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Overgrowth caused by misexpression of a microRNA with dispensable wild-type function

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Abstract

MicroRNAs (miRNAs) represent an abundant class of non-coding RNAs that negatively regulate gene expression, primarily at the post-transcriptional level. miRNA genes are frequently located in proximity to fragile chromosomal sites associated with cancers and amplification of a miRNA cluster has been correlated with the etiology of lymphomas and solid tumors. The oncogenic potential of a miRNA polycistron has recently been demonstrated in vivo. Here, we show that misexpression of the *Drosophila* miRNA *mirvana/mir-278* in the developing eye causes massive overgrowth, in part due to inhibition of apoptosis. A single base substitution affecting the mature miRNA blocks the gain-of-function phenotype but is not associated with a detectable reduction-of-function phenotype when homozygous. This result demonstrates that misexpressed miRNAs may acquire novel functions that cause unscheduled proliferation in vivo and thus exemplifies the potential of miRNAs to promote tumor formation.

Introduction

According to a conservative estimate, the human genome codes for about 220 miRNA genes and the *Drosophila* genome for about 110 (Griffiths-Jones, 2004; Lai et al., 2003). miRNAs act as non-coding single-stranded transcripts of 18 to 24 nucleotides that promote post-transcriptional silencing of mRNAs (Ambros, 2003). This class of small RNAs is processed in a stepwise manner from a large RNA polymerase II-dependent transcript that contains about 70 nucleotide stretches capable of forming stem-loop structures (Lee et al., 2004a). From the longer so-called pri-miRNA, the pre-miRNA hairpin precursor is cleaved off by the RNAase III Drosha and its auxiliary dsRNA-binding protein Pasha/DGCR8 (Denli et al., 2004; Gregory et al., 2004; Han et al., 2004; Landthaler et al., 2004; Lee et al., 2003). The hairpin exhibiting a two to three nucleotide 3′ overhang is recognized and actively exported from the nucleus by Exportin 5 (Lund et al., 2004; Yi et al., 2003). The pre-miRNA is then further processed by the cytoplasmic RNAase III Dicer to yield a siRNA-like double-stranded miRNA/miRNA* duplex (Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001; Knight and Bass, 2001). In *Drosophila*, this step is performed by Dicer-1. Its homologue Dicer-2 is preferentially involved in generating siRNAs from dsRNA (Lee et al., 2004b). In most metazoans, however, both siRNAs and miRNAs are generated by a single Dicer enzyme.

As Dicer functions at a most critical step in the processing of miRNAs, it is a limiting factor of their accumulation. In all systems tested so far, absence of Dicer – and hence of mature miRNAs – does not impair cell viability and may even allow for complete or partial organismal development. *Caenorhabditis elegans* dcr-1 mutants are viable, but sterile (Ketting et al., 2001), zebrafish Dicer mutant embryos display normal axis formation, but abnormal brain development which can be largely rescued by injection of a single miRNA, mir-430 (Giraldez et al., 2005). In the mouse, ES cells without dcr-1 are

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viable, but silencing of heterochromatin is impaired (Kanellopoulou et al., 2005), Dicer knockout mice die between days 12.5 and 14.5 of gestation possibly due to compromised angiogenesis (Yang et al., 2005), and a conditional Dicer knockout interferes with limb morphogenesis, but not patterning (Harfe et al., 2005). In *Drosophila*, absence of Dicer-2 does not impinge on viability indicating that RNA interference is dispensable. Dicer-1, on the other hand, seems to be more critical and its absence is disrupting proper eye development (Lee et al., 2004b).

These data suggest that miRNAs perform modulatory rather than decisive functions and may (apart from being a small target for mutagenesis) explain why functional studies on miRNAs are scarce. Only few of the miRNAs have been identified genetically such as the classical *C. elegans* lin-4 and let-7 genes involved in cell differentiation (Lee et al., 1993; Reinhart et al., 2000) and mir-14 and *bantam* in *Drosophila* (Brennecke et al., 2003; Xu et al., 2003). *bantam*, in fact, was not found because of its loss-of-function phenotype, which is pupal lethality due to inappropriate growth and development of imaginal discs, but rather due to a gain-of-function over-proliferation phenotype when misexpressed (Brennecke et al., 2003; Raisin et al., 2003).

A correlation of miRNA genes with fragile sites and genomic regions that are involved in cancer pathogenesis – like common chromosomal breakpoints, regions of loss of heterozygosity, and amplified regions (amplicons) – has revealed that about half of the human miRNA genes are situated in cancer-associated regions. They are also frequently associated with fragile sites and hotspots for papilloma virus integration sites (Calin et al., 2004). For example, mir-122a was found both in an amplicon as well as close to translocation t (11,18) implicated in MALT lymphoma. It has yet to be determined whether the translocation brings miR-122a into the vicinity of a heterologous promoter. Other reports show that malignant lymphomas are characterized by amplification and overexpression of the mir-17/-18/-19/-20 gene cluster (He et al., 2005; Ota et al., 2004), and that mir-155 is highly expressed in Burkitt and DLBCL lymphomas (Eis et al., 2005; Metzler et al., 2004). Conversely, mir-143 and mir-145 levels are lowered in colorectal neoplasia (Michael et al., 2003). Likewise, the mir-99a/let-7c/mir-125b2 cluster maps to a homozygous deletion found in lung cancers and the homologous cluster mir-100/let-7a/mir-125b to a loss-of-heterozygosity region linked to breast carcinomas (Calin et al., 2004). These data suggest but do not establish a causative effect of miRNA over-, mis-, or under-expression for malignancy.

Here, we show that overexpression of *mir-278/mirvana* causes a *bantam*-like gain-of-function phenotype. We isolated a *mirvana* mutant with a significantly attenuated overexpression phenotype, suggesting that the miRNA function is greatly affected. Surprisingly, the mutant does not exhibit a detectable loss-of-function phenotype. Our finding suggests that miRNAs, when misexpressed, may adopt novel, potentially deleterious functions and add experimental evidence to suspected causative relations of enhanced miRNA levels and cancer.

Results

Genetic identification of a miRNA gene affecting organ growth

We identified *mir-278/mirvana* as a locus that causes massive eye overgrowth when misexpressed by *GMR-Gal4* or *sev-Gal4* (Figs. 1A, B, see Materials and methods). *GMR* and *sev* promoters are only active in a subset of cells in eye imaginal discs of third instar larvae that undergo a single division or are non-dividing. In contrast, expression by *ey-GAL4*, active earlier in eye-development, has little effect (data not shown). Both the C610 "double-headed" transposon containing *UAS* sites at both ends and the "single-headed" EP transposons EP2316 and EP1229 from the Rørth collection (Rorth et al., 1998) promote overgrowth in the presence of *GMR-Gal4* (Fig. 2A). Transcription from the "single-headed" EP elements EP2589, EP2197, and EP2512 that also map to the same genomic site, but are inserted in the reverse orientation, however, did not result in overgrowth. Thus, we concluded that the functionally relevant genomic region is situated between the insertion sites of EP-C610 and EP2316 and the 3′ end of the *fusilli* gene located on the reverse strand (Fig. 2A).

The candidate region comprises approximately 46.5 kb and is devoid of any annotated protein-coding genes. Database searches identified two ESTs, RH5563 and GH5895 mapping to that region. However, they do not encode proteins. Moreover, both cDNAs, when aligned to the genomic DNA, terminate at an A-rich segment and neither of them is spliced (Fig. 2A and data not shown). Thus, the cDNAs may represent fragments amplified from contaminating genomic DNA or, alternatively, fragments from an mRNA too large to be reverse transcribed in its entirety. Next, we adopted an evolutionary approach reasoning that sequences capable of exhibiting such drastic effects may be conserved in other insects. A BLAST search of the candidate region against *Anopheles* and *Drosophila pseudoobscura* genomes did not reveal striking sequence conservation in all three species except for a very small stretch of about 85 nucleotides directly adjacent to the 3′ end of *fusilli* (Fig. 2C). Evaluation of the secondary structure of this conserved sequence by the mfold program (Zuker, 2003) revealed that it has the capacity to fold into a thermodynamically stable RNA hairpin (−31.2 kcal/mol, Fig. 2B). As miRNA genes are generally strictly evolutionarily conserved and are characterized by a potential hairpin structure, these data indicate that the candidate region may encode a miRNA.

While this work was in progress, Aravin et al. (2003) and Lai et al. (2003) predicted and cloned *Drosophila* miRNAs. Both studies identify a miRNA, termed *mir-278*, situated at the very same site and showed that it is highly expressed in all three larval stages, in pupae, and in adult flies (also confirmed by Leaman et al., 2005). *mir-278* does not belong to an miRNA family and appears to be insect-specific (Supplementary Fig. 1).

In order to provide evidence that *mir-278* is responsible for the overgrowth phenotype, we generated *UAS*-transgenes containing 2.7 kb and 0.8 kb of the originally 46.5 kb *mir-278* genomic region (Fig. 2A). Both transgenes behave identically to the EP insertion lines (Fig. 1E and data not

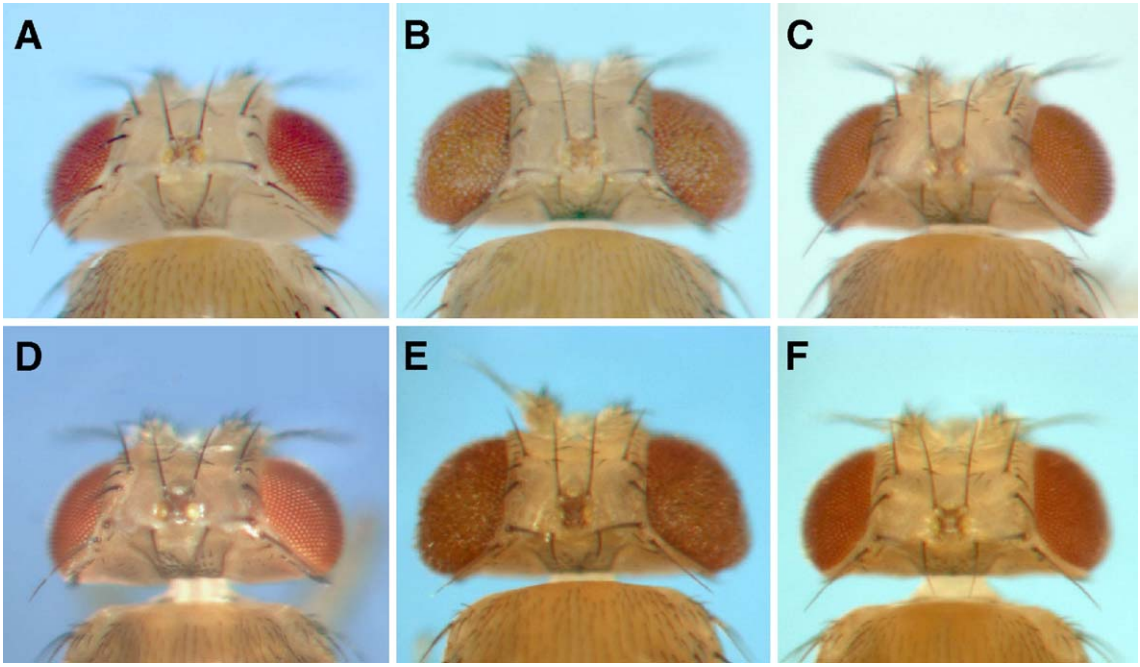

Fig. 1. *mir-278/mirvana* misexpression causes overgrowth. *GMR*-driven overexpression of wild-type *mir-278/mirvana* in the genomic context (B) or from a transgene comprising a 2.7-kb genomic fragment (E, and Fig. 2A) leads to overgrowth (compare to control A). This phenotype is almost completely suppressed by a point mutation at the stem-loop region in the genome or the genomic fragment (C, F). Reversion of the 2.7-kb genomic region relative to the *UAS* site obliterates the growth promoting activity (D). Genotypes are *GMR-GAL4* (A), *GMR-GAL4*, *EP-C610* (B), *GMR-GAL4*, *UAS-mirvana* (*pKN429*) (E), *GMR-GAL4*, *mirvana*^{1} (C), *GMR-GAL4*, *UAS-mirvana*^{1} (*pKN431*) (F), *GMR-GAL4*, *UAS-mirvana* (*reverted*) (*pKN430*) (D).

shown). The overexpression effect is strand-specific: when the orientation of the 2.7-kb fragment was reversed relative to the *UAS* sites, there was no influence on eye and head development (Figs. 1D and 2A). This is in contrast to *bantam* where a *Gal4*-dependent gain-of-function phenotype has also been observed when the locus was in antisense orientation relative to the *UAS* sequence. Presumably, the endogenous promoter contained in the fragment was activated by *Gal4* (Brennecke et al., 2003).

These results indicate that *mir-278* misexpression is sufficient to cause the overexpression phenotype and that the genomic region between the EP integration sites, and the stem-loop is largely dispensable for the phenotype. It is also unlikely that the 2.7-kb *mir-278* fragment contains a promoter with similar characteristics as the *bantam* promoter.

Generation of mirvana^{1}*, a mir-278 mutant*

mir-14 and *bantam* null mutants have been generated by imprecise excisions of P elements situated in the vicinity of the hairpin (Xu et al., 2003; Brennecke et al., 2003). As it is difficult to obtain a 47-kb deletion by a P element jumpout (Robertson et al., 1988), we employed an alternative technique – reversion mutagenesis – to isolate a *mir-278* loss-of-function mutation. The strategy is based on the misexpression phenotype, i.e., overgrowth in the presence of *GMR-GAL4*, which depends on the function of *mir-278*. Reasoning that disruption of this function should result in a reversion of the phenotype, we mutagenized EP-C610 flies with EMS, crossed them to *GMR-GAL4* flies, and screened the progeny for flies *not* exhibiting overgrowth (for details see Materials and methods). Among about 10,000 F_1 flies, we found only one mutant fulfilling this criterion indicating that the gene provides a small target for mutagenesis (Fig. 1C). Sequence analysis of the *mir-278* locus in this mutant revealed a sequence polymorphism that mutates the mature miRNA at position 5 from U to A (Figs. 2A–C). No other mutation was detected in the 2.7 kb surrounding genomic region and the *UAS* sites from the EP transposon remained functional as well (data not shown). As this point mutation was hidden in a genomic context of about 50 kb devoid of any protein-coding gene, we dubbed the mutant allele *mirvana*^{1} (for *mi*cro *R*NA hidden in a nir*vana*). In the following section, *mirvana* is thus used synonymously to *mir-278*.

According to the current sequence annotation, the mature wild-type *mir-278* in *D. pseudoobscura* carries the *mirvana*^{1} mutation. On the other hand, mature *mir-278* from *Anopheles* is identical to *D. melanogaster mirvana*. Considering the extremely high conservation of the stem-loop (Fig. 2C), we regarded a potentially disruptive mutation at the *D. pseudoobscura mir-278* locus unlikely and thus re-sequenced that region. As expected, the published sequence is erroneous and mature *mir-278* is absolutely conserved in the two Drosophilids.

To show that this point mutation was indeed responsible for the reversion of the *mirvana* gain-of-function phenotype, we generated *UAS* transgenes of 2.7 kb and 0.8 kb *mirvana*^{1} genomic context, respectively (Fig. 2A). Misexpression of these transgenes in the eye does not result in overgrowth. However, in the presence of *GMR-GAL4*, both transgenes and the original

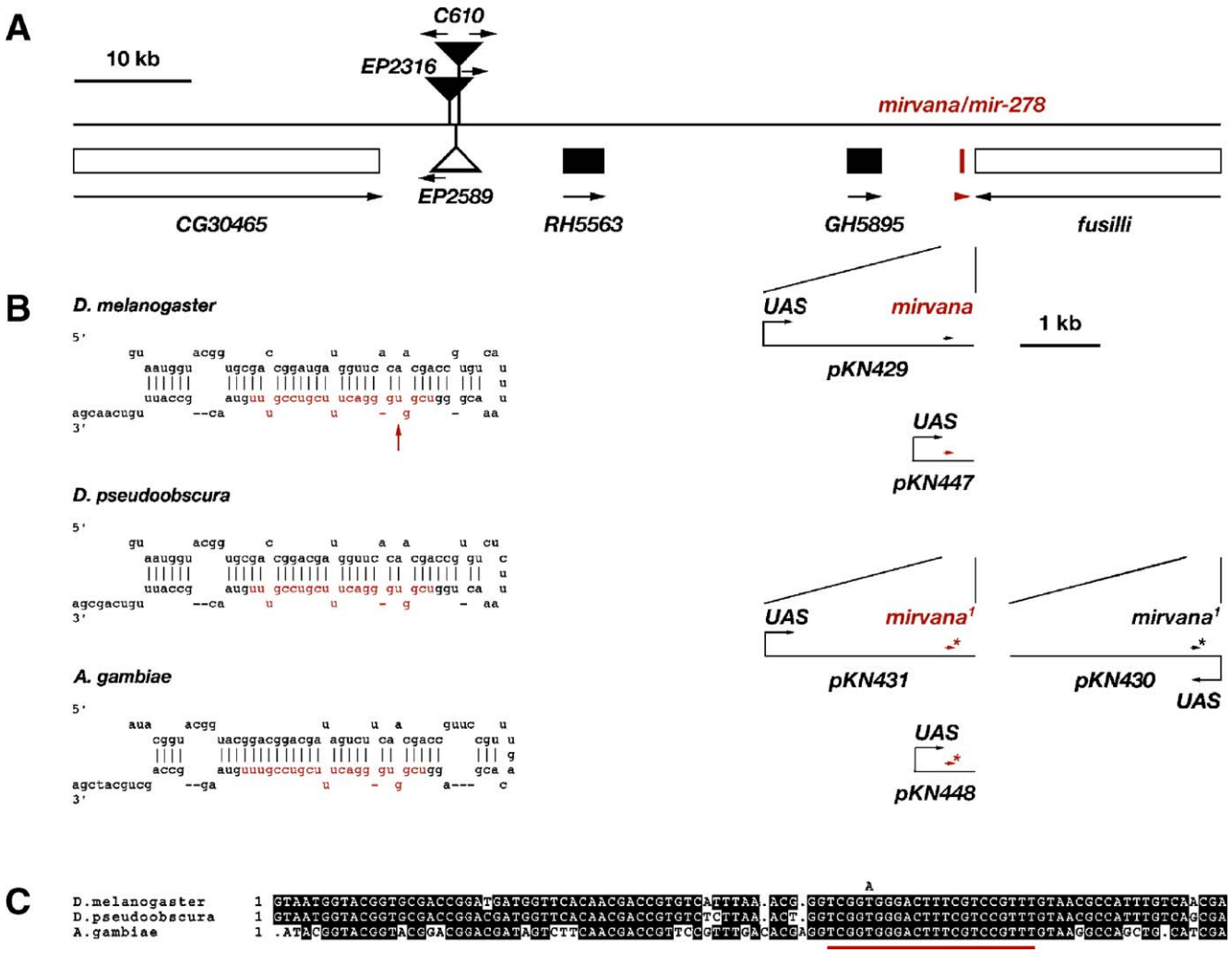

Fig. 2. Genomic organization of the *mir-278/mirvana* locus. (A) The *mirvana* gene is situated between *CG30465* and *fusilli* in a ca. 50-kb region devoid of predicted protein-coding genes. Black boxes indicate putative cDNA fragments. The insertion sites of the bidirectional EP-C610 and of two representative unidirectional Rørth EP elements are shown with arrows indicating *UAS* sites. EP2589 does not lead to overgrowth. The stem-loop genomic region is highlighted as red box. 2.7-kb and 0.8-kb fragments amplified from wild-type and *mirvana*^{1} mutant flies, respectively, and cloned into the *pUAST* vector are shown below at larger scale. (B) Predicted secondary structures of *mir-278* loops. The mature miRNA is in red, and the arrow marks the nucleotide affected by the *mirvana*^{1} mutation. (C) Sequence alignments of the highly conserved genomic region. The sequence of mature *mir-278* is underlined, and the mutation is indicated. Further alignments are given in Supplementary Fig. 1.

mutant cause a slightly rough appearance of the compound eye indicating that some residual miRNA function may remain (Figs. 1C, F). Alternatively, it is also conceivable that due to the altered "seed" sequence *mirvana*^{1} miRNA exhibits an altered target-specificity, thus affecting other genes involved in proper eye development than wild-type *mirvana*.

These results further demonstrate that within the 46.5-kb region, the stem-loop sequence is most responsible for the misexpression phenotype because it is vulnerable to mutagenesis. There are many reports that the 5′ end of a miRNA and especially "seed"-positions 2 to 8 play a key role in target recognition (Brennecke et al., 2005; Doench and Sharp, 2004; Lai, 2002; Lewis et al., 2003; Stark et al., 2003; Lee et al., 1993; Wightman et al., 1993). As the *mirvana*^{1} point mutation maps to position 5 of mature *mir-278*, our data confer additional experimental evidence to the importance of the 5′ region of a miRNA for target regulation.

mirvana^{1} *has strongly reduced activity*

To determine and to quantify potential residual activity of the *mirvana*^{1} mutant miRNA, we generated a sensor of *mirvana* activity. The system has first been developed to assess *bantam* function and relies on the ability of miRNAs to negatively regulate mRNAs by RNAi when there is perfect sequence complementarity (Brennecke et al., 2003). We placed two *mirvana* complementary sites downstream of a GFP gene controlled by the ubiquitously expressed tubulin promoter (Materials and methods). This transgene should reflect the expression of *mirvana* as a negative pattern, i.e., GFP activity should be high when *mirvana* is not expressed and vice versa.

Wild-type and mutant *mirvana* transgenes were misexpressed by a *ptc-GAL4* driver that reflects the expression pattern of the endogenous Ptc protein. Ptc is expressed at low levels in the anterior compartment of wing imaginal discs and is highly

upregulated by Hh in a small stripe along the anterior–posterior compartment boundary (Ingham et al., 1991). Furthermore, we introduced the *mirvana* sensor or a control lacking *mirvana* complementary sites into this genetic background.

GFP is almost completely suppressed in a broad stripe in the anterior compartment when *mirvana* and its sensor are co-expressed (Fig. 3A). *mirvana*^{1} activity is only detected via sensor-GFP repression along the anterior–posterior boundary where *ptc* activity is very high (Fig. 3B). Thus, *mirvana*^{1} activity is only residual. In contrast, GFP expression from a control sensor is high and uniform and unaffected by co-overexpression of *mirvana* confirming that *mirvana* specifically exerts its function via the 3′ UTR of the sensor (Fig. 3C).

The sensor experiment visualizes the drop in miRNA activity by a point mutation and also provides a clue to quantify the relative reduction of activity. Recently, the upregulation of Ptc protein levels at the anterior–posterior compartment boundary relative to the basal expression level in the anterior compartment has been determined to be seven-fold (Casali and Struhl, 2004). When *mirvana*^{1} is overexpressed by *ptc-Gal4*, the sensor readout can only be detected where Ptc levels are high, while *mirvana* wild-type activity is also effective at basal Ptc expression levels. Assuming a linear relationship between expression levels and activities, the *mirvana*^{1} point mutation thus reduces activity by a factor of about seven. This value is higher than the estimate by Brennecke et al. (2005) who systematically introduced mismatches in a 3′ UTR complementary to *mir-278* and found that a bulge in the miRNA—3′ UTR hybrid at position 5 (counted from *mir-278* 5′ end) causes a two-fold reduction of reporter activity. This discrepancy may be explained by different experimental setups that are reciprocal and not directly comparable (see Discussion). Besides, our mutation was selected to show a strong phenotype.

Our results show that in the context of a sensor containing two perfect target sites the *mirvana*^{1} mutation causes a seven-fold activity reduction under overexpression conditions.

mirvana misexpression primarily increases cell number

mirvana mediated overgrowth may be due to increase in cell size or cell number or a combination of both. For comparison, *bantam* expression posterior to the morphogenetic furrow results in overproliferation because of impaired apoptosis of supernumerary cells (Brennecke et al., 2003). Similarly, loss of the tumor suppressor *warts/lats* in the *Drosophila* eye causes tumorous growth due to extra cells (Justice et al., 1995).

To address this issue in a controlled experiment, we sought to compare *mirvana* and *mirvana*^{1} misexpressing and wild-type cells within the same animal and generated mosaic flies that overexpressed the miRNAs in genetically marked clones (see Materials and methods).

We assessed whether there might be additional cells within *mirvana* overexpression clones. For that purpose, we first determined the relative distances between central R7 cells of ommatidia in wild-type tissue and adjacent clones. On average, the distances between ommatidial centers in wild-type tissue and control and *mirvana*^{1} misexpression clones are about equal (Figs. 4B, D, Materials and methods). Ommatidia of *mirvana* misexpression clones, however, are on average 1.23 times more distant to each other than the surrounding wild-type ommatidia (Fig. 4A). This value is comparable to increased distance in *warts/lats* loss-of-function clones relative to heterozygous control tissue measured to be 1.26 (Fig. 4C).

Supernumerary cells between differentiated ommatidia are eliminated by apoptosis during eye-disc development at early pupal stage (Wolff and Ready, 1993). In the absence of *warts,* apoptosis is inhibited and non-eliminated cells fill up mutant

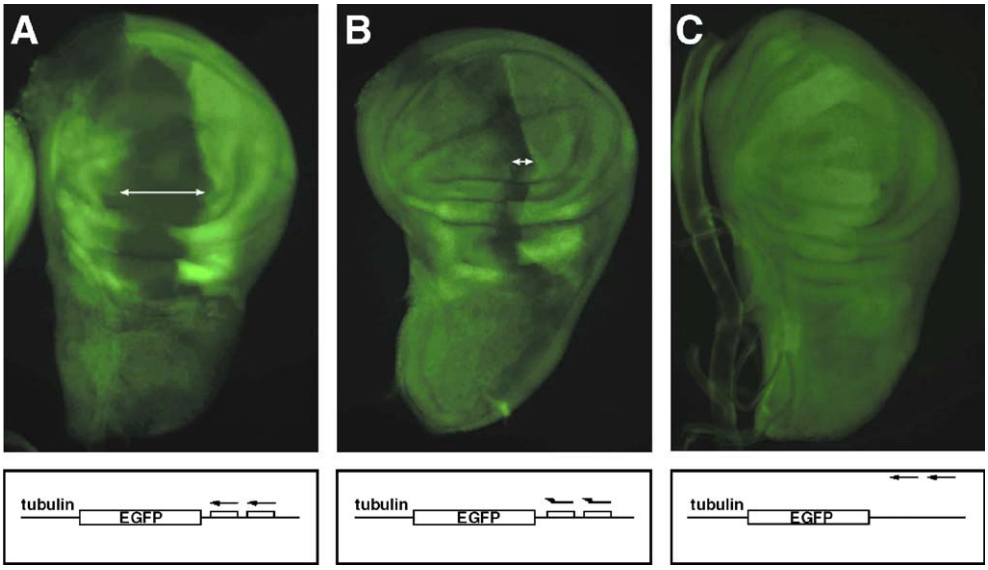

Fig. 3. Activities of *mirvana* and *mirvana*^{1}. *ptc-Gal4*-driven expression of *mirvana* causes a strongly reduced activity of the *tub-EGFP* sensor gene along the anterior–posterior boundary (A). The width of the *ptc* stripe is reduced when *mirvana*^{1} is misexpressed (B). Arrows indicate the width of the ptc > *mirvana* and ptc > *mirvana*^{1} expression domains. GFP levels of a tub-EGFP control sensor containing a 3′ UTR devoid of *mirvana* complementarity are unaffected by *ptc-Gal4, UAS-mirvana* (C). Sketches of the sensor constructs with *mirvana* complementary sites symbolized as boxes and miRNAs as arrows are shown below.

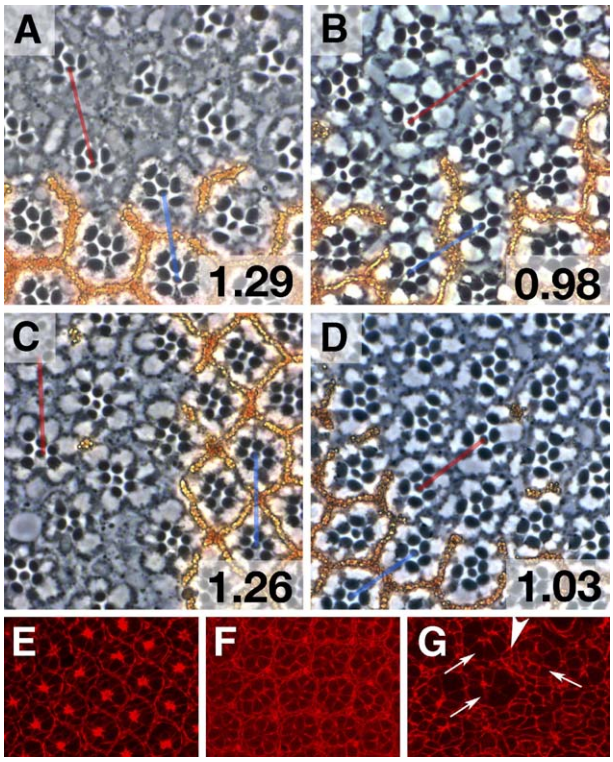

Fig. 4. Increased interommatidial distances and excessive cells in tissues overexpressing *mirvana.* Clones overexpressing *mirvana* (A) and *mirvana*^{1} (B) from 0.8-kb transgenes are marked by the strong reduction of pigment. Control in panel D shows a "FLP-out" clone of a fly devoid of a miRNA transgene. (C) A *warts*^{5Y2} loss-of-function clone (also marked by the absence of pigment). *mirvana* gain-of-function and *warts* loss-of-function clones exhibit increased interommatidial space (A, C) and *mirvana*^{1} misexpression and control clones interommatidial distances comparable to wild-type tissue (B, D). Distances between ommatidia were measured from the central R7 cells in clones (red lines) and in wild-type tissue (blue lines). The ratio between average clonal and wild-type tissue values are given in the lower right corners. Note that cell sizes as judged from the size of darkly staining rhabdomers appear unchanged in *mirvana* overexpression clones (A). (E) Pupal eye imaginal discs of a wild-type control, and of pupae overexpressing *mirvana*^{1} and *mirvana*, respectively, taken at the same microscope settings (F, G). Supernumerary cells (marked by an arrowhead) can be detected between ommatidial clusters (marked by arrows) in *GMR > mirvana* discs (G). Cells in the cluster appear in part larger than in wild-type and *GMR > mirvana*^{1} controls (E, F). Genotypes are *y w hsFLP*; *GMR-FRT-w*$^{+}$*-FRT-GAL4*, *UAS-mirvana* (*pKN447*) (A), *y w hsFLP*; *GMR-FRT-w*$^{+}$*-FRT-GAL4*, *UAS-mirvana*^{1} (*pKN448*) (B), *y w hsFLP*; *FRT82B*, *w*$^{+}$*/FRT82B*, *warts*^{5Y2} (C), *y w hsFLP*; *GMR-FRT-w*$^{+}$*-FRT-GAL4* (D), *y w* (E), *y w*; *GMR-GAL4/mirvana*^{1} (F), *y w*; *GMR-GAL4/EP-C610* (G).

clones, thus explaining greater ommatidial distances (Justice et al., 1995). We therefore sought to determine whether excessive cells persist in pupal eye imaginal discs when *mirvana* is overexpressed. Indeed, while ommatidia were densely packed in wild-type (Fig. 4E) and discs overexpressing *mirvana*^{1} (Fig. 4F), they were disarranged by extra intervening cells in *GMR > mirvana* discs (Fig. 4G). A few extra intraommatidial cells were observed in *GMR > mirvana*^{1} pupal discs confirming some activity of the mutant miRNA (see above). Strikingly, some ommatidial cells of *GMR > mirvana* discs appeared larger than wild-type control cells indicating some size effect on differentiating cells (Figs. 4G, E). In contrast, sagittal eye sections did not reveal a size-difference of wild-type and adjacent *mirvana* misexpressing ommatidial cells as judged from the size of darkly staining rhabdomeres (Fig. 4A).

From these observations, we conclude that *mirvana* misexpression primarily affects cell number to increase the size of an adult eye.

Different effects of mirvana gain and reduction-of-function on apoptosis

The persistence of supernumerary cells in *GMR > mirvana* discs suggests that apoptosis is compromised. To further test for a potential antiapoptotic *mirvana* effect, we assessed its ability to suppress cell death by *hid* and *grim* (Abrams, 1999). Induction of apoptosis by these proapoptotic genes in post-mitotic cells of the eye imaginal discs leads to small and rough adult eyes (Figs. 5A, D). Co-overexpression of *mirvana*, but not *mirvana*^{1}, largely restored eye size indicating that *mirvana* has strong antiapoptotic activity (Figs. 5B, D and 5E, F).

If the wild-type function of *mirvana* were to attenuate *hid* and *grim* induced apoptosis, we in turn would expect that loss of *mirvana* activity would enhance the severity of the eye phenotype. Control experiments utilizing the sensor construct revealed that *mirvana* is ubiquitously expressed in eye-imaginal discs (data not shown). Mutant *mirvana*^{1} flies appear completely viable, fertile, and display normal longevity (data not shown), allowing us to assess the phenotypes in a *mirvana*^{1} background. However, this genetic background had virtually no effect on *GMR-hid* and *GMR-grim* dependent apoptosis (Figs. 5G, H). (Even further reduction of *mirvana* activity in *mirvana*^{1}*/Df(2R)Exel7137* flies did not contribute to the severity of the rough eye, data not shown).

These experiments show that *mirvana* misexpression confers an antiapoptotic function that appears to be neomorphic because we are unable to detect a contribution of endogenous *mirvana* on *hid*- and *grim*-induced apoptosis.

Loss of mirvana function does not attenuate growth

Although the *bantam* and *mirvana* misexpression phenotypes are strikingly similar, the respective mutants behave significantly differently: (1) reduction of *bantam* function enhances *hid*-induced apoptosis. (2) Complete loss of *bantam* function is lethal, but a strong hypomorphic allelic combination allows for viability. The surviving flies are considerably smaller than wild-type flies, thus indicating that both gain and loss of *bantam* functions may deregulate an as yet unidentified negative regulator of cell proliferation (Brennecke et al., 2003).

In the light of our previous findings, we thus explored whether reduction of *mirvana* function would have an impact on cellular or organismal growth. We generated *mirvana*^{1} mutant clones in the compound eye to assess cell sizes in mutant clones and in adjacent heterozygous wild-type tissue. As shown in Fig. 5I, mutant cells do not appear to be significantly smaller. We also assessed the influence of the *mirvana*^{1} mutation on the whole organism. Both male and female mutants are normally sized and have normal weight (Fig. 5J).

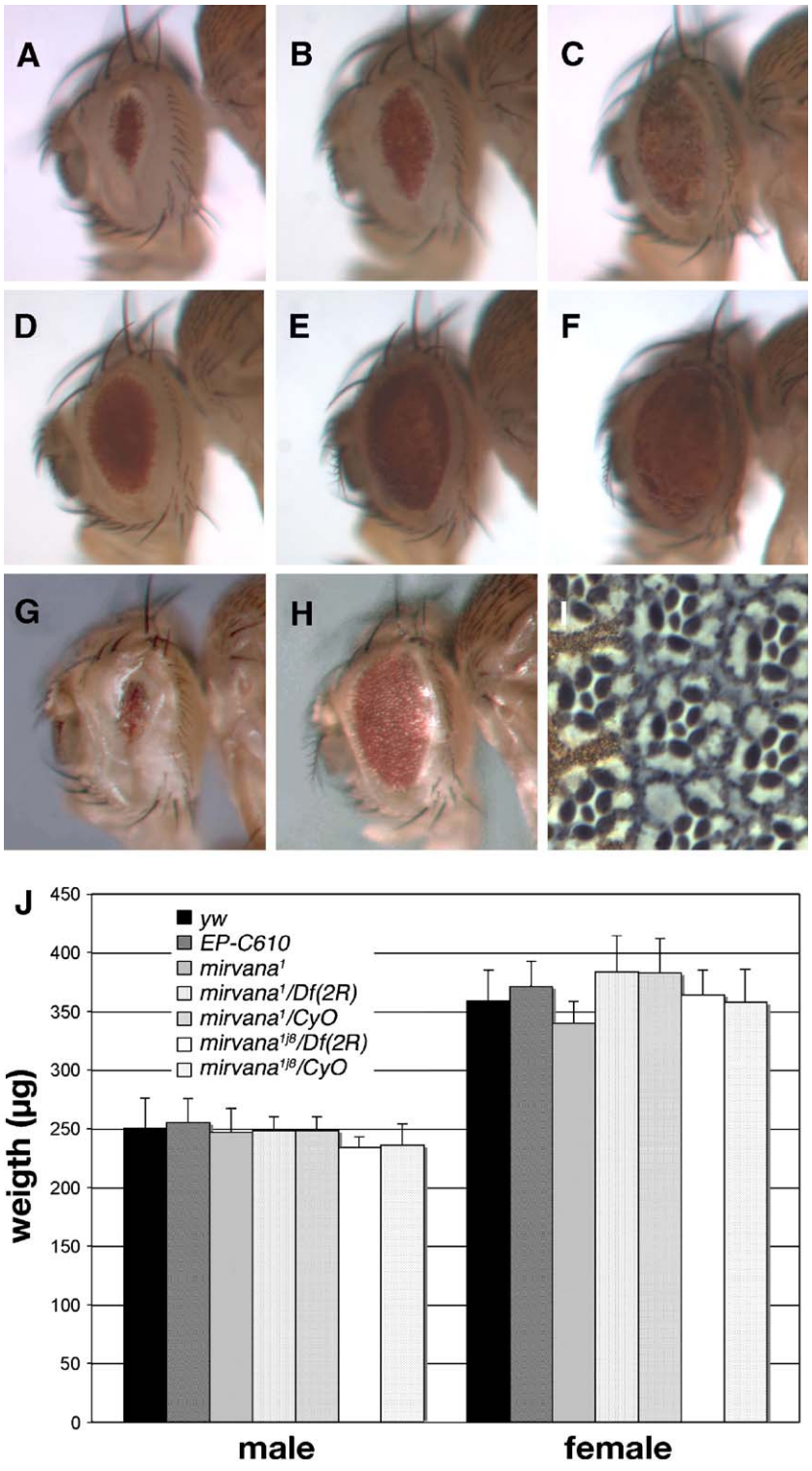

Fig. 5. *mirvana* gain-of-function is antiapoptotic, while reduction-of-function has no obvious phenotypic consequences. *GMR-hid* (A) and *GMR-grim* (D) are slightly suppressed by *mirvana*^{1} overexpression (B, E) and strongly suppressed by *mirvana* overexpression (C, F). In contrast, *GMR-hid* (G) and *GMR-grim* (H) are unaffected in a *mirvana*^{1} homozygous background. (I) Cell sizes (judged from rhabdomer sizes) of *mirvana*^{1} mutant cells (marked by absence of pigment) are not significantly reduced relative to heterozygous wild-type cells (marked by red pigment granules). Loss of *mirvana* function does not influence the dry weight of virgin flies (J). Weights of virgin females (right panel) and males (left panel). Weight of *yw* parental flies is not significantly different from EP-C610, from homozygous *mirvana*^{1}, from hemizygous *mirvana*^{1}/*Df(2R)Exel7137*, from homozygous *mirvana*^{1j8}, and from hemizygous *mirvana*^{1j8}/*Df(2R)Exel7137* (B, ry; Is, dck) flies. When applicable, control balanced flies from the same crosses reared under identical conditions are shown. Genotypes are *y w*; *GMR-hid/+*; *UAS-mirvana* (*pKN447*) (A), *y w*; *GMR-hid/GMR-GAL4*; *UAS-mirvana*^{1} (*pKN448*) (B), *y w*; *GMR-hid/GMR-GAL4*; *UAS-mirvana* (*pKN447*) (C), *y w*; *GMR-grim/+*; *UAS-mirvana* (*pKN447*) (D), *y w*; *GMR-grim/GMR-GAL4*; *UAS-mirvana*^{1} (*pKN448*) (E), *y w*; *GMR-grim/GMR-GAL4*; *UAS-mirvana* (*pKN447*) (F), *y w*; *GMR-hid*, *mirvana*^{1}/*mirvana*^{1} (G), *y w*; *GMR-grim*, *mirvana*^{1}/*mirvana*^{1} (H), *y w hsFLP*; *FRT42D*, *mirvana*^{1}/*FRT42D*, w^{+} (I).

The *mirvana*^{1} chromosome still contains the EP-C610 transposable element. The *UAS* site contains a minimal *hsp70* heat shock promoter, which may confer leaky expression that theoretically could compensate for the reduced *mirvana*^{1} activity. We thus excised the EP to generate allele *mirvana*^{1j8} (Materials and methods). Our strongest mutant combination – flies heterozygous for *mirvana*^{1j8} and deficiency *Df(2R) Exel7137* uncovering the entire *mirvana* locus – exhibits normal weight as well (Fig. 5J).

We have shown that under idealized over- and misexpression conditions, *mirvana*^{1} activity is reduced seven-fold. Although we cannot completely exclude that a single *mirvana*^{1j8} allele may provide sufficient residual function, these data let us conclude that *mirvana* function is largely dispensable for normal growth.

Discussion

The mirvana gene—a vast transcription unit for a small effect

The gene coding for the miRNA *mir-278/mirvana* is remarkably prodigal. Assuming that the hotspot for EP element insertion defines a promoter (judged from the tendency of P-transposons to integrate into promoters (Spradling et al., 1995)), the *mirvana* gene may comprise about 46 to 50 kb. Two presumptive cDNA fragments provide evidence that the *mirvana* pri-miRNA encompasses the region between the promoter and the very distant stem-loop region. The presumably high-energy costs for the transcription of the miRNA are even more remarkable as *mirvana* is not essential. It is thus tempting to speculate that the size of the gene may contribute to a tightly controlled expression to prevent its potentially deleterious side effects.

By a genetic strategy aiming to target a functional unit by screening for revertants of a gain-of-function phenotype, we were able to isolate the *mirvana*^{1} allele that exhibits strongly reduced activity. Previously, we applied this method successfully to identify loss-of-function mutations of a protein-coding gene providing a much better target for mutagenesis (Rintelen et al., 2003). The finding of a miRNA mutant was more fortuitous because the target sequence was just about 7 to 8 nucleotides in length (assuming that only the first third of a miRNA, also termed "seed" sequence, provides essential activity). We also note that the U to A mutation at position 5 does not fall within the mutagenic spectrum of EMS, which is known to cause GC to AT transitions leaving open the possibility that we recovered a spontaneous mutation. The fact that the mutation maps to the "seed" sequence lends in vivo evidence to recent hypotheses that the prime miRNA activity is associated with its 5′ end (Brennecke et al., 2005; Doench and Sharp, 2004; Lai, 2002; Lewis et al., 2003; Wightman et al., 1993).

mirvana^{1} *activity*

Judging from an over- and misexpression phenotype, the mutation was estimated to cause a seven-fold drop in *mirvana* activity. This value is higher than that suggested by Brennecke et al. (2005), who found that a bulge at position 5 of *mir-278* causes a two-fold reduction. The two experimental settings are, however, not directly comparable. Brennecke et al. (2005) varied the target sequence, i.e., the sensor mRNA, rather than the miRNA and their sensor contained only one (imperfect) target site. We calculated the difference of *mirvana* and *mirvana*^{1} binding to be −4.7 kcal/mol per sensor site in our setup (−40.7 and −36.0 kcal/mol, respectively) and −4.8 kcal/mol for the Brennecke setting (−32.6 and −27.8 kcal/mol, respectively). The difference may thus be mainly attributed to the number of sensor target sites. The values have to be interpreted cautiously because the sensor targets are idealized and provide perfect or near-perfect matches. The in vivo targets, however, may only match the short 5′ seed sequence, and this interaction may be much more vulnerable to a distortion by a mismatch.

In a recent study, Leaman et al. (2005) have evaluated the effects of antisense-mediated depletion of miRNA function in *Drosophila*. While loss of some miRNAs and miRNA families greatly affected early development, the depletion of about half of the investigated miRNAs – including *mir-278* – did not lead to discernible phenotypic consequences. These data indicate that the function of every second miRNA is likely to be very subtle. Further, two entirely different approaches suggest that *mirvana* function is non-essential. The lack of obvious miRNA loss-of-function phenotypes will greatly complicate the task of finding regulatory targets.

mirvana targets

For a greater understanding of the *mirvana* overexpression phenotype, it will be important to identify the target genes. This is an extremely difficult task, as there are no biochemical assays for target identification available. Current methodology thus relies on algorithms designed to predict biologically relevant targets by sequence, incorporating features of the few genetically confirmed miRNA target–mRNA pairs, a good complementarity to the 6 to 8 nucleotide "seed" sequence at the 5′ end of the miRNA, sequence conservation, and some structural properties of the 3′ UTR. Scarcity of in vivo interactions and multitude of "seed" matching sequences, however, cause highly divergent predictions: The first three of five programs created to find *Drosophila* miRNA targets (Enright et al., 2003; Rajewsky and Socci, 2004; Rehmsmeier et al., 2004; Robins et al., 2005; Stark et al., 2003) also provide lists of presumptive *mir-278* regulated genes—and these show virtually no overlap.

Maybe not surprisingly, we have unsuccessfully tried to establish epistasis between *mirvana* and genes found by our own prediction program and also genes known to cause phenotypes similar to *mirvana* gain-of-function when mutated.

Inspired by several reports demonstrating that activities of siRNAs and miRNAs can overlap (Jackson et al., 2003; Lim et al., 2005; Yekta et al., 2004), we have also evaluated whether *mirvana* could act as a siRNA and trigger degradation of *warts* mRNA by RNAi: Although the *wts* message contains a site complementary to *mirvana* and although *mirvana* overexpression indeed causes an approximate two-fold reduction of *wts* mRNA, we cannot explain overgrowth by downregulation of *wts* (our unpublished results).

dsRNAs can trigger promoter methylation in plants (Mette et al., 2000), and recently, it was shown that siRNAs are able to induce DNA methylation and gene silencing in mammalian cells, too (Kawasaki and Taira, 2004). Therefore, considering overlapping siRNA and miRNA activities, it is also conceivable that *mirvana* overgrowth would be due to an epigenetic effect on an as yet unidentified promoter.

Antiapoptotic effects of mirvana overexpression

We were able to show that overgrowth can largely be attributed to a strong antiapoptotic effect on supernumerary cells that would otherwise be eliminated.

A recent study reports increased levels of miRNAs of the *mir-17-92* polycistron in various lymphoma samples. In a mouse model, *mir-17-92* expression from a transgene strongly inhibited *c-myc* induced apoptosis and thereby accelerated tumor development leading to the notion miRNAs may act as oncogenes or "oncomirs" (He et al., 2005). As many miRNAs are located at fragile chromosomal sites and cancer-associated genomic regions, many more are to be awaited (Calin et al., 2004).

In the *Drosophila* system, mirRNAs *mir-14* and *bantam* cause cell death suppression as well (Brennecke et al., 2003; Xu et al., 2003). But available data also indicate that there is a fundamental difference between *mirvana* function on the one hand and *bantam* and *mir-14* functions on the other hand: *bantam* and *mir-14* gain- and loss-of-functions are reciprocal, i.e., the gain-of-function mutations promote growth by inhibiting apoptosis, while reduction of function enhances apoptosis and in the case of *bantam* attenuates growth. We could not observe an effect of *mirvana*^{1} on apoptosis, nor could we observe reduced or delayed growth. (Fertility, longevity, male courtship behavior, and innate immunity are unaffected in *mirvana*^{1} flies, too; our unpublished data).

Without a clear loss-of-function phenotype of *mirvana*^{1}, target identification will become even more difficult. Thus, we are unable to decide whether the *mirvana* overexpression phenotype reflects its action on its normal target or whether misexpression creates a neomorphic phenotype. For example, the *mirvana* wild-type function could be limited to a very subtle suppression of cell death that does not result in major developmental defects or, alternatively, only abnormally high *mirvana* levels may suffice to suppress a target providing only imperfect mRNA–miRNA binding potential. Given that miRNAs with highly divergent "seed" sequence, like *mir-14*, *bantam*, and *mirvana*, cause a very similar phenotype, it should even be taken into account that misexpressed miRNAs might trigger an antiapoptotic response by a mechanism unrelated to their primary sequence.

Materials and methods

mirvana gain- and reduction-of-function mutants

EP lines and identification of the mirvana locus

EP elements are P-transposons containing yeast *UAS* sites. This heterologous enhancer is activated by the *Gal4* transcription factor that directs transcription into the genomic region flanking the insertion site (Brand and Perrimon, 1993; Rorth et al., 1998). P elements tend to integrate into promoters and expression via the *GAL4-UAS* system generally leads to overexpression. Thus, insertion of an EP element and activation by a heterologous source of *Gal4* may cause mis- and overexpression of an adjacent gene. EP elements inserted at the *mirvana* locus and overexpressing the miRNA were found in two independent screens for lines exhibiting altered tissue growth: First, the Rørth collection (Rorth et al., 1998) of *white*$^{+}$ marked transposons containing *UAS* site at the 3′ end was crossed to a *sev-GAL4* driver line. Second, in a collaborative effort of the Hafen and Basler laboratories, we created about 10,000 novel insertion strains of a *yellow*$^{+}$ marked EP element featuring *UAS* sites at both the 5′ and 3′ ends (Wittwer et al., 2005), D. Hengartner, unpublished) by Δ2–3 transposase induced mobilization (Robertson et al., 1988). These lines were crossed to *GMR-GAL4* flies, and the progeny was scored for eye overgrowth.

mirvana^{1}

To generate the *mirvana*^{1} mutant, we fed male EP-C610 flies with 25 mM EMS according to Lewis and Bacher (1968) and crossed them to *GMR-GAL4* virgins. The resulting F_1 progeny was screened for flies with normal eye appearance. The *mirvana*^{1} chromosome was recombined five times with the unmutagenized parental *yw* chromosome to purify it from potential second site mutations. The mutation could be followed easily by the tightly linked *yellow*$^{+}$ marker.

mirvana^{1j8}

To excise the EP-C610 transposon from the *mirvana*^{1} chromosome, we mobilized it by Δ2–3 transposase (Robertson et al., 1988) and screened the progeny for loss of the associated *yellow*$^{+}$ marker to yield allele *mirvana*^{1j8}. The transposition of the entire EP element was confirmed by PCR.

Transgenes

UAS constructs

The 2.7-kb and 0.8-kb stem-loop genomic regions of wild-type and *mirvana*^{1} flies were amplified by PCR with primers 5′-GCCACTTATCAAACAGACGC-3′ (2.7 kb) or 5′-GTTACTTGTGCAACCGCTGG-3′ (0.8 kb) and 5′-TTATCCTTGTGCACTCCCAG-3′. The products were subcloned into the TopoTA vector (Invitrogen), sequenced, and then cloned into *pUAST* (Brand and Perrimon, 1993). Germline transformation of a *yw* strain was performed as described (Basler et al., 1991).

Sensor construct

Oligos 5′-CTAGATTACAAACGGACGAAAGTCCCACCGACCCGTTTTTACAAACGGACGAAAGTCCCACCGACCCGTTTC-3′ and 5′-TCGAGAAACGGGTCGGTGGGACTTTCGTCCGTTTGTAAAAACGGGTCGGTGGGACTTTCGTCCGTTTGTAAT-3′ were annealed in 10 mM Tris pH7.5/1 mM $MgCl_2$ and then ligated into XbaI-XhoI-cut JB26 tub-EGFP-MCS-SV40-3′UTR vector (Brennecke et al., 2003). *mirvana* matching sequences are underlined.

Phenotypic analysis

Analysis of pupal discs

46 hours after the formation of the white prepupal stage eye discs were dissected, fixed for 20 min in 4% PFA/PBS, and washed three times for 30 min in PBT. The discs were stained for 2 h in PBT containing 10 mg/ml BSA and 25 μl/ml Phalloidin-Alexa 594 (Molecular Probes), then washed for 1 h in PBT, mounted in VectaShield, and analyzed with a Leica TCS confocal microscope.

Analysis of overexpression clones

Strain *y w hsFLP; GMR-FRT-w*$^{+}$*-FRT-GAL4* (Brogiolo et al., 2001) was crossed to *mirvana* or *mirvana*^{1} EP or pUAST transgenic strains. Mitotic recombination at the FRT sites was induced 48 h after egg laying by a 60 min heat shock at 37°C. The excision of the *w*$^{+}$ marker is accompanied by an activation of *GAL4*. Clones overexpressing the respective miRNAs are thus marked by the absence of pigment. (The *w*$^{+}$ marker of the *pUAST* transgene insertions was only weakly expressed and allowed for distinction of neighboring darker *w*$^{+}$ tissue). Histological eye sections were done as described (Basler and Hafen, 1988). Distances between R7 cells in control and clonal tissue were

measured using Adobe Photoshop. Care was taken that the planes of different sagittal sections were comparable.

Clonal analysis

y w hsFLP; *FRT42D*, w^+ flies were crossed to *y w*; *FRT42D*, *mirvana*^{1} flies and *y w hsFLP*; *FRT82B*, w^+ flies were crossed to *y w*; *FRT82B*, *warts*^{5Y2} flies. Mitotic recombination was induced 48 h after egg laying by a 60-min heat shock at 37°C. *mirvana*^{1} and *warts*^{5Y2} clones in eye section are marked by the absence of the w^+ marker.

Analysis of imaginal discs

Wing imaginal discs of *ptc-GAL4*, *UAS-mirvana*; *tub-GFP-sensor* or *ptc-GAL4*, *UAS-mirvana*^{1}; *tub-GFP-sensor* and eye imaginal discs of *UAS-mirvana*; *tub-GFP-sensor* or *GMR-GAL4*; *UAS-GFP* wandering third instar larvae were fixed in 4% paraformaldehyde and analyzed by fluorescence microscopy.

Fly weight analysis

As the dry weight of flies shows less variation than fresh weight (G. Seisenbacher, personal communication), we dried virgin flies for 5 min at 95°C and weighed them after cooling. At least 25 flies from 2 independent experiments were measured.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2005.11.047.

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