A *Drosophila* genetic screen yields allelic series of core microRNA biogenesis factors and reveals post-developmental roles for microRNAs

PETER SMIBERT,1 FERNANDO BEJARANO,1 DONG WANG,2 DANIEL L. GARAULET,1,3 JR-SHIUAN YANG,1 RAQUEL MARTIN,1,3 DIANE BORTOLAMIOL-BECET,1 NICOLAS ROBINE,1 P. ROBIN HIESINGER,2 and ERIC C. LAI1,4

1Department of Developmental Biology, Sloan-Kettering Institute, New York, New York 10065, USA
2Department of Physiology and Green Center for Systems Biology, University of Texas Southwestern Medical Center, Dallas, Texas 75390, USA

**ABSTRACT**

Canonical animal microRNAs (miRNAs) are ~22-nt regulatory RNAs generated by stepwise cleavage of primary hairpin transcripts by the Drosha and Dicer RNase III enzymes. We performed a genetic screen using an miRNA-repressed reporter in the *Drosophila* eye and recovered the first reported alleles of fly *drosha*, an allelic series of its dsRBD partner *pasha*, and novel alleles of *dicer-1*. Analysis of *drosha* mutants provided direct confirmation that mirtrons are independent of this nuclease, as inferred earlier from *pasha* knockouts. We further used these mutants to demonstrate in vivo cross-regulation of Drosha and Pasha in the intact animal, confirming remarkable conservation of a homeostatic mechanism that aligns their respective levels. Although the loss of core miRNA pathway components is universally lethal in animals, we unexpectedly recovered hypomorphic alleles that gave adult escapers with overtly normal development. However, the mutant photoreceptor neurons exhibited reduced synaptic transmission, without accompanying defects in neuronal development or maintenance. These findings indicate that synaptic function is especially sensitive to optimal miRNA pathway function. These allelic series of miRNA pathway mutants should find broad usage in studies of miRNA biogenesis and biology in the *Drosophila* system.

Keywords: microRNA; Drosha; Pasha; Microprocessor; synapse; Dicer

**INTRODUCTION**

miRNAs (miRNAs) are an abundant class of ~22-nt regulatory RNAs derived from endogenous inverted repeat transcripts, which collectively play substantial roles during development and homeostasis (Flynt and Lai 2008). Biogenesis of canonical animal miRNAs follows a conserved strategy involving stepwise cleavage by the Drosha and Dicer RNase III enzymes (Kim et al. 2009). In the *Drosophila* pathway, hairpins within primary miRNA (pri-miRNA) transcripts are recognized in the nucleus by the dsRNA binding domain (dsRBD) protein Pasha, which positions its partner Drosha to cleave ~10 bp into the stem (Han et al. 2006). The Drosha–Pasha complex is often referred to as the “Microprocessor,” whose activity yields ~55–70-nt pre-miRNA hairpins (Denli et al. 2004; Gregory et al. 2004; Han et al. 2004). These are exported to the cytoplasm by Exportin-5/Ran-GTP (Yi et al. 2003; Bohnsack et al. 2004; Lund et al. 2004) and cleaved again by Dicer-1 in complex with its dsRBD partner Loqs (Forstemann et al. 2005; Saito et al. 2005). One strand of each resulting duplex is preferentially loaded into the miRNA effector Argonaute1 (AGO1) (Forstemann et al. 2007; Tomari et al. 2007; Okamura et al. 2009), which partners with GW182 (Gawky) to repress target transcripts (Liu et al. 2005; Rehwinkel et al. 2005; Eulalio et al. 2008). As in other animals, functional *Drosophila* miRNA targets generally exhibit limited complementarity and frequently pair only to positions 2–8 of the miRNA (Lai 2002; Ruby et al. 2007b), also known as the “seed” region (Bartel 2009). Productive regulatory interactions usually lead to target deadenylation and degradation, although translational inhibition and/or target relocalization also contribute to miRNA-mediated silencing (Lai and Posakony 1997; Lai et al. 1998; Behm-Ansmant et al. 2006; Zdanowicz et al. 2009).

In addition to the canonical Drosha–Dicer pathway, several alternate pathways for miRNA biogenesis have been
documented (Yang and Lai 2011). For example, mirtrons are short hairpin introns that are spliced and debranched to generate pre-miRNA hairpins, thereby bypassing the need for Drosha cleavage (Okamura et al. 2007; Ruby et al. 2007a). Variant mirtrons with 5’ or 3’ tails rely on splicing to generate one hairpin end but require other exonucleases to trim their tails to permit nuclear export and dicing (Babiarz et al. 2008; Flynt et al. 2010). Recently, vertebrate mir-451 was recognized to be Drosha-dependent but Dicer-independent, and instead matures via the catalytic activity of Ago2 (Cheloufi et al. 2010; Cifuentes et al. 2010; Yang et al. 2010). Nevertheless, the majority of miRNAs proceed through the canonical biogenesis pathway.

Most studies of miRNA biogenesis have relied on biochemical approaches and usage of RNA interference to reduce the activity of candidate factors, even in traditionally genetic systems such as Drosophila and Caenorhabditis elegans (Eulalio et al. 2007; Parry et al. 2007; Zhou et al. 2008). Consequently, there are few mutant alleles available for many key miRNA pathway genes. For example, in flies, there are only two alleles of dicer-1 (Lee et al. 2004; Berdnik et al. 2008), two alleles of pasha (Berdnik et al. 2008; Martin et al. 2009), and no mutant alleles described for drosa. The paucity of mutants limits the flexibility of genetic analysis for miRNA pathway function, especially in ways that would otherwise be facilitated by the availability of allelic series. For example, an extensive collection of dicer-2 alleles includes mutants that permit genetic separation of the role of this enzyme for siRNA genesis from its role in siRNA loading into AGO2 complex (Lee et al. 2004; Tomari et al. 2004; Lim et al. 2008).

Forward genetic screening offers a strategy to obtain mutants in an unbiased fashion and has the potential to identify new pathway components and accessory factors. In the present study, we describe a Drosophila genetic screen designed to identify mutants that affect the biogenesis or activity of canonical miRNAs. The efficacy of this screen was demonstrated by recovery of the first reported alleles of Drosophila drosa, the first point mutations of pasha, and several novel alleles of dicer-1. We use these alleles to demonstrate mutual cross-regulation of Drosha and Pasha levels, previously characterized only in cultured cells (Han et al. 2009; Kadener et al. 2009; Triboulet et al. 2009), for the first time in the intact animal. Finally, the availability of allelic series of these genes allowed us to access post-developmental roles of the miRNA pathway. In particular, we find that mild reduction of global miRNA levels leads to a synaptic transmission defect without overtly affecting development or neuronal maintenance.

RESULTS

Development of a simple in vivo visual reporter for miRNA pathway activity

To facilitate large-scale genetic identification of loci required for miRNA biogenesis or function, we designed a system to assay miRNA activity via simple visual inspection of eye pigmentation. A similar strategy was previously used successfully to identify genes involved in RNAi in Drosophila (Lee et al. 2004; Kim et al. 2007; Lim et al. 2008; Lee et al. 2009; Marques et al. 2010). To begin, we reprogrammed the mir-6-1 backbone (Chen et al. 2007) to generate an miRNA that targets a single location in the endogenous 3’ UTR of the white gene (w-miR) (Fig. 1A). To encourage miRNA-like biogenesis and target regulation (Forstemann et al. 2007; Czech et al. 2009; Okamura et al. 2009; Ghildiyal et al. 2010), we designed w-mir to have a central bulge in its pre-miRNA hairpin and mismatches at positions 10–12 relative to the target site (Fig. 1A). This miRNA was placed downstream from DsRed under control of GUS regulatory sequences (Brodsky et al. 2000); this hybrid element combines the GMR enhancer for specific expression in the developing eye and UAS sites for optional Gal4-mediated control. GUS-DsRed-w-miR was carried in a P-element transformation vector containing a standard mini-white construct bearing the endogenous white 3’ UTR.

To assay the regulatory activity of w-miR, we first performed sensor assays in S2 cells. Cotransfection of GUS-DsRed-w-mir and ub-Gal4 specifically repressed a Renilla luciferase reporter bearing the white 3’ UTR, compared with controls (Fig. 1B). We next performed more physiological tests using third instar wing imaginal discs. We prepared a tub-GFP-white 3’ UTR transgene, which is expressed ubiquitously in the animal. When introduced into a background carrying GUS-DsRed-w-mir and ptc-Gal4, thus activating DsRed and w-miR along the anterior–posterior compartment boundary, we correspondingly observed cell-autonomous repression of the GFP-white 3’ UTR sensor (Fig. 1C). Therefore, w-miR was able to silence transcripts bearing a single bulged site in the white 3’ UTR in vivo.

Finally, we assayed the capacity of w-miR to suppress pigmentation. When placed in a background that is mutant for the endogenous white locus, GUS-DsRed-w-miR (hereafter abbreviated as w-mir) is autotargeting in the eye, since it generates both the white gene product from the mini-white transformation marker in the vector backbone and an miRNA that silences this transcript. In principle, if such suppression were sufficiently robust, we may not have been able to score insertions based on standard rescue of eye pigment; we therefore included a DsRed selection marker. It turns out that multiple independent w-mir insertions were easily isolated, and these bore a range of eye colors as is typical for random P integration. On the whole, these independent transgenes had mild levels of pigment (Fig. 1F, cf. wild-type in Fig. 1E and the white-null mutant in Fig. 1D).

To test if w-miR was indeed responsible for this apparent decrease in eye pigment, we generated eye clones for a null mutation of dcr-1 (Lee et al. 2004) in the background of w-miR. The resulting eyes appeared mosaic and had patches of tissue that were small and rough, as is characteristic of dcr-1 clonal tissue. Importantly, these mutant regions
exhibited darker red pigmentation than surrounding tissue, indicating that dcr-1, and by inference the miRNA pathway, was required for w-miR-mediated suppression of eye pigmentation (Fig. 1G). We concluded that w-miR is a suitable reagent with which to carry out forward genetic screening for loci required for the miRNA pathway.

Genetic screening using w-miR recovers novel Microprocessor mutations

We used the suppression of eye pigment by w-miR as the basis of F1 genetic screens of the right arms of chromosome 2 and 3; screening of other chromosome arms is ongoing. We scored for eyes exhibiting mosaicism of eye color, then backcrossed and retested these for germline transmission of mutations and reproducibility of pigment modification. In principle, clonal changes in eye color can result in lighter patches (in which case, the mutation potentially enhances miRNA activity) or darker pigment (in which case, the mutation potentially suppresses miRNA activity). Examples of enhancers and suppressors of w-miR are shown in Figure 1H and 1I, respectively. Of course, it is also possible that clonal changes in eye color were due to mutations in the pigment biosynthetic pathway. Therefore, we performed a secondary test for the dependence of pigment modification on w-miR. Instead of using the eyf lp transgene to generate clones, we used a chromosome with ey-gal4 and UAS-FLP, both of which are carried on transgenes that use white+ as a selectable marker (Stowers and Schwarz 1999). Any mutant alleles that gave eye color mosaicism in this background were discarded because their effect on pigmentation is not w-miR-dependent. A full description of mutants isolated from these screens will be reported elsewhere (P Smibert and EC Lai, in prep.).

Two suppressor mutations recovered from chromosome 3R, Su6B3 and Su14D4, failed to complement each other and also failed to complement a previously described pasha deletion allele, pasha[KO] (Martin et al. 2009). We isolated genomic DNA from larvae trans-heterozygous for these mutations against pasha[KO], and amplified and sequenced the mutant pasha locus. Both alleles had missense mutations in the coding exons of pasha (Fig. 2A,D,E). Three suppressor mutations on chromosome 2R—Su6M1, Su15N1, and Su21K11—also exhibited eye mosaicism with patches of more pigment (Fig. 2L–N). All three failed to complement Df(2R)exc16055, which uncovers 33 genes (http://flybase.org/), including drosha. Sequencing of DNA from

![FIGURE 1](https://example.com/fig1.png)
larvae trans-heterozygous for Df(2R)exel6055 and each mutation revealed that all had nonsynonymous mutations in drosha-coding exons (Fig. 2B). One of these alleles, Su21K11, carried an R662X mutation that results in premature termination of the protein before the RNase III domains and is therefore expected to be a null mutant. This mutant behaves genetically as a null, as homozygotes are indistinguishable from animals trans-heterozygous for this allele and Df(2R)exel6055 (data not shown). To our knowledge, these represent the first reported mutant alleles of drosha in Drosophila.

These Microprocessor mutants were all recovered on the basis of clonal derepression of white+ function in the eye, but different alleles had distinct effects on eye morphology. Eyes bearing null clones for either pasha or drosha were smaller and rough, with disorganized or absent ommatidia (Fig. 2C,L). Eyes bearing clones of pasha[14D4] or drosha[6M1] were slightly less phenotypically severe than their respective null mutants (Fig. 2D,M). In contrast, clones for pasha[6B3] or drosha[15N1] appeared morphologically normal, with no obvious defects in ommatidial size or organization (Fig. 2E,N). Additionally, clones of these mutants occupied ~50% of the eye, compared with the stronger mutants whose clones occupied much less. This indicated a growth disadvantage for strong pasha and drosha alleles that was absent from the corresponding weak alleles.

To assess the overall developmental consequence of flies mutant for our novel alleles, we examined third instar brain
and imaginal disc clusters. To rule out potential contributions from second site mutations, as well as to provide direct comparison of allelic strengths, we placed each pasha or drosha allele in trans to the pasha[KO] or Df(2R)exel6055 deletions, respectively. For simplicity, we refer to these trans-heterozygous allelic combinations by the allele of interest. Homozygous pasha[KO] animals were used because these animals are rescued to viability with a genomic rescue transgene (Martin et al. 2009).

As described previously (Martin et al. 2009), pasha[KO] animals form small third instar larvae that are almost completely devoid of imaginal discs (Fig. 2F). These animals die as larvae or poorly formed early pupae. Some larvae develop rudimentary eye discs, but most animals lack any obvious imaginal discs. pasha[14D4] mutants have rudimentary eye discs that stain for the neural marker Elav (Fig. 2G), and these animals die as early pupae. In contrast, pasha[6B3] animals have a virtually wild-type complement of imaginal discs surrounding a normally sized brain and go on to die as pharate adults (Fig. 2H). Similar to pasha-null animals, drosha[21K11] animals die as third instar larvae or poorly formed early pupae. At the third larval instar, these animals are almost completely lacking imaginal disc structures (Fig. 2O). drosha[6M1] animals exhibit an intermediate phenotype with respect to imaginal tissues at the third larval instar, with rudimentary Elav positive eye discs, but no other imaginal tissues (Fig. 2P). In contrast, drosha[15N1] animals have a full complement of imaginal discs and look morphologically indistinguishable from wild type (Fig. 2Q). While trans-heterozygotes of drosha[15N1] over the deletion die as pharate adults, drosha[15N1] homozygotes occasionally survive to adulthood.

**Differential defects in miRNA biogenesis and function in Microprocessor mutants**

It was unexpected that screening for failure to repress a miRNA-sensitive reporter could yield pasha and drosha alleles that could survive to adulthood, as the miRNA pathway is universally essential for viability in all animals. We therefore conducted a detailed series of functional assays of miRNA biogenesis and function on our allelic series of pasha and drosha mutants. We first generated mitotic clones in the wing imaginal disc and assayed for the levels of previously described sensors of miRNA activity. We used tub-GFP-bantam and tub-GFP-dLMO, which are ubiquitously expressed GFP transgenes driven by the β-tubulin promoter followed by two copies of a sequence complementary to the bantam miRNA, or the 3′ UTR of the miRNA-regulated gene dLMO (Brennecke et al. 2003; Bejarano et al. 2010). Additionally, we stained for endogenous Mei-P26 protein, a miRNA target that is derepressed in dcr-1 and ago1 mutant clones (Herranz et al. 2010). In all alleles examined, homozygosity for the Microprocessor mutations resulted in derepression of both the GFP sensors and Mei-P26 (Fig. 2I–K,R–T). In the case of pasha mutations, the level of derepression of both GFP-bant and Mei-P26 correlated with allele strength with strong derepression for the null mutant and only very mild derepression in the weak pasha[6B3] clones (Fig. 2I–K). However, despite the difference in phenotypic severity of the drosha alleles examined at the level of morphology, no obvious differences in the level of derepression of either GFP-dLMO or Mei-P26 were observed (Fig. 2R–T). In other words, these weak drosha alleles nevertheless exhibited strong derepression of artificial and even endogenous miRNA targets.

The roles of Drosha in miRNA biogenesis, rRNA maturation, and miRNA cleavage have been extensively studied in vitro using biochemistry and knockdowns in cultured cells, but in vivo studies are comparably limited. We took the opportunity to test the requirements for Drosha in RNA metabolism using these first-available Drosophila drosha mutants. The best characterized role for Drosha is to cleave pre-miRNAs from primary miRNA transcripts (Lee et al. 2003; Denli et al. 2004; Gregory et al. 2004; Han et al. 2004; Landthaler et al. 2004). A subset of miRNAs is produced from Drosha-independent substrates, including mirtrons, which are short hairpin introns that use splicing to generate the pre-miRNA (Okamura et al. 2007; Ruby et al. 2007a). Our studies of a pasha knockout verified that this Drosha cofactor was required for in vivo maturation and function of canonical miRNAs but not mirtrons (Martin et al. 2009). Here, we demonstrate the direct involvement of the Drosha nuclease for canonical miRNA biogenesis in the animal. qRT-PCR assays of 5–6-d-old third instar larvae revealed strong accumulation of primary miRNA transcripts in drosha[21K11] animals, comparable to pasha[KO] animals isolated in parallel (Fig. 3A). Northern analysis of the whole panel of Microprocessor mutants showed that levels of mature miR-14, miR-2a, and miR-9a were decreased in all cases, whereas mirtron-derived miR-1003 was unaffected in any of the mutants (Fig. 3B).

Notably, for both pasha and drosha mutants, the level of reduction in mature miRNAs correlated with the strength of developmental and viability defects observed. The strongest loss of mature miRNA species was observed for the null mutations, pasha[KO] and drosha[21K11], followed by the intermediate strength mutations, pasha[14D4] and drosha[6M1]. The weak hypomorphic mutations, pasha[6B3] and drosha[15N1], had only modest reductions in mature miRNA levels, consistent with the modest phenotypes associated with their homozygosity (Figs. 2C–H,L–Q, 3B). Given this correlation, it was surprising that we observed strong derepression of multiple miRNA sensors and even of the endogenous miRNA target Mei-P26 in mutant clones of drosha[15N1], a weak allele that exhibits only subtle effects on absolute mature miRNA levels. We hypothesize that certain readouts and/or targets of miRNA-mediated repression are more sensitive than others, a ge-
netic situation that can be appreciated by availability of an allelic series. We revisit the biological implications of this below in this study.

Drosha was initially identified as an RNase III enzyme required for maturation of ribosomal RNAs. Antisense inhibition of Drosha resulted in accumulation of 12S and 32S rRNA species, interpreted as a partial block in rRNA processing (Wu et al. 2000). Subsequently, loss of the Microprocessor accessory factors, the DEAD-box helicases p68 and p72, was reported to decrease 5.8S rRNA maturation (Fukuda et al. 2007). We analyzed 5.8S rRNA levels across our panel of Microprocessor mutants at the third larval instar, which are presumably depleted of stores of maternal protein inherited 5–6 d earlier. However, we did not observe any obvious differences from control Canton S animals (Fig. 3C). The absence of rRNA biogenesis defects in drosha null flies parallels an analysis from Drosha knockout mouse thymocytes, which indicated no defects in rRNA levels (Chong et al. 2008).

In vivo demonstration of cross-regulation of the Microprocessor

In addition to its major role in nuclear miRNA biogenesis, Drosha also cleaves certain mRNA hairpins. Its most well-characterized mRNA target encodes DGC88, the mammalian ortholog of Pasha (Han et al. 2009; Triboulet et al. 2009). Reciprocally, DGC88/Pasha proteins are required for Drosha stability, thus fulfilling a post-transcriptional and post-translational homeostatic loop that tunes appropriate levels of these core Microprocessor components (Han et al. 2009). Studies in S2 cell culture showed that the capacity of Drosha to cleave and down-regulate pasha transcript is conserved in Drosophila (Han et al. 2009; Kadener et al. 2009).

We used our Microprocessor mutant alleles to perform strict genetic tests of the Microprocessor cross-regulatory loop in the animal. Mitotic clones of drosha[21K11] showed derepression of both an endogenous miRNA target and a sensor for miRNA activity (Fig. 2R) and a strong reduction of Drosha (Fig. 4A). Unfortunately, the available Drosha antibody (Denli et al. 2004) is not entirely specific in tissue staining but exhibits strongly decreased immunoreactivity in drosha-null clones. Mitotic clones of pasha[KO] also showed derepression of both an endogenous miRNA target and a sensor for miRNA activity (Fig. 2I). Furthermore, pasha[KO] clones exhibited strongly reduced Drosha staining equivalent to drosha mutant clones (Fig. 4B), indicating that Drosophila Pasha is required for Drosha stability in the animal.

Knockdown of drosha in S2 cells increases pasha transcript levels (Han et al. 2009), but the effect on Pasha protein was unknown. We generated mitotic clones of null mutants of pasha (Martin et al. 2009), dicer-1 (Lee et al. 2004), and drosha (this work), all of which derepress miRNA sensors and endogenous miRNA targets (Figs. 2, 4D–F), and assayed

FIGURE 3. Effects of Microprocessor mutants on small RNA maturation. (A) Accumulation of pri-miRNA transcripts in null Microprocessor mutants. Total RNA was extracted from Canton S, homozygous pasha[KO], or trans-heterozygous drosha[21K11]/Df(2R)exel6055 animals and assayed by qRT-PCR. The y-axis represents the fold up-regulation of the indicated miRNA relative to the Canton S sample and standard deviation. (B) Mature miRNA levels in Microprocessor mutants. Total RNA was extracted from third instar larvae of the indicated genotypes. For the pasha alleles, indicated alleles were in trans with pasha[KO]. For the drosha alleles, indicated alleles were in trans with Df(2R)exel6055. The small RNA blot was sequentially probed as indicated. 2S rRNA was used as a loading and transferring control. (C) Ribosomal RNA maturation is unaffected in Microprocessor mutants. RNA from the same samples was run simultaneously on two gels, transferred, and probed for 5S and 5.8S rRNA.
Pasha protein levels. As expected, clones homozygous for a null pasha mutant lack Pasha completely (Fig. 4H). Clonal loss of Dcr-1 had no discernible effect on Pasha levels (Fig. 4I), demonstrating that Pasha levels are not significantly affected by global reduction of miRNAs. However, loss of Drosha, which also globally reduces miRNAs and miRNA pathway activity (Figs. 2R, 4F), resulted in a cell-autonomous increase in Pasha (Fig. 4J). Since Pasha protein was not up-regulated in dcr-1 clones, its up-regulation in drosha clones is miRNA-independent and instead consistent with direct cleavage of pasha transcript by Drosha.

We performed a stringent test of this notion by introducing a mutated pasha genomic construct into the pasha deletion background, such that the only source of pasha was transgenically expressed under endogenous transcriptional control. We identified two pasha 5'-UTR hairpins that generated short RNA reads consistent with a low level of miRNA-like biogenesis (Supplemental Fig. 2) and deleted these from the construct. This transgene was inserted onto chromosome 3L and recombinated with pasha[KO]. When introduced this recombinant third chromosome into the FRT42D drosha[21K11] background and generated negatively marked hs-FLP clones. In this experiment, despite the loss of Drosha and the derepression of an endogenous miRNA target (Fig. 4G), the level of Pasha protein was unchanged (Fig. 4K). This confirms that despite the different primary sequences of the hairpins, the molecular logic and precise cross-regulation of Microprocessor components is conserved between Drosophila and mammals.

Synaptic function is sensitive to global reduction of miRNA levels in photoreceptor neurons

In Drosophila, as in other organisms, loss of core components of the miRNA pathway causes a plethora of developmental consequences (Lee et al. 2004; Hatfield et al. 2005; Jin and Xie 2007; Park et al. 2007; Martin et al. 2009). However, it is difficult even with conditional knockout techniques to bypass developmental effects. Our hypomorphic mutants of core miRNA pathway components provided an opportunity to assess the relative sensitivity of development, function, and maintenance on miRNA levels in a specialized cell type. In particular, the observation that the misregulation of certain miRNA targets did not strictly correlate with overall status of
the miRNA pathway suggested that it might be possible to discern specific cellular settings that are especially sensitive to miRNA levels.

In addition to our weak drosha[15N1] and pasha[6B3] alleles, we screen isolated novel alleles of dcr-1, dcr-1[18E6], and dcr-1[19E2], which have missense mutations in the double-stranded RNA binding domain and the second RNase III domain, respectively (Fig. 5A). These hypomorphic mutations exhibited w-miR derepression and adult eye clonal morphology reminiscent of our previously described pasha[6B3] and drosha[15N1] hypomorphic alleles (Figs. 2E,N, 5B–D). They yielded morphologically normal, pharate adult lethal animals with some escapers in trans with either a dcr-1 null mutation, dcr-1[Q1147X], or a deficiency that removes the dcr-1 locus, Df(3R)ED6906 (Lee et al. 2004; Ryder et al. 2007). Together with the hypomorphic pasha[6B3] and drosha[15N1] alleles, we used these reagents to assess post-developmental roles for the miRNA pathway in the eye.

Since external morphology does not reliably report on possible defects of internal structures, we first performed additional analysis on animals bearing whole eye clones of hypomorphic miRNA biogenesis mutants. These tests indicated normal specification of photoreceptor and support cells. Moreover, the mutant photoreceptors retained normal targeting properties and established layer-specific terminals in the optic neuropils lamina and medulla that are indistinguishable from wild type (Fig. 5E–H). These findings demonstrated that development and wiring of the eye was not substantially affected by a mild reduction in miRNA biogenesis.

Photoreceptor neuron function and maintenance (or degeneration) is easily assessed through electroretinogram recordings (Williamson et al. 2010). Electroretinograms (ERGs) are extracellular recordings of the photoreceptor response to a light stimulus that exhibit two main features: The depolarization amplitude, which is reduced when phototransduction or cell health is defective or degrades over time, and the “on” transient, a post-synaptic response that is lost when synaptic transmission is blocked (Fig. 6A). Hence, ERGs provide a measure for both neuronal degeneration and function over time.

We performed ERGs on whole eye clones of our miRNA pathway hypomorphic mutants using the ey3.5FLP system, which selectively renders the pre-synaptic photoreceptor neurons mutant (Fig. 6B; Chotard et al. 2005; Mehta et al. 2005). Whole eye clones of pasha[6B3] showed no substantial difference in the amplitude of depolarization compared with control eyes, indicating that this mutation does not affect the photoreceptor response to stimulation. However, pasha[6B3] ERGs exhibited significantly decreased “on” transients. This defect is significantly rescued by including a single copy of a pasha genomic rescue construct in the pasha[6B3] background. We saw a stronger reduction of “on” transient amplitude in drosha[15N1] whole eye clones, which we could also rescue to near wild-type levels with a single copy of a drosha genomic rescue construct. In addition, our two independent hypomorphic dcr-1 alleles displayed significant reduction in “on” transient amplitude with no significant effect on depolarization (Fig. 6B). The synaptic transmission defects cannot be attributed to post-synaptic neurons because only the pre-synaptic photoreceptors were mutant in these experiments. Taken together, our findings reveal a specific loss of neurotransmission in hypomorphic alleles for drosha, pasha, and dcr-1 that all mildly reduce miRNA levels without affecting development. These data suggest that synaptic transmission is more sensitive to miRNA levels than development of photoreceptor neurons.

It was conceivable that a gradual loss of synaptic transmission precedes neuronal degeneration in the presence of reduced miRNA pathway activity. To test this notion, we compared the depolarization and “on” transients of drosha[15N1] and pasha[6B3] in 1-wk and 3-wk aged flies. As shown in Figure 6B,C, the recordings were virtually identical. We conclude that neither the healthy depolarization upon light stimulation nor the synaptic transmission
transient amplitude that is rescued by a single copy of a genomic rescue
dcr-1 transgene. Two independent alleles of genotype mean depolarization or “on” transient amplitude of >10 flies per of large-scale photoreceptor degradation or death. Values represent the or depolarization in any genotypes examined, demonstrating an absence analysis in 3-wk-old flies revealed no change in “on” transient amplitude to receptor cell bodies was not significantly different from controls. ERG loss of “on” transient amplitude. In all cases, depolarization of the pho-
clones of transients measurements of indicated genotypes. Whole eye alleles (see also Materials and Methods). (A) Illustration of the ERG procedure and description of measurements. Electroretinograms (ERGs) were performed on animals with whole eye clones of the indicated mutant alleles (see also Materials and Methods). (B) Depolarization and (C) “on” transient amplitude measurements of indicated genotypes. Whole eye clones of drosot15SN1] and pasha[6B3] result in significant loss of “on” transient amplitude that is rescued by a single copy of a genomic rescue transgene. Two independent alleles of dcr-1 also exhibited a significant loss of “on” transient amplitude. In all cases, depolarization of the photo-
toceptor cell bodies was not significantly different from controls. ERG analysis in 3-wk-old flies revealed no change in “on” transient amplitude or depolarization in any genotypes examined, demonstrating an absence of large-scale photoreceptor degradation or death. Values represent the mean depolarization or “on” transient amplitude of >10 flies per genotype ± standard error of the mean. ***P < 0.01, *P < 0.05. defect deteriorates over time. These findings suggest that the reduced neurotransmission in the Microprocessor hypomorphs is not secondary to neuronal maintenance defects and, reciprocally, that miRNA function is acutely required for synaptic function.

**DISCUSSION**

**An efficient in vivo genetic screen for miRNA pathway components**

In this study, we describe a forward genetic screen for factors involved in miRNA biogenesis or function and validate its utility by characterizing a series of core miRNA pathway mutants. We used these to investigate Microprocessor cross-regulation in vivo, as well as to study post-develop-
mental roles of miRNAs in neural function. In particular, we provide the first loss-of-function analysis of Drosophila drosot. As expected, we observed a strong block in canonical miRNA biogenesis in the drosot-null mutant, which accumu-
lationary primary miRNA transcripts and was depleted of mature miRNAs, similar to pasha mutants (Martin et al. 2009). In addition, we found mirtron biogenesis to be unaffected by drosot mutation, providing direct evidence that these splicing-derived miRNAs are completely independent of the Drosha nuclease. Animals null for drosot and pasha are generally similar with respect to all phenotypes examined, supporting the obligate nature of these core protein partners within the Microprocessor complex.

Our screen generated allelic series for the key Micropro-
cessor components drosot and pasha. These allowed us for the first time to assess the sensitivities of development versus function in cells with mildly reduced miRNA levels. Compared with full knockouts that give only null states, forward genetic screening can yield allelic series of varying phenotypic severity, which can uncover interesting aspects of pathway and gene function. We note that our hypo-
morphic drosot and pasha mutants have very different effects in different assays, as highlighted by the differences in derepression of both an endogenous miRNA target and a transgenic sensor for miRNA activity between our weak drosot and pasha alleles (Fig. 2). This is the case despite the fact that these mutants have similar effects on total mature miRNA levels as measured by Northern blot (Fig. 3B). The reason for this phenotypic discrepancy is not yet clear but suggests the possibilities that miRNAs are differentially sensitive to availability of the core biogenesis machinery or that specific biological processes are especially sensitive to optimal miRNA biogenesis. Indeed, we have provided electrophysiological evidence of the latter, and both of these hypotheses represent compelling future directions for analysis of these and other mutants that may emerge from genetic screening.

The theme of miRNA pathway autoregulation has emerged at multiple levels in animals (Forman et al. 2008; Han et al. 2009; Kadener et al. 2009; Triboulet et al. 2009; Martello et al. 2010; Bennasser et al. 2011) and in plants (Xie et al. 2003; Vacher et al. 2004; Rajagopalan et al. 2006; Vaucheret et al. 2006). Our in vivo demonstration of the reciprocal regulation of the core Microprocessor compo-
nents reveals that this mechanism is conserved and occurs within the intact animal. The role of Pasha within the Microprocessor to position Drosha catalytic centers is clearly of crucial importance to miRNA biogenesis. Perhaps the instability of Drosha in the absence of Pasha is a biological safeguard to prevent inappropriate cleavage of transcripts by Drosha in the absence of Pasha. Likewise, the capacity of Drosha to cleave pasha transcripts may also limit Drosha
levels by restricting the amount of Pasha for it to associate with.

Our studies to date focused on mutants of known miRNA pathway components. This has been a productive effort, as indicated by our first reported Drosophila alleles of drosa, the first allelic series of pasha in any organism, and novel alleles of dicer-1. Our screening recovered other suppressor mutations that do not map to known pathway components, as well as enhancer mutations that are dependent on the w-miR trigger. We anticipate that the cloning and characterization of these mutations may provide additional insights into the miRNA biogenesis machinery or the mechanism of miRNA-mediated silencing.

**Hypomorphic alleles reveal a synaptic transmission defect caused by mild reduction of miRNA pathway activity**

miRNAs in whole organisms have to date mostly been studied for their roles in development. This is at least in part due to the early developmental defects that potentially mask later functional defects. Yet, miRNAs have long been viewed as a potentially key component of neural function and fine-tuning due to their regulatory potential. One tantalizing property of miRNAs is their ability to regulate gene expression locally. In neurons, where a synapse may be a great distance from the nucleus, this could provide a means for rapid post-transcriptional regulation of targets. How this may be regulated in a signal-dependent manner is a topic of ongoing study (Ashraf et al. 2006; Krol et al. 2010). In this study, we present novel hypomorphic mutants that mildly affect miRNA levels and cause synapse function defects without affecting development.

The requirement of neurons for precise miRNA activity is emphasized by the specific synaptic transmission defects in our hypomorphic miRNA pathway mutants. In weak alleles of either pasha or drosa, we observed only a mild reduction in miRNA biogenesis. While these animals quickly succumb just before or after adult eclosion, they exhibit grossly normal development of all external structures. Using whole eye clone analysis, we documented normal specific projection of mutant photoreceptors, as well as enhancer mutations that are dependent on the w-miR trigger. We anticipate that the cloning and characterization of these mutations may provide additional insights into the miRNA biogenesis machinery or the mechanism of miRNA-mediated silencing.

**MATERIALS AND METHODS**

**Molecular biology and biochemistry**

**Generation of w-miR**

pGUS-DsRed was generated by cloning the DsRed.T4 CDS from Red-H-Pelican (Barolo et al. 2004) as an NcoI/blunt/NotI fragment into pGUS (Brodsky et al. 2000) cut with EcoRI/blunt/NotI. We used the strategy of Hay and colleagues to reprogram the mir-6-1 backbone (Chen et al. 2007) to produce a miR targeted against the white 3' UTR. The partially complementary primers mir6.1 wmiRNA1 For: GGCGAGTACTTACCTTTACCTACACAGCTTT TACGTTTACCACGTGGCAGCTTTA TATC and mir6.1 wmiRNA1 Rev: AATAATGATGTTAGGCACT ATCAAGGAAAATGAAAGTCAAAGTTGGCAGCTTACTTAAAC 9 9 9

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TTA and mir6.1 3’ Xbal: gatctctagaaaaacgccagtttcggt TGGCCCAAAAAAATAATGAGTTAGGAC. This product was digested with NotI and Xbal and cloned into pGUS-DsRed. Transgenic animals were backcrossed to pavar and EMS treated. Small RNA Northern blots were performed using RNA-labeled gels and LNA probes (Exiqon) as described (Okamura et al. 2007).

**Drosophila genetic screen**

We mutagenized flies by collecting about 50–70 males and starved them by placing them in a dry empty vial with Whatman paper for ~8 h. Flies were then fed overnight with a solution of 25 mM MES in 20% sucrose injected onto the Whatman paper. The males were then given 8 h to recover on normal food before being mated with females in a freshly yeasted bottle. Typically, five vials of males were mutagenized in each round, and mated females were allowed to lay on three consecutive sets of bottles for 2 d each. The adult flies were then discarded.

The screen for chromosome 3R was performed as follows. Virgin females of the genotype w; GMR-w-miR, eyFLP; isoFRT82B were crossed with mutagenized males of the genotype w; Sco/CyO: isoFRT82B. F1 progeny with eye color mosaicism were crossed again with w; GMR-w-miR, eyFLP; isoFRT82B to check for germline transmission of the mutation. Progeny displaying mosaicism were crossed to w; Sco/CyO: MKRS/TM6B. As the mutagenized chromosome is superficially impossible to distinguish from the nonmutagenized chromosome, individual males (about 10) were then crossed to w; Sco/CyO: MKRS/TM6B, and stocks were generated that were w; Sco/CyO: FRT82B, mutant!/TM6B. Half of these stocks are expected to have the mutagenized FRT82B chromosome. One stock per original mutant that could reproduce the original phenotype when crossed to w; GMR-w-miR, eyFLP; isoFRT82B was kept. The screen on chromosome 2R was carried out with the same strategy as described for 3R, with the exception that an isogenized FRT42D chromosome and a GMR-w-miR, eyFLP chromosome III were used instead. Images of eye clones in adult animals presented are of the genotype w; GMR-w-miR, eyFLP/Sco or CyO; FRT82 [X]/FRT82, where [X] denotes the appropriate pasha or dcr-1 allele or w; FRT42 [Y]/FRT42; GMR w-miR, eyFLP/MKRS or TM6B, where [Y] denotes the appropriate drosa allele.

**Clonal analysis and immunostaining**

We analyzed the functional properties of mutants isolated in this screen, as well as FRT82B pasha[KO] (Martin et al. 2009) and FRT82B dcr-1[Q1147X] (Lee et al. 2004), by introducing them into backgrounds carrying hs-FLP and the miRNA sensor transgenes tub-GFP-bantam (Brennecke et al. 2003) or tub-GFP-dLMO (Lee et al. 2004). Clonal analysis and immunostaining was performed as previously described (Lai and Rubin 2001). Antibodies used were mouse anti-β-galactosidase (1/50) and rat anti-Elav (1/50) from the Developmental Studies Hybridoma Bank, Chicken anti-GFP (1/1000, Abcam), and Stocks were generated that were w; Sco/CyO: FRT82B, mutant!/TM6B. Half of these stocks are expected to have the mutagenized FRT82B chromosome. One stock per original mutant that could reproduce the original phenotype when crossed to w; GMR-w-miR, eyFLP; isoFRT82B was kept. The screen on chromosome 2R was carried out with the same strategy as described for 3R, with the exception that an isogenized FRT42D chromosome and a GMR-w-miR, eyFLP chromosome III were used instead. Images of eye clones in adult animals presented are of the genotype w; GMR-w-miR, eyFLP/Sco or CyO; FRT82 [X]/FRT82, where [X] denotes the appropriate pasha or dcr-1 allele or w; FRT42 [Y]/FRT42; GMR w-miR, eyFLP/MKRS or TM6B, where [Y] denotes the appropriate drosa allele.

**Clonal analysis and immunostaining**

We analyzed the functional properties of mutants isolated in this screen, as well as FRT82B pasha[KO] (Martin et al. 2009) and FRT82B dcr-1[Q1147X] (Lee et al. 2004), by introducing them into backgrounds carrying hs-FLP and the miRNA sensor transgenes tub-GFP-bantam (Brennecke et al. 2003) or tub-GFP-dLMO 3’ UTR (Bejarano et al. 2010). Chromosome 3R clones were generated in the genotype hs-FLP; GFP-ban+/; FRT82B [X]/FRT82B, arm-lacZ (Fig. 2) or hs-FLP; FRT82B [X]/FRT82B, arm-lacZ (Fig. 5), where [X] denotes the relevant mutant allele. Chromosome 2R clones were generated in the genotype hs-FLP; FRT42D [Y]/FRT42D, arm-lacZ; GFP-dLMO/+ (Fig. 2) or hs-FLP; FRT42D [Y]/FRT42D, arm-lacZ (Fig. 5), where [Y] denotes the appropriate mutant allele. For Figure 5G, the genotype analyzed was hs-FLP; FRT42D, drosa[12K11]/FRT42D, arm-lacZ; attP2+PBP DsRed::ΔHP rescue, pasha[KO].

Immunostaining was performed as previously described (Lai and Rubin 2001). Antibodies used were mouse anti-β-galactosidase (1/50) and rat anti-Elav (1/50) from the Developmental Studies Hybridoma Bank, Chicken anti-GFP (1/1000, Abcam), and mouse anti-β-galactosidase (1/50).
rabbit anti-Mei-P26 (1/1000) (Liu et al. 2009), rabbit anti-Pasha (1/1000), and rabbit anti-Drosha (1/1000) (Denli et al. 2004). We used appropriate secondary antibodies conjugated to Alexa 488, 568, and 647 (Molecular Probes).

Electrophysiology

Electroretinograms (ERGs) were performed as described in Fabian-Fine et al. (2003) with the following modifications: Flies were fixed using Elmer’s nontoxic Glue-All. We used 2 M NaCl in the recording and reference electrodes. Recordings were performed using a Warner IE-210 amplifier and Clampex 10.1 software by Axon Instruments. A post-recording filter was also provided by the Clampex software. Light stimulus was provided in 1-sec pulses by a computer-controlled white LED system (Schott MC1500).

The following genotypes were used:
ey3.5FLP; FRT42D, CL/CyO (FRT42D Ctrl)
ey3.5FLP; FRT42D, CL/FRT42D, drosha[15N1] (FRT42 drosha)
ey3.5FLP; FRT42D, CL/FRT42D, drosha[15N1]; attP2-BDP

drosha+/ (FRT42 drosha rescue)
ey3.5FLP; FRT82B, CL/TM3, Sb (FRT82 Ctrl)
ey3.5FLP; FRT82B, CL/FRT82B, pasha[6B3] (FRT82 pasha 6B3)
ey3.5FLP; FRT82B, CL/attP2-BDP pasha-FlagHA, FRT82B, pasha[6B3] (FRT82 pasha 6B3 rescue)
ey3.5FLP; FRT82B, CL/FRT82B, dcr-1[18E6]
ey3.5FLP; FRT82B, CL/FRT82B, dcr-1[19E2]

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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Authors’ contributions: P.S., P.R.H., and E.C.L. conceived and designed the experiments. P.S., F.B., D.W., D.L.G., J.Y., R.M., D.B.B., and N.R. performed the experiments. P.S., D.W., P.R.H., and E.C.L. analyzed the data. P.S., P.R.H., and E.C.L. wrote the paper.

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