed the expression of the *nos* 3′ UTR-containing reporter, indicating that the effect of these miRNAs was dependent on the miRNA pathway (Fig. 5B). These results indicate that the *nos* 3′ UTR can be a target of miRNAs that are misexpressed in *pgc*$^{-}$ pole cells.

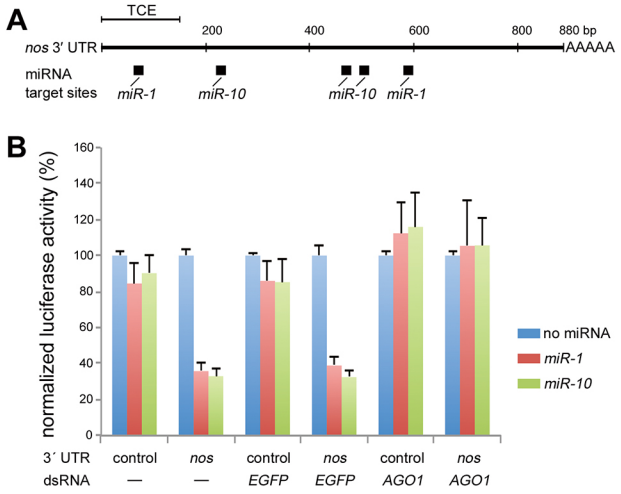

Fig. 5. Both *miR-1* and *miR-10* target the *nos* 3′UTR. (A) Schematic representation of the *nos* 3′ UTR. The *nos* translational control element (TCE), which mediates translational repression of unlocalized *nos* mRNA (Gavis et al., 1996b; Dahanukar and Wharton, 1996), is indicated on the top line. Predicted target regions for *miR-1* and *miR-10* are indicated by black boxes. (B) *Drosophila* S2 cells were co-transfected with a firefly luciferase reporter plasmid containing either the *nos* 3′ UTR or a control 3′ UTR from the pAc5.1 vector, a miRNA-expressing plasmid and a *Renilla* luciferase-containing plasmid, which served as an internal control for transfection variability. Before transfection, S2 cells were treated with the indicated dsRNAs. Expression of firefly luciferase was normalized to that of the *Renilla* luciferase. The values shown are normalized to the firefly luciferase activity produced in the absence of miRNA. Data are mean±s.d.

To test whether *miR-1* and *miR-10* are responsible for *nos* mRNA degradation in *pgc*$^{-}$ pole cells, we examined the effect of the loss of zygotic *mir-1* and *mir-10* on the *pgc* mutant phenotype. The number of pole cells in *pgc*$^{-}$ embryos was statistically unchanged when the zygotic *mir-1* and *mir-10* were simultaneously removed (Table S4). This result indicates that the loss of *mir-1* and *mir-10* is insufficient to promote pole cell survival in *pgc*$^{-}$ embryos.

The miRNA pathway activity is involved in the degradation of germ plasm mRNAs in *pgc*$^{-}$ pole cells

We hypothesized that expression of multiple miRNAs in pole cells may account for the *pgc* mutant phenotype. As many miRNA genes are misexpressed in *pgc*$^{-}$ pole cells, we were unable to directly test the effects of the genetic depletion of all the miRNAs. Instead, we examined genetic interactions between *pgc* and the miRNA pathway components encoded by *AGO1* and *Dicer-1* (*Dcr-1*) to determine whether the inhibition of miRNA pathway activity impacts on pole cell survival in *pgc*$^{-}$ embryos. As both *AGO1* and *Dcr-1* are essential for oogenesis, mutant females lacking these genes do not produce eggs (Hatfield et al., 2005; Yang et al., 2007; Azzam et al., 2012). We therefore removed one copy of *AGO1* or *Dcr-1* in *pgc*$^{-}$ mothers and analyzed the fate of the pole cells in the resulting embryos. Reducing the RNA-induced silencing complex (RISC) activity (achieved by removing one copy of maternal *AGO1*) in *pgc*$^{-}$ embryos resulted in the significant increase in the number of gonadal pole cells (2.4±2.0, *n*=100) compared with that in *pgc*$^{-}$ embryos (1.5±1.8, *n*=100) (Fig. 6A,B,E and Table S6). Similarly, *pgc*$^{-}$ embryos with reduced pre-miRNA processing activity (generated by removing one copy of *Dcr-1*) had more gonadal pole cells (2.7±2.8, *n*=100 and 2.6±2.2, *n*=100 for *pgc*$^{-}$ *Dcr-1*^{Q1147X}/+ and *pgc*$^{-}$ *Dcr-1*^{Q1948X}/+, respectively) compared with *pgc*$^{-}$ embryos (Fig. 6C-E and Table S6). Consequently, the fertility of the adult progeny was restored (Table S6).

We next examined the effects of *Dcr-1* or *gw* knockdown on the *pgc* mutant phenotype. *gw* encodes an AGO1-binding protein, GW182, that facilitates miRNA-dependent mRNA degradation by recruiting the deadenylase complex (Huntzinger and Izaurralde, 2011). When *pgc*$^{-}$ mothers were treated with either *Dcr-1* or *gw* RNAi, their embryos had more gonadal pole cells (3.1±2.4, *n*=100 and 2.6±2.3, *n*=100, respectively) (Fig. 6F-I and Table S6). The *pgc*$^{-}$ *Dcr-1*-RNAi embryos developed into adults with fertility levels higher than untreated *pgc*$^{-}$ controls (Table S6). In contrast, many of *pgc*$^{-}$ *gw*-RNAi embryos did not survive, and the pole cells in these embryos often remained outside the gonads (Fig. 6H), probably owing to defects in somatic development, as observed in *gw*$^{-}$ embryos (Schneider et al., 2006). Finally, we examined the effect of the RNAi treatments on the stability of germ plasm mRNAs in pole cells. In both *pgc*$^{-}$ *Dcr-1*-RNAi and *pgc*$^{-}$ *gw*-RNAi embryos, *nos* and *gcl* mRNAs were stabilized in a fraction of pole cells (Fig. 6J-Y). These results support the idea that the *pgc*-dependent transcriptional repression blocks miRNA action in pole cells, thereby protecting germ plasm mRNAs from degradation to ensure germ cell fate in the embryo.

Development (2019) 146, dev167056. doi:10.1242/dev.167056

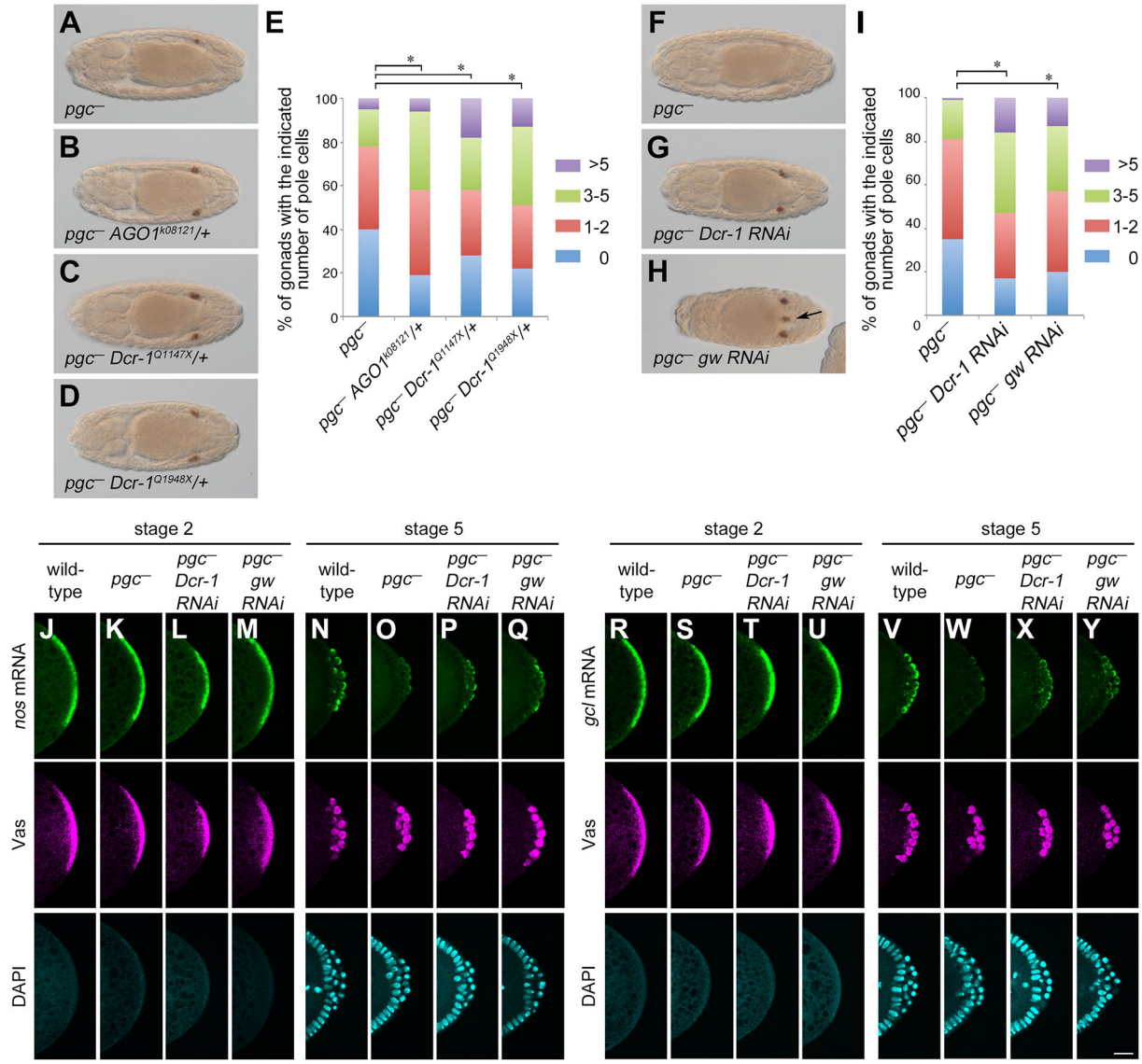

Fig. 6. Pgc protects maternal germ plasm mRNAs from miRNA-mediated degradation. (A-E) Embryos were stained using an anti-Vas antibody, and the pole cells in stage 14-15 embryos were counted. Maternal genotypes are indicated. The percentages of gonads with the indicated pole cell numbers are shown in E. One hundred gonads of each genotype were examined. (F-I) *pgc*⁻ embryos and *pgc*⁻ embryos with RNAi-mediated knockdown of *Dcr-1* (embryos from *pgc*$^{\Delta 1}$*/Df(2R)X58-7; nos-GAL4-VP16/UASp-Dcr-1 RNAi* females – *pgc*⁻ *Dcr-1*-RNAi in the figure) or *gw* (embryos from *pgc*$^{\Delta 1}$*/Df(2R)X58-7; nos-GAL4-VP16/UASp-gw RNAi* females – *pgc*⁻ *gw*-RNAi in the figure) were stained using an anti-Vas antibody. An arrow indicates the pole cells that remained outside the gonads. The percentages of gonads with the indicated pole cell numbers are shown in I. Significance in E and I was calculated using the Wilcoxon rank sum test (*P<0.001). (J-Y) Stage 2 or 5 embryos were analyzed by *in situ* hybridization to visualize *nos* and *gcl* mRNA (green), and stained using an anti-Vas antibody (magenta). Nuclei were counter-stained with DAPI (cyan). Scale bar: 20 µm.

DISCUSSION

In this study, we show that transcriptional repression mediated by Pgc is needed to prevent misexpression of multiple miRNA genes. When misexpressed, these miRNAs appear to be involved in the precocious degradation of germ plasm mRNAs in *pgc*⁻ embryos, as downregulation of the miRNA pathway components partially suppresses defects observed in *pgc*⁻ embryos. These miRNA genes normally become transcriptionally active in the somatic nuclei during the blastoderm stage. Intriguingly, most of them are the regulatory targets of Zelda, a critical transcriptional activator that promotes early zygotic gene expression from the syncytial blastoderm stage (Liang et al., 2008; Fu et al., 2014). As maternally supplied Zelda is distributed uniformly in the syncytial nuclei well before pole cells are formed (Nien et al., 2011), it could be partitioned into pole cells and promote transcription of early zygotic genes. We propose that Pgc represses the misexpression of multiple miRNAs by preventing RNAPII-dependent transcription that is potentially induced by Zelda in pole cells. As germ cell development in *Drosophila* does not require zygotic gene expression during early embryogenesis, this is probably an efficient strategy for accomplishing complete inhibition of miRNA activity in pole cells.

While Pgc is expressed in pole cells to repress mRNA transcription, the MZT takes place in the somatic region. The MZT is a universal event in animal development. During this process, most maternal mRNAs are eliminated and zygotic transcription begins. The extensive remodeling of gene expression results in oogenic (germline) to zygotic (soma) developmental

Development (2019) 146, dev167056. doi:10.1242/dev.167056

reprogramming (Giraldez, 2010; Walser and Lipshitz, 2011). In *Drosophila*, somatic MZT is largely completed by 3 h after fertilization, when the embryo is undergoing cellularization (De Renzis et al., 2007; Thomsen et al., 2010). In contrast, the MZT in pole cells is delayed relative to the soma. In pole cells, zygotic transcription starts during late gastrulation just before the cells become migratory (Van Doren et al., 1998), and the rate of maternal mRNA decay is slower than that in the soma, partly because zygotically encoding factors for mRNA degradation are not synthesized in these cells (Siddiqui et al., 2012). Our results demonstrate that Pgc plays important roles in preventing the MZT: Pgc suppresses zygotically acting mRNA decay machinery and protects maternal mRNAs from degradation. We propose that Pgc-mediated repression of miRNA expression contributes to blocking the MZT in pole cells, thereby maintaining the integrity of the germ plasm that store the information necessary for establishing the germ cell fate.

Pgc is not the only factor that regulates the timing of the MZT in pole cells, as its expression is transient during stages 4-5 (Hanyu-Nakamura et al., 2008). The chromatin-remodeling factor Osa is reported to inhibit the expression of somatic genes in pole cells after Pgc is no longer detectable (Martinho et al., 2004). Furthermore, the loss of a linker Histone H1 variant, dBigH1, show precocious activation of RNAPII in pole cells (Pérez-Montero et al., 2013). Therefore, the timing of MZT in pole cells appears to be determined by a combined action with Pgc-mediated and chromatin-based regulation.

In the somatic region, degradation of maternal mRNAs is affected by multiple mechanisms, including maternally and zygotically acting decay machineries (Bashirullah et al., 1999). Smaug (Smg) is a major component of the maternal decay pathway (Tadros et al., 2007). It is an RNA-binding protein that recruits the deadenylase complex to initiate target mRNA degradation (Semotok et al., 2005; Tadros et al., 2007). In addition to its direct role in targeting mRNA degradation, Smg contributes to the zygotic decay pathway by controlling the zygotic miRNA production (Benoit et al., 2009; Luo et al., 2016). Recently, maternal Brain tumor (Brat), an RNA-binding protein (Loedige et al., 2014; Laver et al., 2015), was reported to participate in maternal mRNA degradation as a component of both maternal and zygotic decay pathways (Laver et al., 2015). Intriguingly, maternal Smg is partitioned into pole cells and implicated in mRNA degradation in these cells (Siddiqui et al., 2012). Oskar (Osk) in the germ plasm binds Smg and prevents the Smg-dependent degradation of *nos* mRNA (Zaessinger et al., 2006). Therefore, the germ plasm contains factors that block both maternal and zygotic decay pathways. These mechanisms would be necessary to ensure sufficient amounts of germ plasm mRNAs for the establishment of the germ cell fate.

Although *mir-309*, *mir-1* and *mir-10* were misexpressed in *pgc*$^{-}$ pole cells, their elimination in *pgc*$^{-}$ pole cells had no discernable impact on pole cell survival. Yet blocking the miRNA pathway partially suppressed the *pgc*$^{-}$ pole cell defect. We interpret these results as meaning that misexpression of multiple miRNAs contributes to the degradation of germ plasm mRNAs. In addition, other non-miRNA mechanisms might operate mRNA degradation in *pgc*$^{-}$ pole cells. As Brat can promote mRNA degradation via a zygotic decay pathway (Laver et al., 2015), it is likely to regulate as yet unidentified zygotic decay pathway factors. Therefore, Pgc might repress the zygotic decay machinery that acts downstream of maternal Brat. Identifying such genes will deepen our understanding of how germ plasm mRNAs are protected from degradation.

Notably, in zebrafish embryos, germ plasm mRNAs, such as *nanos1* and *Tudor domain-containing protein 7* (*tdrd7*), are protected from miRNA-mediated degradation. In contrast to *Drosophila*, where the activity of miRNAs is repressed at the transcriptional level, in zebrafish the maternally provided RNA-binding proteins Dead-end (Dnd) and Daz-like (Dazl) physically interact with the germ plasm mRNAs to counteract the function of *miR-430*, a primary effector of maternal mRNA degradation during the MZT (Mishima et al., 2006; Kedde et al., 2007; Takeda et al., 2009). A similar mechanism was reported in *Xenopus* embryos (Koebernick et al., 2010). Therefore, the protection of germ plasm mRNAs from miRNA-mediated degradation appears to be crucial in both flies and vertebrates, although different mechanisms are used. This is a striking example of convergent evolution and is consistent with the hypothesis that the mode of germ cell specification mediated by a preformed germ plasm arose independently among diverse animal groups (Extavour and Akam, 2003).

MATERIALS AND METHODS

Fly strains and transgenic constructs

The following mutant alleles or transgenic lines were used: *pgc*$^{\Delta 1}$, *UASp-Cdk9-nos* 3′ *UTR*, *UASp-CycT-nos* 3′ *UTR* (Hanyu-Nakamura et al., 2008), *mir-309-6*$^{\Delta 1}$ (Bushati et al., 2008), *mir-1*^{KO} (Sokol and Ambros, 2005) and *mir-10*^{KO} (Chen et al., 2014). *AGO1*^{k08121} was provided by H. Siomi (Keio University, Japan). *Df(2R)X58-7*, *Df(3L)H99*, *nos-GAL4-VP16*, *otu-GAL4-VP16*, *Dcr-1*^{Q1147X}, *Dcr-1*^{Q1948X} and RNAi lines targeting *Dcr-1* (HMS00141) and *gw* (HMS00105) were obtained from the Bloomington *Drosophila* Stock Center. *UASp-nos-pgc 3′ UTR* was constructed by fusing the *nos* CDS to the full-length *pgc* 3′ UTR. P element-mediated germline transformation was performed using a standard method (Spradling and Rubin, 1982).

Immunostaining

Immunostaining was performed using standard procedures (Kabayashi et al., 1999). To detect signals for cleaved Caspase-3, phospho-Histone H3 and CTD pSer2, the vitelline membranes of fixed embryos were hand peeled. For Nos staining, Image-iT FX signal enhancer (Thermo Fisher Scientific) was used as a blocking reagent. The following primary antibodies were used: rabbit anti-Nos (1:1000; laboratory stock), rabbit anti-Vas (1:2500; laboratory stock), rat anti-Vas (1:1000; laboratory stock), rabbit anti-cleaved Caspase 3 (Asp175) lot 15 (1:100) and lot 17 (1:75) (Cell Signaling Technology, 9661), mouse anti-phospho-Histone H3 (Ser10) 6G3 (1:100; Cell Signaling Technology, 9706), and mouse anti-RNAPII H5 (1:1000, Covance, MMS-129R). Antibody detection was performed using either a biotinylated secondary antibody (1:5000, Vector Laboratories, BA-1000) followed by detection with the ABC Kit (Vector Laboratories) and DAB staining, or Alexa Fluor-conjugated secondary antibodies (1:1000; Thermo Fisher Scientific, A11034, A21429, A11037, A21434, A11007, A11029, A21044).

Fluorescent *in situ* hybridization

Whole-mount *in situ* hybridization of embryos with digoxygenin-labeled RNA probes was performed using standard procedures (Lécuyer et al., 2007). To visualize *nos*, *gcl* and *wun2* mRNA, the hybridized signals were detected with a horseradish peroxidase (HRP)-conjugated anti-digoxin antibody (1:18,000; Jackson ImmunoResearch, 200-032-156) and developed with the TSA Fluorescein System (PerkinElmer) for *nos* and *gcl* mRNA, or with the TSA PLUS Fluorescein System (PerkinElmer) for *wun2* mRNA. To visualize *Tao* mRNA, signals were detected using a biotin-conjugated anti-digoxin antibody (1:6000; Jackson ImmunoResearch, 200-062-156), followed by amplification with HRP-conjugated streptavidin (1:15,000; Thermo Fisher Scientific, S911), and development with the TSA Fluorescein System. To detect the primary miRNA transcripts, RNA probes were synthesized from 1.0-1.2 kb genomic fragments. Primers used for amplifying the genomic fragments are listed in Table S5. PCR-amplified fragments were cleaved at restriction enzyme sites introduced in primers (underlined in Table S5) and sub-cloned into pBluescript II. Constructed plasmids were used as templates for *in vitro* transcription.

Development (2019) 146, dev167056. doi:10.1242/dev.167056

Mature *miR-1* signal was detected using a double-digoxygenin-labeled LNA probe (Exiqon, 33002-15). Hybridized signals were detected using a biotin-conjugated anti-digoxin antibody followed by amplification with HRP-conjugated streptavidin, and development with the TSA PLUS Fluorescein System. For *pri-miR-279*, *pri-miR-965*, *pri-miR-996*, *pri-miR-2279*, *pri-miR-4068* and *pri-miR-4974* staining, signals were enhanced with an Alexa Fluor 488-conjugated rabbit anti-fluorescein antibody (1:1000, Thermo Fisher Scientific, A11090). To visualize nuclei, 4,6-diamidino-2-phenylindol (DAPI; Sigma) was added at 1 µg/ml during the secondary antibody incubation. Images were captured using a laser confocal microscope (Leica TCS SP2 AOBS or Olympus FV1000D).

Single-molecule fluorescent *in situ* hybridization (smFISH)

Hybridization of embryos with smFISH probes was performed as described previously (Trcek et al., 2017). A set of Atto565-labeled smFISH probes for *nos* mRNA (Little et al., 2015) was provided by E. R. Gavis (Princeton University, NJ, USA). *Z*-axis serial images were captured using a Leica TCS SP8 laser confocal microscope equipped with a hybrid photodetector (HyD) in the photon-counting mode. Images of 48 optical slices over 25 µm were captured using 40× HC PL APO CORR CS lens (NA 0.85). The sum of photon counts of background regions (ROI2) were subtracted from those of germ plasm (stage 2) or pole cell (stage 5) regions (ROI1). Ten embryos were examined at each stage. Significance was calculated using the Wilcoxon rank sum test on R program.

Cell culture and luciferase assays

Drosophila S2 cells were provided by H. Siomi (Keio University, Japan) and grown at 25°C in Schneider's medium (Thermo Fisher Scientific) supplemented with 2 mM glutamine and 10% fetal calf serum. Typically, transfections were performed in triplicate in 24-well plates using siLentFect reagent (Bio-Rad). The firefly luciferase reporters were constructed by cloning firefly *luc2* with or without the full-length *nos* 3′ UTR into the pAc vector (Thermo Fisher Scientific). To generate a transfection control plasmid, *Renilla hRluc* was subcloned into the pAc vector. The miRNA expression plasmids were constructed by subcloning the genomic fragments containing *pri-miR-1* and *pri-miR-10* into the pAc vector. In both cases, the pre-miRNA hairpins resided in the center of ∼0.8 kb genomic fragments. The following primers were used for amplifying the genomic fragments: 5′-GGGGTACCGTGGGCAAATAAAACGAAAGC-3′ (*mir-1*-F), 5′-GGG-CGGCCGCTTTTTAGCATGGCTCACTTGC-3′ (*mir-1*-R), 5′-GGGGT-ACCTGACTTTCCGGACAGGAACC-3′ (*mir-10*-F) and 5′-GGGCGGC-CGCCACTAAGAATGTGTTAGTTGGC-3′ (*mir-10*-R). The transfection reactions contained 20 ng of firefly luciferase reporter, 20 ng of Rluc plasmid and 200 ng of miRNA-encoding plasmid. The cells were lysed on the second day post-transfection. Firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) with the Lumat LB9507 luminometer (Berthold Technologies). Double-stranded (ds) RNAs of *EGFP* or *AGO1* were introduced into S2 cells by soaking (Clemens et al., 2000). For dsRNA production, the coding regions of the target genes were amplified with the following primers using the corresponding cDNA as a template: 5′-GTAATACGACTCACTATA-GGGACAAGGTGAGCTTGGCCTCG-3′ (*AGO1*-F), 5′-GTAATACGA-CTCACTATAGGGCATGTGGGAATTGTCCCTCG-3′ (*AGO1*-R), 5′-GTAATACGACTCACTATAGGGATGGTGAGCAAGGGCGAGGAG-3′ (*gfp*-F), and 5′-GTAATACGACTCACTATAGGGTTACTTGTACAGCT-CGTCCATG-3′ (*gfp*-R). The PCR products were used as templates for *in vitro* transcription with MEGAscript T7 kit (Thermo Fisher Scientific). The cells were treated with dsRNA on day 0 and again on day 4. Cell transfection was performed on day 5. Each experiment was performed at least three times.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: K.H.-N., S.M.C., A.N.; Methodology: K.H.-N., A.N.; Investigation: K.H.-N., K.M., A.N.; Writing - original draft: K.H.-N., S.M.C., A.N.; Writing - review & editing: K.H.-N., S.M.C., A.N.; Supervision: A.N.; Project administration: K.H.-N., A.N.; Funding acquisition: A.N.

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Supplementary information

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