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A *Drosophila pasha* Mutant Distinguishes the Canonical MicroRNA and Mirtron Pathways▽‡

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Canonical primary microRNA (miRNA) transcripts and mirtrons are proposed to transit distinct nuclear pathways en route to generating mature ~22 nucleotide regulatory RNAs. We generated a null allele of *Drosophila pasha*, which encodes a double-stranded RNA-binding protein partner of the RNase III enzyme Drosha. Analysis of this mutant yielded stringent evidence that Pasha is essential for the biogenesis of canonical miRNAs but is dispensable for the processing and function of mirtron-derived regulatory RNAs. The *pasha* mutant also provided a unique tool to study the developmental requirements for *Drosophila* miRNAs. While *pasha* adult somatic clones are similar in many respects to those of *dicer-1* clones, *pasha* mutant larvae revealed an unexpected requirement for the miRNA pathway in imaginal disc growth. These data suggest limitations to somatic clonal analysis of miRNA pathway components.

MicroRNAs (miRNAs) are endogenous, ~22 nucleotide (nt), regulatory RNAs that associate with Argonaute proteins to repress target transcripts posttranscriptionally (8, 9). miRNAs constitute one of the largest gene families in animal genomes, with over 600 members in humans. Although they can regulate perfectly complementary targets, the vast majority of animal miRNA targets are defined by as little as 7 nt of complementarity to positions 2 to 8 of the miRNA, also known as the miRNA seed (41). Evolutionary conservation of seed matches suggests that 20 to 30% of *Drosophila* and mammalian transcripts actively maintain functional target sites for one or more miRNAs, and presumably many other transcripts contain functional sites that are either not conserved and/or have seed mismatches (41).

In *Drosophila*, as in other animals, miRNA biogenesis proceeds in a stepwise, cell-compartmentalized manner (Fig. 1). Canonical miRNAs are initially transcribed, mostly by RNA polymerase II, as long primary transcripts (pri-miRNAs) bearing one or more miRNA hairpins (32). Most of these hairpins are located in the exons or introns of noncoding RNAs, but approximately one-third are located in the introns of protein-coding genes. Pri-miRNA hairpins contain >30 nt of stem, with the basal hairpin duplex serving to recruit the double-strand RNA-binding domain protein Pasha (also known as DGCR8 in mammals) (7, 14, 17, 27). Pasha binds the nuclear RNase III enzyme Drosha, which "crops" the base of the hairpin ~10 nt away from the junction of its single-stranded flanks to yield the pre-miRNA hairpin (16, 17, 31). The pre-miRNA is exported to the cytoplasm via Exportin-5, where it is cleaved by the cytoplasmic RNase III enzyme Dicer-1 (Dcr-1) (4) and its double-strand RNA-binding domain partner Loquacious (Loqs) (8). From the resultant ~22-nt duplex, one strand preferentially enters an Argonaute-1 (AGO1) complex and guides it to seed-complementary targets (8).

Recently, the analysis of *Drosophila* small RNAs revealed that a subclass of miRNAs derives from atypical hairpin precursors termed mirtrons (38, 44). Their defining feature is that the ends of mirtron hairpins coincide precisely with 5′ and 3′ splice sites of introns of protein-coding genes (Fig. 1). Biogenesis studies carried out primarily using knockdowns of candidate factors in *Drosophila* S2 cells provided evidence that mirtrons use the splicing machinery to bypass Drosha cleavage. Following their linearization by intron lariat debranching enzyme, mirtrons gain access to Exportin-5 and are subsequently treated in the cytoplasm as conventional pre-miRNA hairpins. The mirtron pathway has been most thoroughly studied in *Drosophila*, but the analysis of large-scale small RNA sequence catalogs permitted the confident categorization of mirtrons in nematodes (44), diverse mammals (1), and most recently in chickens (13).

Although the initial studies of mirtron biogenesis were well supported, a potential caveat was their reliance on knockdown strategies. This is potentially significant in light of recent studies of Loqs. This Dcr-1 cofactor was originally classified as a core component of the miRNA biogenesis pathway based on studies of *loqs* knockdown in S2 cells and a hypomorphic *loqs* allele (10, 22, 45). Since these conditions reduced the level of at least some miRNAs and caused pre-miRNA hairpins to accumulate, one might have expected the complete loss of

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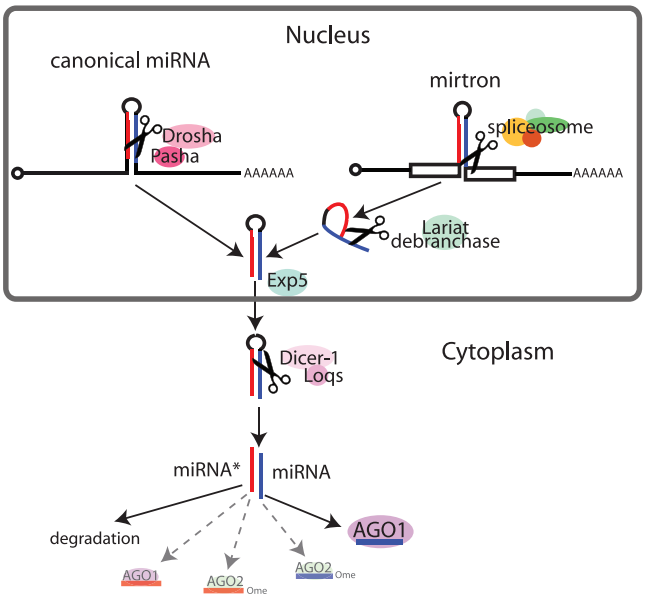

FIG. 1. Canonical miRNA and mirtron pathways in *Drosophila*. Key protein families include RNase III endonucleases (Drosha and Dicer-1), double-stranded RNA-binding domain proteins (Pasha and Loqs) and Argonaute effectors (AGO1 and AGO2). Canonical miRNA precursors are cleaved by the Drosha/Pasha complex in the nucleus, cleaved again by the Dicer-1/Loqs complex in the cytoplasm, and predominantly loaded into AGO1. Mirtrons are short hairpin introns that use the splicing and debranching machinery to bypass the requirement for Drosha/Pasha but are subsequently processed by Dicer-1 to generate miRNA-class regulatory RNAs.

Loqs to confer a stronger effect on miRNA maturation. Perhaps surprisingly then, subsequent analysis of a *loqs* deletion revealed that the biogenesis of many miRNAs was only subtly affected in the *loqs*-null condition (34). This is in strong contrast to the loss of *dcr-1*, for which homozygous mutant cells are unable to generate miRNAs (34).

In the present study, we describe the generation of a *pasha*-null allele and use it to validate the hypothesis that canonical miRNAs and mirtrons transit distinct nuclear pathways. In particular, mirtrons but not canonical miRNAs are produced and can repress targets in *pasha* mutants. Because of its maternal contribution, homozygous *pasha* mutants survive embryogenesis and larval stages. This makes it a particularly useful genetic tool among mutants in core components of the *Drosophila* miRNA pathway. In particular, the *pasha* mutant demonstrates that miRNAs are strictly required for the growth of all imaginal discs, a conclusion that cannot be derived from the clonal analysis of either *pasha* or *dcr-1*. Since the postembryonic functions of invertebrate *dcr-1* and vertebrate *DGCR8* and *Dicer* must be analyzed using mosaics, our findings suggest that caution must be exercised when using this technique to infer whether a given process does or does not require miRNAs.

MATERIALS AND METHODS

Generation of *pasha*^{KO}. We used *PBac{WH}f07241* and *PBac{RB}e00907*, which are inserted 5′ and 3′ of the *pasha* locus, respectively. We induced recombination in transheterozygous pBac flies that carried *hs-FLP* by heat shocking 48- to 72-h-old larvae at 37°C for 1 h. The F_1 female progeny were crossed to *w*; *Dr/TM3*, *Sb* males, and individual F_2 balanced males bearing putative deletion chromosomes were backcrossed to the balancer to generate stocks. We screened for deletion events using PCR, and isolated a recombinant that deleted the ~10-kb interval containing *pasha*; this allele retains a *mini-white*$^+$ insertion at the *pasha* locus. We then recombined *pasha*^{KO} onto a FRT82B chromosome for clonal analysis.

***pasha* rescue construct.** A 4.1-kb fragment including the entire *pasha* gene was amplified by PCR using primers with XhoI linkers: 5′ primer TTTCAAAATG GCCAATAG and 3′ primer CTCGGACTTTCTCTCTGC. After XhoI digestion, the fragment was cloned into pCasper4 in the sense orientation with respect to the P element. Transgenic lines were generated by standard methods, and a second chromosome insertion was used to rescue *pasha*^{KO}. The specific effect of the deletion on the expression of *pasha*, but not the neighboring gene *CG1792*, was verified by using semiquantitative and quantitative reverse transcription-PCR (qRT-PCR) using poly(A)$^+$ RNA and the following primer sets: pasha-F (5′-GTTCAAGGAGCTCCAAAACG-3′)/pasha-R (5′-CCTTGACA TCGGGAATGAGT-3′) and CG1792-F (5′-ACGGCGATTGCTTTCCGGG AAC-3′)/CG1792-R (5′-GGTGTTCCTCGGGTAGAAGGTC-3′).

Analysis of miRNA/mirtron processing. We used TRIzol to isolate total RNA from *Canton S*, *ago2*^{414} (39), *loqs*^{KO} (34), *dcr-2*^{L811fs} (33), and *pasha*^{KO} third-instar larvae, and followed previously described methods (38) for polyacrylamide gel Northern analysis using LNA probes (Exiqon). Probes were complementary to the mature sequences of canonical miRNAs or mirtron-derived miRNAs (http://microrna.sanger.ac.uk/sequences/index.shtml).

qRT-PCR analyses of pri-miRNA segments were performed according to previously described methods (40). We performed six replicate qRT-PCR assays on each of three independently generated cDNA preparations from poly(A)$^+$ RNA, using the following primer sets: miR-1-F (5′-GTTAGCCGCGTTGTGGAAAAT C-3′)/miR-1-R (5′-CATTTCATTACGGTTCTACTTCTG-3′), miR-8-F (5′-AGA ACTTTGAGCTTCCTCTGGC-3′)/miR-8-R (5′-TTTGGTGCTGCTGCTGCTG TTG-3′), miR-10-F (5′-CCGCGATTGCCTAGCGGACTTC-3′)/miR-10-R (5′-TT TCCGCTTGCCATCAGCAACAC-3′), miR-124-F (5′-ACATTGCATAACG ACATAAAGCC-3′)/miR-124-R (5′-AATTTGTCTATTATGATTTCAGG C-3′), miR-263a-F (5′-AGTGCATGCGGGTGAGTAATCC-3′)/miR-263a-R (5′-TAACTTTGAAAGTTTCGGATTTCG-3′), miR-276a-F (5′-AAAAGGGAAAC GCGCTGCCAAG-3′)/miR-276a-R (5′-CGTTTGTCCAGCGTTTTCTCATC-3′), miR-279-F (5′-ATTGAAATTAAAGAGGAGGCGAG-3′)/miR-279-R (5′-AAGT TTGTCAAGAAAACACGTGC-3′), miR-305-F (5′-GAAATGCTCGCAGGCG AGTCC-3′)/miR-305-R (5′-GTTGAACACTTGTATCGGTCGC-3′), miR-317-F (5′-ACGGTTTGTGTCTCTGCTGAGC-3′)/miR-317-R (5′-CTGTGGGGCATT CTCGTTATCC-3′), and miR-bantam-F (5′-CGCTCAGATGCAGATGTTGTT G-3′)/miR-bantam-R (5′-TCGACCATCGGAATGTGGAATG-3′).

For conventional Northern analysis, 20 μg of total RNA was separated on 1% agarose with 18% formaldehyde and 1× morpholinepropanesulfonic acid. After electrophoresis, the RNA was transferred by capillary flow to Hybond N+ membrane and UV cross-linked. Blot hybridizations were performed at 65°C overnight in Church hybridization buffer (0.5 M Church phosphate buffer, 1 mM EDTA, 7% sodium dodecyl sulfate [SDS]) with cDNA probes. Membranes were washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS for 15 min at room temperature and then twice with 0.5× SSC–0.1% SDS for 15 min at 65°C. [α-^{32}P]dCTP-labeled probes were generated by using a random primed DNA labeling kit (Roche) and templates that were amplified from genomic DNA using the following primers: pri-mir-1F (5′-CAG AAGTAGAACCGTAATGAAATG-3′), pri-mir-1R (5′-TGTCGATGGAATT GCTTACGTAC-3′; a 360-nt fragment just downstream of the *mir-1* hairpin), ago2-F (5′-GAGCACTTGCGCGTGTATAA-3′), and ago2-R (5′-AATCGTTC GCTTTGCGTACT-3′; a 700-nt fragment of the coding region).

Analysis of miRNA/mirtron function. We analyzed a number of genotypes. Immunostaining was performed according to a previous report (25), using rat anti-Elav, mouse anti-β-galactosidase (Developmental Studies Hybridoma Bank), or rabbit anti-GFP, followed by Alexa 488- or 568-conjugated secondary antibodies. The following genotypes were evaluated ("*X*" stands for either a control third chromosome arm, *dcr-1*^{Q1147X}, or *pasha*^{KO}): (i) random somatic clones—*hs-FLP*; *FRT82B*, *arm-lacZ*/*FRT82B*, *X*; (ii) small eye clones—*ey-FLP*; *FRT82B*, *arm-lacZ*/*FRT82B*, *X*; (iii) large eye clones—*ey-FLP*; *FRT82B*, *ubi-GFP*, *M(3)*/*FRT82B*, *X* (Dickson method) or *ey-Gal4*, *UAS-FLP*/+; *FRT82B*, *GMR-hid*, *l(3)cl*/*FRT 82B*, *X* (Stowers method); (iv) notum clones—*UAS-FLP*/+; *C684-Gal4*, *FRT82B*/*FRT82B*, *X*; (v) expression of baculovirus p35 in *dcr-1* or *pasha* notum clones—*109-68-Gal4*, *UAS-p35*/*UAS-FLP*; *FRT82B*, *arm-lacZ*/*FRT82B*, *X*; (vi) miRNA sensor test—*hs-FLP*; *tub-GFP-miR-7 sensor*, *UAS-DsRed-mir-7*/+; *tub-Gal4*, *FRT82B*, *tub-Gal80*/*FRT82B*, *X*; (vii) mirtron sensor test—*hs-FLP*; *tub-GFP-miR-1004 sensor*, *UAS-DsRed-mir-1004*/+; *tub-Gal4*,

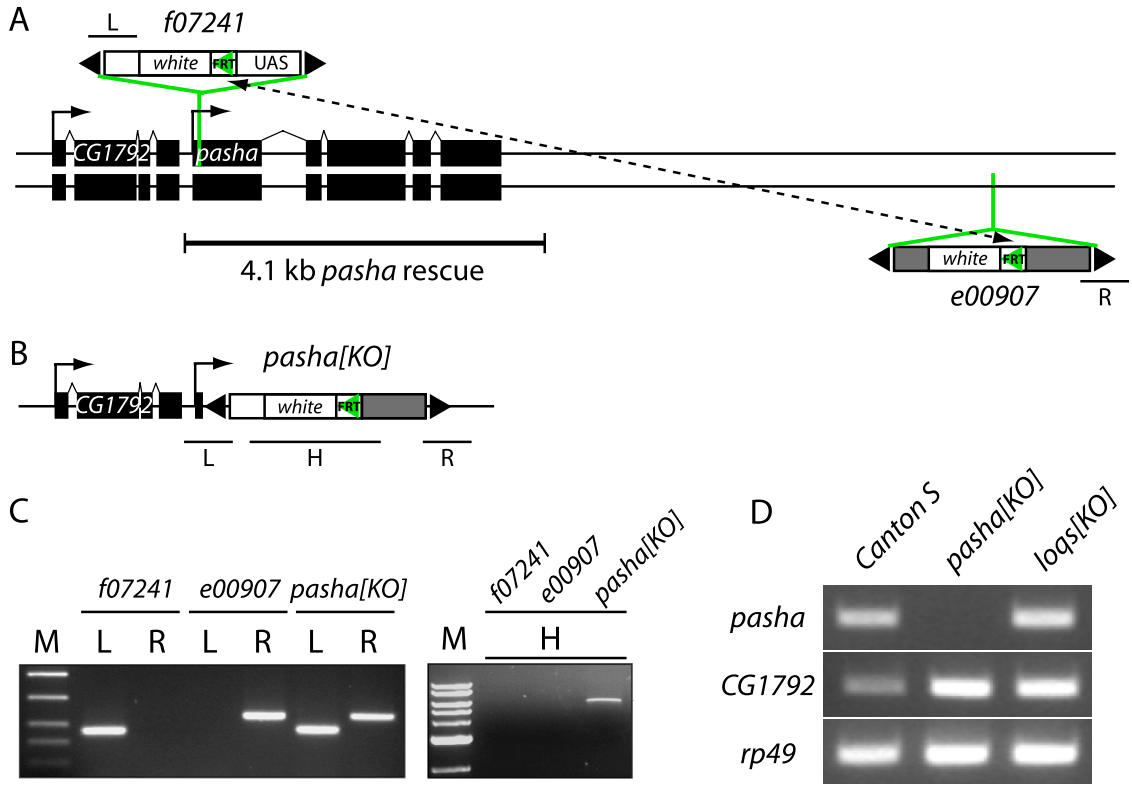

FIG. 2. Scheme for the generation of the *pasha*^{KO} deletion allele. (A) FLP-mediated recombination was used to delete genomic sequence between FRT-bearing piggyBac elements that flank the *pasha* locus. (B) The progenitor chromosomes bear piggyBac insertion in *trans*, which were brought in *cis* as a hybrid element after recombination. (C) PCR analysis verifies that the *pasha*^{KO} allele juxtaposes the left (L) and right (R) arms of the progenitor piggyBacs, while presence of the novel hybrid (H) product reflects the deletion of the intervening *pasha* locus. (D) RT-PCR tests demonstrate that *pasha* transcripts are absent from *pasha*^{KO} larvae, while expression of the neighboring locus *CG1792* is maintained. Quantitative tests indicate 1.65-fold increase in CG1792 mRNA in this mutant (see Fig. S1 in the supplemental material).

FRT82B, *tub-Gal80*/*FRT82B*, *X*; and (viii) germ line clones—*hs-FLP*; *FRT82B*, *ovo*^{D}/*FRT82B*, *pasha*.

RESULTS

Generation of a *pasha*^{KO} deletion allele. We used FLP-mediated recombination to delete ~10 kb of genomic sequence between FRT-bearing piggyBac transposons that flank the *pasha* locus (51) (Fig. 2A). The consequent deletion allele retains a hybrid piggyBac marked by *mini-white* and is not predicted to affect any other known genes (Fig. 2B). PCR analysis showed that the engineered chromosome indeed contains both left and right flanks of the progenitor elements (Fig. 2C). In addition, we were able to detect the unique hybrid product that bridges the hybrid piggyBac present in the deletion allele (Fig. 2C).

Animals homozygous for the deletion died as late third-instar larvae, suggesting that *pasha* is an essential locus. RT-PCR analyses showed that transcription of the neighboring locus CG1792 was still detected in homozygous *pasha* mutants, indicating that the deletion did not adversely affect CG1792 expression (Fig. 2D). In fact, qRT-PCR tests showed a slight increase (~1.65-fold) in CG1792 in the *pasha* mutant (see Fig. S1 in the supplemental material), suggesting that CG1792 is directly or indirectly affected by miRNA depletion. As a stringent test of whether the lethality of the mutant was specifically attributable to the loss of Pasha, we generated a 4.1-kb rescue transgene containing only the *pasha* gene. The *pasha*$^{+}$ insertion rescued deletion homozygotes to adulthood in Mendelian ratio, providing firm confirmation that the deletion solely affects *pasha* function. Consequently, we refer to this allele as *pasha*^{KO}.

***pasha*^{KO} mutants are deficient in processing canonical miRNAs but not mirtrons.** The function of *Drosophila pasha* in small RNA biogenesis has thus far been tested only in tissue culture cells or with in vitro assays. The availability of a genuine *pasha* mutant allowed us to test its endogenous requirement for small RNA biogenesis in the animal. We therefore prepared RNA from Canton S and homozygous mutant *pasha*^{KO}, *loqs*^{KO}, *dcr-2*^{Q1147X}, and *ago2*^{414} third-instar larvae and analyzed their small RNAs using Northern analysis.

Even though Loqs is often portrayed as a core component of the miRNA biogenesis pathway, the steady-state level of only some mature miRNAs is reduced in the absence of Loqs (Fig. 3A) (34). Instead, the predominant effect is the accumulation of pre-miRNA hairpins (Fig. 3A and B), indicative of their suboptimal but detectable processing by Dcr-1. In contrast, we observed that all ten canonical miRNAs tested that were expressed in wild-type larvae were strongly reduced in *pasha*^{KO} mutants, regardless of whether their dependence on Loqs was strong or weak (Fig. 3A and B and see Fig. S2 in the supplemental material). Both mature and pre-miRNA species were

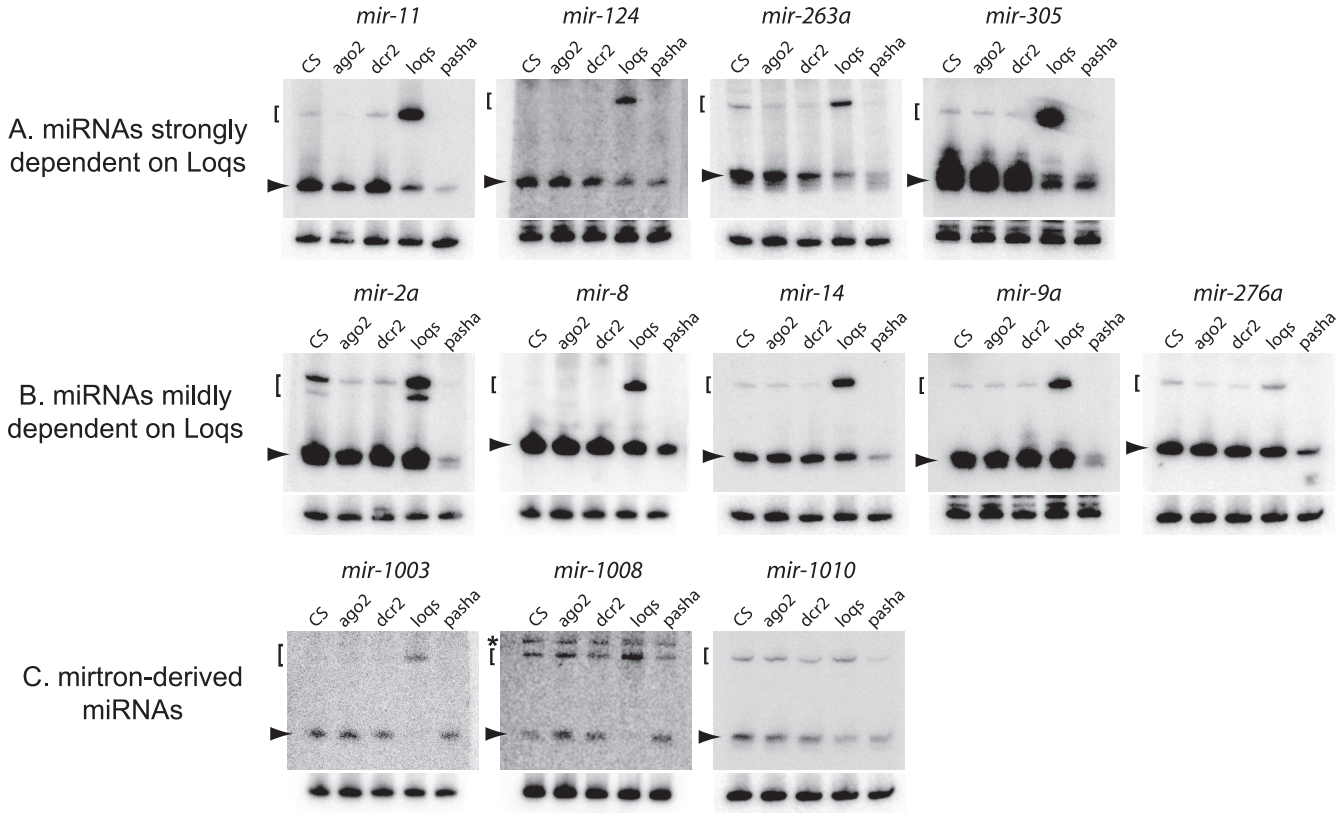

FIG. 3. Small RNA expression in mutants for canonical miRNA and RNAi factors. Total RNAs were extracted from homozygous larvae of the following genotypes: *Canton S* (*CS*) = wild type, *ago2* = $ago2^{414}$; *dcr2* = $dcr\text{-}2^{L811fs}$; *loqs* = $loqs^{KO}$; *pasha* = $pasha^{KO}$. Blots were stripped and probed for 2S rRNA as a loading and transferring control (shown beneath each miRNA blot). Some blots were probed with multiple miRNAs and therefore have the same 2S control. (A) Canonical miRNAs that are strongly dependent on Loqs; i.e., for which the mature species is reduced (arrowhead) and there is accumulation of pre-miRNA hairpins (bracket). All of these show reduced miRNA and pre-miRNA levels in $pasha^{KO}$. (B) miRNAs that are mildly dependent on Loqs; i.e., for which there is pre-miRNA accumulation but mature species are relatively unaffected. All of these still show reduced miRNA and pre-miRNA in *pasha*. (C) Mirtron-derived miRNAs. These are strongly affected in *loqs* but mostly unaffected in *pasha*. There is a slight reduction in *pre-mir-1010* and miR-1010 in *pasha*, although this is potentially due to an effect on the expression of its host gene CG31163.

depleted, data that demonstrate Pasha to be an essential component of the *Drosophila* miRNA biogenesis machinery.

We next analyzed the accumulation of miRNAs derived from mirtron precursors. Previous analyses using dsRNA *pasha* knockdowns in S2 cells suggested that Pasha was not required for mirtron maturation, which was inferred to use the splicing machinery to bypass processing by the Drosha/Pasha complex (38, 44). Indeed, all three of the mirtron-derived miRNAs that we had previously shown to be detectable in third-instar larvae were still expressed in the $pasha^{KO}$ mutant (Fig. 3C). Therefore, the *pasha* mutant distinguishes the processing of canonical miRNAs and mirtrons.

$pasha^{KO}$ mutants accumulate high levels of pri-miRNAs. Our Northern analysis showed that all of the miRNAs tested exhibited lower levels of both mature species and pre-miRNA species, a finding consistent with a failure of "cropping." If so, we might expect miRNA precursors to accumulate as pri-miRNA species, as shown previously using knockdown experiments (7, 14, 27). To test this, we performed qRT-PCRs of poly(A)$^+$ RNA isolated from *Canton S* and $pasha^{KO}$ homozygous larvae. For all 10 loci tested, we observed massive elevation of pri-miRNA species in the *pasha* mutant, usually ranging from 100- to 1,000-fold increases (Fig. 4). By comparison, previous analysis of *Drosophila pasha* using knockdown techniques, which yield only partial target suppression, showed only three- to fivefold elevation in pri-miRNA levels (7, 27). Therefore, our analysis of bona fide *pasha*-null mutants more fully reports its obligate function in cleaving canonical pri-miRNA hairpins.

A consequence of the failure to cleave primary miRNA transcripts might be their accumulation as stable full-length transcripts. Indeed, we observed that a single transcript of at least 8 kb hybridized to a *pri-mir-1* probe in $pasha^{KO}$, but not control *Canton S* larvae (Fig. 4B). Curiously, this is much longer than any of the putative lengths of *pri-mir-1* inferred previously using different strategies. RACE (rapid amplification of cDNA ends) experiments suggested *mir-1* to produce alternative primary transcripts of 548 and 992 nt (47), while tiling microarrays detected a continuous region of transcribed *mir-1* sequence extending for ~2.9 kb (2). Thus, the *pasha* mutant might represent a favorable background to delineate primary miRNA transcripts.

$pasha^{KO}$ distinguishes the activity of canonical miRNAs and mirtrons. We have shown that the maturation of canonical

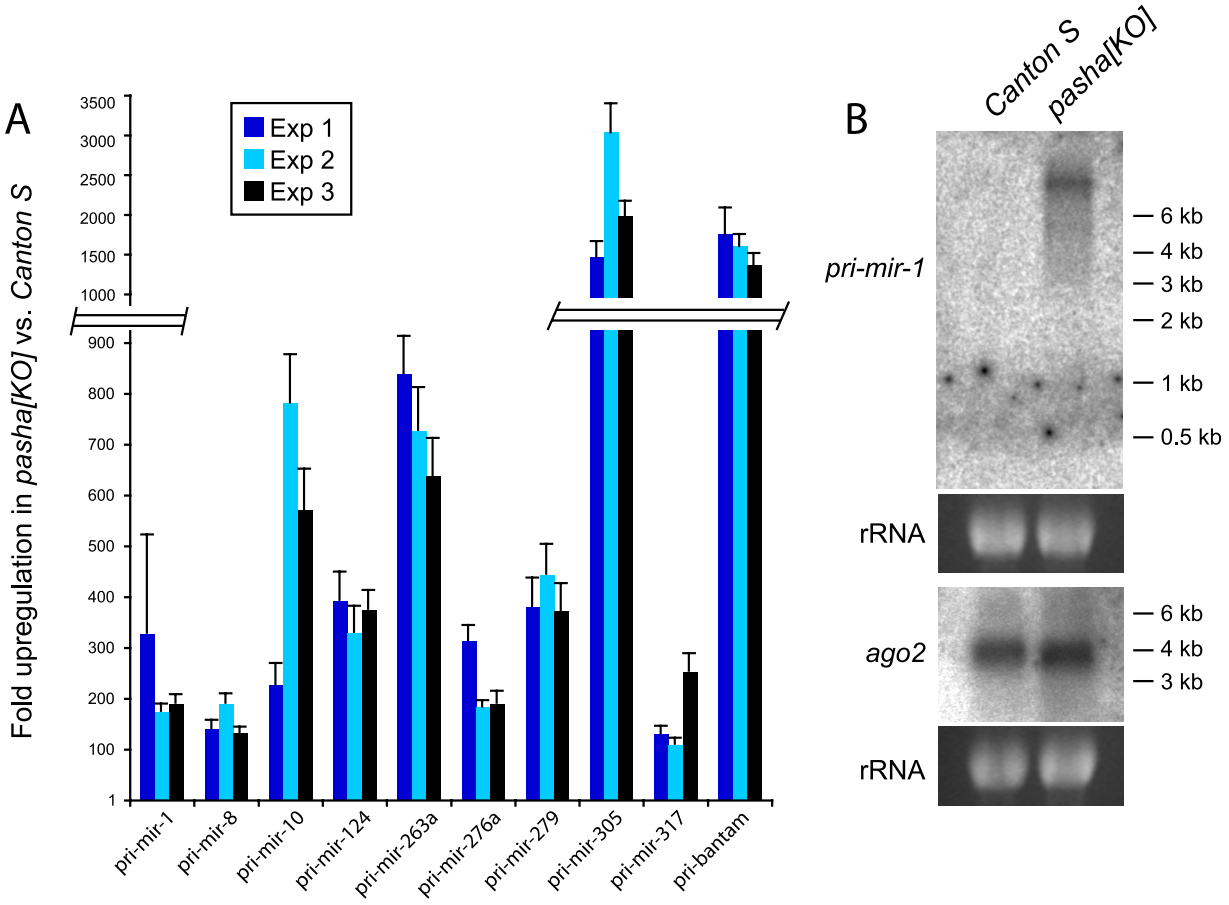

FIG. 4. *Pasha*^{KO} larvae strongly accumulate pri-miRNA species. (A) qRT-PCR) was used to assess pri-miRNA levels in *Canton S* and homozygous *pasha*^{KO} larvae, as normalized to *rp49*. Three independent RNA samples were reverse transcribed for each genotype, and six qPCR measurements were made for each cDNA preparation. The *y* axis depicts the pri-miRNA ratio and standard error determined for each biological replicate. (B) Northern analysis of *pri-mir-1* in *Canton S* and homozygous *pasha*^{KO} larvae revealed the accumulation of an ~8-kb transcript in the pasha mutant. Hybridization with an *ago2* probe and ethidium staining of rRNA served as loading controls.

miRNAs, but not mirtrons, is disrupted in *pasha*^{KO} animals. We decided to perform a functional test of this distinction using a genetic assay of small RNA function. In the standard sensor assay, the Gal4-UAS system is used to ectopically express a canonical miRNA or mirtron as a fusion transcript with a DsRed-encoding marker. In this background, one introduces a ubiquitously expressed green fluorescent protein (GFP) transgene linked to binding sites for the miRNA or mirtron. Successful target repression is observed as a reduction in GFP activity specifically in DsRed-positive cells (26, 38, 48).

We adapted the standard sensor assay for use with the MARCM system (30), in which UAS transgenes can be activated specifically in homozygous somatic clones of mutations of interest. Since both *dcr-1* and *pasha* are located on the right arm of chromosome III, we used FRT82B for somatic recombination and chose the canonical miRNA miR-7 and the mirtron miR-1004 to test against cognate sensors (38, 48). We first analyzed control clones of FRT82B. As expected, control clones expressing miR-7 repressed *tub-GFP-miR-7* (Fig. 5A), while miR-1004-expressing cells repressed *tub-GFP-mir-1004* (Fig. 5D). On the other hand, *dcr-1* clones failed to exhibit either canonical miRNA-mediated or mirtron-mediated repression (Fig. 5B and E) (33). In contrast, *pasha*^{KO} clones were blocked for canonical miRNA-mediated repression (Fig. 5C) but showed efficient mirtron-mediated repression (Fig. 5F). We observed the same trends in both eye and wing imaginal discs (Fig. 5 and data not shown), demonstrating these properties to be spatially general. These data provide convincing genetic evidence that a subclass of miRNA-family regulators remains functional in the absence of an essential component of the canonical miRNA biogenesis pathway.

Phenotypic analysis of *pasha* mutants. Homozygous mutants in *dcr-1* and *ago1* are lethal during embryogenesis (24, 33), while null *loqs* mutants survive to late pupal stages or on occasion even to adulthood (34). The differences in lethal phase appear to be a consequence of their differential requirements for miRNA biogenesis and function: Dcr-1 and AGO1 are core components, while Loqs appears to be an auxiliary factor. Homozygous *pasha*^{KO} mutants survive to an intermediate stage, dying mostly as third-instar larvae. Since we showed that Pasha is a core biogenesis factor likely required for the maturation of all canonical miRNAs, survival of *pasha*^{KO} animals past embryogenesis might reflect its maternal contribution. Inherited protein and RNA stores can allow mutants in

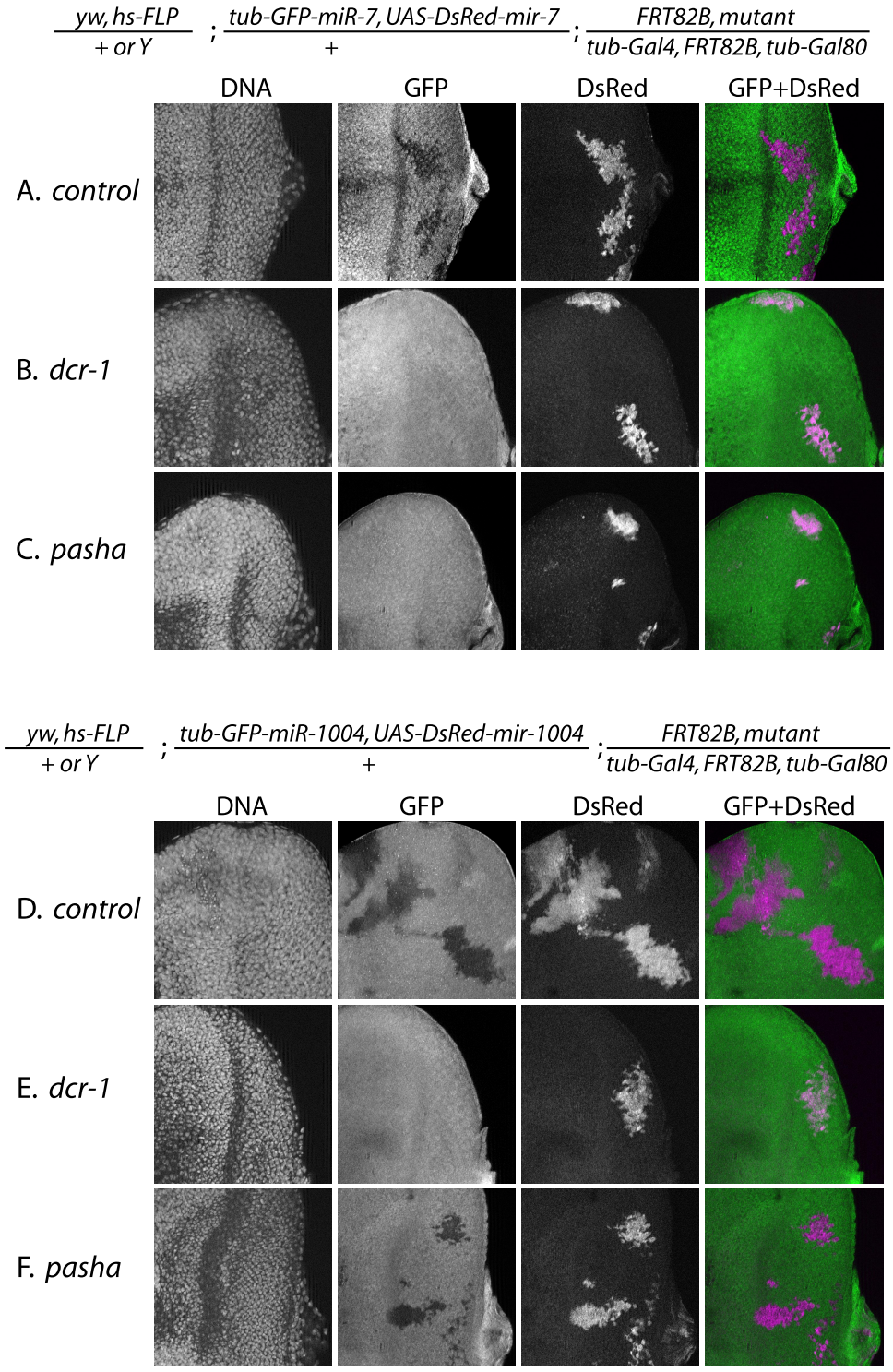

FIG. 5. *pasha* distinguishes the function of canonical miRNAs and mirtrons. Shown are eye imaginal discs stained for DNA and a GFP sensor, with DsRed fluorescence marking active small RNA transgenes; the right panels depict the merged GFP/DsRed channels. (A to C) MARCM clones that are homozygous for a given chromosome 3R and ectopically express a hybrid *DsRed:mir-7* transgene were tested for their ability to repress a miRNA sensor, a ubiquitously transcribed *GFP* target bearing two miR-7 binding sites. miRNA-mediated target repression was detected in control clones (A), but not in *dcr-1* clones (B) or $pasha^{KO}$ clones (C). (D to F) MARCM clones that are homozygous for a given chromosome 3R and ectopically express a hybrid *DsRed:mir-1004* (mirtron) transgene were tested for their ability to repress a mirtron sensor, a ubiquitously transcribed *GFP* transcript bearing two miR-1004 binding sites. Mirtron-mediated target repression was detected in control clones (D) but not *dcr-1* clones (E); $pasha^{KO}$ homozygous cells generated active mirtrons (F).

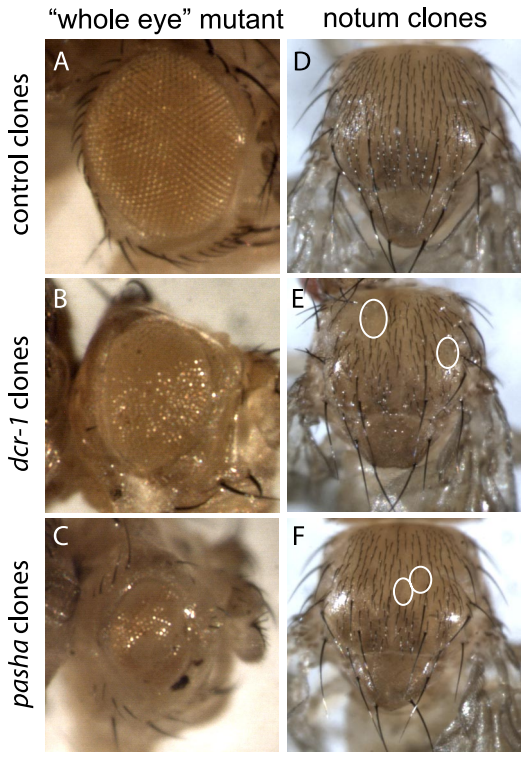

FIG. 6. Phenotypes of *dcr-1* and *pasha*^{KO} adult mutant clones. Compared to wild-type, clonal loss of either Dcr-1 or Pasha results in small rough eyes (A to C) and loss of external mechanosensory bristle structures (D to F). Eye clones were made using the EGUF system (49), which generates eyes that are nearly composed entirely of mutant tissue.

essential *Drosophila* genes to survive to surprisingly late stages in development.

Other miRNA factors are also deposited maternally. For example, loss of both maternal and zygotic *dcr-1* or *ago1* causes developmental arrest at earlier times and with greater pattern abnormalities, compared to the zygotic mutants (19, 24, 36). In addition, their germ line depletion results in substantial defects during oogenesis that compromise egg production (19, 23, 53). Similarly, we observed that removal of *pasha* from the female germ line, using the *FLP/FRT-ovo*^{D} technique, results in their inability to lay eggs (data not shown). Therefore, the later lethal phase of *pasha* zygotic mutants, relative to *dcr-1* or *ago1*, is likely due to either its greater maternal deposition and/or greater stability.

To assess the effects of *pasha* and *dcr-1* loss on adult development, we analyzed somatic FLP clones. In most respects, these revealed similar phenotypes, such as wing blistering, external loss of notum bristle sensory organs, and small, rough eyes (Fig. 6 and data not shown) (33). These rather general phenotypes resemble those caused by defects in cell viability. However, expression of the antiapoptotic baculovirus protein p35, which blocks cell death (20), did not rescue sensory organ formation in either *dcr-1* or *pasha* clones (data not shown). This indicates that these clonal phenotypes were not simply due to cell death caused by miRNA depletion in mutant cells.

One difference between Dcr-1 and Pasha was that *pasha*^{KO} mutant eyes were smaller than those of *dcr-1* (Fig. 6B and C). The apparently stronger eye phenotype of *pasha* was possibly consistent with its more apical position in the canonical miRNA biogenesis pathway, which might lead to a more rapid loss of miRNA production in somatic clones. An alternative interpretation is that there exist mirtrons that antagonize growth, and that these remain active in *pasha* but not *dcr-1* eyes. However, this scenario is not obviously supported by current computational predictions of mirtron targets (e.g., http://www.targetscan.org/fly_12/).

Imaginal disc growth defects in *pasha* mutants. Imaginal disc clones of *dcr-1* are smaller than their wild-type twin-spot clones, implying that cells lacking miRNAs proliferate less effectively than the wild type (11). We similarly observed that random *pasha*^{KO} clones generated with *hs-FLP* were smaller than their wild-type twin-spots (Fig. 7A to C and see Fig. S3 in the supplemental material). These imaginal disc phenotypes were reflected in the adult by the fact that both *dcr-1* and *pasha*^{KO} eye clones, which can be visually marked by absence or presence of eye pigmentation, contribute far less to the adult eye than their respective twin-spots (data not shown). Therefore, cells lacking Pasha or Dcr-1 exhibit similar defects in imaginal disc growth, perhaps reflecting their common loss of canonical miRNAs.

Elegant techniques permit the generation of nearly completely mutant *Drosophila* eye tissue by flipping against a *Minute* mutation (37) or a *GMR-hid* transgene (49). Under these circumstances, twin-spots are cell lethal, while heterozygous cells are at a competitive growth disadvantage and usually contribute little to a mosaic tissue (Fig. 7D′). Such strategies make possible the generation of eyes that are nearly entirely mutant for *dcr-1* (Fig. 6B) (33), suggesting that cells are able to proliferate effectively in the absence of miRNAs provided that cell competition is compromised. Even under these circumstances, however, we observed that *dcr-1* clones competed relatively poorly. In the third-instar eye imaginal disc, control wild-type clones eliminated almost all of the *Minute* heterozygous cells (Fig. 7D′), whereas discs bearing *dcr-1* clones contained substantial regions of GFP^{+}, *Minute* cells (Fig. 7E′). *Pasha* mutant cells were similarly unable to eliminate *Minute* heterozygous cells by the third instar (Fig. 7F′).

The ability of *pasha*^{KO} mutants to survive to late larval stages presented a unique opportunity to assess imaginal disc development in homozygous animals. Interestingly, *pasha* mutant larvae essentially lack imaginal discs, with only rudiments remaining of any discs (Fig. 7G and H). The optic lobes of the brain were also strongly reduced, although the brain stem developed fairly normally. The stronger growth defect exhibited by homozygous *pasha*^{KO} mutants suggests that the clonal loss of Pasha or Dcr-1 reflects only a partial loss-of-function phenotype with respect to imaginal disc growth.

DISCUSSION

Molecular and genetic analysis of a null allele of *Drosophila pasha*. In the present study we described the first loss-of-function analysis of *Drosophila pasha* in the animal, using a deletion allele that removes this locus. We provided evidence for a nearly complete block in the production of canonical miRNAs in this mutant, similar to evidence generated for embryonic stem cells with the *pasha* ortholog, *DGCR8*, deleted (52). We

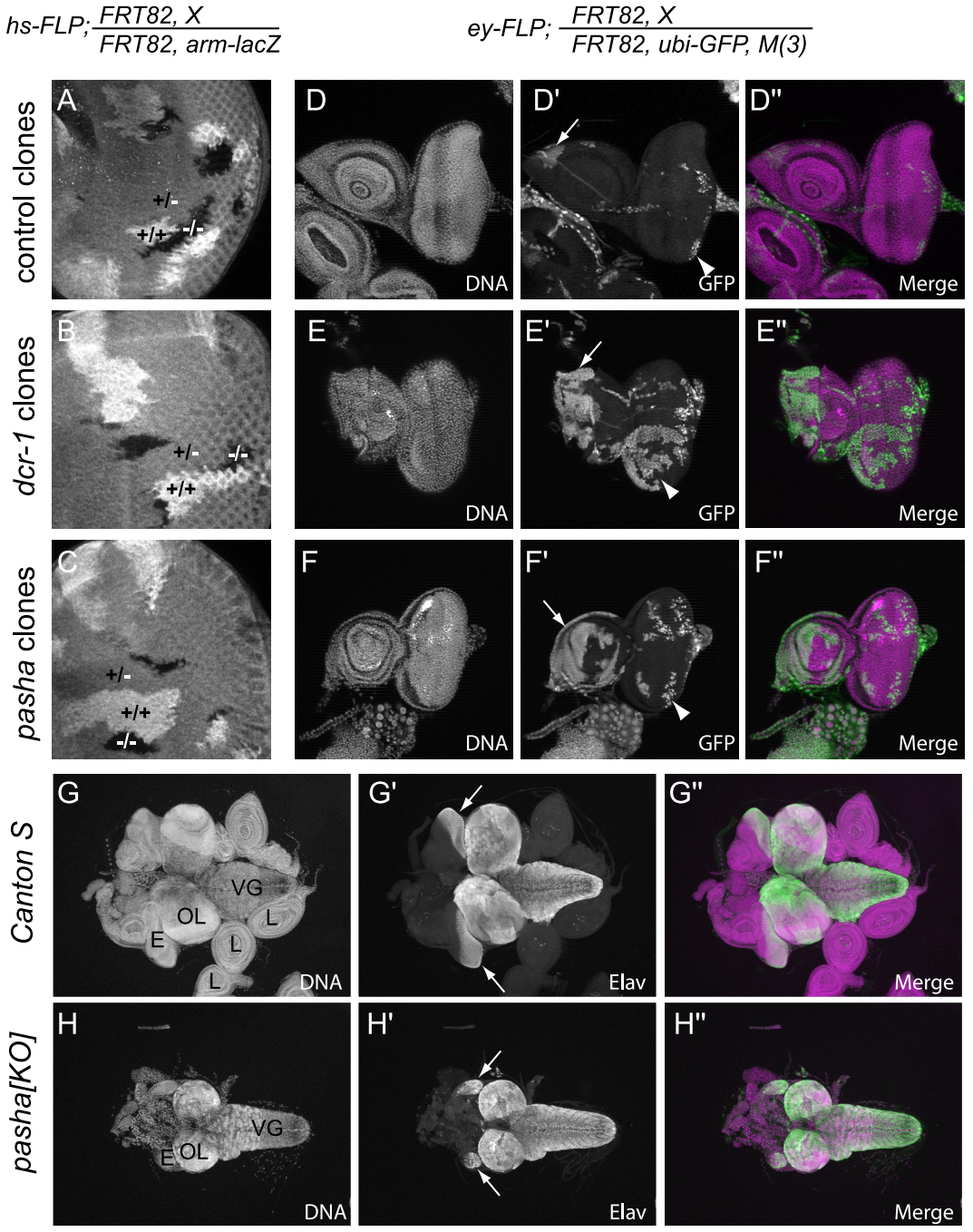

FIG. 7. Distinct growth defects observed in mutant clones compared to homozygous *pasha*^{KO} animals. (A to C) Small retinal clones in third instar eye imaginal discs stained with β-galactosidase antibody. Homozygous mutant cells have no β-galactosidase (−/−), their twin-spots have a high level of β-galactosidase (+/+), and unrecombined heterozygous cells have an intermediate level of β-galactosidase (+/−). Compared to control clones, *dcr-1* and *pasha*^{KO} clones are much smaller than their respective twin-spots. (D to F) Large clones in third-instar eye imaginal discs generated with the *Minute* technique and stained with GFP antibody; the magnification is lower than in panels A to C. *Minute* homozygosity is cell lethal, and *Minute* heterozygous cells have a severe growth disadvantage, thus permitting the recovery of large, GFP$^{-/-}$ mutant clones. (D) Control clones occupy nearly the entire disc; the arrow and arrowhead point to small patches of heterozygous cells in the antenna and retina, respectively. Homozygous *dcr-1* cells (E) and *pasha*^{KO} (F) cells compete poorly even against *Minute* cells, so large GFP^{+} sectors remain. (G) Wild-type brain/imaginal disc complex stained for the neural marker Elav; the olfactory lobe (OL), ventral ganglion (VG), eye-antennal disc (E), and leg disc (L) are indicated. Elav is highly expressed by the brain and the developing retina of the eye disc (arrows, G′). (H) Brain/imaginal disc complex from a homozygous *pasha*^{KO} larva is nearly devoid of imaginal discs and differentiate only small patches of Elav^{+} retina. The *pasha*^{KO} brain exhibits rudimentary optic lobes, but the ventral ganglion is of fairly normal size.

found that the effects of *pasha* deletion on miRNA cropping are more severe than was previously observed using knockdown strategies (7, 27), validating the status of Pasha as an essential component of the canonical miRNA biogenesis pathway.

Importantly, our data provide stringent evidence for the separation of nuclear miRNA sorting pathways in *Drosophila*. Although Pasha is essential for processing of canonical primary miRNA transcripts, it is dispensable for the processing of mirtrons. Indeed, we showed that mirtrons were capable of potent target repression in *pasha*-mutant cells (Fig. 5). The contribution of mirtrons to the miRNA-mediated regulatory network is undoubtedly smaller than that of canonical miRNAs, owing to their generally modest expression levels (1, 44). Nevertheless, in light of the supposition that *DGCR8* mutant cells are specifically lacking miRNA pathway activity (52), it is important to recognize that *Pasha*/*DGCR8*-mutant cells retain this subclass of miRNA regulators.

In theory, canonical miRNAs might be functionally reprogrammed into mirtron backbones, realizing that their 3′ ends would need to be modified into splice sites. This is plausible given that miRNA 3′ ends may be relatively subtly required for major miRNA targeting activities. Despite known roles for miRNA 3′ ends in compensatory pairing (41), all point mutants of endogenous miRNAs isolated in nematodes (*lin-4*, *let-7*, and *lsy-6*) (29, 42, 46) and flies (*mir-278*) (35, 50) invariably affect the seed region. If successful, such a scheme might enable the genetic rescue of *Pasha*/*DGCR8*-mutant phenotypes by single mirtronic-miRNA transgenes, akin to the rescue of maternal-zygotic *Dicer* mutants in zebrafish by injecting individual miRNA duplexes (12). It might even prove to be the case that mirtrons are especially active in *Pasha*/*DGCR8* or *Drosha* mutant cells, given that Dicer would be relieved of its normal role in processing canonical pre-miRNAs in such genetic conditions.

The general fates of pri-miRNA transcripts that escape Drosha processing are incompletely understood at present. In at least some cases, stable transcripts representing full-length pri-miRNA species have been detected (5, 52). We observed the strong accumulation of many pri-miRNA fragments using qPCR analysis and also detected the stable accumulation of an ~8-kb *pri-mir-1* transcript that far exceeded its previously inferred size(s) (which ranged from 0.5 to 1 kb to 3 kb [2, 47]). At the same time, it is relevant to bear in mind that heterogeneous transcripts in the process of degradation (15) are still substrates for qPCR; thus, the accumulation of pri-miRNA species as detected by qPCR need not necessarily be accompanied by single band on a Northern blot. Some invertebrate and vertebrate pri-miRNA transcripts have been suggested to be 50 to 100 kb in length (43, 50), and it would be perhaps remarkable if such long transcripts were completely immune to degradation by one or more RNases. It will therefore be interesting to examine the fates of pri-miRNA transcripts more systematically in *pasha*^{KO}.

Interpreting the consequences of conditional *Dicer* or *pasha*/*DGCR8* loss. It is popularly presumed that the clonal loss of a core miRNA biogenesis component can be used to assess the consequences of removing most, if not all, miRNAs from a given developmental setting. Since mutants in core components of the miRNA biogenesis pathway are lethal in all animals, conditional loss is the only way to examine the effects of miRNA pathway loss-of-function mutations on adult tissues. The activity of residual protein and RNA/miRNA products in these conditions has not often been critically assessed. Notably, miRNAs are highly abundant species and directed tests suggested several miRNAs to be very stable and removed only by dilution in dividing cells (28). The presence of small amounts of mature miRNAs in late third-instar *pasha*^{KO} larvae (Fig. 3), 5 days after their birth, attests to the stability of maternal Pasha and/or mature miRNAs. Double-stranded RNA-mediated knockdown studies carry similar, if not greater caveats, in light of their inherent capability for partial target suppression.

In *Drosophila*, an allele of *dcr-1* was originally isolated in a genetic screen that assayed eye pigment levels in "whole-eye" mutant animals. The very method of its isolation meant that a substantial amount of eye tissue had to be isolated. We similarly observed that although "whole-eye" *pasha*^{KO} mutant adults were substantially reduced in size, homozygous mutant adult tissue was nonetheless recovered. Such observations seemingly suggest that miRNAs are dispensable for imaginal disc growth. This seems unlikely to be the case, since it was reported many years ago that larvae deleted for the bantam miRNA lack imaginal discs (3, 21), similar to what we observed in *pasha* mutant larvae (Fig. 7H). Thus, the strict genetic requirement for bantam does not appear to be revealed through clonal analysis of *dcr-1* or *pasha*.

While it is conceivable that the severe *pasha*^{KO} growth defects are due to a greater reduction of global miRNA activity in imaginal discs relative to clonal experiments, we cannot exclude a nonautonomous role for miRNAs in promoting imaginal disc growth. Nevertheless, our observations serve an important reminder of the necessity for caution in interpreting the consequences of conditional Dicer or Pasha/DGCR8 loss. In particular, many studies of *Dicer* conditional ablation concluded that many specific developmental events do not require miRNAs (6, 18). Instead, residual miRNAs may suffice to drive substantial aspects of development in early clones. Perhaps only later in the age of these clones do miRNA levels fall below a threshold that reveals a phenotype, often during differentiation or survival of mutant cells. The extant catalog of conditional *Dicer* phenotypes is consistent with this interpretation.

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