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Drosophila Argonaute 1 and its miRNA biogenesis partners are required for oocyte formation and germline cell division

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ABSTRACT

Argonaute 1 (Ago1) is a member of the Argonaute/PIWI protein family involved in small RNA-mediated gene regulation. In *Drosophila*, Ago1 plays a specific role in microRNA (miRNA) biogenesis and function. Previous studies have demonstrated that Ago1 regulates the fate of germline stem cells. However, the function of Ago1 in other aspects of oogenesis is still elusive. Here we report the function of Ago1 in developing egg chambers. We find that Ago1 protein is enriched in the oocytes and is also highly expressed in the cytoplasm of follicle cells. Clonal analysis of multiple *ago1* mutant alleles shows that many mutant egg chambers contain only 8 nurse cells without an oocyte which is phenocopied in *dicer-1*, *pasha* and *drosha* mutants. Our results suggest that Ago1 and its miRNA biogenesis partners play a role in oocyte determination and germline cell division in *Drosophila*.

Introduction

Drosophila oogenesis is an excellent system for studying gene regulation during development. Each adult ovary contains about 14–20 ovarioles, each of which contains strings of developing egg chambers (Spradling, 1993). Germline cells are formed in the germarium, at the most anterior part of the ovariole, which contains 2–3 germline stem cells (GSC). Each GSC divides into two daughter cells along the anterior-posterior axis. The anterior daughter cell remains as a stem cell, contacting cap cells in the niche, while the posterior daughter cell differentiates into a cystoblast (Xie and Spradling, 1998). Oocyte determination starts in the germarium when the cystoblast divides four times to produce 16 cyst cells. One cyst cell will become the oocyte, while the other 15 cyst cells become the nurse cells (Spradling, 1993).

The *Drosophila* Argonaute (Ago) family has five members: Ago1, Ago2, Ago3, Piwi and Aubergine (Aub). The Piwi subclade of Argonaute proteins, Piwi, Ago3 and Aub, are devoted to the production and function of piRNAs, a class of small RNAs involved in defending the genome from transposable elements (Girard et al., 2006), and are largely confined to the germline and surrounding somatic tissues (Brennecke et al., 2007). The two remaining members of the Argonaute family, Ago1 and Ago2, are predominantly involved in gene regulation mediated by miRNAs and siRNAs, respectively, although there is some degree of overlap bewteen these two pathways (Czech et al., 2009; Okamura et al., 2009). Ago1, which is conserved from fruit fly to human, binds to mature microRNAs (miRNAs) to form the RNA-induced silencing complex (RISC). Ago2 forms the RISC by binding to siRNAs instead (Okamura et al., 2004).

The predicted molecular weight of the Ago1 protein is approximately 106 kDa comprising 950 amino acids (Kataoka et al., 2001). Argonaute family proteins including *Drosophila* Ago1 contain four main functional domains: The N-terminal domain, the PAZ domain, the MID domain, and the well conserved PIWI domain (Djuranovic et al., 2010; reviewed in Hutvagner and Simard, 2008). The 3′ and 5′ ends of the miRNA bind to the PAZ domain and the MID domain respectively. The PIWI box contains the catalytic centre for cleavage. Yang et al. (2007) showed that Ago1 is required for *Drosophila* germline stem cell (GSC) maintenance. Overexpression of Ago1 leads to GSC over-proliferation while mutation of *ago1* leads to GSC loss.

MicroRNAs (miRNAs) are ~22 nucleotide endogenous short RNAs that regulate gene expression in animals and plants by targeting complimentary mRNAs for degradation or translational repression (Ambros, 2004). RNA polymerase II transcribes the primary transcript (pri-miRNA) which contains one or more hairpin loop structures, a 7-methyl-guanosine cap, and a poly(A) tail (Bartel, 2009). The hairpin structure within the pri-miRNA is recognize and cut by the microprocessor complex, comprising the RNase III enzyme Drosha and its RNA binding domain-containing partner Pasha (Denli et al., 2004; Gregory et al., 2004; Han et al., 2004). This cleavage liberates a 60–70 nucleotide-long precursor miRNA (pre-miRNA), which is then exported to the cytoplasm by Exportin5, a Ran-GTP-dependent cytoplasmic cargo transporter (Bohnsack et al., 2004; Lund et al., 2004; Yi et al., 2003). In the cytoplasm, Dicer-1 (Dcr-1), together with its

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partner, Loquacious, cleaves the hairpin loop of the pre-miRNA to form a miRNA-miRNA* duplex (Forstemann et al., 2005; Saito et al., 2005). Ago1 then directs the unwinding of this duplex and one strand is selected as the mature miRNA and preferentially loaded into Ago1 to form the RISC (Forstemann et al., 2007; Tomari et al., 2007). The strand that is not loaded into Ago1, referred to as the miRNA* strand, is presumably degraded most of the time, but in some cases is loaded into the Ago2-containing RISC where it can function as an siRNA (Czech et al., 2009; Ghildiyal et al., 2010; Okamura et al., 2009). Interactions between the miRNA-RISC (miRISC) and mRNA targets involve binding between the "seed" region of the miRNA (typically nucleotides 2–8) and the complementary target sequence on the mRNA (Bartel, 2009; Lai, 2002). This short recognition sequence means that a single miRNA can target numerous mRNAs and often operate in highly complex regulatory networks in combination with other miRNAs, as most UTRs contain potential target sites for multiple miRNAs (Kim and Nam, 2006). Recent advances from genetic and genomic studies have indicated that miRNAs play important roles in many aspects of cell and animal development such as cell proliferation, differentiation, morphogenesis, and apoptosis (Carrington and Ambros, 2003; Flynt and Lai, 2008; Kim et al., 2006; Nakahara et al., 2005).

Here we investigate the role of Ago1 during oogenesis. We find that Ago1 protein is enriched in the oocyte. Clonal analysis of multiple *ago1* mutant alleles shows that Ago1 is required for proper oocyte formation and germline cell division. We also observe that the germline cell division phenotype manifested in the germarium and the oocyte is determined but failed to be maintained in meiosis stage. Furthermore, mutations in *dcr-1*, *pasha* and *drosha* result in a high incidence of 8-cell cysts, similar to the phenotype observed in *ago1* mutants. Together, our data provide evidence that Ago1 and its miRNA biogenesis pathway partners, Dcr-1, Pasha and Drosha, and by inference microRNAs, have important roles during *Drosophila* oogenesis.

Materials and methods

Fly Stocks

All stocks were raised at 25 °C on standard cornmeal media. *y w* flies were used as a controls in all our experiments, if not stated otherwise. The *ago1* hypomorphic mutant, $ago1^{k08121}$ (Kataoka et al., 2001) and null mutants, $ago1^{14}$ and $ago1^{EMS}$ (Yang et al., 2007) have been described previously. Ago1::GFP protein trap flies were obtained from the Carnegie Protein Trap Collection (Buszczak et al., 2007). The $dcr\text{-}1^{LL06357}$ (Berdnik et al., 2008), $dcr\text{-}1^{Q1147X}$ (Lee et al., 2004), $pasha^{LL03360}$ (Berdnik et al., 2008), $pasha^{KO}$ (Martin et al., 2009) and $drosha^{21K11}$ (Smibert et al., 2011) alleles have been described previously. The inducible GAL4 driver stock *hsFLP, UAS-GFPnls; tub-FRT-GAL80-FRT-GAL4* was used to generate mosaic overexpression (Zecca and Struhl, 2002). UAS-Ago1 T2.1 was used for the overexpression of Ago1 (Dietzl et al., 2007). New mutants of *pasha* and *dcr-1* described in this study were identified as part of an on-going genetic screen against a miRNA reporter that affects eye pigmentation (Smibert et al., 2011). These alleles are: $dcr\text{-}1^{21B2}$, $dcr\text{-}1^{30D2}$, $dcr\text{-}1^{37A1}$, $dcr\text{-}1^{38E3}$ and $pasha^{36B2}$. The molecular lesions associated with these alleles are described in Fig. 6.

Clonal analysis

To generate Ago1 mitotic clones, *hsFLP; FRTG13 Ubi-GFP/CyO* flies were crossed to *y w; FRTG13* $ago1^{k08121}$*/CyO, y w; FRTG13* $ago1^{14}$*/CyO* or *y w; FRTG13* $ago1^{EMS}$*/CyO* flies. Clones of *y w; FRTG13* were used as control. To generate *pasha* or *dcr-1* clones, *hsFLP; FRT82B Ubi-GFP/TM3 Ser* were crossed with *y w; FRT82B* $dicer\text{-}1^{LL06357}$*/TM3Ser, y w; FRT82B* $dcr\text{-}1^{Q1147X}$*/TM3 Ser, w; FRT82B* $dcr\text{-}1^{21B2}$*/TM6B, w; FRT82B* $dicer\text{-}1^{30D2}$*/TM6B, w; FRT82B* $dicer\text{-}1^{37A1}$*/TM6B, w; FRT82B* $dcr\text{-}1^{38E3}$*/TM6B, w; FRT82B* $pasha^{LL03360}$*/TM3 Ser or w; FRT82B* $pasha^{36B2}$*/TM6B. y w; FRT82B* was used as a control. To generate Drosha clones, *hsFLP; FRT42D Ubi-GFP/CyO* flies were crossed with *w; FRT42D* $drosha^{21K11}$*/CyO* and *y w; FRT42D* was used as a control. Eggs were collected in vials for 24 h and heat-shocked after 4 days (3rd instar larvae) in a 37 °C water bath for 1 h, once per day for 3 days. The larvae were left to pupate and newly eclosed flies were collected. The flies were then aged in fresh vials with wet yeast and transferred to new vials every day or every two days. Mutant cells are marked by the absence of GFP.

Immunochemistry and confocal microscopy

Drosophila ovaries were dissected in Grace's insect medium (Invitrogen Cat. #11605045) and fixed with 4% paraformaldehyde for 10 minutes, washed with PBT (PBS + 0.4% Triton X-100), blocked with 5% horse serum for 1 hour and incubated in primary antibody: mouse anti-Ago1 (1B8) (1:1000, gift from Haruhiko Siomi), rabbit anti-Ago1 (1:500, Abcam ab5070), goat anti-GFP (1:3000, Abcam ab6673), Rhodamine-conjugated Phalloidin (1:500, Invitrogen Cat#R415), mouse anti-Orb (6H4) (1:500, Developmental Studies Hybridoma Bank, University of Iowa), mouse anti-HTS (1:20, Developmental Studies Hybridoma Bank, University of Iowa), rabbit anti-Oskar (1:3000), rabbit anti-cyclin-E (1:500, Santa Cruz Biotech, Inc sc-33748), rabbit anti-cleaved-Caspase 3 (1:100, Cell Signaling, Cat # 9661S), rabbit anti-Vasa (1:50, Santa Cruz Biotech, Inc sc-30210) and rabbit anti-Cup (1:1000, gift from A. Nakamura, Riken Center for Developmental Biology, Kobe, Hyogo, Japan) (Nakamura et al., 2004) at room temperature overnight. Samples were washed with PBT and then incubated overnight with the DNA dye Hoechst 33342 and secondary antibodies. Secondary antibodies used in this study were anti-mouse, rabbit, goat, or guinea pig antibodies that were labelled with Alexa Flour® 488, 546 or 633 dyes (Molecular probes), or with Cy5 or Dylight 649 (Jackson ImmunoResearch Laboratories, Inc). All samples were examined and captured using a laser-scanning confocal microscope (Zeiss LSM 510 META, Oberkochen, Germany).

Results

Ago1 protein is expressed in egg chambers and enriched in oocytes

In order to characterize the expression of Ago1 in the *Drosophila* ovary, we analyzed a protein trap line, *CA06914*, in which a *P-element* was inserted in an intron of the *ago1* gene so that a fusion protein (Ago1::GFP) was produced and expressed under the endogenous promoter (Buszczak et al., 2007). In *CA06914* flies, Ago1::GFP was expressed in the cytoplasm of nurse cells, oocytes and follicle cells. Follicle cells showed much higher expression of GFP than nurse cells. In addition, GFP expression was found to be highly enriched in oocytes at all developmental stages (Fig. 1A and A′). The enriched GFP signals were more or less evenly distributed in the ooplasm at the early- and middle-stages but preferentially localized to the posterior cortex of oocytes at stage 9 and 10.

To confirm that Ago1::GFP expression in *CA06914* is indicative of Ago1 localization, we stained endogenous Ago1 protein using multiple antibodies against *Drosophila* Ago1. The expression pattern of Ago1 labelled by two Ago1 antibodies in wild-type ovaries resembled the Ago1::GFP pattern observed in *CA06914* flies (Figs. 1A, B, S1). Double-staining of Ago1 and Oskar revealed that they colocalized at the posterior cortex in stage 10 oocytes (Fig. 1C–E). The expression pattern of Ago1 protein was further confirmed by clonal analysis of *ago1* mutants. The immunoreactivity to Ago1 antibodies was markedly reduced in *ago1* mutant follicle cells and germline cells (Fig. 2A–F). Within the germline, the reduction in Ago1 is most obvious in the oocyte, where the relative enrichment of Ago1 protein compared to the

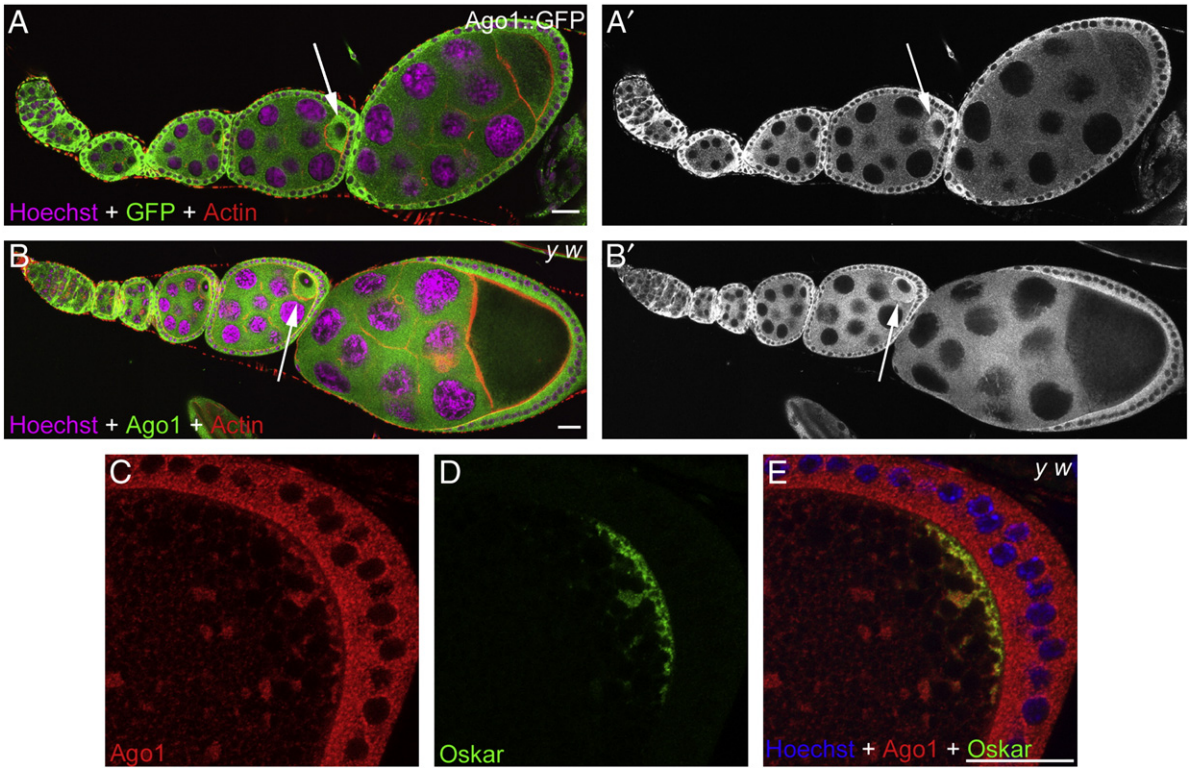

Fig. 1. Ago1 distribution in *Drosophila* egg chambers. (A & A') Ago1::GFP protein trap line and (B & B') Ago1 antibody staining in the *Drosophila* egg chambers showing the distribution of Ago1 protein. Ago1 is distributed in the cytoplasm and enriched in the developing oocyte (arrow) in stage 7 egg chambers. GFP / Ago1 are shown in green, DNA in magenta and F-actin in red. (C-E) Ago1 colocalizes with Oskar at the posterior cortex of a stage 10 oocyte. Ago1 is shown red, Oskar in green, and DNA in blue. Scale bars, 20 μm.

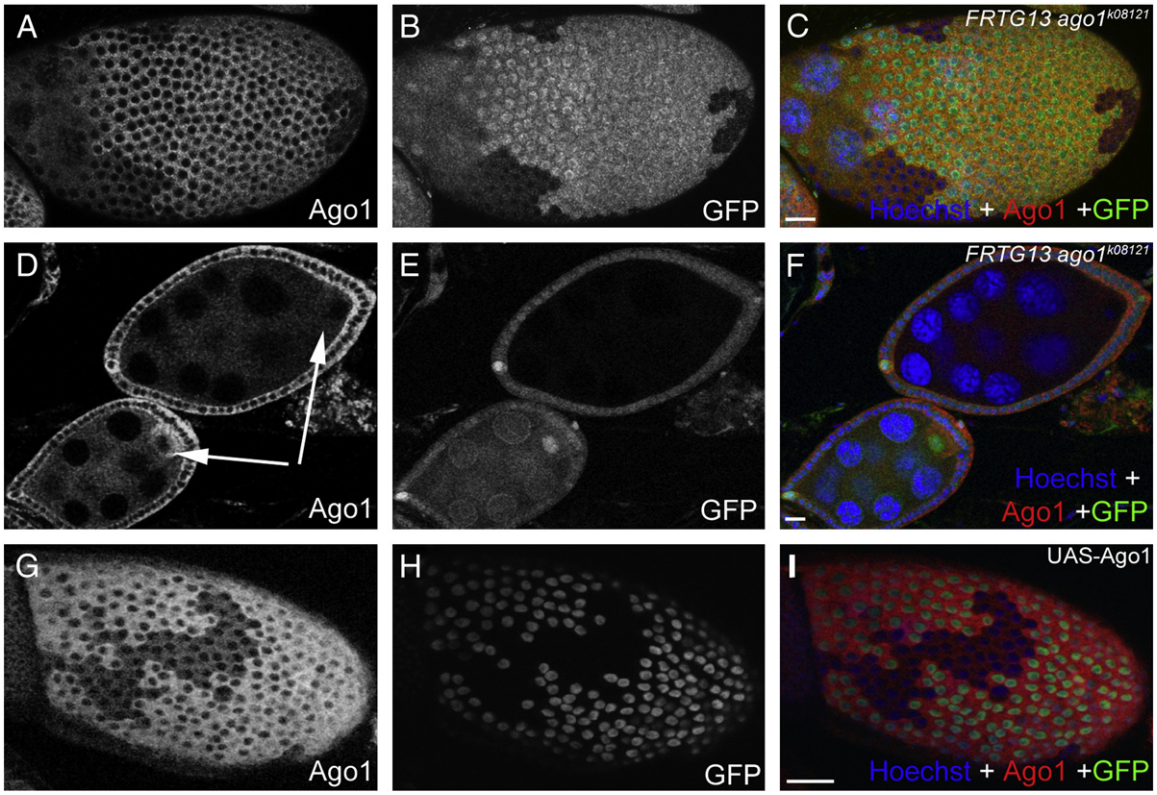

Fig. 2. Ago1 staining is reduced in *ago1* mutants and increases upon overexpression. (A-F) Mutant clones (GFP –ve) show lower expression of Ago1 compared to wild-type (GFP +ve) cells. (A-C) Ago1 expression in follicle cell clones of the $ago1^{k08121}$ mutant. (D-F) Ago1 expression in germline clones. Ago1 enrichment in the oocyte was also dramatically reduced in the $ago1^{k08121}$ mutant clones compared to a heterozygous control egg chamber (arrow). (G-I) Overexpression using an inducible GAL4 driving UAS-Ago1 shows overexpressing clones (GFP +ve) have stronger Ago1 staining compared to wild-type (GFP –ve) cells. Scale bars, 20 μm.

nurse cells (Fig. 1B) was abolished or greatly decreased (Fig. 2D). We made similar observations when analyzing clones of all three *ago1* mutant alleles; $ago1^{k08121}$, $ago1^{14}$, and $ago1^{EMS}$ (Figs. 2, S2).

Finally, we used an inducible GAL4 driver to overexpress Ago1 in "flip out" clones, where the co-expression of a UAS-GFP transgene marks the cells that are overexpressing Ago1 (Zecca and Struhl, 2002). Staining of follicle cells for Ago1 revealed that Ago1 expression in GFP positive cells was clearly higher than that in GFP negative cells (Fig. 2G–I). Together, these data demonstrate that the expression pattern detected by the Ago1 protein trap is representative of endogenous Ago1, and that the antibodies we used are specific to Ago1.

Ago1 mutations affect germline cell division and oocyte formation

To further investigate the role of Ago1 in the egg chambers, we analyzed $ago1^{k08121}$ mutant germline clones. These clones have a variable phenotype of oocyte loss (Table 1). The 1/3 of egg chambers in which the oocyte failed to form still survived and continued to grow in size (Figs. 3A and B). In wild-type egg chambers, follicle cells begin migrating to the posterior side (where the oocyte resides) at stage 9. However, in those egg chambers without an oocyte, follicle cells do not migrate but degenerate at stage 9, resulting in nurse cell-only egg chambers (Fig. 3B). Using an antibody against the oocyte marker Orb, we observed that some egg chambers failed to develop an oocyte (Fig. 3C–J and Table 1). This suggests that oocyte determination might be affected in the absence of Ago1. Despite approximately 2/3 of egg chambers producing oocytes, and in some cases embryos that were successfully laid, in no cases did the *ago1* mutant embryos survive embryogenesis (data not shown). Nurse cells in the egg chambers devoid of oocytes continued to grow.

We analyzed the effects of three *ago1* alleles during *Drosophila* oogenesis by clonal analysis and extended our investigations from 7 days to 14 days after eclosion (DAE) so that transient clones (non-stem cell derived clones), even growing slower, would in theory, not be present in the ovaries (Table 1). Fewer clones were present for all three *ago1* alleles compared to control clones. Both $ago1^{k08121}$ and $ago1^{14}$ had similar phenotypes with approximately 3 clones per ovary at 7 DAE, while $ago1^{EMS}$ had a more severe phenotype with only 0.74 clones per ovary. All alleles had fewer clones than the control groups, which had an average of 9.17 clones per ovary. At 7 DAE, $ago1^{k08121}$ and $ago1^{14}$ clones had a low rate of oocyte-less egg chambers (5% and 1.25%, respectively). The frequency of oocyte-less egg chambers increased dramatically at 14 DAE (38% in $ago1^{k08121}$ and 27% in $ago1^{14}$, Table 1). The most commonly observed developmental arrest amongst the oocyte-less egg chambers was at 8 nurse cells. In the most severe allele, $ago1^{EMS}$, 66% (33/50) of egg chambers exhibited an oocyte-less phenotype, even at 7 DAE. Among the oocyte-less egg chambers, almost half of them (16/33) contained 8 nurse cells, and about 20% have only 2 or 4 nurse cells. This indicates that Ago1 is involved in controlling germline cell division.

The Ago1 phenotype manifests in the germarium

To assess these phenotypes further, we examined the germarium of $ago1^{k08121}$ clones at 14 DAE. First we double stained the germarium against Vasa. Which labels the germ cells, and Hu-li Tai Shao (HTS), the *Drosophila* homologue of Adducin, which labels the fusomes (Fig. 4A–H). Fusomes are an important feature in *Drosophila* cyst division because its large structure which also contain α- and β-spectrin connects the cyst cells through the ring canals (de Cuevas et al., 1996; Lin et al., 1994). In wild-type germarium (Figs. 4A-D), the cystoblasts divide 4 times to produce 16-cell cysts by the time they reach region 2A. However, in *ago1* mutant germarium, we observed that only 8-cell cysts with smaller and less branched fusomes were presented in region 2 (Figs. 4E-H). Furthermore, *ago1* mutant germarium are also smaller, with a noticeably smaller region 2 as shown with an antibody against Orb (Fig. 4L–M, compare to wild-type, Figs. 4I-K). However, the *ago1* mutant clones did not show appreciably different cleaved-caspase 3 staining with no staining detected on $ago1^{k08121}$ (n = 26), $ago1^{14}$ (n = 47), $ago1^{EMS}$ (n = 38) and FRT control (n = 67) germarium clones. Therefore they do not appear to be undergoing apoptosis (Figs. S3A–I). To determine if the phenotype is due to the misregulation of cell division, we stained the clone cysts for Cyclin E. Cyclin E staining was detected in 58% (14/24) of $ago1^{k08121}$, 48% (11/23) of $ago1^{14}$ and 60% (12/20) of $ago1^{EMS}$ region 1 clone cysts in the germarium compared to 70% (19/27) in the FRT control, demonstrating that Cyclin E is not misregulated in the mutant cells (Figs. S3J-L).

Next, we examined Orb and Cup expressions in the germarium to further study the oocyte determination defect. Orb is dynamically expressed in the germarium and its expression starts in region 2A (Fig. 4J) in a relatively ubiquitous pattern (Christerson and Mckearin, 1994; Lantz et al., 1994). As it reaches region 2B, the Orb protein starts to localizes to the pro-oocytes and finally to the determined oocyte. Cup protein however, starts accumulating in region 2B of the oocyte (Piccioni et al., 2005; Zappavigna et al., 2004). Intriguingly, both Orb and Cup are expressed in the egg chambers with 8 nurse cells even though the oocyte is not properly formed (Fig. 5A–D). Orb protein accumulated in region 2A but Cup was not

Table 1
Clonal analysis of *ago1* egg chambers.

7 DAE											
						Clones with no oocyte					
	Total ovaries counted	Ovaries with clones	Clones	Clones per total ovaries	Normal clones	2 NCs	4 NCs	8 NCs	16 NCs	32 NCs	Total clones
Control	58	48	532	9.17	532	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0
$ago1^{k08121}$	47	29	142	3.02	135	0 (0%)	0 (0%)	2 (1.4%)	4 (2.8%)	1 (0.7%)	7
$ago1^{14}$	52	39	160	3.08	158	0 (0%)	0 (0%)	2 (1.3%)	0 (0%)	0 (0%)	2
$ago1^{EMS}$	68	14	50	0.74	17	1 (2%)	5 (10%)	16 (32%)	11 (22%)	0 (0%)	33
14 DAE											
						Clones with no oocyte					
	Total ovaries counted	Ovaries with clones	Clones	Clones per total ovaries	Normal clones	2 NCs	4 NCs	8 NCs	16 NCs	32 NCs	Total clones
Control	81	63	758	9.36	758	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0
$ago1^{k08121}$	84	43	172	2.05	106	2 (3%)	0 (0%)	55 (32%)	8 (4.6%)	1 (0.6%)	66
$ago1^{14}$	94	56	261	2.78	191	0 (0%)	1 (0.4%)	65 (25%)	3 (1.1%)	1 (0.4%)	70
$ago1^{EMS}$	106	31	117	1.10	69	3 (2.6%)	3 (2.6%)	35 (30%)	7 (6%)	0 (0%)	48

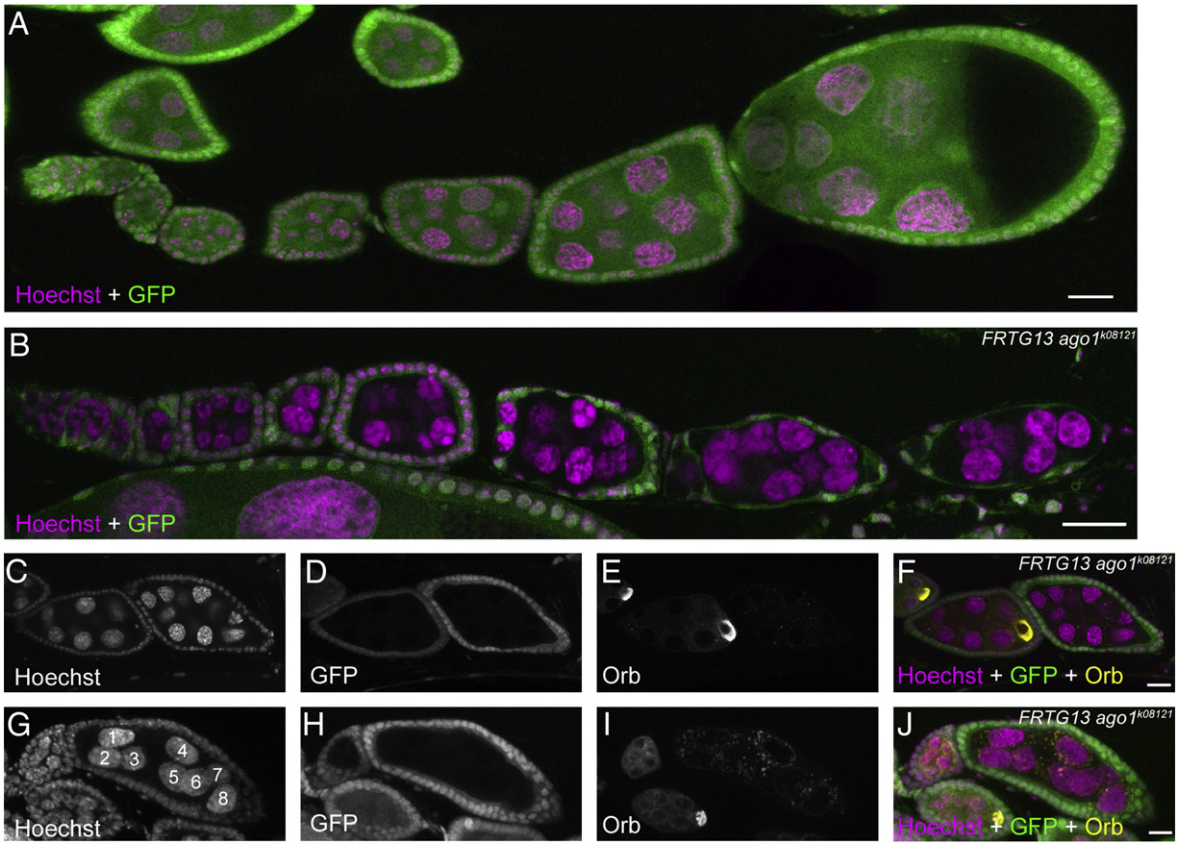

Fig. 3. Loss of Ago1 disrupts oocyte formation and germline cell division. (A) A heterozygous GFP + ve ovariole. (B) Ovariole comprised of germline cell clones of $ago1^{k08121}$. Late stage egg chambers have progressively less follicle cell nuclei surrounding them. (C–F) Two neighbouring *ago1* mutant egg chambers, one with an oocyte and one without (orb staining marks the oocyte). (G–J) $ago1^{k08121}$ clone with only 8 nurse cells and no oocyte formation. Scale bars, 20 μm.

detected until stage 1 egg chamber (Fig. 5A–D). Furthermore, analysis of stage 1 egg chamber mutant clones showing the 8 nurse cell phenotype indicated that although the egg chamber failed to form a proper oocyte, it produced a pseudo-oocyte without a condensed karyosome. However, the accumulation of Orb protein in the pseudo-oocyte seemed to be unaffected (Fig. 5E–H).

dcr-1, pasha and drosha exhibit similar effects on oogenesis as ago1

Since Ago1 is a critical effector of miRNA function, we set out to investigate if mutation of genes that are involved in miRNA biogenesis have similar developmental defects as Ago1. We obtained previously characterized alleles of genes involved in miRNA biogenesis including $dcr\text{-}1^{Q1147X}$, $dcr\text{-}1^{LL06357}$, $pasha^{LL03360}$ and $drosha^{21K11}$ stocks (Berdnik et al., 2008; Lee et al., 2004; Smibert et al., 2011). Additionally, to rule out the effects of genetic background in our assay, we also analyzed newly generated alleles of *dicer-1* and *pasha* (Fig. 6), which we isolated from an on-going genetic screen (Smibert et al., 2011). These mutants, $dcr\text{-}1^{21B2}$, $dcr\text{-}1^{30D2}$, $dcr\text{-}1^{37A1}$, $dcr\text{-}1^{38E3}$ and $pasha^{36B2}$ all contain premature stop codons and are expected to be null for protein function (Fig. 6). All *dcr-1* alleles examined exhibit the same phenotype of egg chambers with 8 nurse cells and lacking an oocyte with 30-53% penetrance, phenocopying $ago1^{EMS}$ mutant at 7 DAE (Fig. 7 and Table 2). One *pasha* mutant allele, $pasha^{LL03360}$, behaved like wild-type in this assay (n = 128, Fig. 7H). However, the newly generated *pasha* mutant, $pasha^{36B2}$ phenocopied $ago1^{EMS}$ and all *dcr-1* mutant alleles with oocyte absence at 50% penetrance (Fig. 7I and Table 2). The $drosha^{21K11}$ mutant also shows similar penetrance of oocyte loss of 48% (Fig. 7J and Table 2). Together, these results suggest a role for the miRNA pathway in oogenesis.

Discussion

Ago1 in oogenesis

Drosophila Ago1 forms a complex with mature miRNAs and acts to repress mRNAs. However, the spatial distribution of Ago1 during development has not been well characterized. The protein trap lines from the Carnegie Protein Trap library provide a powerful way to characterize the spatial and temporal distribution of trapped genes (Buszczak et al., 2007). The distribution of Ago1 in the cytoplasm has been described and shown to be localized in small puncta in the egg chamber (Reich et al., 2009). Our findings using two independent assays for Ago1 localization have shown that Ago1 is enriched in the oocyte and mutant analysis has revealed a role in oocyte formation and germline cell division.

Nurse cells supply nutrition for oocyte growth. The germline cell division defect described here has been previously observed in a *cyclin-E* mutant where 30% of the egg chambers have 8 cells, but the egg chamber still manages to develop an oocyte (Lilly and Spradling, 1996). Mata et al. (2000) have also described 8 cell egg chambers when String is over expressed as well as in a *tribbles* mutant. Both String overexpression and the *tribbles* mutant have 8 cells per egg chamber, but only a proportion fail to develop an oocyte. This defect occurs in the germarium while the cyst cells are undergoing mitosis. In the wild-type situation, the cystoblast divides four times to produce 16 cyst-cells. In the absence of *ago1*, some of the cystoblasts undergo only three divisions, producing 8-cell cysts. However, the *ago1* mutant ovarioles with this phenotype still express Cyclin E, suggesting that mitosis is still occurring although perhaps at a slower rate. Combined with the oocyte formation defect, the resulting

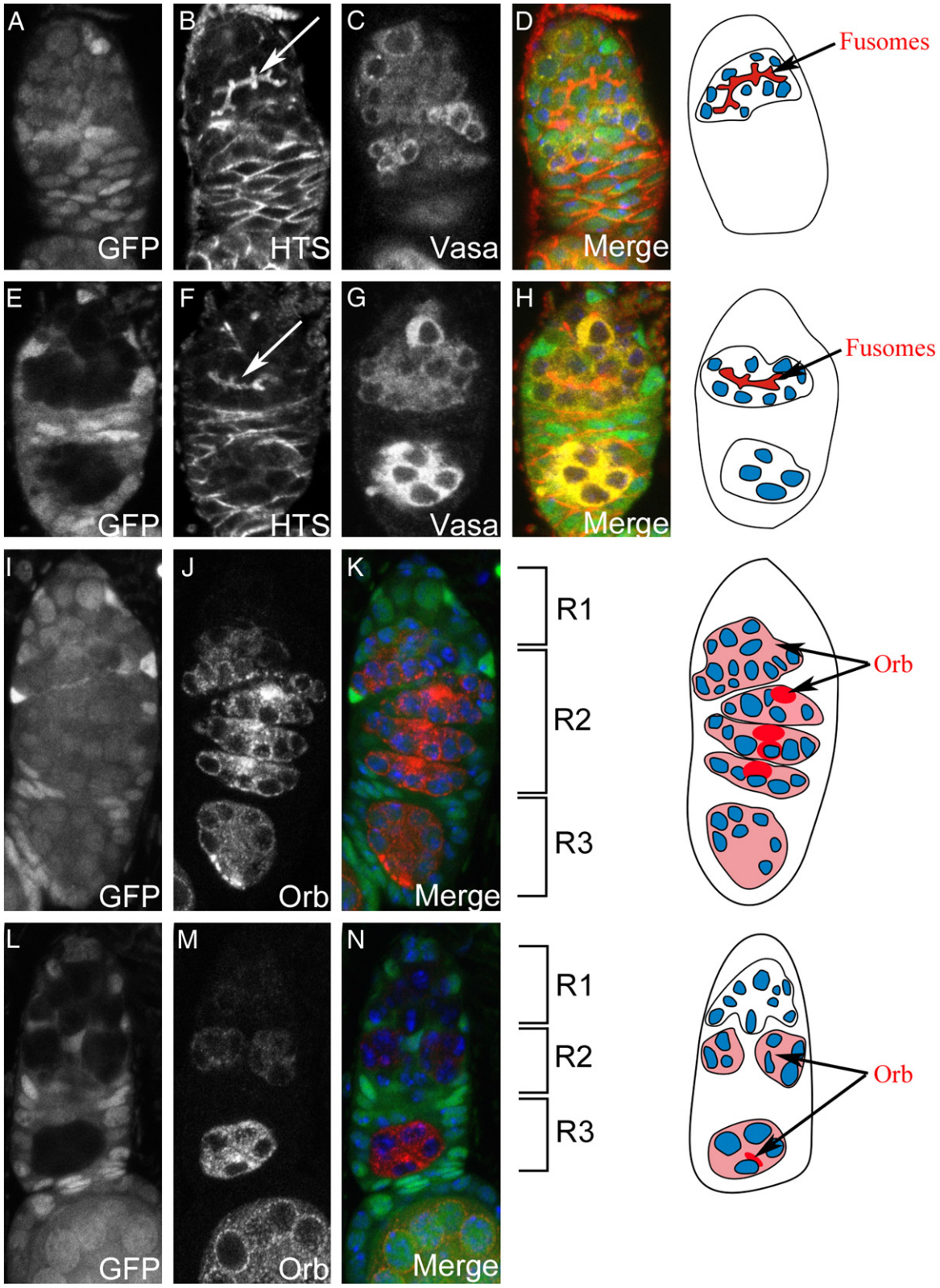

Fig. 4. *ago1* phenotype manifests in the germarium. (A–H) Germarium with GFP + ve heterozygous clones (A–D) showing normal branching of the fusomes (arrow) compared to GFP–ve *ago1* mutant clones (E–H) showing less brancing of the fusomes (arrow). In merge pictures, GFP — green, HTS — red and Vasa — yellow. (I–N) Germarium with GFP + ve wild type clones (I–K) showing normal size germarium compared to the smaller germarium of GFP–ve *ago1* mutant clones (L–N). In merge pictures, GFP — green and Orb — red. R1, R2 and R3 denotes to region 1, region 2 and region 3 in the germarium.

egg chambers only have 8 nurse cells and lack an oocyte. The cyst cell division in the germarium is not well understood. One potential explanation for the observed phenotype is that when Ago1, and presumably miRNA mediated gene regulation, are lost, the signal to stop dividing occurs early. Another possibility is because the egg chamber grows more slowly, the oocyte reaches region 2A before it

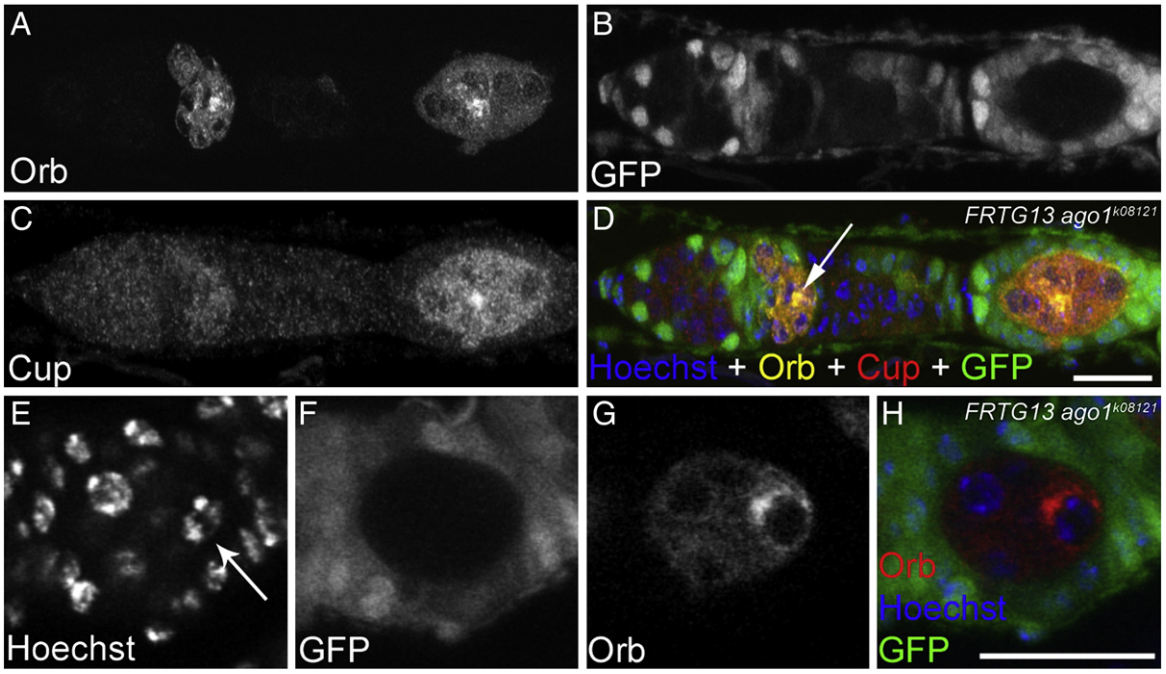

Fig. 5. Orb and Cup staining in the pseudo-oocyte. (A–D) Germarium with GFP–ve *ago1* mutant clones stained with anti-Orb and anti-Cup. Orb protein is present in region 2B (arrow) while Cup can only be seen in stage 1 egg chamber. (E–H) Stage 1 egg chamber with 8 nurse cells stained with anti-Orb showing the Orb protein accumulates at the pseudo-oocyte. Scale bars, 20 μm.

manages to divide 4 times, thus receiving a premature signal to stop dividing, or being prematurely enclosed by the migrating follicle cells. The smaller germarium of *ago1* mutant might also be an effect of cyst-cells dividing slower. The defective egg chamber however still manages to grow. Furthermore, the observation of Orb protein in region 2 of the germarium and in the stage 1 egg chamber could mean that the oocyte is trying to enter meiosis (Senger et al., 2011), or has entered meiosis but is unable to maintain the meiotic state because the Orb accumulation is lost in later stage egg chambers and no oocyte is formed. Oocyte differentiation and maintainance in the meiotic cycle are reliant on microtubule based transport of mRNAs and proteins from the nurse cells to the oocyte (Huynh and St Johnston, 2004). Orb, the germline specific RNA-binding protein starts accumulating in the oocyte at region 2a in a microtubule-dependent manner. *orb* mutant causes the egg chamber to produce 8 nurse cells and no oocyte (Huynh and St Johnston, 2000), similar to the *ago1, dcr-1, drosha* and *pasha* mutant phenotype seen in this study. However, since Orb is still expressed, we could rule out that the phenotype is cause by loss of *orb* function. The inability to maintain the accumulation of Orb in the oocyte in later stages of oogenesis could relate to defect on maintaining the meiotic cycle (Cox et al., 2001; Hong et al., 2003; Huynh and St Johnston, 2004).

Ago1 and senescence

Our results have shown that a greater proportion of older *ago1* flies exhibit the 8-nurse cell phenotype than younger mutant flies. This could be due to the level of Ago1 in older flies decreasing to a certain threshold level to show an obvious phenotype. There is also the possibility that the remaining or leaky (due to hypomorphic allele) Ago1 is diluted through GSC division and maintainance such that GSCs from flies at 14 DAE have less Ago1 than GSCs from flies at 7 DAE. Previous studies suggest that GSC loss in *ago1* mutants are age-dependent (Yang et al., 2007). This could potentially explain the age-dependent 8-nurse cell phenotype that we observed in *ago1* mutants. Self-renewed GSC in the absence of Ago1 could be defective, so cystoblasts produced by defective GSC might not be able to divide normally. Although $ago1^{k08121}$ and $ago1^{14}$ showed a more severe phenotype in older flies, $ago1^{EMS}$, as the strongest allele, showed very severe phenotype even in young flies. This is consistent with a previous study (Yang et al., 2007).

The role of miRNAs in oogenesis

Ago1, Dcr-1, Loquacious and PIWI have roles in small RNA biogenesis and all of them have been shown to be important for germline stem cell maintenance (Forstemann et al., 2005; Hatfield et al., 2005; Jin and Xie, 2007; Megosh et al., 2006; Yang et al., 2007). The role of miRNAs regulating GSC division was first reported by Hatfield et al. (2005) by studying null mutants of *dcr-1*. A similar study looking at *ago1* mutants revealed that Ago1 also regulates the fate of the GSC (Yang et al., 2007). Both of these studies showed a similar phenotypic defect in the germline. Furthermore, there are some cases where mutations in individual miRNA genes show phenotypes in the germline cells. The miRNA bantam has been previously found to be important for GSC maintenance (Shcherbata et al., 2007). Both miR-278 and miR-7 have been shown to regulate GSC division (Yu et al., 2009). Also, miR-184 controls GSC differentiation, dorsoventral patterning of the egg shell and anteroposterior patterning (Iovino et al., 2009). Although the effect in the GSC is quite reproducible from previous studies, it is not uncommon to see this in that knockouts of miRNA biogenesis factors. Bejarano et al. (2010) showed this quite well in the developing wing primordium where clones lacking *mir-9a* upregulate dLMO and induce wing notching. This phenotype is however not fully reproducible in *dcr-1* and *pasha* mutant clones. The effect of removing all miRNA could cancel the effect of a single miRNA mutation.

Our study shows that the *dcr-1*, *pasha* and *drosha* mutants phenocopy the *ago1* mutant during oogenesis. However, one Pasha mutant allele, $pasha^{LL03360}$, did not phenocopy *ago1* and *dcr-1*. This mutant is a piggyBac insertion into the 5'UTR of *pasha* and despite showing a convincing loss of pasha protein in adult neurons

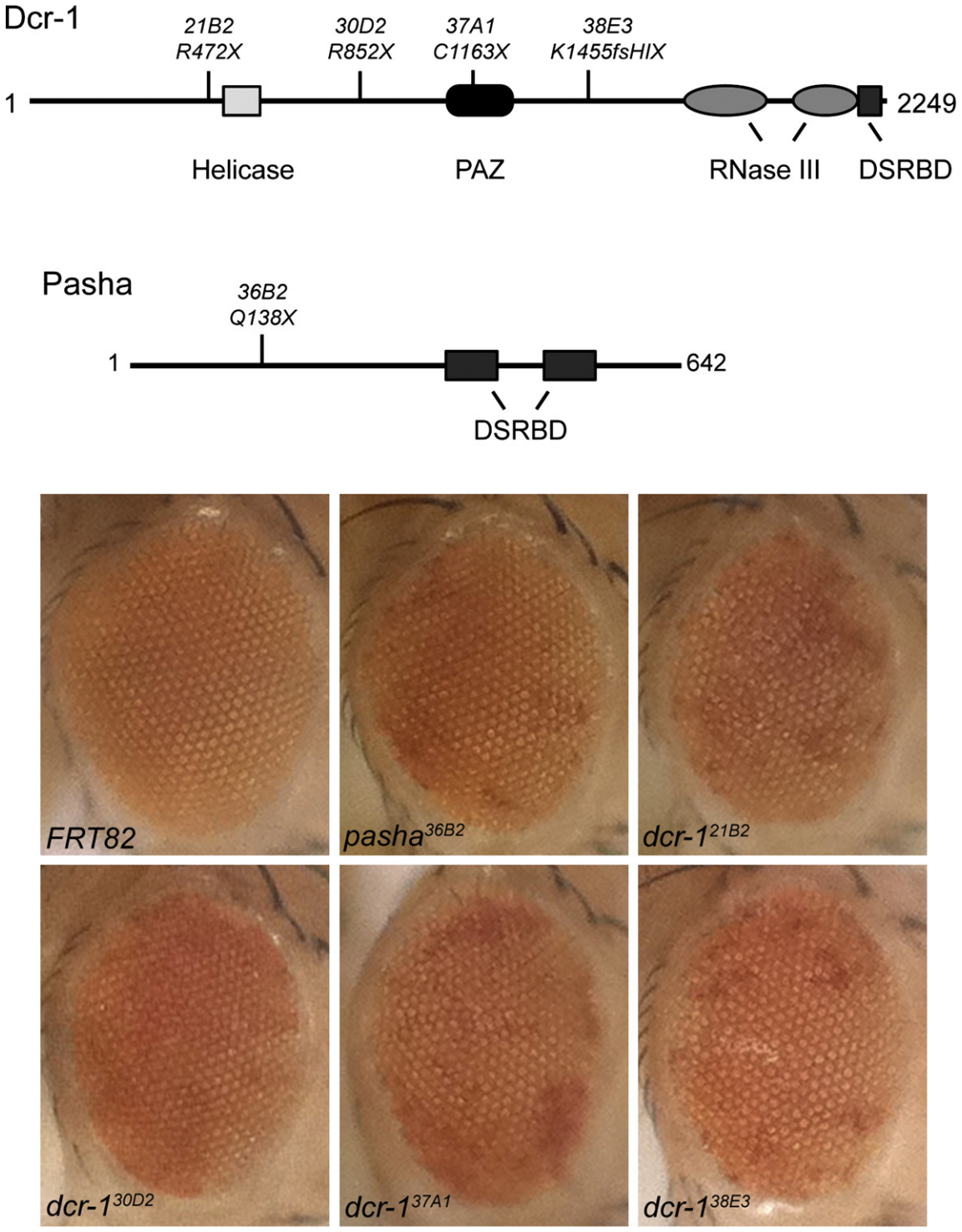

Fig. 6. Identification and characterization of new *dcr-1* and *pasha* alleles. (A) Schematic representations of of Dcr-1 and Pasha proteins showing important domains and locations of the mutations. (B–G) Adult eyes with eyFLP-generated clones of the indicated mutation in the background of GMR-w-miR. Loss of miRNA activity results in more *white* gene product and patches of darker pigmentation.

(Berdnik et al., 2008) it is possible that the allele may only be hypomorphic in the ovary. Pasha has not been studied in the *Drosophila* germline but it has been shown to play a role in olfactory neuron morphogenesis in the *Drosophila* adult brain (Berdnik et al., 2008). In that study, they found that Pasha and Dcr-1 are required for arborization of projection neurons but not Ago1. This argues for Ago1-independent roles of Dcr-1 and Pasha. Alternatively, the ago*1* mutant used in that study and this study, $ago1^{k08121}$ may not be completely null or the protein from the parental cell could be compensating for the loss of Ago1. Recent studies have suggested that neural processes are exquisitely sensitive to miRNA pathway activity (Smibert et al., 2011; Stark et al., 2008) so perhaps a more complete loss of Pasha function is required to produce phenotypic consequences in the ovary compared to neurons. Indeed, the

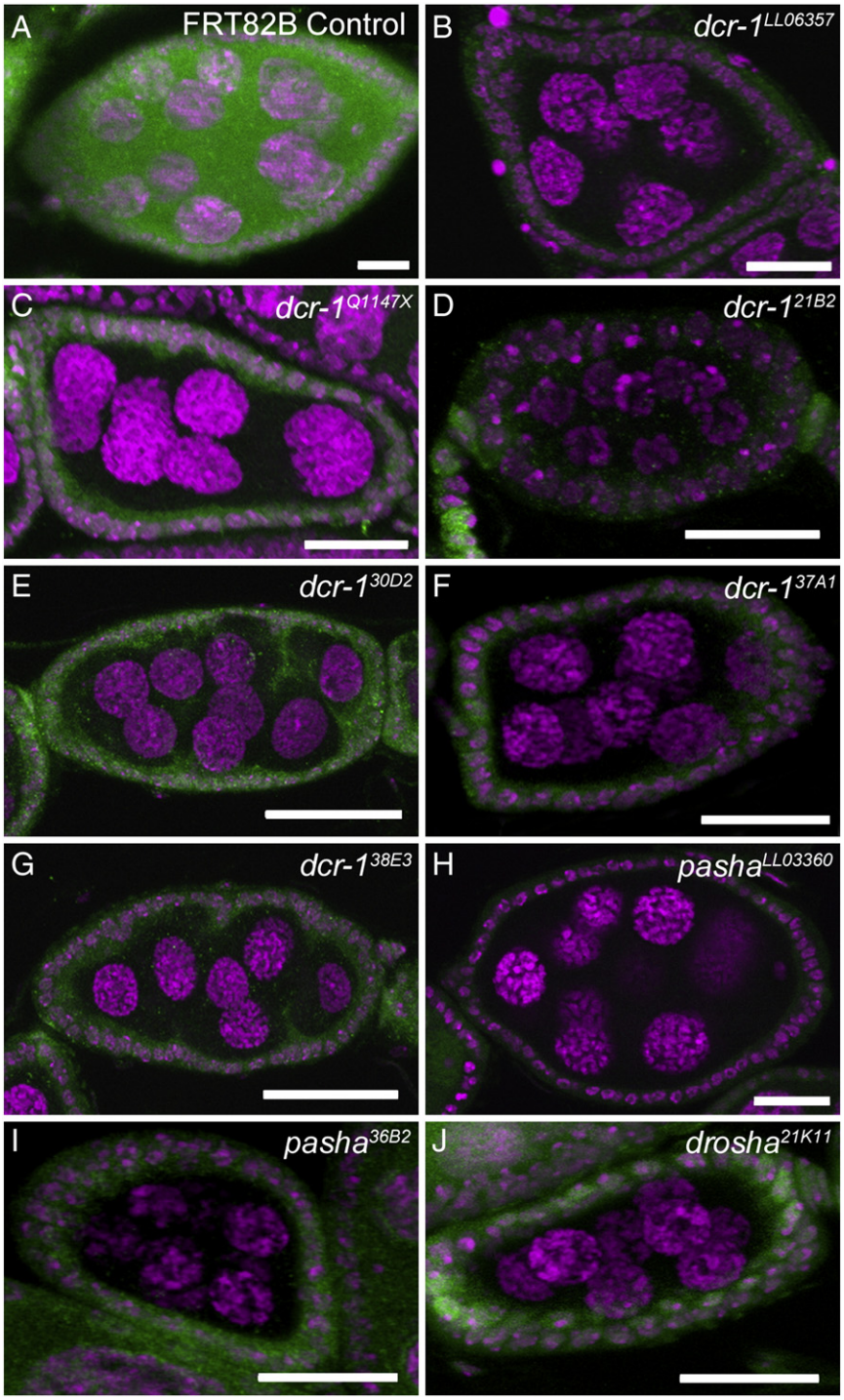

Fig. 7. *dcr-1*, *pasha* and *drosha* null mutants exhibit the same phenotype as Ago-1 mutants. (A) Positive control (GFP + ve) showing a normal egg chamber. (B–G) Selected examples demonstrating the ~30–60% of germline clones for the indicated genotypes that exhibit the oocyte-less 8 nurse cell egg chamber phenotype. Six independent *dcr-1* mutant alleles showing selected egg chamber exhibiting the same germline cell division and oocyte formation defect as in an *ago1* mutant. (H–I) Germline clones of two independent *pasha* alleles. $pasha^{LL03360}$ does not show the same germline cell division or oocyte formation defects as in *ago1*, while $pasha^{36B2}$ phenocopies the *ago1* and *dcr-1* mutant phenotype. (J) *drosha* mutant showing the 8 nurse cells phenotype. DNA is shown in margenta. Scale bars, 20 μm.

relative phenotypic strength of $ago1^{k08121}$ versus $ago1^{EMS1}$ and the null mutants of miRNA biogenesis enzymes argues for the hypomorphic nature of $ago1^{k08121}$. Mirtrons are another class of small RNAs which bypass Pasha/Drosha processing by utilizing the splicing machinery, but are still processed by Dcr-1 and loaded into Ago1 (Okamura et al., 2008; Ruby et al., 2007). However, $drosha^{21K11}$ and the newly generated $pasha^{36B2}$ mutant show the same phenotype, qualitatively and quantitatively, as *ago1* and *dcr-1* mutants. This argues that the majority of the phenotype we observed is due to loss of canonical miRNAs and that miRtrons have a comparably insignificant role (if any) in the phenotypes analysed. Altogether, our study reaffirms that loss of miRNA function at various stages of biogenesis or effector function has important phenotypic consequences for oogenesis.

Table 2
Clonal analysis of *dicer-1*, *pasha* and *drosha* egg chambers.

7 DAE								
			Clones with no oocyte					
	Clones	Normal clones	2 NCs	4 NCs	8 NCs	16 NCs	32 NCs	Total clones
Control FRT82B	187	187	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0
dcr-1^{LL06357}	104	70	0 (0%)	0 (0%)	31 (30%)	3 (0.03%)	0 (0%)	34
dcr-1^{Q1147X}	70	41	0 (0%)	0 (0%)	26 (37%)	3 (0.04%)	0 (0%)	29
dcr-1^{21B2}	42	19	0 (0%)	0 (0%)	19 (45%)	4 (0.1%)	0 (0%)	23
dcr-1^{30D2}	55	31	0 (0%)	0 (0%)	23 (42%)	1 (0.02%)	0 (0%)	24
dcr-1^{37A1}	43	18	0 (0%)	0 (0%)	25 (58%)	0 (0%)	0 (0%)	25
dcr-1^{38E3}	56	20	0 (0%)	0 (0%)	35 (63%)	1 (0.02%)	0 (0%)	36
pasha^{LL03360}	128	128	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0
pasha^{36B2}	121	54	0 (0%)	0 (0%)	59 (49%)	8 (0.07%)	0 (0%)	67
drosha^{21K11}	159	72	1 (0.01%)	3 (0.02%)	77 (48%)	6 (0.04%)	0 (0%)	87

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2012.03.005.

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