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SPECIAL ISSUE RESEARCH ARTICLE

The MicroRNA *miR-8* Is a Positive Regulator of Pigmentation and Eclosion in *Drosophila*

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Background: MicroRNAs (miRNAs) are short, non-coding RNAs that post-transcriptionally silence gene expression by binding to target mRNAs. Previous studies have identified the miRNA miR-8 as a pleiotropic regulator of Drosophila development, controlling body size and neuronal survival by targeting multiple mRNAs. In this study we demonstrate that miR-8 is also required for proper spatial patterning of pigment on the adult abdominal cuticle in females but not males. Results: Female adult flies lacking miR-8 exhibit decreased pigmentation of the dorsal abdomen, with a pattern of pigmentation similar to wild type flies grown at higher temperatures. This pigmentation defect in miR-8 mutants is independent of the previously reported body size defect, and miR-8 acts directly in the developing cuticle to regulate pigmentation patterning. The decrease in pigmentation in miR-8 mutants was more pronounced in flies grown at higher temperatures. We also found that loss of miR-8 dramatically affected the ability to eclose at higher temperatures. Conclusion: Loss of miR-8 increased the sensitivity of Drosophila to higher temperatures for both pigmentation patterning and the ability to eclose. Together, these data suggest that miR-8 acts as a buffer to stabilize gene expression patterns in the midst of environmental variation. *Developmental Dynamics 241:161–168, 2012.* © 2011 Wiley Periodicals, Inc.

Key words: microRNA; miRNA; pigmentation; patterning; phenotypic plasticity; eclosion

Key findings:

- miR-8 is required for proper spatial patterning of pigment on adult female abdomens.
- The pigmentation patterning defect in miR-8 mutants is independent of the small body size defect.
- Loss of miR-8 in the developing cuticle results in cell-autonomous loss of pigmentation.
- MiR-8 is expressed in the epidermis underlying the dorsal abdominal cuticle.
- Loss of miR-8 sensitizes flies to effects of higher growth temperatures on eclosion success and pigmentation patterning.

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INTRODUCTION

The study of adult insect cuticle pigmentation has led to key insights into the genetic differences underlying morphological diversity (Wittkopp and Beldade, 2009). Insects display a wide range of pigmentation patterns, and variation in pigmentation is seen both between species and also within species groups. Body pigments or pigment precursors are produced in the epidermis, and these pigments are then deposited in the hard exoskeleton. The genetic regulation of the pattern and timing of pigment production in adult abdominal segments has been studied most extensively in *Drosophila melanogaster* (Wittkopp et al., 2003). Sexually dimorphic, *D. melanogaster* adult males have fully pigmented posterior abdominal segments A5 and A6, whereas adult females have variable degrees of pigmentation in these segments. In addition to genetic differences, temperature is another factor that affects the degree of pigmentation in the posterior segments of female abdomens (David et al., 1990; Gibert et al., 2000). Growth at lower temperatures causes increased pigmentation, whereas higher temperatures result in less pigmentation.

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MicroRNAs (miRNAs) are one class of genes that have been implicated in buffering developmental processes against the effects of environmental fluctuations such as temperature changes (Hornstein and Shomron, 2006; Wu et al., 2009). The miRNAs are short, noncoding RNAs that regulate gene expression by binding target mRNAs and preventing translation or destabilizing the mRNA (Du and Zamore, 2005). After processing, mature miRNAs, in concert with the RISC complex, generally bind to their target mRNAs by base pairing with complementary regions in the 3′untranslated region (3′UTR). A single miRNA can bind multiple mRNA targets and thus regulate the expression of multiple genes at one time (Bartel, 2009; Smibert and Lai, 2010). The miRNAs have been implicated in many developmental processes, but before this study, no miRNA has been implicated in regulating the complex process of cuticle pigmentation in insects.

Here we report that the miRNA *miR-8* is a positive regulator of pigmentation in *D. melanogaster*, in particular, regulating the spatial patterning of the posterior abdominal segments of adult females. This effect on pigmentation is direct and is independent of the previously reported small body size phenotype of *miR-8* mutants. Overall, loss of *miR-8* affects the thermosensitivity of *Drosophila* for both pigmentation and the ability to eclose, suggesting *miR-8* acts to buffer these complex processes against environmental variation.

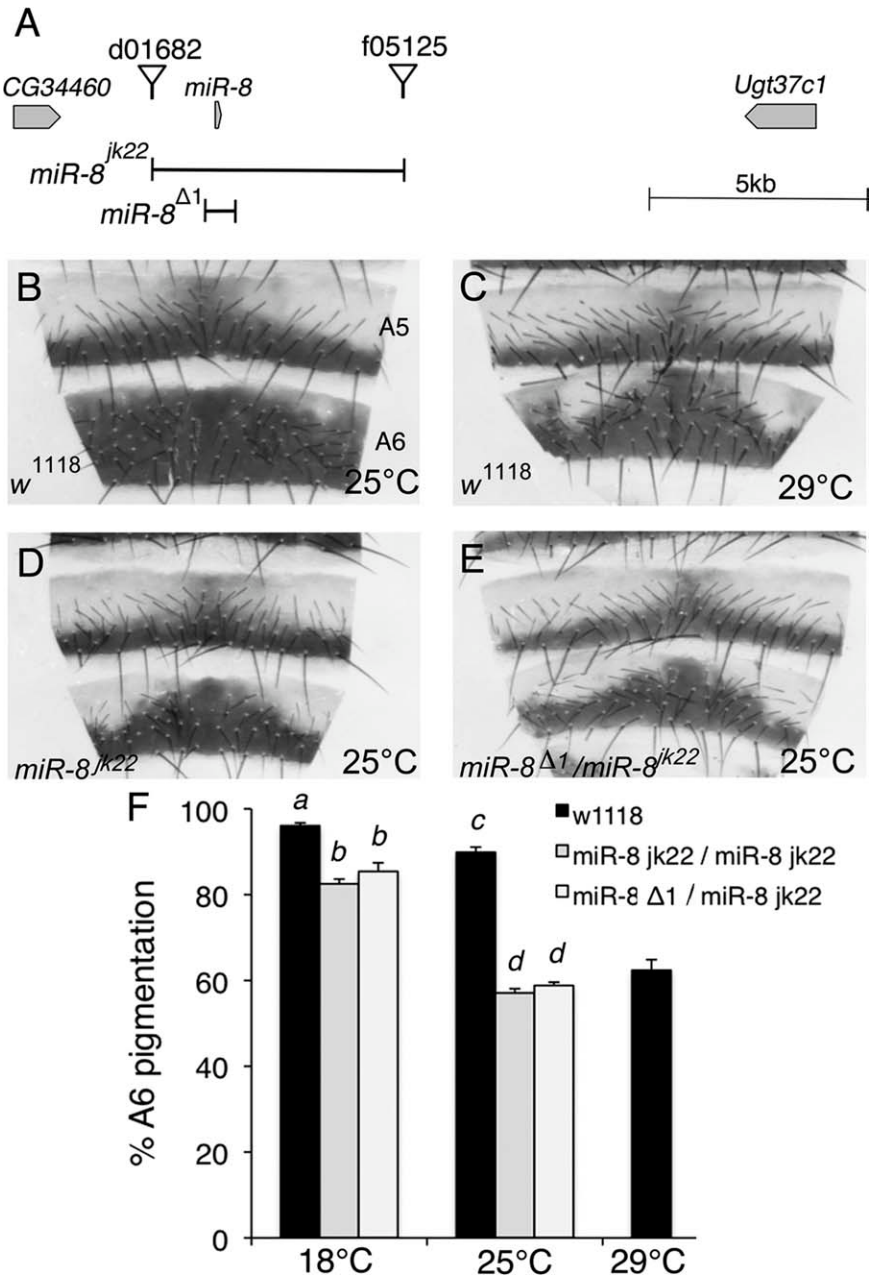

Fig. 1. *MiR-8* is required for proper spatial patterning of pigment on adult female abdomens. **A**: Schematic representation of the genomic region surrounding the *miR-8* locus on chromosome 2R. The null allele *miR-8*^{jk22} was generated with FLP/FRT mediated deletion using the FRT containing elements P{XP}d01682 and PBac{WH}f05125. *MiR-8*^{jk22} lacks the indicated 5.6-kb region containing the *miR-8* hairpin between the two elements. The previously reported *miR-8*$^{\Delta 1}$ allele is missing the 400-bp fragment indicated in the schematic (Karres et al., 2007). B–E: Dorsal abdominal cuticle segments 5 and 6 (A5 and A6) from 4- to 6-day-old adult *Drosophila melanogaster* females. The dorsal midline is in the center of each panel. **B,C**: Wild-type (*w*^{1118}) flies were reared at 25°C (B) or 29°C (C). **D,E**: Flies homozygous for the *miR-8*^{jk22} allele (D) or transheterozygous for the *miR-8*$^{\Delta 1}$ and *miR-8*^{jk22} (E) were reared at 25°C. To control for genetic background, both *miR-8*^{jk22} and *miR-8*$^{\Delta 1}$ lines were backcrossed to *w*^{1118} flies for over 20 generations. **F**: Percent pigmentation of A6 segments was determined by analyzing 15–20 cuticles for each of the indicated genotype/temperature combinations. Mean percent pigmentation is displayed, with error bars representing SEM. Multivariate analysis revealed a *miR-8* × temperature interaction (ANOVA, $F_{2,98} = 15.24$, $P < 0.001$). Shared letters indicate no significant difference (Tukey post hoc, $\alpha = 0.05$).

RESULTS

MiR-8 Is Required for Proper Spatial Patterning of Pigment on Adult Female Abdomens

We previously identified *miR-8* as a negative regulator of Wingless signaling in a misexpression screen (Kennell et al., 2008). To determine the function of *miR-8* in flies, we generated a deletion of the entire predicted *miR-8* locus using the FLP/FRT deletion method (Parks et al., 2004; Thibault et al., 2004). The resulting *miR-8*^{jk22} allele is a deletion that removed 5.6 kb of genomic DNA surrounding the *miR-8* hairpin (Fig. 1A). Consistent with previous reports of *miR-8* mutants, flies homozygous for the *miR-8*^{jk22} allele were proportionately smaller in size, had defective third legs and demonstrated decreased

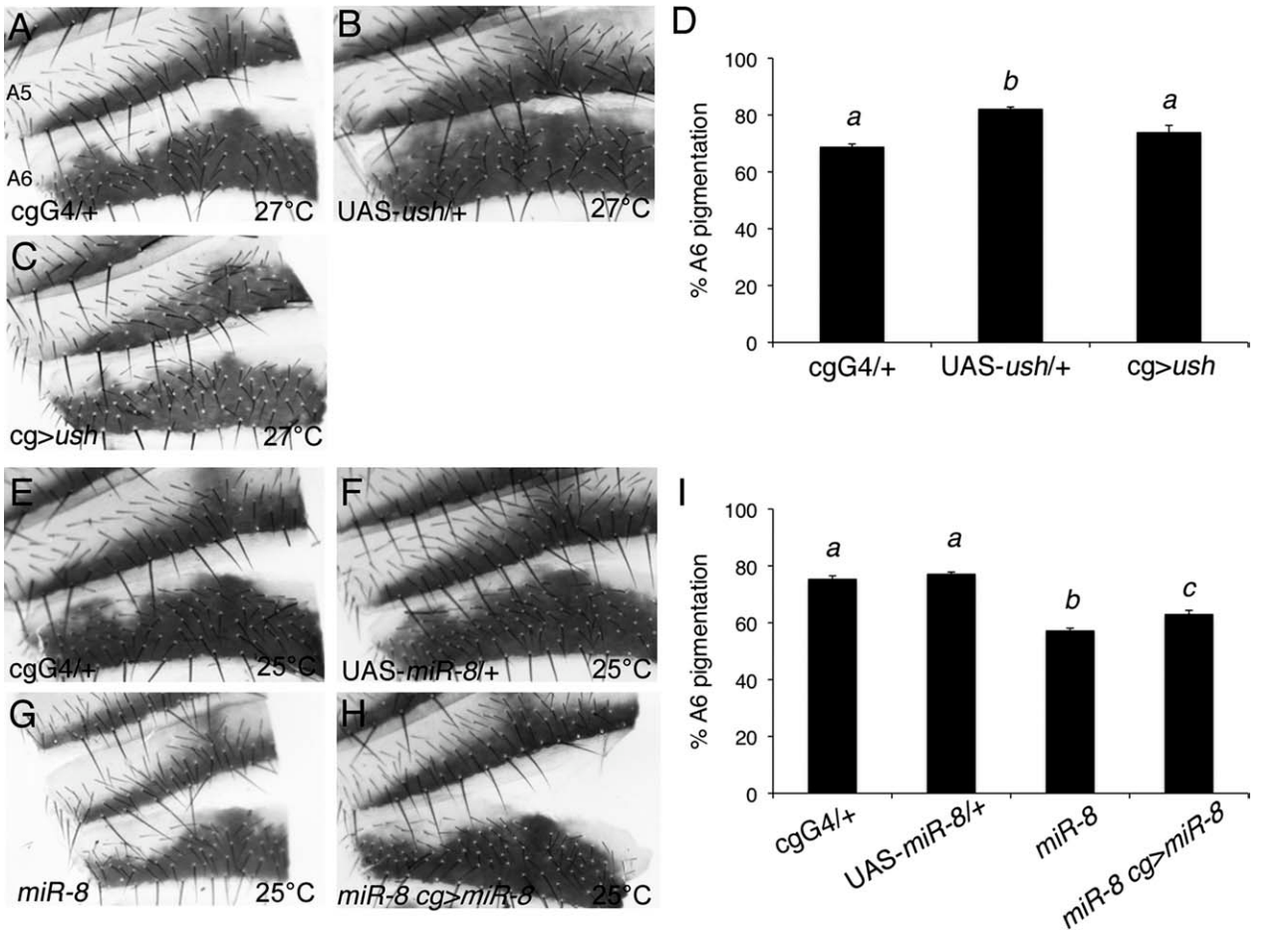

Fig. 2. Spatial pigmentation patterning in *miR-8* mutants is independent of body size. A–C,E–H: Dorsal abdominal cuticle segments A5 and A6 from 4- to 6-day-old adult females. The dorsal midline is toward the right of each panel for better visualization of the region lateral to the midline. **A–C**: cg-*Gal4*/+ (A), UAS-*ush*/+ (B), and (C) cg-*Gal4*/UAS-*ush* flies were reared at 27°C. Expression of *ush* in the fat body by the cg-*Gal4* driver resulted in decreased body size (data not shown). **D**: Quantification of A6 segment pigmentation from 10–12 cuticles per genotype reared at 27°C. Mean percent pigmentation is displayed, with error bars representing SEM. Although there was a statistically significant difference between all three groups (ANOVA, $F^{2,31} = 12.8$, $P < 0.0001$), shared letters indicate no significant difference (Tukey post hoc, $\alpha = 0.05$). **E–H**: cg-*Gal4*/+ (E), UAS-*miR-8*/+ (F), *miR-8*^{jk22}/*miR-8*^{jk22} (G), and *miR-8*^{jk22}, cg-*Gal4*/*miR-8*^{jk22}; UAS-*miR-8*/+ (H) flies were reared at 25°C. Overexpression of *miR-8* in the fat body of *miR-8* mutant flies rescued the body size defect (data not shown). **I**: Quantification of A6 pigmentation from 13–19 cuticles per genotype reared at 25°C. Mean percent pigmentation is displayed, with error bars representing SEM. Although there was a statistically significant difference between all groups (ANOVA, $F^{3,62} = 75.78$; $P < 0.0001$), shared letters indicate no significant difference (Tukey post hoc, $\alpha = 0.05$).

survival and ability to eclose (data not shown; Karres et al., 2007; Hyun et al., 2009). In addition to these previously reported phenotypes, we found that female flies lacking *miR-8* showed alterations in the spatial pigmentation pattern of the dorsal abdomen, most evident in the A6 segment (Fig. 1). At 25°C, the A6 segment of control flies is pigmented throughout most of the segment whereas the A6 segment of *miR-8* mutants is widest at the dorsal midline but tapers off laterally (Fig. 1B vs. D). This decreased pigmentation lateral to the dorsal midline was completely penetrant in *miR-8* mutant females; however, no alteration in pigmentation was evident in mutant adult male fly abdomens (data not shown).

To verify the phenotype was not due to genetic background differences, we also analyzed flies that were transheterozygous for two independent deletions of *miR-8* and found a similar loss of pigmentation (Fig. 1E). Consistent with this finding, flies transheterozygous for the *miR-8*^{jk22} or *miR-8D1* allele and a deficiency on chromosome 2R encompassing *miR-8* (Df(2R)ED2747) had a similar phenotype, with 59% and 52% A6 pigmentation at 25°C, respectively (data not shown).

Of interest, loss of *miR-8* caused a similar pigmentation pattern to rearing control flies at a higher temperature (29°C; Fig. 1C), suggesting that *miR-8* may act as a buffer against the effects of temperature on expression of genes involved in pigmentation. We quantified the percent pigmentation of the A6 segment in flies grown at 18°C and 25°C (Fig. 1F). Consistent with the hypothesis that *miR-8* buffers against the effects of temperature on pigmentation, multivariate analysis revealed a *miR-8*xtemperature interaction (analysis of variance [ANOVA], $F_{2,98} = 15.24$, $P < 0.0001$). Loss of *miR-8* resulted in a statistically significant decrease in A6 pigmentation at both 18°C and 25°C (Tukey post hoc, $\alpha = 0.01$). However, the decrease in pigmentation in *miR-8* mutants compared with controls was more pronounced at 25°C than at 18°C, suggesting that loss of

miR-8 sensitizes the flies to the effects of growth at higher temperatures.

Pigmentation Defect in Flies Lacking *miR-8* Is Independent of the Small Body Size Defect

A previous study suggested that loss of *miR-8* results in decreased body size due to loss of insulin signaling in the fat body, caused by increased expression of the *miR-8* target, *u-shaped* (*ush*; Hyun et al., 2009). To determine whether the pigmentation defect in *miR-8* mutants is related to the proportionately smaller size of the mutants, we analyzed the pigmentation pattern of females overexpressing *ush* in their fat bodies during development (Fig. 2A–D). As predicted, overexpression of *ush* in the larval fat body using the fat body driver cg-*Gal4* at 27°C resulted in flies that were proportionally smaller than control flies (data not shown). Despite the decrease in body size, the pigmentation pattern of female flies overexpressing *ush* in the fat body was similar to cg-*Gal4*/+ controls, with no significant difference in pigmentation of the A6 segment (Fig. 2C,D and data not shown). The parental lines used in these experiments were not introgressed into the w^{1118} stock to control for genetic background. Consequently, the statistically significant difference in pigmentation between UAS-*ush*/+ and the other genotypes is most likely due to effects of unknown genetic modifiers on pigmentation patterning in those strains.

To verify that the pigmentation defect in *miR-8* mutants is not caused by the decrease in body size, we overexpressed *miR-8* in the fat bodies of *miR-8* mutants (Fig. 2E–I). As previously reported, expression of *miR-8* in the larval fat body using the cg-*Gal4* driver rescued the small body size phenotype caused by loss of *miR-8* (data not shown, Hyun et al., 2009). However, the pigmentation defect was still present despite the rescue to normal body size (Fig. 2H), and there was still a statistically significant decrease in A6 segment pigmentation in fat body rescued flies versus either control (Fig. 2I; Tukey post hoc, $\alpha = 0.01$). The slight increase in pigmentation of fat body rescued flies compared with *miR-8* mutants may indicate that some, but not all, of the pigmentation defect is due to nonautonomous effects in the fat body. However, the failure to completely rescue the pigmentation defect suggests that *miR-8* functions independently of its role in the fat body to regulate the spatial pattern of pigmentation in the female abdomen. We were unable to determine whether the pigmentation defect could be rescued by expressing *miR-8* in the developing cuticle. Flies misexpressing *miR-8* in the cuticle with the epidermal drivers pnr-*Gal4* or en-*Gal4* died before eclosion or pupation, respectively, even in the absence of endogenous *miR-8* expression (data not shown).

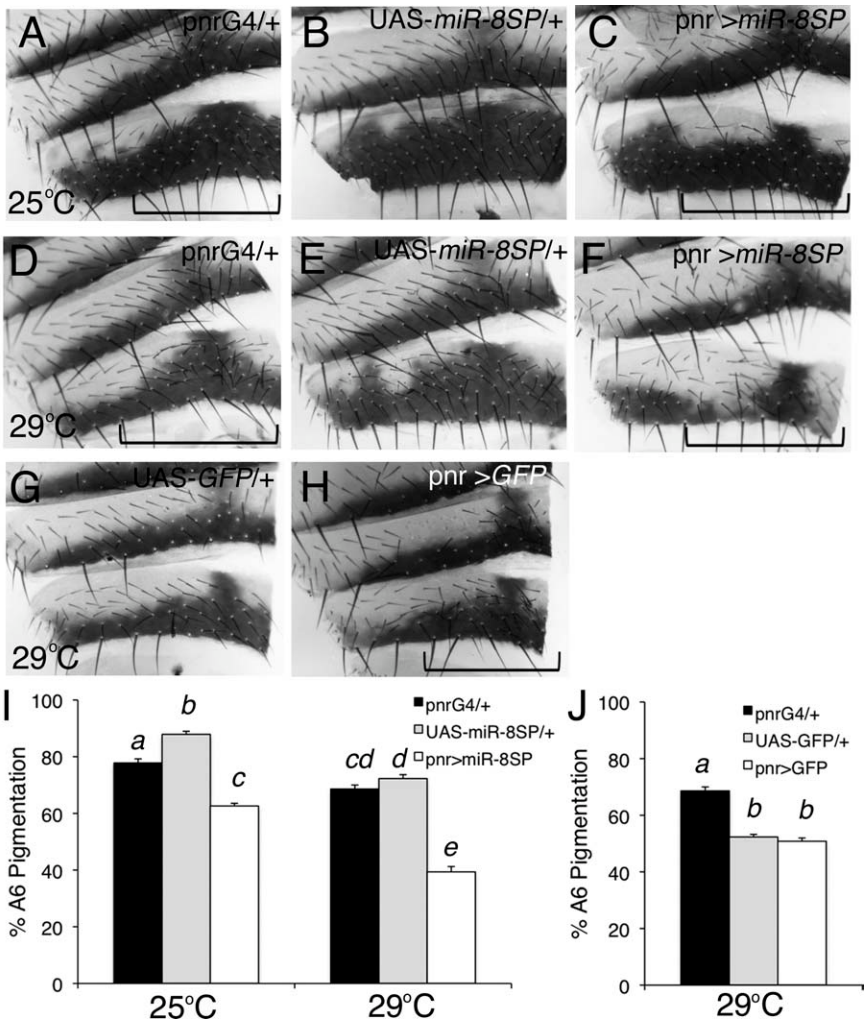

Fig. 3. *MiR-8* acts as a cell-autonomous regulator of pigmentation patterning. Dorsal abdominal cuticle segments A5 and A6 from 4- to 6-day-old adult females. The dorsal midline is toward the right of each panel. The pnr-*Gal4* transgene drives expression along the dorsal midline of the developing animal, and the UAS-*miR-8SP* transgene expressed EGFP with 10 *miR-8* binding sites in its 3′untranslated region (UTR) to soak up endogenous *miR-8*. Approximate regions of pnr-*Gal4* expression are indicated with brackets. **A–F**: pnr-*Gal4*/+ (A,D) UAS-*miR-8SP* (B,E), and UAS-*miR-8SP*/+; pnr-*Gal4*/UAS-*miR-8SP* (C,F) flies were reared at 25°C (A–C) or 29°C (D–F). **G,H**: UAS-*GFP*/+ (G) and pnr-*Gal4*/UAS-*GFP* (H) flies were reared at 29°C. J,K: Percent pigmentation of the A6 segment in the presumed *pnr* expression domain was measured using 15–18 cuticles per genotype/temperature combination. Mean percent pigmentation is displayed, with error bars representing SEM. **J**: Multivariate analysis indicates there is a significant difference between genotypes (ANOVA, $F_{2,93} = 197.92$; $P < 0.0001$). Shared letters indicate no significant difference (Tukey post hoc, $\alpha = 0.05$). **K**: Although there was a statistically significant difference between genotypes (ANOVA, $F = 68.99$; $P < 0.0001$), shared letters indicate no significant difference (Tukey post hoc $\alpha = 0.05$).

Loss of *miR-8* in the Developing Cuticle Results in Cell-Autonomous Loss of Pigmentation

To determine whether loss of *miR-8* in the developing cuticle was sufficient

for the decreased pigmentation seen in mutants, we expressed a *miR-8* sponge to inhibit *miR-8* function directly in the developing cuticle (Fig. 3; Loya et al., 2009). MicroRNA sponges act as competitive inhibitors to prevent the microRNA from targeting endogenous mRNAs (Ebert and Sharp, 2010). The *miR-8* sponge transgene was engineered to express, under the control of the UAS promoter, enhanced green fluorescent protein (EGFP) with 10 target sites complementary to *miR-8* in the EGFP 3′UTR (Loya et al., 2009). Expression of the *miR-8* sponge in a broad stripe along the dorsal midline by pnr-*Gal4* resulted in a decrease in A6 segment pigmentation, similar to the phenotype caused by loss of *miR-8* throughout the entire fly (Fig. 3C,F). Regions proximal to the dorsal midline, in the region of pnr-*Gal4* expression, were affected whereas the central region and lateral edges remained pigmented. Loss of pigmentation is visible in *miR-8* sponge expressing flies reared at either 25°C or 29°C. We quantified this effect by measuring pigmentation only in the presumed *pnr* expression domain of the A6 segment (Fig. 3J). Expression of the *miR-8* sponge caused a greater decrease in pigmentation at 29°C than at 25°C compared with parental line controls (ANOVA, $F_{2,93} = 23.85$, $P < 0.0001$). This difference is consistent with an interaction between *miR-8* and temperature, although this could be explained by greater expression of the *miR-8* sponge at 29°C due to UAS/Gal4 system temperature sensitivity. Misexpression of GFP by pnr-*Gal4* did not cause decreased A6 segment pigmentation in the region proximal to the dorsal midline (Fig. 3I,K), suggesting the effect is specific to the presence of the binding sites for *miR-8* in the sponge transgene and not due to overexpression of GFP alone. In addition, loss of *miR-8* specifically in the developing cuticle by expression of the *miR-8* sponge had no obvious effect on pigmentation patterning in males (data not shown), consistent with the lack of pigmentation defect in male *miR-8* mutants.

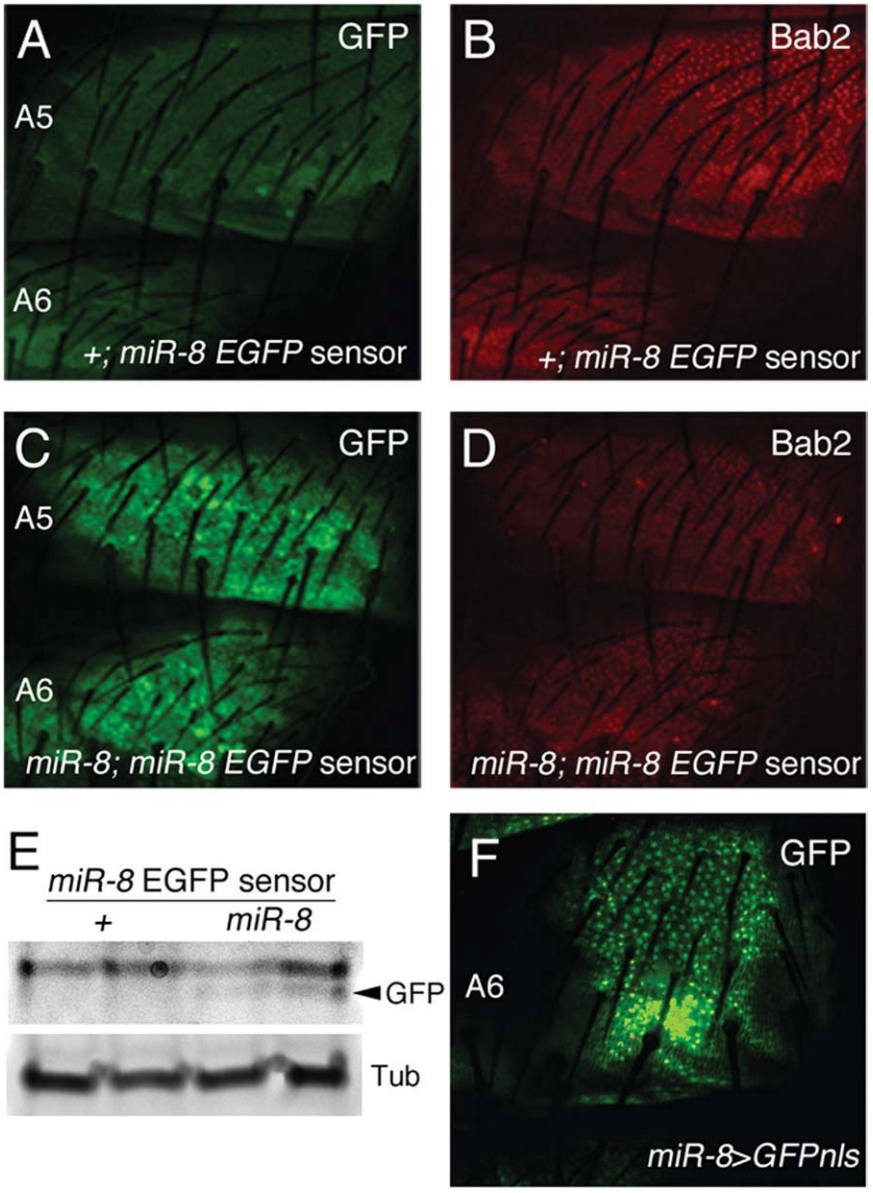

Fig. 4. *MiR-8* is expressed in the epidermis underlying the dorsal abdominal cuticle. A–D,F: Confocal images of the epidermis underlying the dorsal abdominal cuticle in female late pupae (88–96 h APF). All images were taken with the same confocal settings at ×40 magnification. **A–E**: *MiR-8* activity was detected using a *miR-8* enhanced green fluorescent protein (EGFP) sensor transgene. The sensor gene contains EGFP with two binding sites for *miR-8* in its 3′untranslated region (UTR), driven by the α-*tubulin* promoter. A,C: Expression of the *miR-8* sensor in a wild-type background (+/+; *miR-8* EGFP sensor/+; A) was compared with *miR-8* sensor expression in *miR-8* mutants (*miR-8*^{jk22}/*miR-8*^{jk22}; *miR-8* EGFP sensor/+; C). Bab2 expression was used to visualize the epidermis underneath the cuticle (B and D). E: Western blot analysis of GFP and α-Tubulin from isolated dorsal abdominal cuticles of wild-type or *miR-8* mutant flies containing the *miR-8* sensor. The GFP band is indicated by an arrowhead. A strong nonspecific band is located above the GFP band in both wild-type and *miR-8* mutant backgrounds. **F**: GFPnls visualized in dorsal abdominal epidermis of a miR-8-*Gal4*/ UAS-*GFPnls* pupa. The miR-8-*Gal4* enhancer trap line has an element containing the *Gal4* gene upstream of the *miR-8* locus and drives expression of Gal4, and, hence, GFPnls, in a pattern similar to *miR-8* expression (Karres et al., 2007).

MiR-8 Is Expressed in the Epidermis Underlying the Dorsal Abdominal Cuticle

Loss of *miR-8* could cause altered spatial patterning of pigmentation either directly in the developing cuticle or indirectly by altering a target in another tissue that has nonautonomous effects. The result of inhibiting *miR-8* function specifically in the developing cuticle with expression of a *miR-8* sponge is consistent with the hypothesis that *miR-8* is functioning directly in the epidermis underlying the developing cuticle. Pigmentation of the dorsal abdomen is spatially patterned during the late stages of pupal

development (Wittkopp et al., 2003). To determine the location of *miR-8* expression and activity in the developing cuticle of late pupae, we generated an in vivo sensor in which EGFP, containing two *miR-8* binding sites in its 3′UTR, is expressed ubiquitously under the control of the α-*tubulin* promoter (*miR-8* EGFP sensor). We expect decreased expression of the EGFP sensor anywhere *miR-8* is normally expressed during development, due to targeting by *miR-8* (Brennecke et al., 2003; Li and Carthew, 2005). *MiR-8* sensor expression was very low in control flies throughout the abdominal dorsal epidermis but was increased in *miR-8* mutant epidermis (Fig. 4A,C), indicating that *miR-8* is expressed and active when cuticle pigment patterning is occurring. The transcription factor Bab2, a known regulator of the pigmentation pathway (Couderc et al., 2002), was visualized as a control to confirm that *miR-8* was expressed in the epidermal layer. No consistent change in Bab2 expression was observed with the loss of *miR-8* (data not shown), suggesting Bab2 is not a target of *miR-8* in the developing cuticle. Increased expression of the *miR-8* EGFP sensor in *miR-8* mutants was confirmed by immunoblot (Fig. 4E). We also used a previously reported miR-8-*Gal4* enhancer trap line to verify that *miR-8* is expressed in the epidermis of the dorsal abdomen in late pupae (Fig. 4F; Karres et al., 2007). The expression of *miR-8* in the developing cuticle is not sex-specific as *miR-8* is expressed throughout the dorsal abdomen of both males and females (data not shown). A previous study has also reported that *miR-8* is expressed in the cuticle of third instar larvae (Hyun et al., 2009).

Loss of *miR-8* Sensitizes Flies to the Effects of High Temperatures on Eclosion Success

A previous study reported that 14% of *miR-8* mutants failed to eclose or died during eclosion, possibly due to elevated apoptosis in the nervous system and impaired motor coordination (Karres et al., 2007). When investigating the effect of *miR-8* on spatial pigmentation patterns at different rearing temperatures, we were unable to collect *miR-8* mutants grown at 29°C for analysis of cuticle pigmentation. We noticed very few *miR-8* mutants successfully emerged from the pupal cases and those that did eclose died within a few hours. Most of the flies that failed to eclose died as late stage pupae (data not shown). To study more closely the interaction between *miR-8* and temperature in regulating eclosion rates, we quantified the percent eclosion in control and *miR-8* mutant flies reared at different temperatures (Fig. 5). Across the temperatures tested, fewer *miR-8* mutants successfully eclosed than controls. However, the effect was most pronounced when flies were grown at 29°C, as only 15% of *miR-8* mutants successfully eclosed (Fig. 5). Multivariate analysis revealed a strong *miR-8* × temperature interaction for eclosion (ANOVA, $F_{2,30} = 138.75$, $P < 0.001$). These results suggest that *miR-8* mutants are more sensitive to high temperatures, and the underlying defects in leg development and motor activity caused by loss of *miR-8* are more severe when flies are grown at 29°C.

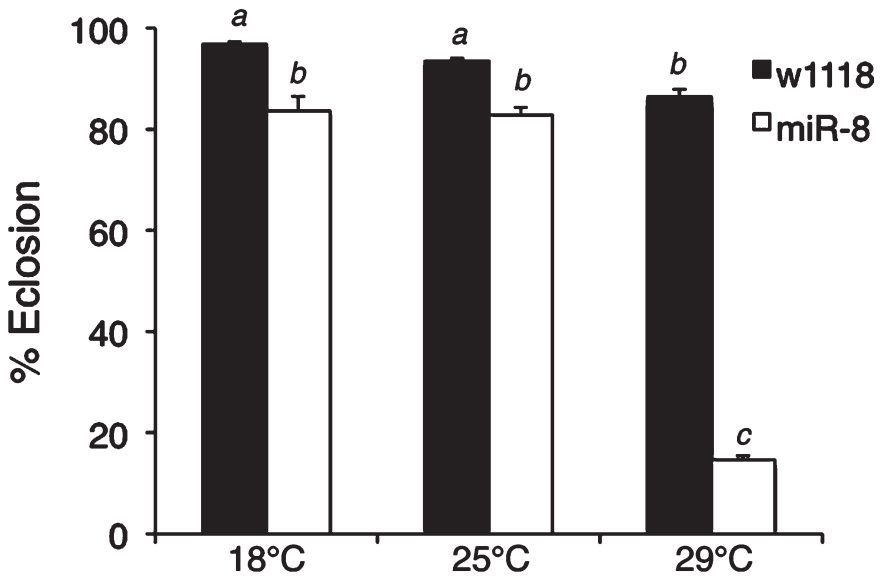

Fig. 5. Loss of *miR-8* sensitizes flies to the effects of high temperatures on eclosion success. The percentage of eclosed flies from wild-type (w^{1118}) and *miR-8* mutants ($miR\text{-}8^{lk22}/miR\text{-}8^{lk22}$) was determined after rearing flies at 18°C, 25°C, or 29°C. Percent eclosion was determined by dividing the number of empty pupal cases by the total number of cases. The experiment was performed with six batches of flies per genotype/temperature combination and the mean percent eclosion was calculated. Error bars indicate SEM. Results are representative of three separate trials. Statistical analysis revealed a strong interaction between *miR-8* and temperature (ANOVA, $F_{2,30} = 138.75$; $P < 0.001$). Shared letters indicate no significant difference (Tukey post hoc, $\alpha = 0.05$).

DISCUSSION

Prior studies have identified *miR-8* as a regulator of growth, apoptosis, and neuronal survival by targeting multiple mRNAs (Karres et al., 2007; Kennell et al., 2008; Hyun et al., 2009; Loya et al., 2009; Vallejo et al., 2011). Our study reveals the previously unidentified role of *miR-8* in regulating the spatial patterning of pigmentation. Indeed, *miR-8* is the first microRNA to be identified as a regulator of pigmentation in insects. The role of *miR-8* in pigmentation patterning appears to be independent of the previously reported growth defect in *miR-8* mutants and highlights the pleiotropic functions of the *miR-8* gene during *Drosophila* development. We found that *miR-8* is expressed in the pupal epidermis when genes involved in pigmentation and patterning are expressed. In addition, tissue-specific inhibition of *miR-8* function by expression of a *miR-8* sponge caused a decrease in pigmentation similar to full body *miR-8* mutants. Together these data suggest that *miR-8* directly regulates the pigmentation pathway in the developing cuticle and does not exert its effects on pigmentation indirectly through effects on another tissue.

Pigmentation is a complex trait and is impacted by multiple factors (Wittkopp et al., 2003). In particular, the

female dorsal abdomen of *D. melanogaster* demonstrates a high degree of phenotypic plasticity in response to changes in temperature (David et al., 1990; Gibert et al., 2000). Of interest, the spatial pigmentation pattern in female *miR-8* mutant abdomens is similar to the pattern of females grown at 29°C. Because *miR-8* mutants fail to eclose/survive at 29°C, we were unable to assess the extent of pigmentation in mutants at this high temperature. However, inhibition of *miR-8* by overexpression of a *miR-8* sponge at 29°C caused cell-autonomous loss of much of the pigment proximal to the dorsal midline. Yet we did not see an obvious difference in the pigmentation patterning of males lacking *miR-8*, which is consistent with temperature having less impact on male pigmentation patterning. Our analysis of the effects of loss of *miR-8* at 18°C versus 25°C suggests that *miR-8* interacts with temperature to regulate spatial patterning of pigmentation, and this possible interaction may be indicative of an unexpected role for *miR-8* in the plasticity and possible evolution of pigmentation patterning in *Drosophila*.

In addition to our finding that *miR-8* may interact with temperature to regulate pigmentation patterning, we found that the previously reported eclosion defect in *miR-8* mutants (Karres et al., 2007) is much more severe when the mutants are grown at 29°C than at 18°C or 25°C. Together these data suggest that *miR-8* may buffer against the effects of temperature on gene expression, and, in the absence of *miR-8*, temperature has a greater impact on the complex genetic pathways regulating pigmentation and eclosure. Other studies of microRNAs have shown a similar role for microRNAs in fine-tuning gene-environment interactions. For example, the microRNA *miR-7* appears to buffer gene expression against the effects of temperature fluctuations in *Drosophila* (Li et al., 2009). Loss of *miR-7* affected sensory organ development in the presence of an environmental stressor (temperature fluctuations) but had very little effect under uniform laboratory conditions. Our results are consistent with a model that some microRNAs, such as *miR-8* and *miR-7*, serve to integrate multiple environmental factors that impact developmental processes and buffer against the destabilizing effects of those factors on genetic networks.

The mechanism for *miR-8* regulation of pigmentation is not yet known. Individual microRNAs have the potential to target multiple mRNAs. In fact, each of the previously identified phenotypes of *miR-8* mutants is caused by up-regulation of a different target mRNA (e.g., *ush* for small body size, *atrophin* for neurodegeneration; Karres et al., 2007; Hyun et al., 2009; Loya et al., 2009; Vallejo et al., 2011). We have found no evidence that up-regulation of the previously identified targets of *miR-8* is causing the pigmentation defect in *miR-8* mutants (data not shown), suggesting that a novel target of *miR-8* is responsible. The increased thermosensitivity of *miR-8* mutants may be indicative of *miR-8* playing a more general role in temperature response, possibly in regulating chaperones and other genes involved more broadly in thermosensitive developmental processes (Gibert et al., 2007). Loss of the chromatin regulators *cramped* or *corto*, or the chaperone *Hsp83*, causes a similar decrease in pigmentation as seen with loss of *miR-8* (Gibert et al., 2007, 2011), suggesting *miR-8* may act in the same pathway as these genes to regulate spatial patterning of pigmentation.

EXPERIMENTAL PROCEDURES

Drosophila Genetics

All flies were maintained at 25°C unless otherwise indicated and all flies contained a mutation in the *white* locus (w^{1118}). pnr-*GAL4*, cg-*Gal4*, UAS-*GFPnls* and Df(2R)ED2747 lines were obtained from the Bloomington Stock Center, miR-8-*GAL4* from the Kyoto Stock Center (stock# NP5247) and P{XP}d01682 and PBac{WH}f05125 from the Exelixis Collection at Harvard. The following lines were kindly provided by various researchers: UAS-*GFPmiR-8SP*#9; UAS-*GFPmiR-8SP*#10 (Loya et al., 2009), $miR\text{-}8^{\Delta 1}$ (Karres et al., 2007) and UAS-*ush* (Tokusumi et al., 2007).

UAS-*miR-8* contains a 500-bp genomic fragment encompassing the *miR-8* locus (Kennell et al., 2008). $miR\text{-}8^{jk22}$ is a null allele that contains a 5.6-kb deletion of the genome that includes the *miR-8* locus. The deletion was generated by FLP/FRT based deletion using P{XP}d01682 and PBac{WH}f05125 lines (Parks et al., 2004). The resulting allele contains a transposable element that is a hybrid of the original XP and WH elements and contains two copies of the mini-white gene. Deletion was verified by polymerase chain reaction. The $miR\text{-}8^{jk22}$ and $miR\text{-}8^{\Delta 1}$ stocks were backcrossed to w^{1118} flies for over twenty generations to isogenize the stocks with the control w^{1118} line. The *miR-8* EGFP sensor line contains two target sites complementary to *miR-8* inserted downstream of the EGFP coding region of tub-*EGFP* in pCaSpeR4 (Brennecke et al., 2003). Transgenic *Drosophila* lines were generated by standard P-element mediated genomic integration (Bestgene Inc.), and multiple lines were analyzed for each construct.

Abdominal Cuticle Preparation and Immunofluorescence

Adult female flies were fixed in 10% glycerol in ethanol 4 to 6 days after eclosion. Abdominal cuticles were dissected, mounted in PVA mounting medium (BioQuip) and imaged with a Leica S8APO dissecting scope and Leica DFC295 camera. All settings were kept constant between images. Quantification of dorsal A6 segment pigmentation was performed using NIH ImageJ software. Images of 10 to 20 cuticles were analyzed for each genotype/temperature combination to determine percent pigmentation. Quantification of pigmentation in Figures 1 and 2 was conducted by measuring the percent pigmentation of the A6 segment for half of the tergite, from the dorsal midline to one lateral edge. Percent pigmentation was determined in Figure 3 for the region from the dorsal midline to halfway to one lateral edge of the tergite to approximate the expression domain of pnr-*Gal4*.

To visualize gene expression, late pupal cuticles (APF 88-96 h; determined by morphological markers) were dissected in 1× phosphate buffered saline, fixed in 4% formaldehyde and blocked with normal donkey serum. Fixed cuticles were incubated with rat anti-Bab2 (1:1,000, Frank Laski)

followed by incubation with Alexa Fluor 568 anti-rat (1:500, Invitrogen). Pupal cuticles were mounted in Vectashield (Vector Labs) and imaged using a Nikon PCM2000 confocal microscope.

Immunoblotting

Dorsal abdominal cuticles from female late pupae (APF 88-96 h) were homogenized in lysis buffer (125 mM Tris, pH 6.8; 6% sodium dodecyl sulfate [SDS]). Following centrifugation, the supernatant was combined with equal amounts of loading buffer (125 mM Tris, pH 6.8; 6% SDS; 20% glycerol; 0.04% Bromophenol Blue; 10% β-mercaptoethanol), and heated to 95°C. Samples were loaded onto Precise Protein gels (Pierce) and transferred to low fluorescence polyvinyl difluoride (PVDF) membrane (Pierce). Primary antibodies used were rabbit anti-GFP (1:500; Santa Cruz) and mouse anti-α-tubulin (1:500; Sigma) followed by Dylight 488 conjugated anti-mouse or anti-rabbit secondary antibodies (1:2,500; Immunoresearch Labs). Dried blots were imaged with a VersaDoc 4000MP (Bio-Rad).

Eclosion Assay

Ten each of males and females were added to new vials and incubated at 25°C for 3 days. Adults were discarded and vials were transferred to the indicated temperature. Pupal cases were counted 15, 16, and 28 days after vials were transferred to 29°C, 25°C, and 18°C, respectively. Empty pupal cases were counted as successful eclosion events, whereas full or partially filled cases were considered unsuccessful. Six vials were prepared for each genotype/temperature combination and the reported results presented are representative of three separate trials.

Statistical Analysis

The calculated values for A6 pigmentation were arcsine transformed before statistical analysis. Multivariate analysis was performed using the statistical tools on the VassarStats website (http://faculty.vassar.edu/lowry/VassarStats.html).

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