Supporting Information

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SI Materials and Methods

***Drosophila* Strains.** Strains used include the following: (*i*) y^1 *w* P{y+, w^ORF::br^3′UTR}; P{ry[+t7.2] = neoFRT}40A*; *P{ry[+t7.2] = ey-FLP.N}6*, (*ii*) y^1 *w* P{y+, w^ORF::br^3′UTR}; P{ry[+t7.2] = neoFRT}42D Mi{ET1}emp[MB02756]*; *P{ry[+t7.2] = ey-FLP.N}6*, (*iii*) y^1 *w* P{y+, w^ORF::br^3′UTR}; P{ry[+t7.2] = ey-FLP.N}5*; *P{neoFRT}82B*, (*iv*) y^1 *w* P{y+, w^ORF::w^3′UTR+6Xlet-7AS}*, (*v*) y^1 *w* P{y+, w^ORF::w^3′UTR+6XmiR-125AS}*, (*vi*) y^1 *w* P{y+, w^ORF::w^3′UTR+6XmiR-100AS}*, (*vii*) *let-7-C^KO1w-* *P{ry[+t7.2] = neoFRT}40A*, (*viii*) *P{neoFRT}82B dcr-1^Q1147X* (gift from R. Carthew), (*ix*) *P{neoFRT}82B pasha^KO* (gift from E. Lai, Memorial Sloan-Kettering Cancer Center, New York), (*x*) *P{ry[+t7.2] = neoFRT}42D drosha^R662X* (gift from E. Lai), (*xi*) *P{ry[+t7.2] = neoFRT}42D drosha^W884X* (gift from R. Carthew, Northwestern University, Evanston, IL), (*xii*) *P{ry[+t7.2] = neoFRT}42D drosha^R1113X*, (*xiii*) *P{ry[+t7.2] = neoFRT}42D drosha^Δ, E859K*, (*xiv*) *P{neoFRT}42D P{Ubi-GFP(S65T)nls}2R* (BL 5626), (*xv*) attP2 > pBDPDrosha (gift from E. Lai), (*xvi*) *P{hsFLP}22, y1 w* P{UAS-mCD8::GFP.L}Ptp4ELL4; P{ry[+t7.2] = neoFRT}42D P{w[+mC] = tubP-GAL80}LL2*; *P{w[+mW.hs] = GawB}ey[OK107]*, (*xvii*) *P{hsFLP}22, y1 w* P{UAS-mCD8::GFP.L}Ptp4ELL4*; *P{ry[+t7.2] = neoFRT}82B P{w[+mC] = tubP-GAL80}LL3*; *P{w[+mW.hs] = GawB}ey[OK107]*, (*xviii*) *y1 w* P{UAS-mCD8::GFP.L}Ptp4ELL4, P{GawB}acj6PG63*; *P{ry[+t7.2] = neoFRT}42D P{w[+mC] = tubP-GAL80}LL2*, (*xix*) PBac{GH146-GAL4.B}; *P{ry[+t7.2] = neoFRT}42D P{w[+mC] = tubP-GAL80}LL2;* P{UAS-mCD8::GFP.L}LL6, (*xx*) P{ry[+t7.2] = hsFLP}122*;* P{GawB}GH146 P{UAS-mCD8::GFP.L}LL5*; P{ry[+t7.2]= neoFRT}82B P{w[+mC] = tubP-GAL80}LL3*, (*xxi*) Df(2R) Exel6055, P{w[+mC]=XP-U}Exel6055/CyO (BL7537), and (*xxii*) P{ry[+t7.2] = neoFRT}42D P{w[+mC]=Ubi-GFP(S65T)nls}2R (BL5626). The *let-7-C^KO1w-* allele is a *white*$^-$ version of *let-7-C^KO1* that was generated by ethylmethylsulfonate (EMS) mutagenesis. P-element containing *UAS-Drosha* transgenes were injected by Rainbow Transgenic Services. Insertions were mapped by standard methods, and at least two different insertions were analyzed for phenotypes.

Eye and Central Nervous System Clones. To generate eye clones, virgins containing eyFLP and either a WT or mutation-bearing *FRT^42D* chromosome were crossed to males containing an *FRT^42D* Ubi-GFP chromosome. Resulting progeny were dissected as third-instar larvae. To generate mosaic analysis with a repressible cell marker (MARCM) clones in mushroom bodies (MBs), *P{hsFLP}22, y1 w* P{UAS-mCD8::GFP.L}Ptp4ELL4; P{ry[+t7.2] = neoFRT}42D P{w[+mC] = tubP-GAL80}LL2*; *P{w[+mW.hs]=GawB}ey[OK107]* or *P{hsFLP}22, y1 w* P{UAS-mCD8::GFP.L}Ptp4ELL4*; *P{ry[+t7.2] = neoFRT}82B P{w[+mC]=tubP-GAL80}LL3*; *P{w[+mW.hs] = GawB}ey[OK107]* virgins were crossed to flies carrying mutation-bearing *FRT^42D* or *FRT^82B* chromosomes. To generate MARCM clones in anterodorsal projection neurons (adPNs), *y1 w* P{UAS-mCD8::GFP.L}Ptp4ELL4, P{GawB}acj6PG63*; *P{ry[+t7.2] = neoFRT}42D P{w[+mC] = tubP-GAL80}LL2* or PBac{GH146-GAL4.B}; *P{ry[+t7.2] = neoFRT}42D P{w[+mC] = tubP-GAL80}LL2;* P{UAS-mCD8::GFP.L}LL6 virgins were crossed to flies carrying mutation-bearing *FRT^42D* chromosomes. Flies were allowed to lay eggs for 6 h and were transferred to new vials. Embryos were then aged for 18–20 h. Newly hatched larvae between 18 and 26 h after egg laying were heatshocked at 38 °C for 1 h to mark MB and adPN neuroblast clones. Single cell MARCM clones in the adPN lineage were generated using a similar method with a 30-min heat shock.

Mutagenesis Screen. Isogenized *FRT^42D* males were fed 25 mM EMS and crossed to *let-7-C* sensor virgins of the genotype y^1 *w* P{y+, w^ORF::br^3′UTR}; FRT^42D Mi{ET1, GFP}emp; eyFLP^6*. Male progeny that displayed eye color mosaicism were backcrossed to the sensor strain, and any of their male progeny that continued to display eye color mosaicism were used to generate balanced GFP negative stocks.

Plasmids. *Drosophila melanogaster* Drosha and Pasha cDNAs were PCR amplified from LD20030 and LD23072 plasmids available from the *Drosophila* Genomic Resource Center. The PCR products were cloned into pENTR/D-TOPO (Invitrogen). Plasmids expressing C-terminal Flag, HA or Myc-tagged versions of Drosha were generated by recombining pENTR-Drosha with pAWF, pAWH, or pAWM Gateway plasmids (T. Murphy, Carnegie Institute for Science, Baltimore; obtained from *Drosophila* Genomic Resource Center, Bloomington, IN), respectively, in an LR Clonase enzyme (Invitrogen) reaction. Plasmids expressing N-terminal Flag or HA-tagged Pasha were similarly generated by recombining pENTR-Pasha with pAFW or pAHW (T. Murphy; obtained from *Drosophila* Genomic Resource Center, Bloomington, IN), respectively. Plasmids expressing C-terminal Flag tagged Drosha containing E859K, Δ, E859K+Δ, or dominant negative (DN) mutations were generated by mutating pENTR-Drosha using the QuikChange Lightning Multi Mutagenesis Kit (Agilent Technologies) and then recombining the mutant version into pAWF. The DN version of Drosha contains two point mutations (E912Q and E1087Q) that are orthologous to the transdominant Drosha mutations described in Heo et al. (1).

Transgenes. *P{w^ORF} reporters*. The pP{w^{ORF}::$w^{UTR+BXG}$} vector, shown in Fig. S2, was derived from the pP{SUPor-P} vector, a gift from P. Geyer (University of Iowa, Iowa City, IA) by inserting a BamHI, XbaI and BglII polycloning site between the *white* ORF and the *white* 3′UTR. pP{w^{ORF}::$w^{UTR+6xlet-7as}$}, pP{w^{ORF}::$w^{UTR+6xmiR-100as}$}, pP{w^{ORF}::$w^{UTR+6xmiR-125as}$} contain six repeats of perfectly complementary sequence to either *let-7*, *miR-100*, or *miR-125* inserted into the XbaI of pP{w^{ORF}::$w^{UTR+BXG}$}, respectively. pP{w^{ORF}::$br^{3'UTR}$} was generated by ligating an error-free PCR amplified version of the *broad-Z3* 3′UTR into the NotI and XbaI sites of pP{w^{ORF}::*MCS*}.

UAS transgenes expressing C-terminal Flag-tagged WT Drosha or Drosha containing E859K, Δ, E859K+Δ, or DN mutations were generated by recombining WT or mutant pENTR-Drosha into pTWF (*Drosophila* Genomic Resource Center, Bloomington, IN).

Northern Blot Analysis. Total RNA was extracted from staged WT and mutant larvae using Tri Reagent (Molecular Research Center). Radioactive Northern blot analysis was performed as previously described. The radioactive signal was detected with a Amersham Bioscience Typhoon 9210 (Physical Biochemistry Instrumentation Facility, Indiana University).

Expression and Affinity Purification of Recombinant Drosha Proteins. Expression plasmids for Flag-Drosha and Flag-Pasha were cotransfected into BG3-c2 cells with Effectene transfection reagent (Qiagen). Cells were plated onto 10-cm dishes at a density of 1×10^6 cells/mL, transfected with 6 μg Flag Drosha and 4 μg of Flag Pasha plasmid DNA along with 50 μL Effectene per dish, and harvested after 72 h. Complexes were purified from cell lysates with anti-Flag M2 affinity gel (Sigma) as described previously (2). The affinity column was eluted with 400 μg/mL of 3×

FLAG peptide (Sigma) at 4 °C for 60 min. The eluate was then concentrated using Microcon concentrator columns (Millipore).

Generation of pri-let-7 Transcript and In Vitro Processing Assay. A 292-bp minimal pri-let-7–containing PCR product was generated containing the T7 sequence and the 78-bp let-7 hairpin and some flanking sequence. This minimal *pri-let-7* was transcribed and labeled with ^{32}UTP (Perkin-Elmer) using the T7 Megashortscript Kit (Invitrogen). The transcript was purified by running the DNase treated reaction on a 4% (wt/vol) denaturing PAGE gel, and the gel piece corresponding to the labeled transcript was excised from the gel and eluted in an Eppendorf thermomixer at 37 °C in a buffer containing 0.3 M sodium acetate, 0.2% SDS, and 1 mM EDTA. Precipitated RNA was refolded by heating at 95 °C for 2 min followed by 37 °C for 1 h. A typical 25-μL processing reaction contained 1–5 μL of the Flag-Drosha/Flag-Pasha immunoprecipitate, 0.5 mM ATP, 10 mM creatine phosphate, 30 μg/μL creatine kinase, 10 ng/μL tRNA, 0.1 U/μL of ribonuclease inhibitor (Invitrogen), and the refolded labeled transcripts (0.5×10^5 cpm). The reaction mixture was incubated at 26 °C for 30–90 min, and RNA was extracted by phenol: chlorofom, followed by ethanol precipitation and analyzed on a 10% (wt/vol) denaturing polyacrylamide gel.

Coimmunoprecipitations and Western Blot Analysis. Transfected Kc-167 cells were sonicated and centrifuged, and the resulting lysate was incubated with 10 μL of anti-Flag M2 affinity gel (Sigma) at 4 °C with nutation. Beads were washed six times with D-K′100 buffer (3) and then used for Western blot analysis. Western blots were probed with primary antibodies, including anti-Flag antibody (1:3,500; Sigma), anti-HA antibody (1:1,000; Santa Cruz Biotechnology), anti-Myc antibody (1:2,000; Cell Signaling Technologies), and anti-Tubulin antibody (1:5,000; Sigma). Primary antibodies were detected with Cy5- or Cy3-conjugated secondary antibodies (1:3,000; Amersham), and fluorescent signal was detected using an Amersham Bioscience Typhoon 9210 (Physical Biochemistry Instrumentation Facility, Indiana University).

1. Heo I, et al. (2008) Lin28 mediates the terminal uridylation of let-7 precursor MicroRNA. *Mol Cell* 32(2):276–284.
2. Ishizuka A, Saito K, Siomi MC, Siomi H (2006) In vitro precursor microRNA processing assays using Drosophila Schneider-2 cell lysates. *Methods Mol Biol* 342:277–286.
3. Han J, et al. (2006) Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. *Cell* 125(5):887–901.

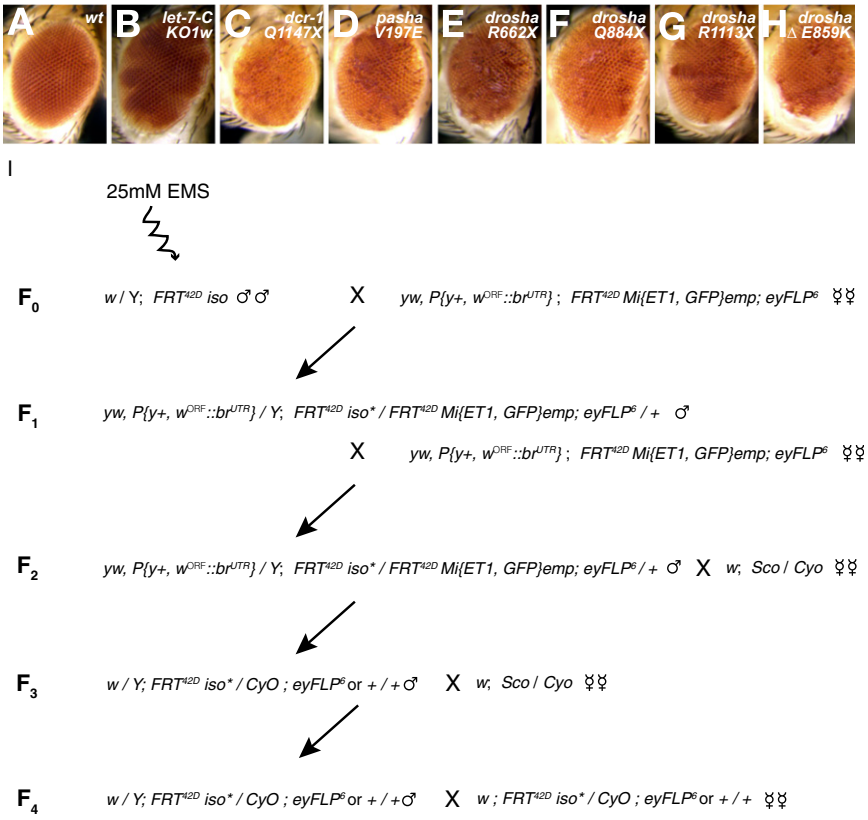

Fig. S1. Genetic screen with *let-7-Complex* miccroRNA reporter. (*A–H*) Eye color mosaicisim of *y, w,* w^{ORF}*::br*$^{3'UTR}$ transgenics containing WT (*A*), *let-7-C*^{KO1w} (*B*), *dcr-1*^{Q1147X} (*C*), *pasha*^{V197E} (*D*), *drosha*^{R662X} (*E*), *drosha*^{Q884X} (*F*), *drosha*^{R1113X} (*G*), and *drosha*$^{\Delta,E859K}$ (*H*) clones. The w^{ORF}*::br*$^{3'UTR}$ reporter is a modified version of the eye pigmentation gene *white* that contains the *broad-Z3* 3′UTR, predicted to contain binding sites for both *let-7* and *miR-125* microRNAs. Flies carrying this transgene exhibited reduced eye pigmentation. Generation of homozygous mutant clones of a *let-7-C* null allele and alleles of core miRNA pathway components, including *dcr-1*^{Q1147X}, *pasha*^{V197E}, *drosha*^{R662X}, *drosha*^{Q884X}, and *dcp1*^{EY16846} mutations restored red pigment to patches of eye tissue. (*I*) Outline of genetic screen using w^{ORF}*::br*$^{3'UTR}$ reporter. See *SI Materials and Methods* for description of screen.

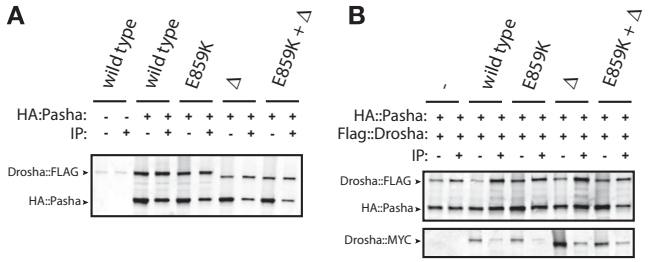

Fig. S2. Drosha$^{\Delta 561–707}$ physically interacts with Pasha and WT Drosha. (*A*) Coimmunoprecipitation of Pasha with WT or mutant Drosha proteins. Western blots containing total extracts (−IP) or FLAG immunoprecipitates (+IP) of KC167 cells cotransfected with HA-Pasha and FLAG-Drosha plasmids. Drosha::FLAG and HA::Pasha were detected with anti-FLAG and anti-HA antibodies, respectively. FLAG::Drosha to HA::Pasha ratio indicates that the deletion of amino acids 561 through 707 did not affect Drosha's physical interaction with Pasha. (*B*) Coimmunoprecipitation of Drosha with WT or mutant Drosha proteins. Western blots containing total extracts (−IP) or FLAG immunoprecipitates (+IP) of KC167 cells cotransfected with HA-Pasha, FLAG-Drosha, and Myc-Drosha plasmids. Drosha::FLAG, HA::Pasha, and Drosha::MYC were detected with anti-FLAG, anti-HA antibodies and anti-MYC antibodies, respectively. Detection of Myc::Drosha indicates that the 561–707 amino acid deletion does not abolish Drosha dimerization.

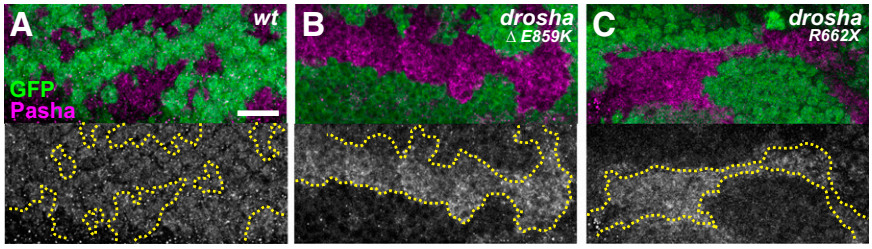

Fig. S3. Pasha levels are elevated in both *drosha*$^{\Delta\ E859K}$ and *drosha*^{R662X} clones. Eye discs from third-instar larvae containing WT (*A*), *drosha*$^{\Delta\ E859K}$ (*B*), and *drosha*^{R662X} (*C*) clones costained with anti-GFP (green) and anti-Pasha antibodies (magenta in upper panels; white in lower panels). Clones are indicated by the absence of GFP in upper panels and outlined by dotted yellow lines in lower panels. Although Pasha levels were similar in WT clones and surrounding cells, they were noticeably increased in both *drosha*$^{\Delta\ E859K}$ clones and, as has been shown previously, in *drosha*^{R662X} clones (1). (Scale bar, 10 μm.)

1. Smibert P, et al. (2011) A Drosophila genetic screen yields allelic series of core microRNA biogenesis factors and reveals post-developmental roles for microRNAs. *RNA* 17(11):1997–2010.

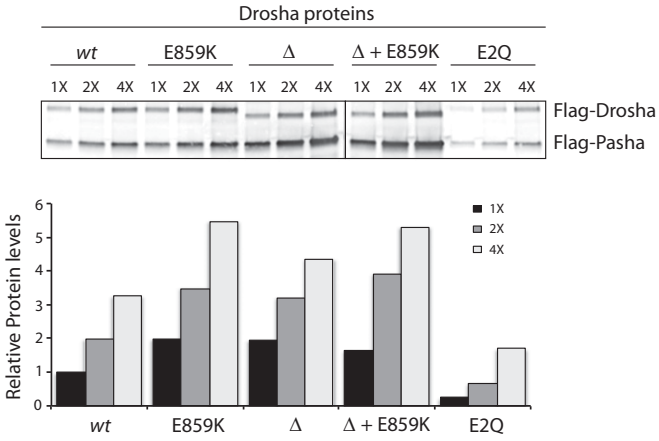

Fig. S4. Transfected cells express comparable levels of WT and mutant Drosha proteins. Western blots containing total extracts of BG3-C2 cells cotransfected with FLAG-Pasha and FLAG-Drosha plasmids. Tagged proteins were detected with anti-FLAG antibodies. Note the increased mobility of the smaller Drosha$^{\Delta}$ and Drosha$^{\Delta\ +E859K}$ proteins. Increasing volumes (1×, 2×, and 4×) of lysates were loaded, and the in vitro processing assays shown in Fig. 2*B* were performed with 2× lysate. Graph at bottom depicts quantification of Flag-Drosha bands of Western blot.

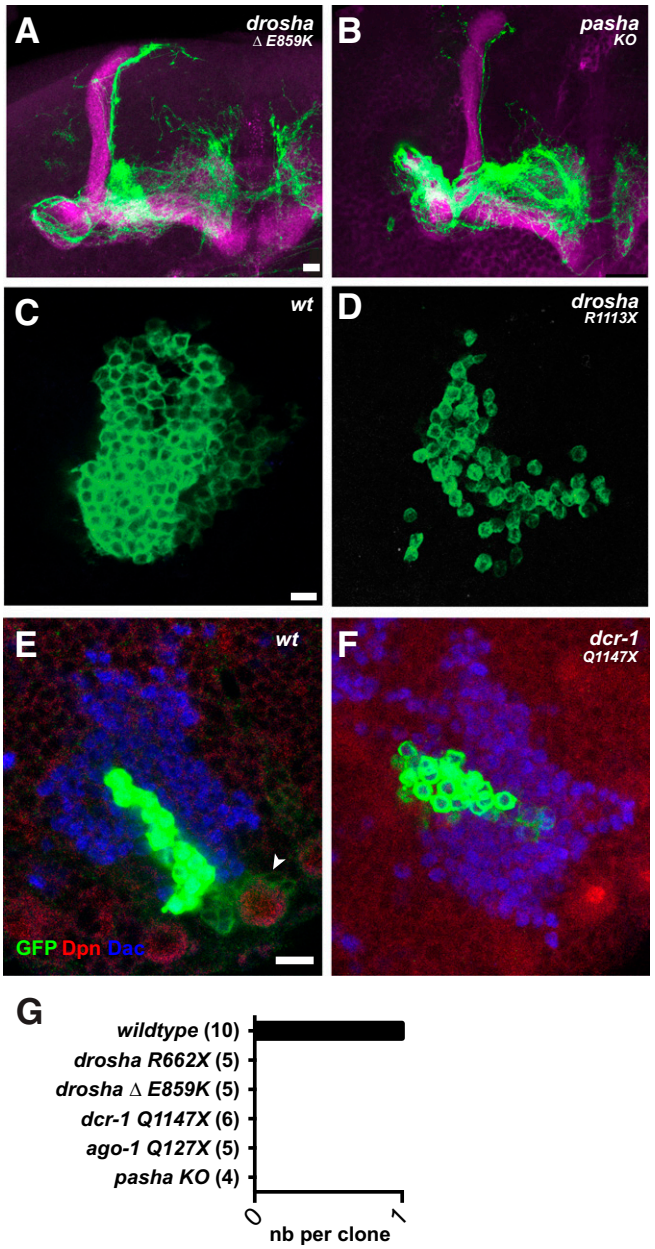

Fig. S5. *OK107-Gal4*, *UAS-mCD8::GFP*-labeled $drosha^{\Delta,\ E859K}$ (*A*) and $pasha^{KO}$ (*B*) adult MB neuroblast clones generated in newly hatched larvae and stained with anti-FasII antibodies. Panels *A* and *B* are uncropped versions of the panels in Fig. 2 that highlight the neuronal overextension. *OK107-Gal4*, *UAS-mCD8::GFP*-labeled WT (*C*) and $drosha^{R1113X}$ (*D*) adult MB neuroblast clones generated in newly hatched larvae clones. Panels *C* and *D* are representative examples of the cell body images used for cell number quantification shown in Fig. 2*H*. *OK107-Gal4*, *UAS-mCD8::GFP*-labeled WT (*E*) and $dcr\text{-}1^{Q1147X}$ (*F*) larval MB clones generated in newly hatched larvae and stained with anti-Dpn (red) and anti-Dac (blue) antibodies. This NB displays characteristic features, including large size, weak GFP expression, presence of NB marker Deadpan (Dpn), and absence of differentiated MB neuron marker, Dachshund (Dac). Note that a cell displaying these characteristics is not found in *F*. (Scale bar for *A*–*F*, 10 μm.) (*G*) Number of neuroblasts in larval MB clones of indicated genotypes. Error bars are not shown, because all WT and mutant clones contained one and zero neuroblasts, respectively. Numbers in parentheses indicate number of clones analyzed.

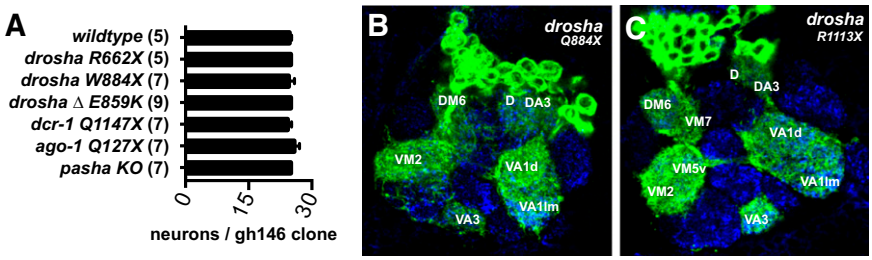

Fig. S6. Additional analysis of adPNs NB clones. (*A*) Average number of cells in adult adPN clones of indicated genotypes labeled with *GH146-GAL4*, marking only early larval born adPN neurons. Numbers in parentheses indicate number of clones analyzed. Anterior sections of *Acj6-Gal4, UAS-mCD8::GFP*-labeled adult $drosha^{Q884X}$ (*B*) and $drosha^{R1113X}$ (*C*) NB clones generated in newly hatched larvae and stained with anti-GFP (green) and anti-nc82 (magenta) antibodies.

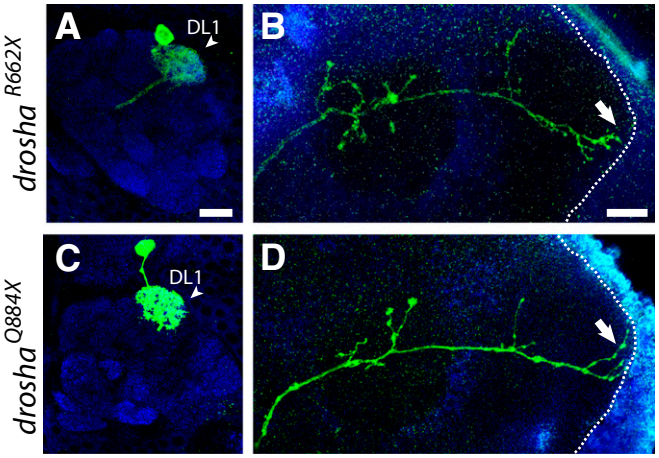

Fig. S7. *Acj6-Gal4, UAS-mCD8::GFP*-labeled, $drosha^{R662X}$ (*A* and *B*) and $drosha^{Q884X}$ (*C* and *D*) adult DL1 clones generated in newly hatched larvae and stained with anti-GFP (green) and anti-nc82 (blue) antibodies.

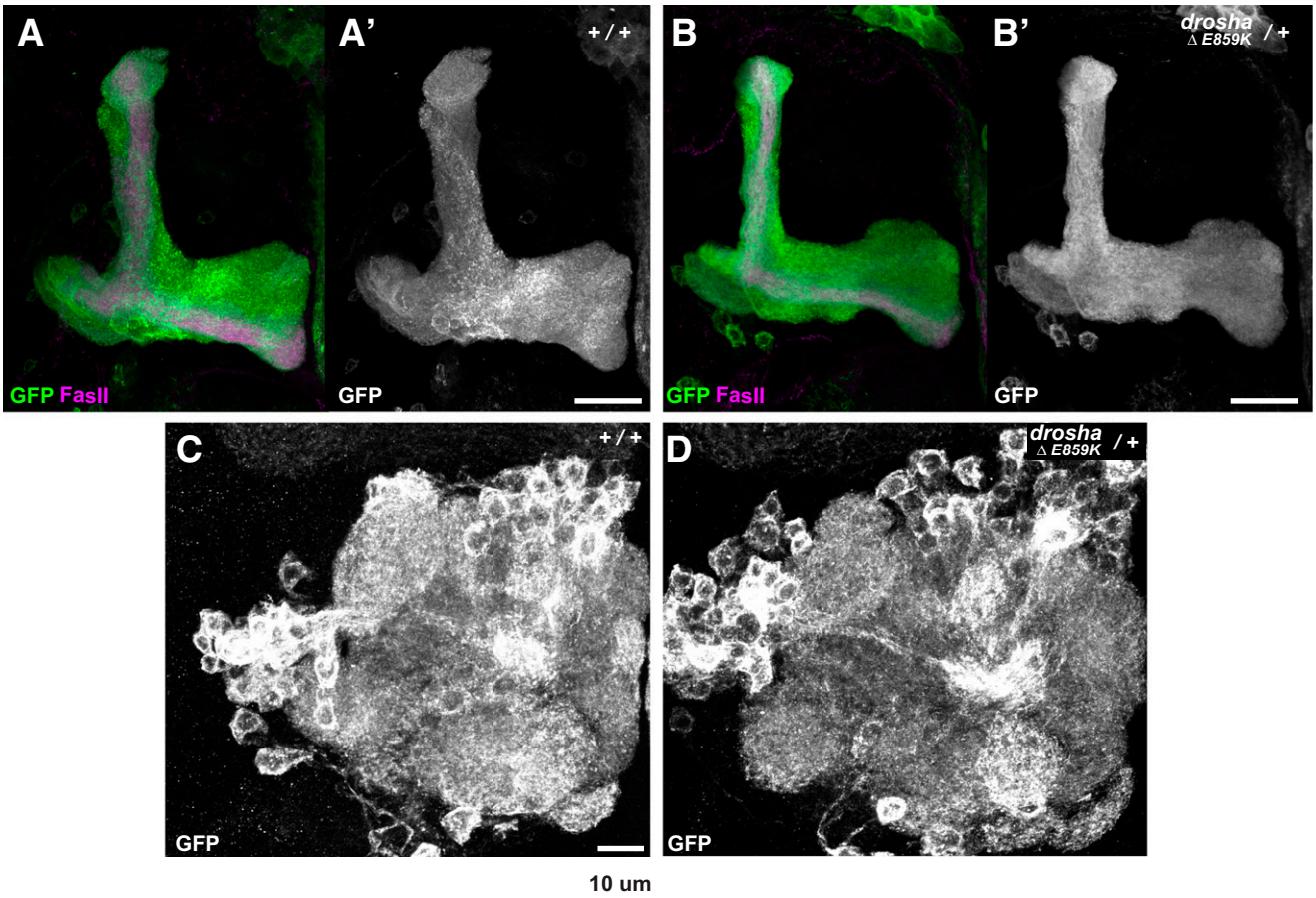

Fig. S8. The structure of MBs and antennal lobes from *drosha*$^{\Delta,\ E859K}$ heterozygote animals are comparably normal. Entire MB structure labeled with *OK107-GAL4*, UAS-mCD8GFP from (*A*) WT and (*B*) *drosha*$^{\Delta,\ E859K}$ heterozygotes. The antennal lobe structure of (*C*) WT and (*D*) *drosha*$^{\Delta,\ E859K}$ heterozygotes, labeled with GH146-GAL4, UAS-mCD8GFP. (Scale bar, 10 μm.)