Natural variation in *Drosophila melanogaster* diapause due to the insulin-regulated PI3-kinase

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This study links natural variation in a *Drosophila melanogaster* overwintering strategy, diapause, to the insulin-regulated phosphatidylinositol 3-kinase (PI3-kinase) gene, *Dp110*. Variation in diapause, a reproductive arrest, was associated with *Dp110* by using *Dp110* deletions and genomic rescue fragments in transgenic flies. Deletions of *Dp110* increased the proportion of individuals in diapause, whereas expression of *Dp110* in the nervous system, but not including the visual system, decreased it. The roles of phosphatidylinositol 3-kinase for both diapause in *D. melanogaster* and dauer formation in *Caenorhabditis elegans* suggest a conserved role for this kinase in both reproductive and developmental arrests in response to environmental stresses.

evolutionary genetics

Little is known about genes that harbor ecologically relevant allelic variation in natural populations or the degree of conservation of such variation across species (1–3). Arrests in development are widespread. For example, mammals hibernate, insects enter diapause, and nematodes form dauer larvae all in response to adverse conditions. *Caenorhabditis elegans* arrests development to form dauer larvae in response to harsh environmental conditions such as food depletion and overcrowding (4); dauer larvae have decreased metabolism and increased fat storage. When confronted with low temperature and short days (SDs), *Drosophila melanogaster* enters an ovarian reproductive diapause where adult females have immature ovaries and exclusively previtellogenic oocytes (5, 6). It is not known whether common mechanisms underlie these arrests because, although much is known about the genetic underpinnings of dauer formation in *C. elegans*, little is known about genes involved in reproductive diapause.

Ecological studies show that adult reproductive diapause is a powerful overwintering strategy for many insects including *Drosophila* (7). Diapause is advantageous in northern climates because it enables females to survive for several months through harsh winter conditions and then emerge and lay eggs when temperatures increase and days get longer. Arrests in response to adverse conditions are of interest because they can provide us with an evolutionary framework in which to interpret existing genetic variation both within and between species. Diapause is associated with resource allocation trade-offs involved in life history strategies, for example, whether to allocate resources to growth and survivorship or reproduction. *D. melanogaster* lines that vary in latitudinal origin differ in the proportion of individuals in diapause (8) as well as in a suite of fitness-related traits that are genetically correlated to diapause. These traits include life span, age-specific mortality, fecundity, resistance to cold and starvation stress, lipid content, development time, and egg-to-adult viability (8, 9). We wondered what gene(s) or gene pathways play a role in natural variation in diapause and potentially underlie this pleiotropy.

We used two natural diapause variants of *D. melanogaster* as a basis to identify genetic regulators of diapause (10). The Windsor (W) natural variant from Canada exhibits an autosomal-recessive high-diapause phenotype compared with the Cartersville (C) natural variant from the Southern U.S., which confers a fully dominant low-diapause phenotype (10). Crosses between the variants suggested that diapause may be influenced by relatively few genes (10).

Results

Gene(s) Involved in the W and C Diapause Phenotypes Maps to Chromosome-3. The W and C natural variants were obtained by measuring the proportion of flies in diapause in 23–30 females from each of 10 isofemale lines (families of flies derived from one inseminated female) collected from four localities in North America (by T. Long, University of California, Irvine, CA). Significant genetic variation was found within [$F_{(3,36)} = 6.8$, $P < 0.0001$] and between [$F_{(3,883)} = 24.2$, $P < 0.0001$] sites. The mean proportion of flies in diapause from Windsor, ON, Canada (W; 54%) was significantly higher than from the more southern locale, Cartersville, GA (C; 32%) ($G = 28.30$, $P < 0.05$). The W and C variants were each chosen as representative isofemale lines from each of the two locations.

By using segregation analysis, we mapped the gene(s) involved in the W and C variant diapause phenotypes to chromosome-3. Deletion mapping enabled us to further localize this variation to the third chromosome deficiency *Df(3R)H-B79*, *e/TM2*, which spans 92B03–92F12 and deletes a number of genes including the *D. melanogaster* insulin-regulated phosphatidylinositol 3-kinase (PI3-kinase) gene, *Dp110* (11, 12). The *Dp110* orthologue in *C. elegans*, *age-1*, plays a role in dauer formation, which, like diapause, is an arrest in response to environmental stress (13). Thus, we chose to focus our efforts on *Dp110* as a candidate gene for the regulation of *D. melanogaster* diapause.

Reduction of *Dp110* Gene Dosage Increases the Proportion of Flies in Diapause. To test whether the W and C variation in diapause localizes to *Dp110*, we used quantitative complementation, a method of deletion mapping originally developed for testing quantitative effects (14). We crossed W and C to four strains: two deficiency strains ($Dp110^A$ and $Dp110^B$), which remove *Dp110* function, and two strains bearing a deficiency plus a *Dp110* genomic

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Abbreviations: C, Cartersville; W, Windsor; PI3-kinase, phosphatidylinositol 3-kinase; LD, long day; SD, short day.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. DQ792599 and DQ792600).

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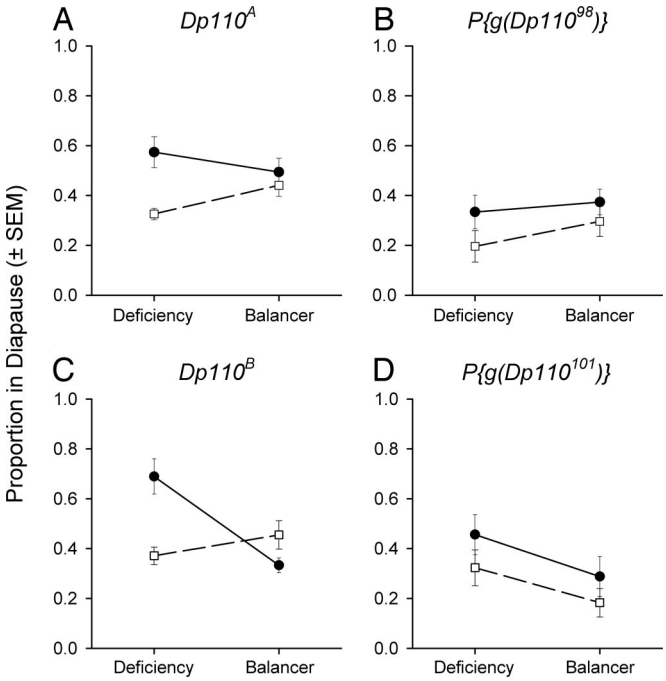

Fig. 1. Diapause natural variants map to *Dp110*. Wild-type W (filled circles) or C (open squares) flies were crossed to two deletions, which remove *Dp110*{*yw*;*P*[*ry*+,*gH*],*Dp110*^{A}/*TM3*,*Ser*,*y*$^{+}$ (A) and *yw*;*P*[*ry*$^{+}$,*gH*], *Dp110*^{B}/*TM3*,*Ser*,*y*$^{+}$ (*C*)} and to two rescue lines each bearing a deletion plus a genomic *Dp110* rescue fragment [*yw*;*P*{*w*$^{+}$,*g*(*Dp110*^{98})},*Dp110*^{A}/*TM3*,*Ser*,*y*$^{+}$ (*B*) and *yw*;*P*{*g*(*Dp110*^{101})};*Dp110*^{B}/*TM6B*,*Tb*,*Sb* (*D*)]. From each of the four crosses, two populations of flies were recovered: one in which C or W was heterozygous with the deletion (or deletion plus rescue fragment) and one in which C or W was heterozygous with the balancer chromosome. The interaction plots show the mean proportion of flies in diapause (±SEM) for the progeny of each cross. There were significant statistical interactions between the proportions in diapause for deletion and the balancer [*Dp110*^{A}: $F_{(1,24)} = 4.53$, $P < 0.04$; *Dp110*^{B}: $F_{(1,24)} = 30.47$, $P < 0.0001$]. The lack of parallel lines found for the crosses to the deletion strains *Dp110*^{A} and *Dp110*^{B} indicate statistical interaction, demonstrating positive quantitative complementation (13). The interactions disappeared when the *Dp110* genomic rescue fragments were present, indicating restoration of the phenotype with the genomic fragments [*P*{*g*(*Dp110*^{98})} $F_{(1,17)} = 0.03$, $P = 0.86$; *P*{*g*(*Dp110*^{101})} $F_{(1,15)} = 0.00$, $P = 0.95$].

rescue fragment [*Dp110*^{A}; *P*{*g*(*Dp110*^{98})} and *Dp110*^{B}; *P*{*g*(*Dp110*^{101})} (12). All deletions were maintained heterozygous with balancer chromosomes, which did not carry mutations in *Dp110*. In quantitative complementation, the W and C deletion heterozygotes are compared with W and C balancer heterozygotes (using the balancer chromosome from the deficiency strain as a common genetic background control). The balancer heterozygotes control for effects of natural variation in the genetic background that can result in increases or decreases in diapause (14). Variation in genetic background in this case could arise from genes other than *Dp110*, which have small effects on the diapause phenotypes. These small effect or minor genes could be segregating in the deletion, transgenic, and/or natural diapause strains. {An example of a genetic background effect is seen in the C/deletion heterozygotes and C/balancer heterozygotes, which significantly differed in one [Fig. 1*A*; ANOVA, $F_{(1,11)} = 7.33$, $P = 0.02$] of three comparisons (Fig. 1 *B–D*; $P > 0.05$).} The balancer heterozygotes enable us to control for these genetic background effects and determine statistically if a gene of large effect is uncovered by the *Dp110* deletion strains.

A lack of quantitative complementation is identified as a two-way statistical interaction between the variant (W or C) and test genotype (deletion or control) in which the variant phenotypes are most distinguishable between heterozygotes carrying the deletion chromosome and less divergent in the control genotype. Indeed, we found just such a two-way statistical interaction occurs when each of the deletion strains was crossed to W and C (Fig. 1 *A* and *C*). Both deletions of *Dp110*, *Dp110*^{A} and *Dp110*^{B}, uncovered the recessive high-diapause, W, phenotype. These findings demonstrate that natural variation in diapause between the W and C variants maps to *Dp110*. Further evidence is provided by introducing a *Dp110* genomic rescue fragment into each of the deletion strains. In this case, interactions such as those shown in Fig. 1 *A* and *C* were not observed, demonstrating lack of complementation when *Dp110* function is restored in the deletion strains (Fig. 1 *B* and *D*). We conclude that *Dp110* is responsible for the differences in diapause between the W and C strains and that a reduction in the dosage of *Dp110* in a deletion heterozygote increases the proportion of flies in diapause. These results show that natural variation in *Dp110* can have a significant effect on diapause, a phenotype of great adaptive significance.

Augmenting *Dp110* in the Nervous System Decreases Diapause. The deletion results led to the prediction that an increase in *Dp110* would decrease diapause. To examine this hypothesis, we used two independent *UAS-Dp110* lines called *UAS-Dp110*^{II} and *UAS-Dp110*^{III} and an *elav-GAL4* driver to drive expression of *Dp110* in neurons (15–17). We chose to express the *UAS-Dp110* transgenes in the nervous system because *in situ* hybridization showed that *Dp110* is widely expressed in the visual system and the brain of the adult fly (data not shown) and also in the larval eye discs (11). We first confirmed that the *UAS-Dp110* lines exhibit a diapause response in SD compared with long day (LD) conditions at 11°C (Fig. 2*A*). Then, we excised each transgene to generate background control lines, *UAS-Dp110*^{IIex} and *UAS-Dp110*^{IIIex}, for our targeted expression studies. We crossed *Dp110*^{II}, *UAS-Dp110*^{III}, *UAS-Dp110*^{IIex}, and *UAS-Dp110*^{IIIex} to *elav-GAL4* flies, thereby expressing *Dp110* in neurons only when the *UAS* and *GAL4* were present together in the same fly.

A significant reduction in diapause resulted when we neuronally expressed either of the two *UAS-Dp110* transgenes (II and III) compared with control excision fly crosses (Fig. 2*B*). Because diapause involves a photoperiodic response, we tested the phenotypic effect of targeted *Dp110* expression in the visual system. To this end, we crossed each of our *UAS-Dp110* lines and their *UAS-Dp110*^{ex} control lines to a *GMR-GAL4* driver, which expresses in the visual system (18). No significant differences were detected when using this driver (Fig. 2*C*). Thus, contrary to our results with the neuronal *elav-GAL4* driver, we found that expressing *Dp110* in the visual system did not significantly affect diapause.

Analysis of RNA Abundance and DNA Sequence Variation of the W and C Variants. With the genetic analysis in mind, we hypothesized that the higher-diapausing W strain might have lower levels of *Dp110* RNA than the lower-diapausing C strain. To address this hypothesis, we measured *Dp110* mRNA levels in W and C flies from diapause inducing conditions. Fly heads and bodies were quantified separately by using real-time quantitative RT-PCR. We were unable to detect significant differences in *Dp110* RNA abundance between the W and C heads [mean counts ± SE (n); W = 3.42 ± 0.37 (8), C = 3.04 ± 0.18 (8); one-tailed, paired, two sample, Student's t test: $t_{(7)} = 0.97$, $P = 0.18$] or bodies [W = 2.66 ± 0.26 (8), C = 2.46 ± 0.17 (8); one-tailed, paired, two sample t test: $t_{(7)} = 0.81$, $P = 0.22$]. One interpretation of these data is that *Dp110* may be required in only a subset of neurons for diapause, and our quantification of RNA in whole heads (or bodies) was not able to detect small localized differences in RNA levels between W and C. Alternatively, the genetic differences

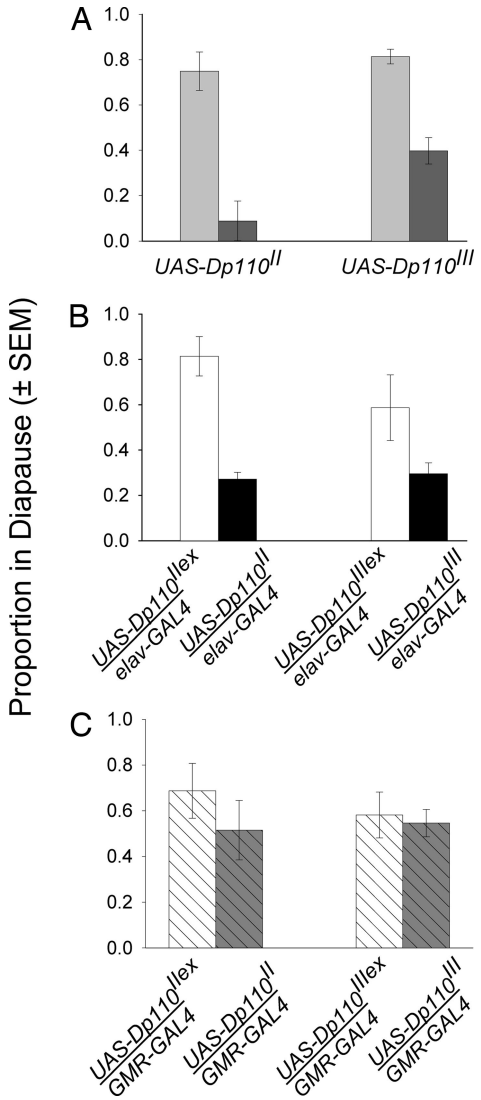

Fig. 2. Expression of *Dp110*. (*A*) Expression of *Dp110* in the nervous system decreases diapause. Independently generated homozygous viable transgenic lines *UAS-Dp110^{II}* and *Dp110^{III}* with inserts on chromosomes-2 and -3, respectively, are used here. We first confirm that the two *UAS-Dp110* lines generated had a photoperiodic response. We measure diapause under SD (light gray bars) and LD (dark gray bars) conditions, an accepted test of photoperiodic diapause (5). Our expectation that under SD conditions more flies would diapause than under LD conditions was met for both the *UAS-Dp110* lines [ANOVA $F_{(1,6)} = 10.7$, $P = 0.02$ and $F_{(1,9)} = 12.9$, $P = 0.006$]. (*B*) Pan-neuronal expression of *UAS-Dp110^{II}* and *UAS-Dp110^{III}* by using the *elav-GAL4* driver under SD conditions (open bars) significantly lowered the proportion of flies in diapause by comparison with the two control excision lines generated from *UAS-Dp110^{IIex}* and *UAS-Dp110^{IIIex}* and crossed to *elav-GAL4* (filled bars) [two-way ANOVA: $F_{(1,17)} = 5.59$, $P < 0.03$]. (*C*) Expressing *UAS-Dp110^{II}* and *UAS-Dp110^{III}* by using a GMR-GAL4 driver under SD conditions (white hatched bars) did not significantly affect the proportion of flies in diapause when compared with the two control excision lines (dark gray hatched bars) [two-way ANOVA: $F_{(1,31)} = 0.68$, $P = 0.41$].

we report here may not result in differences in *Dp110* RNA abundance between W and C.

To look further at the DNA, we sequenced a 6.5-kb genomic region containing the entire *Dp110* gene from both W and C (GenBank accession nos. DQ792599 and DQ792600) to assess whether nucleotide sequence differences in the coding and untranslated regions (UTRs) of the gene exist between the two variants. A total of 20 nucleotide sequence differences were identified over the 6.5-kb stretch, 18 of which were base substitutions occurring within the first three exons and introns of the gene and its 3′ UTR. Although nine of these substitutions were found in exons, none of them would lead to an amino acid change in the deduced protein sequence, suggesting that differences in PI3-kinase expression between W and C variants may instead be regulated by one or a combination of the sequence differences (six substitutions, two deletions) within the noncoding regions of *Dp110*.

Discussion

The data presented here are consistent with a model in which reducing signaling via the insulin/PI3-kinase pathway increases *D. melanogaster* diapause. Our finding that altered PI3-kinase expression in neurons alone can affect fly diapause is reminiscent of the observation that expression of PI3-kinase in *C. elegans* neurons can restore dauer formation to wild-type levels in *age-1* (PI3-kinase) null mutant worms (19). Furthermore, mutations that lower nematode insulin signaling promote dauer larval formation (19) suggesting the exciting possibility of a shared mechanism for developmental and reproductive arrests in *D. melanogaster* ovarian diapause and nematode dauer formation between these species' distant relatives. Our results suggest that insulin-signaling genes known to play important roles in dauer formation in *C. elegans* are excellent candidate genes for further investigation into the mechanisms involved in diapause in *D. melangaster* and the many other species that undergo developmental and reproductive arrests.

As described above, various fitness-related phenotypes are genetically correlated to *D. melanogaster* diapause in nature (9). This genetic correlation could be explained by pleiotropic effects of the *Dp110* gene. Notably, mutational analyses of the insulin-regulated PI3-kinase and other genes in the insulin-signaling pathway in *D. melanogaster* have revealed affects on lifespan (20–22), development (22), body size and growth (23, 24), nutrient stress (25), lipid content (26), locomotor activity (27), and egg chamber development (28). Our findings suggest that *Dp110* is not only an important regulator of diapause in *D. melanogaster* but also, through its pleiotropic effects, may influence the suite of life-history trade-offs associated with diapause in natural populations.

Few genes are known to affect reproductive diapause (29–33). Our discovery of a role for *Dp110* in reproductive diapause in *D. melanogaster* is significant because this species is amenable to mutational analyses, transgenic manipulations, and genomic investigations of the various phases of diapause including its photoperiodic induction, maintenance, and termination. Additionally, once genes that affect diapause have been identified, they can be used as candidate genes for the investigation of diapause in other species (34). From an evolutionary perspective, diapause is an important adaptive trait linked to many life-history parameters important for survivorship; the identification of genes involved in diapause is an entry point into studies of the molecular evolution of these genes. Finally, from an applied perspective, genetic manipulations of diapause can be engineered to control pest species and maximize the efficiency of their biological control agents.

Diapause is an adaptive trait critical for survival in temperate climates. Our identification of a single major gene regulator of diapause in nature provides us with a tantalizing example of the small but growing body of literature that reveals that major adaptive phenotypes are affected by variation in genes with large effects (single genes) (35). This finding is in conflict with the historically prominent notion of Fisher, Haldane, and Wright (45) who envisioned hundreds of interactive genes, each having tiny additive effects on a trait. These genes by definition could not be localized or identified because their effect sizes were so small. However, contrary to this model, recent data suggest that

genes can have large effects on adaptive phenotypes and that they exist along with genes with small effects. Challenges for the future are to identify both large and smaller effect genes and understand how they interact with the environment to generate variation in traits of adaptive significance.

Materials and Methods

Assay for Ovarian Diapause. Flies were grown under standard conditions at 25 ± 0.5°C on a 12-h light/12-h dark (12L:12D) light cycle as described in (10). Male and female flies were collected <8 h after eclosion and placed at 11 ± 0.5 °C under SD conditions (10L:14D). Ovaries were dissected in insect Ringer's solution at 4°C after 17 ± 1 days and scored for the maximum stage of ovary development (36). An average of 30 flies was dissected per vial, and diapause was measured as the mean proportion of flies (in more than three vials per strain) with maximum ovary development less than stage 8 of oogenesis (36). The photoperiod-induced diapause responses of the *UAS-Dp110* lines were confirmed at 11 ± 0.5 °C using SD (10L:14D) and LD (16L:8D) conditions.

Deletion Mapping. $Dp110^{A}$ and $Dp110^{B}$, two small deletions that remove *Dp110* and *Hairless* (H), a neighboring gene, were derived from the imprecise excision of H^{D179} (12). The $Dp110^{A}$ deletion strain is *yw*; *P[ry*$^{+}$, *gH]*, $Dp110^{A}/TM3$, *Ser*, y^{+} in which (*P[ry+, gH]*) is a *Hairless* genomic rescue construct. The $Dp110^{B}$ deletion strain is *yw*; *P[ry+, gH]*, $Dp110^{B}/TM3$, *Ser*, y^{+}. These deletion strains form the basis of the rescue lines $P\{g(Dp110^{98})\}$ and $P\{g(Dp110^{101})\}$, which each bear an insertion *P[w*$^{+}$, *gDp110]* of genomic *Dp110* DNA (12).

Transgenic Expression. Two *UAS-Dp110* lines ($UAS\text{-}Dp110^{II}$ and $UAS\text{-}Dp110^{III}$) were crossed to the pan-neuronal driver *elav-GAL4* (15) and a *GMR-GAL4* line (18) that drives expression in the visual system. To control for possible effects of different genetic backgrounds of the *UAS* and the W and C lines, excision lines of the *UAS* P-elements were generated ($UAS\text{-}Dp110^{IIex}$ and $UAS\text{-}Dp110^{ex}$).

Immunohistochemistry. The expression pattern of *elav-GAL4* was confirmed by using confocal microscopy. *elav-GAL4* was crossed to *UAS-tau-lacZ*, and the nervous system, eye discs, and ovaries were dissected from third-instar larvae, pupae, or adults carrying both *GAL4* driver and *UAS-tau-lacZ* transgenes (37). We confirmed that *elav-GAL4* was restricted to neurons and that no expression could be detected in ovaries where PI3-kinase could act to disrupt diapause (data not shown) (28, 38, 39).

Statistical Analysis. All analyses of diapause were done by using ANOVA, GLM (SAS Institute, Cary, NC) (40), of arcsine square-root transformed proportions. A Student–Neuman–Keuls test (SNK) was applied as an *a posteriori* test to determine whether differences between groups were significant ($P = 0.05$) (41).

mRNA Expression Analysis. We measured *Dp110* mRNA levels for 8 groups of 30 fly heads or 15–20 bodies with real-time quantitative RT-PCR with TaqMan technology (Applied Biosystems, Foster City, CA) (3). Flies were flash-frozen in liquid nitrogen followed by rapid vortexing to separate heads from rest of body parts. RNA was extracted with the miniRNeasy kit according to manufacturer instructions with on-column DNase I treatment (Qiagen, Valencia, CA). The reverse-transcription reaction (RT) was performed with random hexamers on 100 ng of total RNA according to the TaqMan RT-PCR kit protocol. The PCR was performed with gene-specific primers and dual-labeled TaqMan probes. Primers and probe for *Dp110* were as follows: forward primer, AGTCCACCTCCA-CAAGTCGAT; reverse primer, TGTGCAGCGTCAACT-GAAAG; and probe, CTCGCTGTGGGACATGGGCAA. PCR conditions were the default settings of the ABI TaqMan 7000 SDS machine (Applied Biosystems). We determined the cycle threshold (Ct) during the geometric phase of the PCR amplification plots as recommended by the manufacturer. Relative differences in *Dp110* transcripts were quantified by using the ΔΔCt method (42) with the *rp49* mRNA as a "housekeeping" gene loading control. *rp49* is widely used in this way in *Drosophila* and other organisms (43, 44).

***Dp110* Amplification and Sequence Determination.** Genomic DNA from W and C flies was used as template for the amplification of *Dp110* fragments using PCR. Reactions (20 μl) contained of 1 ng of template DNA mixed with a 1 μM concentration of each primer, 200 μM concentration of each dNTP, 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, and 1 unit of AmpliTaq DNA polymerase (Applied Biosystems). Samples were thoroughly mixed by repeated pipetting and incubated at 95°C for 5 min before thermal cycling. PCR amplifications were carried out for 35 cycles, each consisting of three stages: denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, and extension at 72°C for 45 s. An additional extension step at 72°C for 5 min was performed for all reactions. Amplicons were electrophoresed, purified from gel slices by using the QIAEX II Gel Extraction Kit (Qiagen), and sequenced by using an ABI Prism 373A Gene Analyzer (Applied Biosystems).

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