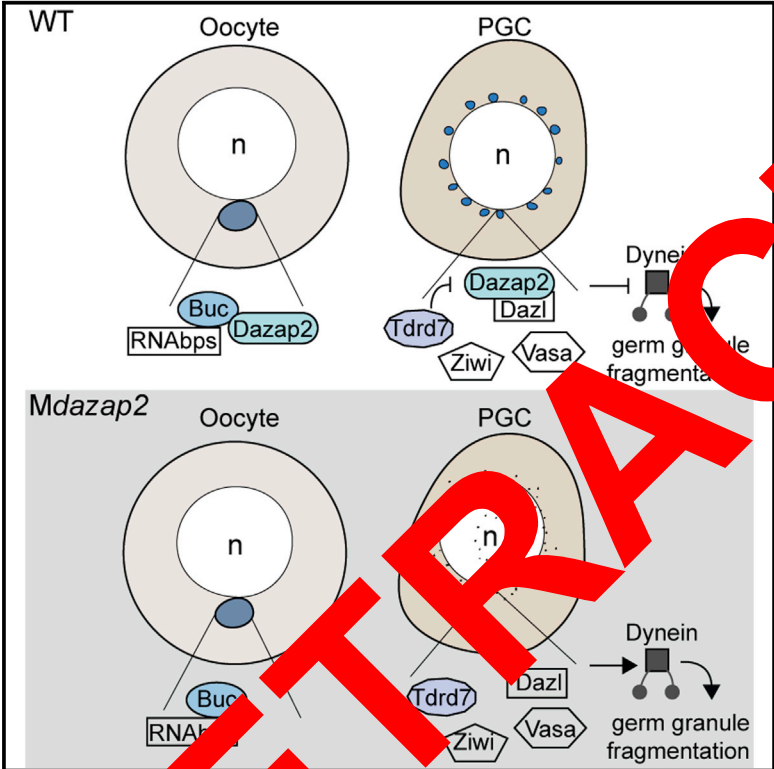# Cell Reports

Maternal *dazap2* Regulates Germ Granules by Counteracting Dynein in Zebrafish Primordial Germ Cells

Graphical Abstract

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In Brief

Forbes and colleagues identify a requirement for maternal Dazap2 in germ-granule maintenance in the germline stem cells of zebrafish. They show that Dazap2 binds the germ-plasm factor Bucky ball and localizes to germ plasm where it promotes germ-granule maintenance by acting epistatic to Tdrd7 and antagonizing Dynein.

Highlights

- The RNA-binding protein Dazap2 binds Bucky ball and localizes to the Balbiani body

- Germ-granule maintenance in zebrafish primordial germ cells requires MDazap2

- Maternal Dazap2 acts antagonistically to Dynein to maintain germ granules

- Maternal Dazap2 is epistatic to Tdrd7

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Maternal *dazap2* Regulates Germ Granules by Counteracting Dynein in Zebrafish Primordial Germ Cells

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SUMMARY

Primordial germ cells (PGCs) are the stem cells of the germline. Generally, germline induction occurs via zygotic factors or the inheritance of maternal determinants called germ plasm (GP). GP is packaged into ribonucleoprotein complexes within oocytes and later promotes the germline fate in embryos. Once PGCs are specified by either mechanism, GP components localize to perinuclear granule-like bodies. Although components of zebrafish PGC germ granules have been studied, the maternal factors regulating their assembly and distribution to germ cell development are unknown. Here, we show that the scaffold protein Dazap2 binds to Bucky ball, an essential regulator of oocyte polarity and GP assembly, and colocalizes with the GP in oocytes and in PGCs. Mutational analysis revealed a requirement for maternal Dazap2 (mDazap2) in germ-granule maintenance. Through molecular epistasis analyses, we show that mDazap2 is epistatic to Tdrd7 and maintains germ granules in the embryonic germline by counteracting Dynein activity.

INTRODUCTION

Germ cell specification occurs via two primary mechanisms (Extavour and Akam, 2003). In most mammals, the germline is determined by inductive signaling during early embryogenesis, whereas in others it is determined via inheritance of maternally derived cytoplasm known as germ plasm (GP) (Extavour and Akam, 2003). In these organisms, GP produced in oocytes is recruited to subcellular locations within oocytes and later is incorporated into the primordial germ cells (PGCs) of the embryos. Studies in model organisms such as flies and fish have shown that GP is both necessary and sufficient for germ cell specification, and, during the germline life cycle, GP adopts distinct

morphologies and undergoes cycles of assembly and dispersal (Hartung and Marlow, 2014).

In all animals examined, an evolutionarily conserved structure known as the Balbiani body (Bb) or mitochondrial cloud forms in early oocytes (Kloc et al., 2014). The Bb is a non-membrane structure that contains organelles, proteins, and RNAs and that in some vertebrates serves as a hub for GP assembly and to transport its cargo, including GP to the vegetal pole of oocytes (Kloc et al., 2014); however, the mechanisms underlying Bb function are poorly understood. The only gene known to be necessary and sufficient for Bb assembly in zebrafish or any other vertebrate thus far is *bucky ball* (*buc*) (Bontems et al., 2009; Heim et al., 2014; Marlow and Mullins, 2008). Buc protein is perinuclear at zygotene stage, prior to Bb formation (Heim et al., 2014), and, in *buc* mutant oocytes, GP components fail to localize to the Bb (Bontems et al., 2009; Marlow and Mullins, 2008). Consequently, embryos from *buc* mutant females lack animal-vegetal (AnVg) polarity and arrest during cleavage stages (Dosch et al., 2004; Marlow and Mullins, 2008), whereas excess Buc induces ectopic Bbs and disrupts polarity (Heim et al., 2014), indicating that tight regulation of *buc* is essential.

After zebrafish eggs are fertilized, GP components localize to the cleavage furrows in a cytoskeleton-dependent manner and then are incorporated into the PGCs (Nair et al., 2013). Following PGC specification, germ-cell-specific proteins, Vasa and Ziwi, localize to perinuclear granules in PGCs (Houwing et al., 2007; Knaut et al., 2000). Microinjection of RNAs encoding fluorescently tagged *nanos3*, *dead-end*, and tudor-repeat-containing7 (*tdrd7*) revealed the germ-granule localization of these proteins in PGCs, and, consistent with their PGC localization, these GP components are essential for germ cell development or survival (Knaut et al., 2000; Strasser et al., 2008; Beer and Draper, 2013; Draper et al., 2007; Hartung et al., 2014; Köprunner et al., 2001; Weidinger et al., 2003). Although the subcellular localization of most GP-RNAs in PGCs is unknown, the RNAs that encode Vasa and Nanos3 do not colocalize with Vasa protein in germ granules (Campbell et al., 2015; Gross-Thebing et al., 2014; Knaut et al., 2000). Germ granules are first detected in zebrafish at 4 hpf (Strasser et al., 2008), just after zygotic genome activation (ZGA). Germ granules are dynamic structures: their size and
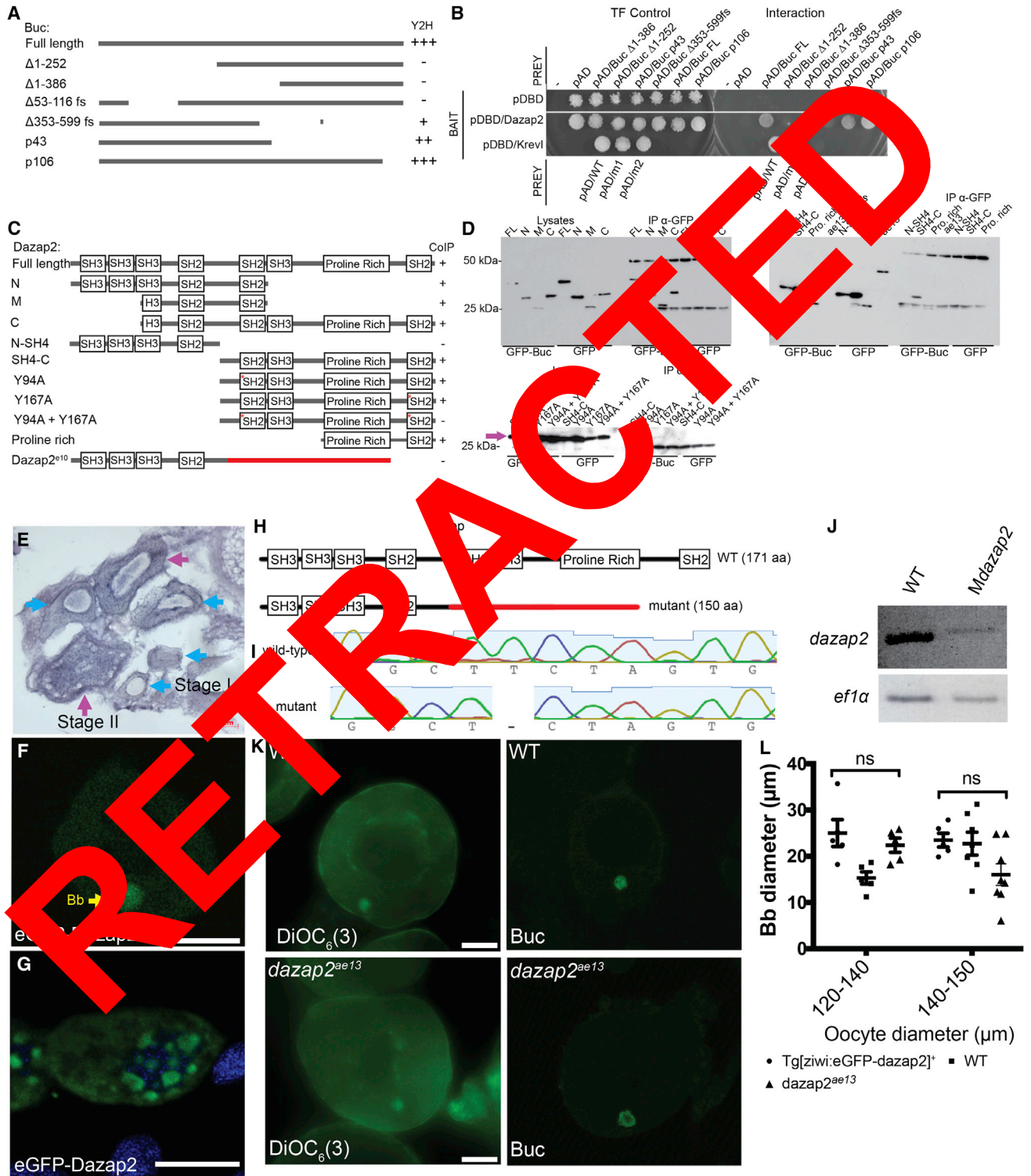
Figure 1. eGFP-Dazap2 Localizes to Germ Plasm of Early Oocytes and PGCs and Binds Buc Protein

(A) Buc deletion constructs used in the Y2H experiments in (B).

(B) Y2H assay shows that Dazap2 only interacts with Buc truncations that contain the N terminus.

(C and D) Dazap2 deletions used in the IP experiments in (D) show Dazap2 interacts with Buc via the C terminus of Dazap2. The pink arrow indicates MT-Dazap2 SH4-C truncations.

(legend continued on next page)

distribution varies during PGC migration, but germ granules become more uniform once the PGCs reach the gonad anlagen ∼30 hpf (Strasser et al., 2008). Germ-granule size and numbers depend on microtubule dynamics and involve Dynein motor protein activity (Strasser et al., 2008). Inhibition of Dynein motor function by overexpressing the p50 subunit of the Dynactin complex or morpholino knockdown of the germ-granule component, *tdrd7*, leads to a reduced population of granules that are larger than those of wild-type (WT) PGCs (Strasser et al., 2008). Because *tdrd7* knockdown in zebrafish did not perturb Dynein or microtubules two independent pathways regulating germ-granule formation were proposed, but the genes with essential roles in assembly or maintenance of germ granules in zebrafish PGCs are not known.

In this study, we identified the scaffold protein Dazap2 as a binding partner of Buc, an essential regulator of oocyte polarity and GP assembly (Bontems et al., 2009; Marlow and Mullins, 2008). Although *dazap2* transcripts are not localized in zebrafish oocytes, eGFP-Dazap2 protein colocalizes with the GP in primary oocytes. Later, in the embryo, eGFP-Dazap2 protein accumulates in the PGCs by a mechanism that depends on the Dazap2:Buc interaction domains of Dazap2. We generated *dazap2* maternal-effect mutants (M*dazap2*) and found that M*dazap2* is dispensable for AnVg polarity, and for PGC specification and migration; however, PGCs lacking M*dazap2* are devoid of perinuclear germ granules based on analysis of components. Consistent with its necessary function in germ-granule development, overexpression (OE) of eGFP-*dazap2* in WT embryos causes larger germ granules to form, and returning Dazap2 to M*dazap2* mutant eggs rescues germ granules. We show that microtubules are intact in M*dazap2* germ cells and that M*dazap2* is epistatic to *tdrd7*. Moreover, we show that inhibiting Dynein in M*dazap2* mutants restores germ granules. Together, these results uncover a role for maternal *dazap2* in germ-granule maintenance by limiting their Dynein-dependent fragmentation.

RESULTS

Dazap2 Protein Binds Bucky Ball and eGFP-Dazap2 Localizes to the Bb of Primary Oocytes and the Germ Granules

Buc encodes a vertebrate-specific protein that is required for Bb assembly, GP assembly, and AnVg axis formation by a mechanism that is not fully understood (Bontems et al., 2009; Heim et al., 2014; Marlow and Mullins, 2008). Through the yeast two-hybrid approach (Y2H), we identified an interaction between Buc and the highly conserved Proline-rich Deleted in Azoo-spermia Associated Protein, and Dazap2 and determined that Dazap2 binds to the N-terminal portion of Buc (Figures 1A and 1B). To confirm the interaction observed in the Y2H assay and to map the region of Dazap2 that binds to Buc, we co-transfected HEK293 cells with expression vectors for eGFP-Buc or eGFP as a control and Myc-Dazap2 or Myc-Dazap2 truncations as shown in Figure 1C. Our coimmunoprecipitation (coIP) experiments showed that eGFP-Buc binds to Myc-Dazap2 via two SH2 binding sites within the C terminus of Dazap2 (Figure 1D), suggesting these may be important functional regions of the protein. Moreover, mutation of the Tyrosines within the SH2.2 and SH2.3 binding domains to test whether these potentially phosphorylated residues might be necessary for Buc binding revealed that either SH2 binding site is sufficient for Buc binding (Figure 1D) and thus may also be important for Dazap2 localization to germ granules.

Dazap2 binds to the germline-specific proteins Dazl (Tsui et al., 2000) and Buc; however, the localization of maternal *dazap2* RNA and protein in zebrafish oocytes and embryos is unknown. M*dazap2* transcripts are expressed throughout the early embryo as previously reported (Thisse and Thisse, 2004) (Figure 1J). Based on its interaction with Buc we expected *dazap2* RNA or protein might localize to the Bb. To determine whether this was the case, we performed in situ hybridization on cryosectioned ovary tissue. *dazap2* transcripts were expressed in oocytes but were not asymmetrically localized (Figure 1E). To examine Dazap2 protein localization in oocytes, we generated stable transgenic zebrafish lines that express eGFP-*dazap2* controlled by the germline-specific *ziwi* promoter (Leu and Draper, 2010). In transgenic ovaries (n = 3), eGFP-Dazap2 localized to a spherical structure that resembled the Bb of WT oocytes. Further analysis showed that maternally provided eGFP-Dazap2 localized to the perinuclear granules of PGCs in the progeny of transgenic females (Figures 1F and 1G).

dazap2 Is Required for Germ-Granule Development in PGCs but Not Oocytes

To investigate the function of Dazap2 and examine its role in the Buc pathway that regulates AnVg axis development and GP assembly, we used CRISPR-Cas targeted mutagenesis (Hwang et al., 2013) to generate mutations disrupting zebrafish *dazap2*. We identified a founder fish with a 1-bp deletion in exon 3, *dazap2^ae13^*, which produced a frameshift predicted to yield a truncated protein (Figures 1H and 1I). This mutation affects amino acids within the C-terminal region, which is required for binding to Buc. *dazap2^ae13^* was transmitted through the germline, and all anticipated genotypes were recovered at the

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(E) In situ hybridization on sections of *dazap2* transcripts, which are expressed but not localized in oocytes. Blue arrowheads indicate stage I oocytes. Magenta arrowheads indicate stage II oocytes. Scale bar, 50 μm.

(F) In stage I oocytes of Tg[*ziwi*:eGFP-dazap2;*cmlc2*:mCherry]⁺, eGFP-Dazap2 fusion protein localizes to the Bb. Scale bar, 50 μm.

(G) In WT embryos at 30 hpf, eGFP-dazap2 localizes to perinuclear granules in the PGCs. Scale bar, 8 μm.

(H) Schematic of Dazap2 protein in WT and *dazap2^ae13^* mutants. The red color indicates altered amino acids due to the frameshift mutation, which is predicted to generate a truncated protein.

(I) Sequence traces of the WT and *dazap2* mutant alleles.

(J) RT-PCR of WT and M*dazap2* embryos.

(K) DiOC₆(3) and Buc staining of WT and *dazap2^ae13^* mutant oocytes. Scale bar, 50 μm.

(L) Quantification of Bb diameter in WT and *dazap2^ae13^* mutant oocytes. All p values are >0.05.

expected Mendelian frequencies with no obvious (Figure S2) phenotypes through adulthood, indicating that zygotic *dazap2* is not required for viability or fertility. Examination of *dazap2*[ae13] transcripts of 2 hpf M*Zdazap2*[ae13] embryos (before ZGA) with RT-PCR revealed that maternal *dazap2* expression was reduced in the progeny of mutant females, suggesting that mutant transcripts are less stable and that little if any mutant protein is produced (Figure 1J).

In zebrafish, GP is produced in oocytes and is maternally inherited (Hartung and Marlow, 2014). Based on its localization to the Bb of oocytes, to the germ granules of PGCs, and its interaction with Buc (Figures 1A–1D) and other GP components (Tsui et al., 2000), we reasoned that maternal Dazap2 might contribute to Bb assembly, maintenance, or disassembly. Examination of Bb components revealed no differences in $DiOC_6(3)$ and Buc protein localization between *dazap2*[ae13] and WT ovaries (Figures 1K and 1L), and we found no differences in the diameter of Buc domains of WT and *dazap2*[ae13] oocytes, indicating M*dazap2* is not required to regulate Bb assembly or size (Figure 4L). Moreover, we found that M*dazap2* was not required to localize the GP-RNA *dazl* RNA to the Bb (data not shown). Taken together, these results indicate that, although maternal Dazap2 localizes to the GP in oocytes, it is not essential for GP assembly in oocytes.

To investigate whether maternal Dazap2 was required later in the embryo, specifically in PGC development, we examined endogenous Vasa protein, a conserved marker of the germ line, in M*dazap2* mutant embryos. Based on Vasa immunostaining, PGCs were specified and migrated to the presumptive gonad at 30 hpf (n > 15 embryos) in WT and mutants. In WT PGCs endogenous Vasa protein accumulated in perinuclear aggregates >1 μm (Figures 2A and 2B). No similar Vasa aggregates >1 μm were observed in M*dazap2* PGCs (Figures 2A and 2B), indicating that, although Vasa expression in PGCs does not depend on Dazap2, its localization to perinuclear granules does.

Failed Vasa accumulation in perinuclear granules could be due to a specific requirement for Dazap2 in Vasa recruitment or may reflect a broader defect in germ-granule assembly. To distinguish between these possibilities, we examined another germ-granule component, Ziwi, in M*dazap2* PGCs at 30 hpf (Figure 2A). Like Vasa, Ziwi protein accumulated in >1-μm perinuclear granules of *dazap2*[ae13] but not M*dazap2* PGCs (Figures 2A and 2B). Taken together, these results indicate that M*dazap2* is required to maintain the germinal granule localization of Vasa and Ziwi in PGCs.

Germ-granule formation in zebrafish involves assembly and subsequent fragmentation of larger granules to generate uniformly sized granules of 2 μm at stages after the PGCs have reached the gonad anlage (Strasser et al., 2008). To distinguish between a role for Dazap2 in assembly versus maintenance of granules and to pinpoint when the germ-granule defect occurs, we examined PGC granules at critical time points during germ-granule development (Strasser et al., 2008). We found that Vasa immunostaining was comparable in the PGCs of M*dazap2* and WT embryos at 4 hpf when granules first appear (Figures 2C and 2D). As development progresses, the larger granules fragment to produce smaller granules in WT (Strasser et al., 2008). We also observed no significant change in Vasa granule

morphology in the PGCs of M*Zdazap2* embryos at 10 hpf, a time when PGCs have a larger proportion of smaller germ granules (Figures 2C and 2D)(Strasser et al., 2008). This suggests that initial germ-granule assembly is intact in M*dazap2* mutants, whereas maintenance of the germ granules after the PGCs reach the gonad anlage depends on M*dazap2*. Consistent with normal assembly at these early stages, we confirmed that the perinuclear microtubule cytoskeleton cage was intact at 10 and 30 hpf based on comparable CLIP170-GFP colocalization in WT and mutant PGCs (Figure 2E). Similarly, at 30 hpf, the distribution of Dynll2b-GFP (Strasser et al., 2008) labeled Dynein complexes within PGCs of WT and M*dazap2* embryos were comparable, indicating that loss of maternal *dazap2* does not perturb dynein localization but may affect Dynein activity (Figure 2F).

To understand how germ-granule loss impacts germ cell development and fertility, we attempted to raise progeny from *dazap2* mutant females. Analysis of Vasa protein in 14 dpf M*Zdazap2* mutants revealed the presence of PGCs at this time (n = 15/15), indicating that proper germ granules may not be essential for germ cell survival or identity up to this point (Figures 2G and 2H). Lethality of M*Zdazap2* mutants precluded assessment of germ cell viability and maintenance of PGC identity beyond 14 dpf (Figures S3A–S3C).

eGFP-Dazap2 Is Sufficient to Rescue Germ-Granule Formation in M*dazap2* Mutants

To determine whether Dazap2 was required in oocytes or in embryos, we injected 100 pg of eGFP-*dazap2* RNA into WT and M*dazap2*[ae13] mutant embryos. At 30 hpf, eGFP-Dazap2 colocalized with Vasa in cytoplasmic granules of WT and M*Zdazap2*[ae13] PGCs (Figure 3A). Furthermore, compared to GFP-injected controls, at 30 hpf Vasa and Ziwi positive granules were larger in WT embryos overexpressing eGFP-Dazap2 (Figure 3B). Based on the rescue data and the larger granules of WT embryos expressing eGFP-Dazap2, we conclude that the germ-granule defect is specific to loss of maternal *dazap2*, and that Dazap2 is required in the embryo to maintain germ granules in zebrafish PGCs.

To identify the region of Dazap2 that mediates its recruitment to perinuclear germ granules, we generated eGFP-Dazap2 truncation mutants and examined their localization within PGCs. Based on our domain mapping data (Figure 1) and the cell-culture studies of others that indicate the C-terminal two-thirds of Dazap2 is sufficient for its localization to stress granules (Kim et al., 2008), we used the Dazap2 fragments from the coIP assays to test whether the C terminus of Dazap2 was sufficient for its germ-granule localization and to rescue the M*dazap2* germ-granule maintenance defect. We injected WT and M*dazap2*[ae13] mutants with RNA encoding each truncation-eGFP fusion. All of the truncated proteins except for Dazap2 Y94A and Y167A single and double mutants and the Dazap2[ae13] mutant protein localized to germ granules (Figures 3B and 3C). Notably, most of the fragments that localized to germ granules were also sufficient for Buc binding, with the exception of the Dazap2 Y94A and Y167A single mutants, which could bind Buc protein but did not localize to germ granules (Figures 1D, 1E, and 3C). The Proline-rich and N fragments were sufficient to localize to granules, however, failed to induce larger granules (Figures 3B and 3C). Notably, the Dazap2[ae13] mutant protein,
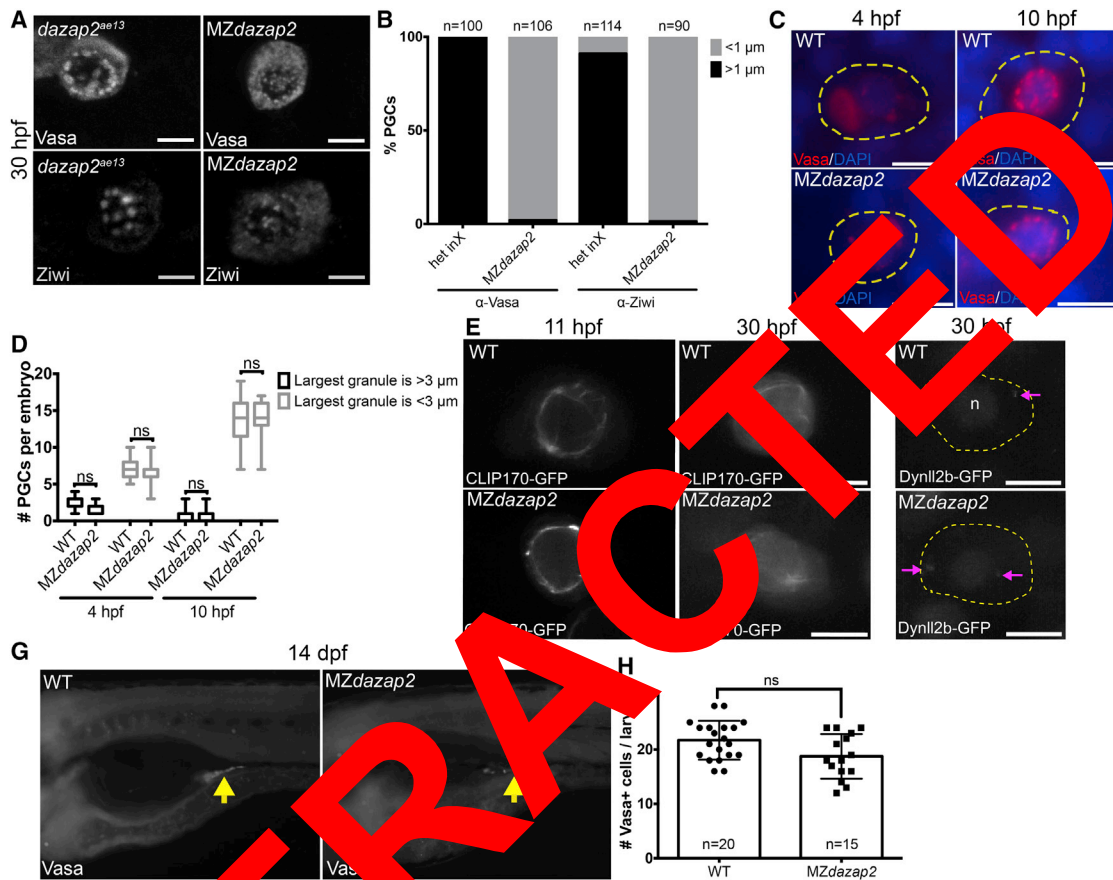
**Figure 2. Maternal *dazap2* Is Required for Maintenance of Germ Granules in PGCs**

(A) Endogenous Vasa and Ziwi protein localization in *dazap2^ae13^* and MZ*dazap2* mutant PGCs at 30 hpf. Both Vasa and Ziwi protein fail to localize to perinuclear germ granules in MZ*dazap2* mutants. Scale bar, 5 µm.

(B) Quantification of PGC germ granules of each genotype indicates %PGCs with granules greater than or less than 1 µm. Quantification of Vasa+ and Ziwi+ granules from heterozygous intercrosses (het inX) and MZ*dazap2* embryos represent n > 90 PGCs from ten embryos for each condition.

(C) Germ-granule morphology at 4 and 10 hpf using Vasa protein to label germ granules. Scale bar, 15 µm. Yellow dotted line marks PGC.

(D) Quantification of germ-granule size and its variation at 4 and 10 hpf. All p values are >0.05.

(E) Live imaging of PGCs at 11 and 30 hpf expressing CLIP170-GFP reveals microtubules of WT and MZ*dazap2* mutants. Scale bar, 8 µm.

(F) Live imaging of PGCs at 30 hpf expressing Dynll2b-GFP reveals the localization of Dynein motor protein complexes in WT and MZ*dazap2* PGCs. Dashed yellow line outlines the PGC and "n" denotes the PGC nucleus.

(G) Vasa protein [...]

(H) Quantification of Vasa positive PGCs at 14 dpf. p value = 0.0566.

which lacks the C terminus and does not interact with Buc, failed to localize to germ granules, suggesting that the mutant gene product may not be functional and/or that localization to granules is necessary for Dazap2 activity (Figure 3C). To determine whether the truncations were functional, we tested their ability to rescue germ granules in MZ*dazap2* mutants. Whereas our binding and localization studies showed that fragments containing SH2 binding sites 2 and 3 were sufficient for Buc binding and localization, rescue experiments indicated that only fragments containing both C-terminal SH2 binding sites with intact Tyrosine residues were able to rescue germ-granule formation in MZ*dazap2* PGCs (Figures 3B and 3D). Altogether, these experiments indicate that the C-terminal region of Dazap2 is necessary and sufficient for Dazap2 localization to germ granules. Furthermore, our studies identify distinct SH2 binding sites

that may be functionally important, as proteins with truncations of or point mutants within these domains fails to restore germ granules in MZ*dazap2* mutant PGCs.

dctn2 Overexpression but Not *tdrd7* Knockdown Rescues Germ-Granule Formation in MZ*dazap2* PGCs

Currently, there are only two known modulators of germ-granule dynamics in zebrafish, the microtubule motor protein Dynein and the germ-granule component Tdrd7. Dynein is thought to facilitate fragmentation and segregation of germ granules as overexpressing the Dynactin subunit Dynactin2 (Dctn2), formerly called p50 or Dynamitin, which inhibits Dynein function (Echeverri et al., 1996), reduces germ-granule numbers with a concomitant increase in granule size (Strasser et al., 2008). A similar but Dynein- and microtubule-independent phenotype is observed when
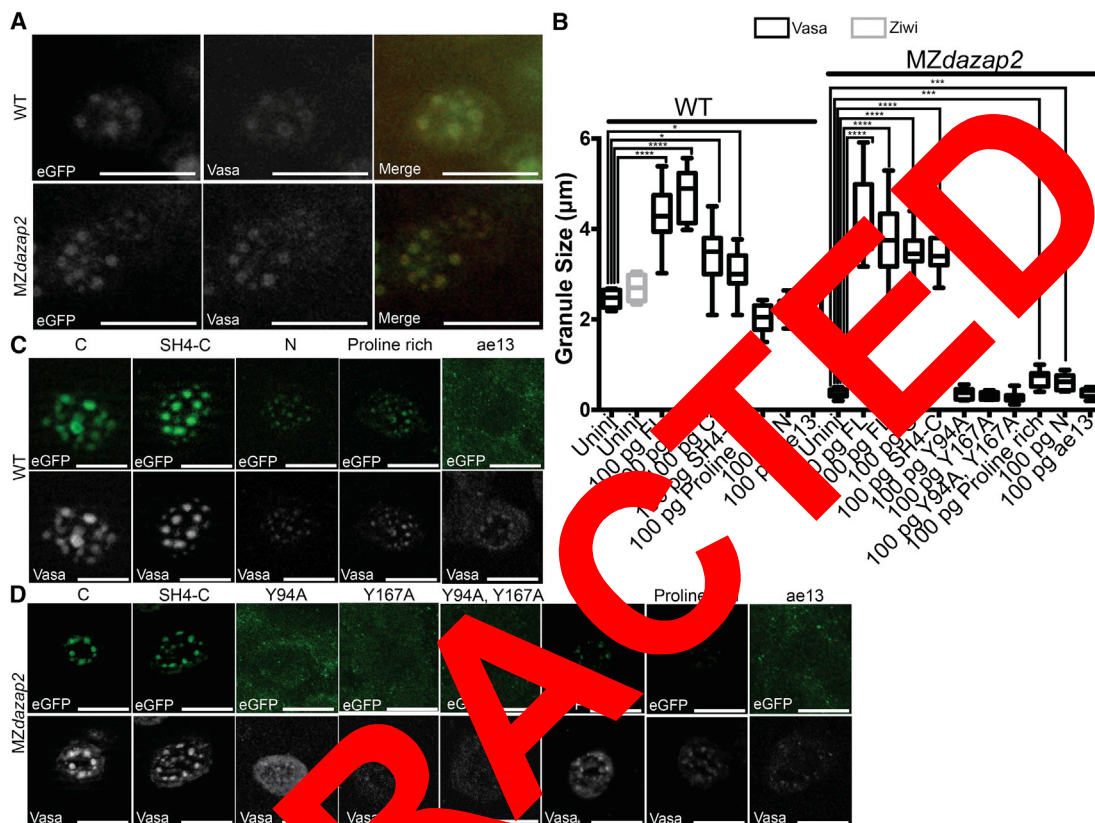
Figure 3. eGFP-Dazap2 Rescues Germ-Granule Defects in M*dazap2* Mutants
(A) 24 hpf PGCs of WT and MZ*dazap2* embryos injected with 100 pg *eGFP-dazap2* RNA. *eGFP-dazap2* colocalizes with Vasa (red) in the germ granules.
(B) Quantification of germ granules for each condition. Graph only depicts statistically significant differences. ****p < 0.0001; ***p = 0.0004; *p = 0.01.
(C) PGCs of WT embryos injected with RNA encoding the specified truncations fused to eGFP.
(D) PGCs of M*dazap2* embryos injected with RNA encoding the specified truncations fused to eGFP.

tdrd7 is knocked down (Strasser et al., 2008). Our time course suggested that loss of granules in MZ*dazap2* mutants was likely due to hyperfragmentation since germ granules were present and comparable between WT and mutants at early stages but were lost from mutants at later stages. Therefore, we tested whether excess activity of the Dynein, Tdrd7, or both pathways was responsible for failed granule maintenance. To determine whether the germ-granule defect was due to increased *tdrd7* function, we injected WT and MZ*dazap2* embryos with the *tdrd7* morpholino (Strasser et al., 2008). Consistent with the previous study, *tdrd7* knockdown in WT resulted in granules of increased size and led to decreased total numbers of granules per PGC (Figures 4A–4C). However, *tdrd7* knockdown in the *dazap2* maternal mutants did not restore germ-granule size, suggesting that *dazap2* is epistatic to *tdrd7*.

To determine whether hyperfragmentation due to excess Dynein activity caused loss of granules in M*dazap2* mutants, we injected progeny of WT and M*dazap2^ae13^* mutants with *dctn2* RNA (Strasser et al., 2008). Based on Vasa protein localization, we quantified the number of germ granules per PGC and measured the size of each Vasa-positive granule (n = 10 embryos per condition; n > 200 PGCs per condition) (Figure 4). As previously reported (Strasser et al., 2008), *dctn2* overexpression

increased the size of germ granules in WT embryos (Figures 4A–4C), but, in contrast to *tdrd7* knockdown, *dctn2* restored germ-granule size in MZ*dazap2* mutants (Figures 4A–4C). This result indicates that Dazap2 promotes germ-granule maintenance by a mechanism that involves inhibition of Dynein activity in PGCs. Alternatively, Dazap2 and Dynein could act in parallel pathways with Dazap2 limiting or counteracting Dynein mediated fragmentation of granules.

DISCUSSION

In this study, we describe a role for *dazap2* as a maternal-effect gene that is required to maintain germ granules in a vertebrate organism. Prior cell-culture studies had identified a role for *dazap2* in the assembly of stress granules which, like germ granules, contain RNAs and proteins (Anderson and Kedersha, 2008; Buchan, 2014; Voronina et al., 2011). We show that Dazap2 binds to a key regulator of GP assembly, Buc (Bontems et al., 2009; Marlow and Mullins, 2008), via its C terminus. Consistent with this protein interaction eGFP-Dazap2 protein localizes to the Bb of oocytes and later localizes to germ granules of PGCs. Surprisingly, maternal *dazap2* is required in PGCs but not in oocytes, suggesting that Dazap2 contributes to a distinct
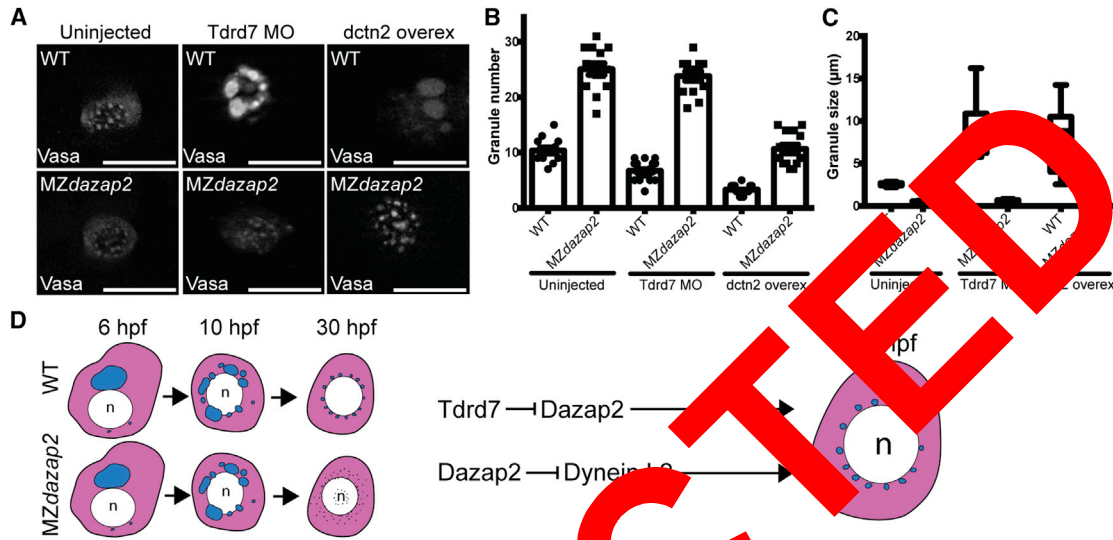
Figure 4. *dazap2* Is Epistatic to Tdrd7 and *dctn2* Overexpression Rescues Germ Granules of M*dazap2* Mutants

(A) Representative images of PGCs of WT and MZ*dazap* embryos uninjected or injected with Tdrd7 morpholino or *dctn2* RNA at 24 hpf. Scale bars, 10 μm.

(B) Quantification of germ-granule number.

(C) Quantification of germ-granule size.

(D) Schematic depicts germ-granule development in WT and MZ*dazap2* PGCs to 30 hpf (when the PGCs reside in the gonad analgen). Epistasis analyses indicate that Tdrd7 regulates granules by repressing Dazap2, whereas Dynein and Dazap2 have antagonistic roles in regulating germ-granule maintenance and size.

mechanism that promotes GP maintenance in PGCs. Moreover, our rescue data map Dazap2 interaction with Buc protein, germ-granule localization, and rescue activity to Buc binding sites within the Dazap2 C terminus. Notably, cell culture studies have implicated the same region of Dazap2 in stress-granule formation in overexpression assays (Kim et al., 2008). Our data suggest a model in which Dazap2 is recruited to the Bb, likely via its interaction with Buc protein, possibly to preserve it for its future role in maintaining germ granules within PGCs of the embryo.

Previous work suggested Tdrd7 and Dynein regulate distinct mechanisms that mediate germ-granule development (Strasser et al., 2008) (Figure 4D). However, what regulates Dynein and how Tdrd7 regulates germ-granule size were unclear. Our findings that Dazap2 is epistatic to Tdrd7 and that inhibiting Dynein function restores germ granules in *dazap2* maternal mutants suggest a potential model in which Tdrd7 could regulate germ-granule morphology by repressing Dazap2. Dazap2 may then directly or indirectly repress Dynein function to maintain germ granules possibly acting as a scaffolding protein to limit their Dynein-induced fragmentation (Figure 4D). Consistent with this model, the germ-cell-specific RNA binding protein, Deleted in azoospermia-like (Dazl), a GP component (Hashimoto et al., 2004; Kosaka et al., 2007) and Dazap2 interaction partner (Tsui et al., 2000), binds to the dynein motor complex and regulates stress granule dynamics in cell culture (Kim et al., 2012; Lee et al., 2006). However, the role of Dazl in germ-granule formation in zebrafish PGCs remains to be determined.

In this study, we were unable to assess the consequence of impaired germ-granule formation on subsequent PGC development because M*dazap2* is required for viability beyond 14 dpf. This lethality suggests that M*dazap2* may modulate the dy-

namics of RNP complexes in other cell types that were not examined in this study, such as neurons or immune cells. Consistent with this notion *dazap2* transcripts are broadly expressed in zebrafish embryos, including in the CNS. Identification of Dazap2 as an essential maternal factor for proper germ-granule formation in the germline stem cells of zebrafish embryos is significant because germ granules are conserved structures in PGCs, and the factors that are required for germ granule formation are largely unknown in vertebrates. Moreover, our finding that MDazap2 is epistatic to Tdrd7 and promotes germ-granule development by a microtubule-independent mechanism that involves inhibition of or counteracts Dynein provides mechanistic insight into maternal regulation of these conserved PGC structures.

EXPERIMENTAL PROCEDURES

Animals

Construction of *dazap2* Gateway Expression and Transgenesis Vectors

The corresponding sequences for *dazap2* are available in a publically available database and can be found at http://zfin.org/ (ZDB-GENE-030131-3036). Gateway recombination-based cloning was utilized. Full-length (FL) *dazap2* cDNA was PCR amplified from *dazap2* cDNA (Open Biosystems Clone MDR1734-7598613) with *dazap2_ATG* and *dazap2_stop* primers and Easy-A Hi-Fi Enzyme (600400, Agilent). The PCR product was gel purified (28704, QIAGEN) and then cloned into pCR8/GW/TOPO (K250020, Invitrogen). eGFP and Myc fusions were made by recombining *pCR8-dazap2 FL* and *pCS3MTdest* or *pCS3eGFPdest*, respectively (Villefranc et al., 2007).

Transgenic constructs were made by PCR amplifying *eGFP-dazap2 FL* from *pCS3eGFP-dazap2 FL*. PCR products were TOPO cloned into pCR8/GW/TOPO (K250020, Invitrogen) as described above. The *eGFP-dazap2* FL cassette was recombined downstream of the *ziwi* promoter fragment (Leu and Draper, 2010) with the multi-site destination vector pBH-R4/R2 (Heim et al., 2014).

Dazap2 truncations were generated by PCR amplification of the indicated fragments from pCR8-*dazap2*-FL. The N fragment was constructed with *dazap2_ATG* and *dazap2_294bp* primers. The M fragment was constructed with *dazap2_93bp* and *dazap2_294bp* primers. The C fragment was constructed with *dazap2_93bp* and *dazap2_STOP* primers. The N-SH4 fragment was constructed with *dazap2_ATG* and *dazap2_195bp* primers. The SH4-C fragment was constructed with *dazap2_180bp* and *dazap2_STOP* primers. The Proline-rich fragment was constructed with *dazap2_364bp* to *dazap2_STOP* primers. The ae13 mutant was cloned from cDNA prepared from homozygous mutant fish and amplified with *dazap2_ATG* and *dazap2_ae13_STOP* primers. All truncations were recombined into *pCS3MTdest* or *pCS3eGFPdest* vector.

The SH2.2 and SH2.3 point mutations were generated by performing Quik-Change Site Directed Mutagenesis (200519, Stratagene) of eGFP-Dazap2 SH4-C and MT-Dazap2 SH4-C to create Y94A and Y167A single and double mutants.

dazap2 Mutant and Transgenic Lines
Transgenic fish were generated by microinjecting 1 nl of a solution containing 50 ng/μl of plasmid DNA and 25 ng/μl of transposase RNA transcribed from *pCS2FA-transposase* (Kwan et al., 2007) into the WT AB strain. Embryos were raised to adulthood and screened for germline transmission of the transgene. Founders were outcrossed to WT males to produce the stable line Tg[ziwi:eGFP-dazap2;cmlc2:mCherry]. Localization of the fusion protein in oocytes was carried out with dissected ovaries from three founder fish.

Targeted mutagenesis and recovery of alleles were performed with CRISPR-Cas Systems, described in Hwang et al. (2013), and are detailed in the Supplemental Experimental Procedures.

Genotyping
Genomic DNA was isolated from clutches or single embryos and genotyping of the *buc^p43* mutant allele was performed as in Bontems et al. (2009). Genotyping of the *dazap2^ae13* mutant allele was performed by amplifying genomic DNA flanking the *dazap2^ae13* mutation with *dazap2_XbaI_dCAPs_F* and *dazap2_294bp* primers in Table S1 to create an XbaI site in the mutant allele.

RNA Extraction, cDNA Generation, and RT-PCR
For RT-PCR of embryos, RNA was isolated from 20 pooled embryos (prior to the MZT) from the same single cross with Trizol (15596-026, Life Technologies), followed by oligo(dT) cDNA first-strand synthesis (18080-051, Invitrogen). RT-PCR was performed (primers: *dazap2_ATG* and *dazap2_364bp_R*) and other primers described in Heim et al. (2014).

Construct In Vitro Transcription and Microinjection
The indicated plasmids were linearized and transcribed with the mMESSAGE mMACHINE SP6 Transcription Kit (AM1340, Invitrogen). For *eGFP-dazap2* plasmids and *clip170-eGFP*, 0.5 nl of a solution at 200 ng/μl RNA was injected. For eGFP-*dynll2b* and *dctn2* (Strasser et al., 2008), 1 nl of a solution at 600 ng/μl was injected. For *granulito* and *tdrd7*, 1 nl of 600 ng/μl solution was injected.

Protein Binding Studies: Yeast Two-Hybrid and Co-Immunoprecipitation Assays
PJ169 (Clontech) was used for Y2H assays. Bait and prey were prepared from ovary cDNA as described in Heim et al. (2014) were sequenced and then recombined into pDEST32 or pDEST22 vectors (Invitrogen).

HEK293 cells were transfected with 3 μg of *pCS3eGFP-buc*, *pCS3MT-dazap2-FL*, or the specified *pCS3MT-dazap2* truncations, and IP was performed with 1 μg of anti-GFP antibody (A11120, Invitrogen), and blotting and detection were as in Heim et al. (2014) and described in detail in the Supplemental Experimental Procedures.

Immunohistochemistry
Zebrafish embryos and larvae were euthanized in Tricaine and fixed overnight (ON) in 4% PFA/1 × PBS. Dissected ovaries were fixed ON in 4% PFA/PBS and then washed in 1 × PBS.

DiOC$_6$(3) staining (D-273, Life Technologies) of oocytes was performed as in Heim et al. (2014).

Antibody staining was performed as in Heim et al. (2014) and as detailed in the Supplemental Experimental Procedures. Samples were mounted in 1% LMA or VECTASHIELD Mounting Medium with DAPI (H-1200, Vector Labs) and then imaged with Zeiss AxioObserver Apotome fluorescence microscope or Zeiss LSM5 LIVE Duoscan confocal microscope.

In Situ Hybridization
Whole-mount in situ hybridization (WISH) of embryos was performed as in Thisse and Thisse (2008). In situ hybridization on cryosections were performed as in Santos et al. (2013). Images were acquired with an Olympus SZ61 dissecting microscope with a digital camera (model S97809, Olympus America) and Picture Frame 3.0 software (Optronics) or a Zeiss Axioskop2 plus with a Zeiss AxioCam MRc camera and Zeiss AxioVision Rel. 4.6 software.

Statistical Analysis
GraphPad Prism was used for statistical analysis. Error bars represent ± SD unless otherwise stated, and p values were determined by either two-tailed paired Student's t test to compare two populations or two-way ANOVA followed by Tukey multiple comparisons test.

Quantification of Germ Granules
ImageJ was used to quantify Vasa positive germ granules and to quantify germ-granule sizes by measuring the perimeter of Vasa and Ziwi granules in the center of each PGC.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.06.010.

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