

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

Karen D. Williams*, Macarena Busto*, Maximiliano L. Suster*†, Anthony K.-C. So*, Yehuda Ben-Shahar***, Sally J. Leever§, and Marla B. Sokolowski*¶

*Department of Biology, University of Toronto, 3359 Mississauga Road, Mississauga, ON, Canada L5L 1C6; and †Growth Regulation Laboratory, Cancer Research UK London Research Institute, P.O. Box 123, 44 Lincoln's Inn Fields, London WC2A 3PX, United Kingdom

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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).

†Present address: Centre for Research in Neuroscience, Montreal General Hospital, Neurology L7–120, 1650 Cedar Avenue, Montreal, QC, Canada H3G 1A4.

**Present address: Howard Hughes Medical Institute, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, IA 52242.

¶To whom correspondence should be addressed. E-mail: msokolow@utm.utoronto.ca.

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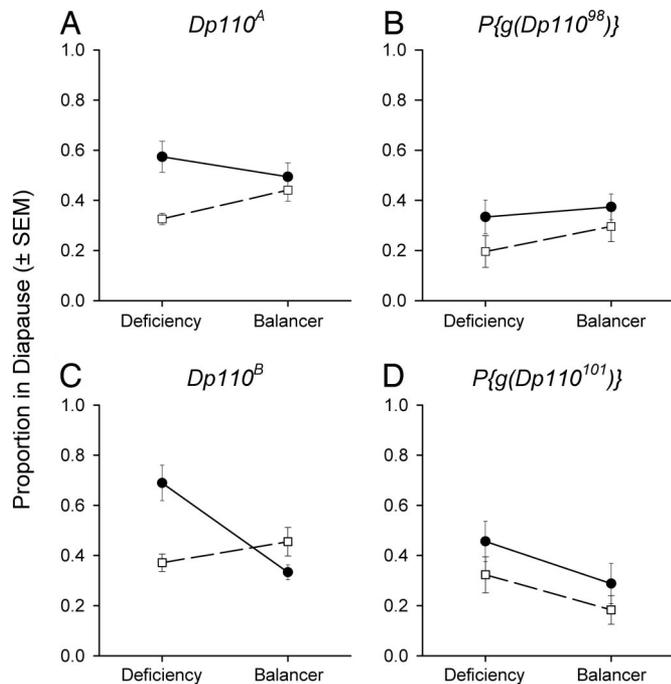


Fig. 1. Diapause natural variants map to *Dp110*. Wild-type W (filled circles) or C (open squares) flies were crossed to two deletions, which remove *Dp110*{*yw*; *P*{*ry*⁺, *gH*}, *Dp110*^A/*TM3*, *Ser*, *y*⁺ (A) and *yw*; *P*{*ry*⁺, *gH*}, *Dp110*^B/*TM3*, *Ser*, *y*⁺ (C) and to two rescue lines each 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. 1A; ANOVA, $F_{(1,11)} = 7.33$, $P = 0.02$] of three comparisons (Fig. 1B–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 pheno-

types 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. 1A 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. 1A and C were not observed, demonstrating lack of complementation when *Dp110* function is restored in the deletion strains (Fig. 1B 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. 2A). Then, we excised each transgene to generate background control lines, *UAS-Dp110*^{II}*exc* and *UAS-Dp110*^{III}*exc*, for our targeted expression studies. We crossed *Dp110*^{II}, *UAS-Dp110*^{III}, *UAS-Dp110*^{II}*exc*, and *UAS-Dp110*^{III}*exc* 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. 2B). 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*^{exc} control lines to a *GMR-GAL4* driver, which expresses in the visual system (18). No significant differences were detected when using this driver (Fig. 2C). 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

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 $25 \pm 0.5^\circ\text{C}$ 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 $11 \pm 0.5^\circ\text{C}$ 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 $11 \pm 0.5^\circ\text{C}$ 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(Dp110⁹⁸)}* and *P{g(Dp110¹⁰¹)}*, which each bear an insertion *P[w⁺, gDp110]* of genomic *Dp110* DNA (12).

Transgenic Expression. Two *UAS-Dp110* lines (*UAS-Dp110^{II}* and *UAS-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-Dp110^{IIex}* and *UAS-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, CTCGCTGTGGACATGGGCAA. 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 $\Delta\Delta\text{Ct}$ 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 MgCl₂, 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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