An analysis of putative roles for the CCR4-NOT deadenylase-complex subunit Regena (NOT2) in microRNA-mediated gene silencing in *Drosophila melanogaster*

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ABSTRACT

microRNAs (miRNAs) are one class of small non-coding ribonucleic acid (RNA) molecule essential to development and homeostasis in plants and animals. miRNAs silence gene expression through complementary base pairing with target gene messenger RNAs and association with the miRNA-induced Silencing Complex (miRISC). The identification and characterization of cellular factors required for miRNA-mediated gene silencing is incomplete.

A forward genetic screen was carried out in *Drosophila melanogaster* to generate flies defective for gene silencing. Silencing was assayed by expression of a Green Fluorescent Protein (GFP) reporter fused to the *Brd* gene 3 'UTR, which is regulated by miRNAs. Genetic analysis revealed that the CCR4-NOT deadenylase-complex subunit Regena (NOT2) is required for miRNA-mediated silencing of the reporter. In addition, perturbation of the *Regena* gene altered *Drosophila* eye development and resulting adult eye morphology.

miRNAs are thought to silence target gene expression through a combination of translational repression and target mRNA degradation, though the detailed mechanism of this process is a matter of controversy.

Novel genetic reagents to explore miRNA function *in vivo* have been generated and characterized. Ongoing efforts aim to explore whether Regena is required to silence other miRNA targets *in vivo*, and whether Regena is required for miRNA-mediated gene silencing at different stages of the *Drosophila* life cycle. Elucidation of the lesion in the *Regena* (*NOT2*) gene and the molecular nature of GFP reporter silencing will contribute to an understanding of the mechanism of miRNA-mediated gene silencing *in vivo*.

The microRNA pathway

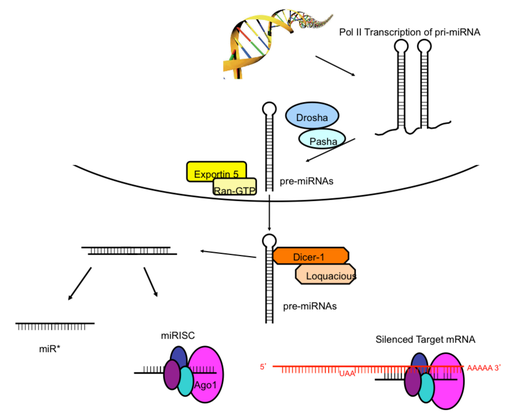

Figure 1: Gene silencing requires the generation of a functional miRNA-induced Silencing Complex (miRISC). MiRNA genes are transcribed from genomic DNA and processed to yield short ~22 nucleotide double-stranded RNAs. A single miRNA strand is incorporated into the miRNA-induced Silencing Complex (miRISC), which binds to complementary target mRNAs and silences gene expression by means of mRNA degradation or translation block.

EXPERIMENTAL APPROACH

A forward genetics screen to identify new genes required for miRNA-mediated gene silencing

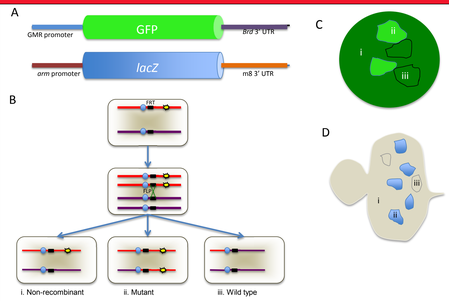

Figure 2: Isolation of mutants and detection of miRNA activity. The GFP and *lacZ* reporters (A). Mitotic recombination is used to generate clones of cells homozygous for a mutation in *Regena* (B). Resulting GFP expression in the adult eye, and resulting *lacZ* expression in larval eye discs; phenotypes and corresponding genotypes as depicted in B (C and D).

A forward genetics screen was conducted to isolate mutants with altered gene silencing. A GFP reporter was used to assay miRNA activity. The GFP reporter is sensitive to miRNA activity because it is fused to a 3' untranslated region (3'UTR) regulated by miRNAs in the *Drosophila* eye. An increase in GFP fluorescence indicates a loss of gene silencing (Figure 2C). An alternate *lacZ* reporter is also being used to evaluate gene silencing (Figure 2D). An increase in *lacZ* expression indicates a loss of gene silencing.
Given that a mutation disrupting miRNA function is likely to be lethal, mitotic recombination was used to generate clones of cells in the eye that are homozygous for the mutation, and can be used to assay GFP fluorescence in adult eyes, or *lacZ* expression in larval eye discs.

Discovery of the *Regena* gene

We analyzed one mutant from the screen (U3-2) and performed deficiency mapping and complementation testing in order to determine the identity of the gene that is essential for silencing the GFP reporter (Figure 3). Deficiency mapping narrowed the genomic region of interest to a ~16.4kb region containing 5 genes. We tested the ability of characterized alleles of these genes to complement the genetic lesion in our mutant. Noncomplementation was observed when our mutant flies were crossed with flies containing a nonfunctional allele of the *Regena* gene. This suggests that Regena is required for gene silencing by miRNAs.

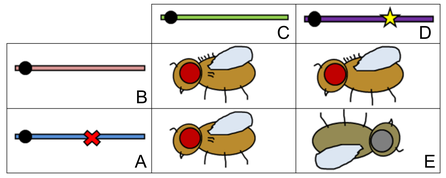

Figure 3: Complementation testing Chromosome containing a nonfunctional allele of a known gene (A). Balancer chromosomes (B,C). Chromosome with the lethal mutation generated in the forward genetic screen (D). We crossed flies hererozygous for a known mutation with flies heterozygous for a lethal mutation in the gene we were attempting to identify. When noncomplementation is observed, progeny heterozygous for both of these mutations are lethal (E), suggesting that we have discovered our gene of interest.

The CCR4-NOT deadenylase complex is essential for gene silencing

We have identified *Regena*(NOT2) as an essential gene required for microRNA activity *in vivo*. Regena has been previously characterized as a subunit of the CCR4-NOT deadenylase complex, a highly-conserved, multi-subunit complex implicated in gene silencing via target mRNA transcript degradation and translational repression.
Controversy exists as to whether translational repression necessarily precedes transcript degradation. Further analysis of our mutant is likely to inform this debate.

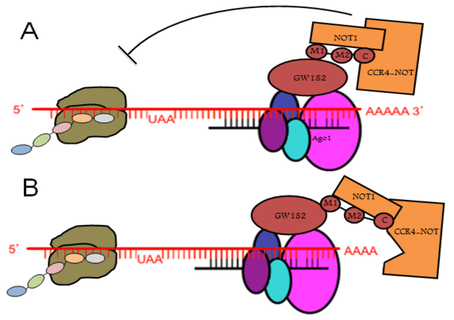

Figure 4: Function of the deadenylase complex in miRNA-mediated silencing. (A) Translational repression. (B) Deadenylation of the target mRNA. (Adapted from Braun et al.)

The role of Regena in cell viability and normal eye development

We assayed the role of Regena in eye development by generating whole eyes homozygous for the mutation in *Regena*, using mitotic recombination and a nonfunctional allele of the cell lethal gene *hid.*
The results of this perturbation are seen as malformation of some ommatidia, or eye facets (arrow). Loss of Regena function has a mild effect on *Drosophila* eye morphology.

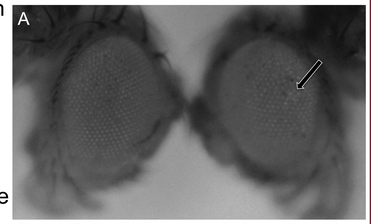

Figure 5: A weak eye morphology defect is observed in the absence of Regena function. A completely wild type *Drosophila* eye (A). A *Drosophila* eye homozygous for a mutation in *Regena.* Malformation of the ommatidia is indicated (arrow).

Exploration of a putative role for Regena in gene silencing during early development

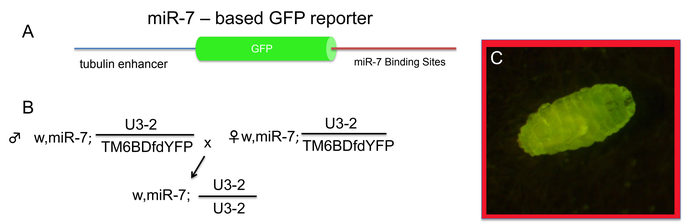

Figure 6. Generation of a new tool to assay miRNA activity during early development. A ubiquitously expressed GFP reporter with miR-7 binding sites in the 3 'UTR, which is sensitive to regulation by miRNAs (A). Generation of larvae that contain the GFP reporter and are homozygous for a mutation in *Regena* (B). Partial silencing of the GFP reporter in wild type larvae (C).

Larvae homozygous for a mutation in *Regena* will be compared to wild type larvae to determine whether Regena is indeed essential for miRNA-mediated gene silencing during early development. Further, the importance of the *Regena* gene to larval viability will be assayed by means of a lethal phase analysis of homozygous larvae.

FUTURE PLANS

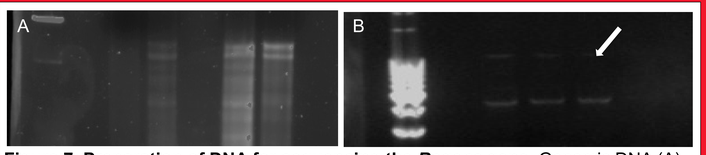

Figure 7. Preparation of DNA for sequencing the *Regena* gene. Genomic DNA (A). PCR Amplification of the *Regena* coding region, ~4.3 kb (B).

After identifying the mutation in *Regena*, we will analyze the effect of this mutation on the silencing machinery and gene silencing in general. To accomplish this, we will 1) Examine the silencing of additional sensors of miRNA activity in the *Drosophila* eye, 2) Use the newly-generated *Drosophila* line to assay miRNA-mediated gene silencing in early development,
3) Determine the effect of the mutation in *Regena* on larval viability, and 4) Carry out a biochemical analysis of the silencing machinery in the absence of Regena function.