Drosophila microRNAs 263a/b Confer Robustness during Development by Protecting Nascent Sense Organs from Apoptosis

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Abstract

miR-263a/b are members of a conserved family of microRNAs that are expressed in peripheral sense organs across the animal kingdom. Here we present evidence that miR-263a and miR-263b play a role in protecting Drosophila mechanosensory bristles from apoptosis by down-regulating the pro-apoptotic gene head involution defective. Both microRNAs are expressed in the bristle progenitors, and despite a difference in their seed sequence, they share this key common target. In miR-263a and miR-263b deletion mutants, loss of bristles appears to be sporadic, suggesting that the role of the microRNAs may be to ensure robustness of the patterning process by promoting survival of these functionally specified cells. In the context of the retina, this mechanism ensures that the interommatidial bristles are protected during the developmentally programmed wave of cell death that prunes excess cells in order to refine the pattern of the pupal retina.

Introduction

Organogenesis requires the organization of different cell types into precise spatial patterns. The Drosophila compound eye has proven to be a useful model system in which to investigate how such ordered patterns are established and maintained. The mature retina consists of ~750 regular units, called ommatidia. Each ommatidium consists of eight photoreceptors, four cone cells, and two primary pigment cells. Individual ommatidia are separated by a layer of secondary and tertiary pigment cells. The “interommatidial” lattice also includes sense organs called interommatidial bristles (IOB). The IOB are mechanosensory hair cells, which may help the fly to avoid damage to the eye surface. IOB develop from a distinct set of sensory organ precursors (SOP), specified at discrete positions among the array of interommatidial cells [1].

In the developing eye imaginal disc, a field of naı¨ve cells is produced by proliferation and the requisite number of ommatidial precursors is selected in a process of spatially patterned cell-type specification [2]. Short-range signaling by the initially specified R8 photoreceptor cell determines the fate of surrounding cells to make the full complement of neuronal cells needed for the ommatidium. Accessory cells, such as pigment cells, are then selected from the surrounding field of interommatidial cells. As in most developing neuronal systems, progenitor cells are over-produced, and excess cells eliminated by apoptosis after the correct pattern has been generated. In the eye imaginal disc, excess interommatidial cells are removed by two waves of programmed cell death during early pupal stages to produce the near-perfect array of ommatidia found in the adult eye [3,4]. A patterning process based on “pruning” of excess cells requires a mechanism to protect important functionally specified cells.

Mechanisms to ensure robustness are an important feature of developmental systems that can be subject to perturbation. MicroRNAs (miRNAs) have been proposed to play a role in conferring robustness during development [5,6]. This is exemplified by miR-7, which has been shown to contribute to the robustness of regulatory networks that ensure correct sense organ specification in Drosophila [7]. Although miR-7 is not required under normal conditions, SOP patterning was compromised when miR-7 mutant flies were subjected to environmentally challenging conditions. miRNAs act as post-transcriptional regulators that limit levels of target gene expression. This property makes them well suited to buffer fluctuating levels of gene activity. It may also make them well suited to serve a protective function during patterned tissue pruning.
miRNAs Confer Robustness to Sense Organ Survival

In spite of continuous challenges from the ever-changing environment, biological systems exhibit incredible stability in their developmental and physiological processes. In addition to extrinsic variability caused by environmental fluctuations, cells face intrinsic variability arising from the inherent noise of gene expression and of other molecular processes. microRNAs, which act as post-transcriptional regulators of gene expression, are beginning to be recognized for their ability to confer robustness to biological systems by buffering the effects of noisy gene expression. Although noise often is viewed as destabilizing, some biological processes make use of noise in order to make stochastic decisions. In this paper, we describe a role for microRNAs in preventing the stochastic elimination of excess cells in the developing fly retina. After the sense organs that make up the eye have been specified, pruning of excess cells occurs through the action of the gene hid, the expression of which triggers cell death. Specific mechanisms are needed to protect specialized cells which need to be maintained to ensure that only excess cells are eliminated. We report that a pair of related microRNAs, miR-263a/b, protect sense organs during this pruning process by directly acting upon and limiting the expression of the proapoptotic gene hid. This example, illustrates a novel function for miRNAs in ensuring developmental robustness during apoptotic tissue pruning.

In this report we present evidence that the miR-263a/b family of miRNAs contributes to the robustness of sense organ development. During apoptotic tissue pruning, functionally specified cells such as photoreceptors and mechanosensory organs are protected, while excess cells are eliminated. Mechanisms to ensure survival of specific cells are needed. Tissue pruning in the developing retina depends on activity of the pro-apoptotic gene hid [8], however the mechanisms that govern the decision as to which cells are lost are not fully understood. In the absence of miR-263a/b sensory bristles are lost, like other cells, in a stochastic manner. Through a process of experimental validation we identify hid, among over 30 candidates examined in vivo, as a biologically important target of miR-263a/b in this context. While hid and other proapoptotic genes are targeted by other miRNAs, including bantam and the miR-2 family [5,9,10], none of these interactions has been shown to affect apoptotic pruning. Thus miR-263a/b may have a dedicated antiapoptotic role to ensure the robustness of sense organ development in a fluctuating developmental landscape.

Results

Loss of Sense Organs in Flies Lacking miR-263a and miR-263b

miR-263a is located near the bereft locus on chromosome 2L (Figure 1A). cDNA evidence has indicated that bereft encodes a spliced transcript, however, one without an obvious protein-coding region [11]. Expression of this cDNA in transgenic flies did not rescue the defects that were attributed to bereft mutants [11]. In this light, we asked if miR-263a/b might be the functional product of the bereft locus. To address this, ends-out homologous recombination was used to generate a small deletion removing miR-263a. Three hundred and fifty nucleotides including the miRNA hairpin were replaced with a mini-white gene cassette (Figure 1A) [12]. The absence of the mature miR-263a miRNA was confirmed by Northern blot using total RNA isolated from adult flies homozygous mutant for the targeted allele (A263a, Figure 1B). Mature miR-263a was also missing in flies carrying the bereft24 allele in trans to the A263a deletion allele (A263a/bf, Figure 1B), as well as in other bereft mutants (Figure S1). The bereft24 allele is a 2.8 kb

Figure 1. miR-263a and miR-263b mutants. (A) Schematic representation of the bereft/miR-263a locus. miR-263a is located 2.7 Kb downstream of the 3' end of the annotated bereft transcript. The extent of the 2.8 Kb deletion in the bereft allele and that of the 350 nt deletion in the miR-263a deletion allele (A263a) are indicated. (B) Northern blot showing mature miR-263a RNA levels in total RNA extracted from adult control flies (WT) and two combinations of miR-263a mutant alleles. The most abundant product of miR-263a detected by sequencing is 24 nt in length [25]. A263a denotes the targeted miR-263a deletion, bft denotes the bft24 allele described in [11]. A probe for Valine tRNA was used to monitor loading. (C) Scanning electron micrographs (SEM) of adult eyes from miR-263a/+ heterozygous control and miR-263a mutant flies. + denotes the wild-type chromosome. Scale bars = 50 μm. (D) Quantification of IOB in miR-263a and miR-263b single mutants and miR-263a/miR-263b double mutant flies. “Def” denotes Df(3L)X-21.2. Error bars represent mean ± SD. N = 10–30 flies per genotype. [*] and [**] = p<0.001 using two-tailed unpaired Student’s t test comparing to the miR-263a/+ control [*] or the miR-263a single mutant [**].

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deletion that removes the first exon of the berto transcript (Figure 1A). The absence of mature miR-263a in these flies suggests that miR-263a is the functional product of the berto locus.

berto mutants show defects in the formation of a variety of external sense organs, including loss of the IOB of the eye [11]. In miR-263a homozygous mutants and in A263a/berto flies ~80% of IOB were missing (Figure 1C, 1D). The Drosophila genome encodes a second miRNA closely related in sequence to miR-263a (Figure S2A). We generated a miR-263b deletion allele (A263b) by homologous recombination and confirmed that mature miR-263b was absent in the mutant (Figure S2B). IOB numbers were only modestly reduced in flies lacking miR-263b alone (A263b/Df(3L)X-21.2, Figures S2C, 1D). However, we observed a significant increase in the loss of IOB in flies lacking both miR-263a and miR-263b compared to miR-263a alone (Figures S2C, 1D). These observations suggest that both the miR-263a and miR-263b miRNAs contribute to IOB formation, with miR-263a playing the major role.

In addition to the IOB phenotype, the miR-263a and miR-263b mutants exhibit other milder defects. The number of large mechanosensory bristles (macrochaetae) on the head and thorax was reduced in miR-263a mutant flies compared to controls (Figure S3). Although the magnitude of the reduction in bristle number was small, the difference was statistically significant. There was no significant enhancement of this phenotype in the miR-263a miR-263b double mutant (Figure S3). In addition, the miR-263a mutant showed reduced viability compared to control flies. Although miR-263b showed little effect alone, the miR-263a miR-263b double mutant showed a stronger viability phenotype (Figure S4). Our further analysis focused on the bristle phenotypes.

In order to verify that the bristle phenotypes are due to loss of the miRNAs, we performed genetic rescue experiments. To this end, we produced Gal4 "knock-in" alleles of the miRNAs, we performed genetic rescue experiments. To determine when bristle development fails, we examined pupal retinas using an antibody to the cell junction protein DEC- cadherin [16]. At 24 h after puparium formation (APF), the hexagonal array of ommatidia is clearly defined; bristle progenitor cells are visible at alternate corners in the hexagonal array of ommatidia (arrows, Figure 3A). At this stage, miR-263a mutant

miRNAs Confer Robustness to Sense Organ Survival

Figure 2. The miR-263a phenotype is rescued by expression of a miR-263a or overexpression of a miR-263b transgene. (A) SEM of adult eyes from miR-263a and miR-263b mutants expressing the UAS-miR-263a transgene. Δ263a denotes the targeted miR-263a deletion, Δ263a-G4 denotes the miR-263a-Gal4 knock-in allele, UAS-263a denotes the UAS-miR-263a transgene, and btf is btf+/+. Scale bars = 5 μm. (B) Quantification of IOB numbers (gray bars, left scale) and normalized miR-263a miRNA levels measured by miRNA qRT-PCR (green bars, scale at right). Genotypes as indicated in (A), and a wild-type control for comparison. Error bars represent mean ± SD. N = 20–25 flies per genotype. [*] p<0.001, Student’s t test comparing to the miR-263a mutant. (C) SEM of adult eyes from miR-263a mutant flies expressing a UAS-miR-263b transgene under the control of miR-263b-Gal4 (right) or without a Gal4 driver (left). (D) Quantification of IOB numbers and miR-263b miRNA levels. Error bars represent mean ± SD. N = 20–30 flies per genotype. [∗] p<0.001, Student’s t test comparing to Δ263a/btf; UAS-263b/+.

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retinas were indistinguishable from the controls and bristle shaft progenitor cells were present in normal numbers (Figure 3B).

Approximately one third of the interommatidial cells present at 24 h APF undergo apoptosis during the following 12 h [1,3]. By 40 h APF, a single row of interommatidial cells surrounds each ommatidium. Bristle shaft progenitor cells appear as brightly labeled cells at three of the six corners (arrows, Figure 3C, 3D). In miR-263a mutant retinas, the majority of these cells were missing at 40 h APF (arrowsheads, Figure 3E, 3F). Pax2 protein expression marks the nuclei of bristle shaft and sheath cells of external sensory organs (Figure 3G; [17,18]). The bristle shaft cell grows by an unusual type of cell cycle called endoreplication, in which DNA replication takes place without cell division [19,20]. These cells have increased ploidy and therefore larger nuclei (arrows, Figure 3G) than the sheath cells (arrowheads). In miR-263a mutant retinas many of the larger Pax2 positive nuclei were missing, consistent with bristle shaft cell loss (Figure 3H). We made use of the Gal4 knock-in alleles to direct UAS-GFP reporter expression in the endogenous miR-263a and miR-263b expression domains. Triple labeling to visualize GFP and Pax2, together with DE-cadherin (Figure 3I, 3J), showed that both miRNAs are expressed in the bristle shaft cells during the developmental window in which bristles are lost in the mutants.

The loss of bristle shaft cells by 40 h APF raised the possibility that they might be eliminated during the normal wave of apoptotic pruning of interommatidial cells. To test this possibility, we made use of miR-263a-Gal4 to express the anti-apoptotic protein p35 in miR-263a expressing cells, p35 has been shown to be effective as an inhibitor of apoptosis in Drosophila [21,22]. Expression of UAS-p35 using miR-263a-Gal4 suppressed IOB loss in miR-263a mutant flies (Figure 4A, 4B). Similarly, over-expression of the anti-apoptotic protein DIAP1, a direct target of the proapoptotic protein Hid [23], was able to prevent IOB loss in miR-263a flies (Figure 4A, 4B). We also monitored programmed cell death in the pupal retina at 35 h APF by visualizing double strand DNA breaks caused by apoptotic endonucleases. In control retinas, we did not observe apoptosis of the Pax2-expressing bristle shaft or sheath cells (Figure 4C). However, apoptotic Pax2-expressing nuclei corresponding to bristle shaft cells were seen in miR-263a mutants (arrows, Figure 4D). The total number of apoptotic cells/ommatidium was not significantly elevated in the mutant (Figure 4E), but there was a statistically significant increase in the number of apoptotic cells that were Pax2-expressing bristle shaft cells (p<0.001). These findings indicate that miR-263a acts to protect these sense organs from the wave of programmed cell death that sweeps over the retina during early pupal development.

Identification of miR-263a Targets

miR-263a has several hundred computationally predicted targets [24,25]. Among these are genes involved in cell proliferation and cell death and a broad range of other biological processes. Endogenous targets are often upregulated in miRNA mutants. Therefore over-expression of a biologically important target in miR-263a expressing cells would be expected to result in IOB loss, phenocopying the miR-263a mutant phenotype. miR-263b-Gal4 was used to drive over-expression of target genes in this series of experiments because it has higher Gal4 activity than miR-263a-Gal4. We selected 56 predicted targets for analysis (Table S1). Only two of the candidates caused bristle loss when expressed under control of miR-263b-Gal4: Cyclin E and head involution defective (hid).

Cyclin E is an essential cell cycle regulator, required for normal cell proliferation and for endoreplication [26]. Endoreplication plays an important role in the growth of bristle shaft and socket cells [19]. Over-expression of Cyclin E has been shown to interfere with endoreplication [27,28] and can suppress bristle shaft cell growth [29]. If Cyclin E over-expression is the cause of the bristle loss in miR-263a mutants, limiting their capacity to express Cyclin E should suppress this phenotype. Bristle loss occurs between 24 and 40 h APF in the mutants. RNA was prepared from pupae at 30 h APF, because the majority of the bristles are not yet lost at this stage. Cyclin E mRNA levels were elevated by ~2.5-fold in RNA samples from miR-263a mutants (Figure S5). Removing one copy of the Cyclin E gene restored the mRNA to near normal levels but did not rescue the IOB loss phenotype (Figure S5). Although Cyclin E is upregulated in the miR-263a mutant, this does not appear to contribute to the bristle loss phenotype.
miRNAs Confer Robustness to Sense Organ Survival

The other candidate, *hid*, encodes an inducer of cell death in Drosophila. *hid* has been shown to play a role in the late stage cell death pathway in the retina [22]. *hid* expression under miR-263b-Gal4 control caused loss of IOB (Figure 5A). To determine whether *hid* might be a biologically relevant target of miR-263a in vivo, we compared *hid* mRNA levels in RNA samples from mutant and control pupal eye discs. *hid* mRNA was 1.6-fold higher in the mutants (Figure 3B). This difference was abolished in *miR-263a* mutant flies rescued by expression of *UAS-miR-263a* under *miR-263a-Gal4* control.

To test whether *hid* over-expression is the cause of bristle loss, we reduced *hid* activity in the *miR-263a* mutant background by introducing the *hid*<sup>591/4</sup> loss of function allele [22]. This genetic combination restored IOB numbers to ~60% of normal levels (Figure 5C, 5D). To further reduce *hid* activity we made use of the *W<sup>0</sup>* allele, which expresses an antimorphic form of *hid* [30], and found a further restoration of IOB number (Figure 5C, 5D). Similarly, reducing *hid* levels by expression of a *UAS-hid-RNAi* transgene under the control of miR-263b-Gal4 produced a strong suppression of the *miR-263a* mutant phenotype (Figure 5C, 5D). Taken together, these data suggest that *miR-263a* serves to prevent apoptosis in the IOB precursors by limiting *hid* expression during the wave of interommatidial cell pruning.

**hid** Is a Direct Target of miR-263a/b

The *hid* 3′UTR contains four potential *miR-263a* binding sites (Figure 6A). To address whether *hid* is a direct target of *miR-263a*, we generated luciferase reporter constructs carrying the full length endogenous *hid* 3′UTR or mutant versions in which two nucleotides of each predicted *miR-263a* site were mutated to compromise pairing to the miRNA seed region (Figure 6A, in red). In S2 cells, co-expression of the luciferase reporter carrying the intact sites with *miR-263a* significantly reduced luciferase activity (Figure 6B, *p*<0.001). This was attributable to reduced luciferase mRNA levels (Figure 6C). These effects were not observed in cells expressing the mutant form of the *hid* reporter (Figure 6B, 6C). We also analyzed the effect of *miR-263b* on the 3′UTR of *hid*. Although *miR-263b* differs from *miR-263a* by three residues, including position 1 of the seed region, *hid* is also a predicted target of *miR-263b* (Figure S6; [24,25]). Coexpression of *miR-263b* also significantly reduced luciferase activity from the reporter carrying the intact *hid* 3′UTR but not from the reporter in which the *miRNA* sites were mutated (Figure 6B). Therefore, *miR-263b* and *miR-263a* can each act directly via these sites to regulate *hid* mRNA levels. Differences in the quality of the sites for the two miRNAs may contribute to the apparent difference in their relative potency observed in vivo.

To further assess the functionality of these sites in vivo, we generated transgenic flies expressing the two *hid* 3′UTR luciferase reporters. Luciferase activity levels were compared in pupal retinas dissected from control animals and *miR-263a* mutants. There was no difference in luciferase activity for the transgene carrying the mutant form of the *hid* reporter, but the reporter with the intact sites clearly showed increased luciferase activity in the *miR-263a* mutant (Figure 6D). A similar increase was observed in the level of luciferase mRNA from the *hid* reporter with the intact sites, but not from the reporter with the mutated sites (Figure 6E). Comparable results were obtained comparing GFP reporter transgenes with intact and mutated sites in control and *miR-263a* mutants (Figure S7). We also observed an increase in the level of the endogenous mature *hid* mRNA in the mutant, but not in the level of the *hid* primary transcript, measured by qRT-PCR using intron-specific primers (Figure 6F). Taken together these experiments provide evidence that *miR-263a* acts directly via the sites identified in the 3′UTR to regulate *hid* mRNA levels in vivo. These effects are posttranscriptional, most likely reflecting destabilization of *hid* transcripts.
Figure 5. *hid* downregulation by miR-263a is required for IOB formation. (A) SEM of an adult eye expressing the endogenous *hid* gene from the EP line P[XP]d10274 under miR-263b-Gal4 control. (B) Normalized *hid* mRNA levels measured by qRT-PCR. RNA was extracted from pupal eye discs of the indicated genotypes at 30 h APF (before the IOB cells are lost in the mutant). Error bars represent mean ± SD of three independent biological replicates for each genotype. [*p* < 0.001, Student’s *t* test compared to the control (WT)]. (C) SEM of adult eyes from miR-263a mutant flies with reduced Hid activity. Left panel: miR-263a mutant with one copy of *hid*05014; middle panel: miR-263a mutant with one copy of the antimorphic *hid* allele W1; right panel: miR-263a mutant expressing a UAS-*hid*-RNAi transgene under control of miR-263b-Gal4. Scale bars = 50 μm. (D) Quantification of IOB numbers from flies of the genotypes shown in (C), and miR-263a mutant for comparison. Error bars represent mean ± SD for *N* = 20–25 flies per genotype. [*p* < 0.001, Student’s *t* test comparing to the miR-263a mutant.]

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Figure 6. miR-263a acts on binding sites in the *hid* 3′ UTR. (A) Predicted miR-263a target sites in the *hid* 3′ UTR. Pairing to the miRNA seed sequence is shaded in grey. Nucleotides changed to generate the target site mutant UTR are shown in red. (B) Normalized firefly luciferase activity from S2 cells transfected to express control and mutated *hid* 3′ UTR transgenes. Cells were cotransfected to express miR-263a or miR-263b or with a vector-only control, and with a plasmid expressing Renilla luciferase as a transfection control. (C) Normalized luciferase mRNA levels measured by qRT-PCR on RNA samples from cells transfected as in (B). (B, C) Error bars: SD based on six independent biological replicates for miR-263a and three independent biological replicates for miR-263b. (D) Normalized luciferase activity. Cell lysates were obtained from 30 h pupal eye imaginal discs from flies expressing a luciferase reporter carrying the *hid* 3′ UTR, or the mutated version of it, in a miR-263a mutant or wild-type background. (E) Normalized luciferase mRNA levels. RNA was extracted from samples as in (D). (F) *hid* RNA levels measured using intron-specific primers (left) and exon-specific primers (right). RNA was extracted from pupal eye imaginal discs from control and miR-263a mutants at 30 h APF. Unless otherwise indicated, error bars: SD of three independent experiments. [*p* < 0.001, Student’s *t* test comparing to the control levels.

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It has been reported previously that excess hid activity eliminates photoreceptors and pigment cells, effectively ablating the eye, while sparing the IOB [22,31]. The result was a tuft of IOB and undifferentiated cuticle in place of the eye. Further increase of hid activity led to loss of these cells as well. Based on these observations, it was proposed that IOB might contain high levels of a negative regulator of cell death. To ask whether miR-263a/b might be responsible for this activity, we compared the effects of reducing miR-263a/b activity in animals expressing the constitutively active form of Hid, hid(Ala5), in the eye. Reducing miR-263a levels by removing one copy of the miR-263a gene led to fewer IOB, producing a more sparse appearance in the tuft of bristles (Figure 7A, 7B). Because the morphology of the hid(Ala5) eyes is strongly perturbed, the effect of reducing the miRNA can be quantified most reliably by counting the number of empty sockets. Every socket should have an IOB hair cell, so the empty socket indicates a specified sense organ lacking the bristle shaft cell. Although the variance of this measure is high, there was a statistically significant increase of ~2-fold in the proportion of empty sockets (p<0.005), indicating loss of IOB due to reduced miR-263a activity. Thus reduced activity of the miRNA enhanced apoptosis due to elevated Hid activity. To address this we miR-263a mutant flies and found that mechanosensory bristle loss was also a consequence of apoptosis due to elevated hid activity. To address this we introduced the antimorphic allele of hid, W4, into miR-263a miRNA mutant flies and found that mechanosensory bristle loss on head and thorax was also significantly suppressed (Figure S3; p<0.001). These observations suggest that miR-263a/b play a protective role, preventing the loss of mechanosensory cells due to hid-induced apoptosis.

Discussion

microRNAs and Robustness

Robustness of a biological system can be thought of in terms of the mechanisms that ensure stability. In developmental terms, perturbation can come in the form of fluctuating levels of intercellular signaling and/or gene expression and can be intrinsic or of environmental origin. Gene regulatory networks have properties that can help to confer stability by buffering the effects of fluctuations in gene expression [reviewed in [32]]. Computational analysis has suggested that miRNAs are over-represented in gene regulatory networks in animals, suggesting that they confer useful regulatory possibilities [33,34]. However, to date few examples have been investigated experimentally in terms of biological processes that confer robustness during development of multicellular organisms. A recent study has provided compelling evidence for a miRNA acting to confer robustness to sensory organ specification in Drosophila [7]. In this report we examine the role of miR-263a/b in conferring robustness to sensory organ survival during a developmental pruning process.

miR-7 was shown to act in two molecularly distinct feed-forward loops required for sense organ specification [7]. One pair of feedforward motifs involves the transcription factors Yan and Pointed, which mediate Notch and EGF signaling to control R8 photoreceptor specification. The second involves the transcription factors E(spl) and Atonal to control SOP specification. In both examples miR-7 is induced by one of the transcription factors to confer repression on the other. The requirement for miR-7 activity in these patterning processes is not evident under normal, environmentally stable, conditions. However, it can be revealed under destabilizing conditions, including severe environmental fluctuation, or in sensitized genetic backgrounds. Thus miR-7 acts to provide stability to these molecular networks.

We have explored the possibility that miR-263a/b might function in a regulatory feed-forward network to control hid both directly and indirectly. The RAS/MAPK pathway has been reported to regulate hid activity at two levels. Hid activity is controlled by direct phosphorylation mediated by MAPK [31]. Signalling through the EGF/RAS/MAPK pathway protects cells of the developing eye from apoptosis, through MAPK mediated regulation of Hid activity. In addition MAPK signalling represses hid transcription [8]. Intriguingly, an upstream element of the MAPK pathway, Ras85D, is a predicted target of miR-263a. In this scenario, miR-263a would act directly to repress hid and indirectly via the RAS/MAPK pathway (illustrated in Figure S8). Negative regulation of Ras85D by miR-263a would repress MAPK activity and alleviate repression of hid transcription and of Hid protein activity. The result is a so-called “incoherent” motif [32], in which the two branches have opposing effects on their shared target. A prediction of this model is that hid transcription should decrease in the miR-263a mutant due to elevation of MAPK activity. However, as shown in Figure 6F, hid primary transcript levels were not significantly affected in the miR-263a mutant, although mature hid mRNA levels increased due to loss of miRNA direct mediated repression. As a further test, Ras85D mRNA levels were measured in pupal eye discs dissected from control animals and miR-263a mutants. There was no significant difference (Figure S8). Although the topology of this predicted network suggested a potential role in control of hid activity, we do not find sufficient evidence to support the biological relevance of this network in vivo. This example highlights the importance of experimental validation in vivo in assessing such predictions.

Figure 7. miR-263a limits Hid activity during eye development. (A, B) SEM of adult eyes that express the activated form of hid under GMR control. (A) GMR:hid(Ala5) alone (B) GMR:hid(Ala5) lacking one genomic copy of miR-263a. (C) Quantification of missing IOB from flies of the genotypes shown in (A) and (B). The number of IOB socket cells devoid of a shaft cell was normalized to the total number of socket cells. Error bars represent mean ± SD for N = 10–15 flies per genotype. [*] p<0.005, Student’s t test comparing to GMR:hid(Ala5)+. doi:10.1371/journal.pbio.1000396.g007
Our findings suggest a role for the miR-263a/b miRNAs in conferring robustness of a different sort, ensuring the survival of sense organ cells, after they have been specified by the developmental patterning process. In this scenario the fluctuating cellular landscape derives from triggering cell death through randomly variable activity of the pro-apoptotic gene hid. Under normal conditions, this rarely, if ever, causes bristle loss. However in miR-263a/b mutants sporadic bristle loss was seen and was attributable to elevated hid activity. As in the case of the macrochaetae, loss of individual interommatidial cells is a stochastic process. In each of the single mutants we observed a variable loss of IOB, suggesting that the chance of any given nascent IOB cell succumbing to apoptosis has increased in the absence of the protective effect of miR-263a/b. The overall robustness of the pruning process is impaired.

In the miR-7 case, the mutants do not show any defect under normal conditions, but the limits to the robustness of the system can be revealed by environmental perturbation. This is consistent with a scenario in which robustness derives from a gene regulatory network designed to buffer molecular perturbation. Based on the observations of Li et al. [7], we examined whether the severity of the miR-263a/b mutant phenotype would be affected by environmental fluctuation to increase noise but found no effect (unpublished data). We also did not find evidence that miR-263a/b act in the context of a gene regulatory network. Instead, miR-263a/b appears to function in a different context, acting as a buffer in a biological process that is inherently stochastic. In this way miRNA activity is used to ensure that apoptotic cell death is not allowed to compromise specific cells. It is noteworthy that as little as 20% of normal miR-263a levels are sufficient to support IOB development. This implies a considerable buffering capacity to ensure that the process of IOB formation is robust in a fluctuating developmental landscape.

**microRNAs and Apoptosis**

Several other microRNAs have been implicated in the control of apoptosis in Drosophila. bantam miRNA functions during tissue growth and regulates hid, to prevent proliferation induced apoptosis [9]. However, under normal conditions bantam regulation of hid does not appear to impact upon apoptotic pruning or on survival of sense organs (our unpublished observation). miR-14 has also been reported to be anti-apoptotic [35]. miR-14 mutants do not impact on IOB apoptosis ([36] and our unpublished observations). Similarly, miR-8 mutants show apoptosis in the CNS [37] but do not have an IOB phenotype. Finally, members of the miR-2 family of miRNAs have been shown to regulate the propaptotic genes, wapre, geyn, and sickle in S2 cell over-expression assays or in reporter transgene assays in vivo [10,38]. Injection of antisense oligonucleotides to deplete members of this family have been reported to cause apoptosis in the embryo [39], but none of the mutants that affect the members of this family have yet shown any role in apoptotic pruning ([40] and our unpublished observations). We do not exclude the possibility that potential phenotypes might be masked by functional redundancy among family members. The available evidence suggests that miR-263a/b may have a dedicated role in controlling hid-induced apoptosis during developmental pruning of interommatidial cells.

**microRNA Targets**

Most miRNAs are predicted computationally to have many possible targets. Yet our survey of over 50 candidates yielded only one target, hid, for which we have functional evidence in vivo. It may be of interest in this context to consider data from analysis of Drosophila miRNA target predictions. A high proportion of predicted targets are regulated in cell-based miRNA over-expression assays (sample refs: [5,41–44]). This provides evidence that the miRNA, when present abundantly, can regulate the predicted target site. There are fewer examples in which over-expression of a predicted target can be shown to contribute directly to causing a specific miRNA mutant phenotype in vivo. In most such cases only one or a few targets have been implicated (reviewed in [45,46]; see also [47–49]). For miRNA mutants that have been studied in detail, evidence has begun to emerge that different aspects of the mutant phenotype may result from misregulation of different targets in different tissues. mir-8 is a good example, with well characterized phenotypes linked to different targets in three different tissues: Atrophin for neurodegeneration in the CNS [37], Enabled for neuromuscular junction development [48], and U-shaped in the adipose tissue to control tissue growth [49]. Finding a few different key targets in different tissues may prove to be a common theme.

The genetic evidence presented here identifies hid as a key target of the miR-263 family in supporting bristle development. A priori we cannot exclude that there might be other important targets. But we note that only hid, of the 56 candidates tested, fulfilled two essential criteria: (1) being able to mimic the miRNA mutant phenotype when over-expressed in the miRNA expressing cells and (2) being able to suppress the miRNA mutant phenotype when its level was reduced in the miRNA expressing cells. Further investigation of the miR-263 family miRNA mutants may lead to identification of targets important for other aspects of the miRNA function, such as the reduced viability observed in the double mutants.

**A Conserved Family of microRNAs Implicated in Maintenance of Sense Organ Survival**

Drosophila miR-263a and miR-263b are members of a conserved family of miRNAs, including mammalian miR-183, miR-96 and miR-182, and miR-228 in C. elegans. Interestingly, members of this family display conservation of expression in ciliated sensory organs in vertebrate and invertebrate organisms [50]. miR-183, miR-96, and miR-182 are expressed in sensory hair cells in mammalian auditory and vestibular organs, as well as in sensory cells of the eye and ear in zebrafish and chicken [51–55]. C. elegans miR-228 is expressed in chemosensory and mechanosensory sensilla [50]. Drosophila miR-263a and miR-263b are expressed in sense organ precursors in embryos [11,56] and in mechanosensory organs of the eye, antenna, and haltere ([11,50], this report). The high degree of sequence conservation and expression in sensory organs across phyla raises the possibility that a common ancestor of these miRNAs was associated with sensory cell development and function [50].

Further support for the idea of conservation of function comes from the observation that mutations affecting miR-96 have been identified as the cause of auditory hair cell degeneration and nonsyndromic progressive hearing loss in mice and humans [57,58]. Depletion of all miRNAs using conditional dicer mutants in the mouse also leads to defects in inner ear hair cell development [59,60]. Whether there is more than a coincidental similarity to the role of miR-263a/b in support of sensory hair development in Drosophila remains to be determined. Superficially the way in which these sense organs are lost appears to differ. In the fly, the mechanosensory cells are lost due to apoptosis in the miR-263a/b mutants. In the mammalian systems, mature differentiated sensory cells appear to be lost through degeneration. In the case of the miR-96 mutant this could be due to inappropriate regulation of genes that are not normally miR-96 targets due to the change in sequence of the mutant miRNA seed, but in the case of dicer
conditional mutants it is more likely due to loss of normal miRNA mediated target regulation. Whether this involves apoptosis is not known. Intriguingly, there is evidence suggesting an anti-apoptotic role for miR-192 and related family members in human cancers [61,62]. So the possibility of an underlying conservation of mechanism exists.

**Methods**

**Plasmids and Fly Strains**

Canton-S flies were used as the wild-type control. EP lines were obtained from the Bloomington, Sezged, and Exelixis stock centres. The hid UAS-RNAi strain was obtained from the VDRC. bft23, bft235, and bft24 were provided by Rolf Bodmer. hidP5014 and GMR hid(Aa5) were provided by Hermann Steller. W1, Df(3L)X-21.2, UAS-p35, and UAS-Diap1 were obtained from the Bloomington stock centre. UAS-miR-263a and UAS-miR-263b lines were made by cloning a 300 bp base pair genomic fragment containing the miRNA hairpin into the 3’UTR of dsRed in pUAST, as described in [10]. The GFP and luciferase hid 3’UTR reporters were made by cloning the 2.2 kb hid 3’UTR after GFP or luciferase, under control of the tubulin promoter [5,9]. hid UTR reporters with mutated miR-263a/b sites were generated by PCR using primers designed to change the seed region from TGCCA to CAGCCGCTA and AGCCTTCGGGCATGGC.

**Mutant Generation**

Ends-out homologous recombination was performed as described [12]. As miR-263b is located in an intron of CG32150, we removed the intron-containing mini-white gene cassette and confirmed that splicing of CG32150 mRNA was not affected in the Amir-263b mutants by comparing the level of spliced mRNA in total RNA extracted from adult flies, measured by miRNA qPCR. miR-263a and miR-263b Ga4 knock-in alleles were made using a modified targeting vector [13].

**Cell Transfection and Luciferase Assays**

Luciferase reporters and miRNA expression plasmids were expressed under the control of the tubulin promoter. S2 cells were transfected in 6-well plates with 1,000 ng of miRNA expression plasmid or empty vector, 500 ng of firefly luciferase reporter plasmid, and 500 ng of Renilla luciferase DNA as a transfection control. Transfections were performed with triplicate technical replicates in at least three independent experiments. At 60 h post-transfection, dual luciferase assays (Promega) were performed on a portion of the transfected cells. The other portion was pelleted and dissolved in Trizol reagent (Invitrogen) for total RNA extraction. For luciferase assays on pupal retinas, retinal tissue was dissected and immediately lysed in passive lysis buffer (Promega). Luciferase activity was normalized to total protein content, measured on the same sample using the Bradford method (Bio-Rad).

**RNA Analysis**

Northern blots on small RNA were carried out according to [63]. 5 µg of total RNA extracted from adult flies were loaded per lane. The blot was probed with an oligonucleotide complementary to miR-263a, 5’end-labeled with [32]-P. For miRNA qRT-PCR, primer sets designed to amplify mature miR-263a and miR-263b were obtained from Applied Biosystems. Reverse transcription was done on 100 ng of total RNA. mRNA levels were calculated relative to miR-8, after having confirmed that miR-8 levels remain constant in the relevant fly strains. For miRNA qRT-PCR, total RNA was treated with RNase-free DNase (Promega) to eliminate DNA contamination. First strand synthesis used random hexamer primers and SuperScript RT-III (Invitrogen). Samples were RNaseH-treated after the RT reaction and used for qRT-PCR. Measurements were normalized to mitochondrial large ribosomal RNA (mtlRNA1, AAAAACAGTAGCACCGCTGAT and AAAAGAACCTGGCTTACACG) or to the transfection control Renilla luciferase mRNA (GGGAGGAGATTCCCTTCTTTT and TTGGAGAAAAATGAAAGACCT). Primers for hid pre-miRNA: TGGAGGTGTTCTCCGATTCG and ATCTCTCAGAG-GTCTTTTA. Primers for mature hid mRNA: GAGAAGCGA-CAAAAAGGCAAG and CAAGCAGAAXGCTGCTA. Firefly luciferase primers: CCGAGTGTTTGGTTTGTG and CTGGGCGCACTTTCGCCC. GFP primers: GCAATGGCTTTACGCGGCTA and AGCCCTCGGCGATGGCC.

**Immunocytochemistry**

To stage pupae, white prepupa were collected and aged at 25°C until dissection. Pupal eye imaginal discs were dissected and fixed in 4% formaldehyde for 20 min on ice. The following primary antibodies were used: rat anti-Dcad2 1:40 (Developmental Studies Hybridoma Bank), rabbit anti-DPax2 1:50 (a gift from Erich Frei and Markus Noll), and chicken anti-GFP 1:1000 (Abcam). Fluorescent secondary antibodies were from Jackson Laboratories. Samples were mounted in Vectashield (Vector Laboratories). Detection of apoptotic cells in pupal eye discs was done using the Apoptag ISOL dual fluorescence kit (Chemicon). Immunofluorescence images were collected using a Leica SPE confocal microscope and processed using ImageJ. Quantification of apoptotic nuclei was done using z-projections of confocal sections. Total apoptotic nuclei as well as apoptotic shaft cell nuclei were counted and normalized to the total number of ommatidia analyzed.

**Supporting Information**

**Figure S1 miR-263a is absent in bft lines.** Northern blot showing mature miR-263a in total RNA extracted from adult control flies (WT) and the three bft homozygous mutants described in Hardiman et al. 2002 [11]. A probe for Valine tRNA was used to monitor loading. Found at: doi:10.1371/journal.pbio.1000396.s001 (0.47 MB TIF)

**Figure S2 miR-263b contributes to IOB formation.** (A) Aligned sequences of miR-263a and miR-263b. The three differing nucleotides are highlighted in red. The seed region (grey shading) comprises nucleotides 2 to 8 of the miRNA. (B) Normalized miR-263b levels in adult flies, measured by miRNA qPCR. Δ263b/miR-263b knockout allele, Δ263b-G4/Δ263b-miR-263b/Ga4 knock-in allele in trans with the genomic deficiency Df(3L)X-21.2. (C) SEM of adult eyes from miR-263b single mutant (two representative examples) and miR-263a miR-263b double mutant flies. Scale bars = 50 µm. Found at: doi:10.1371/journal.pbio.1000396.s002 (0.45 MB TIF)
Figure S3  Absence of miR-263 causes loss of bristles on head and thorax. Quantification of macrochaetae on head and thorax of adult flies: wild-type (WT), miR-263a mutant (Δ263a/bft), miR-263a mutant expressing an UAS-miR-263a transgene (rescue flies: Δ263a-G4/bft; UAS-263a/+), miR-263a miR-263b double mutant ( Δ263a/bft; Δ263b/Def, where Def represents the genomic deficiency Df(3L)X-21.2), miR-263a mutant with one copy of the antimorphic hid allele W+ (Δ263a/bft; W1/+). Error bars represent mean ± SD for N = 50 flies per genotype. [*] p < 0.001, using two-tailed unpaired Student’s t test comparing to Δ263a/bft flies. Macrochaetae numbers in the miR-263a miR-263b double mutant differed slightly, but not statistically significantly, from those in miR-263a mutants. Single mutant: 93.3%, double mutant 94.4%, p = 0.12 using two-tailed unpaired Student’s t test comparing the single and double mutants.

Found at: doi:10.1371/journal.pbio.1000396.s003 (0.11 MB TIF)

Figure S4  Viability of miR-263a and miR-263b mutants. Viability of different miR-263a and miR-263b mutant lines. Numbers indicate the percentage of flies observed relative to what is expected if fully viable. Hatched: percentage of embryos that hatched (n = 500 embryos counted); pupated: percentage of the resulting first instar larvae that pupated; eclosed: percentage of adult flies that emerged from these pupae. 1-d-old is the percentage of adult flies surviving 1 d after eclosion. For ease of comparison, the numbers in each category for w1118 were set to 100%. A1118 flies were used as a control. Δ263a/bft; miR-263a mutant; Δ263b/Def: miR-263b mutant, where Def represents the genomic deficiency Df(3L)X-21.2; Δ263a/bft; Δ263b/Def; miR-263a miR-263b double mutant.

Found at: doi:10.1371/journal.pbio.1000396.s004 (0.38 MB TIF)

Figure S5  CycE over-expression does not cause the IOB phenotype. (A) Normalized Cyclin E mRNA levels in flies with the indicated genotype. RNA was extracted from wild-type (WT), miR-263a mutant (Δ263a/bft), trans-heterozygous miR-263a mutant (Δ263a/CycEΔ655/bft; miR-263a/bft mutant carrying one copy of CycEΔ655), a null allele of CycE. Bars represent mean ± SD of three independent batches of pupae. (B) SEM of adult eyes from flies with the indicated genotype. Scale bars = 50 μm. CycE is elevated in miR-263a mutants. Reducing the dosage of CycE to wild-type levels does not rescue bristle loss, which indicates that over-expression of CycE is not the cause of the miR-263a phenotype.

Found at: doi:10.1371/journal.pbio.1000396.s005 (0.44 MB TIF)

Figure S6  miR-263b target sites in the hid 3’ UTR. Predicted miR-263b target sites in the hid 3’UTR. Pairing to the mRNA seed sequence is shaded in grey. Nucleotides changed to represent mean ± SD of three independent experiments. [*] p < 0.001 using two-tailed unpaired Student’s t test comparing to the control levels.

Found at: doi:10.1371/journal.pbio.1000396.s007 (0.11 MB TIF)

Figure S7  miR-263a regulates a GFP transgene carrying the hid 3’UTR. Normalized GFP mRNA levels measured by qRT-PCR. RNA was extracted from 30 h pupal eye imaginal discs from flies expressing a GFP reporter carrying the hid 3’UTR or a mutated version of it, in a miR-263a mutant or wild-type control background. Bars represent mean ± SD of three independent experiments. [*] p < 0.001 using two-tailed unpaired Student’s t test comparing to the control levels.

Found at: doi:10.1371/journal.pbio.1000396.s008 (0.10 MB TIF)

Table S1  List of tested candidate genes, with the corresponding EP lines and results (IOB loss: yes or no) when expression is driven with miR-263b-Gal4.

Found at: doi:10.1371/journal.pbio.1000396.s009 (0.09 MB DOC)

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Author Contributions

The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: VH NB SMC. Performed the experiments: VH NB. Analyzed the data: VH NB SMC. Wrote the paper: VH NB SMC.

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